UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Malwina Brożyna

Rozprawa doktorska w oparciu o monotematyczny cykl publikacji w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki farmaceutyczne

Określenie zdolności wybranych olejków eterycznych do eradykacji biofilmu tworzonego przez drobnoustroje izolowane z zakażeń kości i ran

Determination of the ability of selected essential oils to eradicate biofilm formed by microorganisms isolated from bone and wound infections

Rozprawa doktorska wykonana
w Katedrze i Zakładzie Mikrobiologii Farmaceutycznej i Parazytologii
Uniwersytetu Medycznego we Wrocławiu

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składam serdeczne podziękowania za nieocenioną pomoc i wsparcie oraz ogromną cierpliwość, życzliwość, motywację, i poświęcony czas.

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dziękuję za pomoc i wiele cennych rad.

Dziękuję mojej **Rodzinie i Przyjaciołom**

za wsparcie i wiarę we mnie.

Spis treści

PUBLIKACJE NAUKOWE WCHODZĄCE W SKŁAD CYKLU STANOWIĄCEGO ROZPRAWĘ DOKTORSKĄ

PUBLIKACJA P1

Brożyna Malwina, Żywicka Anna, Fijałkowski Karol, Gorczyca Damian, Oleksy-Wawrzyniak Monika, Dydak Karolina, Migdał Paweł, Dudek Bartłomiej, Bartoszewicz Marzenna, Junka Adam. The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol). *Applied Sciences-Basel*, 2020, vol. 10, nr 20, art.7343.

IF $_{2020}$ = 2,679; 100 pkt. MEiN

PUBLIKACJA P2

Brożyna Malwina, Paleczny Justyna, Kozłowska Weronika, Chodaczek Grzegorz, Dudek-Wicher Ruth, Felińczak Anna, Gołębiewska Joanna, Górniak Agata, Junka Adam. The antimicrobial and antibiofilm *in vitro* activity of liquid and vapour phases of selected essential oils against *Staphylococcus aureus*. *Pathogens*, 2021, vol. 10, nr 9, art.1207.

IF $_{2021}$ = 4,531; 100 pkt. MEiN

PUBLIKACJA P3

Brożyna Malwina, Paleczny Justyna, Kozłowska Weronika, Ciecholewska-Juśko Daria, Parfieńczyk Adam, Chodaczek Grzegorz, Junka Adam. Chemical composition and antibacterial activity of liquid and volatile phase of essential oils against planktonic and biofilm-forming cells of *Pseudomonas aeruginosa*. *Molecules*, 2022, vol. 27, nr 13, art.4096.

IF $_{2022}$ = 4,927; 140 pkt. MEiN

PUBLIKACJA P4

Brożyna Malwina, Kozłowska Weronika, Malec Katarzyna, Paleczny Justyna, Detyna Jerzy, Fabianowska-Majewska Krystyna, Junka Adam. Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity. Publikacja została udostępniona w bazie bioRxiv jako preprint, DOI: 10.1101/2023.06.21.545846.

Łączna liczba punktów za cykl prac opublikowanych stanowiących rozprawę doktorską według wykazu czasopism naukowych MEiN, zgodny z rokiem ukazania się prac wynosi **340 punktów**. Sumaryczny Impact Factor (IF) wymienionych prac zgodny z rokiem ich ukazania **wynosi 12,137**.

Badania naukowe, których wyniki zostały zaprezentowane w publikacjach wykonano w ramach projektów Preludium 20 (nr 2021/41/N/NZ6/03305) oraz Opus 14 (nr 2017/27/B/NZ6/02103) finansowanych ze źródeł Narodowego Centrum Nauki, a także projektu dla Młodych Naukowców (nr STM.D230.20.126) finansowanego ze środków Uniwersytetu Medycznego we Wrocławiu.

W przygotowaniu wszystkich artykułów naukowych, wchodzących w skład cyklu stanowiącego rozprawę doktorską, miałam wiodący udział. Polegał on na opracowaniu koncepcji badań i metodologii, wykonaniu eksperymentów, przeprowadzeniu analiz statystycznych, opracowaniu szkicu manuskryptu. Odpowiadałam również za przygotowanie ostatecznych wersji prac i pozyskanie finansowania badań.

STRESZCZENIE

Rozprzestrzenianie się wśród drobnoustrojów mechanizmów oporności na antybiotyki prowadzi do istotnego obniżenia ich skuteczności oraz stanowi globalne zagrożenie dla ochrony zdrowia. Ze względu na niewielką liczbę wdrażanych antybiotyków, prowadzone są obecnie intensywne prace nad wprowadzeniem nieantybiotykowych środków terapeutycznych mających służyć zwalczaniu infekcji.

Infekcje powodowane przez biofilmy *Staphylococcus aureus* i *Pseudomonas aeruginosa* są jednym z najczęstszych powikłań niegojących się ran i zakażeń kości. Biofilm cechuje się wysoką tolerancją na konwencjonalne środki przeciwdrobnoustrojowe (antybiotyki, środki antyseptyczne, dezynfekujące), mechanizmy obrony immunologicznej człowieka oraz czynniki zewnętrzne. Jednym z rozwiązań proponowanych do zwalczania zakażeń wywołanych obecnością biofilmów jest zastosowanie olejków eterycznych. Cechują się one silnymi właściwościami przeciwdrobnoustrojowymi i przeciwbiofilmowymi. Jednakże metodyki oceny ich działania wobec drobnoustrojów charakteryzują się wysokim stopniem niespójności. Metody te nie odzwierciedlają również warunków panujących w miejscu infekcji, przez co w ograniczonym tylko stopniu określają rzeczywistą aktywność olejków eterycznych w warunkach *in vivo.*

Celem pracy było opracowanie testu służącego ocenie aktywności przeciwbiofilmowych lotnych związków, ocena *in vitro* właściwości przeciwdrobnoustrojowych i przeciwbiofilmowych olejków eterycznych oraz określenie wpływu składu medium hodowlanego na cechy biofilmu i skuteczność przeciwdrobnoustrojową olejków eterycznych.

Badaniom poddano szczepy wzorcowe i kliniczne *Staphylococcus aureus* i *Pseudomonas aeruginosa* izolowane z zakażeń kości i ran. Testowano siedem olejków eterycznych pochodzących ze źródeł komercyjnych. Drobnoustroje hodowano w standardowej pożywce mikrobiologicznej lub w medium imitującym środowisko niegojącej się rany. Analizowano właściwości przeciwdrobnoustrojowe i przeciwbiofilmowe frakcji płynnych i lotnych olejków wykorzystując metodologie standardowe oraz autorską metodę badawczą.

Otrzymane wyniki wykazały, iż opracowana metodologia badawcza pozwala na ocenę właściwości przeciwbiofilmowych lotnych substancji, w tym olejków eterycznych. Olejki tymiankowy i rozmarynowy cechowały się najwyższą efektywnością przeciwdrobnoustrojową względem badanych patogenów. Skład medium hodowlanego wpływał na cechy strukturalne i funkcjonalne biofilmu, a tym samym na poziom aktywności przeciwdrobnoustrojowej testowanych związków. Wrażliwość na olejki eteryczne komórek w formie planktonicznej była niższa w medium imitującym środowisko rany niż w standardowej pożywce hodowlanej, natomiast na poziom wrażliwości biofilmów wpływ miał także zastosowany układ badawczy oraz rodzaj użytego olejku.

W celu właściwej oceny skuteczności przeciwdrobnoustrojowej olejków eterycznych niezbędna jest standaryzacja metodologii badawczej *in vitro*, szczególnie w odniesieniu do analizy aktywności frakcji lotnych. Stosowany układ badawczy powinien odzwierciedlać warunki panujące w miejscu infekcji.

ABSTRACT

The spreading of antibiotic resistance mechanisms among microorganisms significantly reduces their effectiveness and threatens public health care worldwide. As the number of newly implemented antibiotics is limited, efforts are being undertaken to introduce non-antibiotic therapeutic agents for combating infections.

The major causative factors of non-healing wounds and bone infections are *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Biofilms are specific microbial structures exhibiting high tolerance to antimicrobials (antibiotics, antiseptics, disinfectants), human immune defense mechanisms, and environmental factors.

Essential oils [EOs] are considered a promising therapeutic option for treating biofilm-related infections. Potent antimicrobial and antibiofilm properties characterize these plant-derived substances. However, the *in vitro* methodologies for assessing their antimicrobial activity are inconsistent and do not reflect the conditions at the site of infection. Therefore, the results of these methods do not provide the data of EOs *in vivo* effect.

The aim of the present study was to develop a test to evaluate the antibiofilm effectiveness of volatile compounds, assess *in vitro* the antimicrobial and antibiofilm properties of EOs, and determine the effect of the composition of culture medium on biofilm features and antimicrobial activity of EOs.

Standard and clinical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from bone and wound infections were tested. Seven EOs from commercial sources were analyzed. The microorganisms were cultured in a standard microbiological medium or a medium imitating a non-healing wound milieu. The antimicrobial and antibiofilm activity of liquid and volatile fractions of EOs was evaluated using standard methodologies and a self-developed method.

According to the results, thyme and rosemary oils exhibited the highest antimicrobial effectiveness against the tested pathogens. The composition of culture medium affected the biofilm features and the antibacterial activity of the analyzed compounds. The susceptibility of planktonic cells to EOs was lower in the wound medium than in the standard culture medium, while biofilm tolerance varied. The developed research methodology allows for the assessment of the antibiofilm properties of volatile substances.

The standardization of the *in vitro* methodology, especially evaluating the volatiles, is required to assess the factual EOs antimicrobial activity. Moreover, the applied models should reflect the infection environment.

AUTOREFERAT

1. Wprowadzenie

Problemem naukowym analizowanym w niniejszej rozprawie doktorskiej była ocena *in vitro* aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej frakcji lotnych i płynnych wybranych olejków eterycznych względem patogenów izolowanych z zakażeń kości i ran.

Rozprzestrzenianie się wśród drobnoustrojów mechanizmów oporności na antybiotyki prowadzi do istotnego obniżenia ich skuteczności oraz stanowi globalne zagrożenie dla ochrony zdrowia. Ze względu na niewielką liczbę wdrażanych antybiotyków, prowadzone są obecnie intensywne prace nad wprowadzeniem nieantybiotykowych środków terapeutycznych mających służyć zwalczaniu infekcji [1].

Infekcje powodowane przez biofilmy *Staphylococcus aureus* i *Pseudomonas aeruginosa* są jednym z najczęstszych powikłań niegojących się ran i zakażeń kości [2]. Biofilm to zróżnicowana społeczność komórek drobnoustrojów otoczonych macierzą zewnątrzkomórkową. Komórki w strukturze biofilmu wykazują odmienny metabolizm, wzrost i transfer genów oporności w porównaniu do ich odpowiedników w formie planktonicznej (komórek niezagregowanych, wolno-pływających) [3]. Biofilm cechuje się wysoką tolerancją na konwencjonalne środki przeciwdrobnoustrojowe (antybiotyki, środki antyseptyczne, dezynfekujące), mechanizmy obrony immunologicznej człowieka oraz czynniki zewnętrzne [4]. W związku z tym istnieje potrzeba opracowania nowych rozwiązań terapeutycznych służących zwalczaniu infekcji wywołanych przez obecność biofilmu [5].

Jedną z metod wydaje się być zastosowanie związków pochodzenia naturalnego, do których należą między innymi olejki eteryczne [6]. Są to wieloskładnikowe metabolity roślinne, charakteryzujące się wysoką lipofilnością, lotnością oraz szerokim spektrum działania przeciwdrobnoustrojowego, również względem patogenów antybiotykoopornych. Olejki eteryczne posiadają zdolność do wiązania się ze ścianą komórkową oraz strukturami błonowymi mikroorganizmów, co prowadzi do zniszczenia ich integralności, a w efekcie do lizy komórek. Cząsteczki wchodzące w skład olejków eterycznych mogą również zakłócać wielorakie procesy zachodzące w biofilmach lub podczas ich tworzenia, takie jak adhezja, quorum-sensing oraz modulacja ekspresji genów. Ponadto, poszczególne olejki eteryczne charakteryzują się niską toksycznością, biodegradowalnością, a także właściwościami przeciwzapalnymi i immunostymulującymi. Wymienione cechy predysponują olejki eteryczne do stosowania jako adjuwanty antybiotyków lub nawet w monoterapii. Zarówno ciekłe, jak i lotne frakcje olejków mogą wykazywać działanie przeciwdrobnoustrojowe i przeciwbiofilmowe [7-11].

Jednakże poszczególne metodyki oceny aktywności lotnych frakcji wobec drobnoustrojów charakteryzują się wysokim stopniem niespójności zarówno w aspekcie warunków przeprowadzenia eksperymentu, jak również badanych form mikroorganizmów (formy planktoniczne, biofilm). Ponadto, ze względu na liczne czynniki (położenie geograficzne i warunki uprawy rośliny, metodę ekstrakcji olejku, część i gatunek rośliny, z której są pozyskiwane, zmienność wewnątrzgatunkową), skład ilościowy oraz jakościowy olejków eterycznych cechować się może istotnym zróżnicowaniem [12-15].

Pomimo iż liczne badania *in vitro* wskazują na silne właściwości przeciwdrobnoustrojowe olejków eterycznych, to prawdopodobnie dane te w ograniczonym tylko stopniu odzwierciedlają rzeczywistą aktywność tych związków w warunkach *in vivo* [16-17]. Zdecydowana większość testów przystosowana jest do określenia skuteczności olejków względem form planktonicznych, a nie wobec biofilmów tworzonych przez drobnoustroje. Poza tym, w testach tych stosowane są przede wszystkim standardowe pożywki mikrobiologiczne zamiast mediów, których skład odzwierciedla płyny występujące w miejscu infekcji (np. wysięk z rany). Najnowsze badania podkreślają natomiast, że przeprowadzenie analiz w warunkach imitujących środowisko zakażenia ma istotny wpływ na cechy wytworzonego biofilmu takie jak struktura czy grubość (wysokość biofilmu), co przekłada się na poziom aktywności przeciwdrobnoustrojowych testowanych substancji [18-19].

Wymienione czynniki przyczyniają się do braku jednoznacznych danych dotyczących skuteczności przeciwdrobnoustrojowych olejków eterycznych. Skutkiem tego jest brak ich zastosowania klinicznego w terapii infekcji bakteryjnych takich jak zakażenia kości i ran. Niezbędne jest zatem opracowanie i standaryzacja modeli *in vitro* oceniających właściwości przeciwbiofilmowe olejków w formie płynnej i lotnej. Modele te powinny w wysokim stopniu odzwierciedlać środowisko infekcji, wykluczać możliwie największą liczbę zmiennych wpływających na aktywność przeciwdrobnoustrojową olejków eterycznych oraz tolerancję drobnoustrojów na badane związki.

2. Cele pracy

Głównym celem badawczym publikacji wchodzących w skład osiągnięcia naukowego i stanowiących podstawę ubiegania się o stopień naukowy doktora była ocena *in vitro* aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej frakcji lotnych i płynnych wybranych olejków eterycznych względem patogenów izolowanych z zakażeń kości i ran.

W pierwszej pracy stanowiącej cykl publikacyjny przedstawiono autorską metodę oceny aktywności przeciwbiofilmowej substancji lotnych, w tym olejków eterycznych - „AntiBioVol" (ang. *Antibiofilm Activity of Volatile Compounds*). Metodologia umożliwia uzyskanie powtarzalnych wyników bez konieczności użycia skomplikowanego i kosztownego sprzętu badawczego. W celu wykazania skuteczności metody wykorzystano trzy olejki eteryczne, których aktywność analizowano względem szczepów wzorcowych drobnoustrojów z gatunków *S. aureus, P. aeruginosa, C. albicans*. Właściwości przeciwdrobnoustrojowe olejków potwierdzono także wykonując szereg testów kontrolnych stosując standardowe techniki badawcze (**publikacja P1**).

Celem drugiej i trzeciej pracy wchodzących w skład rozprawy doktorskiej była ocena aktywności przeciwdrobnoustrojowych i przeciwbiofilmowych frakcji lotnych i płynnych siedmiu olejków eterycznych wobec szczepów klinicznych i wzorcowych *S. aureus* i *P. aeruginosa* izolowanych z zakażeń kości i ran hodowanych w standardowym medium mikrobiologicznym. Zastosowano szerokie spektrum metod badawczych, wykorzystując zarówno standardowe techniki laboratoryjne, jak również metodologię „AntiBioVol" oraz model wykorzystujący biocelulozę jako nośnik olejku eterycznego. Ponadto, w celu dokładnego scharakteryzowania testowanych olejków eterycznych, dokonano analizy zawartości procentowych ich składników oraz wielkości kropel emulsji olejków (**publikacje P2, P3**).

Celem czwartej pracy była ocena wpływu składu chemicznego mediów hodowlanych na biofilm szczepów *S. aureus* izolowanych z zakażeń ran. W badaniach zastosowano standardową pożywkę mikrobiologiczną lub medium imitujące środowisko rany, które zawierało surowicę bydlęcą, elementy macierzy komórkowej i czynniki wytarzane w organizmie ludzkim w odpowiedzi na infekcję bakteryjną. Scharakteryzowano następujące cechy biofilmu: poziom tworzonej biomasy, aktywność metaboliczną, grubość i strukturę przestrzenną oraz liczbę komórek. Ponadto, na podstawie wyników uzyskanych w poprzednich pracach, wybrano dwa olejki eteryczne wykazujące najwyższą skuteczność przeciwdrobnoustrojową – tymiankowy i rozmarynowy. Analizowano właściwości przeciwbakteryjne frakcji płynnych tych olejków względem form planktonicznych i biofilmów gronkowca złocistego hodowanego w układach badawczych zawierających medium standardowe lub odzwierciedlające środowisko rany (**publikacja P4**).

3. Metodyka badań

3.1. Charakterystyka mikroorganizmów

Do oceny aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej olejków eterycznych wykorzystano szczepy referencyjne ATCC (ang. *American Type Culture Collection*) mikroorganizmów należących do gatunków *S. aureus* ATCC 6538 (**publikacje P1, P2, P4**) oraz ATCC 33591 (**publikacja P2**), *P. aeruginosa* ATCC 15442 (**publikacje P1, P3**), *C. albicans* ATCC 10231 (**publikacja P1**), oraz 24 izolaty kliniczne *S. aureus* (**publikacje P2, P4**) i 14 izolatów klinicznych *P. aeruginosa* (**publikacja P3**) będących częścią kolekcji szczepów Katedry i Zakładu Mikrobiologii Farmaceutycznej i Parazytologii UMW.

Celulozę bakteryjną wytworzono wykorzystując szczep referencyjny bakterii należącej do gatunku *K. xylinus* ATCC 53524 (**publikacja P2, P3**).

3.2. Olejki eteryczne

W doświadczeniach zastosowano następujące olejki eteryczne pochodzące ze źródeł komercyjnych:

- olejek eukaliptusowy (*Eucalyptus globulus* Labill.) (PharmaTech) (**publikacje P1, P2, P3**);
- olejek tymiankowy (*Thymus vulgaris* L.) (Etja lub Instytut Aromaterapii) (**publikacje P1, P2, P3, P4**);
- olejek z drzewa herbacianego (*Melaleuca alternifolia* Cheel.) (PharmaTech) (**publikacje P1, P2, P3**);
- olejek bazyliowy (*Ocimum basilicum* L.) (Nanga) (**publikacje P2, P3**);
- olejek rozmarynowy (*Rosmarinus officinalis* L.) (Nanga lub Instytut Aromaterapii) (**publikacje P2, P3, P4**);
- olejek miętowy (*Mentha arvensis* L.) (Optima Natura) (**publikacje P2, P3**);
- olejek lawendowy (*Lavandula angustifolia* Mill.) (Kej) (**publikacje P2, P3**).

3.3. Media hodowlane

Drobnoustroje wykorzystane do oceny aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej olejków eterycznych hodowano w dwóch mediach:

- bulionie tryptozowo-sojowym (TSB, ang. *Tryptic Soy Broth*) (**publikacje P1, P2, P3, P4**);
- medium odzwierciedlającym środowisko rany (IVWM, ang. *In Vitro Wound Milieu*). Medium przygotowano według przepisu przedstawionego w publikacji Kadam i wsp. [20]. Jego składnikami były: surowica bydlęca, fibronektyna, fibrynogen, laktoferyna, kwas mlekowy, kolagen i sól fizjologiczna (**publikacja P4**).

3.4. Określenie składu procentowego olejków eterycznych

Analizę zawartości procentowej poszczególnych składników olejków eterycznych wykonano przy użyciu metody gazowej chromatografii sprzężonej ze spektrometrią mas (GC-MS, ang. *Gas Chromatography-Mass Spectrometry*, Agilent 7890B GC połączony z systemem 7000GC/TQ i autosamplerem PAL RSI85, Agilent Technologies, Stany Zjednoczone Ameryki) (**publikacje P1, P2, P3, P4**). Badania przeprowadzono we współpracy

z dr inż. Weroniką Kozłowską z Katedry Biologii i Biotechnologii Farmaceutycznej Uniwersytetu Medycznego we Wrocławiu.

3.5. Ocena poziomu biomasy oraz aktywności metabolicznej biofilmów badanych szczepów drobnoustrojów

W celu wytworzenia biofilmów szczepy bakterii z gatunków *S. aureus* i *P. aeruginosa* hodowano w warunkach statycznych w płytkach polistyrenowych w medium TSB (**publikacja P2, P3, P4**) oraz szczepy *S. aureus* również w medium IVWM (**publikacja P4**). Następnie określano poziom biomasy biofilmów za pomocą barwienia fioletem krystalicznym, dokonując odczytów absorbancji przy długości fali 550 nm. Aktywność metaboliczną biofilmów *S. aureus* oceniano stosując test redukcji soli tetrazolowej (TTC, ang. *2,3,5-triphenyl-2H-tetrazolium chloride*), wykonując pomiary absorbancji przy długości fali 490 nm. Aktywność metaboliczną biofilmów *P. aeruginosa* oceniano przy użyciu testu opartego na redukcji soli sodowej resazuryny, dokonując pomiarów absorbancji przy długościach fali 570 nm i 600 nm.

3.6. Ocena właściwości biofilmu

Za pomocą barwników SYTO-9 i jodku propidyny barwiono komórki biofilmów *S. aureus* i *P. aeruginosa* wytworzonych w medium TSB (**publikacje P3, P4**) oraz *S. aureus* w medium IVWM (**publikacja P4**), które następnie poddano analizie z użyciem mikroskopu konfokalnego (Leica SP8, Leica Microsystems, Niemcy). Określono grubość biofilmu *S. aureus* oraz stosunek komórek żywych do komórek martwych na poszczególnych poziomach wysokości struktur biofilmów tworzonych w obu mediach. Stosunek komórek żywych do martwych w biofilmie obliczono z wykorzystaniem oprogramowania do opracowania danych liczbowych z danych graficznych ImageJ. Ponadto, przy pomocy posiewów ilościowych określono liczbę komórek bakteryjnych gronkowca złocistego w biofilmie tworzonym w medium TSB lub IVWM (**publikacja P4**). Wykonano również wizualizację biofilmów *S. aureus, P. aeruginosa, C. albicans* w medium TSB (**publikacje P1, P4**) oraz *S. aureus* także w medium IVWM (**publikacja P4**) z wykorzystaniem skaningowej mikroskopii elektronowej (SEM, ang. *Scanning Electron Microscopy,* Auriga 60, ZEISS, Niemcy). Analizy mikroskopowe przeprowadzono we współpracy z dr inż. Pawłem Migdałem z Katedry Higieny Środowiska i Dobrostanu Zwierząt Uniwersytetu Przyrodniczego we Wrocławiu oraz dr hab. Grzegorzem Chodaczkiem z Laboratorium Bioobrazowania Sieci Badawczej ŁUKASIEWICZ – PORT we Wrocławiu.

3.7. Ocena aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej lotnych frakcji olejków eterycznych

3.7.1. Ocena aktywności przeciwdrobnoustrojowej frakcji lotnych olejków eterycznych względem form planktonicznych drobnoustrojów

Oceny aktywności przeciwdrobnoustrojowej frakcji lotnych olejków eterycznych względem form planktonicznych badanych drobnoustrojów (*S. aureus, P. aeruginosa, C. albicans*) dokonano stosując metodę „Inverted Petri dish". Technika ta polegała na posiewie drobnoustrojów na podłoże agarowe MH (ang. *Mueller–Hinton*) znajdujące się w szalce Petriego, umieszczeniu krążka bibułowego nasączonego olejkiem eterycznym na wieczku szalki i inkubacji układu. Skuteczność przeciwdrobnoustrojową olejków eterycznych określano mierząc strefy zahamowania wzrostu mikroorganizmów (**publikacje P1, P2, P3**).

3.7.2. Ocena aktywności przeciwbiofilmowej frakcji lotnych olejków eterycznych

Aktywność przeciwbiofilmową lotnych frakcji olejków eterycznych oceniono wykorzystując autorską metodę „AntiBioVol" (ang. *Antibiofilm Activity of Volatile Compounds*) (**publikacje P1, P2, P3**). W płytce testowej 24-dołkowej umieszczono podłoże agarowe wymieszane z bulionem BHI (ang. *Brain Heart Infusion*), z którego wycięto krążki o średnicy mniejszej niż średnica dołka płytki i przeniesiono je do świeżej płytki. Następnie krążki zalano zawiesinami badanych drobnoustrojów (*S. aureus, P. aeruginosa, C. albicans*), przygotowanymi w medium TSB. Całość inkubowano w celu wytworzenia biofilmu. Po inkubacji krążki z biofilmami umieszczono ponownie w dołkach agarowych, z których zostały uprzednio wycięte i całą płytkę testową umieszczono nad osobną płytką 24-dołkową zawierającą olejki eteryczne. Układ uszczelniono i inkubowano. Po zakończeniu inkubacji płytki rozdzielono i wyjęto krążki agarowe. W celu określenia efektywności przeciwbiofilmowej lotnych frakcji olejków eterycznych względem bakterii, oceniono poziom aktywności metabolicznej biofilmów bakteryjnych (używając testów redukcji barwników TTC lub resazuryny) lub liczbę jednostek tworzących kolonie biofilmów *S. aureus* i *C. albicans* (metodą posiewu ilościowego) poddanych działaniu olejków eterycznych i biofilmów nietraktowanych olejkami eterycznymi. Obliczono procentową redukcję żywotności komórek biofilmu po ich ekspozycji na olejki eteryczne w odniesieniu do biofilmów nieeksponowanych na ich działanie.

3.8. Ocena aktywności przeciwdrobnoustrojowej i przeciwbiofilmowej płynnych frakcji olejków eterycznych

3.8.1. Ocena aktywności przeciwdrobnoustrojowej frakcji płynnych olejków eterycznych względem form planktonicznych drobnoustrojów

Właściwości przeciwdrobnoustrojowe frakcji płynnych olejków eterycznych względem form planktonicznych badanych drobnoustrojów (*S. aureus, P. aeruginosa, C. albicans*) określono przy użyciu metody dyfuzyjno-krążkowej. Aktywność przeciwdrobnoustrojową olejków eterycznych wyznaczano mierząc strefę zahamowania wzrostu drobnoustrojów wokół krążka bibułowego nasączonego badanym olejkiem i umieszczonego na podłożu agarowym MH zawierającym posiany drobnoustrój (**publikacje P1, P2, P3**). Ponadto oceniono właściwości przeciwdrobnoustrojowe płynnych frakcji olejków eterycznych w formie emulsji przy zastosowaniu metody mikrorozcieńczeń w płytkach polistyrenowych 96 dołkowych poprzez wyznaczenie wartości minimalnego stężenia hamującego (%) (MIC, ang. *Minimal Inhibitory Concentration*). Szczepy bakterii z gatunków *S. aureus* i *P. aeruginosa* oraz grzyba z gatunku *C. albicans* hodowano w medium TSB (**publikacje P1, P2, P3, P4**); szczepy *S. aureus* również w medium IVWM (**publikacja P4**).

3.8.2. Ocena aktywności przeciwbiofilmowej frakcji płynnych olejków eterycznych

Szczepy bakterii *S. aureus* i *P. aeruginosa* oraz grzyba z gatunku *C. albicans* hodowano w warunkach statycznych w płytkach polistyrenowych w medium TSB (**publikacje P1, P2, P3, P4**); szczepy *S. aureus* również w medium IVWM (**publikacja P4**) w celu wytworzenia biofilmów. Następnie biofilmy poddano działaniu emulsji olejków eterycznych metodą mikrorozcieńczeń. Wyznaczono wartości minimalnego stężenia eradykującego biofilm (MBEC, ang. *Minimal Biofilm Eradication Concentration*) przy zastosowaniu barwników TTC

lub resazuryny. Określono także procentową redukcję żywotności komórek biofilmów (używając barwników TTC, resazuryny lub SYTO-9 i jodku propidyny) po ich ekspozycji na działanie frakcji płynnych olejków eterycznych w odniesieniu do biofilmów nietraktowanych olejkami eterycznymi.

Dodatkowo oceniono aktywność przeciwbiofilmową frakcji płynnych olejków eterycznych aplikowanych na nośniku – biocelulozie, z wykorzystaniem zmodyfikowanej metody „A.D.A.M." (ang. *Antibiofilm Dressing's Activity Measurement*) (**publikacje P2, P3**). Biofilmy szczepów *S. aureus* i *P. aeruginosa* hodowano na krążkach agarowych w medium TSB, następnie poddano działaniu olejków eterycznych uwalnianych z biocelulozy. W celu określenia skuteczności przeciwbiofilmowej płynnych frakcji olejków eterycznych wyznaczono poziom aktywności metabolicznej biofilmów (używając testów redukcji barwników TTC lub resazuryny) poddanych działaniu olejków eterycznych i biofilmów nietraktowanych olejkami eterycznymi. Obliczono procentową redukcję żywotności komórek biofilmów po ich ekspozycji na olejki eteryczne w odniesieniu do biofilmów nieeksponowanych na ich działanie.

3.9. Określenie cech emulsji olejków eterycznych

Wykonano analizę wielkości kropel emulsji olejków eterycznych za pomocą metody dynamicznego rozpraszania światła (DLS, ang. *Dynamic Light Scattering*, Zetasizer Nano ZS ZEN3600, Malvern Instruments, Wielka Brytania). Badania przeprowadzono we współpracy z dr inż. Agatą Górniak z Pracowni Analizy Elementarnej i Badań Strukturalnych Uniwersytetu Medycznego we Wrocławiu oraz mgr Katarzyną Malec z Katedry i Zakładu Technologii Postaci Leku Uniwersytetu Medycznego we Wrocławiu.

3.10. Analiza statystyczna

Obserwacje odstające identyfikowano testem Hampel'a (**publikacja P4**). Testu Shapiro-Wilk'a (**publikacje P3, P4**) lub D'Agostino–Pearson'a (**publikacja P1**) użyto w celu oceny rozkładu normalnego, a testu Levene'a do oceny jednorodności wariancji (**publikacja P4**). Poziomy istotności $p < 0,05$ uznano jako istotne statystycznie (**publikacje P1, P3, P4**). Różnice w efektywności analizowanych olejków eterycznych porównywano stosując nieparametryczny test ANOVA Kruskal-Wallis'a z analizą post-hoc Dunn'a (**publikacja P3**) lub Tukey'a (**publikacja P1**). Istotność różnic pomiędzy poszczególnymi parametrami cechującymi biofilm analizowano testami parametrycznymi (test-t lub test-t Welch'a) lub nieparametrycznym testem Mann-Whitney'a (**publikacja P4**). Korelację liniową Pearson'a zastosowano w celu oceny zależności pomiędzy biomasą biofilmu a jego aktywnością metaboliczną. Wpływ poszczególnych czynników na efektywność olejków eterycznych oceniano przeprowadzając wieloczynnikową analizę wariancji (**publikacja P4**).

4. Wyniki badań

Wszystkie etapy badań przedstawione w czterech pracach wchodzących w skład rozprawy doktorskiej stanowią powiązany ze sobą cykl eksperymentalny.

W pierwszym etapie zaprezentowano protokół opracowanej metodologii służącej do oceny *in vitro* aktywności przeciwbiofilmowej lotnych związków, w tym lotnych frakcji olejków eterycznych. Ze względu na wysoką powtarzalność otrzymanych wyników, niskie koszty, łatwość wykonania, użyteczność względem szerokiego spektrum drobnoustrojów oraz możliwość przeprowadzenia badań bez konieczności użycia zaawansowanego sprzętu badawczego, technika ta może stanowić skuteczne narzędzie analityczne szeroko wykorzystywane w laboratoriach badawczych. W metodzie wyeliminowano liczne czynniki wpływające na obniżenie powtarzalności i odtwarzalności uzyskanych wyników, które posiadają stosowane standardowo techniki (**publikacja P1**).

W kolejnym kroku dokonano oceny aktywności przeciwdrobnoustrojowych i przeciwbiofilmowych form płynnych i lotnych siedmiu olejków eterycznych względem *S. aureus* i *P. aeruginosa* hodowanych w standardowej pożywce mikrobiologicznej (**publikacje P1, P2**). Niezależnie od zastosowanej metody, zaobserwowano różnice w tolerancji szczepów należących do tego samego gatunku drobnoustroju na poszczególne olejki eteryczne. Różnice te są wynikiem zmienności wewnątrzgatunkowej. Olejek tymiankowy w formie lotnej jako jedyny wykazał wysoką skuteczność wobec form planktonicznych wszystkich badanych szczepów gronkowca złocistego. Cechował się również najsilniejszymi właściwościami przeciwbiofilmowymi względem szczepów wzorcowych bakterii. Frakcje lotne olejku rozmarynowego silnie hamowały wzrost form planktonicznych 38% badanych szczepów oraz redukowały żywotność 60% biofilmów tworzonych przez określone szczepy *S. aureus*. Nie wykazano wpływu frakcji lotnych olejku bazyliowego na wzrost komórek planktonicznych bakterii, mimo obserwowanej redukcji żywotności biofilmu. Frakcje lotne pozostałych olejków eterycznych były nieaktywne lub cechowały się niską skutecznością wobec form planktonicznych *S. aureus,* natomiast wobec biofilmu ich aktywność dotyczyła 40-60% z testowanych szczepów. Olejek tymiankowy aplikowany również w formie płynnej cechował się najwyższą efektywnością przeciwdrobnoustrojową i przeciwbiofilmową względem gronkowca złocistego. W formie emulsji, w stężeniu równym lub niższym niż 0,1% (v/v) całkowicie eradykował biofilm szczepów klinicznych. Olejki tymiankowy i rozmarynowy uwalniane z krążka biocelulozowego redukowały żywotność biofilmów o około 90%. Emulsje pozostałych olejków eterycznych hamowały wzrost form planktonicznych bakterii w stężeniach równych lub niższych niż 6,3% (v/v). Zastosowanie emulsji zwierających olejek lawendowy lub eukaliptusowy w stężeniu 50% (v/v) nie wystarczyło do całkowitej eradykacji biofilmu gronkowca złocistego. Redukcja żywotności komórek biofilmów *S. aureus* uzyskana pod wpływem działania tych olejków uwalnianych z krążka celulozowego wynosiła ≥ 27% (**publikacja P2**).

Analiza aktywności przeciwbakteryjnych płynnych frakcji olejków eterycznych względem form planktonicznych *P. aeruginosa* wykazała, iż olejek rozmarynowy cechował się najwyższą skutecznością spośród testowanych związków. 0,8% stężenie olejku w emulsji całkowicie hamowało wzrost 93% badanych szczepów. Wartości MIC olejku bazyliowego wynosiły 1,6-12,5% (v/v), natomiast strefy zahamowania wzrostu ocenione metodą dyfuzyjno-krążkową zaobserwowano tylko względem 27% badanych szczepów.

Dla pozostałych olejków eterycznych określono strefy zahamowania wzrostu wobec części analizowanych szczepów (wobec 87% szczepów dla olejku tymiankowego, 53% dla olejku z drzewa herbacianego, 60% dla olejku eukaliptusowego, 33% dla olejku miętowego, 7% dla olejku lawendowego) oraz wartości MIC głównie w zakresie stężeń 12,5-25% (v/v). Pomimo iż wartości MBEC nie zostały wyznaczone dla żadnego z badanych olejków, emulsje olejków tymiankowego, z drzewa herbacianego, bazyliowego i rozmarynowego istotnie wpłynęły na obniżenie żywotności biofilmów *P. aeruginosa*. Skuteczność przeciwbiofilmowa nieemulgowanych olejków była zależna od szczepu, chociaż w odniesieniu do wszystkich olejków z wyjątkiem bazyliowego, dla poszczególnych szczepów odnotowano 60% lub wyższą redukcję żywotności komórek. Strefy zahamowania wzrostu from planktonicznych *P. aeruginosa* osiągnięte względem więcej niż jednego szczepu wykazano tylko po ekspozycji na działanie lotnych frakcji olejku rozmarynowego i eukaliptusowego. Obserwowano wysoką zmienność w tolerancji biofilmów na działanie lotnych frakcji olejków eterycznych w zależności od badanego szczepu. Najwyższą redukcję żywotności komórek biofilmów uzyskano pod wpływem działania lotnych frakcji olejku miętowego (**publikacja P3**).

W ostatnim etapie pracy dokonano oceny różnic w biofilmach szczepów gronkowca złocistego tworzonych w standardowym bulionie mikrobiologicznym (TSB) lub w medium odzwierciedlającym środowisko rany (IVWM). Wykazano, iż poziom biomasy biofilmu, jego aktywność metaboliczna, liczba komórek, stosunek komórek żywych do martwych były istotnie niższe w medium IVWM niż w TSB. Biofilm utworzony w medium IVWM cechował się natomiast większą grubością (wysokością) niż w TSB. Zaobserwowano także różnice w morfologii – biofilmy hodowane w medium TSB pokrywały równomiernie całą powierzchnię studzienek; natomiast w medium IVWM rosły w formie agregatów komórkowych o określonym kształcie (w literaturze anglojęzycznej określane jako „mushroom-like structures", „struktury grzybo-podobne") i zróżnicowanych wielkościach, rozmieszczone nierównomiernie w studzienkach. Ponadto analizowano właściwości przeciwbakteryjne frakcji płynnych olejków tymiankowego i rozmarynowego względem form planktonicznych i biofilmów gronkowca złocistego hodowanego w pożywce TSB lub IVWM. Przebadane olejki wykazały istotną aktywność wobec *S. aureus*, jednakże ich skuteczność była zróżnicowana w zależności od użytego podłoża. Olejki eteryczne działały słabiej wobec form planktonicznych bakterii hodowanych w medium IVWM niż w pożywce TSB. Olejek tymiankowy silniej redukował żywotność komórek biofilmu w pożywce IVWM niż w podłożu TSB. Odwrotny efekt zaobserwowano względem komórek biofilmów traktowanych olejkiem rozmarynowym (**publikacja P4**).

5. Wnioski

1. Opracowana metoda badawcza „AntiBioVol" jest łatwą w przeprowadzeniu techniką umożliwiającą ocenę właściwości przeciwbiofilmowych lotnych substancji z wykorzystaniem podstawowego sprzętu laboratoryjnego. Zastosowane zmiany w stosunku do standardowych metodologii wpływają na zwiększenie powtarzalności otrzymanych wyników (**publikacja P1**).
2. Badane szczepy *S. aureus* oraz *P. aeruginosa* cechowały się zróżnicowaniem wewnątrzgatunkowym w aspekcie tolerancji na działanie frakcji płynnych i lotnych olejków eterycznych (**publikacje P2, P3, P4**).
3. Analizowane szczepy gronkowca złocistego charakteryzowały się wyższą wrażliwością na działanie frakcji lotnych olejków eterycznych niż szczepy *P. aeruginosa* (**publikacje P2, P3**).
4. Badane szczepy *S. aureus* charakteryzowały się niższą tolerancją na działanie frakcji płynnych olejków eterycznych niż szczepy *P. aeruginosa* (**publikacje P2, P3**).
5. Płynne frakcje olejku tymiankowego cechowały się najsilniejszym efektem przeciwdrobnoustrojowym i przeciwbiofilmowym względem gronkowca złocistego. Frakcje lotne olejku wykazywały najwyższą skuteczność wobec form planktonicznych bakterii oraz biofilmów szczepów wzorcowych (**publikacja P2**).
6. Spośród testowanych olejków, olejek rozmarynowy wykazał najsilniejsze działanie przeciwdrobnoustrojowe w formie płynnej i lotnej spośród testowanych olejków względem form planktonicznych istotnej większości badanych szczepów *P. aeruginosa* (**publikacja P3**).
7. W stosunku do biofilmu *P. aeruginosa* nie wskazano olejku, którego aktywność przeciwbiofilmowa frakcji lotnych lub płynnych była istotnie wyższa niż pozostałych olejków. Efektywność działania olejków różniła się w zależności od zastosowanej metody badawczej oraz analizowanego szczepu bakterii. Jest to wynikiem przede wszystkim zmienności wewnątrzgatunkowej drobnoustrojów oraz różnic w formulacjach olejków oddziałujących na komórki biofilmu (jako emulsje aplikowane bezpośrednio na biofilm lub olejki uwalniane stopniowo z nośnika) (**publikacja P3**).
8. Wszystkie oceniane parametry charakteryzujące biofilm (poziom biomasy, aktywność metaboliczna i liczba komórek, grubość, stosunek komórek żywych do martwych, morfologia struktury) różniły się istotnie dla gronkowca złocistego hodowanego w medium TSB od hodowanego w medium IVWM (**publikacja P4**).
9. Powyższe różnice przekładały się na obniżoną wrażliwość komórek form planktonicznych oraz zróżnicowaną tolerancję biofilmów na działanie olejków eterycznych w medium IVWM. Jest to prawdopodobnie wynikiem zwiększenia grubości ścian komórkowych bakterii na skutek obecności w medium IVWM związków o działaniu przeciwdrobnoustrojowym, różnej zdolności olejków eterycznych do penetracji przez biofilm lub oddziaływania składowych IVWM z substancjami aktywnymi olejków. Dalsze badania są wymagane w celu poznania mechanizmu(ów) stojących za obserwowanym zjawiskiem (**publikacja P4**).

10. Aktywność przeciwdrobnoustrojowa olejków eterycznych względem gronkowca złocistego w warunkach *in vitro* zależy od składu medium hodowlanego; opracowane metody badawcze *in vitro* powinny odzwierciedlać środowisko infekcji (**publikacja P4**).

11. W celu właściwej oceny skuteczności przeciwdrobnoustrojowej olejków eterycznych niezbędna jest standaryzacja metodologii badawczej, szczególnie w odniesieniu do analizy aktywności frakcji lotnych.

6. Cytowana literatura

1. Mühlen, S.; Dersch, P. Anti-virulence strategies to target bacterial infections. *Curr Top Microbiol Immunol* 2016, 398, 147–183.
2. dos Santos Ramos, M.A.; da Silva, P.B.; Spósito, L.; de Toledo, L.G.; vidal Bonifácio, B.; Rodero, C.F.; dos Santos, K.C.; Chorilli, M.; Bauab, T.M. Nanotechnology-Based Drug Delivery Systems for Control of Microbial Biofilms: A Review. *Int. J. Nanomed*. 2018, 13, 1179–1213.
3. Hall, C.W.; Mah, T.F. Molecular Mechanisms of Biofilm-Based Antibiotic Resistance and Tolerance in Pathogenic Bacteria. *FEMS Microbiol. Rev*. 2017, 41, 3, 276–301.
4. Vestby, L.K.; Grønseth, T.; Simm, R.; Nesse, L.L. Bacterial Biofilm and Its Role in the Pathogenesis of Disease. *Antibiotics* 2020, 9, 2, 59.
5. Verderosa, A.D.; Totsika, M.; Fairfull-Smith, K.E. Bacterial Biofilm Eradication Agents: A Current Review. Front. Chem. 2019, 7, 1–17.
6. Jafri, H.; Ansari, F.A.; Ahmad, I. Prospects of Essential Oils in Controlling Pathogenic Biofilm. In New Look to Phytomedicine; Khan, M.S.A., Ahmad, I., Chattopadhyay, D., Eds.; Elsevier Inc.: New York, NY, USA, 2019; pp. 203–236. ISBN 978-0-12-814619-4.
7. Chouhan, S.; Sharma, K.; Guleria, S. Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives. *Medicines* 2017, 4, 3, 58.
8. Peterfalvi, A.; Miko, E.; Nagy, T.; Reger, B.; Simon, D.; Miseta, A.; Czéh, B.; Szereday, L. Much More than a Pleasant Scent: A Review on Essential Oils Supporting the Immune System. *Molecules* 2019, 24, 24, 4530.
9. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential Oils, A New Horizon in Combating Bacterial Antibiotic Resistance. *Open Microbiol. J*. 2014, 8, 6–14.
10. Mulyaningsih, S.; Sporer, F.; Reichling, J.; Wink, M. Antibacterial activity of essential oils from Eucalyptus and of selected components against multidrug-resistant bacterial pathogens. *Pharm. Biol*. 2011, 49, 9, 893–899.
11. Reichling, J. Anti-biofilm and Virulence Factor-Reducing Activities of Essential Oils and Oil Components as a Possible Option for Bacterial Infection Control. *Planta Med* 2020, 86, 8, 520–537.
12. Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for In Vitro Evaluating Antimicrobial Activity: A Review. J. *Pharm. Anal*. 2016, 6, 2, 71–79.
13. Yala, J.F.; Mabika, R.M.; Camara, B.; Tuo, S.; Souza, A.; Lepengue, A.N.; Koné, D.; M'batchi, B. Assessment of the Antibacterial Activity of Four Essential Oils and the Biobactericide Neco. *Int. J. Phytomed*. 2017, 9, 443–450.
14. Budzynska, A.; Sadowska, B.; Paszkiewicz, M.; Rózalska, B. Assessment of essential oils activity used in liquid or volatile phase against biofilm cultures of Staphylococcus aureus and Candida albicans. The methodological study. *Med. Doswiadczalna* Mikrobiol. 2011, 63, 4, 327–331.

15. Sadgrove, N.; Nicolas, J. A Contemporary Introduction to Essential Oils: Chemistry, Bioactivity and Prospects for Australian Agriculture. *Agriculture* 2015, 5,1, 48–102.
16. Orchard, A.; Sandasi, M.; Kamatou, G.; Viljoen, A.; van Vuuren, S. The in vitro Antimicrobial Activity and Chemometric Modelling of 59 Commercial Essential Oils against Pathogens of Dermatological Relevance. *Chem Biodivers* 2017, 14,1.
17. Patterson, J. E.; McElmeel, L.; Wiederhold, N. P. In vitro activity of essential oils against gram-positive and gram-negative clinical isolates, including carbapenem-resistant Enterobacteriaceae. *Open Forum Infect Dis* 2019, 6,12.
18. Thaarup, I. C.; Bjarnsholt, T. Current in vitro biofilm-infected chronic wound models for developing new treatment possibilities. *Advances in Wound Care (New Rochelle)* 2021, 10,2, 91–102.
19. Vyas, H. K. N.; Xia, B.; Mai-Prochnow, A. Clinically relevant in vitro biofilm models: A need to mimic and recapitulate the host environment. *Biofilm* 2022, 4.
20. Kadam, S.; Madhusoodhanan, V.; Dhekane, R.; Bhide, D.; Ugale, R.; Tikhole, U.; Kaushik, K. Milieu matters: An in vitro wound milieu to recapitulate key features of, and probe new insights into, mixed-species bacterial biofilms. *Biofilm* 2021, 3.

OSIĄGNIĘCIA NAUKOWE

Wrocław, 16.06.2023 r.

Malwina Brożyna

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp.	Opis bibliograficzny	IF	Punkty
1.	**Brożyna Malwina**, Żywicka Anna, Fijałkowski Karol, Gorczyca Damian, Oleksy-Wawrzyniak Monika, Dydak Karolina, Migdał Paweł, Dudek Bartłomiej, Bartoszewicz Marzenna, Junka Adam: The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol), Applied Sciences-Basel, 2020, vol. 10, nr 20, art.7343 [19 s.], DOI:10.3390/app10207343	2,679	100
2	Zielińska Sylwia, Dziągwa-Becker Magdalena, Piątczak Ewelina, Jezierska-Domaradzka Anna, **Brożyna Malwina**, Junka Adam, Kucharski Mariusz, Cicek Serhat Sezai, Zidorn Christian, Matkowski Adam: Phytochemical composition and antimicrobial activity of Corydalis solida and Pseudofumaria lutea, Molecules, 2020, vol. 25, nr 16, art.3591 [12 s.], DOI:10.3390/molecules25163591	4,412	140
3	Dydak Karolina, Junka Adam, Dydak Agata, **Brożyna Malwina**, Paleczny Justyna, Fijałkowski Karol, Kubielas Grzegorz, Aniołek Olga, Bartoszewicz Marzenna: In vitro efficacy of bacterial cellulose dressings chemisorbed with antiseptics against biofilm formed by pathogens isolated from chronic wounds, International Journal of Molecular Sciences, 2021, vol. 22, nr 8, art.3996 [43 s.], DOI:10.3390/ijms22083996	6,208	140
4	Zielińska Sylwia, Dziągwa-Becker Magdalena, Junka Adam, Piątczak Ewelina, Jezierska-Domaradzka Anna, **Brożyna Malwina**, Paleczny Justyna, Sobiecka Aleksandra, Słupski Wojciech, Mess Eleonora, Kucharski Mariusz, Çiçek Serhat Sezai, Zidorn Christian, Matkowski Adam: Screening Papaveraceae as novel antibiofilm natural-based agents, Molecules, 2021, vol. 26, nr 16, art.4778 [20 s.], DOI:10.3390/molecules26164778	4,927	140
5	Dudek-Wicher Ruth, Paleczny Justyna, Kowalska-Krochmal Beata, Szymczyk-Ziółkowska Patrycja, Pachura Natalia, Szumny Antoni, **Brożyna Malwina**: Activity of liquid and volatile fractions of essential oils against biofilm formed by selected reference strains on polystyrene and hydroxyapatite surfaces, Pathogens, 2021, vol. 10, nr 5, art.515 [22 s.], DOI:10.3390/pathogens10050515	4,531	100

Lp.	Opis bibliograficzny	IF	Punkty
6	**Brożyna Malwina**, Paleczny Justyna, Kozłowska Weronika, Chodaczek Grzegorz, Dudek-Wicher Ruth, Felińczak Anna, Gołębiewska Joanna, Górniak Agata, Junka Adam: The antimicrobial and antibiofilm in vitro activity of liquid and vapour phases of selected essential oils against Staphylococcus aureus, Pathogens, 2021, vol. 10, nr 9, art.1207 [25 s.], DOI:10.3390/pathogens10091207	4,531	100
7	Paleczny Justyna, Junka Adam, **Brożyna Malwina**, Dydak Karolina, Oleksy-Wawrzyniak Monika, Ciecholewska-Juśko Daria, Dziedzic Ewelina, Bartoszewicz Marzenna: The high impact of Staphylococcus aureus biofilm culture medium on in vitro outcomes of antimicrobial activity of wound antiseptics and antibiotic, Pathogens, 2021, vol. 10, nr 11, art.1385 [26 s.], DOI:10.3390/pathogens10111385	4,531	100
8	Simińska-Stanny Julia, Nizioł Martyna, Szymczyk-Ziółkowska Patrycja, **Brożyna Malwina**, Junka Adam, Shavandi Amin, Podstawczyk Daria: 4D printing of patterned multimaterial magnetic hydrogel actuators, Additive Manufacturing, 2022, vol. 49, art.102506 [14 s.], DOI:10.1016/j.addma.2021.102506	11,632*	200
9	Paleczny Justyna, **Brożyna Malwina**, Dudek-Wicher Ruth, Dydak Karolina, Oleksy-Wawrzyniak Monika, Madziała Marcin, Bartoszewicz Marzenna, Junka Adam: The medium composition impacts Staphylococcus aureus biofilm formation and susceptibility to antibiotics applied in the treatment of bone infections, International Journal of Molecular Sciences, 2022, vol. 23, nr 19, art.11564 [21 s.], DOI:10.3390/ijms231911564	6,208*	140
10	Krasowski Grzegorz, Migdał Paweł, Woroszyło Marta, Fijałkowski Karol, Chodaczek Grzegorz, Czajkowska Joanna, Dudek Bartłomiej, Nowicka Joanna, Oleksy-Wawrzyniak Monika, Kwiek Bartłomiej, Paleczny Justyna, **Brożyna Malwina**, Junka Adam: The assessment of activity of antiseptic agents against biofilm of staphylococcus aureus measured with the use of processed microscopic images, International Journal of Molecular Sciences, 2022, vol. 23, nr 21, art.13524 [20 s.], DOI:10.3390/ijms232113524	6,208*	140
11	**Brożyna Malwina**, Paleczny Justyna, Kozłowska Weronika, Ciecholewska-Juśko Daria, Parfieńczyk Adam, Chodaczek Grzegorz, Junka Adam: Chemical composition and antibacterial activity of liquid and volatile phase of essential oils against planktonic and biofilm-forming cells of Pseudomonas aeruginosa, Molecules, 2022, vol. 27, nr 13, art.4096 [22 s.], DOI:10.3390/molecules27134096	4,927*	140
12	Oleksy-Wawrzyniak Monika, Junka Adam, **Brożyna Malwina**, Migdał Paweł, Kwiek Bartłomiej, Nowak Maciej, Mączyńska Beata, Bartoszewicz Marzenna: The in vitro ability of Klebsiella pneumoniae to form biofilm and the potential of various compounds to eradicate it from urinary catheters, Pathogens, 2022, vol. 11, nr 1, art.42 [24 s.], DOI:10.3390/pathogens11010042	4,531*	100
13	Bąchor Urszula, Junka Adam, **Brożyna Malwina**, Mączyński Marcin: The in vitro impact of isoxazole derivatives on pathogenic biofilm and cytotoxicity of fibroblast cell line, International Journal of Molecular Sciences, 2023, vol. 24, nr 3, art.2997 [18 s.], DOI:10.3390/ijms24032997	6,208*	140

** IF 2021*

1.2 Publikacje w czasopiśmie bez IF

Lp.	Opis bibliograficzny	Punkty
1	Oleksy-Wawrzyniak Monika, **Brożyna Malwina**, Piątkowska Elżbieta, Niewińska Kinga, Bartoszewicz Marzenna: Profilaktyka zakażeń u wcześniaków i noworodków - jaki antyseptyk wybrać?, Forum Zakażeń, 2020, vol. 11, nr 2, s. 67-79, [Publikacja w czasopiśmie spoza listy MNiSW], DOI:10.15374/FZ2020012	5
2	Paleczny Justyna, **Brożyna Malwina**, Junka Adam, Bartoszewicz Marzenna, Dudek-Wicher Ruth: Modifications of bacterial cellulose in wound care, Polimery w Medycynie, 2021, vol. 51, nr 2, s. 77-84, DOI:10.17219/pim/143330	70

2. Monografie naukowe

2.1 Książka autorska -
2.2 Książka redagowana -

2.3 Rozdziały

Lp.	Opis bibliograficzny	Punkty
1	Piątkowska Elżbieta, Włodarczyk Maciej, Kominek Filip, Chwiećko Aleksandra, **Brożyna Malwina**: Aktywność przeciwbakteryjna wybranych substancji pochodzenia roślinnego względem Staphylococcus aureus, w obecności i przy braku emulgatora - Tween 80, W: Nauka, badania i doniesienia naukowe 2018 : nauki przyrodnicze i medyczne, (red.) Tobiasz Wysoczański, Świebodzice 2018, Idea Knowledge Future, s. 210-220, ISBN 978-83-945311-7-1, [Publikacja w wydawnictwie spoza listy MNiSW]	5

3. Abstrakty

Lp.	Opis bibliograficzny
1	Kominek Filip, Chwiećko Aleksandra, **Brożyna Malwina**, Piątkowska Elżbieta, Włodarczyk Maciej: Porównanie metod określania aktywności przeciwbakteryjnej wybranych wyciągów roślinnych i olejków eterycznych względem S. aureus, W: III Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 9 kwietnia 2018 r. Książka abstraktów 2018, 27 poz.P10, [[Dostęp 12.04.2018]. Dostępny w: http://www.farmacja.wroclaw.pl/images/ksi%C4%85%C5%BCka%20abstrakt%C3%B3w%20kwiecie%C5%84%202018.pdf]
2	Kuś Piotr, Kominek F., Chwiećko A., Starzec Aneta, **Brożyna Malwina**: Plant substances in dermatological medications and cosmetics - source of safe bioactive compounds or potential danger?, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 114 poz.P60, ISBN 978-83-65932-64-8
3	Piątkowska Elżbieta, **Brożyna Malwina**, Starzec Aneta, Kominek F.: Cooperation is the key to success - synergistic combinations of essential oils, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 145 poz.P91, ISBN 978-83-65932-64-8

Lp.	Opis bibliograficzny
4	Starzec Aneta, **Brożyna Malwina**, Kominek F., Fecka Izabela: Self-preserving cosmetics - is it possible?, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 164 poz.P110, ISBN 978-83-65932-64-8
5	Starzec Aneta, **Brożyna Malwina**, Fecka Izabela: Metody analityczne stosowane w przemyśle kosmetycznym, W: IV Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 12 kwietnia 2019 r. Książka abstraktów 2019, [40], [[Dostęp 18.04.2019]. Dostępny w: http://www.farmacja.wroclaw.pl/images/ksia%CC%A8z%CC%87ka-abstrakto%CC%81w-kwiecien%CC%81-2019.pdf]
6	Starzec Aneta, **Brożyna Malwina**, Fecka Izabela: Naturalne konserwanty w produktach kosmetycznych, W: XVI Konferencja Naukowa BioMedTech Silesia 2019. Zabrze, 05.04.2019 roku. Streszczenia : referaty - junior 2019 - profesjonalna [online] 2019, poz.03
7	**Brożyna Malwina**, Starzec Aneta: Charakterystyka biofilmu, W: XVI Konferencja Naukowa BioMedTech Silesia 2019. Zabrze, 05.04.2019 roku. Streszczenia : referaty - junior 2019 - profesjonalna [online] 2019, poz.09
8	Dydak Karolina, Paleczny Justyna, **Brożyna Malwina**, Junka Adam, Bartoszewicz Marzenna: The antimicrobial effectiveness of bacterial cellulose dressings chemisorbed with commonly used wounds irrigation agents against chosen opportunistic pathogens, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 100-102, ISBN 978-83-66489-37-0
9	Paleczny Justyna, **Brożyna Malwina**, Dydak Karolina, Junka Adam, Bartoszewicz Marzenna: Efficacy of biofilm eradication of Staphylococcus aureus strains isolated from wounds by the antimicrobials commonly applied to treat wound infections, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 187-188, ISBN 978-83-66489-37-0
10	Starzec Aneta, **Brożyna Malwina**, Kotyra Łukasz, Fecka Izabela: Anti-inflammatory and skin regenerating properties of vegetable oils, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 225, ISBN 978-83-66489-37-0
11	**Brożyna Malwina**, Paleczny Justyna, Dydak Karolina, Starzec Aneta, Junka Adam: Antimicrobial activity of thyme, tea tree and eucalyptus essential oils against Staphylococcus aureus biofilm, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 90-91, ISBN 978-83-66489-37-0
12	**Brożyna Malwina**, Paleczny Justyna, Junka Adam: The antibiofilm potential of vapor fractions of selected essential oils against Pseudomonas aeruginosa, Medical Sciences Forum, 2022, vol. 12, nr 1, art.3 [2 s.], [2nd International Electronic Conference on Antibiotics - Drugs for Superbugs: Antibiotic Discovery, Modes of Action and Mechanisms of Resistance. Online, 15-30 June 2022], DOI:10.3390/eca2022-12702

Lp.	Opis bibliograficzny
13	Paleczny Justyna, **Brożyna Malwina**, Bartoszewicz Marzenna, Junka Adam Feliks: Solid-phase microextraction as an antibiotic resistance detector in Staphylococcus aureus strains, Medical Sciences Forum, 2022, vol. 12, nr 1, art.30 [1 s.], [2nd International Electronic Conference on Antibiotics - Drugs for Superbugs: Antibiotic Discovery, Modes of Action and Mechanisms of Resistance. Online, 15-30 June 2022], DOI:10.3390/eca2022-12697
14	Junka Adam, **Brożyna Malwina**: Is it possible to obtain cohesive results of antimicrobial activity of essential oils?, W: PSE 2022 Meeting "Natural Products in Drug Discovery and Development – Advances and Perspectives". Iasi, Romania, September 19-22, 2022. Abstracts book [online] 2022, [92]
15	Zielińska Sylwia, Suśniak K, Krysa M, Sroka-Bartnicka A, **Brożyna Malwina**, Dydak Karolina, Sobiecka Aleksandra, Matkowski Adam, Dziągwa-Becker M, Wójciak M, Sowa I, Czerwińska M, Junka Adam: Bio-elicitation stimulated isoquinoline alkaloids production in Chelidonium majus cells cultured on bio-nano-cellulose, Planta Medica, 2022, vol. 88, nr 15, 1492-1493 poz.P-160, [70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). Thessaloniki, Greece, 28.-31.08.2022. Abstracts], DOI:10.1055/s-0042-1759137
16	**Brożyna Malwina**, Junka Adam: The development of conclusive toolbox for analysis of in vitro antimicrobial activity of Essential Oils, W: The last word belongs to microbes – Celebrating the 200th anniversary of the birth of Louis Pasteur. Warsaw, Poland, November 29-30, 2022 2022, 124 poz.P052
17	Malec Katarzyna, **Brożyna Malwina**, Junka Adam, Karolewicz Bożena, Nartowski Karol: Addressing the global challenge of resistant bacteria – the composition optimization of the emulsions with incorporated essential oils effective against MRSA, W: 4th European Conference on Pharmaceutics. Marseille, France, 20-21 March 2023. Files [USB-Drive] 2023, International Association for Pharmaceutical Technology, poz.4KAZM-8LU8Q-ECCCS-GSWZ7-GCKKK

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Punkty ministerialne : 1760

Wykaz projektów naukowych

1. Grant Preludium 20 Narodowego Centrum Nauki pt. „Ocena aktywności przeciwbiofilmowej olejków eterycznych wobec drobnoustrojów izolowanych z zakażeń ran przewlekłych przeprowadzona w środowisku odzwierciedlającym środowisko rany przewlekłej", 2021/41/N/NZ6/03305, **Kierownik projektu**
2. Projekt w ramach subwencji Uniwersytetu Medycznego we Wrocławiu 2022 pt. „Układy micelarne z inkorporowanymi olejkami eterycznymi jako systemy dostarczania substancji o zwiększonej aktywności przeciwbakteryjnej", SUBK.D230.22.040, **Kierownik projektu**
3. Projekt w ramach subwencji Uniwersytetu Medycznego we Wrocławiu 2022 pt. „Określenie aktywności przeciwdrobnoustrojowej wybranych składników olejków eterycznych oraz ocena ich wpływu na ścianę komórkową metycylinoopornych szczepów gronkowca złocistego (MRSA)", SUBK.D190.22.006, **Członek zespołu badawczego**
4. Projekt w ramach subwencji Uniwersytetu Medycznego we Wrocławiu 2022 pt. „Wpływ chlorynu sodu aktywowanego kwasami organicznymi na wzrostu drobnoustrojów chorobotwórczych oraz wybranych szczepów probiotycznych w formie biofilmu", SUBK.D230.22.074, **Członek zespołu badawczego**
5. Projekt Dolnośląscy Liderzy Medycyny - wdrożenie zintegrowanego programu podnoszenia kompetencji studentów, doktorantów, kadry dydaktycznej i administracyjnej Uniwersytetu Medycznego im Piastów Śląskich we Wrocławiu" Zadanie 5 – Wdrożenie Programu Stypendiów Dydaktycznych jako działania uzupełniającego Program Studiów Doktoranckich, **Realizator projektu**
6. Projekt dla Młodych Naukowców 2020 pt. „Ocena działania protekcyjnego i regeneracyjnego bezkomórkowych supernatantów uzyskanych z hodowli bakterii z rodzaju *Lactobacillus* i *Bifidobacterium* względem neuronów poddanych działaniu ß-amyloidu", STM.D230.20.126, **Młody Naukowiec**
7. Projekt dla Młodych Naukowców 2020 pt. „Ocena skuteczności działania bakteriostatycznego i przeciwbiofilmowego antyseptyków i antybiotyków wykorzystywanych w leczeniu infekcji ran i kości względem szczepów *Staphylococcus aureus* opornych i wrażliwych na działanie metycyliny", STM.D230.20.127, **Członek zespołu badawczego**
8. Projekt dla Młodych Naukowców 2020 pt. „Wykorzystanie bakteryjnej bionanocelulozy jako nowoczesnej bazy do opatrunków dedykowanych pacjentom cierpiącym z powodu ran niegojących się, oparzeniowych oraz owrzodzeń", STM.D230.20.053, **Członek zespołu badawczego**

Wykaz nagród

1. Nagroda JM Rektora (za osiągnięcia 2021) Zespołowa II stopnia za publikację
2. Nagroda Główna w konkursie „Aromaterapia i Nauka 2021" za pracę magisterską
3. Laureatka konkursu Preludium 20 Narodowego Centrum Nauki NCN

Staże zagraniczne

1. Max-Planck Institut für Molekulare Planzenphysiologie, Department of Small-Molecule Signalling, Poczdam, Niemcy, 07.2018- 09.2018

PUBLIKACJE WCHODZĄCE W SKŁAD CYKLU STANOWIĄCEGO ROZPRAWĘ DOKTORSKĄ

PUBLIKACJA P1

applied sciences

MDPI

Article

The Novel Quantitative Assay for Measuring the Antibiofilm Activity of Volatile Compounds (AntiBioVol)

Malwina Brożyna [1], Anna Żywicka [2], Karol Fijałkowski [2], Damian Gorczyca [3], Monika Oleksy-Wawrzyniak [1], Karolina Dydak [1], Paweł Migdał [4,5], Bartlomiej Dudek [6,7], Marzenna Bartoszewicz [1] and Adam Junka [1,7,*]

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Abstract: Herein, we present a new test, dubbed AntiBioVol, to be used for the quantitative evaluation of antibiofilm activity of volatile compounds in vitro. AntiBioVol is performed in two 24-well plates using a basic microbiological laboratory equipment. To demonstrate AntiBioVol usability, we have scrutinized the activity of volatilized eucalyptus, tea tree, thyme essential oils, and ethanol (used for method suitability testing) against biofilms of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. We have also compared AntiBioVol with the standard disc volatilization method, placing a special stress on evaluating the impact of various technical parameters on the outcomes of the latter method. The obtained results indicate that AntiBioVol allows analyzing the antibiofilm activity of volatile compounds in a high number of repeats and provides semi-quantitative or quantitative results of high repeatability. In comparison to disc volatilization, AntiBioVol is a more space- and cost-effective method that allows analyzing various types of microbial aggregates. Moreover, we have indicated that the possible reasons for the discrepancies in the results obtained by means of the standard disc volatilization method may be related to various parameters of the testing dishes used (height, volume, diameter) and to various volumes of the agar medium applied. In turn, the application of a 24-well plate and a strictly defined AntiBioVol protocol provide a higher control of experimental conditions. Therefore, the application of AntiBioVol may enable an optimization of and introduction of volatile compounds to the fight against infective biofilms.

Keywords: antibiofilm activity; volatile compounds; biofilm; essential oils; quantitative measurements

1. Introduction

Biofilm is a diversified and adaptive community of microbial cells that displays a high tolerance to conventional antimicrobials (antibiotics, antiseptics, disinfectants) due to the presence of a protective extracellular matrix, diversification of metabolism within specific biofilm layers, and coordinated reactivity to stimuli [1]. High tolerance to stressors and antibiotic resistance mechanisms displayed by microbial cells within the biofilm make the structure highly persistent and are the reasons why science has recognized the biofilm's central role in the pathogenesis of infective diseases [2–4]. Such opportunistic pathogens as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* are particularly frequently associated with serious biofilm-related infections and strong antibiotic resistance [5]. The health complications resulting from infections caused by the above-mentioned pathogens are of severe and life-threatening nature. Moreover, they are also a significant economic burden for healthcare systems [6].

Therefore, the introduction of novel antibiofilm countermeasures is one of the most pressing needs of contemporary medicine [7]. The use of such antimicrobial substances of plant origin as essential oils (EOs) in biofilm eradication is considered a promising direction to follow [8]. Thanks to their high lipophilicity, EOs bind to and break the integrity of microbial cell walls and membrane structures, which results in cell lysis through a mechanism resembling the one displayed by antiseptics [9]. Moreover, individual EOs are characterized by low toxicity, a broad spectrum of effectiveness, biodegradability, as well as anti-inflammatory and immune-stimulating properties [10]. The above-mentioned features also predispose EOs to be used either in monotherapy or as adjuvant substances for antibiotics [11]. We have chosen the essential oils of confirmed biological activity, including eucalyptus oil (*Eucalyptus globulus* Labill.), which exhibits antibacterial, antifungal, analgesic, and anti-inflammatory properties and has also been widely used in pharmaceutical, food, and cosmetics products [12]; thyme oil (*Thymus vulgaris* L.), which is known for its anti-inflammatory and antibacterial properties [13], and tea tree oil (*Melaleuca alternifolia* Cheel.), which is employed largely for its antimicrobial properties and incorporated as an active ingredient in many topical formulations used to treat cutaneous infections [14].

Both liquid and volatile fractions of EOs may display antimicrobial (and antibiofilm) activity. However, while the testing methods for EOs liquid fractions rely on well-defined EUCAST (European Committee for Antimicrobial Susceptibility Testing) and pharmacopeial recommendations [15,16], the methodology of testing EOs' volatile activity against microbes is highly diversified. These differences concern not only the experimental setting itself but also the various types of microbial aggregates scrutinized (lawn, biofilm) [17–19]. Moreover, also, the EOs are highly intra-species differentiated (with regard to the composition and concentration of antimicrobial compounds) caused by geographic and location factors, seasonal effects, and genetic factors, which determine the so-called chemotypes commonly found in EO-bearing plant species [20]. The above may explain why various scientific studies of the activity of the same type of EO against the same microorganism obtained using a single testing method produce varying data [21,22]. Such a lack of standardization impedes the introduction of EOs to the ensemble of clinical, anti-infective measures. Since there is an urgent need for new "anti-biofilm" compounds, the development of an in vitro test for screening the antibiofilm activity of volatile fractions of EOs is of high importance.

The main aim of the current study was to improve the consistency of EO analysis and to design a sensitive, cost-effective, and easy-to-perform test of antibiofilm activity of volatile compounds (later abbreviated as AntiBioVol). We also wanted to evaluate the AntiBioVol usability to test the aforementioned EOs' activity against biofilms formed by *S. aureus*, *P. aeruginosa*, and *C. albicans*. AntiBioVol was also compared with the standard disc volatilization method.

2. Materials and Methods

2.1. Microorganisms and Essential Oils

1. For experimental purposes, the following reference strains from the American Type Culture Collection (ATCC) were applied: *S. aureus* 6538, *P. aeruginosa* 15442, and *C. albicans* 10321.
2. The EOs chosen for the experiment purposes were as follows:

    - Eucalyptus oil (*Eucalyptus globulus* Labill.), (PharmaTech, Poland), later referred to as E-EO;
    - Thyme oil (*Thymus vulgaris* L.), (Etja, Poland), later referred to as T-EO;
    - Tea tree oil (*Melaleuca alternifolia* Cheel.), (PharmaTech, Poland), later referred to as TT-EO.
    - The above-mentioned EOs were chosen for experimental purposes because of their confirmed antimicrobial activity. This fact allows comparing the results presented in this work with the results of other research teams.

3. Gas Chromatography-Mass Spectrometry Analysis of the Tested EOs Composition

TT-EOs, T-EOs, and E-EOs were diluted with hexane (JTB, Great Britain), vortexed, and immediately analyzed. The analysis was performed using the Agilent 7890B GC system coupled with the 7000GC/TQ system connected to PAL RSI85 autosampler (Agilent Technologies, Palo Alto, CA, USA). The column used was HP-5 MS; 30 m × 0.25 mm × 0.25 μm (J & W, Agilent Technologies, Palo Alto, CA, USA) with helium as a carrier gas at a total flow of 1 mL/min. Chromatographic conditions were as follows: split injection at a ratio of 100:1, the injector was set at 250 °C, the oven temperature program was 50 °C held for 1 min, then 4 °C/min up to 130 °C, 10 °C/min to 280 °C and then isothermal for 2 min. The MS detector operated in the electronic impact ionization mode at 70 eV. The transfer line, source, and quadrupole temperatures were set at 320, 230, and 150 °C, respectively. Masses were registered in the range from 30 to 400 m/z. The peaks were identified in the MassHunter Workstation Software Version B.08.00 coupled with the NIST17 mass spectra library and accomplished by a comparison with linear retention indexes. The relative abundance of each EO constituent was expressed as a percentage content based on the peak area normalization. All analyses were performed in triplicate.

2.2. Antibiofilm Activity of Volatile Fraction Test (AntiBioVol) Test Performance

The general principles of the AntiBioVol test are presented in Figure 1, while a photographic presentation of the AntiBioVol test performance is presented in Figure S2.

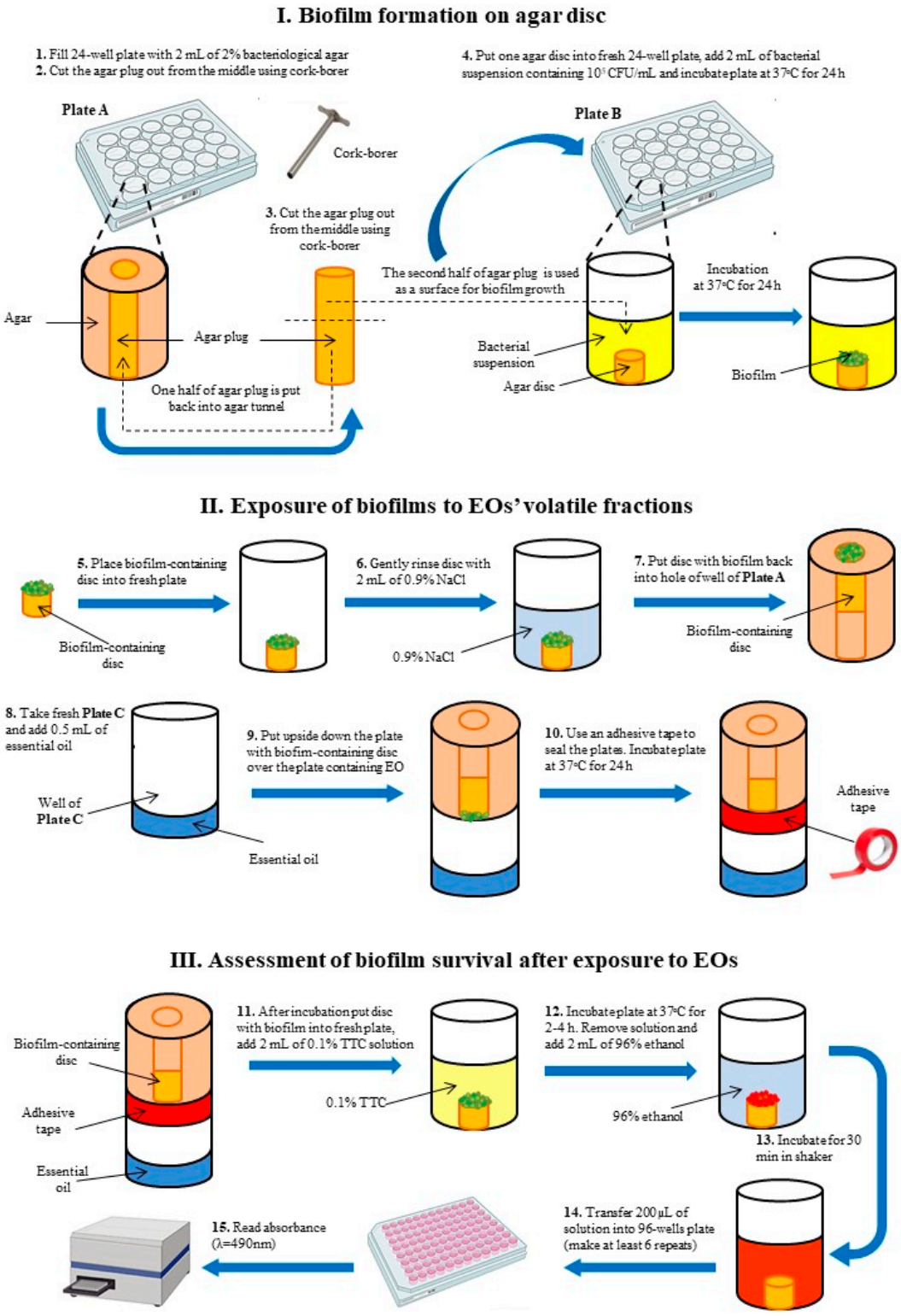


Figure 1. Schematic diagram of AntiBioVol test performance. For picture clarity, the version using the semi-quantitative method of biofilm assessment is presented. For a version using the direct quantitative method (colony forming units counting), please refer to Section 2.2.4 of Materials and Methods. I–III: the main operating blocks of experiment performance.

2.2.1. Preparation of Agar Plugs

First, 2.5 mL of 2% Brain Heart Infusion (BHI, Graso, Poland) agar was poured into the wells of a 24-well plate (BioFil, Warsaw, Poland). The plate was left in a sterile chamber until the agar has solidified (this plate is further referred to as Plate A, where "A" stands for "Agar"). Next, the agar plugs were cut out using a cork-borer (ChemLand, Warsaw, Poland) with a diameter of 8 mm. Subsequently, the plugs were cut evenly crosswise into two discs. One disc was placed in a well of a new 24-well plate (later referred to as Plate B, where "B" stands for "Biofilm") and another disc was placed back to the agar-containing well of Plate A (Figure 1, Part I). The latter plate was sealed with an adhesive tape and kept refrigerated until further analysis.

2.2.2. Biofilm Formation on Agar Disc in Plate B

Using a densitometer (Densitomat II, BioMerieux, Poland), the analyzed strain's suspension of 0.5 McFarland (MF) density in Tryptic Soya Broth (TSB, Graso Biotech, Poland) medium was obtained and subsequently diluted (1500x for bacteria and 14x for fungi) to obtain approximately 10^5 Colony Forming Units (CFU)/mL. In the next step, 2 mL of the strain's suspension was added to Plate B wells containing the agar disc. Plate B was incubated for 24 h at 37 °C. After incubation, the discs were taken gently out using tweezers (Conbest, Warsaw, Poland) so as not to disrupt the agar surface and the biofilm structure on it. Next, the discs were rinsed twice with 0.9% saline (Stanlab, Wroclaw, Poland) to remove non-adhered microorganisms. The discs were subsequently placed in the agar wells of Plate A on top of the sterile discs to fill the hole of the well (Figure 1, Part II).

2.2.3. Exposure of Biofilms to EOs' Volatile Fractions

First, 0.5 mL of the tested EO was added to a well of a fresh 24-well plate, which is later referred to as Plate C (EO-Containing plate). Then, Plate A with biofilm-containing agar discs was put upside down on Plate C in such a manner that the EO-containing well was placed directly under the biofilm-containing well. Next, the rims of both plates were taped around using an adhesive tape (Diall, Poland) and incubated for 24 h at 37 °C (Figure 1, Part II).

Control Settings

To check whether the applied experimental setting itself had no potentially impeding effect on the microorganisms' growth, 0.9% saline was applied instead of EOs. Thus, the aforementioned setting served as a control of the microorganism's growth (positive control, latter abbreviated as "C+"). To confirm the method's suitability, 96% ethanol (Stanlab, Wroclaw, Poland), whose volatile form has a well-recognized antimicrobial activity, was applied. The above-mentioned control samples were performed in separate plates. Each experimental and control setting was tested in six replicates.

2.2.4. Assessment of Bacterial Biofilm Survival after Exposure to EOs

Following the exposure described in Section 2.2.3 of Materials and Methods, bacterial biofilm-containing discs were transferred to fresh wells of a 24-well plate. Next, 2 mL of 0.1% tetrazolium chloride solution (2,3,5-triphenyl-2H-tetrazolium chloride, TTC) (PanReac AppliChem, Darmstadt, Germany) was introduced gently in order to not de-attach the formed biofilm from the disc surface. The plates were incubated for 1.5 h at 37 °C. TTC turns into red formazan in the presence of metabolically active microorganisms. Next, the TTC-containing medium was removed, and 2 mL of 96% ethanol was introduced to the wells to extract red formazan crystals out of the biofilm-forming cells. Subsequently, the plates were incubated for 30 min at room temperature in a microplate shaker (Schuttler MTS-4, IKA, Germany) at a speed of 300 rpm/min. After this time, 200 µL of formazan-containing solution was transferred to the wells of a 96-well plate (Biofil, Warsaw. Poland). Absorbance was measured at 490 nm using a MultiScan Go spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Then, the values of absorbance measured for formazan in the samples

of EO-treated biofilms were compared to the corresponding values obtained for biofilms incubated with 0.9% saline (Figure 1, Part III). In order to confirm the linear relationship between the values from TTC assay and the number of CFU, standard calibration curves were performed. A calibration curve for absorbance measurements at 490 nm versus the number of CFU has been established before the experiment (Figure S3).

2.2.4.1. Assessment of Fungal Biofilm Survival after Exposure to EOs

Since the use of tetrazolium salt-based assays in studies of *Candida* biofilm has significant limitations [23], we used this fact as an opportunity to present the possibility of direct quantitative assessment within the AntiBioVol setting. Following the exposure described in Section 2.2.3 of Materials and Methods, fungal biofilm-containing discs were transferred to 1 mL of 0.5% mild detergent saponin solution (Merck, KenilWorth, NJ, Germany) and subjected to vigorous vortex-shaking for 1 min to de-attach the biofilm-forming cells from the agar surface. Next, serial dilutions of the obtained suspension were cultured on the Sabouraud agar plates (Graso, Poland) and incubated for 24 h at 37 °C. The CFU number was counted on the next day.

2.2.5. Application of the AntiBioVol Test for Various Concentrations of EOs in Single vs. Separate Test Plates

The aim of this experimental setting was to investigate whether a liquid EO introduced to a single 24-well plate (to which other concentrations of the same type of liquid EO were also introduced) may get across, after volatilization, to another test well. We hypothesized that if such phenomenon occurs, we would observe a higher biofilm eradication in the well in which a lower EO concentration was applied in comparison to the setting in which only one EO concentration in a single test plate was used. To check it, two concentrations of TT-EO (25%, 50%) *v/v* diluted in polyethylene glycol (Pol-Aura, Zabrze, Poland) and an un-diluted TT-EO (100% *v/v*) were applied in a single AntiBioVol setting against *S. aureus* biofilm.

Then, 25%, 50%, 100% TT-EO concentrations were introduced to a 24-well plate in such a manner that the 6 wells containing 100% TT-EO were placed between the 6 wells containing the 25% concentration and between the 6 wells containing the 50% concentration of the TT-EO [Figure S3]. Additionally, the same experiments were performed in separate AntiBioVol settings (single plate/single EO concentration). The remaining steps of biofilm exposure and further quantification were performed according to the procedures presented in Section 2.2.3, Section 2.2.4, and Section 2.2.4.1 of Materials and Methods. The additional set of controls applied in this experiment included the use of EOs' solvent—50% and 75% (*v/v*) of polyethylene glycol (PEG); this control set was performed in a separate plate for *S. aureus* as the microorganism tested.

2.3. Additional Control Experiments

This part of the manuscript is deliberately presented at the end of this section because the techniques presented below were not part of the standard AntiBioVol test, and they were performed to provide additional data confirming the correctness of the AntiBioVol setting.

2.3.1. Assessment of Antimicrobial Activity of Liquid and Volatile EO Fractions Using Standard Methods

To compare AntiBioVol with the methods previously developed for the assessment of antimicrobial activity of liquid EO fractions, we used the disc diffusion method [24], while to assess the activity of volatile fractions, we applied the inverted Petri plate method (also referred to as the Disc Volatilization Method [25]).

This part of the experiment was performed as a proof of concept for *S. aureus* biofilm vs. T-EO, because the activity of this oil was the highest against this particular pathogen, and it could be hypothesized that positive outcomes (with regard to the halo zone) might also be obtained.

In the case of the disc diffusion method, microbial suspension at 0.5 MF density was cultured on a Muller–Hinton (BioCorp, Warsaw, Poland) agar plate (diameter of 9 cm, height of 1.4 cm). Next, standard paper discs (6 mm in diameter, thickness of 0.5 mm) saturated with EOs were placed on the agar plates (one disc per plate). Then, the plates were incubated for 24 h at 37 °C. After incubation, microbial growth inhibition zones were measured (in mm) using a ruler (Leniar, Krakow, Poland).

In the inverted Petri plate method, paper discs saturated with EOs were placed on the inside of Petri-dish lids (one disc per plate). Next, the lids were placed on the base of the dish containing microbial-seeded agar. Subsequently, the whole dishes (lids and agar-containing bases) were incubated in such a manner that the lids were lying on the incubator shelf. Such a setting prevented EO-saturated discs and EO droplets from falling down on the microbial lawn formed on the agar. Inhibition of growth (if occurred) was visible as zones of reduced microbial growth and was measured using a ruler. Moreover, in order to evaluate the correlation between the parameters relating to Petri dish sizes and the antimicrobial activity of the EOs applied, this version of the experiment was performed on Petri dishes 15, 9, and 6 cm in diameter; plate height was equal to 2.5 cm (for plates 15 cm in diameter) and 1.4 cm (for plates 6 and 9 cm in diameter); poured agar height was 1.5, 0.5, and 0.3 cm resulting in agar volume equal to 100, 20, and 10 mL, respectively. When the test involved dishes 9 cm in diameter specifically, the range of agar volume was 30, 20, and 10 mm.

2.3.2. Scanning Electron Microscopy Analysis of the Strains' Biofilm-Forming Ability in the Applied In Vitro Setting

The aim of this procedure was to confirm the microorganisms' ability to form biofilm on the agar surface. Fresh, 24-h liquid cultures of *S. aureus*, *P. aeruginosa,* and *C. albicans* strains were diluted to 10^5 CFU/mL. Next, 2 mL of such a suspension was introduced to the wells of a 24-well plate containing agar discs, which was prepared as described in Section 2.2.1 of Materials and Methods of this manuscript. The plates with the discs and microbial suspensions were incubated for 24 h at 37 °C. Subsequently, the discs were carefully taken out and rinsed twice with 0.9% saline to remove non-adhered microorganisms. Next, the agar plugs were fixed by immersion in 2% glutarate (ChemPur, Piekary, Poland) for 4 h at 4 °C. After incubation, the samples were rinsed three times (for 2 min) with distilled water to remove the fixative. Subsequently, 10 min dehydration for each of the following ethanol concentrations (10%, 25%, 50%, 70%, 80%, 90%) was performed; the last dehydration lasted for 15 min and was performed in 100% EtOH. After dehydration, the ethanol was removed and the samples were dried at 37 °C. Then, the biofilm-containing agar discs were sputtered with Au:Pd mixture using a sputter device (Quorum International, Fort Worth, TX, USA) and examined using a scanning electron microscope (Auriga 60, ZEISS, Germany).

2.3.3. Evaluation of Minimal Inhibitory and Minimal Biofilm Eradication Concentrations of Liquid EOs

MIC assessment was prepared in 96-well titration microplates. The wells of the plate were filled with 100 µL of TSB medium. Next, 100 µL of undiluted EO was added to the first of the wells and mixed with the medium. Subsequently, geometric dilutions of the EOs in TSB were performed. Next, 100 µL of the bacterial suspension (10^5 CFU/mL) was introduced to each well containing different concentrations of the EOs. The plate, wrapped with adhesive tape, was incubated for 24 h at 37 °C in a microplate shaker. The culture with only the medium added served as a positive control of the microorganisms' growth, while the well containing only the medium served as the sterility control of the experiment. After incubation, 5 µL of TTC was added to each well and incubated for 5 h at 37 °C. The EO concentration in the first colorless well, neighboring the red well, was taken as the MIC value.

The minimum biofilm eradication concentration was estimated in a manner similar to the MIC analysis. Briefly, 100 µL of the microbial suspension (10^5 CFU/mL) was introduced to each well and incubated for 24 h at 37 °C without shaking. Next, the whole medium (containing non-adhered microorganisms) was removed, leaving only biofilm-forming organisms attached to the bottom of the

96-well plate. Subsequently, geometric dilutions of EOs in the medium were applied to the wells and left for another 24 h at 37 °C. The following procedures containing TTC introduction and subsequent analysis were performed similarly to the ones described for MIC assessment. In the case of *C. albicans*, due to the aforementioned limitations of tetrazolium salt-based assays [23], a different check of cell viability was performed. Namely, fungus-containing suspensions were removed from the 96-well plates and spotted on stable Sabouraud agar plates (dedicated for fungi growth and cultivation) and incubated for 24 h. The presence of living colonies on the agar indicated the lack of viable cells, while the absence of colonies in the place where spotting was performed confirmed the survival of *Candida* cells.

3. Statistical Analysis

Calculations were performed using the GraphPad Prism (version 7) software. Normality distribution was calculated by means of D'Agostino–Pearson omnibus test. Since all values were non-normally distributed, the Kruskal–Wallis test with post-hoc Tukey's analysis was applied. The results of statistical analyses were considered significant if they produced p-values < 0.05.

4. Results

In the first line of the investigation, we have scrutinized the E-, T-, and TT-EOs with regard to their content of antimicrobial substances. The GC-MS analysis confirmed the presence of specific active compounds within E-EO (including 1,8-cineole and γ—terpinene), T-EO (including thymol and *p*-cymene), and TT-EO (including γ—terpinene and terpinen-4-ol). The full list of active compounds identified is presented in Figure S1 and Table S1. Having proven that the tested EOs contain antimicrobial substances, we have analyzed the antimicrobial and antibiofilm activity of EOs' liquid fractions against microorganisms in planktonic and biofilm forms using the microdilution method. The rationale behind this preliminary research was the fact that the crucial EOs' components (such as thymol) display activity in both liquid and volatile fractions. Thus, the confirmation of antimicrobial activity of the liquid fraction would be a strong assumption for the potential activity of the volatile fraction. The results presented in Table S2 indicate a higher activity of all the tested liquid EOs against the applied microorganisms in planktonic than in biofilm form. Next, we analyzed the ability of *S. aureus*, *C. albicans*, and *P. aeruginosa* strains to form biofilm (Figure 2) on the agar surface. A confirmation of the presence of a settled, multi-cellular biofilm formed on agar was a prerequisite condition for the performance of the core part of this research, namely AntiBioVol test for the E-, T-, and TT-EOs.

The results of the performed AntiBioVol test suitability with the use of ethanol (which is a substance of known volatile antimicrobial activity) showed the correctness of the applied experimental setting—the number of biofilm-forming cells remaining after exposure to ethanol was lower than in the positive control of biofilm growth, regardless of the microorganism applied (Figure 3A–C). With regard to EOs, the results presented in Figure 3A–C show that none of the applied volatile fractions of EOs were able to completely eradicate bacterial or yeast biofilm, although the number of biofilm-forming cells after the exposure to all types of EOs was lower than in non-treated control settings. The volatile T-EO acted more efficiently against *S. aureus* biofilm than TT- and E-EO, while TT- and T-EO was stronger against *P. aeruginosa* biofilm than E-EO.

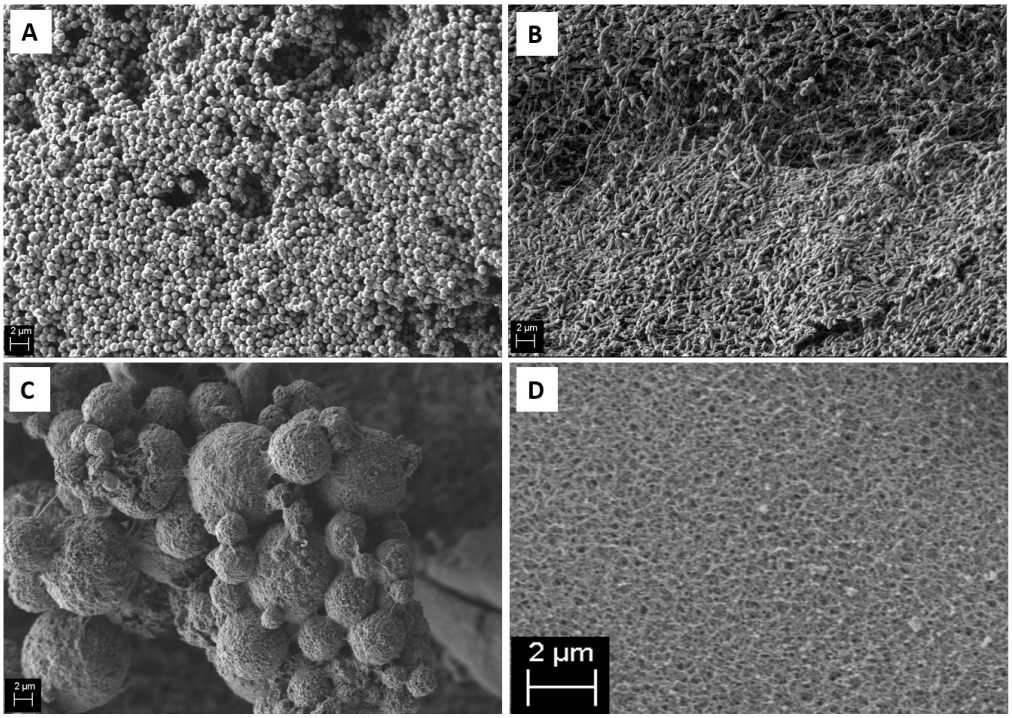


Figure 2. Confirmation of the analyzed pathogens' ability to form biofilm in vitro. (**A**)—biofilm of *S. aureus* formed on the agar surface; (**B**)—biofilm formed by *P. aeruginosa*, (**C**)—biofilm formed by *C. albicans*, (**D**)—sterile agar surface. SEM Zeiss Auriga 60, (magnification 5000x).

Next, we have measured the activity of three concentrations of TT-EO against *S. aureus* biofilm within a single measurement plate [Figure 4]. The comparison of TT-EO's antibiofilm activity in a single vs. separate settings revealed a lack of statistical significance between them (K–W test $p < 0.05$, with post-hoc Tukey's analysis) indicating no transmission of TT-EO volatile fractions between various plate wells within a single test plate. Similarly, no transmission of TT-EO solvent (PEG) was detected in the control setting (Figure S5).

Finally, we have performed the conventional assessment of EOs' volatile fraction's antimicrobial activity referred to as the inverted Petri plate method to check whether such initial technical parameters as plate diameter, plate height, poured agar volume, and agar height translate into an outcome such as the size of the microbial growth's inhibition zone (Tables 1 and 2). Moreover, the reader can find a comparison of the inverted Petri dish method and the conventional disc diffusion method (which, being referred to as the aromatogram [26], is also applied for EOs' volatile fraction activity measurement) in Figures S6 and S7.

As can be seen in Table 1, the differences between agar height obtained in Petri dishes of 15, 9, and 6 cm translate into differences in air volume between the agar surface and the lid of the dish. In the case of dishes 15 cm in diameter vs. dishes 9 and 6 cm in diameter, the aforementioned parameter differed by 75% and 83%, respectively. In turn, the difference in air volume between the 9 and 6 cm diameter dish was 31%. It translated into differences in the concentrations of volatile compound in experimental settings and finally into ambiguous results of the microbial growth inhibition zone (from 0 cm observed if a 15 cm diameter dish was applied vs. a 4 cm inhibition zone observed when a 6 cm diameter dish was applied).

When Petri dishes were of the same diameter and height but various agar volumes were poured (the difference between them was 10 mL, see Table 2), a relatively small difference in agar height was observed (2 mm). Nevertheless, it translated into 19–36% of difference in air volume between the plates and differences in inhibition zone outcomes from 0 cm (lack of volatile compound's antimicrobial activity) to 2.7 cm (detection of antimicrobial activity).

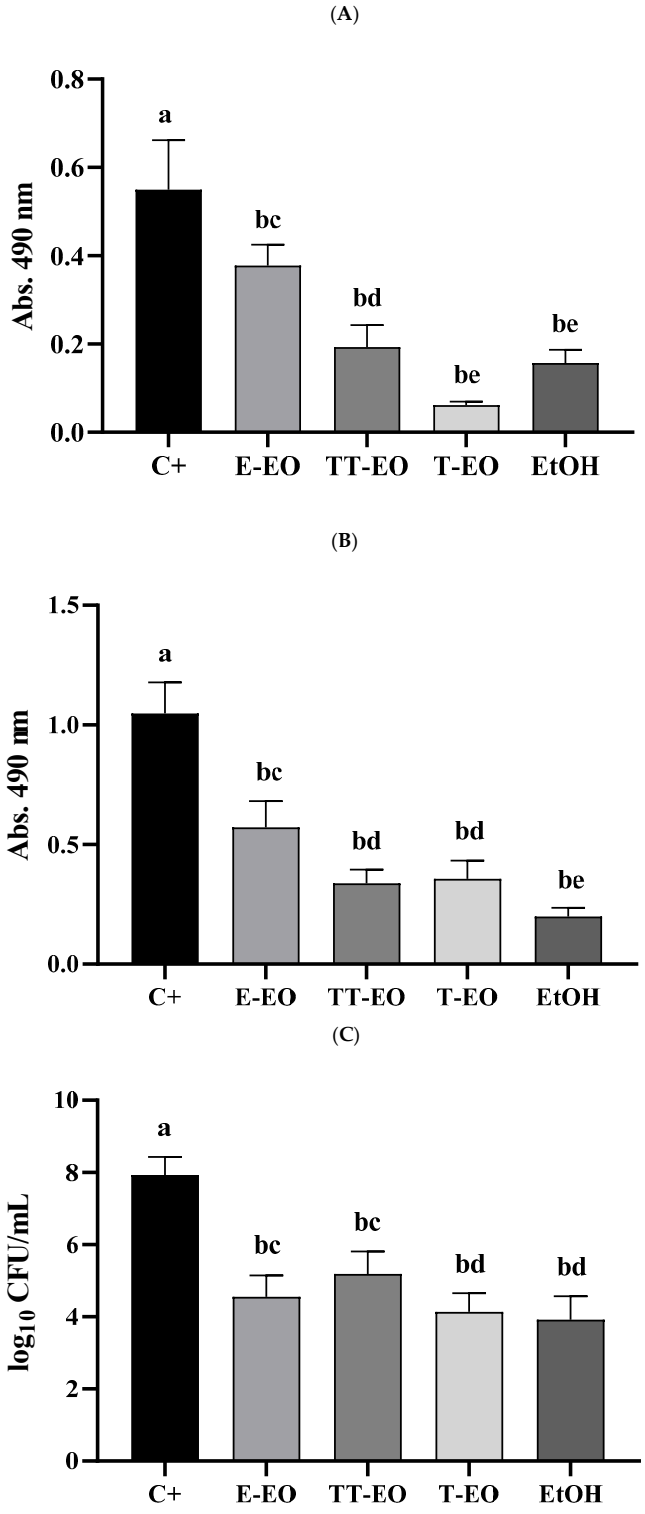


Figure 3. Antibiofilm activity of volatile fractions of essential oils (EOs) and ethanol against (**A**) *S. aureus*, (**B**) *P. aeruginosa*, and (**C**) *C. albicans* biofilm. C+—positive control of growth, i.e., biofilm treated with 0.9% saline, EtOH—suitability control setting, i.e., biofilm after treatment with ethanol fumes, E-EO—biofilm after treatment with volatile fraction of eucalyptus oil, TT-EO—biofilm after treatment with volatile fraction of tea-tree oil, T-EO—biofilm after treatment with volatile fraction of thyme oil. Values with different letters are significantly different ($p > 0.05$, K–W test, followed by Tukey's analysis): a, b—statistically significant differences between C+ and volatile fractions of EOs or ethanol; c, d, e—statistically significant differences between volatile fractions of EOs and ethanol.

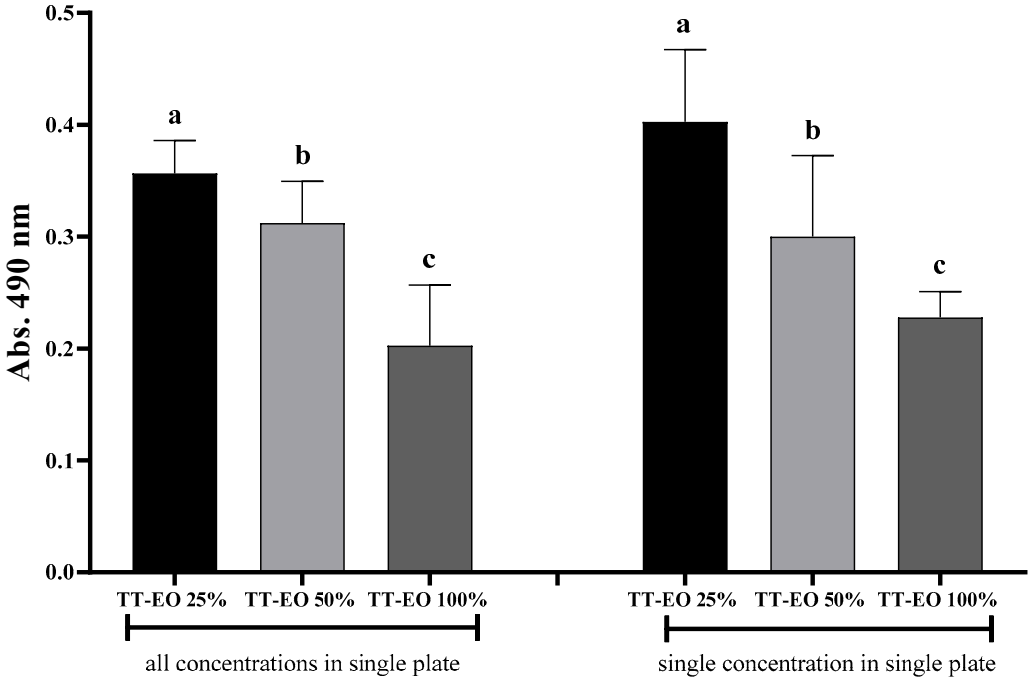


Figure 4. Comparison of antibiofilm activity of three concentrations of TT-EO against *S. aureus* biofilm. The test was performed in a single plate for three TT-EO concentrations and in separate plates for each concentration. TT-EO 25%, 50%, 100%—biofilm treated with 25%, 50% or undiluted (100%) tea-tree oil, respectively. Values with the same letters show a lack of significant difference ($p < 0.05$, K–W test, followed by Tukey's analysis) between the impact of the same concentrations of TT-EO within two experimental settings applied (all TT-EO concentrations in a single plate vs. single TT-EO concentration in a single plate).

Table 1. Dependence between Petri dish diameter and size of *S. aureus* growth inhibition zone after exposure to T-EO. Inverted Petri dish method (also known as disc volatilization method).

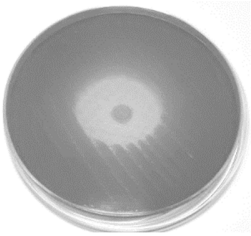
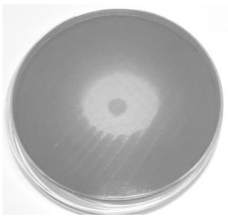
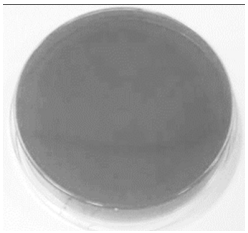
			
Plate Diameter (cm)	15.0	9.0	6.0
Plate Height (cm)	2.5	1.4	1.4
Agar Volume (mL)	100	30	10
Agar Height (cm)	1.5	0.7	0.3
Air Volume (cm^3)	177	45	31
Inhibition Zone (cm)	**0.0**	**2.7**	**4.0**

Table 2. Dependence between agar height in the Petri dish and the size of *S. aureus* growth inhibition zone after exposure to T-EO. Inverted Petri dish method (also known as disc volatilization method).

			
Plate Diameter (cm)	9.0	9.0	9.0
Plate Height (cm)	1.4	1.4	1.4
Agar Volume (mL)	30	20	10
Agar Height (cm)	0.7	0.5	0.3
Air Volume (cm^3)	45	57	70
Inhibition Zone (cm)	**2.7**	**0.5**	**0.0**

5. Discussion

The aim of this work was to develop a reliable and easy-to-perform test for the evaluation of antibiofilm activity of volatile fractions of antimicrobial compounds. We have chosen EOs as an example because, due to their plant-derived origin resulting in a variability of microbiological outcomes, EOs present a higher challenge than compounds obtained in a strictly defined process of chemical synthesis [20]. Another reason behind choosing EOs were promising reports on their antimicrobial and antibiofilm activity [9,27]. To stay in line with methodological requirements, we have also scrutinized ethanol fumes of known antimicrobial activity (usability control). Moreover, to allow other research teams to replicate our experiment, we have confirmed the presence of antimicrobial substances in the analyzed EOs and presented the results in Figure S1 and Table S1.

While the methodology for liquid EOs' antimicrobial activity is practically based on the protocol for antibiotic-susceptibility testing provided by EUCAST [15], the assessment of EOs' volatile fraction activity against settled forms of microbial aggregates is performed by means of diverse approaches, which are fraught with certain disadvantages [28–31]. Therefore, a methodology is needed allowing the performance of a high number of technical repeats within a single screening test, providing results in the form of a parametric ratio of microbial cells' eradication (and not in the form of the size of the halo zone, as is the case for the inverted Petri dish method, see Section 2.2.4.1 of Materials and Methods). Such methodology should provide standardized test conditions and the ability to analyze the impact of volatile compounds against (among others) microbial biofilm, which is basically missing in the standard test methods.

The prerequisite condition for AntiBioVol performance was to obtain a robust biofilm in in vitro conditions. As can be seen in Figure 2, the Gram-positive bacteria, Gram-negative bacteria, and the fungus formed multicellular biofilms on the agar surface. A confirmation of this fact allowed us to perform the analyses presented in Figures 3 and 4. It should be emphasized that methods simpler than Scanning Electron Microscopy can also be used to visualize biofilm presence, including dyeing with tetrazolium salts or quantitative culturing. These methods are sufficient for the above purpose, as we have indicated in our previous research [31–33]. Although we have performed an analysis of EOs' activity specifically against biofilms, the AntiBioVol methodology (thanks to using agar discs) also enables testing such surface-attached cellular aggregates as microbial lawns or even single agar colonies, unlike standard Petri dish-based methods, where only planktonic cells seeded on agar are used.

The results presented in Figure 3 indicate that the application of AntiBioVol translates into low standard errors of mean (expressed as box whiskers in Figure 3) in outcomes of technical repeats. The number of *S. aureus* biofilm-forming cells of the growth control differed maximally by 27.6%

between six biofilm samples used to produce the average value presented in Figure 3A. The difference in outcomes measured for four out of these six samples did not exceed 8% (please refer to the corresponding raw data of the experiment in Tables S3 and S4). One of the reasons behind these low deviations in outcomes is the fact that the AntiBioVol test is performed not in Petri dishes (as in standard methods) but in 24-well plates. This simple change allows performing 24 assays and obtaining 24 outcomes using only one test plate, while a similar assessment performed by means of the inverted Petri dish method requires 24 vessels. The high number of easy-to-achieve technical repeats gained owing to the AntiBioVol test correlates positively with the quality of data obtained and processed during post-laboratory statistical calculations. This is of paramount importance with regard to biofilm studies, where high deviations in outcomes is a common phenomenon hindering the obtaining of statistically significant results [34].

The above-mentioned reduction of experimental setting size observed for AntiBioVol vs. various Petri dish-based methods translates also into significant space savings in the microbiological incubator [Figure 5] and into materials' cost savings.

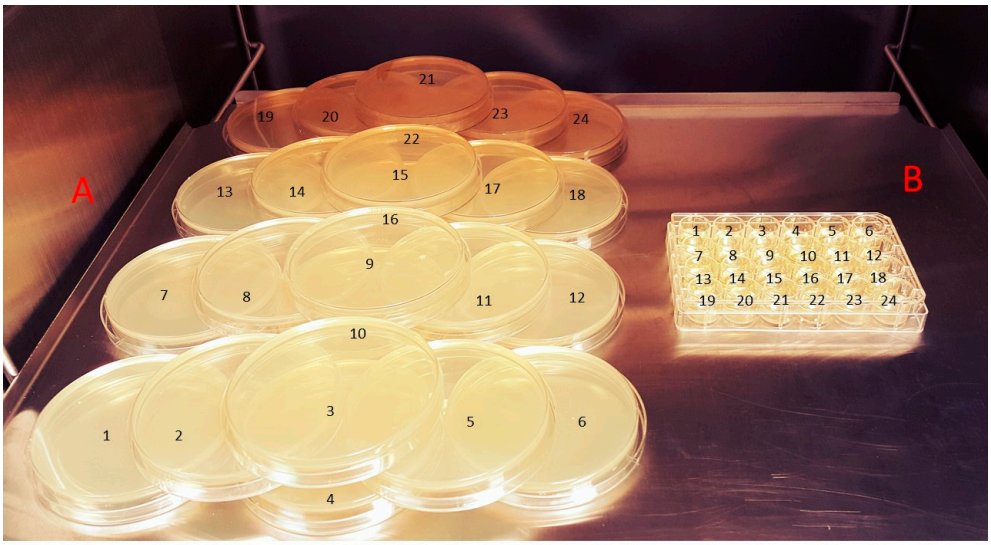


Figure 5. Comparison of place and resource consumption for the conventional Petri dish method of EOs testing (**A**) and the AntiBioVol (**B**) test.

One of the key challenges of in vitro biofilm testing is its low reproducibility of results due to aggressive pipette-based washing. It frequently causes a random de-attachment of large amounts of biofilm [35]. Therefore, in our test, we put special stress on the careful performance of the procedures concerning agar discs rinsing, washing, and transferring (please refer to Materials and Method Sections 2.2.2 and 2.2.4). Moreover, we have deliberately chosen agar discs in the AntiBioVol test as a surface for biofilm growth. While a majority of other in vitro biofilm-oriented tests apply various types of polished polystyrene/polypropylene inserts or plugs for this purpose [36], we hypothesized that a porous agar surface used in the AntiBioVol test may be more appropriate for enabling the cells' adhesion. The correlation between higher biofilm adherence and surface porosity has already been demonstrated by other research teams [37]. Indeed, as shown in Figure 6, the microorganisms used agar pores to anchor to the surface.

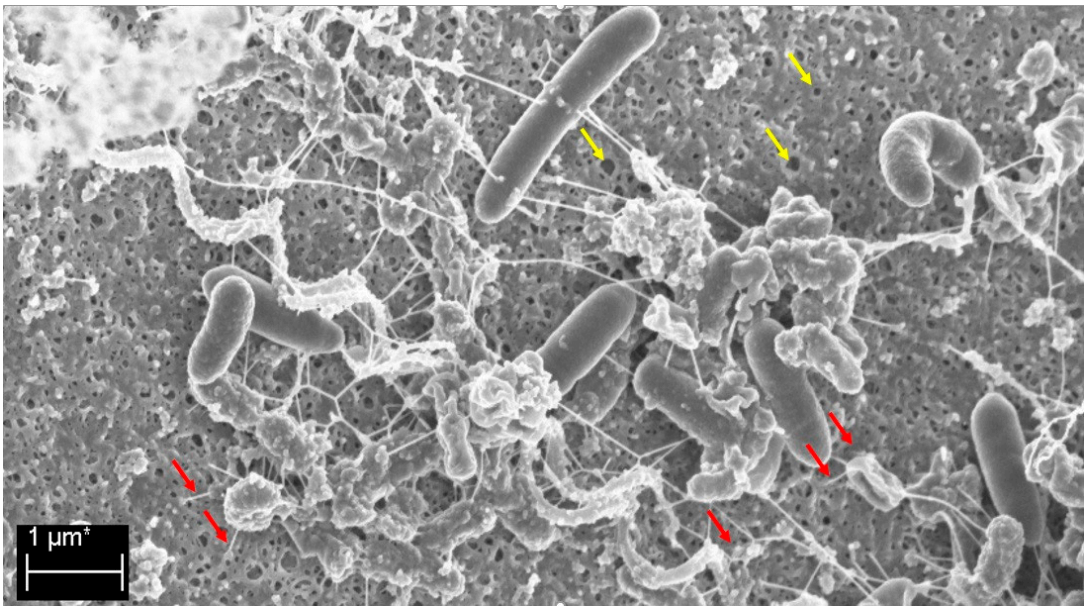


Figure 6. Cells of *P. aeruginosa* attached to agar surface (magnification 5000x). Yellow arrows indicate pores within agar facilitating the adhesion process; red arrows indicate the cells' adhesive structures anchored to the pores in the agar. SEM Zeiss Auriga 60.

Another issue related to the testing of volatile compounds activity against microorganisms is the possibility of obtaining biased results due to the natural tendency of fumes to spread in the air and to get across from one experimental setting to another. To prevent the passing of volatile EOs from the plates to the microbiological incubator, we have wrapped the two AntiBioVol plates up tightly (using an adhesive tape) (A and C) (Figure S2, II). After 24 h of AntiBioVol test incubation, no EO scent was organoleptically detected in the incubator. The aforementioned wrapping resulted in another beneficial effect, i.e., the relevant wells of plate A and C were placed directly one over another (Figure 1, Part III). We hypothesized that such a setup would prevent EO volatile fractions placed in a specific well from reaching another well and from cross-reacting with other biofilm-covered agar discs. To test this hypothesis, we have introduced three different concentrations (25%, 50%, and 100%) of TT-EO to a single AntiBioVol setting and checked whether the obtained results of biofilm reduction differed from the results obtained in settings where each of the aforementioned TT-EO concentrations was tested in separate plates (for TT-EO concentration distribution, please refer to Figure S4). Moreover, we have confirmed that EO solvent does not affect the microorganisms' viability [Figure S5]. As shown in Figure 4, the differences in these two set-ups with regard to the level of biofilm reduction were statistically insignificant (K–W test, $p > 0.05$). Therefore, it can be concluded that AntiBioVol allows testing various concentrations of volatile compounds within a single experimental setting. It should be pointed out that the issue of a possible interference of various volatile compounds in a single experimental set-up, resulting in a potentially biased outcome, is often neglected in other in vitro tests. For example, in a test referred to as the aromatogram, not only various concentrations, but also various types of EOs are analyzed in a single Petri dish [38,39]. Although the aromatogram is designed basically to assess the antimicrobial activity of liquid fractions of EOs, one should be aware that during incubation lasting for 24 h at 37 °C, also volatile fractions of EOs will be released and will interact with the microbial cells. Thus, the observed outcome of the aromatogram (expressed as the halo zone) would be an effect of a combined activity of liquid EO fractions and mixed-up plethora of volatile compounds. Therefore, a general principle with regard to standard Petri dish-based tests should be to apply a single type of EO, in a single concentration, per one Petri dish. This statement significantly supports the considerations concerning the advantage of AntiBioVol vs. Petri dish-based tests in the aforementioned context of space savings in the microbiological incubator.

As already mentioned above, the variety of Petri dish-based assays on antimicrobial testing translates into inconsistent results reported in the literature. For example, Tyagi et al. indicated that E-EO vapors are effective against *P. aeruginosa* [21], while Kloucek et al. [22] reported that the volatile E-EO had no activity against this pathogen. The standard disc volatilization method, and the modification of this method, where one Petri dish was divided into four parts, were applied by the first and the second team, respectively. Chao et al. [40] observed a reduction in the viability of *P. aeruginosa* cells following exposure to TT-EO, while the study performed by Lopez et al. showed no effect of volatile TT-EO against *P. aeruginosa* [28]. Among other methods, both teams applied disc-diffusion methods. These opposite outcomes concerning EOs activity may not only be a result of variances in EOs' composition and various testing methods; they may be a consequence of differences in such seemingly trivial technical parameters as Petri dish diameter and the height of agar poured. These parameters correlate with the volume of air between the agar surface and the lid, translating into the concentration of volatilized compound inside the dish, and finally into the obtained size of the microbial growth inhibition zone. It should be noted that commercially available Petri dishes of the same diameter often vary in height (from 14 through 16 to 20 mm [41–43], most typically), and this difference has a substantial impact on the air volume within them and on the final concentration of the applied volatilized compound.

To investigate the relationship between the above parameters and the obtained halo zone size, we have performed a disc volatilization method using Petri dishes of various diameters, agar volumes, and heights (Table 1) or of the same diameter but of various agar volumes (Table 2). As can be seen in Table 1, along with the decrease in air volume, the final outcome (halo zone) increased. It is worthwhile to note that the outcomes obtained for dishes of 9 vs. 15 cm were opposite (confirmation of antimicrobial activity vs. lack of antimicrobial activity, respectively). Moreover, a similar discrepancy of results was observed if Petri dishes of the same diameter but of various agar volumes were scrutinized (Table 2). Agar heights differed from each other by 2 mm only, which may easily be overlooked during the test performance. In conclusion, such technicalities as dish height (or dish diameter) contributing to the final parameter (halo zone) are frequently not reported in the methods sections of manuscripts [44–46], and we believe it may be one of the reasons for the observed result discrepancies. The above-presented consideration explicitly indicates the need to standardize the test, which the AntiBioVol setting offers. The differences in the diameter to height ratio (which serves to calculate air volume) in the wells of the 24-well plate used in the AntiBioVol test are too faint to have a substantial impact on the outcome, and hence at least one important factor of variability is eliminated. Moreover, we have explicitly indicated the volume of agar needed to be poured to the well (2.5 mL, please refer to Section 2.2.1 of Materials and Methods).

Although the AntiBioVol test provides many advantages as compared to standard Petri dish tests, we are aware of certain limitations of our methodology. First of all, AntiBioVol is not designed to analyze anaerobic microorganisms, which are a frequent etiological factor of oral, bone, and chronic wound infections [47]. An adaptation of the AntiBioVol methodology for the analysis of anaerobic biofilm would require an implementation of expensive anaerobic chambers and nucleic acid sequencing methods, significantly increasing the time and cost of the procedure. Secondly, the biofilms cultured in laboratory conditions, as described in Section 2.2.2 of Materials and Methods, do not fully resemble the ones infecting a patient's body in terms of cell number, matrix composition, and dimensional structures [48]. Therefore, one should be careful with translating the results obtained by means of AntiBioVol into clinical conclusions. Thirdly, the basic AntiBioVol setting relies on a TTC-based assay whose sensitivity differs depending on the specific microbial strain applied (and its ability to reduce tetrazolium salts). However, as we have shown in Figure S3, although the TTC test is able to detect from 10 to up 10^{10} microbial cells, the performance of additional tests to evaluate a strain's ability to metabolize TTC may be required, especially for strains able to switch their metabolism from aerobic to anaerobic. Another limitation of our study, strictly related to the previous one, is the fact that microorganisms form various types of biofilm (as regards cell number and yield of the exopolymeric

matrix) in various types of culturing media [49]. This fact makes us lean toward a conclusion that a more labor-consuming version of AntiBioVol (in which quantitative culturing (QC) is performed, please refer to Figure 3C) would be of higher usability than a quick, TTC-based version of AntiBioVol (please refer to Figure 3A,B). In such case, a plausible solution would be to use the TTC-based version of AntiBioVol for the rapid screening of a high number of strains and, subsequently, the QC-based version of AntiBioVol for further analyses of selected strains. Thus, the AntiBioVol methodology and study is of preliminary character and should be investigated further.

Despite the above-mentioned limitations, the AntiBioVol test set provides a powerful tool for a consisted, rapid analysis of already used or newly designed volatile antimicrobials of known or suspected anti-biofilm activity. Bearing in mind the fact that biofilm often exists in body sites hardly reached by liquid or stable medicinal products, the application of EOs and the data provided by AntiBioVol on their efficacy may be of importance for not only basic but also clinical studies on the eradication of biofilm formed in the pathogenesis of infections.

6. Conclusions

In this paper, the protocol for a test we dubbed AntiBioVol is provided. Furthermore, the antibiofilm activity of volatilized EOs against pathogenic biofilm was scrutinized in vitro using the AntiBioVol methodology. This method allowed a high number of repeats to be performed in a space- and cost-efficient manner. AntiBioVol has been compared to the standard Petri dish-based test. Not only do the results obtained by AntiBioVol display low standard deviations but also the use of resources is lower in comparison to standard tests. No expensive/advanced equipment is required for AntiBioVol performance. The application of AntiBioVol facilitates and improves the assessment of the effectiveness of antibiofilm volatile compounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/20/7343/s1, Figure S1: GC-MS chromatograms of main EO ingredients. E-EO—eucalyptus oil; TT-EO—tea tree oil; T-EO—thyme oil. Results are presented as percentage content based on the peak area normalization. Figure S2: Photographic presentation of AntiBioVol test performance. Figure S3: Calibration curves for absorbance measurements at 490 nm (TTC assay) versus number of log10 colony-forming units (CFU)/mL [A] S. aureus, [B] *P. aeruginosa*. Figure S4: Distribution of 3 TT-EO's concentrations in a single test plate. TT-EO—tea tree oil; 25%, 50%, 100%: applied concentrations [*v/v*] of TT-EO. Figure S5: Lack of PEG antimicrobial activity against *S. aureus* in the AntiBioVol experimental setting. C+—positive control; 50%, 25% PEG—concentrations (*v/v*) of polyethylene glycol. No significant difference ($p < 0.05$, K–W test, followed by Tukey's analysis) between control and PEGs was detected with relation to S. aureus viability. Figure S6: Antibacterial activity of thyme essential oil liquid against S. aureus determined by the disc diffusion method (A) and inverted Petri plate method (B). Figure S7: Antibacterial activity of thyme essential oil liquid against *P. areuginosa* determined by the disc diffusion method (A) and inverted Petri plate method (B). Table S1: Composition of main ingredients of tested EOs. E-EO—eucalyptus essential oil; TT-EO—tea tree essential oil; T-EO—thyme essential oil; RI—retention index; RT—retention time. Table S2: Minimal Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) of eucalyptus, thyme, tea-tree EOs (E-EO, T-EO, and TT-EO, respectively) and ethanol (EtOH). Table S3: Antibiofilm activity of volatile fractions of EOs and ethanol against *S. aureus*, *P. aeruginosa*, and *C. albicans* biofilm. Table S4: Comparison of antibiofilm activity of three concentrations of TT-EO against *S. aureus* biofilm.

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References

1. Muhammad, M.H.; Idris, A.L.; Fan, X.; Guo, Y.; Yu, Y.; Jin, X.; Qiu, J.; Guan, X.; Huang, T. Beyond Risk: Bacterial Biofilms and Their Regulating Approaches. *Front. Microbiol.* **2020**, *11*, 1–20. [CrossRef] [PubMed]
2. Römling, U.; Kjelleberg, S.; Normark, S.; Nyman, L.; Uhlin, B.E.; Åkerlund, B. Microbial Biofilm Formation: A Need to Act. *J. Intern. Med.* **2014**, *276*, 98–110. [CrossRef]
3. Rabin, N.; Zheng, Y.; Opoku-Temeng, C.; Du, Y.; Bonsu, E.; Sintim, H.O. Biofilm Formation Mechanisms and Targets for Developing Antibiofilm Agents. *Future Med. Chem.* **2015**, *7*, 493–512. [CrossRef] [PubMed]
4. Ventola, C.L. The Antibiotic Resistance Crisis. *Pharm. Ther.* **2015**, *40*, 277–283. [CrossRef]
5. European Committee on Antimicrobial Susceptibility Testing. Clinical Breakpoints—Breakpoints and Guidance. Available online: https://eucast.org/clinical_breakpoints/ (accessed on 29 July 2020).
6. Dewasthale, S.; Mani, I.; Vasdev, K. Microbial Biofilm: Current Challenges in Health Care Industry. *J. Appl. Biotechnol. Bioeng.* **2018**, *5*, 156–160. [CrossRef]
7. Verderosa, A.D.; Totsika, M.; Fairfull-Smith, K.E. Bacterial Biofilm Eradication Agents: A Current Review. *Front. Chem.* **2019**, *7*, 1–17. [CrossRef]
8. Jafri, H.; Ansari, F.A.; Ahmad, I. Prospects of Essential Oils in Controlling Pathogenic Biofilm. In *New Look to Phytomedicine*; Khan, M.S.A., Ahmad, I., Chattopadhyay, D., Eds.; Elsevier Inc.: New York, NY, USA, 2019; pp. 203–236. ISBN 978-0-12-814619-4.
9. Chouhan, S.; Sharma, K.; Guleria, S. Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives. *Medicines* **2017**, *4*, 58. [CrossRef]
10. Peterfalvi, A.; Miko, E.; Nagy, T.; Reger, B.; Simon, D.; Miseta, A.; Czéh, B.; Szereday, L. Much More than a Pleasant Scent: A Review on Essential Oils Supporting the Immune System. *Molecules* **2019**, *24*, 4530. [CrossRef]
11. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential Oils, A New Horizon in Combating Bacterial Antibiotic Resistance. *Open Microbiol. J.* **2014**, *8*, 6–14. [CrossRef]
12. Mulyaningsih, S.; Sporer, F.; Reichling, J.; Wink, M. Antibacterial activity of essential oils from Eucalyptus and of selected components against multidrug-resistant bacterial pathogens. *Pharm. Biol.* **2011**, *49*, 893–899. [CrossRef]
13. De Oliveira, J.; de Jesus Viegas, J.; Martins, A.; Carvalho, C.; Soares, C.; Camargo, S.; Jorge, A.; de Oliveira, L. *Thymus vulgaris* L. extract has antimicrobial and anti-inflammatory effects in the absence of cytotoxicity and genotoxicity. *Arch. Oral Biol.* **2017**, *82*, 271–279. [CrossRef] [PubMed]
14. Carson, D.; Hammer, K.; Riley, V. Melaleuca alternifolia (Tea Tree) Oil: A Review of Antimicrobial and Other Medicinal Properties. *Clin. Microbiol. Rev.* **2006**, *19*, 50–62. [CrossRef] [PubMed]
15. European Committee on Antimicrobial Susceptibility Testing. EUCAST Reading Guide for Broth Microdilution. Available online: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Reading_guide_BMD_v_2.0_2020.pdf (accessed on 29 July 2020).
16. Moser, C.L.; Meyer, B.K. Comparison of Compendial Antimicrobial Effectiveness Tests: A Review. *Aaps Pharmscitech* **2011**, *12*, 222–226. [CrossRef] [PubMed]
17. Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for In Vitro Evaluating Antimicrobial Activity: A Review. *J. Pharm. Anal.* **2016**, *6*, 71–79. [CrossRef]
18. Yala, J.-F.; Mabika, R.M.; Camara, B.; Tuo, S.; Souza, A.; Lepengue, A.N.; Koné, D.; M'batchi, B. Assessment of the Antibacterial Activity of Four Essential Oils and the Biobactericide Neco. *Int. J. Phytomed.* **2017**, *9*, 443–450. [CrossRef]
19. Budzyńska, A.; Sadowska, B.; Paszkiewicz, M.; Rózalska, B. Assessment of essential oils activity used in liquid or volatile phase against biofilm cultures of Staphylococcus aureus and Candida albicans. The methodological study. *Med. Doswiadczalna Mikrobiol.* **2011**, *63*, 327–331.
20. Sadgrove, N.; Nicolas, J. A Contemporary Introduction to Essential Oils: Chemistry, Bioactivity and Prospects for Australian Agriculture. *Agriculture* **2015**, *5*, 48–102. [CrossRef]
21. Tyagi, A.K.; Malik, A. Antimicrobial Potential and Chemical Composition of Eucalyptus Globulus Oil in Liquid and Vapour Phase against Food Spoilage Microorganisms. *Food Chem.* **2011**, *126*, 228–235. [CrossRef]
22. Kloucek, P.; Smid, J.; Frankova, A.; Kokoska, L.; Valterova, I.; Pavela, R. Fast Screening Method for Assessment of Antimicrobial Activity of Essential Oils in Vapor Phase. *Food Res. Int.* **2012**, *47*, 161–165. [CrossRef]

23. Kuhn, D.M.; Balkis, M.; Chandra, J.; Mukherjee, P.K.; Ghannoum, M.A. Uses and Limitations of the XTT Assay in Studies Of. *J. Clin. Microbiol.* **2003**, *41*, 506–508. [CrossRef]
24. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disc Susceptibility Tests; Approved Standard M2-A9. Available online: https://clsi.org/media/1631/m02a12_sample.pdf (accessed on 29 July 2020).
25. Bueno, J. Models of Evaluation of Antimicrobial Activity of Essential Oils in Vapour Phase: A Promising Use in Healthcare Decontamination. *Nat. Volatiles Essent. Oils* **2015**, *2*, 16–29.
26. Bismarck, D.; Dusold, A.; Heusinger, A.; Müller, E. Antifungal In Vitro Activity of Essential Oils against Clinical Isolates of Malassezia Pachydermatis from Canine Ears: A Report from a Practice Laboratory. *Complement. Med. Res.* **2020**, *27*, 143–154. [CrossRef] [PubMed]
27. Zhang, D.; Gan, R.Y.; Zhang, J.R.; Farha, A.K.; Li, H.-B.; Zhu, F.; Wang, X.H.; Corke, H. Antivirulence Properties and Related Mechanisms of Spice Essential Oils: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 1018–1055. [CrossRef]
28. López, P.; Sánchez, C.; Batlle, R.; Nerín, C. Vapor-Phase Activities of Cinnamon, Thyme, and Oregano Essential Oils and Key Constituents against Foodborne Microorganisms. *J. Agric. Food Chem.* **2007**, *55*, 4348–4356. [CrossRef]
29. Ashraf, S.A.; Al-Shammari, E.; Hussain, T.; Tajuddin, S.; Panda, B.P. In-Vitro Antimicrobial Activity and Identification of Bioactive Components Using GC–MS of Commercially Available Essential Oils in Saudi Arabia. *J. Food Sci. Technol.* **2017**, *54*, 3948–3958. [CrossRef]
30. Amatiste, S.; Sagrafoli, D.; Giacinti, G.; Rosa, G.; Carfora, V.; Marri, N.; Tammaro, A.; Bovi, E.; Rosati, R. Antimicrobial Activity of Essential Oils against Staphylococcus Aureus in Fresh Sheep Cheese. *Ital. J. Food Saf.* **2014**, *3*, 1696. [CrossRef]
31. Junka, A.; Szymczyk, P.; Ziółkowski, G.; Karuga-Kuzniewska, E.; Smutnicka, D.; Bil-Lula, I.; Bartoszewicz, M.; Mahabady, S.; Sedghizadeh, P.P. Bad to the Bone: On In Vitro and Ex Vivo Microbial Biofilm Ability to Directly Destroy Colonized Bone Surfaces without Participation of Host Immunity or Osteoclastogenesis. *PLoS ONE* **2017**, *12*, e0169565. [CrossRef]
32. Junka, A.F.; Żywicka, A.; Szymczyk, P.; Dziadas, M.; Bartoszewicz, M.; Fijałkowski, K.A.D.A.M. Test (Antibiofilm Dressing's Activity Measurement)—Simple Method for Evaluating Anti-Biofilm Activity of Drug-Saturated Dressings against Wound Pathogens. *J. Microbiol. Methods* **2017**, *143*, 6–12. [CrossRef]
33. Junka, A.; Bartoszewicz, M.; Smutnicka, D.; Secewicz, A.; Szymczyk, P. Efficacy of Antiseptics Containing Povidone-Iodine, Octenidine Dihydrochloride and Ethacridine Lactate against Biofilm Formed by Pseudomonas Aeruginosa and Staphylococcus Aureus Measured with the Novel Biofilm-Oriented Antiseptics Test. *Int. Wound J.* **2014**, *11*, 730–734. [CrossRef]
34. Kragh, K.N.; Alhede, M.; Kvich, L.; Bjarnsholt, T. Into the Well—A Close Look at the Complex Structures of a Microtiter Biofilm and the Crystal Violet Assay. *Biofilm* **2019**, *1*, 100006. [CrossRef]
35. Tasse, J.; Cara, A.; Saglio, M.; Villet, R.; Laurent, F. A Steam-Based Method to Investigate Biofilm. *Sci. Rep.* **2018**, *8*, 13040. [CrossRef] [PubMed]
36. Azeredo, J.; Azevedo, N.F.; Briandet, R.; Cerca, N.; Coenye, T.; Costa, A.R.; Desvaux, M.; di Bonaventura, G.; Hébraud, M.; Jaglic, Z.; et al. Critical Review on Biofilm Methods. *Crit. Rev. Microbiol.* **2017**, *43*, 313–351. [CrossRef] [PubMed]
37. Bordeleau, E.; Mazinani, S.A.; Nguyen, D.; Betancourt, F.; Yan, H. Abrasive Treatment of Microtiter Plates Improves the Reproducibility of Bacterial Biofilm Assays. *RSC Adv.* **2018**, *8*, 32434–32439. [CrossRef]
38. Djelloul, R.; Mokrani, K.; Hacini, N. Study of the Antibacterial Activity of the Extract from the Essential Oil of Eucalyptus Globulus and Rosmarinus Officinalis on Three Bacterial Strains. *Int. J. Appl. Environ. Sci.* **2017**, *12*, 47–56.
39. Institute of Microecology. Aromatogram: Essential Oils against Infections. Available online: https://www.mikrooek.de/en/laboratory-diagnostics/for-the-medical-community/extras/aromatogram/ (accessed on 29 July 2020).
40. Chao, S.C.; Young, D.G.; Oberg, C.J. Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *J. Essent. Oil Res.* **2000**, *12*, 639–649. [CrossRef]
41. Katalog Gama Group A.S. Available online: https://www.gama.cz/en/diameter-of-petri-dishes-90-mm (accessed on 29 July 2020).

42. Merk. BRAND® Disposable Petri Dish with Lid. Available online: https://www.sigmaaldrich.com/catalog/product/aldrich/br452005?lang=pl&region=PL&cm_sp=Insite-_-caContent_prodMerch_cooccuranceModel-_-prodMerch10-5 (accessed on 29 July 2020).
43. Amazon. Simport D210-7 Polystyrene Petri Dish. Available online: https://www.amazon.com/Simport-D210-7-Polystyrene-Sterile-Diameter/dp/B001P4FFLY (accessed on 29 July 2020).
44. Bammou, M.; Bouhlali, E.D.T.; Sellam, K.; Ibijbijen, J.; el Rhaffari, L.; Nassiri, L. Liquid and Vapour-Phase Bioactivity of Hertia Maroccana (Batt.) Maire Essential Oil: An Endemic Asteraceae from Morocco. *J. Appl. Pharm. Sci.* **2016**, *6*, 131–136. [CrossRef]
45. Dobre, A.; Gagiu, V.; Petru, N. Antimicrobial Activity of Essential Oils Against Food-Borne Bacteria Evaluated by Two Preliminary Methods. *Rom. Biotechnol. Lett.* **2011**, *16*, 119–125.
46. Tyagi, A.K.; Bukvicki, D.; Gottardi, D.; Tabanelli, G.; Montanari, C.; Malik, A.; Guerzoni, M.E. Eucalyptus Essential Oil as a Natural Food Preservative: In Vivo and In Vitro Antiyeast Potential. *BioMed Res. Int.* **2014**, *2014*. [CrossRef]
47. Thaarup, I.C.; Bjarnsholt, T. Current In Vitro Biofilm-Infected Chronic Wound Models for Developing New Treatment Possibilities. *Adv. Wound Care* **2020**. [CrossRef]
48. Vuotto, C.; Donelli, G. Biofilm-based healthcare-associated infections, advances in experimental medicine and biology. In *Anaerobes in Biofilm-Based Healthcare-Associated Infections*; Donelli, G., Ed.; Springer: Cham, Switzerland, 2014. [CrossRef]
49. Haney, E.; Trimble, M.; Cheng, J.; Vallé, Q.; Hancock, R. Critical Assessment of Methods to Quantify Biofilm Growth and Evaluate Antibiofilm Activity of Host Defence Peptides. *Biomolecules* **2018**, *8*, 29. [CrossRef]

Supplementary data

The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol)

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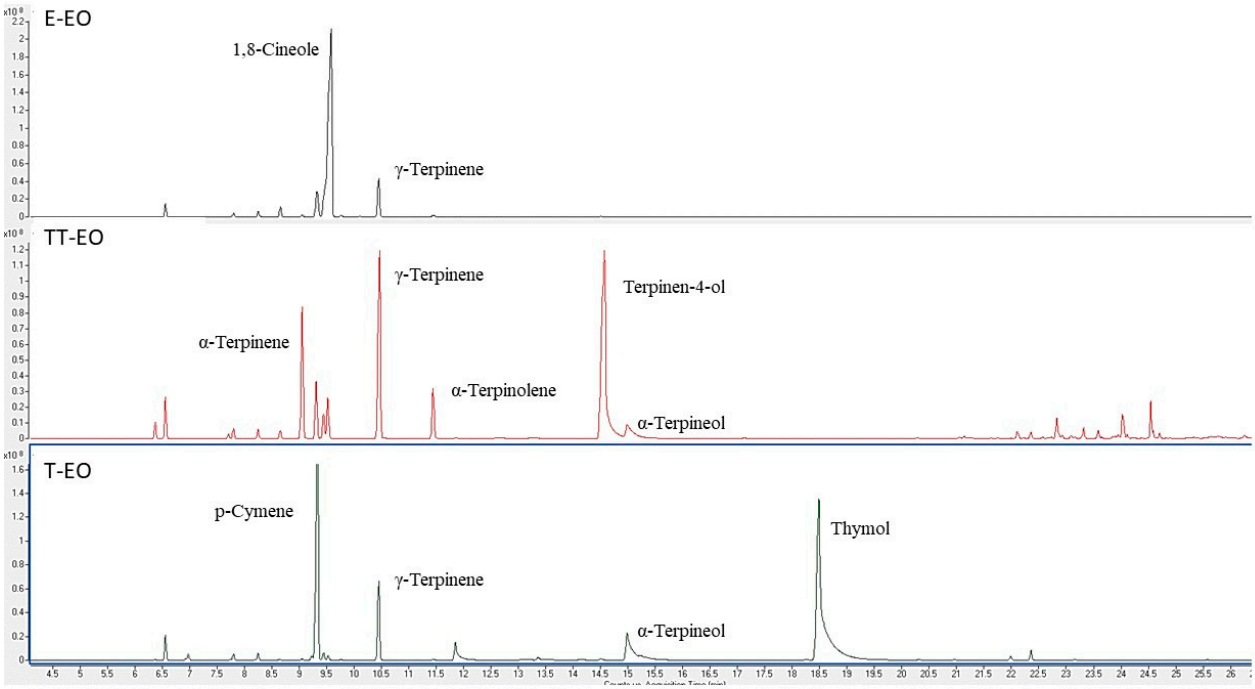


Fig.S1. GC-MS chromatograms of main EO ingredients. **E-EO** – eucalyptus oil; **TT-EO** – tea tree oil; **T-EO** – thyme oil. Results are presented as percentage content based on the peak area normalization.

RI	RT	Compound	E-EO	TT-EO	T-EO
929	6.37	α-Thujene		1.11 ± 0.02	
937	6.56	α-Pinene	2.30 ± 0.04	2.85 ± 0.06	2.20 ± 0.09
949	6.98	Camphene			0.73 ± 0.04
975	7.80	Sabinene		0.75 ± 0.02	0.64 ± 0.03
979	8.25	β-Pinene	1.09 ± 0.02	0.69 ± 0.01	0.81 ± 0.05
1005	8.65	α-Phellandrene	2.02 ± 0.02	0.61 ± 0.01	
1017	9.06	α-Terpinene		11.07 ± 0.17	
1025	9.31	p-Cymene	6.89 ± 0.07	4.69 ± 0.07	26.91 ± 0.99
1028	9.45	Limonene		2.08 ± 0.05	0.77 ± 0.04
1031	9.52	1,8-Cineole	79.10 ± 0.61	3.34 ± 0.06	
1060	10.47	γ-Terpinene	8.16 ± 0.07	19.07 ± 0.27	8.60 ± 0.03
1088	11.44	α-Terpinolene		4.34 ± 0.06	
1096	11.85	Linalool			3.45 ± 0.15
1141	13.36	Camphor			0.66 ± 0.06
1177	14.58	Terpinen-4-ol		33.27 ± 0.79	
1189	14.99	α-Terpineol		3.26 ± 0.13	7.84 ± 0.30
1289	18.49	Thymol			44.00 ± 0.46
1419	22.36	β-Caryophyllene		0.53 ± 0.01	1.00 ± 0.05
1440	22.83	Aromadendrene		1.83 ± 0.03	
1460	23.32	Alloaromadendrene		0.82 ± 0.02	
1496	24.03	Viridiflorene		2.35 ± 0.04	
1518	24.55	β-Cadinene		2.78 ± 0.03	

Tab.S1. Composition of main ingredients of tested EOs. **E-EO** – eucalyptus essential oil; **TT-EO** – tea tree essential oil; **T-EO** – thyme essential oil; **RI** - retention index; **RT** – retention time

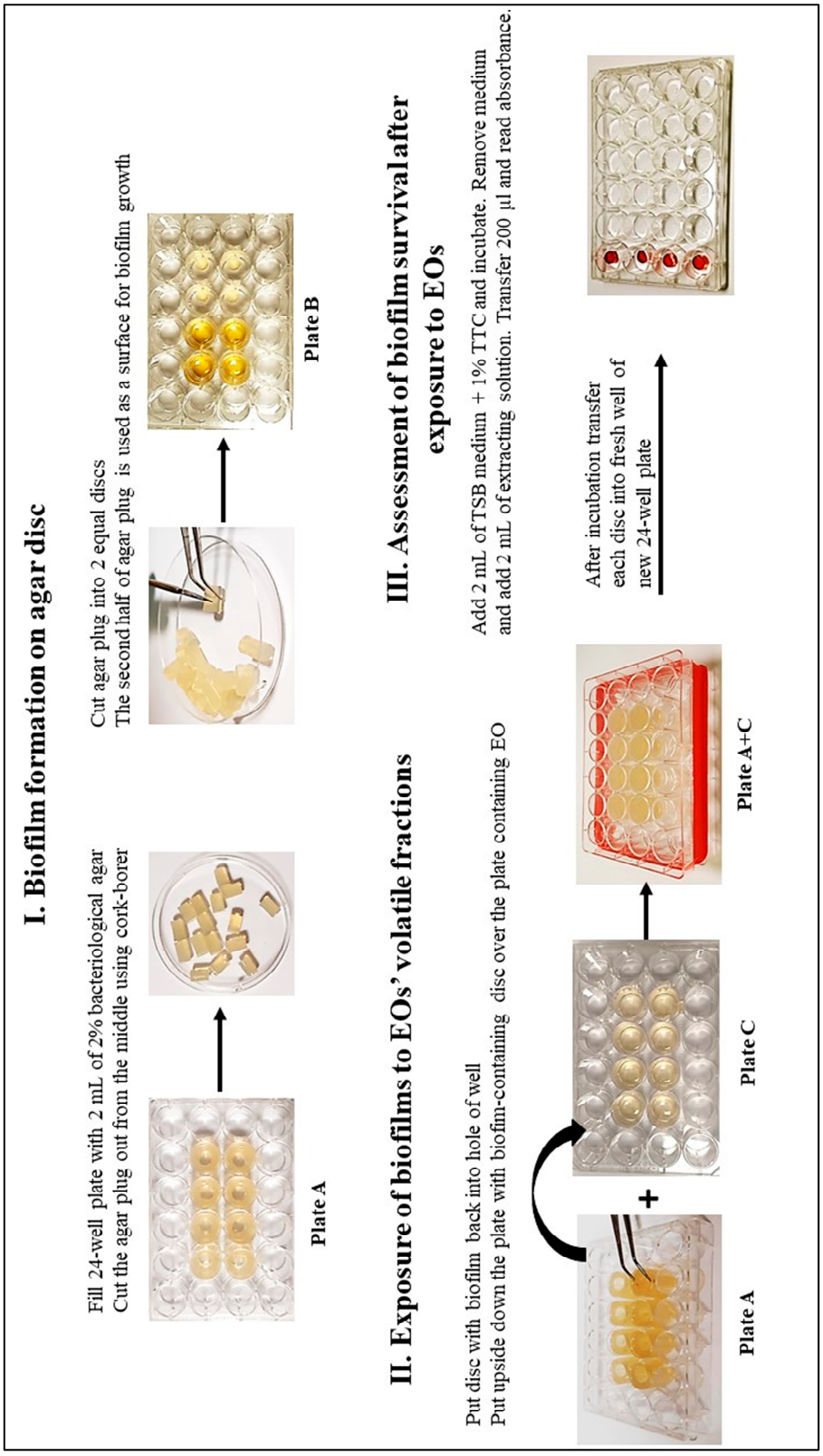
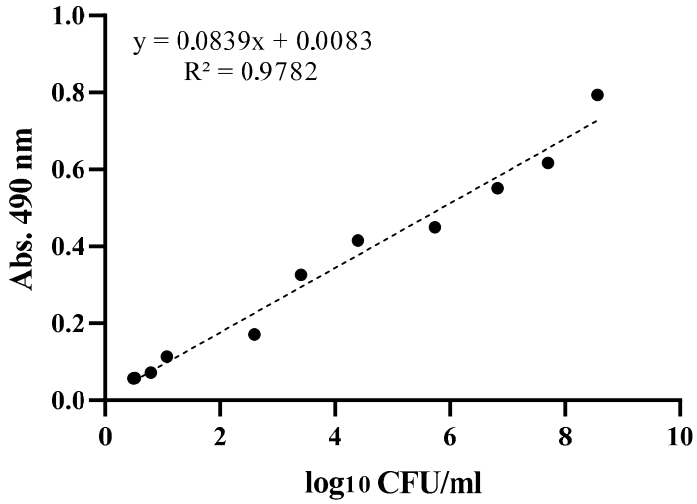


Fig. S2. Photographic presentation of AntiBioVol test performance.

A



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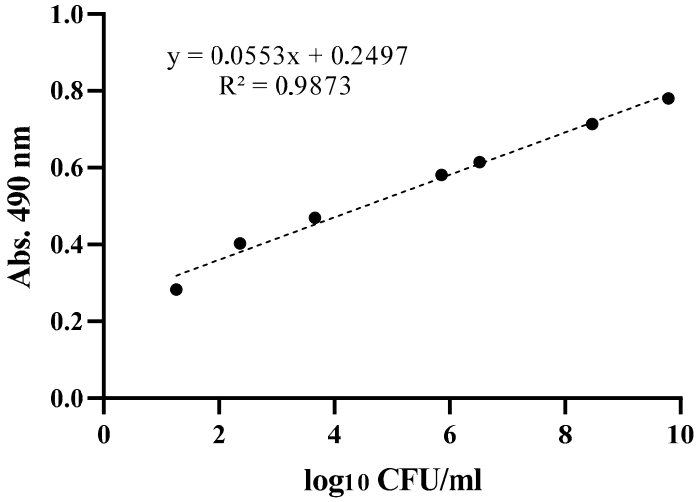

Fig. S3. Calibration curves for absorbance measurements at 490 nm (TTC assay) versus number of log10 colony forming units (CFU) / mL **[A]** *S. aureus*, **[B]** *P. aeruginosa*.

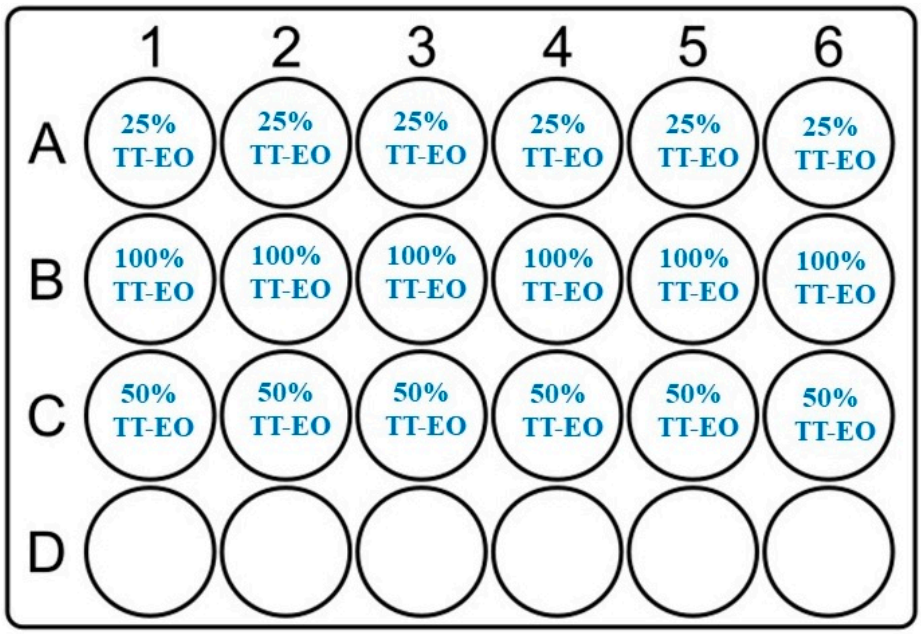

Fig. S4. Distribution of 3 TT-EO's concentrations in a single test plate. **TT-EO** – tea tree oil; 25%, 50%, 100%: applied concentrations [v/v] of TT-EO.

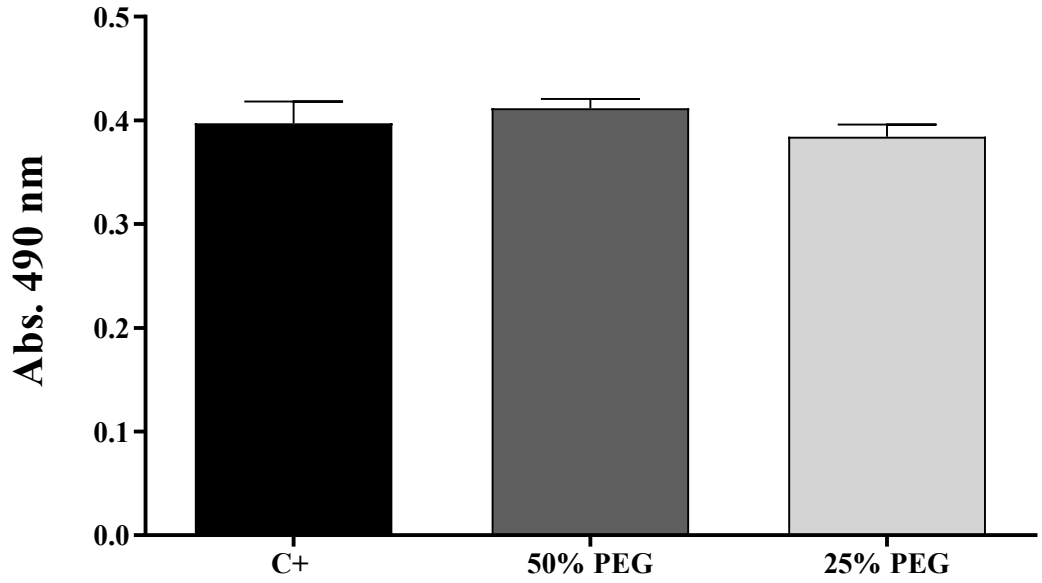

Fig. S5. Lack of PEG antimicrobial activity against *S. aureus* in the AntiBioVol experimental setting. C+ – positive control; 50%, 25% PEG – concentrations (v/v) of polyethylene glycol. No significant difference ($p<0.05$, K-W test, followed by Tukey's analysis) between control and PEGs was detected with relation to *S. aureus* viability.

Tab. S2. Minimal Inhibitory Concentration [**MIC**] and Minimum Biofilm Eradication Concentration [**MBEC**] of eucalyptus, thyme, tea-tree EOs [**E-EO**, **T-EO**, **TT-EO**, respectively] and ethanol [**EtOH**].

Microorganisms	E-EO		T-EO		TT-EO		EtOH
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC
S. aureus	**12.5**	**n-m**	**0.02**	**0.19**	**6.25**	**12.5**	**12.5**
C. albicans	**1.5**	**n-m**	**0.02**	**0.38**	**0.78**	**n-m**	**3.12**
P. aeruginosa	**25**	**n-m**	**50**	**n-m**	**25**	**50**	**12.5**

"n-m" abbreviation stands for "non-measurable" and it is used when **no** MIC or MBEC values were observed when the highest possible concentration of specific EO was applied. The numbers given in the **Tab. S1** are expressed as the percentage volume of EO within total volume consisting of medium and EO in plate's well.

Description of the results presented in **Tab. S1:** All liquid EOs acted stronger against the planktonic forms of the tested microorganisms than against their biofilmic counterparts. Moreover, liquid EOs acted stronger against thick-walled cells of *S. aureus* and *C. albicans* than against thin-walled *P. aeruginosa*. Liquid E-EO was inactive (within tested range of concentrations) against biofilm of all analyzed microorganisms; liquid TT-EO displayed measurable activity against *S. aureus* and *P. aeruginosa* but not against *C. albicans* biofilm, while T-EO was active against *S. aureus* and *C. albicans* but not against *P. aeruginosa* biofilm.

A **B**

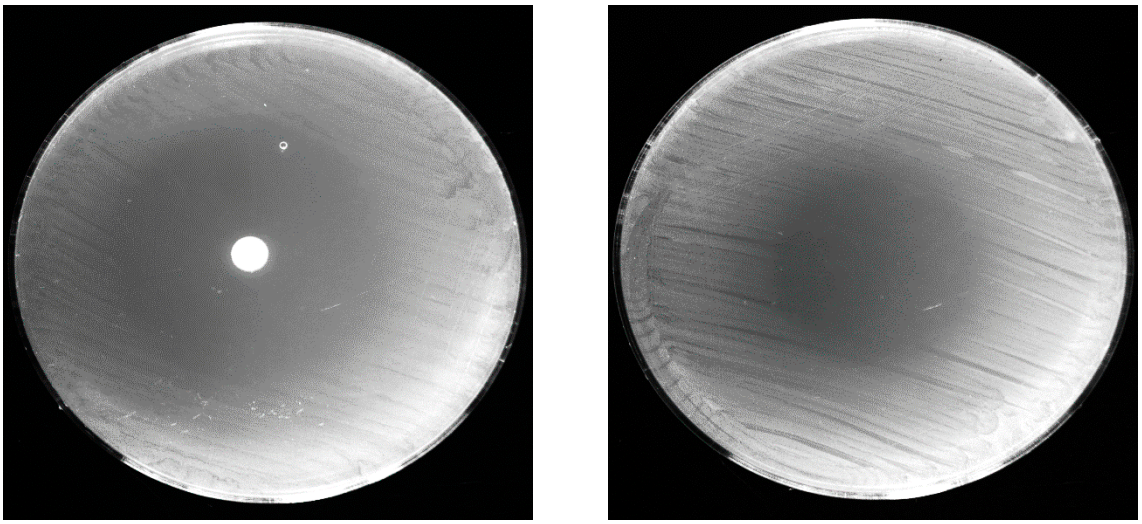

Fig. S6. Antibacterial activity of thyme essential oil liquid against *S. aureus* determined by disc diffusion method **(A)** and inverted Petri plate method **(B).**

A **B**

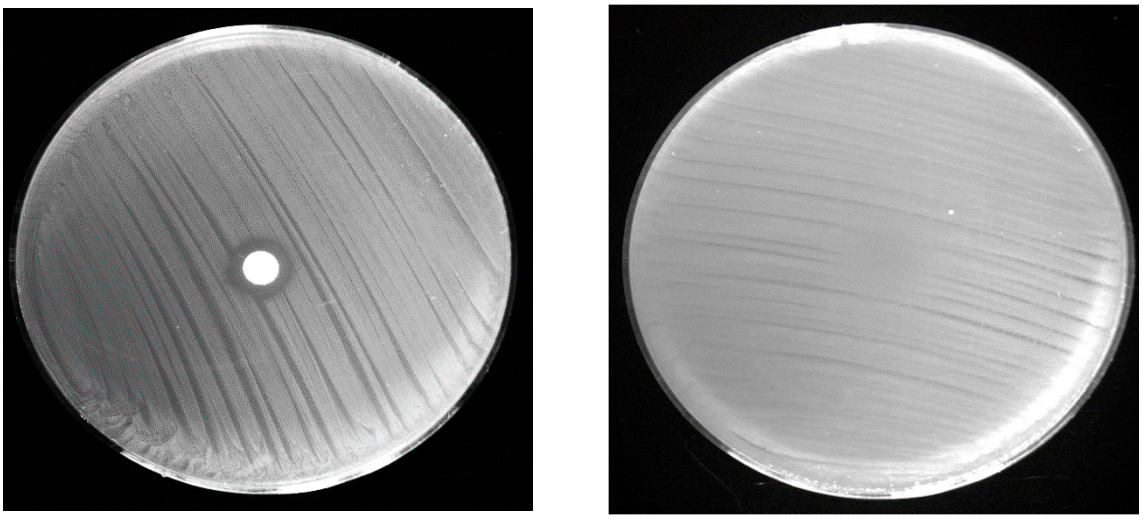

Fig. S7. Antibacterial activity of thyme essential oil liquid against *P. areuginosa* determined by disc diffusion method **(A)** and inverted Petri plate method **(B).**

Tab. S3. Antibiofilm activity of volatile fractions of EOs and ethanol against *S. aureus, P. aeruginosa*, *C. albicans* biofilm.

Volatile fractions	***S. aureus***		***P. areuginosa***		***C. albicans***	
	Mean	SEM	Mean	SEM	Mean	SEM
T-EO	0.377	0.048	0.572	0.109	4.555	0.592
TT-EO	0.193	0.049	0.338	0.057	5.190	0.618
E-EO	0.062	0.007	0.357	0.076	4.134	0.522
EtOH	0.157	0.029	0.199	0.036	3.917	0.657
C+	0.549	0.112	1.048	0.129	7.928	0.501

Data are presented as a mean ± standard error of the mean (SEM) of absorbance value (TTC assay) for *S. aureus*, *P. aeruginosa* and $\log_{10}$ CFU/mL for *C. albicans*. **C+** – positive control of growth. **EtOH** – usability control.

Tab. S4. Comparison of antibiofilm activity of 3 concentrations of TT-EO against *S. aureus* biofilm.

Volatile fractions	**Single plate**		**Separate plate**	
	Mean	SEM	Mean	SEM
TT-EO 25%	0.357	0.028	0.403	0.063
TT-EO 50%	0.312	0.036	0.299	0.069
TT-EO 100%	0.203	0.052	0.228	0.022

Data are presented as a mean of absorbance ± standard error of the mean (SEM) of absorbance value (TTC assay).

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Article

The Antimicrobial and Antibiofilm In Vitro Activity of Liquid and Vapour Phases of Selected Essential Oils against *Staphylococcus aureus*

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Abstract: The high resistance of staphylococcal biofilm against antibiotics and developing resistance against antiseptics induces a search for novel antimicrobial compounds. Due to acknowledged and/or alleged antimicrobial activity of EOs, their application seems to be a promising direction to follow. Nevertheless, the high complexity of EOs composition and differences in laboratory protocols of the antimicrobial activity assessment hinders the exact estimation of EOs effectiveness. To overcome these disadvantages, in the present work we analysed the effectiveness of volatile and liquid forms of seven EOs (derived from thyme, tea tree, basil, rosemary, eucalyptus, lavender, and menthol mint) against 16 staphylococcal biofilm-forming strains using cohesive set of in vitro techniques, including gas chromatography–mass spectrometry, inverted Petri dish, modified disk-diffusion assay, microdilution techniques, antibiofilm dressing activity measurement, AntiBioVol protocol, fluorescence/confocal microscopy, and dynamic light scattering. Depending on the requirements of the technique, EOs were applied in emulsified or non-emulsified form. The obtained results revealed that application of different in vitro techniques allows us to get a comprehensive set of data and to gain insight into the analysed phenomena. In the course of our investigation, liquid and volatile fractions of thyme EO displayed the highest antibiofilm activity. Liquid fractions of rosemary oil were the second most active against *S. aureus*. Vapour phases of tea tree and lavender oils exhibited the weakest anti-staphylococcal activity. The size of emulsified droplets was the lowest for T-EO and the highest for L-EO. Bearing in mind the limitations of the in vitro study, results from presented analysis may be of pivotal meaning for the potential application of thymol as a antimicrobial agent used to fight against staphylococcal biofilm-based infections.

Keywords: *S. aureus*; biofilm; essential oils

1. Introduction

Biofilm is a cohesive and complex community consisting of microbial cells, embedded within a self-produced matrix that displays protective and nutritional features. The matrix also enables the integrity of biofilm, and in the case of the sessile type of this structure because it facilities adhesion to biotic and abiotic surfaces. Bacteria within the biofilm, compared to their planktonic (non-aggregated) counterparts, demonstrate specific patterns

of growth rate, gene transcription, and metabolic activity. It translates into (among others) highly elevated biofilm tolerance/resistance to environmental stress and eradication with antimicrobials [1]. Thus, biofilm is a significant causative factor in a number of persistent, hard-to-heal infections, including these occurring in chronic wounds and bones [2]. Due to biofilm's persistence, even systemic high-dose antibiotic therapy displays low efficacy; in turn, topical application of antibiotics to treat biofilm-based infections is associated with numerous adverse effects. Therefore, the treatment of biofilm-based, chronic bone and wound infections requires (if possible) surgical intervention and application of antiseptics [3]. As numerous reports indicate microbial resistance to not only antibiotics but also to antiseptics, a growing interest in new antimicrobials and novel ways of their administration is presently observed [4].

Essential oils (EOs) are plant-derived liquids containing numerous compounds of acknowledged and/or alleged antimicrobial activity. Many of the compounds display broad and unspecific mechanisms of action (for example, interaction with the lipids of the cell membrane of microorganism, resulting in metabolic damages and cell death) making them effective against antibiotic-resistant strains and biofilms [5]. The complexity of EOs composition hinders, to some extent, exact understanding of the interplay between their specific components, because various types of interactions, as synergy, antagonism, addition or indifference may occur. On the other hand, this diversity contributes to EOs omnidirectional influence on biofilm (manifested as inhibition of Quorum Sensing (QS), reduction of virulence factors' expression, or inhibition of biofilm adhesion). Moreover, because various EOs components target diverse sites of microbial cell structure, the application of EOs is not associated with the risk of the development of bacterial resistance [6].

It was reported that the combined use of various EOs modulates bacterial resistance to antibiotics, for example, by targeting efflux pumps, stabilizing molecule form, and by protecting antibiotics against bacterial enzymes [7]. The synergistic action of EOs with antibiotics and antiseptics ("boosting effect") was also indicated. It was revealed that the application of rosemary, eucalyptus, and thyme oils increases antimicrobial activity of povidone-iodine antiseptic against methicillin-resistant *Staphylococcus aureus* strains up to 136 times [8].

It is worth noting that not only liquid but also volatile forms of EOs display antimicrobial activity. The application of the vapour of EOs provides a high concentration of active compounds to the infection site and limits side effects related to systemic administration and the toxicity being result of direct contact between antimicrobial substances and the issue [9].

Studies on an animal model have confirmed that topical application of EOs promotes the wound healing process. The use of lavender, rosemary, eucalyptus, and basil oils on wounds translates into more favourable results of collagen deposition, closure rate, fibroblasts proliferation, and exudate level [10]. Other research indicates that thyme oil reduces the amount of nitric oxide released in response to burn injuries and facilitates wound healing [11]. The clinical trial has demonstrated the potential of tea tree EO in the therapy of osteomyelitis and wound infection [12]. In turn, 1,8-cineole, a compound found in rosemary and eucalyptus oils, has been reported to act synergistically with amoxicillin and gentamicin in combating MRSA-induced osteomyelitis in rabbits [13]. It should also be noted that such commonly used EOs as St. John's wort, cinnamon, thyme, rosemary, white poplar, ginger, and notopterygium root, have a beneficial impact on bone features, including mineral turnover normalization, inhibition of bone loss, enhancement of plasma calcium and vitamin D3 level, bone mineral-density improvement, and drop of inflammation and oxidative stress level [14]. Moreover, ylang-ylang, rosemary, eucalyptus, frankincense, tea tree, and wintergreen EOs are able to improve biocompatibility and bone regeneration ability and to prevent microbial colonization [14]. Since EOs are extensively metabolized in the human organism, their bioavailability as a potential systemic agent is limited [15]. However, this obstacle is of rather low meaning in the case of wound treatment, where local effectiveness is primarily required [9]. Taking into consideration the wide

range of antimicrobial activity of EOs and their low cytotoxicity, the application of these plant-derived substances as alternatives to antibiotics and antiseptics in the treatment of chronic wound and bone infections is a direction worth to follow and to investigate [16–18]. Therefore, the present study aimed to evaluate the antimicrobial and antibiofilm in vitro activity of volatile and liquid fractions of selected EOs against *S. aureus* methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) clinical and reference strains (the key factors of wound and bone infections).

2. Results

2.1. Assessment of EOs Compositions Using Gas Chromatography Mass Spectrometry

Each EO consists of numerous components; therefore, in the first line of experiment, EOs' content with regard to presence of antimicrobial substances was analysed using GCMS technique. Thymol and p-cymene were confirmed to be the main components of T-EO. Terpinen-4-ol and γ-terpinene were primarily presented in TT-EO. B-EO was comprised of methyl chavicol and linalool; the main components of R-EO were 1,8-cineole, camphor, and limonene. 1,8-cineole and γ-terpinene predominated in E-EO. M-EO was mainly composed of menthol, menthone, isomenthone, and L-EO of linalyl acetate and linalool. The detailed list of EOs composition is presented in Table S1 in the Supplementary Materials.

2.2. Assessment of Biofilm Biomass Level Using Crystal Violet Assay and Biofilm Metabolic Activity Level Using Tetrazolium Chloride Staining

After confirmation of presence of antimicrobial substances in tested EOs, the ability of all *S. aureus* strains to form biofilm in applied in vitro setting was checked. The results presented in Figure 1 indicate that all staphylococcal strains possess the ability to form in vitro biofilms displaying metabolic activity.

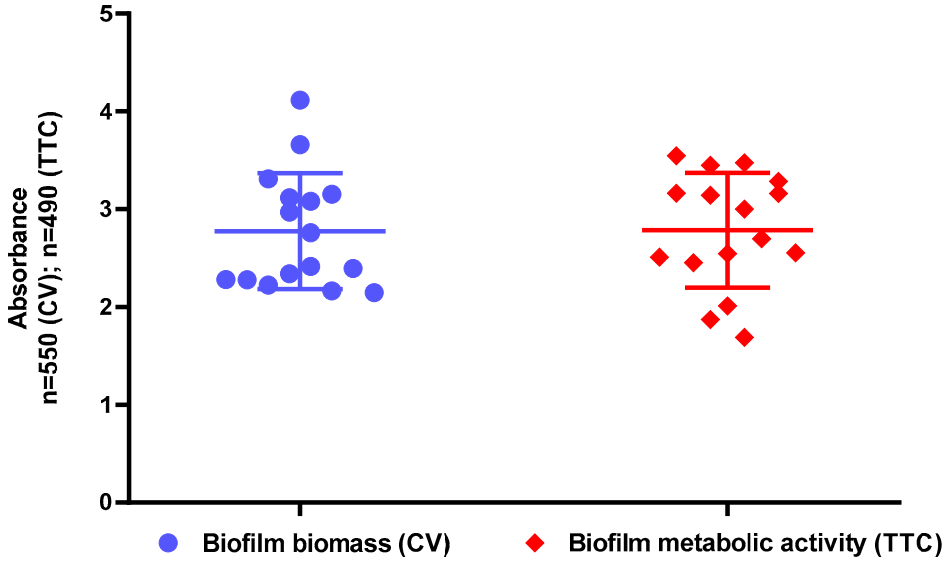

Figure 1. Ability of analysed *S. aureus* strains to form biofilm and assessed with crystal violet (CV) and tetrazolium chloride (TTC) staining.

2.3. Antimicrobial Activity of All EOs Using Disc Diffusion Method and Inverted Petri Dish Method

Next, the antimicrobial activity of EOs' liquid and volatile fractions was evaluated with standard techniques referred to as the disc diffusion and inverted Petri dish methods, respectively. The representative results from techniques applied are shown in Figure 2. With regard to liquid phases, T-EO and R-EO were the most effective against staphylococcal cells. B-EO, E-EO and L-EO exhibited the lowest antimicrobial activity among tested EOs.

In case of specific strains, only zones of partial growth inhibition were observed. The mean diameters of growth inhibition zones being result of exposure of staphylococci to liquid EOs are presented in Table 1. By means of the inverted Petri dish method, volatile fractions of TT-EO, B-EO, and L-EO were characterized as ineffective against the majority of tested strains. Among the investigated EOs, vapours of T-EO displayed the most potent anti-staphylococcal activity. The mean diameters of growth inhibition zones being result of exposure of staphylococci to vapour phases of EOs are presented in Table 2.

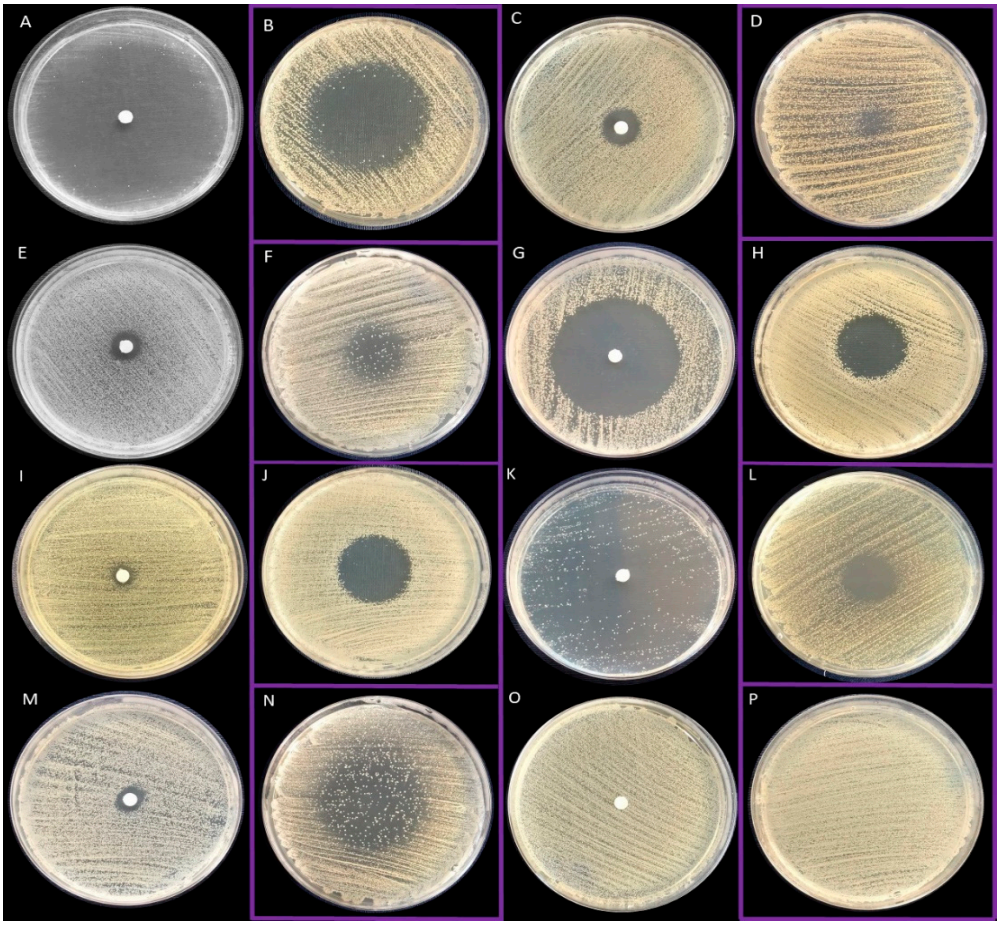

Figure 2. Zones of growth inhibition after the treatment of bacteria with volatile and liquid fractions of EOs assessed with the inverted Petri dish method and disc diffusion technique, respectively. Results of volatile fractions activity are marked with purple frames. (**A**,**B**)—thyme oil (SA 2, SA ATCC 33591, respectively); (**C**,**D**)—tea tree oil (SA 35, SA ATCC 33591, respectively); (**E**,**F**)—basil oil (SA 32, SA 33, respectively); (**G**,**H**)—rosemary oil (SA 33, SA 10, respectively); (**I**,**J**)—eucalyptus oil (SA 5, SA 28, respectively); (**K**,**L**)—menthol mint oil (SA 33, M SA ATCC 33591, respectively); (**M**,**N**)—lavender oil (SA 35, SA 33, respectively); (**O**,**P**)—sodium chloride 0.9% (control setting) (SA 26, SA 27, respectively). The picture (**F**) shows the zone of the partial inhibition of growth.

Table 1. Mean diameters of inhibition zones [mm]/mean radii of zones of partial growth inhibition (mm) (bolded values) after treatment with liquid fractions of EOs. T-EO—thyme oil, TT-EO—tea tree oil, B-EO–basil oil, R-EO—rosemary oil, E-EO—eucalyptus oil, M-EO—menthol mint oil, L-EO—lavender oil.

Zones of Growth Inhibition (mm) after Treatment with Liquid Fractions of EOs							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**M-EO**	**L-EO**
2	56 (±9.29)	8 (±6.66)	8 (±1.15)	41 (±0.58)	**0 (±0.00)/4.5**	15 (±2.65)	9 (±1.00)
4	57(±16.50)	**9 (±1.15)/3.3**	9 (±1.00)	32 (±1.53)	**0 (±0.00)/4.8**	14 (±2.31)	8 (±1.15)
5	36 (±4.04)	15 (±2.89)	10 (±1.00)	40 (±3.46)	8 (±0.58)	12 (±2.31)	9 (±1.00)
6	35 (±3.06)	15 (±1.73)	8 (±0.58)	32 (±0.58)	8 (±1.15)	11 (±1.53)	12 (±2.65)
7	55 (±13.87)	**20 (±10.79)/2.0**	17 (±0.58)	45 (±0.00)	**0 (±0.00)/6.7**	13 (±1.73)	9 (±1.15)
10	61 (±2.31)	14 (±1.15)	9 (±2.31)	35 (±0.58)	**0 (±0.00)/4.7**	12 (±1.53)	9 (±0.58)
26	43 (±3.79)	14 (±3.21)	14 (±2.08)	42 (±0.58)	**0 (±0.00)/5.7**	15 (±0.58)	9 (±1.00)
27	**60 (±13.86)/7.0**	17 (±3.06)	9 (±0.58)	27 (±3.06)	**8 (±0.58)/2.0**	13 (±2.00)	10 (±1.15)
28	**39 (±6.56)/4.2**	19 (±4.04)	12 (±5.29)	33 (±1.53)	**10 (±2.08)/2.5**	15 (±1.15)	12 (±2.00)
29	51 (±5.13)	13 (±1.15)	9 (±1.53)	38 (±3.21)	**3 (±5.20)/6.0**	10 (±0.00)	9 (±2.65)
32	50 (±9.87)	18 (±1.53)	13 (±1.15)	35 (±0.00)	11 (±0.58)	14 (±0.58)	10 (±0.58)
33	**56 (±1.73)/17.0**	24 (±1.53)	18 (±0.00)	51 (±1.00)	**0 (±0.00)/5.5**	**19 (±6.56)/6.5**	**14 (±2.89)/37.0**
34	40 (±6.35)	10 (±1.00)	18 (±0.58)	34 (±0.58)	11 (±1.15)	10 (±0.58)	8 (±1.00)
35	39 (±5.69)	13 (±1.53)	18 (±0.58)	43 (±1.73)	**8 (±0.58)/2.3**	14 (±3.61)	9 (±0.58)
ATCC 33591	43 (±3.06)	17 (±3.51)	9 (±0.00)	37 (±0.00)	**3 (±4.62)/4.0**	17 (±10.44)	10 (±1.00)
ATCC 6538	77(±12.58)	30(±5.00)	12 (±0.58)	14 (±5.13)	30(±0.5.8)	14 (±3.21)	10 (±0.58)

Table 2. Mean diameters of inhibition zones [mm]/mean radii of zones of partial growth inhibition (mm) (bolded values) after treatment with volatile fractions of EOs. T-EO—thyme oil, TT-EO—tea tree oil, B-EO—basil oil, R-EO—rosemary oil, E-EO—eucalyptus oil, M-EO—menthol mint oil, L-EO—lavender oil.

Zones of Growth Inhibition (mm) after Treatment with Volatile Fractions of EOs							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**M-EO**	**L-EO**
2	30 (±4.93)	0 (±0.00)	0 (±0.00)	13 (±8.66)	0 (±0.00)	11 (±1.73)	0 (±0.00)
4	32 (±1.00)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/10.5**	**0 (±0.00)/9.3**	7 (±1.15)	0 (±0.00)
5	24 (±1.15)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/11.5**	**8 (±6.93)/9.2**	2 (±3.46)	0 (±0.00)
6	24 (±1.15)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/11.2**	**0 (±0.00)/12.3**	0 (±0.00)	0 (±0.00)
7	31 (±0.00)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/13.0**	**6 (±9.81)/7.0**	2 (±4.04)	0 (±0.00)
10	31 (±1.15)	0 (±0.00)	0 (±0.00)	29 (±2.65)	27 (±1.73)	11 (±0.00)	0 (±0.00)
26	28 (±2.08)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/13.5**	**15 (±13.61)/12.5**	2 (±4.04)	0 (±0.00)
27	30 (±3.06)	0 (±0.00)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/7.5**	9 (±2.00)	0 (±0.00)
28	29 (±4.51)	0 (±0.00)	0 (±0.00)	29 (±2.52)	26 (±0.58)	8 (±7.00)	0 (±0.00)
29	31 (±2.08)	0 (±0.00)	0 (±0.00)	**0 (±0.00)/14.2**	**7 (±11.55)/10.5**	**0 (±0.00)/3.8**	0 (±0.00)
32	27 (±1.00)	0 (±0.00)	0 (±0.00)	**9 (±15.59)/21.5**	**0 (±0.00)/12.7**	**0 (±0.00)/5.2**	0 (±0.00)
33	**30 (±1.73)/13.2**	**0 (±0.00)/7.5**	**0 (±0.00)/13.0**	**28 (±1.15)/1.5**	**0 (±0.00)/13.8**	**0 (±0.00)/13.8**	**0 (±0.00)/22.8**
34	25 (±2.65)	0 (±0.00)	0 (±0.00)	**20 (±1.15)/2.8**	18 (±1.73)	0 (±0.00)	0 (±0.00)
35	21 (±1.15)	14 (±12.77)	0 (±0.00)	**0 (±0.00)/13.5**	**0 (±0.00)/6**	0 (±0.00)	0 (±0.00)
ATCC 33591	**37 (±0.58)/3.7**	**0 (±0.00)/5.5**	0 (±0.00)	**4 (±6.35)/10.0**	**0 (±0.00)/6.2**	12 (±4.93)	0 (±0.00)
ATCC 6538	43 (±5.03)	15 (±6.81)	0 (±0.00)	49 (±5.29)	0 (±0.00)	18 (±2.65)	0 (±0.00)

2.4. Evaluation of the Minimal Inhibitory Concentration (MIC) of Liquid Fractions of All EOs Emulsions in Tween 20 Using Serial Microdilution Method

The aim of this part of the study was to determine the MIC (minimal inhibitory concentration) of liquid fractions of EOs using microdilution method in 96-well plates. Due to the poor solubility of EOs in hydrophilic media such as Tryptic Soy Broth, emulsions in Tween 20 were applied. Firstly, the influence of different Tween 20 concentrations on the growth of planktonic forms of *S. aureus* ATCC 6538 strain was evaluated. The results, presented in Figure S1 in the Supplementary Materials, indicated that addition of up to 1% (*v/v*) Tween 20 did not affect staphylococcal growth. All tested EOs emulsions displayed antimicrobial activity against planktonic forms of analysed clinical and reference *S. aureus* strains. All tested strains were sensitive to EOs emulsions in concentrations equal to or lower than 6.3% (*v/v*). The lowest (the most favourable ones with regard to antimicrobial activity) MIC values were obtained for thyme oil (T-EO) emulsion, while the highest for B-EO and E-EO. Interestingly, clinical MRSA planktonic strains were more susceptible to

T-EO emulsions than a reference *S. aureus* ATCC 33591 strain. The MIC values of all EOs emulsions against all strains are presented in Table 3.

Table 3. Antimicrobial activity of liquid fractions of tested EOs' emulsions in Tween 20 against planktonic (MIC (%) (*v/v*)) and biofilm cells (MBEC (%) (*v/v*)) of clinical (SA 2–SA 35) and reference (SA ATCC 6538 and SA ATCC 33591) strains of *S. aureus*. Dashes (-) indicate EOs where MIC and MBEC values were not reached in the highest concentration (25% (*v/v*) and 50% (*v/v*), respectively) of EOs applied. T-EO—thyme oil, TT-EO—tea tree oil, B-EO—basil oil, R-EO—rosemary oil, E-EO—eucalyptus oil, M-EO—menthol mint oil, L-EO—lavender oil.

	T-EO		TT-EO		B-EO		R-EO		E-EO		M-EO		L-EO	
Strain	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**	**MIC (%)**	**MBEC (%)**
2	0.05	0.05	0.1	1.6	1.6	25	0.4	12.5	1.6	-	0.05	50	0.2	-
4	0.05	0.05	0.2	1.6	1.6	50	0.4	6.3	0.8	-	0.05	0.2	0.8	-
5	0.05	0.05	0.8	1.6	1.6	12.5	0.8	6.3	0.8	-	0.4	50	0.8	-
6	0.1	0.1	0.4	1.6	3.1	-	0.8	3.1	1.6	-	0.2	-	3.1	-
7	0.05	0.05	0.4	3.1	3.1	-	0.8	50	1.6	-	0.1	-	1.6	-
10	0.05	0.05	0.2	3.1	0.8	-	0.4	50	1.6	-	0.1	-	0.4	-
26	0.05	0.05	0.2	6.3	0.8	12.5	0.4	6.3	0.8	-	0.1	3.1	0.8	-
27	0.025	0.05	0.8	6.3	1.6	-	0.1	25	6.3	-	0.2	50	1.6	-
28	0.05	0.1	0.2	3.1	0.8	25	0.4	6.3	1.6	-	0.1	0.2	0.4	-
29	0.05	0.1	0.8	1.6	3.1	50	0.8	-	1.6	-	0.1	0.4	1.6	-
32	0.05	0.1	0.2	6.3	0.8	12.5	0.4	6.3	0.8	-	0.2	-	0.8	-
33	0.05	0.1	0.1	6.3	1.6	-	0.4	50	0.1	-	0.05	-	0.2	-
34	0.05	0.1	0.4	6.3	0.4	25	0.4	-	3.1	-	0.2	50	0.2	-
35	0.05	0.1	0.2	1.6	1.6	25	0.4	50	1.6	-	0.2	50	0.8	-
ATCC 33591	0.1	0.1	0.2	3.1	1.6	50	0.4	12.5	0.8	-	0.1	0.8	0.4	-
ATCC 6538	0.025	0.4	0.1	0.8	0.8	12.5	0.4	6.3	0.8	-	0.1	0.2	0.2	1.6

2.5. Evaluation of the Minimal Biofilm Eradication Concentration (MBEC) and the Minimal Bactericidal Concentration for Biofilm (MBC-B) of Liquid Fractions of All EOs Emulsions in Tween 20

The ability of liquid phases of EOs to eradicate bacterial biofilms was assessed with an MBEC (minimal biofilm eradication concentration) assay. Similar to the MIC assay, dilutions of EOs were performed using Tween 20 as an emulsifier. E-EO and L-EO emulsions exhibited no antibiofilm activity. T-EO emulsion was the most effective among the tested EOs against *S. aureus* biofilms. Minimal biofilm eradication concentrations of T-EO were equal to inhibition values against eight staphylococcal strains. Except for E-EO, liquid fractions of all EOs emulsions exhibited bactericidal activity against individual strains. In the case of nine staphylococcal strains, T-EO emulsions demonstrated bactericidal activity in concentrations equal to MBEC values. The MBEC values of each EO emulsions and all strains are presented in Table 3. The MBC-B (minimal bactericidal concentration for biofilm) values are presented in Table S2 in the Supplementary Materials.

2.6. Evaluation of Antibiofilm Activity of All Non-Emulsified EOs' Liquid Fractions Measured with Modified Antibiofilm Dressing's Activity Measurement Assay

The antibiofilm activity of liquid fractions of all non-emulsified and non-diluted EOs against *S. aureus* was determined using a modified A.D.A.M. (antibiofilm dressing's activity measurement) method. Based on the results of microdilution assays, three different clinical strains for each EO were selected and examined. To provide other research teams with the possibility of performance of this analysis, reference staphylococcal strains were also included. As a substance of proven antimicrobial activity, liquid phases of 96% (*v/v*) ethanol were applied (as controls of test usability). The concentration of EOs released from biocellulose discs was 65.8%. All EOs displayed an ability to eradicate biofilms (from 27% up to 92%). T-EO and R-EO were the most effective against all tested strains. T-EO and R-EO exhibited stronger antibiofilm activity against the *S. aureus* ATCC 33591 strain than ethanol, which served as reference substance. The antibiofilm activity of liquid fractions of non-emulsified EOs and ethanol against selected strains is depicted in Figure 3.

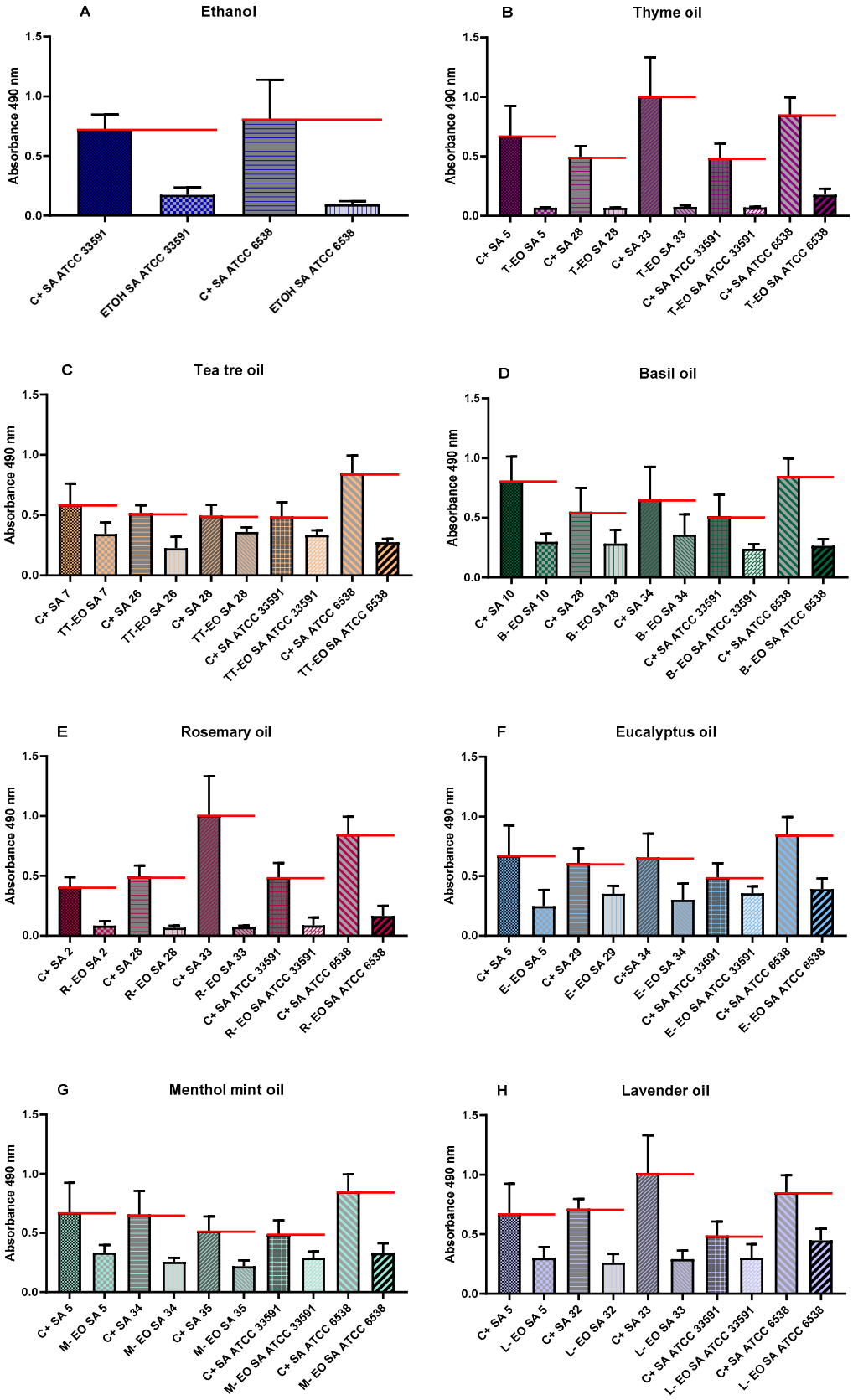

Figure 3. Antibiofilm activity of liquid fractions of non-emulsified EOs and ethanol against *S. aureus* measured with modified A.D.A.M. (antibiofilm dressing's activity measurement) assay. (**A**–**H**)—results of TTC assay. ETOCH—ethanol, T-EO—thyme oil, TT-EO—tea tree oil, B-EO—basil oil, R-EO—rosemary oil, E-EO—eucalyptus oil, M-EO—menthol mint oil, L-EO—lavender oil, C+ control of growth. Absorbance of growth controls samples are marked with red lines.

2.7. Evaluation of Antibiofilm Activity of All Non-Emulsified Eos' Volatile Fractions Measured with AntiBioVol Assay

Antibiofilm activity of volatile fractions of all non-emulsified and non-diluted EOs against *S. aureus* was determined using AntiBioVol (antibiofilm activity of volatile compounds) method against strains investigated in the modified A.D.A.M. test. As a substance of proven antimicrobial activity, the volatile phase of 96% (*v/v*) ethanol was investigated against reference strains. The differentiated antibiofilm efficacy was observed depending on analysed bacterial strains and the type of oil applied. Volatile fractions of B-EO were the only ones that reduced the number of biofilm cells of all tested strains. In turn, T-EO's volatile fractions exhibited the strongest antibiofilm activity against reference strains. Growth of SA 5 biofilm was increased after exposure to vapours of all applied EOs (T-EO, E-EO, M-EO, and L-EO). TT-EO and L-EO only slightly eradicated biofilm but in specific cases they enhanced the growth of biofilm formed by clinical strains. In order to determine the influence of volatile fractions of EOs on bacterial cell numbers, quantitative culturing was also performed for two strains exposed to four Eos. SA 5 and SA ATCC 6538 were chosen as the strains in which the opposite impact of EOs was observed in the AntiBioVol assay. The decrease in the number of SA ATCC 6538 cells was observed, while in the case of SA 5, the number of cells was higher or scarcely lower than in control setting. The results of the AntiBioVol (antibiofilm activity of volatile compounds) test are presented in Figure 4.

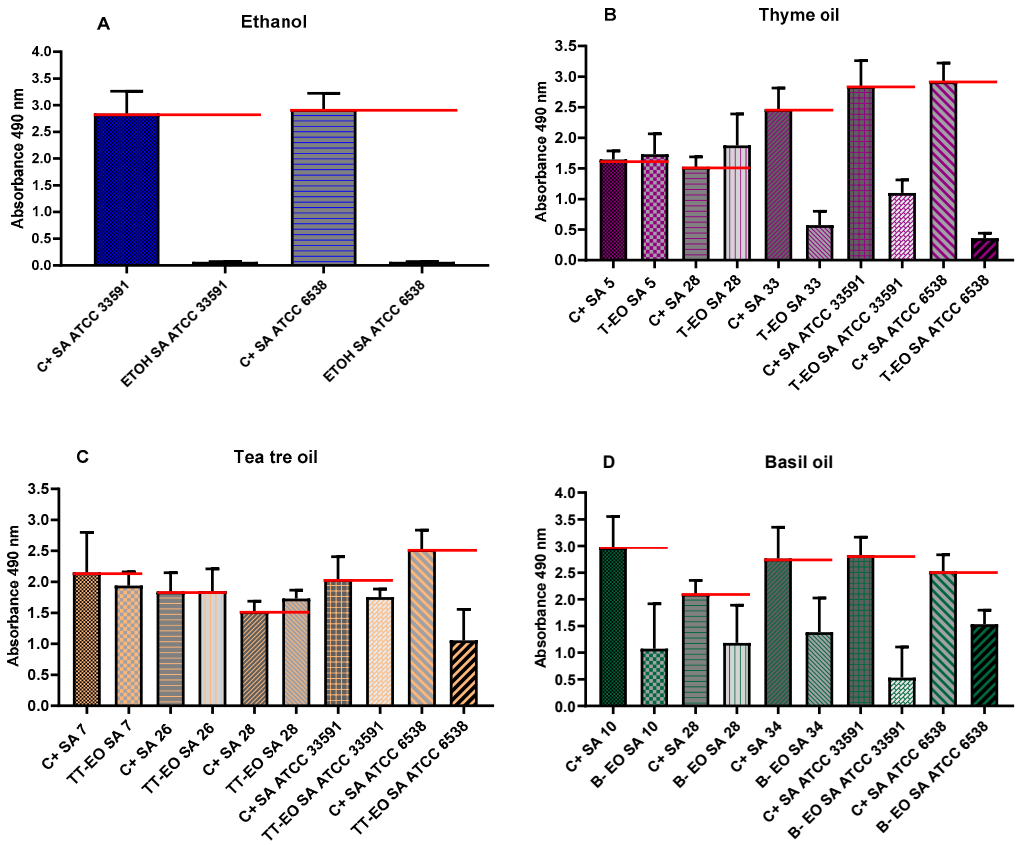

Figure 4. *Cont.*

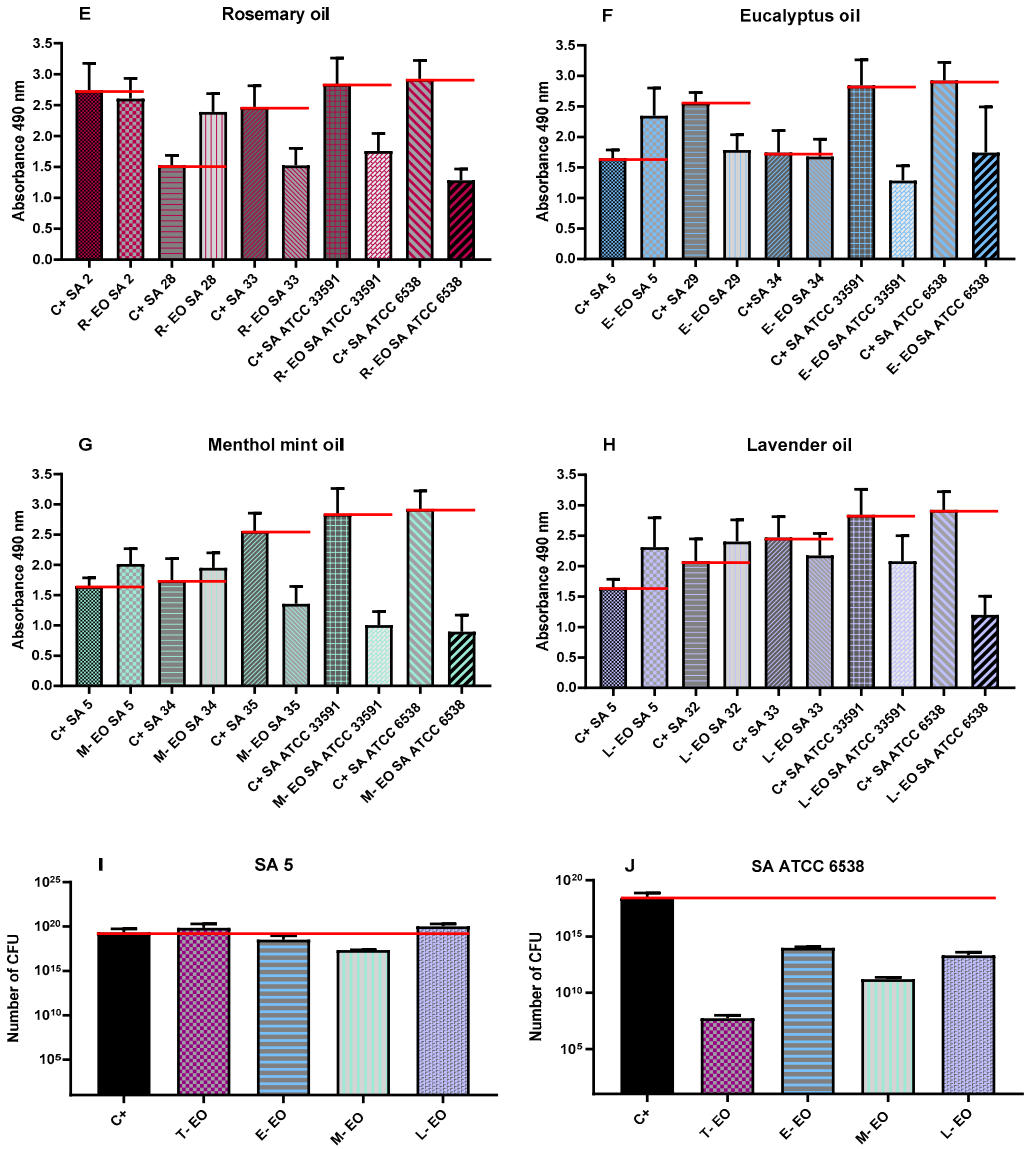

Figure 4. Antibiofilm activity of volatile fractions of non-emulsified EOs and ethanol against *S. aureus* measured with AntiBioVol (antibiofilm activity of volatile compounds) method. (**A**–**H**)—results of TTC assay, (**I**,**J**)—results of quantitative culturing. ETOCH—ethanol, T-EO—thyme oil, TT-EO—tea tree oil, B-EO—basil oil, R-EO—rosemary oil, E-EO—eucalyptus oil, M-EO—menthol mint oil, L-EO—lavender oil, C+ control of growth. Absorbance of growth controls samples are marked with red lines.

2.8. Visualisation of Impact of T-EO Emulsions' Liquid Fractions on Staphylococcal Biofilm Using LIVE/DEAD Staining and Epifluorescence Microscopy

Based on the above-presented results, T-EO emulsion, as the most potent EO against *S. aureus* biofilms, was chosen for study using fluorescent microscopy. The assessment of antibiofilm activity of the emulsion's liquid fractions against a reference ATCC 6538 strain, examined with a LIVE/DEAD dye, indicated 57% biofilm cell reduction in concentration 0.4% (*v*/*v*) (concentration equal to MBEC value evaluated with a TTC indicator). Visualisation of the strain ATCC 6538 biofilm stained with LIVE/DEAD dye is presented in Figure 5. Graphical comparison of ATCC 6538 biofilm viability treated with liquid phases of T-EO emulsion evaluated with LIVE/DEAD and TTC dyes is presented in Figure 6. An interesting phenomenon manifested by significant drop of metabolic activity occurred after exposure to T-EO concentration >0.1% (*v*/*v*) with maintaining level of staphylococcal cell integrity (at level of ~35%) was observed.

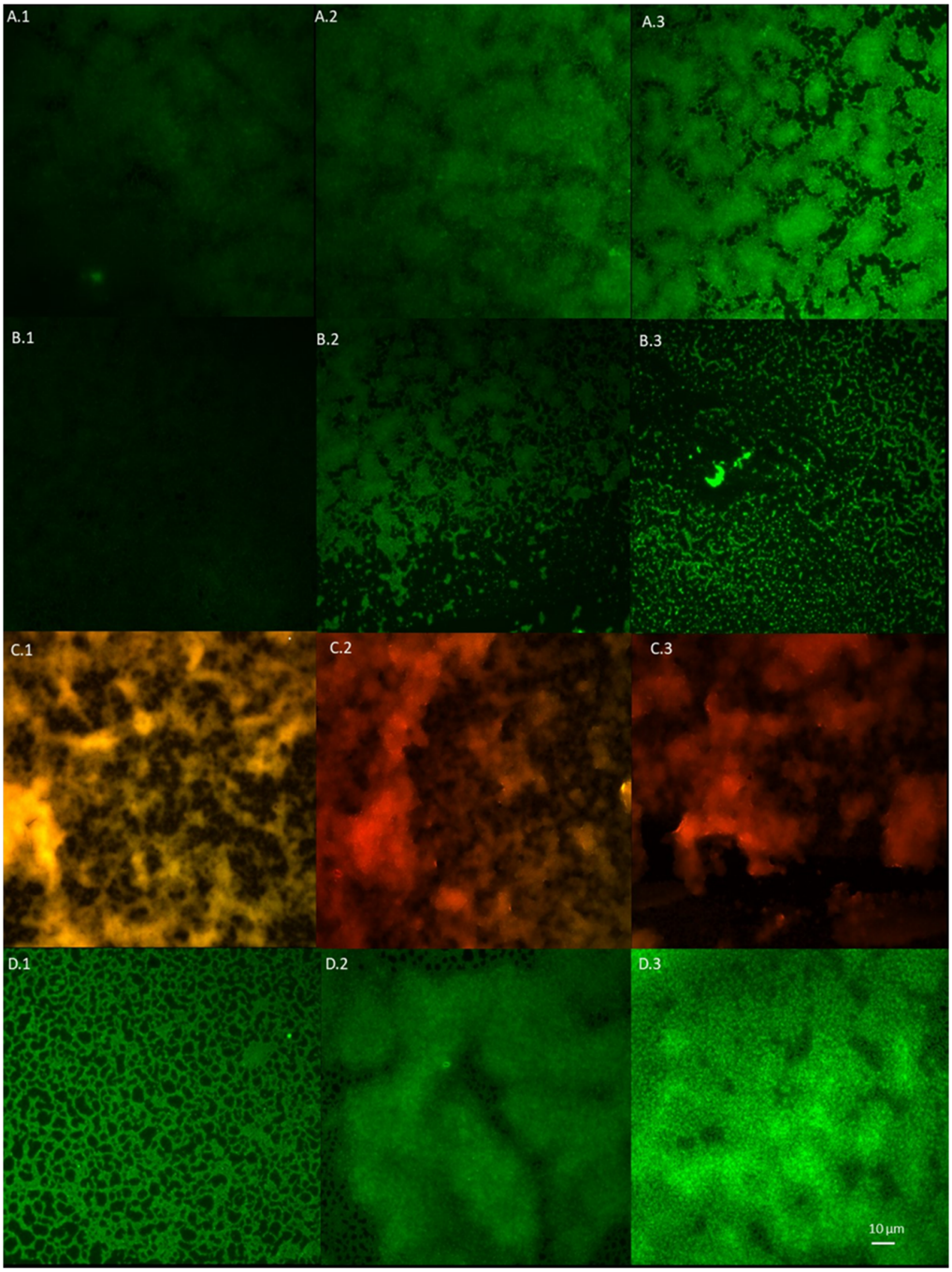

Figure 5. Microphotography of the *S. aureus* ATCC 6538 reference strain biofilm stained with LIVE/DEAD dye. (**A**,**B**)—biofilm exposed to liquid fractions of thyme oil emulsions in concentration 1.6% (*v*/*v*) (**A.1**–**A.3**) and 0.8% (*v*/*v*) (**B.1**–**B.3**); (**C.1**–**C.3**)—biofilm treated with 0.1% octenidine and 2% phenoxyethanol solution; (**D.1**–**D.3**)—untreated cells. The red/orange colour shows staphylococcal cells altered/damaged in result of exposure to liquid T-EO emulsion, while green-coloured cells are non-altered, viable cells. Fluorescence microscope Etaluma 600 (magnification 20×).

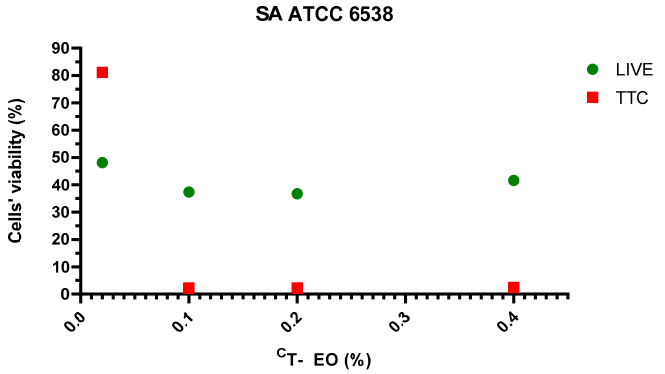

Figure 6. Viability (%) of *S. aureus* ATCC 6538 biofilm treated with liquid fractions of T-EO (thyme oil) emulsions assessed with LIVE (**green colour**) and TTC (**red colour**) dyes.

2.9. Three Dimensional Visualization of Alterations of Staphylococcal Biofilm Exposed to Volatile Fractions of R-EO

The impact of vapour R-EO on *S. aureus* ATCC 6538 biofilm was analysed using 3D confocal microscopy and parametric processing of visual data obtained (Figure 7). Volumetric images revealed high reduction of staphylococcal biofilm after exposure to R-EO (manifested in the form of loss of biofilm (Figure 7B, fragments pointed with number "2") and higher share of damaged/compromised cells (Figure 7D) compared to the staphylococcal biofilm untreated with R-EO (Figure 7A,C).

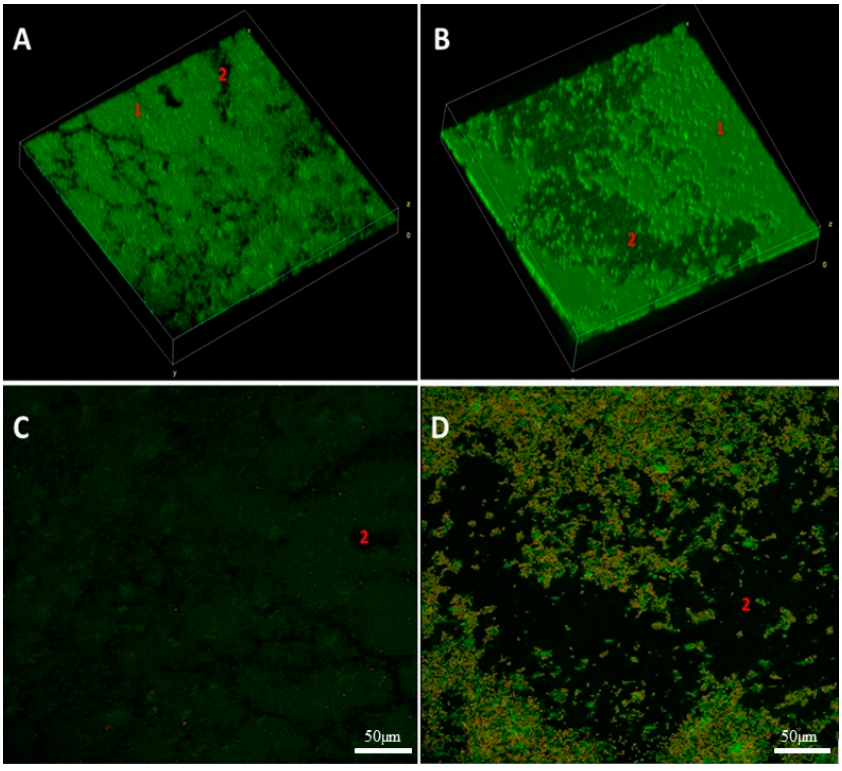

Figure 7. Impact of vapour phase of R-EO on *S. aureus* ATCC 6538 biofilm. (**A**,**B**)—volumetric data showing untreated and treated biofilm, respectively. (**1**)—non-altered fragment of biofilms; (**2**)—loss of biofilm volume. (**C**,**D**)—staphylococcal biofilm cells treated and untreated with R-EO, respectively. The red/orange colour shows staphylococcal cells altered/damaged in result of exposure to vapour R-EO, while green-coloured cells are non-altered, viable cells. Moreover, the more dark (less green) picture is, the less live cells are captured in this particular field of vision.

2.10. Size of Emulsified EOs Droplets

Finally, because the correlation between EO emulsion droplet size was recently shown to be another factor of impact on antimicrobial activity, the average diameters of droplets of emulsified EOs were measured. The results (in increasing order of diameter) were as follows: T-EO: 637 ± 287 nm; R-EO: 783 ± 69 nm; TT-EO: 1079 ± 59 nm; M-EO: 1515 ± 116 nm; B-EO: 2172 ± 813 nm; E-EO:2201 ± 110 nm; L-EO: 3531 ± 204 nm.

3. Discussion

The results of numerous studies indicate that EOs are a promising alternative to antibiotics thanks to their broad spectrum of antimicrobial activity and unspecific mode of action, which correlates with low risk of microbial resistance emergence [19,20]. As highly lipophilic substances, EOs bind to and disrupt the integrity of microbial cell walls and membrane structures, resulting in cell lysis [21]. It is reported that EOs may also exhibit such other mechanisms of action against biofilms as blocking the quorum-sense system, inhibiting the transcription of flagellar genes, interfering with bacterial motility, reducing the bacterial adherence to inert surfaces, increasing the oxidative stress in microbial cells, and blocking the productions of enzymes [22].

Essential oils are characterized by complex and variable composition, high volatility, and poor water solubility [23]. There are many factors which have impacts on the molecular composition of EOs, including seasonal climatic variations, intraspecies variability, and the method of oil extraction [24]. It has been found that two or three major classes of substances (and their concentrations) determine EOs biological activity to the major extent [23]. EOs contain a high level of phenolic compounds, e.g., carvacrol, eugenol, and thymol, which are substances with a proven, strong antibacterial effect [25]. Therefore, in the first part of the study we have evaluated the composition of each EO and confirmed the presence of compounds of antimicrobial activity (Table S1). It is noteworthy that vapour forms of EOs are reported to have higher antimicrobial effect than the liquid fractions [26–29]. It is suggested that lipophilic molecules in the EOs' aqueous phase associate, form micelles, and restrain the attachment of EOs to microorganisms. The vapour phase is devoid of this disadvantage that allows antimicrobials to be easily released and to strongly attach to microbial structures [30]. Due to the aforementioned volatility and water immiscibility of EOs, evaluation of their antimicrobial activity using in vitro assays displays certain limitations. Therefore, we have analysed antibacterial effectiveness of EOs' both fractions and have compared results using differentiated methodological approaches. In the present study, the antimicrobial activity of seven essential oils has been investigated against fourteen clinical and two reference *S. aureus* strains. The strains forming biofilm in the most robust manner have been selected (Figure 1) for further analyses. First, the evaluation of antimicrobial activity of liquid and vapour fractions of EOs was performed using disc diffusion method and inverted Petri dish assay, respectively. Results of the inverted Petri dish assay demonstrated that the strongest antimicrobial activity was displayed by the liquid phases of T-EO and R-EO; a moderate effect was shown for TT-EO, M-EO; and the weakest effect for B-EO, E-EO and L-EO (Figure 2, Table 1). In turn, Chao et al., analysing antibacterial effect of EOs against a MRSA reference strain, indicated the following (in decreasing order) activity of liquid fractions of these substances: T-EO > TT-EO > R-EO = L-EO > B-EO > E-EO [31]. The discrepancies of results presented by Chao et al. with outcomes presented in this work (concerning difference in activity of R-EO but not T-EO) may be caused by the different volume of used EOs, resulted in various level of diffusion of these substances through the agar medium. It is noteworthy that other studies have also confirmed the significant antimicrobial activity of T-EO liquids against clinical and reference MRSA and MSSA strains [32–35], similar to the results presented in this study. The zones of growth inhibition of reference and clinical staphylococcal strains after exposure to T-EO liquid fractions, presented in work of Tohidpour et al., where the disc diffusion method was applied, ranged from 12 to 35 mm, while Kryvtsova et al., using a well diffusion assay, observed formation of zones of 45–66 mm diameter [36,37]. Contrary to these results and

results shown in this work, Mardafkan et al. have presented no activity of T-EO liquid phases against *S. aureus* [38]. The different thymol content—the main T-EO's compound responsible for antimicrobial activity—may be the reason standing behind the observed differences in outcomes. T-EO, applied in the research of Mardafkan et al., contained thymol in concentrations of 30% and benzene in concentrations of 14%, whereas T-EO applied in our study comprised a higher concentration of thymol (44%) and p-cymene (27%) but not benzene (Table S1). Lemos et al. have shown that T-EO where the thymol concentration was equal to 53% exerted an MIC against *S. aureus* equal to 0.02 mg/mL, while the 40% concentration of thymol, corresponded with an MIC value of 0.17 mg/mL [39].

Similarly, the results obtained for liquid phases of EOs when vapour phases were also analysed showed that the greatest zones of microbial growth inhibition were detected for T-EO. No zones of growth inhibition were demonstrated for B-EO and L-EO (Figure 2, Table 2). These results cannot be directly compared with results from other studies due to diversity of Inverted Petri Dish assay protocols applied. Nevertheless, the anti-staphylococcal efficacy of vapour phases of thyme oil has been proven in numerous studies [40–47]. On the other hand, some studies have also indicated significant activity of TT-EO, E-EO, B-EO, L-EO vapour against *S. aureus* [40,42,47–49], whereas other studies are consistent with our results [40,41]. Differences in applied methodologies such as volume and concentration of oil used, addition of oil solvents, testing several samples on the same plate, paper disc diameter, or agar surface height, are suggested to explain the discrepancies in outcomes. As it has been reported in our previous study, all these factors may have a significant impact on the diameter of the obtained zone of growth inhibition [50]. The above conclusion is also strongly upheld by the data provided by Aber et al. who showed dose-dependent antibacterial activity of essential oils' vapour fractions [51].

In the subsequent step of our investigation, we have evaluated antibacterial effect of liquid fractions of EO emulsions on planktonic and biofilm cells of *S. aureus* using standard microdilution methods. Tween 20 was used as non- ionic surfactant to enhance solubilization and reduce evaporation of EOs [52]. According to our previous research, addition of 0.5% (*v/v*) Tween 20 improved anti-staphylococcal activity of TT-EO liquid fractions [53]. As it is presented in Figure S1 in Supplementary Materials, Tween 20 did not inhibit growth of *S. aureus* planktonic cells in the concentrations used for emulsion preparation. The liquid fractions of all tested EOs emulsions effectively inhibited growth of *S. aureus* planktonic forms (Table 3). The lowest (most favourable outcome) MIC—minimal inhibitory concentration—values were obtained for T-EO and M-EO emulsions. Numerous studies have confirmed our results [54–56]. In case of antibiofilm activity of EOs liquid phases, T-EO and TT-EO emulsions were the most potent ones. B-EO, R-EO, and M-EO emulsions eradicated biofilms of particular strains, whereas E-EO and L-EO emulsions were inactive (Table 3). Except for T-EO, MBEC (minimal biofilm eradication concentration) values of EOs were higher than MIC. Such results stay in line with generally accepted fact of protective function of biofilm matrix resulting, among others, in increased tolerance of cells on antimicrobial substances [57]. Oussalah et al. and Horváth et al. have reported the same MIC values against reference *S. aureus* strain as these presented in this research for T-EO and M-EO emulsions, respectively [54,58]. It is noteworthy that the major components and their concentrations in T-EO applied by these research teams were comparable (Table S1). Similarly, in our previous research, the same MIC and MBEC values of non-emulsified T-EO have been recorded against *S. aureus* reference strain. Considering the above, it is suggested that an emulsifier, if applied in an appropriate concentration, does not increase the oil's liquid fraction antimicrobial activity. As mentioned before, thymol is thought to be the compound accountable for T-EO properties. Li et al. have indicated that, in case of thymol, surfactant addition may even reduce its antimicrobial activity [59]. One of proposed explanations of this phenomenon is the trapping thymol by the surfactant at the micelle oil-water interface. This phenomenon reduces the soluble thymol content in the aqueous phase. However, low Tween 20 concentration in our samples may explain why reduction of activity was not observed. It has been reported that thymol's activity

depends on the bacterial species that is applied against and physical properties of the molecule. The presence of the hydroxyl group and a system of delocalized electrons plays an important role in the antimicrobial activity of thymol (and its isomer—carvacrol), by disturbing bacterial membrane functions, altering lipid barrier, depleting ATP, and finally, causing bacterial cell dead, [60]. This complex mechanism may be responsible for observed antibiofilm effect of T-EO emulsion against several *S. aureus*, when applied concentrations of T-EO were equal of these defined as minimal inhibitory ones (Table 3, Table S2). Gömöri et al. have demonstrated that minimal bactericidal concentration (MBC) of T-EO against MRSA and MSSA reference strains were only 2-fold higher than MIC values [61]. In our study, liquid fractions of TT-EO emulsions exerted antibiofilm effect in concentration ranges from 6.3 to 0.8% (*v/v*) (Table 3). These results are consistent with results of our previous studies in which we observed that liquid fractions of the EOs are able to reduce viability of staphylococcal biofilm cells in microdilution assays [50,53]. Feng et al. have indicated complete removal of mature MRSA biofilm after the exposure to 0.32% TT-EO [62]. In turn, MBEC and MBC-B values of liquid fractions of emulsified R-EO, M-EO, and B-EO differed with regard to staphylococcal strains they were applied against (Table 3). Analysing influence of R-EO on biofilm of clinical MSSA and MRSA clinical and reference strains, other researchers revealed analogical trends to these shown in this work [8,63]. Nazir et al. have found that B-EO in 5% (*v/v*) concentration inhibited 20% staphylococcal biofilm cells, whereas concentrated oil was able to inhibit 55% of these cells [64]. No MBEC value was also obtained when oil concentration of 50 µL/mL was applied [65]. Kifer et al. have found that MBEC value of menthol, a main component of M-EO, ranged from 3.21 to 6.35 mg/mL against MRSA and MSSA clinical and reference strains [66]. In the same study MBEC values of the component predominating in E-EO (1,8-cineole) were in the concentration range from 128 to 254 mg/mL [66]. These data stay in line with results from our study in which we showed that M-EO emulsions liquid fractions display higher antibiofilm activity than E-EO (Table 3). On the other hand, Merghni et al. have demonstrated staphylococcal biofilm reduction above 80% after the treatment with liquid phases of 0.2 mg/mL E-EO as well as 0.2 mg/mL 1,8-cineole [67]. Liquid phases of L-EO emulsions tested in this research effectively eradicated only biofilm of a *S. aureus* ATCC 6538 strain at minimal concentration equal to 1.6% (*v/v*) (Table 3). Research provided by other teams have indicated a 3-fold higher MBEC value against a MRSA reference strain and MBEC equal to 12.5 µL/mL against the susceptible ones [65,68]. The low reduction of biofilms formed by clinical MRSA and reference MSSA strains was demonstrated after the exposure to liquid fractions of L-EO, linalyl acetate, and linalool [69,70]. To gain broader insight into phenomena analysed, we have performed tests of antibiofilm activity of non-emulsified liquid and volatile fractions of EOs against selected strains of *S. aureus* using another set of methodological settings. We have applied that recently developed and modified A.D.A.M. (antibiofilm dressing's activity measurement) assay and AntiBioVol (antibiofilm activity of volatile compounds) methodology for the assessment of liquid and volatile fractions, respectively. Both models use agar as a surface for biofilm culturing and provide semi-quantitative type of data. According to the results received in the modified A.D.A.M. assay, liquid fractions of all essential oils possessed the ability to reduce viability of staphylococcal biofilms. The T-EO and R-EO were the most effective ones (Figure 3). Antibiofilm activity of non- emulsified T-EO and E-EO liquid fractions was assessed in our previous study with a similar methodology, though the oil-soaked dressing was applied directly on biofilm cells [71]. The previous study also demonstrated about 60% and 50% reduction of biofilm cells viability for T-EO and E-EO, respectively [71]. The outcomes of AntiBioVol assay have revealed that only volatile fractions of B-EO reduced viability of biofilms of all tested strains (Figure 4). Antibiofilm activity of vapour of other EOs was strain-dependent. Interestingly, volatile fractions of all EOs possessed the ability to decrease level of metabolically active biofilm cells of SA ATCC 6538 strain (and cell number in case of four oils), whereas the level of metabolic viability and cell number of SA 5 biofilm cells increased after exposure to each tested oil (Figure 4). As the analyses were

performed in high number of repeats and displayed high cohesion, this specific result additionally underlines inter-species variability in answer of staphylococcal strains to exposure to specific EOs. It also shows importance of using the appropriate number of strains for evaluation of EOs activity to not omit such important phenomena. Finally, the impact of liquid fractions of T-EO emulsion on SA ATCC 6538 biofilm cells viability and membrane integrity was evaluated (Figures 5 and 6). The significant drop of metabolic activity occurred after exposure to T-EO concentration >0.1% (*v/v*) with maintaining level of staphylococcal cell integrity (at level of ~35%) was observed. Considering the fact that liquid fractions of T-EO emulsion demonstrated bactericidal activity in concentration 0.4% (*v/v*) (Table S2), it may be assumed that the oil affects bacterial cells not only by targeting membrane but also via other mechanisms of more bacteriostatic nature. It was recently proposed that thymol may exert antibiofilm activity by inhibiting virulence factors such as PIA and hemolysin synthesis [72] and to act in similar manner as its isomer, carvacrol, by affecting genes coding for quorum sensing process [60].

In the present study, the broad spectrum of methods for the assessment of EOs' antimicrobial activity was applied. In case of non-biofilm forms of microbial communities, the significantly lower activity of liquid fractions of TT-EO, B-EO, E-EO, M-EO, and L-EO has been demonstrated using disc diffusion method comparing to the MIC assay. The discrepancies may result from the fact that usability of disc diffusion technique for such lipophilic substances as essential oils is limited. Furthermore, application of the emulsifier in MIC assessment may have improved the efficacy of EOs components.

No antibiofilm activity of E-EO and L-EO emulsions' liquid fractions has been indicated with use of microdilution method, whereas reduction of biofilm cells' viability after exposure to these non- emulsified EOs was demonstrated using modified A.D.A.M. methodology. It should be stressed that different surfaces for biofilm formation (polystyrene in MBEC assay and agar in the modified A.D.A.M. test) may influence biofilm adhesion, density and contribute to level of EOs' effectiveness. Moreover, in MBEC assay EOs emulsions are in constant contact with bacterial cells, while in A.D.A.M. assay, EOs are gradually released from biocellulose discs to the medium in which bacteria are immersed in. In turn, rate of EOs components release from biocellulose may have an effect on eradication activity of particular EOs.

There were no zones of growth inhibition obtained with inverted Petri dish method after the exposure of *S. aureus* cells to B-EO, though significant reduction of biofilm cells viability after exposure to this EO was measured with AntiBioVol test. More potent activity of B-EO's vapour fractions against mature biofilm than against microbial planktonic cells suggests that activity of this oil may affect various stages of biofilm life-cycle. As it was mentioned, numerous factors have an impact on inverted Petri dish method's accuracy. Furthermore, in AntiBioVol technique EOs are applied directly under the entire biofilm surface, while in inverted Petri dish EOs vapour are spread over the 90-mm Petri dish. Different volumes of EOs used in both assays are also of high impact for obtained results.

The strongest antimicrobial and antibiofilm activity were determined for thyme oil. Rosemary and menthol mint oils also displayed significant anti-staphylococcal activity. The main components of the oils (thymol in thyme oil, 1,8-cineole in rosemary oil, and menthol in menthol mint oil) are, to a major extent, accountable for their activity. Studies indicated that the antimicrobial effect of monoterpenes such as (+)-menthol and thymol is partially observed due to the disruption of the lipid fraction of the plasma membrane, causing a changed permeability and leakage of intracellular materials [73]. The research of Li et al. [74] showed that 1,8-cineole changed the shape and size of the bacterial cell (for both Gram-negative and Gram-positive bacteria). In addition, bacterial cells treated with this compound underwent apoptosis, because they showed a strong condensation of nuclear chromatin located in the central part of the nucleoplasm [74]. 1,8-cineole is the component also presented in eucalyptus oil; it may be assumed that better antimicrobial activity of rosemary oil is the result of synergistic action of eucalyptol and other compounds of the oil, as was suggested by Bajalan et al. [75]

In this work, we applied a broad spectrum of analytical techniques to assess the impact of EOs on staphylococcal biofilm. The reason behind this agenda was the fact that there are numerous variables related to this seemingly easy-to-perform research, including variances in EOs' composition, intraspecies variability, and crucial differences in methodological approaches (related to fraction of EO tested, type of surface used for growth, and the various volumes of EOs used, to name just the most important ones). It should be mentioned that such an approach is increasingly being applied, especially in the studies on the impact of various antimicrobials against biofilms [76]. Therefore, we hypothesized that application of prolific in vitro techniques allows us to overcome these challenges and indicates that antibiofilm activity of specific (or some) EOs prevail over others when the abovementioned specific methodologies are applied as a whole. Indeed, the data obtained in this study indicates T-EO (consisting mostly of thymol) as the most potent one. It is noteworthy that Multu-Inglok et al. [77] presented the reverse correlation between the size of emulsified EO and its antimicrobial activity (the smaller the size of droplets, the higher the antimicrobial potential). In our study, the emulsified T-EO droplets were of the lowest, 637 nm diameter, while the size of emulsified L-EO droplets (which displayed low antimicrobial activity in majority of tests) were over 5 times bigger (3531 nm), confirming data presented by above-mentioned research team. It explicitly shows that the number of variables that should be taken under consideration during analysis of interactions of EOs with microorganisms (in biofilm form, especially) may be even higher than previously assumed. Nevertheless, bearing in mind all limitations of the in vitro study, we believe that this conclusion may be of pivotal meaning in subsequent lines of investigation and potential application of thymol in the character of new, antimicrobial agent used to fight against staphylococcal biofilm-based infections.

4. Materials and Methods

4.1. Microorganisms and Culture Conditions

Two reference strains, *Staphylococcus aureus* 6538 and 33591 from the American Type and Culture Collection (ATCC) and fourteen clinical isolates from bone (7 strains) and wound infections (7 strains) were analysed in this study. Three strains from each group were MSSA strains (methicillin-susceptible *Staphylococcus aureus*), and four were MRSA strains (methicillin-resistant *Staphylococcus aureus*). The list of the strains is presented in Table 4. The strains are part of the Strain and Line Collection of Pharmaceutical Microbiology and Parasitology Department of the Medical University of Wroclaw. All clinical strains applied in this study are part of collection of strains of Department of Pharmaceutical Microbiology and Parasitology of Wroclaw Medical University. The strains were obtained in year 2016 during the internal Wroclaw Medical University SUB. D198.16.001 project: "The insight into biofilm-related properties of clinical microorganisms and possibilities of their eradication". All patients provided a written consent to participate in the trial and allowed the material obtained during the study (exudate, bioptates, microorganisms) to be used for scientific purposes. The study was approved by the Bioethical Committee of Wroclaw Medical University, protocol # 8/2016.

Table 4. Type and origin of clinical strains used in the study.

Wound Infection Strains		Bone Infection Strains	
MSSA	**MRSA**	**MSSA**	**MRSA**
SA 2	SA 26	SA 6	SA 32
SA 4	SA 27	SA 7	SA 33
SA 5	SA 28	SA 10	SA 34
	SA 29		SA 35

All strains were cultured overnight before the experiments at 37 °C in Tryptic Soy Broth medium (TSB, Biomaxima, Lublin, Poland). Subsequently, 0.5 McFarland suspensions were

established in saline (NaCl, Stanlab, Lublin, Poland) using a densitometer (Densilameter II Erba Lachema, Brno, the Czech Republic) and applied in each test.

4.2. Essential Oils

In this study, the antimicrobial activity of volatile and liquid fractions of EOs was investigated. Due to the volatility of EOs, individual essential oils and control settings were examined on separate plates.

The following seven commercial EOs were examined in this study:

- Thyme oil (T-EO, obtained from *Thymus vulgaris* L. herb) was purchased from Etja, Elblag, Poland;
- Tea tree oil (TT-EO, obtained from *Melaleuca alternifolia* Cheel. leaves) was purchased from Pharmatech, Zukowo, Poland;
- Basil oil (B-EO, obtained from *Ocimum basilicum* L. leaves and flowers) was purchased from Nanga, Zlotow, Poland;
- Rosemary oil (R-EO, obtained from *Rosmarinus officinalis* L. flowering shoots) was purchased from Nanga, Zlotow, Poland;
- Eucalyptus oil (E-EO, *obtained from Eucalyptus globulus* Labill. leaves and twigs) was purchased from Pharmatech, Zukowo, Poland;
- Lavender oil (L-EO, obtained from *Lavandula angustifolia* Mill. flowering herb) was purchased from Kej, Cirkowice, Poland;
- Menthol mint oil (M-EO, obtained from *Mentha arvensis* L. leaves) was purchased from Optima Natura, Grodki, Poland.

4.3. GC-MS (Gas Chromatography Mass Spectrometry) Analysis of the Tested EOs Composition

4.3.1. Essential Oil Preparation

Essential oils (EO) were diluted with hexane (JTB, GB), vortexed, and immediately analysed. All analyses were performed in triplicate.

4.3.2. GC-MS Analysis

Analysis was performed using Agilent 7890B GC system coupled with 7000GC/TQ system connected to PAL RSI85 autosampler (Agilent Technologies, Palo Alto, CA, USA). The column used was HP-5 MS; 30 m × 0.25 mm × 0.25 µm (J&W, Agilent Technologies, Palo Alto, CA, USA) with helium used as a carrier gas at the total flow of 1 mL/min. Chromatographic conditions were as follows: split injection in a ratio 100:1, the injector was set on 250 °C, oven temperature program was: 50 °C held for 1 min, then 4 °C/min up to 130 °C, 10 °C/min to 280 °C, and then isothermal for 2 min. The MS detector operated in the electronic impact ionization mode at 70 eV, transfer line, source, and quadrupole temperatures were set at 320, 230, and 150 °C, respectively. Masses were registered in a range from 30 to 400 *m/z*. Peaks were identified in MassHunter Workstation Software Version B.08.00 coupled with the NIST17 mass spectra library and accomplished by comparison with linear retention indexes. The relative abundance of each EO constituent was expressed as percentage content based on the peak area normalization. Due to the obtained results, none of the analysed EOs is of pharmacopeial grade. However, the analysis was not performed in accordance with normalization procedure from Polish Pharmacopea XI (different column and different temperature program).

4.4. Assessment of Biofilm Biomass Level Using Crystal Violet Assay

To assess total biofilm mass, crystal violet staining was applied. In the first step, 100 µL of all 0.5 MF bacterial suspensions diluted 1000 times in TSB (Tryptic Soy Broth, Biomaxima, Lublin, Poland) medium were added to the wells of 96-well plates (Wuxi Nest Biotechnology, Wuxi, China) and incubated under static conditions for 24 h at 37 °C. After the supernatant fluid was removed, the plate was dried for 10 min (37 °C), and the biofilm was dyed with 100 µL of 20% (*v/v*) crystal violet solution (Aqua-med, Lodz, Poland). The plate was kept at RT for 10 min, biofilm cells were washed twice with 100 µL of saline,

and the plate was returned to the incubator (37 °C) for 10 min once more. Subsequently, 100 µL of 30% (*v/v*) acetic acid (Chempur, Piekary Slaskie, Poland) solution was poured into the wells, and the plate was stirred for 30 min at 350 rpm shaker (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). The plate's content was transferred to a fresh 96-well plate, and absorbance was measured at 550 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). The experiment was performed twice with six replicates.

4.5. Assessment of Biofilm Activity Level Using TTC Staining

Biofilm culturing was performed as described in the crystal violet assay. Subsequently, tetrazolium chloride assay was carried out as follows: 100 µL of 0.1% (*w/v*) TTC solution (2,3,5-triphenyl-tetrazolium chloride, AppliChem Gmbh, Damstadt, Germany) in TSB (Tryptic Soy Broth, Biomaxima, Lublin, Poland) was added to the cells for 2 h (37 °C). The suspension was aspirated, the plate was dried (10 min/37 °C), and 100 µL of methanol was added (Stanlab, Lublin, Poland). The plate was shaken (30 min at 350 rpm), and the solution was carried to the fresh wells of a 96-well plate. Absorbance was measured at 490 nm. The experiment was performed twice with six replicates.

4.6. Evaluation of the Antimicrobial Activity of All EOs Using Disc Diffusion Method and Inverted Petri Dish Method

The experiments were performed using Mueller–Hinton agar (Biomaxima, Lublin, Poland) plates (90 mm diameter, 14.2 mm height, Noex, Komorniki, Poland). The agar layer was 5 mm thick. Standard paper discs (diameter of 6 mm, 0.5 mm of thickness) were placed in a 48-well plate (Thermo Fisher Scientific, Waltham, MA, USA), soaked with 0.2 mL of each EOs or saline (as control of bacterial growth) (NaCl, Stanlab, Lublin, Poland), wrapped with tape and kept refrigerated for 30 min. The abovementioned suspensions of all strains (two references and fourteen clinical) (0.5 McFarland) were cultured onto the plates. To evaluate the antimicrobial activity of liquid fractions of all EOs, the paper discs were placed onto the agar, while to assess the activity of vapour fractions onto the plate lids. The plates were sealed with parafilm and incubated for 24 h at 37 °C. Microbial growth inhibition zones were measured (in mm) afterwards using a ruler. Zones of partial growth inhibition were evaluated (in mm) if no total inhibition was observed. In the case of unequal zones, a shorter diameter was included. Each experimental setting was performed in triplicate, and the mean diameter was calculated.

4.7. Evaluation of Minimal Inhibitory and Minimal Biofilm Eradication Concentrations of Liquid Fractions of EOs Emulsions

The minimal inhibitory concentration (MIC) and minimal biofilm eradication concentration (MBEC) were assessed for liquid fractions of all EOs against each tested strain. There were three replicates performed in two separate repetitions.

Due to the poor solubility of EOs in a water medium, Tween 20 (Zielony Klub, Kielce, Poland) was used as an emulsifier. Each dilution of the EOs was prepared in a separate 15 mL falcon tube (Flmedica, Padova, Italy). First, 2-fold dilution of EO was performed in Tryptic Soy Broth (TSB, Biomaxima, Lublin, Poland) with 1% (*v/v*) Tween 20 and vortexed (Micro-shaker type 326 m, Premed, Marki, Poland) for 30 min/RT. Other geometric dilutions were prepared in TSB and vortexed for 30 s. For the minimal inhibitory concentration (MIC) purpose, the 0.5 MacFarland suspensions of all strains were diluted 1000× in TSB, and 100 µL were added to 96-well plates (Jet Bio-Filtration Co. Ltd., Guanzhou, China). Subsequently, 100 µL of EOs emulsions were poured, and the plates were incubated for 24 h at 37 °C with continuous shaking at 350 rpm (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). After incubation, 20 µL of 1% (*w/v*) TTC (2,3,5-triphenyl-tetrazolium chloride, AppliChem Gmbh, Damstadt, Germany) solution in TSB was added, incubation has proceeded for 2 h under the same conditions. Absorbance was measured at wavelength λ = 580 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland) before and right after the incubation with EOs. In the MBEC assay (minimal biofilm eradication concentration), the microorganisms' suspensions were prepared likewise, and 100 µL were

poured into the wells of 96-well plates and 100 µL of TSB was added. The plates were incubated for 24 h/37 °C to form biofilm. Next, the medium was replaced with 200 µL of EOs emulsions (in the concentration range 50–0.02% (*v/v*)), and the plates were incubated again for 24 h at 37 °C. EOs emulsions were removed, 200 µL of 0.1% (*w/v*) TTC solution in TSB was poured into the biofilm wells, and the plates were incubated for 2 h at 37 °C. The medium was replaced with the same volume of methanol (Stanlab, Lublin, Poland) and glacial acetic acid solution (Chempur, Piekary Slaskie, Poland) (in a ratio of 9:1), and the plates were shaken at 350 rpm for 30 min at RT. A total of 100 µL was transferred to fresh 96-well plates and absorbance was measured at 490 nm wavelength. The MIC and MBEC (%) (*v/v*) values were assessed as the first well where no colour was observed after incubation with TTC. In both assays, controls of microorganisms' growth and controls of medium sterility were applied. Moreover, the antimicrobial activity of Tween 20 (in the concentration range 1–0.002% (*v/v*)) against *S. aureus* 6538 planktonic forms was investigated.

Additionally, the content of the wells corresponding to MBEC values was transferred to glass tubes with 5 mL of TSB medium and incubated overnight at 37 °C. The MBC-B (minimal bactericidal concentration for biofilm) values were assessed in the tubes where no visible growth was observed.

Based on the MIC and MBEC results, three clinical strains the most susceptible to the liquid fractions of EOs and two reference *S. aureus* strains were selected for further antimicrobial experiments.

4.8. Evaluation of Antibiofilm Activity of All Non-Emulsified EOs' Liquid Fractions Using Modified A.D.A.M. (Antibiofilm Dressing's Activity Measurement) Assay

The assay was a modification of the protocol presented in our previous study [78]. The following steps of the experiment were performed:

4.8.1. Biofilm Plugs Preparation

Brain Heart Infusion Broth (BHI, Biomaxima, Lublin, Poland) and 2% (*w/v*) of Bacteriological Lab Agar (Biomaxima, Lublin, Poland) were used for filling the wells of a 24-well plate (further referred to as Plate 1) (Wuxi Nest Biotechnology, Wuxi, China) to half their height. The plate was left for agar solidification. Subsequently, agar plugs 8 mm in diameter were cut out of each well using a cork-borer. The plugs were divided into two equal parts. One part of the divided plugs was discarded. The second part was placed in a new 24-well plate (later referred to as Plate 2). The selected strains' suspensions, prepared as described in the Section 4.1, were then diluted one thousand times in Tryptic Soy Broth medium (TSB, Biomaxima, Lublin, Poland), and 2 mL was added to the plugs—containing wells of Plate 2 and incubated at 37 °C for 24 h. During the incubation, biofilm was formed at the top of the plugs.

4.8.2. Treatment with EOs

To assess the antibiofilm activity of all tested EOs, biocellulose dressings were prepared as follows:

A *Komagataeibacter xylinus* ATCC 53524 strain was cultured stativity in Herstin–Schramm (H-S) medium for 7 days at 28 °C for cellulose production. The medium was composed of 2% (*w/v*) glucose (Chempur, Piekary Slaskie, Poland), 0.5% (*w/v*) yeast extract (VWR, Radnor, PA, USA), 0.5% (*w/v*) bacto-peptone (VWR, Radnor, PA, USA), 0.115% (*w/v*) citric acid monohydricum (POCH, Gliwice, Poland), 0.27% (*w/v*) Na_2HPO_4 (POCH, Gliwice, Poland), 0.05% (*w/v*) $MgSO_4 \cdot 7H_2O$ (POCH, Gliwice, Poland), and 1% (*v/v*) ethanol (Chempur, Piekary Slaskie, Poland). Subsequently, the bacteria were removed from the cellulose by shaking. To obtain 14 mm biocellulose (BC) discs, 1 mL of H-S medium was poured into the wells of a 24-well plate (Wuxi Nest Biotechnology, Jiangsu, China). The wells were inoculated with the released bacteria and incubated for 7 days/28 °C. Afterwards, the BC discs were taken out, cleansed with 0.1 M NaOH (Chempur, Piekary Slaskie, Poland) at 80 °C, and rinsed with double-distilled water until neutral pH was reached.

Finally, the BC discs were sterilized in an autoclave. Six BC discs were weighed, dried for 24 h at 37 °C, and weighed again. The average volume of water in discs was approximately 0.76 g. The BC discs were soaked with 1 mL of EOs or saline (positive control) or 96% (*v/v*) ethanol (for the reference strains only) and incubated for 24 h at 4 °C. The concentration of substances absorbed into the BC discs was calculated by the formula:

$$\text{Compound concentration (\%)} = [EV/((WBC - DBC) + EV)] * 100$$

EV—a volume of essential oil (mL).
WBC—the weight of wet BC disc (g).
DBC—the weight of dry BC disc (g).

Once the plugs were covered with biofilm (Plate 2), they were placed in the empty agar hollows of Plate 1, and 120 µL of TSB (Tryptic Soy Broth medium, Biomaxima, Lublin, Poland) was added to fill up the hollows' space. The EOs/saline/ethanol-containing BC discs were placed on the top of the wells of the Pate 1. The plate was sealed with tape and incubated for 24 h/37 °C.

4.8.3. Viability Measurement

As incubation was completed, the BC discs and the medium over the biofilm were removed. A total of 1 mL of 0.1% (*w/v*) solution of tetrazolium chloride (TTC, 2,3,5-triphenyl-tetrazolium chloride, AppliChem Gmbh, Damstadt, Germany) in TSB was added for 2 h (37 °C) (Plate 3). Next, the solution was gently removed, and 1 mL of methanol (Stanlab, Lublin, Poland): acetic acid (Chempur, Piekary Slaskie, Poland) mixture (9:1) was added. The plate was shaken for 30 min/400 rpm at RT. From each well, three samples for 100 µL were transferred to 96-well plates (Jet Bio-Filtration Co. Ltd., Guanzhou, China), and absorbance was measured at 490 nm with a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). Each EO and control were tested in six replicates. Compared to the biofilms treated with NaCl, reduction in biofilm metabolic activity was defined as a percentage.

4.9. Evaluation of Antibiofilm Activity of All Non-Emulsified EOs' Volatile Fractions Measured with AntiBioVol Assay (Antibiofilm Activity of Volatile Compounds)

AntiBioVol test was performed as demonstrated in our previous paper [50]. The following steps of the experiment were similar to the modified A.D.A.M. assay and were conducted as follows:

4.9.1. Biofilm Plugs Preparation

The first step of the experiment was performed similarly as in the modified A.D.A.M. methodology, but the wells of a 24-well plate (Plate 1) were filled with the BHI agar to the full. Moreover, one part of the divided plugs was replaced to the agar hollows of Plate 1, and the plate was kept at 8 °C. The second part was used for biofilm culturing (Plate 2). The same strains as in the modified A.D.A.M. assay were tested.

4.9.2. Treatment with EOs

In the next step of the experiment, the biofilm plugs were gently transferred to Plate 1 and put on the biofilm-free plugs. All tested, undiluted essential oils were poured into a separate 24-well plate (later referred to as Plate 3) in volume 0.5 mL. Plate 1 was put upside down on Plate 3 (the agar wells were set directly above the EOs wells). The plates were sealed with tape and incubated at 37 °C for 24 h. For growth control, a 0.9% solution of NaCl (Stanlab, Lublin, Poland) was applied instead of EOs. Furthermore, the antimicrobial activity of 96% (*v/v*) ethanol (Stanlab, Lublin, Poland) was tested against the reference strains.

4.9.3. Viability Measurement

After the exposure to the tested EOs, the upper plugs were gently transferred to a fresh 24-well plate (Plate 4), poured over with 2 mL of 0.1% (*w/v*) solution of tetrazolium chloride TTC (2,3,5-triphenyl-tetrazolium chloride, AppliChem Gmbh, Damstadt, Germany) in TSB and incubated for 2 h/37 °C. The solution was gently aspirated, and 2 mL of methanol (Stanlab, Lublin, Poland) and glacial acetic acid solution (Chempur, Piekary Slaskie, Poland) (9:1 ratio) was added. The plate was agitated at RT for 30 min/400 rpm using a shaker (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). Three samples for 200 µL were transferred to 96-well plates (Jet Bio-Filtration Co. Ltd., Guanzhou, China) from each well, and absorbance measurement was performed at 490 nm wavelength using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). Each EO and control were examined in six replicates. Compared to the biofilms treated with NaCl, a reduction in biofilm metabolic activity (representing metabolically active cells) was defined as a percentage.

Based on the results obtained by the AntiBioVol method, two strains (the reference strain ATCC 6538 and strain referred to as SA 5) and four EOs (T-EO, E-EO, M-EO, and L-EO) were chosen for cell number quantification. The AntiBioVol assay was re-conducted, although instead of the TTC staining step, quantitative culturing was performed. For this purpose, the agar plugs treated for 24 h with volatile fractions of EOs were transferred to 1 mL of a 0.1% (*w/v*) saponin (VWR, Leuven, Belgium) solution and agitated for 30 s using a vortex mixer (Micro-shaker type 326 m, Premed, Marki, Poland). Subsequently, the serial dilutions of the suspension were cultured onto Mueller–Hinton agar (Biomaxima, Lublin, Poland) and Petri dishes (Noex, Komorniki, Poland), and then incubated for 24 h at 37 °C; finally, the CFU number was counted. The samples exposed to EOs were compared to grow control (0.9% NaCl) samples. The test was performed in three replicates.

Moreover, vapour fractions of R-EO were applied against *S. aureus* ATCC 6538 biofilm and analysed using 3D confocal microscopy. The data applied for volumetric visualization was obtained using SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) and processed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

4.10. Fluorescence Microscopy of Biofilms Visualised with Use of LIVE/DEAD Staining

Antibiofilm activity of liquid fractions of thyme oil (T-EO) emulsion against *S. aureus* ATCC 6538 strain was examined using a fluorescence microscope (Etaluma lumascope 600, San Diego, CA, USA). Biofilm culturing and treatment with thyme oil emulsions in concentrations (*v/v*): 0.4%, 0.2%, 0.1%, 0.02%, 0.01%, and 0.006% was carried out as described in the MBEC assay. The cells exposed to 0.1% octenidine and 2% phenoxyethanol solution (Octenisept, Schulke, Wien, Austria) were used as the negative control, whereas untreated cells were used as a positive control. Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Thermo Fischer Scientific, Waltham, MA, USA) prepared according to manufacturer's instruction was applied as a dye to assess membrane integrity. A total of 10 µL of the reagent was added to each well of a 96-well plate (Jet Bio-Filtration Co. Ltd., Guanzhou, China) for 15 min (RT, darkness). Next, the cells were washed once with 200 µL of double-distilled water, and the plate was dried for 15 min at 37 °C. Biofilms were then analysed using a fluorescence microscope Etaluma 600 (an object lens with magnification 20×).

4.11. Analysis on the Size of EOs Emulsion Droplets

The analysis on size of emulsion's droplets (the hydrodynamic diameter) were performed by dynamic light scattering (DLS) method using Zetasizer Nano ZS ZEN3600 device (Malvern Instruments, Malvern, UK). The 1000× diluted samples of 1 mL volume were introduced to disposable polystyrene cuvettes, equilibrated at 25 °C and determined with the detection angle of 173°. Data were acquired in automatic mode. The results were presented in the form of average droplets' diameter (nm), and droplets size distribution.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10091207/s1, Figure S1: Influence of different concentrations of Tween 20 [(%) (*v/v*)] on planktonic forms of *S. aureus* ATCC 6538 strain. C+ untreated cells., Table S1: Ingredients of tested EOs measured with GC-MS (Gas Chromatography Mass Spectrometry), Table S2: The MBC-B [(%) (*v/v*)] (minimal bactericidal concentration for biofilm) values of EOs emulsions.

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References

1. Hall, C.W.; Mah, T.F. Molecular Mechanisms of Biofilm-Based Antibiotic Resistance and Tolerance in Pathogenic Bacteria. *FEMS Microbiol. Rev.* **2017**, *41*, 276–301. [CrossRef]
2. dos Santos Ramos, M.A.; da Silva, P.B.; Spósito, L.; de Toledo, L.G.; vidal Bonifácio, B.; Rodero, C.F.; dos Santos, K.C.; Chorilli, M.; Bauab, T.M. Nanotechnology-Based Drug Delivery Systems for Control of Microbial Biofilms: A Review. *Int. J. Nanomed.* **2018**, *13*, 1179–1213. [CrossRef]
3. Schultz, G.; Bjarnsholt, T.; James, G.A.; Leaper, D.J.; McBain, A.J.; Malone, M.; Stoodley, P.; Swanson, T.; Tachi, M.; Wolcott, R.D. Consensus Guidelines for the Identification and Treatment of Biofilms in Chronic Nonhealing Wounds. *Wound Repair Regen.* **2017**, *25*, 744–757. [CrossRef]
4. Jamal, M.; Ahmad, W.; Andleeb, S.; Jalil, F.; Imran, M.; Nawaz, M.A.; Hussain, T.; Ali, M.; Rafiq, M.; Kamil, M.A. Bacterial Biofilm and Associated Infections. *J. Chin. Med. Assoc.* **2018**, *81*, 7–11. [CrossRef]
5. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential Oils, A New Horizon in Combating Bacterial Antibiotic Resistance. *Open Microbiol. J.* **2014**, *8*, 6–14. [CrossRef] [PubMed]
6. Becerril, R.; Nerín, C.; Gómez-Lus, R. Evaluation of Bacterial Resistance to Essential Oils and Antibiotics after Exposure to Oregano and Cinnamon Essential Oils. *Foodborne Pathog. Dis.* **2012**, *9*, 699–705. [CrossRef] [PubMed]
7. Chaves, T.P.; Pinheiro, R.E.E.; Melo, E.S.; Soares, M.J.D.S.; Souza, J.S.N.; de Andrade, T.B.; de Lemos, T.L.G.; Coutinho, H.D.M. Essential oil of *Eucalyptus Camaldulensis* Dehn Potentiates β-Lactam Activity against *Staphylococcus Aureus* and *Escherichia coli* Resistant Strains. *Ind. Crops Prod.* **2018**, *112*, 70–74. [CrossRef]
8. Ghellai, L.; Hassaine, H.; Khelil, N.K.; Nas, F.; Aissaoui, N.; Hoceini, A.; Ziouane, S.; Zing, W. Antibacterial Efficacy of Essential Oils of Three Aromatic Plants in Combination with Povidone-Iodine against Staphylococcus Aureus Grown in Biofilm Cultures. *J. Appl. Pharm. Sci.* **2014**, *4*, 88–93. [CrossRef]
9. Builders, P.F. Herbal Medicine. In *Herbal Medicine*; Builders, P.F., Ed.; IntechOpen: London, UK, 2019; pp. 122–138. [CrossRef]
10. Pérez-Recalde, M.; Ruiz Arias, I.E.; Hermida, É.B. Could Essential Oils Enhance Biopolymers Performance for Wound Healing? A Systematic Review. *Phytomedicine* **2018**, *38*, 57–65. [CrossRef]
11. Dursun, N.; Liman, N.; Özyazgan, I.; Güneş, I.; Saraymen, R. Role of Thymus Oil in Burn Wound Healing. *J. Burn Care Rehabil.* **2003**, *24*, 395–399. [CrossRef]
12. Lawal, T.O.; Soni, K.K.; Adeniyi, B.A.; Doyle, B.J.; Mahady, G.B. Botanicals for Adjunct Therapy and the Treatment of Multidrug-Resistant Staphylococcal Infections. *Fight. Multidrug Resist. Herb. Extr. Essent. Oils Compon.* **2013**, 135–147. [CrossRef]
13. Hriouech, S.; Akhmouch, A.A.; Mzabi, A.; Chefchaou, H.; Tanghort, M.; Oumokhtar, B.; Chami, N.; Remmal, A. The Antistaphylococcal Activity of Amoxicillin/Clavulanic Acid, Gentamicin, and 1,8-Cineole Alone or in Combination and Their Efficacy through a Rabbit Model of Methicillin-Resistant Staphylococcus Aureus Osteomyelitis. *Evid.-Based Complementary Altern. Med.* **2020**, *2020*, 4271017. [CrossRef]
14. Chircov, C.; Miclea, I.I.; Grumezescu, V.; Grumezescu, A.M. Essential Oils for Bone Repair and Regeneration—Mechanisms and Applications. *Materials* **2021**, *14*, 1867. [CrossRef]
15. Baptista-Silva, S.; Borges, S.; Ramos, O.L.; Pintado, M.; Sarmento, B. The Progress of Essential Oils as Potential Therapeutic Agents: A Review. *J. Essent. Oil Res.* **2020**, *32*, 279–295. [CrossRef]

16. Reichling, J.; Schnitzler, P.; Suschke, U.; Saller, R. Essential Oils of Aromatic Plants with Antibacterial, Antifungal, Antiviral, and Cytotoxic Properties—An Overview. *Forsch. Komplement.* **2009**, *16*, 79–90. [CrossRef] [PubMed]
17. Adorjan, B.; Buchbauer, G. Biological Properties of Essential Oils: An Updated Review. *Flavour Fragr. J.* **2010**, *25*, 407–426. [CrossRef]
18. Pereira dos Santos, E.; Nicácio, P.H.M.; Coêlho Barbosa, F.; Nunes da Silva, H.; Andrade, A.L.S.; Lia Fook, M.V.; Farias Leite, I. Chitosan/Essential Oils Formulations for Potential Use as Wound Dressing: Physical And. *Materials* **2019**, *12*, 2223. [CrossRef]
19. Chouhan, S.; Sharma, K.; Guleria , S. Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives. *Medicines* **2017**, *4*, 58. [CrossRef]
20. Wińska, K.; Mączka, W.; Łyczko, J.; Grabarczyk, M.; Czubaszek, A.; Szumny, A. Essential Oils as Antimicrobial Agents—Myth or Real Alternative? *Molecules* **2019**, *24*, 2130. [CrossRef]
21. Sakkas, H.; Papadopoulou, C. Antimicrobial Activity of Basil, Oregano, and Thyme Essential oils. *J. Microbiol. Biotechnol.* **2017**, *27*, 429–438. [CrossRef]
22. Nuță, D.C.; Limban, C.; Chiriță, C.; Chifiriuc, M.C.; Costea, T.; Ioniță, P.; Nicolau, I.; Zarafu, I. Contribution of Essential Oils to the Fight against Microbial Biofilms—A Review. *Processes* **2021**, *9*, 537. [CrossRef]
23. Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological Effects of Essential Oils—A Review. *Food Chem. Toxicol.* **2008**, *46*, 446–475. [CrossRef]
24. Langeveld, W.T.; Veldhuizen, E.J.A.; Burt, S.A. Synergy between Essential Oil Components and Antibiotics: A Review. *Crit. Rev. Microbiol.* **2014**, *40*, 76–94. [CrossRef] [PubMed]
25. Dhifi, W.; Bellili, S.; Jazi, S.; Bahloul, N.; Mnif, W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines* **2016**, *3*, 25. [CrossRef]
26. Inouye, S.; Takizawa, T.; Yamaguchi, H. Antibacterial Activity of Essential Oils and Their Major Constituents against Respiratory Tract Pathogens by Gaseous Contact. *J. Antimicrob. Chemother.* **2001**, *47*, 565–573. [CrossRef] [PubMed]
27. Fisher, K.; Phillips, C. Potential Antimicrobial Uses of Essential Oils in Food: Is Citrus the Answer? *Trends Food Sci. Technol.* **2008**, *19*, 156–164. [CrossRef]
28. Tullio, V.; Nostro, A.; Mandras, N.; Dugo, P.; Banche, G.; Cannatelli, M.A.; Cuffini, A.M.; Alonzo, V.; Carlone, N.A. Antifungal Activity of Essential Oils against Filamentous Fungi Determined by Broth Microdilution and Vapour Contact Methods. *J. Appl. Microbiol.* **2007**, *102*, 1544–1550. [CrossRef]
29. Goñi, P.; López, P.; Sánchez, C.; Gómez-Lus, R.; Becerril, R.; Nerín, C. Antimicrobial Activity in the Vapour Phase of a Combination of Cinnamon and Clove Essential Oils. *Food Chem.* **2009**, *116*, 982–989. [CrossRef]
30. Nadjib, B.M.; Amine, F.M.; Abdelkrim, K.; Fairouz, S.; Maamar, M. Liquid and Vapour Phase Antibacterial Activity of Eucalyptus Globulus Essential Oil = Susceptibility of Selected Respiratory Tract Pathogens. *Am. J. Infect. Dis.* **2014**, *10*, 105–117. [CrossRef]
31. Chao, S.; Young, G.; Oberg, C.; Nakaoka, K. Inhibition of Methicillin-Resistant Staphylococcus Aureus (MRSA) by Essential Oils. *Flavour Fragr. J.* **2008**, *23*, 444–449. [CrossRef]
32. Patterson, J.E.; McElmeel, L.; Wiederhold, N.P. In Vitro Activity of Essential Oils against Gram-Positive and Gram-Negative Clinical Isolates, Including Carbapenem-Resistant Enterobacteriaceae. *Open Forum Infect. Dis.* **2019**, *6*, 1–4. [CrossRef]
33. Vasile, C.; Sivertsvik, M.; Miteluț, A.C.; Brebu, M.A.; Stoleru, E.; Rosnes, J.T.; Tănase, E.E.; Khan, W.; Pamfil, D.; Cornea, C.P.; et al. Comparative Analysis of the Composition and Active Property Evaluation of Certain Essential Oils to Assess Their Potential Applications in Active Food Packaging. *Materials* **2017**, *10*, 45. [CrossRef]
34. Soković, M.; Glamočlija, J.; Marin, P.D.; Brkić, D.; van Griensven, L.J.L.D. Antibacterial Effects of the Essential Oils of Commonly Consumed Medicinal Herbs Using an in Vitro Model. *Molecules* **2010**, *15*, 7532–7546. [CrossRef]
35. Uzair, B.; Niaz, N.; Bano, A.; Khan, B.A.; Zafar, N.; Iqbal, M.; Tahira, R.; Fasim, F. Essential Oils Showing in Vitro Anti MRSA and Synergistic Activity with Penicillin Group of Antibiotics. *Pak. J. Pharm. Sci.* **2017**, *30*, 1997–2002.
36. Tohidpour, A.; Sattari, M.; Omidbaigi, R.; Yadegar, A.; Nazemi, J. Antibacterial Effect of Essential Oils from Two Medicinal Plants against Methicillin-Resistant Staphylococcus Aureus (MRSA). *Phytomedicine* **2010**, *17*, 142–145. [CrossRef]
37. Kryvtsova, M.V.; Salamon, I.; Koscova, J.; Bucko, D.; Spivak, M. Antimicrobial, Antibiofilm and Biochemichal Properties of Thymus Vulgaris Essential Oil against Clinical Isolates of Opportunistic Infections. *Biosyst. Divers.* **2019**, *27*, 270–275. [CrossRef]
38. Mardafkan, N.; Iranmanesha, M.; Larijani, K.; Mahasti, P.; Nazari, F.; Zojaji, M. Chemical Components and Antibacterial Activities of Essential Oils Obtained From Iranian Local Lavandula Officinalis and Thymus Vulgaris Against Pathogenic Bacteria Isolated From Human. *J. Food Biosci. Technol.* **2015**, *5*, 31–36.
39. Lemos, M.F.; Lemos, M.F.; Pacheco, H.P.; Guimarães, A.C.; Fronza, M.; Endringer, D.C.; Scherer, R. Seasonal Variation Affects the Composition and Antibacterial and Antioxidant Activities of Thymus Vulgaris. *Ind. Crops Prod.* **2017**, *95*, 543–548. [CrossRef]
40. Voravuthikunchai, S.P.; Minbutra, S.; Goodla, L.; Jefferies, J.; Voravuthikunchai, S. Mixtures of Essential Oils in an Air Conditioning Prototype to Reduce the Prevalence of Airborne Pathogenic Bacteria. *J. Essent. Oil-Bear. Plants* **2012**, *15*, 739–749. [CrossRef]
41. Ács, K.; Bencsik, T.; Böszörményi, A.; Kocsis, B.; Horváth, G. Essential Oils and Their Vapors as Potential Antibacterial Agents against Respiratory Tract Pathogens. *Nat. Prod. Commun.* **2016**, *11*, 1709–1712. [CrossRef]
42. Frankova, A.; Kloucek, P.S.J.; Nedorostova, L. Testing the Antimicrobial Activity of Essential Oils. *Acta Agrar. Debr.* **2011**, *44*, 71–74. [CrossRef]

43. Nedorostova, L.; Kloucek, P.; Urbanova, K.; Kokoska, L.; Smid, J.; Urban, J.; Valterova, I.; Stolcova, M. Antibacterial Effect of Essential Oil Vapours against Different Strains of Staphylococcus Aureus, Including MRSA. *Flavour Fragr. J.* **2011**, *26*, 403–407. [CrossRef]
44. Dobre, A.A.; Gagiu, V.; Petru, N. Antimicrobial Activity of Essential Oils against Food-Borne Bacteria Evaluated by Two Preliminary Methods. *Rom. Biotechnol. Lett.* **2011**, *16*, 119–125.
45. Ghabraie, M.; Vu, K.D.; Tata, L.; Salmieri, S.; Lacroix, M. Antimicrobial Effect of Essential Oils in Combinations against Five Bacteria and Their Effect on Sensorial Quality of Ground Meat. *LWT Food Sci. Technol.* **2016**, *66*, 332–339. [CrossRef]
46. López, P.; Sánchez, C.; Batlle, R.; Nerín, C. Vapor-Phase Activities of Cinnamon, Thyme, and Oregano Essential Oils and Key Constituents against Foodborne Microorganisms. *J. Agric. Food Chem.* **2007**, *55*, 4348–4356. [CrossRef]
47. Nedorostova, L.; Kloucek, P.; Kokoska, L.; Stolcova, M.; Pulkrabek, J. Antimicrobial Properties of Selected Essential Oils in Vapour Phase against Foodborne Bacteria. *Food Control* **2009**, *20*, 157–160. [CrossRef]
48. Lisin, G.; Safiyev, S.; Craker, L.E. Antimicrobial Activity of Some Essential Oils. *Acta Hortic.* **1999**, *501*, 283–287. [CrossRef]
49. Andrade, B.F.M.T.; Barbosa, L.N.; Alves, F.C.B.; Albano, M.; Rall, V.L.M.; Sforcin, J.M.; Fernandes, A.A.H.; Fernandes, A. The Antibacterial Effects of Melaleuca Alternifolia, Pelargonium Graveolens and Cymbopogon Martinii Essential Oils and Major Compounds on Liquid and Vapor Phase. *J. Essent. Oil Res.* **2016**, *28*, 227–233. [CrossRef]
50. Brożyna, M.; Żywicka, A.; Fijałkowski, K.; Gorczyca, D.; Oleksy-Wawrzyniak, M.; Dydak, K.; Migdał, P.; Dudek, B.; Bartoszewicz, M.; Junka, A. The Novel Quantitative Assay for Measuring the Antibiofilm Activity of Volatile Compounds (Antibiovol). *Appl. Sci.* **2020**, *10*, 7343. [CrossRef]
51. Abers, M.; Schroeder, S.; Goelz, L.; Sulser, A.; st. Rose, T.; Puchalski, K.; Langland, J. Antimicrobial Activity of the Volatile Substances from Essential Oils. *BMC Complementary Med. Ther.* **2021**, *21*, 124. [CrossRef]
52. Martin, M.J.; Trujillo, L.A.; Garcia, M.C.; Alfaro, M.C.; Muñoz, J. Effect of Emulsifier HLB and Stabilizer Addition on the Physical Stability of Thyme Essential Oil Emulsions. *J. Dispers. Sci. Technol.* **2018**, *39*, 1627–1634. [CrossRef]
53. Dudek-Wicher, R.; Paleczny, J.; Kowalska-Krochmal, B.; Szymczyk-Ziółkowska, P.; Pachura, N.; Szumny, A.; Brożyna, M. Activity of Liquid and Volatile Fractions of Essential Oils against Biofilm Formed by Selected Reference Strains on Polystyrene and Hydroxyapatite Surfaces. *Pathogens* **2021**, *10*, 515. [CrossRef]
54. Murbach Teles Andrade, B.F.; Nunes Barbosa, L.; da Silva Probst, I.; Fernandes Júnior, A. Antimicrobial Activity of Essential Oils. *J. Essent. Oil Res.* **2014**, *26*, 34–40. [CrossRef]
55. Sakkas, H.; Economou, V.; Gousia, P.; Bozidis, P.; Sakkas, V.A.; Petsios, S.; Mpekoulis, G.; Ilia, A.; Papadopoulou, C. Antibacterial Efficacy of Commercially Available Essential Oils Tested against Drug-Resistant Gram-Positive Pathogens. *Appl. Sci.* **2018**, *10*, 2201. [CrossRef]
56. Hussain, A.I.; Anwar, F.; Nigam, P.S.; Ashraf, M.; Gilani, A.H. Seasonal Variation in Content, Chemical Composition and Antimicrobial and Cytotoxic Activities of Essential Oils from Four Mentha Species. *J. Sci. Food Agric.* **2010**, *90*, 1827–1836. [CrossRef]
57. Sadekuzzaman, M.; Mizan, M.F.R.; Kim, H.S.; Yang, S.; Ha, S.-D. Activity of Thyme and Tea Tree Essential Oils against Selected Foodborne Pathogens in Biofilms on Abiotic Surfaces. *LWT Food Sci. Technol.* **2018**, *89*, 134–139. [CrossRef]
58. Horváth, P.; Koščová, J. In Vitro Antibacterial Activity of Mentha Essential Oils against Staphylococcus Aureus. *Folia Vet. I* **2017**, *61*, 71–77. [CrossRef]
59. Li, J.; Chang, J.W.; Saenger, M.; Deering, A. Thymol Nanoemulsions Formed via Spontaneous Emulsification: Physical and Antimicrobial Properties. *Food Chem.* **2017**, *232*, 191–197. [CrossRef] [PubMed]
60. Kachur, K.; Suntres, Z. The Antibacterial Properties of Phenolic Isomers, Carvacrol and Thymol. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 3042–3053. [CrossRef] [PubMed]
61. Gömöri, C.; Vidács, A.; Kerekes, E.B.; Nacsa-Farkas, E.; Böszörményi, A.; Vágvölgyi, C.; Krisch, J. Altered Antimicrobial and Anti-Biofilm Forming Effect of Thyme Essential Oil Due to Changes in Composition. *Nat. Prod. Commun.* **2018**, *13*, 483–487. [CrossRef]
62. Feng, C.H.; Yu, L.; Zhen, Y.; Yanan, M.O.; Ruofeng, S.; Baocheng, H.; Xuehong, W.; Jianping, L. Effect of Tea Tree Oil on Methicillin-Resistant Staphylococcus Aureus Biofilm. *Sci. Technol. Food Ind.* **2021**, *42*, 107–112. [CrossRef]
63. Endo, E.H.; Costa, G.M.; Makimori, R.Y.; Ueda-Nakamura, T.; Nakamura, C.V.; Dias Filho, B.P. Anti-Biofilm Activity of Rosmarinus Officinalis, Punica Granatum and Tetradenia Riparia against Methicillin-Resistant Staphylococcus Aureus (MRSA) and Synergic Interaction with Penicillin. *J. Herb. Med.* **2018**, *14*, 48–54. [CrossRef]
64. Nazir, U.; Javaid, S.; Awais, H.; Bashir, F.; Shahid, M. Biochemical Potential and Screening of Bioactive Components of Ocimum Basilicum. *Pure Appl. Biol.* **2021**, *10*, 1004–1013. [CrossRef]
65. Ivopol, M.; Vlăsceanu, G.; Dune, A.; Popa, M.; Măruţescu, L.; Ionescu, D.; Ivopol, G.-C.; Crişan, I.; Chifiriuc, C. In Vitro Investigation of Some Plant Essential Oils for Potential Antimicrobial Activity. In *The National Congress of Pharmacy*, 17th ed.; Filodiritto Editore: Bucharest, Romania, 2018; p. 9.
66. Kifer, D.; Mužinić, V.; Klaric, M.Š. Antimicrobial Potency of Single and Combined Mupirocin and Monoterpenes, Thymol, Menthol and 1,8-Cineole against Staphylococcus Aureus Planktonic and Biofilm Growth. *J. Antibiot.* **2016**, *69*, 689–696. [CrossRef] [PubMed]

67. Merghni, A.; Noumi, E.; Hadded, O.; Dridi, N.; Panwar, H.; Ceylan, O.; Mastouri, M.; Snoussi, M. Assessment of the Antibiofilm and Antiquorum Sensing Activities of Eucalyptus Globulus Essential Oil and Its Main Component 1,8-Cineole against Methicillin-Resistant Staphylococcus Aureus Strains. *Microb. Pathog.* **2018**, *118*, 74–80. [CrossRef]
68. Kavanaugh, N.L.; Ribbeck, K. Selected Antimicrobial Essential Oils Eradicate Pseudomonas Spp. And Staphylococcus Aureus Biofilms. *Appl. Environ. Microbiol.* **2012**, *78*, 4057–4061. [CrossRef]
69. Blažeković, B.; Yang, W.; Wang, Y.; Li, C.; Kindl, M.; Pepeljnjak, S.; Vladimir-Knežević, S. Chemical Composition, Antimicrobial and Antioxidant Activities of Essential Oils of Lavandula × intermedia 'Budrovka' and L. Angustifolia Cultivated in Croatia. *Ind. Crops Prod.* **2018**, *123*, 173–182. [CrossRef]
70. Kot, B.; Wierzchowska, K.; Grużewska, A.; Lohinau, D. The Effects of Selected Phytochemicals on Biofilm Formed by Five Methicillin-Resistant Staphylococcus Aureus. *Nat. Prod. Res.* **2018**, *32*, 1299–1302. [CrossRef]
71. Junka, A.; Żywicka, A.; Chodaczek, G.; Dziadas, M.; Czajkowska, J.; Duda-Madej, A.; Bartoszewicz, M.; Mikołajewicz, K.; Krasowski, G.; Szymczyk, P.; et al. Potential of Biocellulose Carrier Impregnated with Essential Oils to Fight Against Biofilms Formed on Hydroxyapatite. *Sci. Rep.* **2019**, *9*, 1–13. [CrossRef]
72. Valliammai, A.; Selvaraj, A.; Yuvashree, U.; Aravindraja, C.; Karutha Pandian, S. SarA-Dependent Antibiofilm Activity of Thymol Enhances the Antibacterial Efficacy of Rifampicin Against Staphylococcus Aureus. *Front. Microbiol.* **2020**, *11*, 1–13. [CrossRef]
73. Trombetta, D.; Castelli, F.; Sarpietro, M.G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano, G. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* **2005**, *49*, 2474–2478. [CrossRef]
74. Bajalan, I.; Rouzbahani, R.; Ghasemi Pirbalouti, A.; Maggi, F. Antioxidant and antibacterial activities of the essential oils obtained from seven Iranian populations of Rosmarinus officinalis. *Ind. Crops Prod.* **2017**, 305–311. [CrossRef]
75. Li, L.; Li, Z.W.; Yin, Z.Q.; Wei, Q.; Jia, R.Y.; Zhou, L.J.; Xu, J.; Song, X.; Zhou, Y.; Du, Y.H.; et al. Antibacterial activity of leaf essential oils and its constituents from Cinnamomum longe paniculatum. *Int. J. Clin. Exp. Med.* **2014**, *7*, 1721–1727. [PubMed]
76. Maillard, J.Y.; Kampf, G.; Cooper, R. Antimicrobial stewardship of antiseptics that are pertinent to wounds: The need for a united approach. *JAC Antimicrob. Resist.* **2021**, *3*, dlab027. [CrossRef]
77. Mutlu-Ingok, A.; Firtin, B.; Karbancioglu-Guler, F.; Altay, F. A study on correlations between antimicrobial effects and diffusion coefficient, zeta potential and droplet size of essential oils. *Int. J. Food Eng.* **2020**, *16*, 20190354. [CrossRef]
78. Junka, A.F.; Żywicka, A.; Szymczyk, P.; Dziadas, M.; Bartoszewicz, M.; Fijałkowski, K.A.D.A.M. Test (Antibiofilm Dressing's Activity Measurement)—Simple Method for Evaluating Anti-Biofilm Activity of Drug-Saturated Dressings against Wound Pathogens. *J. Microbiol. Methods* **2017**, *143*, 6–12. [CrossRef] [PubMed]

Supplementary materials

The antimicrobial and antibiofilm i*n vitro* activity of liquid and vapour phases of selected Essential Oils against *Staphylococcus aureus*

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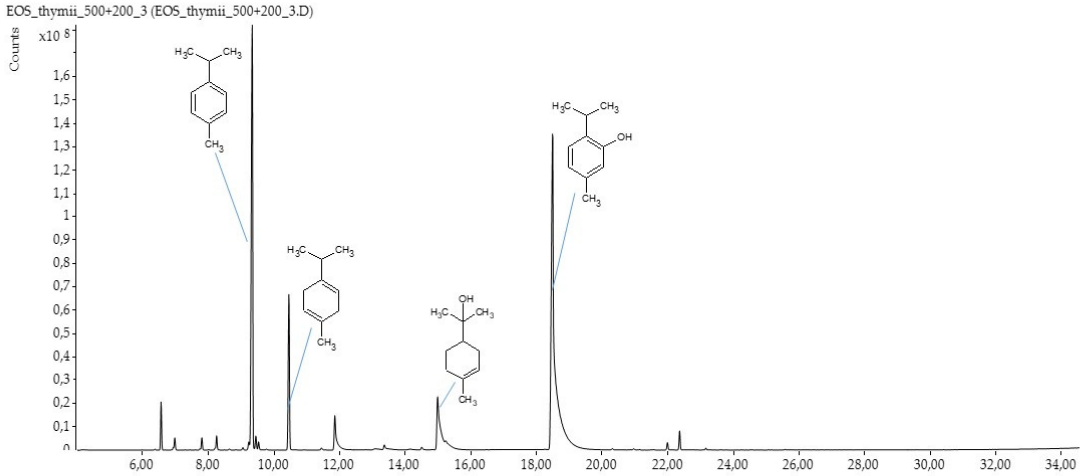
Table S1. Ingredients of tested EOs measured with GC-MS (Gas Chromatography Mass Spectrometry). Components which are in line with Polish Pharmakopea XI standards are marked green colour, the ones which are not in line are marked red. **RI** - retention index, **RT** – retention time

A. Thyme oil



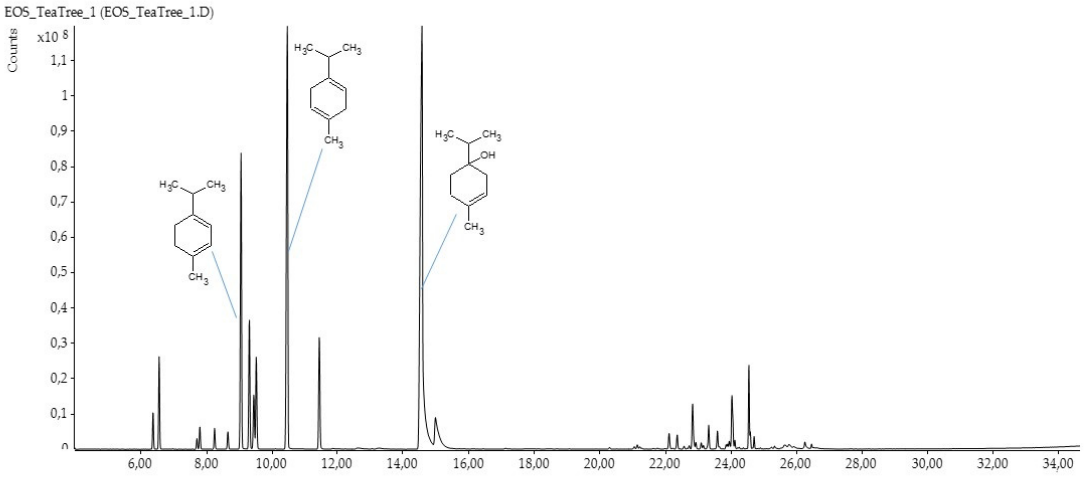
RI	RT	Compound	*T. vulgaris*
937	6.56	α-Pinene	2.20 ± 0.09
949	6.98	Camphene	0.73 ± 0.04
975	7.80	Sabinene	0.64 ± 0.03
979	8.25	β-Pinene	0.81 ± 0.05
1025	**9.31**	**p-Cymene**	**26.91 ± 0.99**
1028	9.45	Limonene	0.77 ± 0.04
1060	10.47	γ-Terpinene	8.60 ± 0.03
1096	11.85	Linalool	3.45 ± 0.15
1141	13.36	Camphor	0.66 ± 0.06
1189	14.99	α-Terpineol	7.84 ± 0.30
1289	**18.49**	**Thymol**	**44.00 ± 0.46**
1419	22.36	β-Caryophyllene	1.00 ± 0.05

Polish Pharmakopea XI ranges:

α– thujene: 0.2 per cent to 1.5 per cent
β-myrcene: 1.0 per cent to 3.0 per cent
α– terpinene: 0.9 per cent to 2.6 per cent
p- cymene: 14.0 per cent to 28.0 per cent
γ-terpinene: 4.0 per cent to 12.0 per cent
linalool: 1.5 per cent to 6.5 per cent
terpinen-4-ol: 0.1 per cent to 2.5 per cent
carvacrol methyl ether: 0.05 per cent to 1.5 per cent
thymol: 37.0 per cent to 55.0 per cent

carvacrol: 0.5 per cent to 5.5 per cent

B. Tea tree oil



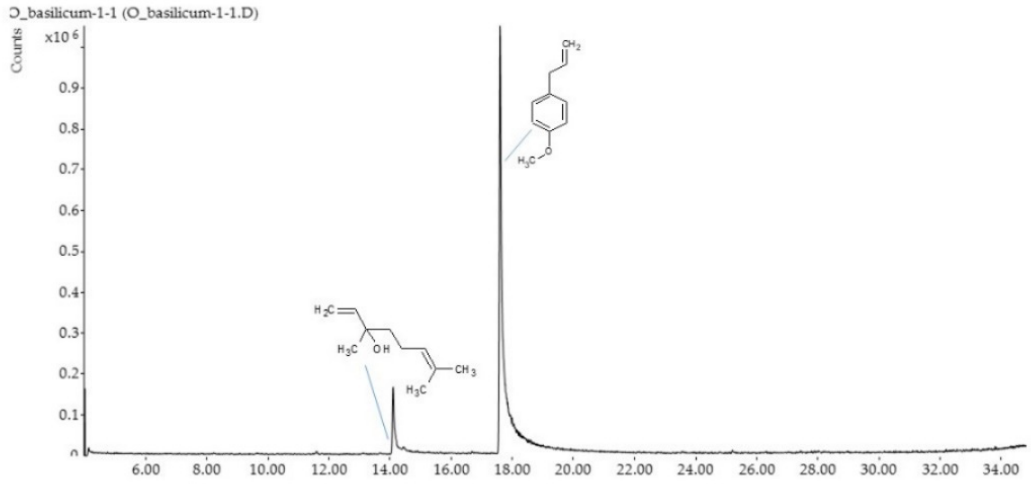
RI	RT	Compound	*M. alternifolia*
929	6.37	α-Thujene	1.11 ± 0.02
937	6.56	α-Pinene	2.85 ± 0.06
975	7.80	Sabinene	0.75 ± 0.02
979	8.25	β-Pinene	0.69 ± 0.01
1005	8.65	α-Phellandrene	0.61 ± 0.01
1017	**9.06**	**α-Terpinene**	**11.07 ± 0.17**
1025	9.31	p-Cymene	4.69 ± 0.07
1028	9.45	Limonene	2.08 ± 0.05
1031	9.52	1,8-Cineole	3.34 ± 0.06
1060	**10.47**	**γ-Terpinene**	**19.07 ± 0.27**
1088	11.44	α-Terpinolene	4.34 ± 0.06
1177	**14.58**	**Terpinen-4-ol**	**33.27 ± 0.79**
1189	14.99	α-Terpineol	3.26 ± 0.13
1419	22.36	β-Caryophyllene	0.53 ± 0.01
1440	22.83	Aromadendrene	1.83 ± 0.03
1460	23.32	Alloaromadendrene	0.82 ± 0.02
1496	24.03	Viridiflorene	2.35 ± 0.04
1518	24.55	β-Cadinene	2.78 ± 0.03

Polish Pharmakopea XI ranges:

α-pinene: 1.0 per cent to 6.0 per cent
sabinene: maximum 3.5 per cent
α– terpinene: 5.0 per cent to 13.0 per cent

limonene: 0.5 per cent to 4.0 per cent
cineole: maximum 15.0 per cent
γ-terpinene: 10.0 per cent to 28.0 per cent
p- cymene: 0.5 per cent to 12.0 per cent
terpinolene: 1.5 per cent to 5.0 per cent
terpinen-4-ol: minimum 30.0 per cent
aromadendrene: maximum 7.0 per cent
α– terpineol: 1.5 per cent to 8.0 per cent

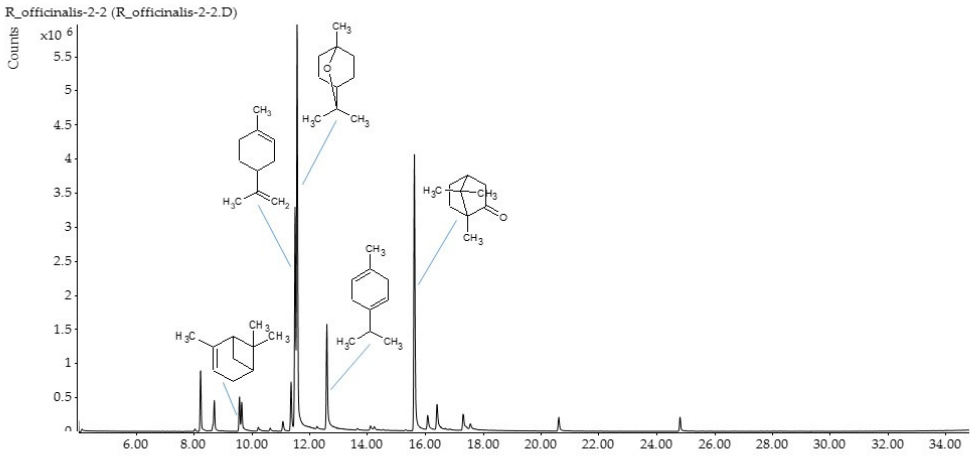
C. Basil oil



RI	RT	Compound	*O.basilicum*
1095	**14.08**	**Linalool**	**10.69±1.13**
1192	**17.59**	**Methyl chavicol [Estragole]**	**89.31±1.13**

Not included in Polish Pharmakopea XI

D. Rosemary oil



RI	RT	Compound	*R.officinalis*
896	8.22	Cyclofenchene	4.99±0.48
908	8.69	2-Bornene	3.00±0.29
924	9.57	β-Thujene	2.33±0.11
932	9.65	α-Pinene	2.58±0.28
988	10.22	Myrcene	0.38±0.14
1002	10.63	α-Phellandrene	0.23±0.07
1014	11.07	α-Terpinene	0.80±0.04
1022	11.35	o-Cymene	3.15±1.45
1024	**11.49**	**Limonene**	**14.26±0.99**
1026	**11.57**	**1,8-Cineole [Eucalyptol]**	**30.12±1.74**
1054	12.59	γ-Terpinene	8.21±0.35
1141	**15.64**	**Camphor**	**21.97±0.77**
1156	16.08	Isoborneol	1.53±0.06
1165	16.40	Borneol	2.69±0.09
1186	17.32	α-Terpineol	1.56±0.39
1284	20.61	Bornyl acetate	1.17±0.09
1417	24.80	Caryophyllene	0.85±0.21

Polish Pharmakopea XI ranges:

For rosemary oil, **Spanish type,** the percentages are within the following ranges:

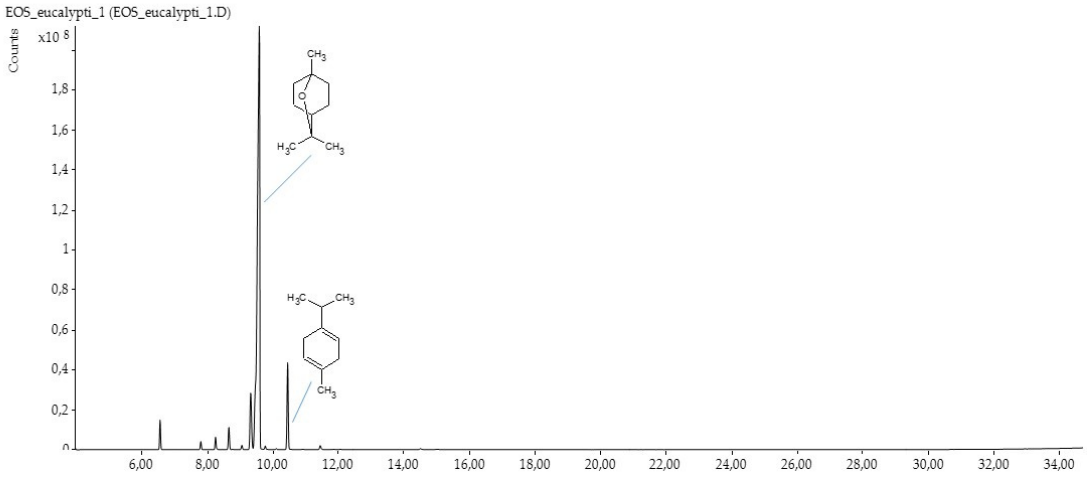
α-pinene: 18 per cent to 26 per cent
camphene: 8.0 per cent to 12.0 per cent
β-pinene: 2.0 per cent to 6.0 per cent
β-myrcene: 1.5 per cent to 5.0 per cent
limonene: 2.5 per cent to 5.0 per cent
cineole: 16.0 per cent to 25.0 per cent
p-cymene: 1.0 per cent to 2.2 per cent

camphor: 13.0 per cent to 21.0 per cent
bornyl acetate: 0.5 per cent to 2.5 per cent
α-terpineol: 1.0 per cent to 3.5 per cent
borneol: 2.0 per cent to 4.5 per cent
verbenone: 0.7 per cent to 2.5 per cent

For rosemary oil, **Moroccan and Tunisian type**, the percentages are within the following ranges:

α-pinene: 9.0 per cent to 14.0 per cent
camphene: 2.5 per cent to 6.0 per cent
β-pinene: 4.0 per cent to 9.0 per cent
β-myrcene: 1.0 per cent to 2.0 per cent
limonene: 1.5 per cent to 4.0 per cent
cineole: 38.0 per cent to 55.0 per cent
p-cymene: 0.8 per cent to 2.5 per cent
camphor: 5.0 per cent to 15.0 per cent
bornyl acetate: 0.1 per cent to 1.5 per cent
α-terpineol: 1.0 per cent to 2.6 per cent
borneol: 1.5 per cent to 5.0 per cent
verbenone: maximum 0.4 per cent

E. Eucalyptus oil

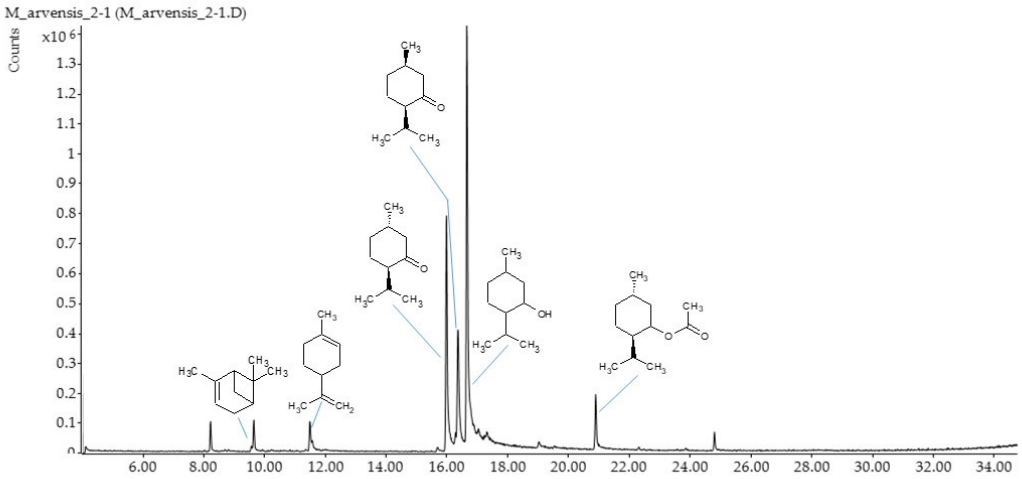


RI	RT	Compound	*E. globulus*
937	6.56	α-Pinene	2.30 ± 0.04
979	8.25	β-Pinene	1.09 ± 0.02
1005	8.65	α-Phellandrene	2.02 ± 0.02
1025	9.31	p-Cymene	6.89 ± 0.07
1031	**9.52**	**1,8-Cineole**	**79.10 ± 0.61**
1060	**10.47**	**γ-Terpinene**	**8.16 ± 0.07**

Polish Pharmakopea XI ranges

α-pinene: 0.05 per cent to 10.0 per cent
β-pinene: 0.05 per cent to 1.5 per cent
sabinene: maximum 0.3 per cent
α-phellandrene: 0.05 per cent to 1.5 per cent
limonene: 0.05 per cent to15.0 per cent
1,8-cineole: minimum 70.0 per cent
camphor: maximum 0.1 per cent

F. Menthol mint oil

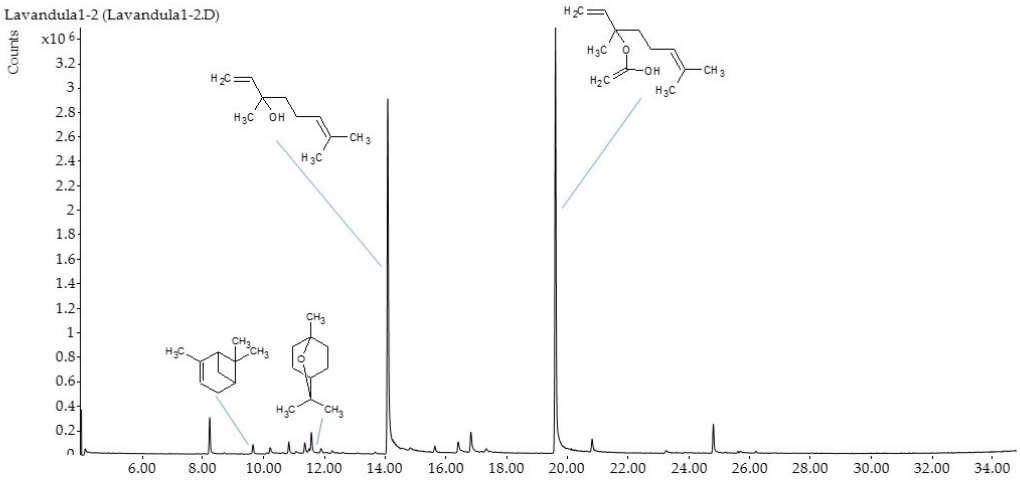


RI	RT	Compound	*M.arvensis*
896	8.22	Cyclofenchene	2.68±0.17
932	9.65	α-Pinene	3.01±0.17
1024	11.49	Limonene	3.78±0.27
1148	**15.98**	**Menthone**	**24.53±0.23**
1158	**16.36**	**Isomenthone**	**13.54±1.75**
1167	**16.66**	**Menthol**	**45.57±2.21**
1294	20.89	Menthyl acetate	5.61±0.36
1417	24.80	Caryophyllene	1.27±0.57

Polish Pharmakopea XI ranges:

limonene: 1.5 per cent to 7.0 per cent
cineole: maximum 1.5 per cent
menthone: 17.0 per cent to 35.0 per cent
isomenthone: 5.0 per cent to 13.0 per cent
menthyl acetate: 1.5 per cent to 7.0 per cent
isopulegol: 1.0 per cent to 3.0 per cent
menthol: 30.0 per cent to 50.0 per cent
pulegone: maximum 2.5 per cent
carvone: maximum 2.0 per cent
The ratio of cineole content to limonene content is less than 1.

G. Lavender oil



RI	RT	Compound	*L. angustifolia*
896	8.22	Cyclofenchene	3.66±0.35
932	9.65	α -Pinene	0.94±0.04
988	10.22	Myrcene	0.86±0.13
1008	10.83	3-Carene	1.19±0.12
1022	11.35	o-Cymene	1.19±0.12
1026	11.57	1,8-Cineole [Eucalyptol]	2.56±0.39
1095	**14.08**	**Linalool**	**37.76±1.18**
1141	15.64	Camphor	0.84±0.10

1165	16.40	Borneol	2.04±0.56
1174	16.82	Terpinen-4-ol	3.03±0.46
1254	**19.60**	**Linalyl acetate**	**41.13±0.40**
1288	20.81	Lavandulyl acetate	1.80±0.22
1417	24.80	Caryophyllene	3.47±0.66

Polish Pharmakopea XI ranges

limonene: maximum 1.0 per cent
1,8-cineole: maximum 2.5 per cent
3-octanone: 0.1 per cent to 5.0 per cent
camphor: maximum 1.2 per cent
linalool: 20.0 per cent to 45.0 per cent
linalyl acetate: 25.0 per cent to 47.0 per cent
terpinen-4-ol: 0.1 per cent to 8.0 per cent
lavandulyl acetate: minimum 0.2 per cent
lavandulol: minimum 0.1 per cent
α-terpineol: maximum 2.0 per cent

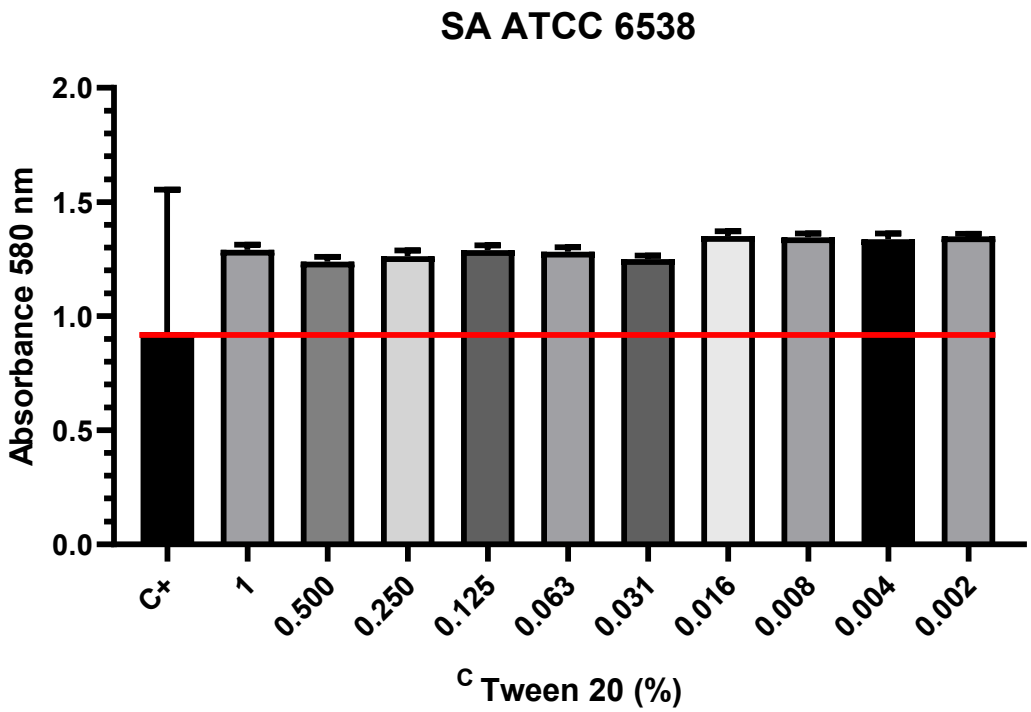


Figure S1. Influence of different concentrations of Tween 20 [(%) (v/v)] on planktonic forms of *S. aureus* ATCC 6538 strain. C+ untreated cells.

MBC-B (%)

T- EO		TT-EO		B-EO		R-EO		M-EO		L-EO	
Strain	**MBC-B**	**Strain**	**MBC-B**	**Strain**	**MBC-B**	**Strain**	**MBC-B**	**Strain**	**MBC-B**	**Strain**	**MBC-B**
SA 5	0.05	SA 2	25	SA 4	50	2	12.5	SA 32	50	SA 2	25
SA 6	0.1	SA 4	12.5	SA 29	50	4	6.3	SA 34	50		
SA 7	0.05	SA 5	25	SA 32	12.5	7	50	SA 35	50		
SA 10	0.05	SA 27	50	SA 34	25	27	12.5	SA ATCC 33591	25		
SA 26	0.05	SA 28	3.1	SA ATCC 33591	25	33	25	SA ATCC 6538	50		
SA 27	0.05	SA 32	6.3	SA ATCC 6538	12.5	34	25				
SA 32	0.1	SA 33	6.3			35	50				
SA 33	0.1	SA 34	6.3			SA ATCC 33591	12.5				
SA ATCC 6538	0.4	SA ATCC 33591	1.6								

Table S2. The MBC- B [(%) (v/v)] (minimal bactericidal concentration for biofilm) values of EOs emulsions. T-EO- thyme oil, TT-EO- tea tree oil, B- EO- basil oil, R-EO- rosemary oil, M-EO- menthol mint oil, L- EO- lavender oil

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Chemical Composition and Antibacterial Activity of Liquid and Volatile Phase of Essential Oils against Planktonic and Biofilm-Forming Cells of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen causing life-threatening, hard-to-heal infections associated with the presence of a biofilm. Essential oils (EOs) are promising agents to combat pseudomonal infections because of the alleged antimicrobial activity of their volatile fractions and liquid forms. Therefore, the purpose of this paper was to evaluate the antibacterial efficacy of both volatile and liquid phases of seven EOs (thyme, tea tree, basil, rosemary, eucalyptus, menthol mint, lavender) against *P. aeruginosa* biofilm and planktonic cells with the use of a broad spectrum of analytical in vitro methods. According to the study results, the antibacterial activity of EOs in their liquid forms varied from that of the volatile fractions. Overall, liquid and volatile forms of rosemary EO and tea tree EO displayed significant antibiofilm effectiveness. The outcomes indicate that these particular EOs possess the potential to be used in the therapy of *P. aeruginosa* infections.

Keywords: biofilm; *Pseudomonas aeruginosa*; essential oil; EOs in liquid form; volatile fractions; antimicrobial activity

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1. Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic bacterium responsible for a growing number of serious nosocomial infections. As an example, such different populations as patients suffering from chronic wounds, cystic fibrosis, catheter-related infections, or AIDS are at risk of developing a severe pseudomonal infection, accounting for high mortality and morbidity rates [1]. The production of surface factors, flagella, pili, lipopolysaccharide, toxin secretion and biofilm formation are considered primary virulence determinants of *P. aeruginosa* [2]. Biofilms are complex microbial communities in which cells display characteristic spatial localization and phenotypic and biochemical features differentiating them from their free-swimming (planktonic) counterparts. Cells in the biofilm are encased in extracellular polymeric substances (EPS), which serve as a scaffold for the structural integrity of the microbial community and as a barrier protecting cells from detrimental factors. The biofilm may form on living tissue and indwelling medical devices, including catheters, tracheal tubes, implants, etc. Cells in the biofilm are highly tolerant to antimicrobials (antibiotics, especially) and are resistant to host immune defense mechanisms [3]. Therefore, therapeutic options for the treatment of pseudomonal, biofilm-based infections are limited. Thus, numerous attempts have been made to devise novel strategies

for combating *Pseudomonas* infections [2]. Among them, the application of various kinds of phytochemical molecules has been reported to be a promising direction with regard to *P. aeruginosa* biofilm eradication and overcoming this bacteria's resistance to antibiotics [4].

One of the types of such phytochemical molecules is referred to as essential oils (EOs). These mixtures of plants' secondary metabolites belong mainly to groups of terpenes, sesquiterpenes, and phenylpropanoids. Their broad spectrum of biological activity covers antibacterial, antifungal, anti-inflammatory, antiviral, antioxidant, and anticancer properties. Overall, EO activity depends on the content of the constituents, which is, to a major extent, impacted by such variables as plant origin, cultivation conditions, and extraction techniques [5]. EOs are characterized by high lipophilicity and volatility. Due to the lipophilic nature, they exhibit an unspecific mode of antimicrobial action, which relies on binding to the cell wall or the membrane and damaging its integrity. Concerning this broad mechanism of action and already proven effectiveness against multidrug-resistant microorganisms/biofilms, EOs are suggested to be the alternative approach to combat bacterial infections [6]. However, the topical administration of EOs may cause allergic reactions and skin irritation; thus, undiluted EOs cannot be applied directly to body parts altered by the infection process [7]. From the manufacturing perspective, EOs' lipophilic properties also restrain the development of physically stable, non-harmful skin formulations [8]. Therefore, volatile fractions of EOs are more and more considered to be suitable for the treatment of specific bacterial infections (e.g., of the respiratory tract, skin, or wounds) [9].

The already performed research demonstrated that the volatile forms of EOs may display a stronger antimicrobial effect than their liquid phases used in direct contact [10]. It is suggested that hydrophobic molecules in the aqueous phase associate and form micelles, which hinder the attachment of EOs to microorganisms. The EOs' volatile fractions are devoided of this disadvantage [11]. In addition, because EOs' volatile fractions may be administered locally (not via systemic dosage), the risk of side effects and interactions with other drugs is exiguous [8]. However, the majority of research is still mainly focused on EOs' antimicrobial activity through direct contact with the oil in its liquid form. Only a limited number of studies on antimicrobial effectiveness of the volatile forms of EOs are available [12]. Therefore, the purpose of this paper was to evaluate the antibacterial potential of both volatile and liquid phases of seven commercially available EOs against *P. aeruginosa* biofilm and planktonic cells with the use of a broad spectrum of analytical in vitro methods.

2. Results

2.1. GC–MS (Gas Chromatography–Mass Spectrometry) Analysis of the Tested EOs Composition

The percentage composition of EOs' constituents was assessed using gas chromatography–mass spectrometry. The two main ingredients of thyme oil (T-EO) are thymol and p-cymene. Terpinen-4-ol and γ-terpinene predominate in tea tree oil (TT-EO). Basil oil (B-EO) is composed of methyl chavicol and linalool only. Camphor and 1,8-cineole are the main components of rosemary oil (R-EO), while 1,8-cineole and γ-terpinene are primarily presented in eucalyptus oil (E-EO). Menthol mint oil (MM-EO) contains mostly menthol and menthone, and linalyl acetate and linalool are the main ingredients of lavender oil (L-EO). The compositions of EOs were compared to the Polish Pharmacopoeia XI standards. TT-EO was the only one whose components (and their percentage) were directly within pharmacopeial ranges. B-EO is not included in the Pharmacopoeia. Table 1 presents a list of the EOs' detected compounds. Chromatograms of the tested EOs are presented in Figure S1 in the Supplementary Materials.

Table 1. The content (% ± standard deviation) of compounds in essential oils. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. Dashes (-) indicate the compounds not presented in the specific EO. The components in line with Polish Pharmacopoeia XI standards are marked in green color.

Retention Time (min)	Compound	Mean Concentration (%) ± SD						
		T-EO	TT-EO	B-EO	R-EO	E-EO	MM-EO	L-EO
6.37	α-Thujene	-	1.11 ± 0.02	-	-	-	-	-
6.56	α-Pinene	2.20 ± 0.09	2.85 ± 0.06	-	2.58 ± 0.28	2.30 ± 0.04	3.01 ± 0.17	0.94 ± 0.04
6.98	Camphene	0.73 ± 0.04	-	-	-	-	-	-
7.8	Sabinene	0.64 ± 0.03	0.75 ± 0.02	-	-	-	-	-
8.22	Cyclofenchene	-	-	-	4.99 ± 0.48	-	2.68 ± 0.17	3.66 ± 0.35
8.25	β-Pinene	0.81 ± 0.05	0.69 ± 0.01	-	-	1.09 ± 0.02	-	-
8.65	α-Phellandrene	-	0.61 ± 0.01	-	0.23 ± 0.07	2.02 ± 0.02	-	-
8.69	2-Bornene	-	-	-	3.00 ± 0.29	-	-	-
9.06	α-Terpinene	-	11.07 ± 0.17	-	0.80 ± 0.04	-	-	-
9.31	p-Cymene	26.91 ± 0.99	4.69 ± 0.07	-	-	6.89 ± 0.07	-	-
9.45	Limonene	0.77 ± 0.04	2.08 ± 0.05	-	14.26 ± 0.99	-	3.78 ± 0.27	-
9.52	1.8-Cineole	-	3.34 ± 0.06	-	30.12 ± 1.74	79.10 ± 0.61	-	2.56 ± 0.39
9.57	β -Thujene	-	-	-	2.33 ± 0.11	-	-	-
10.22	Myrcene	-	-	-	0.38 ± 0.14	-	-	-
10.22	Myrcene	-	-	-	-	-	-	0.86 ± 0.13
10.47	γ-Terpinene	8.60 ± 0.03	19.07 ± 0.27	-	8.21 ± 0.35	8.16 ± 0.07	-	-
10.83	3-Carene	-	-	-	-	-	-	1.19 ± 0.12
11.35	o-Cymene	-	-	-	3.15 ± 1.45	-	-	1.19 ± 0.12
11.44	α-Terpinolene	-	4.34 ± 0.06	-	-	-	-	-
11.85	Linalool	3.45 ± 0.15	-	10.69 ± 1.13	-	-	-	37.76 ± 1.18
13.36	Camphor	0.66 ± 0.06	-	-	21.97 ± 0.77	-	-	0.84 ± 0.10
14.58	Terpinen-4-ol	-	33.27 ± 0.79	-	-	-	-	3.03 ± 0.46
14.99	α-Terpineol	7.84 ± 0.30	3.26 ± 0.13	-	1.56 ± 0.39	-	-	-
15.98	Menthone	-	-	-	-	-	24.53 ± 0.23	-
16.08	Isoborneol	-	-	-	1.53 ± 0.06	-	-	-
16.36	Isomenthone	-	-	-	-	-	13.54 ± 1.75	-
16.40	Borneol	-	-	-	2.69 ± 0.09	-	-	2.04 ± 0.56
16.66	Menthol	-	-	-	-	-	45.57 ± 2.21	-
17.59	Methyl chavicol	-	-	89.31 ± 1.13	-	-	-	-
18.49	Thymol	44.00 ± 0.46	-	-	-	-	-	-
19.60	Linalyl acetate	-	-	-	-	-	-	41.13 ± 0.40
20.61	Bornyl acetate	-	-	-	1.17 ± 0.09	-	-	-
20.81	Lavandulyl acetate	-	-	-	-	-	-	1.80 ± 0.22
20.89	Menthyl acetate	-	-	-	-	-	5.61 ± 0.36	-
22.36	β-Caryophyllene	1.00 ± 0.05	0.53 ± 0.01	-	-	-	-	-
22.83	Aromadendrene	-	1.83 ± 0.03	-	-	-	-	-
23.32	Alloaromadendrene	-	0.82 ± 0.02	-	-	-	-	-
24.03	Viridiflorene	-	2.35 ± 0.04	-	-	-	-	-
24.55	β-Cadinene	-	2.78 ± 0.03	-	-	-	-	-
24.80	Caryophyllene	-	-	-	0.85 ± 0.21	-	1.27 ± 0.57	3.47 ± 0.66

2.2. Biofilm Biomass and Activity Level Assessment

In the next line of investigation, the level of *P. aeruginosa* biofilm biomass and its cells' metabolic activity were assessed. All tested strains were metabolically active and able to form biofilm under applied in vitro conditions; however, differences in both biofilm biomass and metabolic activity were observed between specific strains (Figure 1).

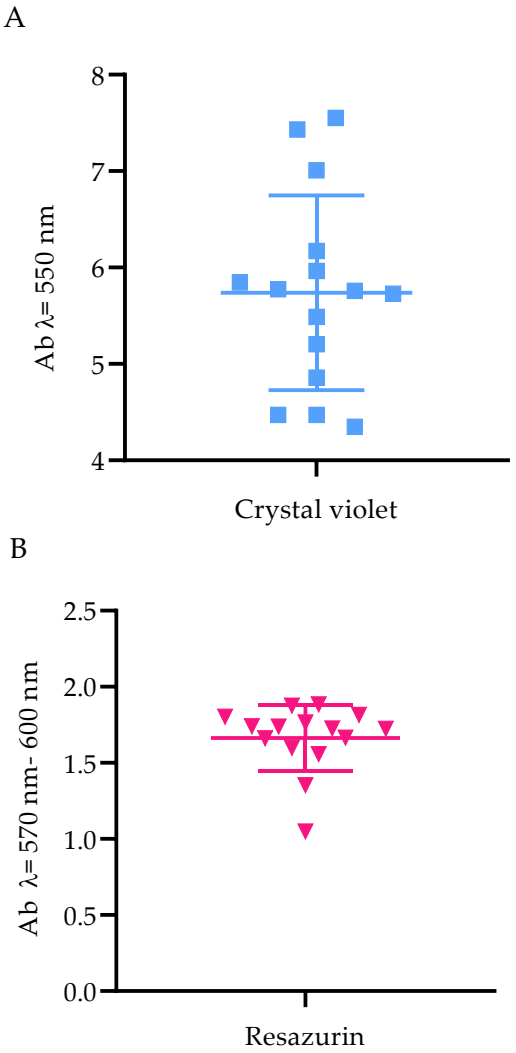

Figure 1. The ability of *Pseudomonas aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains to form biofilm. (**A**) Biofilm biomass level assessed with the crystal violet method. (**B**) Metabolic activity of biofilm-forming cells, determined with resazurin staining. Ab, absorbance. The average and standard deviations are marked.

2.3. Antimicrobial Activity of EOs in Their Liquid Forms against P. aeruginosa Planktonic Forms

Two standard techniques were performed to evaluate the antimicrobial effectiveness of EOs in their liquid forms toward planktonic cells: disc diffusion, in which non-emulsified EOs were applied, and minimal inhibitory concentration (MIC) estimation, in which EOs emulsions were used. The results from the first methodology mentioned are presented in Table 2. R-EO exhibited the highest antimicrobial activity, and zones of growth inhibition (mm) were obtained for all strains. T-EO and E-EO displayed moderate antibacterial efficacy against most of the strains. L-EO was almost inactive against *P. aeruginosa* strains. Because Tween 20 was used as an emulsifying agent in the MIC assay, its antibacterial potential against planktonic forms of *P. aeruginosa* ATCC 15442 strain was also evaluated. The aforementioned substance did not influence *P. aeruginosa* cell growth in concentrations from 1.0% to 0.002% (*v*/*v*), as it is presented in Figure S2 in the Supplementary Materials. R-EO emulsion was the most active among the tested EOs against planktonic pseudomonal cells. MIC values of B-EO ranged from 12.5% to 1.6% (*v*/*v*). In other EOs, MIC values were detected mostly in concentrations of 12.5–25.0% (*v*/*v*). Moreover, particular strains were not sensitive to MM-EO and T-EO even if a 25.0% (*v*/*v*) concentration of these oils was applied. The detailed list of the determined MIC (%) values is presented in Table 3.

Table 2. Mean diameters of inhibition zones (mm ± standard deviation) after treatment of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains with non-emulsified EOs in their liquid forms assessed with the disc diffusion method. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. According to their susceptibility to a particular oil, the strains were divided into two groups of seven or eight samples per group. In the case of B-EO, MM-EO, and L-EO, the zone equal to 0 mm was the parameter for the low-susceptibility group and higher than 0 mm for the high-susceptibility one. The groups are marked as follows: red, low susceptibility among the tested strains; green, high susceptibility among the tested strains.

Mean Zones of Growth Inhibition (mm ± SD) after Treatment with Non-Emulsified EOs in Their Liquid Forms							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
PA 1	9 ± 1	8 ± 1	10 ± 2	18 ± 5	18 ± 1	8 ± 7	7 ± 1
PA 2	13 ± 3	0 ± 0	12 ± 1	17 ± 5	18 ± 2	0 ± 0	0 ± 0
PA 3	3 ± 5	13 ± 1	0 ± 0	15 ± 4	11 ± 1	0 ± 0	0 ± 0
PA 4	9 ± 1	0 ± 0	0 ± 0	29 ± 2	0 ± 0	0 ± 0	0 ± 0
PA 5	0 ± 0	0 ± 0	0 ± 0	20 ± 2	8 ± 2	6 ± 6	0 ± 0
PA 6	5 ± 5	6 ± 5	0 ± 0	20 ± 1	9 ± 1	7 ± 6	0 ± 0
PA 7	8 ± 7	0 ± 0	0 ± 0	19 ± 1	0 ± 0	11 ± 2	0 ± 0
PA 13	13 ± 4	4 ± 8	10 ± 1	15 ± 4	14 ± 1	0 ± 0	0 ± 0
PA 14	7 ± 1	0 ± 0	0 ± 0	15 ± 1	0 ± 0	0 ± 0	0 ± 0
PA 15	8 ± 0	0 ± 0	8 ± 1	14 ± 1	14 ± 1	0 ± 0	0 ± 0
PA 16	3 ± 6	9 ± 1	0 ± 0	19 ± 0	0 ± 0	0 ± 0	0 ± 0
PA 17	11 ± 1	9 ± 1	0 ± 0	19 ± 1	10 ± 8	0 ± 0	0 ± 0
PA 18	10 ± 1	14 ± 1	0 ± 0	15 ± 0	0 ± 0	4 ± 7	0 ± 0
PA 19	0 ± 0	0 ± 0	0 ± 0	13 ± 2	11 ± 0	0 ± 0	0 ± 0
ATCC 15442	7 ± 0	5 ± 4	0 ± 0	12 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 3. Minimal inhibitory concentration (%) (*v*/*v*) of emulsified EOs in their liquid forms against *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains assessed with the microdilution method. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. R symbols indicate EOs where the minimal inhibitory concentration values were not reached in the highest concentration (25.0% (*v*/*v*)) of applied emulsions.

Minimal Inhibitory Concentration (%) of Emulsified EOs in Their Liquid Forms							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
PA 1	6.3	12.5	6.3	0.4	25.0	25.0	25.0
PA 2	25.0	25.0	12.5	0.8	25.0	25.0	25.0
PA 3	25.0	12.5	3.1	0.4	25.0	25.0	25.0
PA 4	25.0	12.5	6.3	0.4	25.0	25.0	25.0
PA 5	12.5	12.5	3.1	0.4	25.0	25.0	25.0
PA 6	25.0	25.0	6.3	0.8	25.0	25.0	25.0
PA 7	0.2	12.5	1.6	6.3	25.0	R	25.0
PA 13	25.0	12.5	1.6	0.8	25.0	R	25.0
PA 14	25.0	25.0	3.1	0.4	25.0	25.0	25.0
PA 15	25.0	25.0	3.1	0.8	25.0	25.0	25.0
PA 16	R	25.0	12.5	0.8	25.0	25.0	25.0
PA 17	R	25.0	12.5	0.8	25.0	25.0	25.0
PA 18	25.0	25.0	3.1	0.8	25.0	12.5	25.0
PA 19	12.5	25.0	3.1	0.4	12.5	6.3	25.0
ATCC 15442	R	25.0	12.5	0.4	25.0	R	25.0

2.4. Antimicrobial Activity of EOs in Their Liquid Forms against P. aeruginosa Biofilms

The antibiofilm activity of EOs in their liquid forms was determined using the standard microdilution method and the modified A.D.A.M. (antibiofilm dressing's activity measure-

ment) technique. To evaluate the MBEC values (minimal biofilm eradication concentration) of the emulsified EOs in their liquid forms, resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) staining was applied as an indicator of the presence of metabolically active cells. Due to the observed inconsistency of the results (data not shown) obtained for strains treated with T-EO and TT-EO, and dyed with resazurin, TTC (2,3,5-triphenyltetrazolium chloride) solution was used instead (for these above-mentioned EOs). As the MBEC values were not found within the applied concentration spectrum, changes in the biofilm-forming cells viability (%) after exposure to selected EO concentrations are presented in the study. The significant changes in the biofilm-forming cells viability were assessed after the treatment of pseudomonal biofilm with T-EO and TT-EO emulsions in concentrations of 25.0%–6.3% (*v*/*v*). B-EO and R-EO emulsions, both in concentrations of 25.0%–12.5% (*v*/*v*) and L-EO emulsion in a concentration of 25.0% (*v*/*v*) exhibited potent antibiofilm effectiveness. The percentage changes in the pseudomonal biofilm-forming cells viability after the application of 25.0% (*v*/*v*), 12.5% (*v*/*v*), and 6.3% (*v*/*v*) oils emulsions are presented in Figure 2 and Figures S3 and S4 in the Supplementary Materials, respectively.

Finally, the antibiofilm effect exerted by volatile phases of non-emulsified EOs was determined with the AntiBioVol (antibiofilm activity of volatile compounds) methodology. As can be seen in Table 4, PA 6, PA 17–19 and the ATCC 15442 strains were the most susceptible among all tested strains to volatile EOs. Reductions of biofilm cells viability ranged from 5.40% to 52.99% were obtained for the strains after the exposure to MM-EO. Volatile fractions of 96% (*v*/*v*) ethanol reduced 100.00% of pseudomonal biofilm.

Table 4. Changes in biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains after treatment with volatile non-emulsified EOs assessed with the AntiBioVol (antibiofilm activity of volatile compounds) method. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. The strains were grouped by their susceptibility to the particular oil. The groups are marked as follows: red, lowest susceptibility; purple, moderate susceptibility; green, highest susceptibility among the tested strains. The negative values indicate an increase in biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells).

Changes in the Biofilm-Forming Cells Viability (%) after Treatment with Volatile Fractions of Non-Emulsified EOs							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
PA 1	18.13	11.08	13.23	2.65	13.21	26.86	4.49
PA 2	29.21	24.08	6.18	−0.11	22.29	40.82	2.53
PA 3	10.29	19.17	10.73	−17.52	3.75	25.85	−0.78
PA 4	12.94	14.66	−1.71	−28.46	6.75	33.56	−6.11
PA 5	1.71	50.34	15.55	45.10	18.26	9.98	34.20
PA 6	9.22	53.81	−12.83	37.88	19.24	52.99	32.67
PA 7	3.51	20.04	−83.01	12.43	−96.64	5.40	−81.17
PA 13	8.11	−1.63	22.75	−4.50	3.69	17.62	−2.68
PA 14	12.30	−3.61	−17.21	−5.96	−8.91	19.66	−4.45
PA 15	15.10	−12.49	45.13	−10.29	−1.21	37.22	−3.83
PA 16	18.57	0.17	−3.85	−3.40	0.01	9.49	1.27
PA 17	6.38	22.34	20,84	14.73	13.74	11.22	42.97
PA 18	9.12	27.69	33.45	16.28	12.29	15.00	33.00
PA 19	3.58	27.20	7.97	18.32	11.94	19.00	40.84
ATCC 15442	13.08	53.11	22.80	43.74	−7.53	46.76	−1.71

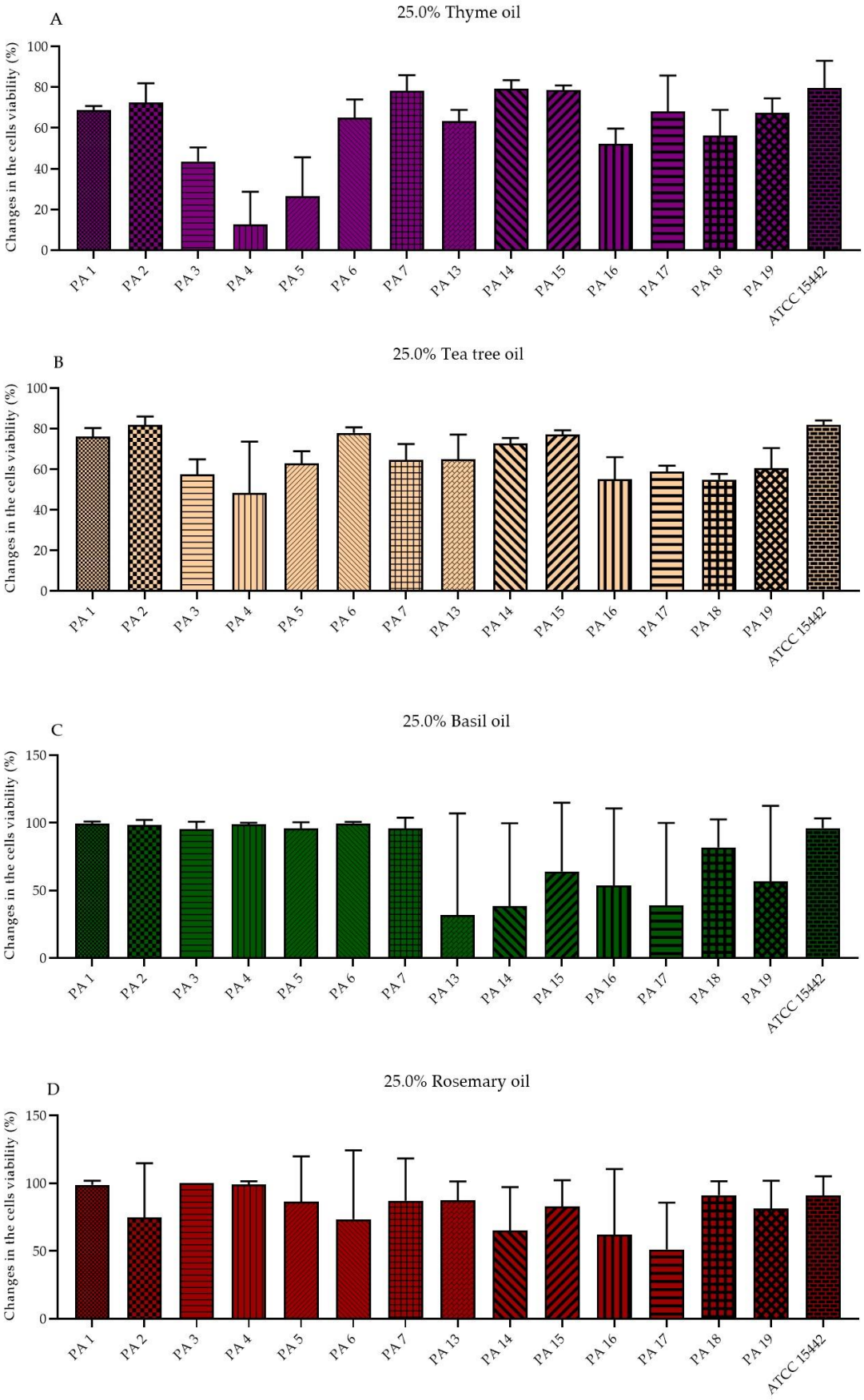

Figure 2. *Cont.*

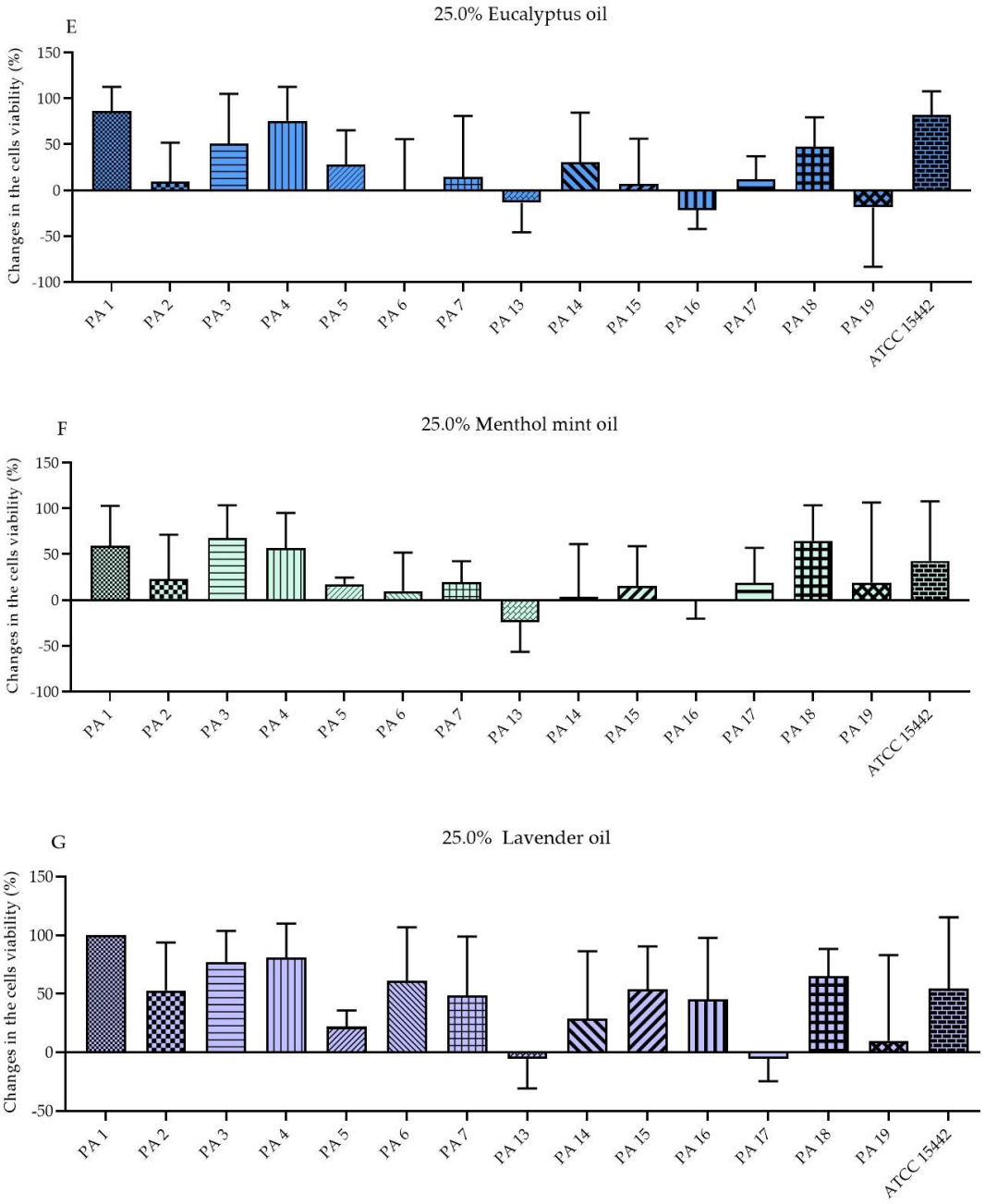

Figure 2. Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains after treatment with emulsified essential oils in their liquid forms in the concentration of 25.0% (*v*/*v*). Results of microdilution methodology with (**A**,**B**) TTC and (**C**–**G**) resazurin staining. Standard deviations are marked. The negative values indicate an increase in biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells).

To perform the modified A.D.A.M. technique, biocellulose discs were soaked with the non-emulsified EOs. The concentration of EOs released from the biocellulose discs was approximately 65.8%. The biofilms of PA 1–7 and PA 14–16 strains were the most prone to the activity of the EOs in their liquid forms (Table 5). The antibiofilm effectiveness of individual EOs was strain-dependent, although regarding all EOs except for B-EO, the reduction of cells viability equal to 60.11% or more was reported for selected strains. The mentioned B-EO displayed the lowest antibiofilm activity, and 96% (*v*/*v*) ethanol was

applied against the reference strain to prove the method's usability. The mean obtained reduction of the biofilm cells viability after the treatment with ethanol was 90.33%.

Table 5. Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains after treatment with non-emulsified essential oils in their liquid forms assessed with the A.D.A.M. (antibiofilm dressing's activity measurement) method. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. The strains were grouped by their susceptibility to the particular oil. The groups are marked as follows: red, lowest susceptibility; purple, moderate susceptibility; green, the highest susceptibility among the tested strains. The negative values indicate an increase in biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells).

Changes in the Biofilm-Forming Cells Viability (%) after Treatment with Non-Emulsified EOs in Their Liquid Forms							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
PA 1	47.23	50.57	3.49	69.55	33.14	26.11	43.93
PA 2	52.79	54.53	32.19	52.64	38.57	35.23	38.61
PA 3	63.13	37.74	22.23	87.56	64.39	79.78	69.11
PA 4	38.37	58.28	10.05	81.75	52.14	27.31	61.08
PA 5	69.45	50.36	37.78	70.46	19.31	53.54	54.64
PA 6	60.11	60.23	25.06	37.05	29.49	72.11	60.74
PA 7	36.34	44.24	20.36	38.93	20.23	64.88	40.90
PA 13	51.79	44.36	−34.87	55.01	−16.98	−13.07	20.92
PA 14	49.02	81.27	16.06	31.39	31.71	25.78	27.83
PA 15	63.80	85.39	25.56	36.98	23.81	44.93	31.14
PA 16	77.03	80.88	37.35	53.77	21.97	−12.46	50.29
PA 17	13.07	44.30	36.54	31.32	44.17	43.41	51.57
PA 18	−49.39	−39.67	−138.15	−152.19	−168.89	−110.24	−127.17
PA 19	56.21	83.26	4.13	17.92	1.78	46.16	34.75
ATCC 15442	41.11	47.06	12.50	14.56	11.33	49.87	57.36

2.5. Antimicrobial Activity of EOs' Volatile Fractions against P. aeruginosa

The influence of non-emulsified EOs' volatile phases on pseudomonal planktonic cells was assessed with the inverted Petri dish method. All of the examined strains (PA 1-7, PA 13–19, ATCC 15442) were resistant to the volatile of L-EO and B-EO. Almost all of the tested strains were resistant to T-EO (PA 1-6, PA 13-19, ATCC 15442), TT-EO (PA 1-7, PA 13–17, PA 19, ATCC 15442), MM-EO (PA 1-4, PA 6-7, PA 13-19, ATCC 15442). Mean zones of growth inhibition obtained for R-EO ranged from 0 to 23 mm. Table 6 presents the mean diameters of inhibition zones (mm) measured after the treatment of planktonic cells with the volatile fractions of EOs.

Table 6. Mean diameters of inhibition zones (mm ± standard deviation) after treatment of planktonic forms of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains with volatile fractions of non-emulsified EOs assessed with the inverted Petri dish method. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil. According to their susceptibility to R-EO, the strains were divided into two groups for seven or eight samples per group. For the rest of the EOs, the zone equal to 0 mm was the parameter for the low susceptibility group and higher than 0 mm for the high susceptibility one. The groups are marked as follows: red, low susceptibility among the tested strains; green, high susceptibility among the tested strains.

Mean Zones of Growth Inhibition (mm ± SD) after Treatment with Volatile Fractions of Non-Emulsified EOs							
Strain	**T-EO**	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
PA 1	0 ± 0	0 ± 0	0 ± 0	18 ± 15	22 ± 2	0 ± 0	0 ± 0
PA 2	0 ± 0	0 ± 0	0 ± 0	13 ± 13	16 ± 2	0 ± 0	0 ± 0
PA 3	0 ± 0	0 ± 0	0 ± 0	13 ± 6	16 ± 2	0 ± 0	0 ± 0
PA 4	0 ± 0	0 ± 0	0 ± 0	23 ± 2	0 ± 0	0 ± 0	0 ± 0
PA 5	0 ± 0	0 ± 0	0 ± 0	19 ± 3	0 ± 0	3 ± 6	0 ± 0
PA 6	0 ± 0	0 ± 0	0 ± 0	15 ± 2	0 ± 0	0 ± 0	0 ± 0
PA 7	3 ± 5	0 ± 0	0 ± 0	15 ± 3	0 ± 0	0 ± 0	0 ± 0
PA 13	0 ± 0	0 ± 0	0 ± 0	3 ± 6	3 ± 5	0 ± 0	0 ± 0
PA 14	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PA 15	0 ± 0	0 ± 0	0 ± 0	5 ± 4	0 ± 0	0 ± 0	0 ± 0
PA 16	0 ± 0	0 ± 0	0 ± 0	12 ± 4	0 ± 0	0 ± 0	0 ± 0
PA 17	0 ± 0	0 ± 0	0 ± 0	16 ± 5	0 ± 0	0 ± 0	0 ± 0
PA 18	0 ± 0	7 ± 6	0 ± 0	14 ± 6	0 ± 0	0 ± 0	0 ± 0
PA 19	0 ± 0	0 ± 0	0 ± 0	3 ± 5	0 ± 0	0 ± 0	0 ± 0
ATCC 15442	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

2.6. Microscopic Visualization of Biofilm

The R-EO's high activity against *P. aeruginosa* biofilms was additionally confirmed by fluorescence microscopy (Figure 3). While the high amount of live bacterial, biofilm-forming cells (dyed green) was observed in the untreated control setting (Figure 3A,C), the exposure of biofilm to the liquid (Figure 3B) or the volatile (Figure 3D) R-EO resulted in the high increase in dead/damaged biofilm-forming cells (dyed red/orange).

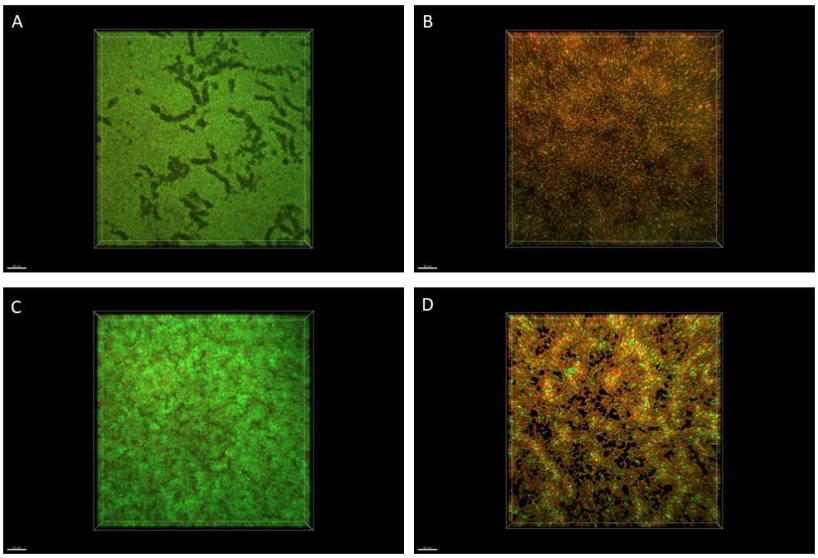

Figure 3. Impact of R-EO on *P. aeruginosa* ATCC 15442 biofilm. (**A**,**B**) Pseudomonal biofilm untreated and treated with R-EO in its liquid form, assessed with the modified A.D.A.M. (antibiofilm dressing's activity measurement) method. (**C**,**D**) Pseudomonal biofilm untreated and treated with R-EO volatiles, assessed with the AntiBioVol (antibiofilm activity of volatile compounds) assay. The red/orange color shows pseudomonal cells altered/damaged as the result of exposure to R-EO, while green-colored cells are non-altered, viable cells. Moreover, the darker (less green) picture shows that fewer live cells are captured in this particular field of vision.

2.7. Statistical Analysis

Statistical analysis was performed to evaluate statistically significant differences between EOs' antimicrobial activity. A summary of significance levels for each method is presented in Table 7.

Table 7. Significance levels of differences in changes in pseudomonal biofilm cells viability after treatment with EOs in their liquid forms and volatile fractions obtained with three methods. The differences were statistically significant for $p < 0.05$ and are referred to as $p < 0.03$ (*), $p < 0.006$ (**), $p < 0.00003$ (***); ns refers to difference being statistically insignificant. T-EO, thyme oil; TT-EO, tea tree oil; B-EO, basil oil; R-EO, rosemary oil; E-EO, eucalyptus oil; MM-EO, menthol mint oil; L-EO, lavender oil.

Comparison of the Changes in the Biofilm-Forming Cells Viability after Treatment with Emulsified EOs in Their Liquid Forms in the Concentration of 25.0% (*v*/*v*)

	T-EO	TT-EO	B-EO	R-EO	E-EO	MM-EO	L-EO
T-EO	-	ns	***	***	ns	ns	ns
TT-EO	ns	-	**	***	*	**	ns
B-EO	***	**	-	ns	***	***	***
R-EO	***	***	ns	-	***	***	***
E-EO	ns	*	***	***	-	ns	ns
MM-EO	ns	**	***	***	ns	-	ns
L-EO	ns	ns	***	***	ns	ns	-

Table 7. *Cont.*

Comparison of the Changes in the Biofilm-Forming Cells Viability after Treatment with Emulsified EOs in Their Liquid Forms in the Concentration of 12.5 (*v*/*v*)							
	T-EO	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
T-EO	-	ns	ns	ns	***	***	***
TT-EO	ns	-	ns	ns	***	***	***
B-EO	ns	ns	-	ns	***	***	***
R-EO	ns	ns	ns	-	***	***	**
E-EO	***	***	***	***	-	ns	ns
MM-EO	***	***	***	***	ns	-	ns
L-EO	***	***	***	**	ns	ns	-
Comparison of the Changes in the Biofilm-Forming Cells Viability after Treatment with Emulsified EOs in Their Liquid Forms in the Concentration of 6.3 (*v*/*v*)							
	T-EO	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
T-EO	-	ns	***	***	***	***	***
TT-EO	ns	-	***	**	***	***	***
B-EO	***	***	-	ns	ns	ns	ns
R-EO	***	**	ns	-	***	ns	*
E-EO	***	***	ns	***	-	ns	ns
MM-EO	***	***	ns	ns	ns	-	ns
L-EO	***	***	ns	*	ns	ns	-
Comparison of the Changes in the Biofilm-Forming Cells Viability after Treatment with Non-Emulsified EOs in Their Liquid Forms							
	T-EO	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
T-EO	-	ns	***	ns	**	ns	ns
TT-EO	ns	-	***	ns	***	*	ns
B-EO	***	***	-	***	ns	**	***
R-EO	ns	ns	***	-	**	ns	ns
E-EO	**	***	ns	**	-	ns	*
MM-EO	ns	*	**	ns	ns	-	ns
L-EO	ns	ns	***	ns	*	ns	-
Comparison of the Changes in the Biofilm-Forming Cells Viability after Treatment with Volatile Fractions of Non-Emulsified EOs							
	T-EO	**TT-EO**	**B-EO**	**R-EO**	**E-EO**	**MM-EO**	**L-EO**
T-EO	-	ns	ns	ns	ns	**	ns
TT-EO	ns	-	ns	**	**	ns	**
B-EO	ns	ns	-	ns	ns	**	ns
R-EO	ns	**	ns	-	ns	***	ns
E-EO	ns	**	ns	ns	-	***	ns
MM-EO	**	ns	**	***	***	-	***
L-EO	ns	**	ns	ns	ns	***	-

3. Discussion

EOs are volatile plant derivatives of global medical interest due to their high antimicrobial activity. However, the number of studies focused specifically on the antimicrobial/antibiofilm potential of EOs' volatile forms is still limited. EO antimicrobial activity depends on a plethora of factors: EO state of matter (volatile or liquid one), chemical composition, hydrophilicity/hydrophobicity, species/strain of microorganism they act against, but also on the type of methodology applied to analyze the aforementioned potential [13]. Therefore, the aim of this paper was to investigate and compare the anti-pseudomonal efficacy of seven EOs in their liquid forms and their volatile fraction with the use of a diversified spectrum of techniques in order to obtain cohesive data. First, the content of the EOs' constituents was determined to confirm the presence of molecules recognized as those exhibiting antimicrobial potential (Table 1, Figure S1 in the Supplementary Ma-

terials). Next, the pseudomonal strains' ability to form biofilm was assessed (Figure 1). Having preliminary tests performed, the evaluation of the EOs in their liquid forms' antimicrobial activity against *P. aeruginosa* planktonic cells was made with the use of two different techniques. Their results indicated that R-EO was the most effective one among the tested EOs (Tables 2 and 3). In addition, it is suggested that the strong antibacterial potential of R-EO is associated with the activity of its main component, 1,8-cineole [14–16]. However, 1,8-cineole is also the major compound of E-EO (Table 1); this specific oil exhibited significantly lower anti-pseudomonal efficacy than R-EO. Other research teams indicated that the antimicrobial effectiveness of crude R-EO and E-EO was stronger than 1,8-cineole applied as a self-reliant antimicrobial agent [17,18]. Therefore, the synergistic (or at least additive) effect of remaining R-EO constituents may also account for the overall oil's activity against *P. aeruginosa*. It is hypothesized that the smaller the droplets of EO emulsions, the higher the antimicrobial effect that occurs [19]. As we have shown earlier, the droplet diameters of the E-EO emulsion were 2201 nm, while R-EO was 783 nm [20]. The minimal inhibitory concentration (MIC) values (Table 3) of L-EO were 25% (*v*/*v*). MIC values of E-EO were: 12.5% (*v*/*v*) for PA 19 strain and 25% (*v*/*v*) for the rest of the strains, whereas the zones of growth inhibition being the result of antimicrobial activity of L-EO vs. E-EO were 0-7 and 0-18 mm, respectively (Table 2). This interesting observation requires additional experiments to be elucidated because a broad spectrum of possible variables may contribute to the discrepancy between outcomes measured by two testing methods. As an example, the differences in water solubility of the main EO ingredients also have an impact on diffusion through agar. Because agar is mainly composed of water, the higher the aqueous solubility of the compounds, the better their diffusion across the agar. The aqueous solubility of 1,8-cineole, which predominates in E-EO, is 2.63 mg/mL at 293 K, while the solubility of linalool (the main component of L-EO) is 1.34 mg/mL [21]. Linalool diffuses more poorly through the agar than 1,8-cineole, thus, the growth inhibition zones of L-EO are less than E-EO.

To evaluate the antibiofilm efficacy of EOs in their liquid forms, the microdilution method and the modified A.D.A.M. (antibiofilm dressing's activity measurement) assay were conducted. In the microdilution assay, significant biofilm-forming cells viability reduction was demonstrated for the emulsions of T-EO, TT-EO, B-EO, R-EO and L-EO in their liquid forms at a concentration of 25% (*v*/*v*). In the modified A.D.A.M. methodology, levels of biofilm-forming cells viability reduction were diversified and dependent on the EO applied and on the specific strain exposed to the EO's activity. Nevertheless, by means of both methods, R-EO, T-EO and TT-EO in their liquid forms can be pointed out as the most potent against the pseudomonal biofilm, which is in line with the results of other research teams. It was reported that above 90% reduction of *P. aeruginosa* biofilm was obtained after the incubation with T-EO and that 1,8-cineole, the main component of R-EO, greatly affected *P. aeruginosa* biofilm formation and disrupted the mature biofilm [18,22–24]. The volatile fractions of R-EO and TT-EO also displayed high effectiveness against *P. aeruginosa* biofilm, which may result from their multiple mechanisms of anti-biofilm activity.

In the case of volatile fraction assays, not only does the volatility of the components determine EO activity but also the number and concentration of particular molecules adhered to the agar surface where biofilm forms. In the standard inverted Petri dish method, the EOs are applied to a small stretch (6 mm in diameter paper disc). Therefore, the obtained zones of growth inhibition also depend on the volatiles spreading. The volume of the EOs used in the inverted Petri dish method is lower, and the tightness of the setting is poorer than in the AntiBioVol (antibiofilm activity of volatile compounds) technique; thus, the loss of volatiles is higher.

As mentioned above, volatile fractions of EOs exhibited higher antimicrobial activity than those EOs in their liquid forms in some of the research reports [25]. However, opposite results are reported in the present paper (Tables 4 and 6). In turn, outcomes of other studies stay in line with the data provided in our work and indicate that EOs in their liquid forms are more active against bacteria than in volatiles, due to the direct contact of molecules

with the microorganism [9]. It was also suggested that the antimicrobial activity of EOs' volatiles is related to the volatility of the EOs' compounds and their adsorption into the agar surface, which is associated with its hydrophilic character [26,27]. In the previous paper, we demonstrated that the adsorption of EO compounds to the agar changes with the time of exposure [28]. Moreover, the maximum concentration of molecules adsorbed into the agar surface was approximately 40% [28]. Therefore, the working concentration of the active substances is much lower than in the liquid tests. The obtained differences in both fractions' activity may result from various times of biofilm incubation with the applied indicators of cell metabolic activity (resazurin and tetrazolium chloride). According to the AntiBioVol results, TT-EO, R-EO, MM-EO, and L-EO were the most potent ones among the analyzed EOs. The speed of EOs evaporation is related to their vapor pressure; the higher the vapor pressure, the faster they evaporate. The vapor pressures of EOs main compounds at 25 °C are: thymol, 2.2 Pa; terpinen-4-ol, 6.4 Pa; methyl chavicol, 22.0 Pa; 1,8-cineole, 253.0 Pa; menthol, 19.0 Pa; linalyl acetate, 61.0 Pa. In our study, we found no correlation between the above-mentioned parameter and the level of antibacterial activity of EOs. Such a phenomenon may be explained by the fact that besides vapor pressure, EO's particular antimicrobial mechanism of action and affinity to agar surface should be considered to analyze the total level of antimicrobial activity. Additionally, strain-dependent tolerance to EOs was observed when AntiBioVol was applied, similar to what was observed when the modified A.D.A.M. method was used. The EOs examined in the current study were also scrutinized in our previous line of investigation toward a Gram-positive bacterium, *S. aureus* [20]. Comparing results from the recent and the present work, it has to be stated that both fractions of all EOs (except for R-EO) displayed higher antimicrobial activity against *S. aureus* than *P. aeruginosa*. It may be related to the fact that, because of the hydrophobic nature, EOs react with lipids of the cell membrane and lead to a leak of intracellular substances and to damage of the cell. Gram-negative bacteria hydrophilic cell walls hinder the penetration of lipophilic EOs, resulting in their higher tolerance to EOs than in the case of Gram-positive pathogens. Furthermore, Gram-negative *P. aeruginosa* is a ubiquitous, opportunistic microorganism, forming a robust biofilm on solid surfaces at the water–air interface [29–31]. It thrives there by developing a vast spectrum of adaptive resistance to various antimicrobial agents, probably including those secreted by plants. [32]. Contrary to other EOs, MIC values of R-EO in its liquid emulsified form were equal for *S. aureus* and *P. aeruginosa*. (Table 3) [20]. An explanation of this phenomenon may be the hypothesis that the charge of the bacterial cell surfaces and the cell shape play a role in their tolerance to EOs as well [33–35]. Hajlaouli et al. demonstrated a more evident reduction of the bacterial cells' negative charge for the Gram-negative versus Gram-positive bacteria after the treatment with EOs [34]. Rod-shaped bacterial cells were also more susceptible to EOs than the coccoid ones [35]. In the present study, EOs in their liquid forms and their volatile fractions were investigated because the antimicrobial effect of EOs may alter among different in vitro conditions. R-EO exhibited the highest antibacterial effectiveness against *P. aeruginosa* of all tested EOs. The reported data confirm the high potency of EOs against *P. aeruginosa* biofilms and planktonic cells. Therefore, we are convinced that the data presented in this paper, showing EOs as a promising alternative to the current (performed by means of antibiotics and antiseptics, mostly) anti-pseudomonal therapies, will finally pave the way for novel solutions and approaches aiming to significantly reduce the risk associated with pseudomonal biofilm-related infections.

4. Materials and Methods

4.1. Bacterial Strains and Culture Conditions

For research purposes, one reference strain of *Pseudomonas aeruginosa* 15442 from the American Type and Culture Collection (ATCC) and fourteen clinical strains (later referred to as PA 1–7 and PA 13–19) of this bacterial species were applied. The strains were part of the Strain and Line Collection of Pharmaceutical Microbiology and Parasitology Department of the Medical University of Wroclaw. The clinical strains were obtained in the year 2016

during the internal Wroclaw Medical University SUB. D198.16.001 project: "The insight into biofilm-related properties of clinical microorganisms and possibilities of their eradication". All patients provided written consent to participate in the trial and allowed the material obtained during the study (exudate, biopsy specimen, microorganisms) to be used for scientific purposes. The study was approved by the Bioethical Committee of Wroclaw Medical University, protocol # 8/2016.

In each of the performed experiments, the microorganisms' overnight cultures in the TSB medium (Tryptic Soy Broth, Biomaxima, Lublin, Poland) were prepared, and 0.5 McFarland suspensions were established afterward in a 0.9% (*w/v*) solution of sodium chloride (NaCl, Stanlab, Lublin, Poland) using a densitometer (Densilameter II Erba Lachema, Brno, the Czech Republic).

4.2. Essential Oils

Seven commercially available essential oils in their liquid forms and their volatile fractions were tested in the research. The applied EOs are listed in Table 8.

Table 8. List of the essential oils analyzed in the paper.

Common Name of EO	Plant Origin	Part of the Plant	Abbreviation	Manufacturer, City, Country
thyme oil	*Thymus vulgaris* L.	herb	T-EO	Etja, Elblag, Poland
tea tree oil	*Melaleuca alternifolia* Cheel.	leaves	TT-EO	Pharmatech, Zukowo, Poland
basil oil	*Ocimum basilicum* L.	flowers	B-EO	Nanga, Zlotow, Poland
rosemary oil	*Rosmarinus officinalis* L.	flowering shoots	R-EO	Nanga, Zlotow, Poland
eucalyptus oil	*Eucalyptus globulus* Labill.	leaves and twigs	E-EO	Pharmatech, Zukowo, Poland
lavender oil	*Lavandula angustifolia* Mill.	flowering herb	L-EO	Kej, Cirkowice, Poland
menthol mint oil	*Mentha arvensis* L.	leaves	MM-EO	Optima Natura, Grodki, Poland

Due to the volatility of EOs, individual essential oils and control settings were examined on separate plates. Moreover, to prevent EO evaporation, in the experiments where their volatile phases were investigated, plates were sealed with parafilm.

4.3. GC–MS (Gas Chromatography–Mass Spectrometry) Analysis of the Tested EOs Composition

Essential oils (EOs) were diluted with hexane (JTB, GB), vortexed, and immediately analyzed. All analyses were performed in triplicate.Analysis was carried out using the system Agilent 7890B GC coupled with 7000GC/TQ system connected to PAL RSI85 autosampler (Agilent Technologies, Palo Alto, CA, USA).

The applied column was HP-5 MS; 30 m × 0.25 mm × 0.25 µm (J&W, Agilent Technologies, Palo Alto, CA, USA). Helium was used as a carrier gas at a total flow of 1 mL/min. Chromatographic conditions were applied as follows: split injection in a ratio 100:1, the injector was set on 250 °C, oven temperature program was: 50 °C held for 1 min, then 4 °C/min up to 130 °C, 10 °C/min to 280 °C, and then isothermal for 2 min. The MS detector operated in the electronic impact ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set at 320, 230, and 150 °C, respectively. Masses were registered in a range from 30 to 400 m/z. Peaks identification was performed using MassHunter Workstation Software Version B.08.00 coupled with the NIST17 mass spectra library and accomplished by comparison with linear retention indexes. The relative abundance of each EO constituent was expressed as percentage content based on the peak area normalization. Regarding the obtained outcomes, only TT-EO met the requirement of pharmacopeial standards. Although, the analysis was not performed in accordance with the normalization procedure from Polish Pharmacopea XI (different column and different temperature program).

4.4. Biofilm Biomass Level Assay

The crystal violet assay was performed in 96-well plates (Wuxi Nest Biotechnology, Wuxi, China) to evaluate the total biofilm mass. Briefly, 0.5 McFarland bacterial suspensions, prepared as described in Materials and Methods Section 4.1., were diluted 1000 times in TSB (Tryptic Soy Broth, Biomaxima, Lublin, Poland) medium, and 100 µL was poured into the wells of the plates. The plates were then incubated under static conditions for 24 h at 37 °C. Subsequently, the medium above the biofilm was gently pipetted out, and the plates were dried for 10 min at 37 °C. Then, 100 µL of 20% (*v*/*v*) aqueous crystal violet solution (Aqua-med, Lodz, Poland) was added to each well for 10 min at room temperature. Biofilm cells were rinsed twice with 100 µL of 0.9% (*w/v)* solution of sodium chloride (NaCl, Stanlab, Lublin, Poland) to remove unbounded cells and excess stain. The plates were transferred to the incubator (37 °C) for 10 min. Next, 100 µL of 30% (*v*/*v*) water solution of acetic acid (Chempur, Piekary Slaskie, Poland) was introduced to the wells, and the plates were shaken for 30 min at room temperature at 450 rpm (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). The solution was transferred to fresh 96-well plates, and the absorbance was measured at 550 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). One independent experiment was performed with six technical replicates.

4.5. Biofilm Activity Level Assessment

The presented protocol of biofilm culturing in Materials and Methods Section 4.4. was applied. After the biofilm formation, 10 µL of 0.1% (*w/v*) resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Acros Organics, Geel, Belgium) solution in TSB (Tryptic Soy Broth, Biomaxima, Lublin, Poland) was added to the wells, and the plates were incubated at 37 °C for two hours. The solution was transferred to fresh 96-well plates (Wuxi Nest Biotechnology, Wuxi, China), and its absorbance was measured at 570 and 600 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). To assess the cells viability, the absorbance value at 600 nm was subtracted from the value at 570 nm. One independent experiment was carried out with six technical repetitions.

4.6. Methods for the Assessment of the Activity of EOs in Their Liquid Forms

4.6.1. The Disc Diffusion Method

In the experiment, 90 mm diameter, 14.2 mm height Petri dishes (Noex, Komorniki, Poland) with 5 mm thick Mueller–Hinton agar layers (Biomaxima, Lublin, Poland) were used. Standard paper discs (diameter of 6 mm, 0.5 mm thickness) were introduced to the wells of 48-well plates (Thermo Fisher Scientific, Waltham, MA, USA), and 0.2 mL of each non-emulsified EOs or saline (control of bacterial growth) (NaCl, Stanlab, Lublin, Poland) was added. The plates were wrapped with tape and kept at 4 °C for 30 min to soak the discs. In the second step of the tests, the Petri dish plates were inoculated with the bacterial suspensions at density 0.5 McFarland prepared as described in Materials and Methods Section 4.1. Next, the soaked paper discs were placed onto the agar layer for assessing the antimicrobial activity of pure EOs in their liquid forms. The dishes were incubated for 24 h at 37 °C, and bacterial growth inhibition zones were measured in mm with a ruler. If no total inhibition was obtained, zones of partial growth inhibition were assessed (in mm). When unequal zones were observed, a shorter diameter was included. One independent experiment with three repetitions was performed, and the mean diameters were calculated.

4.6.2. Assessment of the Minimal Inhibitory Concentrations of EOs Emulsions

To determine the minimal inhibitory concentration (MIC) values of the EOs in their liquid forms, their emulsions in Tween 20 (Zielony Klub, Kielce, Poland) were prepared. In the first step, each EO was combined with the solution of 1.0% (*v*/*v*) Tween 20 in TSB medium (Tryptic Soy Broth, Biomaxima, Lublin, Poland) in ratio 1:1 and mixed with a vortex (Micro-shaker type 326 m, Premed, Marki, Poland) for 30 min at room temperature. Following, geometric dilutions were prepared in TSB and shaken for 30 s. Subsequently, 0.5 MacFarland bacterial suspensions were made according to the description in Materials

and Methods Section 4.1. and diluted 1000 times in the TSB medium. Next, 100 µL of the suspensions was added to the wells of 96-well plates (Jet Bio-Filtration Co. Ltd., Guangzhou, China), and the same volume of diluted emulsions was poured. Therefore, the final concentration of each EO applied in the test ranged from 25.0% (*v*/*v*) to 0.01% (*v*/*v*). The following additional samples were included: control of bacterial growth (bacteria with medium), control of medium sterility (medium only), control of 1.0–0.002% (*v*/*v*) Tween 20 antimicrobial activity and samples of emulsions only. The absorbance of the solution was measured at 580 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). During the 24 h incubation at 37 °C, the plates were shaken at 350 rpm (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). The absorbance was measured immediately after the incubation at the same wavelength. The MIC value was assessed in the concentration (%) (*v*/*v*) in which the difference between the absorbance after and before the sample's incubation was equal to or lower than zero. Two independent experiments were performed, each with three technical replicates.

4.6.3. Assessment of the Minimal Biofilm Eradication Concentrations of EOs Emulsions

The EOs emulsions and bacterial suspensions applied to assess the Minimal Biofilm Eradication Concentration (MBEC) values were prepared as was elaborated in Materials and Methods Section 4.6.2.. On the first day of the experiment, 100 µL of the aforementioned suspensions was added to the wells of 96-well plates (Jet Bio-Filtration Co. Ltd., Guangzhou, China) containing 100 µL of TSB medium (Tryptic Soy Broth, Biomaxima, Lublin, Poland). The plates were incubated at 37 °C for 24 h under static conditions. Subsequently, the medium was aspirated, and 200 µL of EOs emulsions geometric dilutions were added to the wells. The concentration of each EO applied in the test ranged from 25.0% (*v*/*v*) to 0.01% (*v*/*v*). Control of bacterial growth (bacteria with medium) and medium sterility (medium only) were also prepared. The plates were re-incubated for the next 24 h. Based on the results of the preliminary study (data not shown), the MBEC values of T-EO and TT-EO were determined using TTC (2,3,5-triphenyl-tetrazolium chloride, AppliChem Gmbh, Darmstadt, Germany) as the indicator, while resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Acros Organics, Geel, Belgium) was applied to other EOs. The following steps were executed for the resazurin assay: 40 µL of 0.05% (*w*/*v*) resazurin solution in TSB medium was added to the biofilm wells treated with EOs emulsions and to the control wells. The plates were incubated for 2 h at 37 °C with continuous shaking at 400 rpm (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia). The absorbance was measured at 570 and 600 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). To calculate the final absorbance of the tested substance, the absorbance value at 600 nm was subtracted from the value at 570 nm. The MBEC values were determined as the lowest concentration of the emulsions where the obtained difference was equal to or lower than zero. For the TTC methodology, 0.2% (*w*/*v*) TTC solution in TSB was added in the volume of 150 µL to the biofilm after its incubation with EOs emulsions. The plates were incubated for 2 h at 37 °C. Next, the MBEC value was visually determined in the lowest concentration where no red color was observed. Due to the fact that in no case was the MBEC assessed, the three highest concentrations of each EO emulsion for each strain were chosen for further analysis. The contents of the mentioned wells were transferred to 1.5 mL Eppendorf tubes, and 350 µL of 0.1% (*w*/*v*) solution of saponin (VWR Chemicals, Radnor, PA, USA) was added, and the Eppendorf tubes were vortexed for 1 min. Then, 700 µL of methanol (Stanlab, Lublin, Poland) was added, and the Eppendorf tubes were vortexed for 30 min. Finally, the Eppendorf tubes were centrifuged for 1 min at 3000 rpm, and 100 µL of the supernatant was transferred in three replicates to the wells of the 96-well plates. The same procedure was performed for the control growth wells. The absorbance was measured at 490 nm.

The level of the reduction of biofilm-forming cells viability was calculated according to the formula.

$$\text{Cells viability reduction } (\%) = 100\% - \left(\frac{\text{AbS}}{\text{AbC}} * 100\%\right) \quad (1)$$

AbS, absorbance of the tested substance;
AbC, mean absorbance of growth control.
Two independent experiments were performed, each with three technical replicates.

4.6.4. Evaluation of Antibiofilm Activity of Non-Emulsified EOs Using the Modified A.D.A.M. (Antibiofilm Dressing's Activity Measurement) Assay

The methodology was a modification of the procedure displayed in our previous research [36]. The following steps of the experiment were performed:

Preparation before the Experiment

To treat the biofilm with the EOs, biocellulose discs (BC) were produced. A *Komagataeibacter xylinus* ATCC 53524 (American Type and Culture Collection) strain was used for this purpose. A Herstin–Schramm (H-S) medium composed of 2% (*w/v*) glucose (Chempur, Piekary Slaskie, Poland), 0.5% (*w/v*) yeast extract (VWR, Radnor, PA, USA), 0.5% (*w/v*) bactopeptone (VWR, Radnor, PA, USA), 0.115% (*w/v*) citric acid monohydric (POCH, Gliwice, Poland), 0.27% (*w/v*) Na2HPO4 (POCH, Gliwice, Poland), 0.05% (*w/v*) MgSO4·7H2O (POCH, Gliwice, Poland), and 1% (*v*/*v*) ethanol (Chempur, Piekary Slaskie, Poland) was used for bacterial culturing. Then, 1 mL of H-S medium was added to the wells of a 24-well plate (Wuxi Nest Biotechnology, Wuxi, China) and inoculated with *K.xylinus*. The plate was incubated for 7 days at 28 °C to obtain 14 mm BC discs. Then, the discs were removed and washed with 0.1 M NaOH (Chempur, Piekary Slaskie, Poland) at 80 °C. Next, BC discs were washed with double-distilled water to neutralize their pH and autoclaved. To evaluate the concentration of substances adsorbed into the BC discs, six of them were weighed, dried at 37 °C and weighed again. The average concentration (%) of the adsorbed liquid was calculated with the formula:

$$\text{Compound concentration } (\%) = [\text{EV}/((\text{WBC} - \text{DBC}) + \text{EV})] * 100\% \quad (2)$$

EV, a volume of essential oil (mL);
WBC, the weight of wet BC disc (g);
DBC, the weight of dry BC disc (g).

First Day of the Experiment

In the first line of the investigation, 1.5 mL of Brain Heart Infusion Broth (BHI, Biomaxima, Lublin, Poland) with 2% (*w/v*) of Bacteriological Lab Agar (Biomaxima, Lublin, Poland) was poured into the wells of a 24-well plate (further referred to as Agar Plate). After the agar solidifies, 8 mm in diameter plugs were cut out of each well using a cork-borer. The plugs were removed and discarded to make 8 mm in diameter tunnels in agar, and the Agar Plate was kept refrigerated for the next day. Simultaneously, the same agar formulation was used to prepare a 6 mm in height agar layer on a Petri dish (Noex, Komorniki, Poland). Then, agar plugs 8 mm in diameter were cut out from the agar Petri dish and placed in a fresh 24-well plate (further referred to as Plugs Plate). The microorganisms' suspensions, prepared according to the description in Materials and Methods Section 4.1., were diluted 1000 times in TSB medium (Tryptic Soy Broth, Biomaxima, Lublin, Poland), introduced to the wells of the Plugs Plate in the volume of 2 mL and transferred to the incubator (37 °C) for biofilm formation. The BC discs, prepared as described above, were placed in a fresh 24-well plate (further referred to as Discs Plate), and 1 mL of undiluted, non-emulsified essential oils or saline (control of bacterial growth), or 96% (*v*/*v*) ethanol

(Chempur, Piekary Slaskie, Poland) was added, and the plate was sealed with parafilm and placed at 4 °C. Both plates (Plugs Plate 2 and Discs Plate) were incubated for 24 h.

Second Day of the Experiment

Subsequently, biofilm plugs were taken out from the Plugs Plate and placed in the bottom of agar tunnels in the Agar Plate so that the biofilm was on the top of the plugs. Then, 50 µL of TSB medium was added to fill up the space in the tunnels. The BC discs soaked with non-emulsified EOs/saline/ethanol were transferred from the Disc Plate and placed on the top of the agar wells in the Agar Plate. The biofilm was adhered to the plug and had no direct contact with the BC. Substances were gradually released from the BC to the medium. The experimental setting was incubated for 24 h/ 37 °C under static conditions.

Third Day of the Experiment

After the biofilm treatment with the tested substances, the BC discs were removed. The content of each agar tunnel (medium and agar plugs) was transferred to the wells of 48-well plates (Wuxi Nest Biotechnology, Wuxi, China) and 1 mL of 0.002% (*w/v*) resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Acros Organics, Geel, Belgium) in TSB was added. The plates were shaken at 350 rpm (Mini-shaker PSU-2T, Biosan SIA, Riga, Latvia) for 4 h and 15 min at 37 °C. Then, 100 µL of the color solution was transferred to 96-well plates (Jet Bio-Filtration Co. Ltd., Guangzhou, China) in three replications from each 48-well. The absorbance was measured at 570 and 600 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). To calculate the final absorbance of the tested substance, the absorbance at 600 nm was subtracted from the value at 570 nm. The formula calculated the reduction of cells viability:

$$\text{Cells viability reduction } (\%) = 100\% - \left(\frac{\text{AbS}}{\text{AbC}} * 100\%\right) \quad (3)$$

AbS, absorbance of the tested substance;
AbC, mean absorbance of growth control.

One independent experiment was performed with six technical replicates. Antibiofilm activity of ethanol was examined only against the *P. aeruginosa* 15442 strain.

4.7. Methods for the Assessment of EOs' Volatile Fractions Activity

4.7.1. The Inverted Petri Dish Methodology

The assay was performed similarly to the method presented in Materials and Methods Section 4.6.1. The paper disc soaked with the non-emulsified tested substances was solely placed onto the lid of the Petri dish.

4.7.2. Evaluation of Antibiofilm Activity of Non-Emulsified EOs Using the AntiBioVol (Antibiofilm Activity of Volatile Compounds) Method

The assay was performed based on the protocol presented in our previous study [37].

First Day of the Experiment

In this part of the investigation, the Agar Plate, the Petri dish with the plugs and the Plugs Plate were prepared as mentioned in Materials and Methods Section 4.6.4. The following modifications were made. First, wells of the Agar Plate were filled with BHI (Brain Heart Infusion Broth, Biomaxima, Lublin, Poland) and agar to full. Second, twice as many agar plugs were cut out from the Petri dish, and part of them remained sterile. They were placed on the bottom of the agar tunnels (Agar Plate) and kept refrigerated until the next day.

Second Day of the Experiment

Biofilm plugs were taken out from the Plugs Plate and placed in the agar tunnels of the Agar Plate on the top of the sterile ones. Then, 0.5 mL of undiluted, non-emulsified EOs or saline (control of growth) or ethanol (Chempur, Piekary Slaskie, Poland) was added to the wells of a fresh 24-well plate (later referred to as Substance Plate) (Wuxi Nest Biotechnology, Wuxi, China). The Agar Plate was placed upside down on the Substance Plate, and the agar wells were set directly above the wells with the tested substances. The plugs' diameters were the same as the tunnels in which they were placed, and plates were gently transferred to protect the plugs from dropping into the wells of the Substance Plate. The plates were sealed and incubated for 24 h at 37 °C under static conditions.

Third Day of the Experiment

As the incubation was completed, the plates were separated, and the upper plugs (containing biofilms) were transferred to fresh 48-well plates (Wuxi Nest Biotechnology, Wuxi, China), and 1 mL of 0.002% (*w/v*) resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Acros Organics, Geel, Belgium) in TSB was poured. The incubation was continued for 2 h/ 37 °C with shaking at 350 rpm. Then, 100 µL of the color solution was transferred to 96-well plates (Jet Bio-Filtration Co. Ltd Guangzhou, China)) in three replications from each 48-well. The absorbance was measured at 570 and 600 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). The absorbance at 600 nm was subtracted from the value at 570 nm. The reduction of cells viability was calculated by the formula:

$$\text{Cells viability reduction } (\%) = 100\% - \left(\frac{\text{AbS}}{\text{AbC}} * 100\%\right) \quad (4)$$

AbS, absorbance of the tested substance;
AbC, mean absorbance of growth control.

One independent experiment was performed with six technical replicates. Antibiofilm activity of ethanol was examined only against the *P. aeruginosa* 15442 strain.

4.8. Microscopic Visualization of Biofilm

The pseudomonal biofilm (ATCC 15442 strain) treated with R-EO or saline by means of the A.D.A.M. (antibiofilm dressing's activity measurement) or the AntiBioVol (antibiofilm activity of volatile compounds) technique (as presented in Materials and Methods Section 4.6.4. or Section 4.7.2, respectively) was immersed in 1 mL of Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Invitrogen, Thermo Fisher Scientific, USA) solution and incubated at room temperature for 15 min. After incubation, the solution was removed, and the biofilms were gently rinsed once with sterile water. The biofilms were analyzed using a confocal microscope (Leica, SP8, Wetzlar, Germany) with a 25× water dipping objective, using sequential mode for 488 nm laser line and 500–530 nm emission to detect SYTO-9 and 552 nm laser line and 575–627 nm emission to detect propidium iodide (PI) within microbial cells.

4.9. Statistical Analysis

Statistical analysis was performed using Statistica (Version 13; TIBCO Software Inc., Palo Alto, CA, USA). Normality distribution was assessed with the Shapiro–Wilk test. To compare EOs' efficacy, a non-parametric ANOVA Kruskal–Wallis test and post hoc Dunn's analysis were performed. Results with a significance level $p < 0.05$ were considered significant. The statistical analysis is presented in Table 7.

5. Conclusions

The antimicrobial activity of EOs' volatile fractions varies from that of EOs in their liquid forms. The antimicrobial effectiveness of EOs may depend on the type of methodology applied to analyze the antimicrobial activity. Rosemary and tea tree EOs in their liquid

forms and their volatile fractions displayed significant antibiofilm effectiveness against *P. aeruginosa*. Volatile fractions of menthol mint EO exhibited the most potent antibiofilm effect among all tested EOs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27134096/s1, Figure S1: Chromatograms of tested EOs measured with GC–MS (Gas Chromatography–Mass Spectrometry); Figure S2: The antimicrobial activity of different concentrations (%) (*v*/*v*) of Tween 20 against planktonic forms of *P. aeruginosa* ATCC 15442 strains. Ab, absorbance; C+, untreated cells. The absorbance of untreated cells is marked with a red line; Figure S3: Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1-7, PA 13-19) and the reference (ATCC 15442) strains after treatment with emulsified essential oils in their liquid forms in the concentration of 12.5% (*v*/*v*). Results of microdilution methodology with (A,B) TTC and (C–G) resazurin staining. Standard deviations are marked. The negative values indicate an increase in biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells); Figure S4: Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1–7, PA 13–19) and the reference (ATCC 15442) strains after treatment with emulsified essential oils in their liquid forms in the concentration of 6.3% (*v*/*v*). Results of microdilution methodology with (A,B) TTC and (C–G) resazurin staining. Standard deviations are marked. The negative values indicate an increase in biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells).

Author Contributions: Conceptualization, M.B. and A.J.; methodology, M.B. and A.J.; software, M.B.; validation, M.B. and A.J.; formal analysis, M.B. and A.J.; investigation, M.B., W.K., J.P., G.C., D.C.-J., A.P., and A.J.; resources, M.B. and A.J.; data curation, M.B.; writing—original draft preparation, M.B.; writing—review and editing, M.B. and A.J.; visualization, M.B.; supervision, A.J.; project administration, M.B. and A.J.; funding acquisition, M.B. and A.J. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All necessary data are presented in the manuscript and in the supplementary data and can be provided from the authors upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the essential oils analyzed in this research are available from the authors upon reasonable request.

References

1. Bassetti, M.; Vena, A.; Croxatto, A.; Righi, E.; Guery, B. How to Manage Pseudomonas Aeruginosa Infections. *Drugs Context* **2018**, *7*, 212527. [CrossRef] [PubMed]
2. Harmsen, M.; Yang, L.; Pamp, S.J.; Tolker-Nielsen, T. An Update on Pseudomonas Aeruginosa Biofilm Formation, Tolerance, and Dispersal. *FEMS Immunol. Med. Microbiol.* **2010**, *59*, 253–268. [CrossRef] [PubMed]
3. Vestby, L.K.; Grønseth, T.; Simm, R.; Nesse, L.L. Bacterial Biofilm and Its Role in the Pathogenesis of Disease. *Antibiotics* **2020**, *9*, 59. [CrossRef] [PubMed]
4. Bharadwaj, K.K.; Rabha, B.; Choudhury, B.K.; Rosalin, R.; Sarkar, T.; Baishya, D.; Chanu, N.B.; Singh, Y.D.; Panda, M.K.; Pati, S. Current Strategies in Inhibiting Biofilm Formation for Combating Urinary Tract Infections: Special Focus on Peptides, Nano-Particles and Phytochemicals. *Biocatal. Agric. Biotechnol.* **2021**, *38*, 102209. [CrossRef]
5. Baptista-Silva, S.; Borges, S.; Ramos, O.L.; Pintado, M.; Sarmento, B. The Progress of Essential Oils as Potential Therapeutic Agents: A Review. *J. Essent. Oil Res.* **2020**, *32*, 279–295. [CrossRef]
6. Yu, Z.; Tang, J.; Khare, T.; Kumar, V. The Alarming Antimicrobial Resistance in ESKAPEE Pathogens: Can Essential Oils Come to the Rescue? *Fitoterapia* **2020**, *140*, 104433. [CrossRef]
7. Sarkic, A.; Stappen, I. Essential Oils and Their Single Compounds in Cosmetics-a Critical Review. *Cosmetics* **2018**, *5*, 11. [CrossRef]
8. Das, S.; Horváth, B.; Šafranko, S.; Jokić, S.; Széchenyi, A.; Koszegi, T. Antimicrobial Activity of Chamomile Essential Oil: Effect of Different Formulations. *Molecules* **2019**, *24*, 4321. [CrossRef]
9. Ács, K.; Balázs, V.L.; Kocsis, B.; Bencsik, T.; Böszörményi, A.; Horváth, G. Antibacterial Activity Evaluation of Selected Essential Oils in Liquid and Vapor Phase on Respiratory Tract Pathogens. *BMC Complementary Altern. Med.* **2018**, *18*, 227. [CrossRef]

10. Amat, S.; Baines, D.; Alexander, T.W. A vapour phase assay for evaluating the antimicrobial activities of essential oils against bovine respiratory bacterial pathogens. *Lett. Appl. Microbiol.* **2017**, *65*, 489–495. [CrossRef]
11. Sakkas, H.; Papadopoulou, C. Antimicrobial Activity of Basil, Oregano, and Thyme Essential Oils. *J. Microbiol. Biotechnol.* **2017**, *27*, 429–438. [CrossRef] [PubMed]
12. Abers, M.; Schroeder, S.; Goelz, L.; Sulser, A.; St. Rose, T.; Puchalski, K.; Langland, J. Antimicrobial Activity of the Volatile Substances from Essential Oils. *BMC Complementary Med. Ther.* **2021**, *21*, 124. [CrossRef] [PubMed]
13. Reyes-Jurado, F.; Franco-Vega, A.; Ramírez-Corona, N.; Palou, E.; López-Malo, A. Essential Oils: Antimicrobial Activities, Extraction Methods, and Their Modeling. *Food Eng. Rev.* **2015**, *7*, 275–297. [CrossRef]
14. de Sousa, J.P.; de Azerêdo, G.A.; de Araújo Torres, R.; da Silva Vasconcelos, M.A.; da Conceição, M.L.; de Souza, E.L. Synergies of Carvacrol and 1,8-Cineole to Inhibit Bacteria Associated with Minimally Processed Vegetables. *Int. J. Food Microbiol.* **2012**, *154*, 145–151. [CrossRef]
15. de Azeredo, G.A.; Stamford, T.L.M.; Nunes, P.C.; Gomes Neto, N.J.; de Oliveira, M.E.G.; de Souza, E.L. Combined Application of Essential Oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to Inhibit Bacteria and Autochthonous Microflora Associated with Minimally Processed Vegetables. *Food Res. Int.* **2011**, *44*, 1541–1548. [CrossRef]
16. de Sousa, J.P.; de Araújo Torres, R.; de Azerêdo, G.A.; Figueiredo, R.C.B.Q.; da Silva Vasconcelos, M.A.; de Souza, E.L. Carvacrol and 1,8-Cineole Alone or in Combination at Sublethal Concentrations Induce Changes in the Cell Morphology and Membrane Permeability of Pseudomonas Fluorescens in a Vegetable-Based Broth. *Int. J. Food Microbiol.* **2012**, *158*, 9–13. [CrossRef] [PubMed]
17. Jiang, Y.; Wu, N.; Fu, Y.J.; Wang, W.; Luo, M.; Zhao, C.J.; Zu, Y.G.; Liu, X.L. Chemical Composition and Antimicrobial Activity of the Essential Oil of Rosemary. *Environ. Toxicol. Pharmacol.* **2011**, *32*, 63–68. [CrossRef] [PubMed]
18. Hendry, E.R.; Worthington, T.; Conway, B.R.; Lambert, P.A. Antimicrobial Efficacy of Eucalyptus Oil and 1,8-Cineole Alone and in Combination with Chlorhexidine Digluconate against Microorganisms Grown in Planktonic and Biofilm Cultures. *J. Antimicrob. Chemother.* **2009**, *64*, 1219–1225. [CrossRef]
19. Mutlu-Ingok, A.; Firtin, B.; Karbancioglu-Guler, F.; Altay, F. A Study on Correlations between Antimicrobial Effects and Diffusion Coefficient, Zeta Potential and Droplet Size of Essential Oils. *Int. J. Food Eng.* **2020**, *16*, 1–9. [CrossRef]
20. Brożyna, M.; Paleczny, J.; Kozłowska, W.; Chodaczek, G.; Dudek-Wicher, R.; Felińczak, A.; Gołębiewska, J.; Górniak, A.; Junka, A. The Antimicrobial and Antibiofilm in Vitro Activity of Liquid and Vapour Phases of Selected Essential Oils against Staphylococcus Aureus. *Pathogens* **2021**, *10*, 1207. [CrossRef]
21. Cal, K. Aqueous Solubility of Liquid Monoterpenes at 293 K and Relationship with Calculated Log P Value. *Yakugaku Zasshi* **2006**, *126*, 307–309. [CrossRef] [PubMed]
22. Alibi, S.; Ben Selma, W.; Ramos-Vivas, J.; Smach, M.A.; Touati, R.; Boukadida, J.; Navas, J.; Ben Mansour, H. Anti-Oxidant, Antibacterial, Anti-Biofilm, and Anti-Quorum Sensing Activities of Four Essential Oils against Multidrug-Resistant Bacterial Clinical Isolates. *Curr. Res. Transl. Med.* **2020**, *68*, 59–66. [CrossRef] [PubMed]
23. Ceylan, O.; Uğur, A.; Saraç, N.; Ozcan, F.; Baygar, T. The in Vitro Antibiofilm Activity of *Rosmarinus officinalis* L. Essential Oil against Multiple Antibiotic Resistant *Pseudomonas* sp. and *Staphylococcus* sp. *J. Food Agric. Environ.* **2014**, *12*, 82–86.
24. Karuppiah, V.; Thirunanasambandham, R.; Thangaraj, G. Anti-Quorum Sensing and Antibiofilm Potential of 1,8-Cineole Derived from Musa Paradisiaca against Pseudomonas Aeruginosa Strain PAO1. *World J. Microbiol. Biotechnol.* **2021**, *37*, 66. [CrossRef]
25. Janssen, A.M.; Chin, N.L.J.; Scheffer, J.J.C.; Svendsen, A.B. Screening for Antimicrobial Activity of Some Essential Oils by the Agar Overlay Technique—Statistics and Correlations. *Pharm. Weekbl. Sci. Ed.* **1986**, *8*, 289–292. [CrossRef]
26. Cavanagh, H.M.A. Antifungal Activity of the Volatile Phase of Essential Oils: A Brief Review. *Nat. Prod. Commun.* **2007**, *2*, 1297–1302. [CrossRef]
27. Goñi, P.; López, P.; Sánchez, C.; Gómez-Lus, R.; Becerril, R.; Nerín, C. Antimicrobial Activity in the Vapour Phase of a Combination of Cinnamon and Clove Essential Oils. *Food Chem.* **2009**, *116*, 982–989. [CrossRef]
28. Dudek-Wicher, R.; Paleczny, J.; Kowalska-Krochmal, B.; Szymczyk-Ziółkowska, P.; Pachura, N.; Szumny, A.; Brożyna, M. Activity of Liquid and Volatile Fractions of Essential Oils against Biofilm Formed by Selected Reference Strains on Polystyrene and Hydroxyapatite Surfaces. *Pathogens* **2021**, *10*, 515. [CrossRef]
29. Reyes-Jurado, F.; Navarro-Cruz, A.R.; Ochoa-Velasco, C.E.; Palou, E.; López-Malo, A.; Ávila-Sosa, R. Essential Oils in Vapor Phase as Alternative Antimicrobials: A Review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 1641–1650. [CrossRef]
30. Gagné-Thivierge, C.; Barbeau, J.; Levesque, R.C.; Charette, S.J. A New Approach to Study Attached Biofilms and Floating Communities from Pseudomonas Aeruginosa Strains of Various Origins Reveals Diverse Effects of Divalent Ions. *FEMS Microbiol. Lett.* **2018**, *365*, fny155. [CrossRef]
31. Azam, M.W.; Khan, A.U. Updates on the Pathogenicity Status of Pseudomonas Aeruginosa. *Drug Discov. Today* **2019**, *24*, 350–359. [CrossRef] [PubMed]
32. Poole, K. Pseudomonas Aeruginosa: Resistance to the Max. *Front. Microbiol.* **2011**, *2*, 65. [CrossRef] [PubMed]
33. Kim, J.; Marshall, M.R.; Wei, C.-i. Antibacterial Activity of Some Essential Oil Components against Five Foodborne Pathogens. *J. Agric. Food Chem.* **1995**, *43*, 2839–2845. [CrossRef]
34. Hajlaoui, H.; Trabelsi, N.; Noumi, E.; Snoussi, M.; Fallah, H.; Ksouri, R.; Bakhrouf, A. Biological Activities of the Essential Oils and Methanol Extract of Tow Cultivated Mint Species (Mentha Longifolia and Mentha Pulegium) Used in the Tunisian Folkloric Medicine. *World J. Microbiol. Biotechnol.* **2009**, *25*, 2227–2238. [CrossRef]

35. Cristani, M.; D'Arrigo, M.; Mandalari, G.; Castelli, F.; Sarpietro, M.G.; Micieli, D.; Venuti, V.; Bisignano, G.; Saija, A.; Trombetta, D. Interaction of Four Monoterpenes Contained in Essential Oils with Model Membranes: Implications for Their Antibacterial Activity. *J. Agric. Food Chem.* **2007**, *55*, 6300–6308. [CrossRef] [PubMed]
36. Junka, A.F.; Żywicka, A.; Szymczyk, P.; Dziadas, M.; Bartoszewicz, M.; Fijałkowski, K.A.D.A.M. Test (Antibiofilm Dressing's Activity Measurement)—Simple Method for Evaluating Anti-Biofilm Activity of Drug-Saturated Dressings against Wound Pathogens. *J. Microbiol. Methods* **2017**, *143*, 6–12. [CrossRef]
37. Brożyna, M.; Żywicka, A.; Fijałkowski, K.; Gorczyca, D.; Oleksy-Wawrzyniak, M.; Dydak, K.; Migdał, P.; Dudek, B.; Bartoszewicz, M.; Junka, A. The Novel Quantitative Assay for Measuring the Antibiofilm Activity of Volatile Compounds (Antibiovol). *Appl. Sci.* **2020**, *10*, 7343. [CrossRef]

molecules

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Supplementary materials

Chemical Composition and Antibacterial Activity of Liquid and Volatile Phase of Essential Oils Against Planktonic and Biofilm-Forming Cells of *Pseudomonas aeruginosa*

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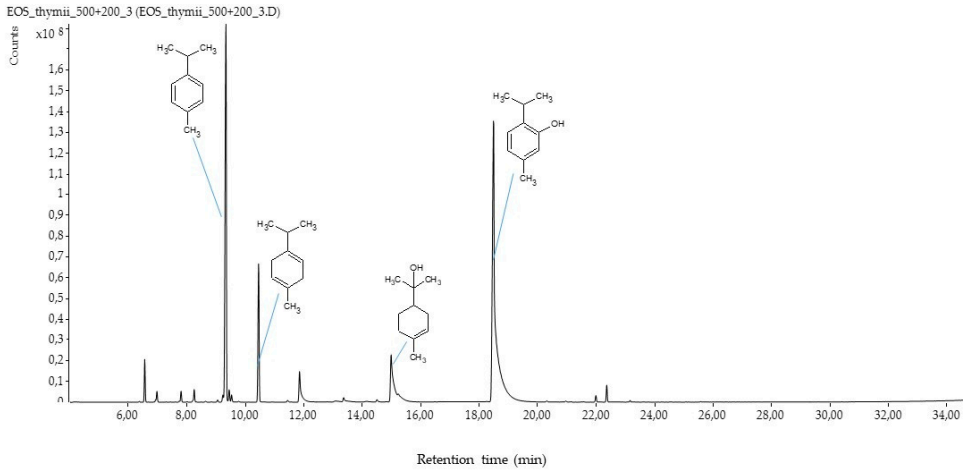
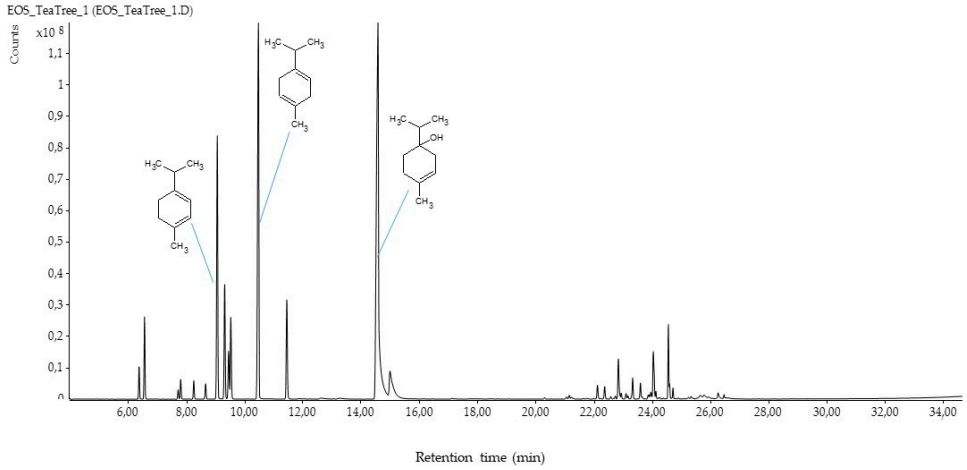
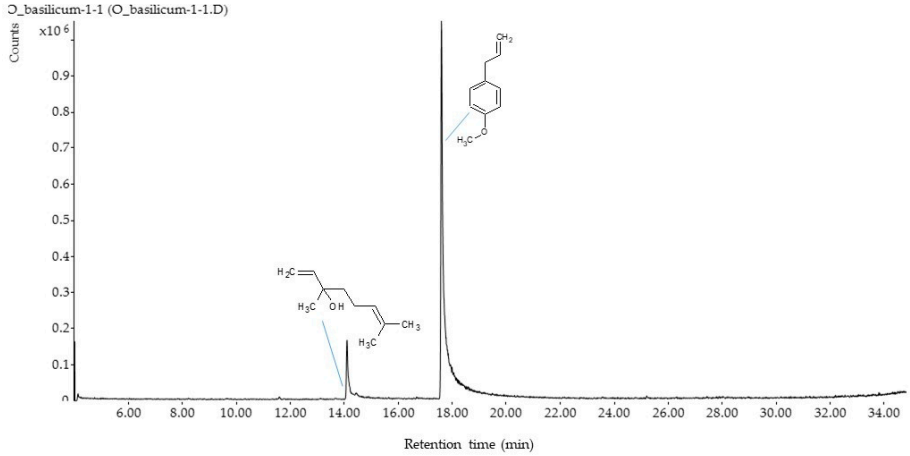
A. Thyme oil



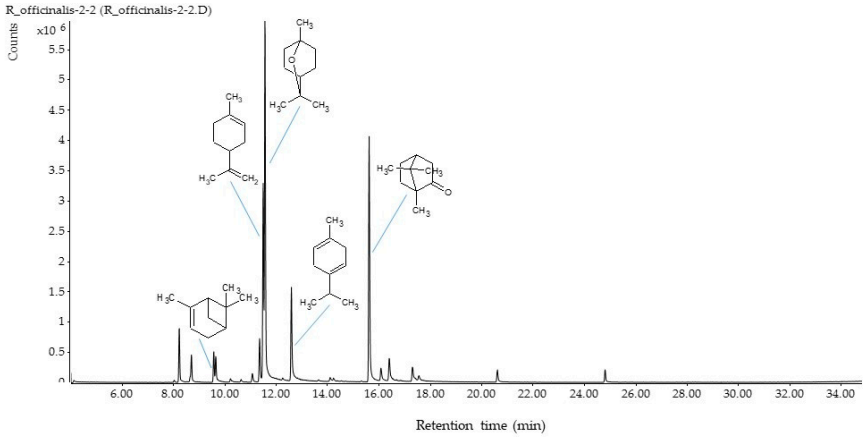
B. Tea tree oil



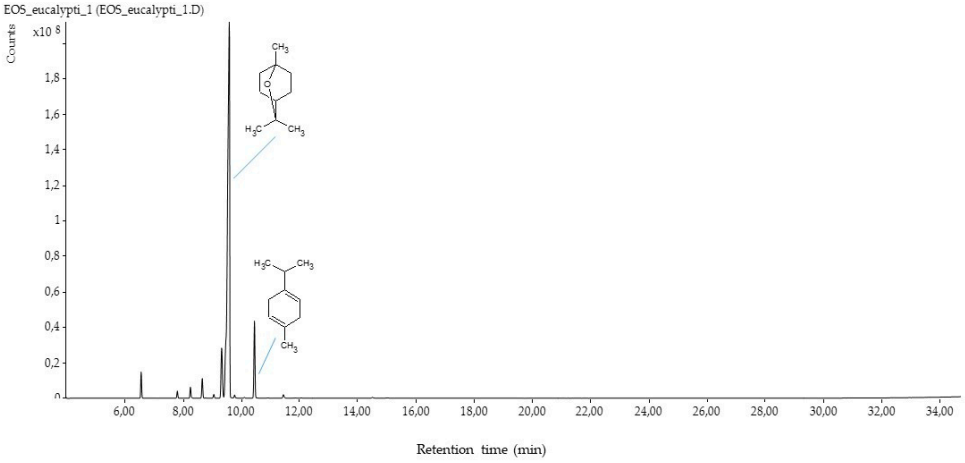
C. Basil oil



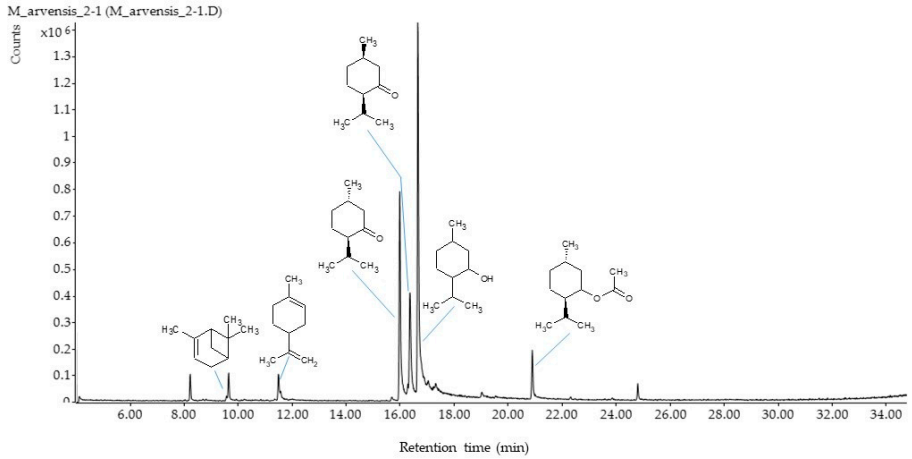
D. Rosemary oil



E. Eucalyptus oil



F. Menthol mint oil



G. Lavender oil

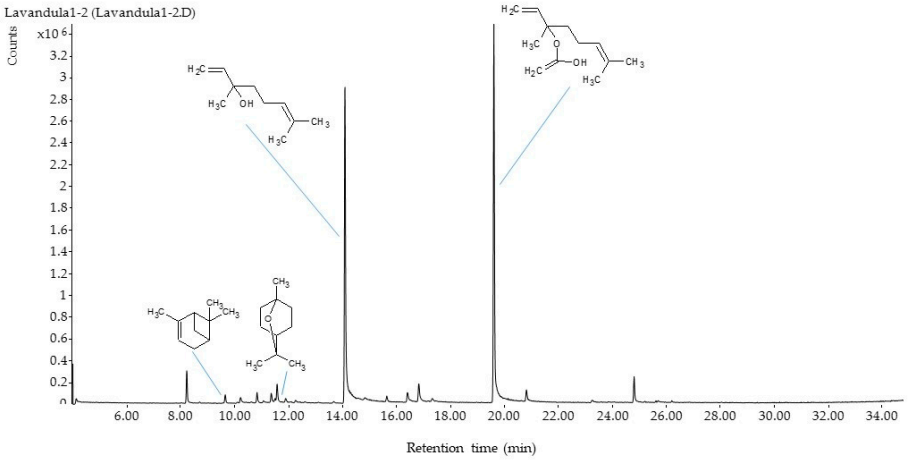


Figure S1. Chromatograms of tested EOs measured with GC-MS (Gas Chromatography-Mass Spectrometry).

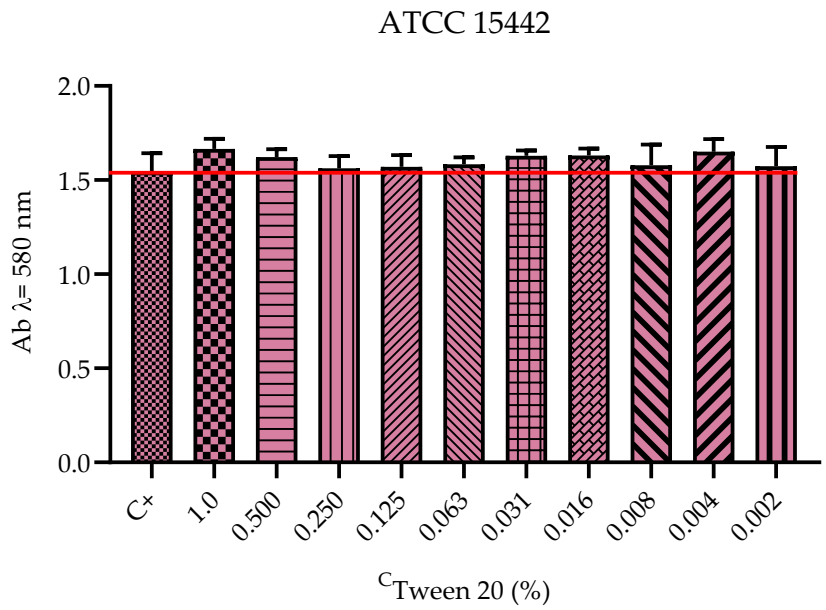
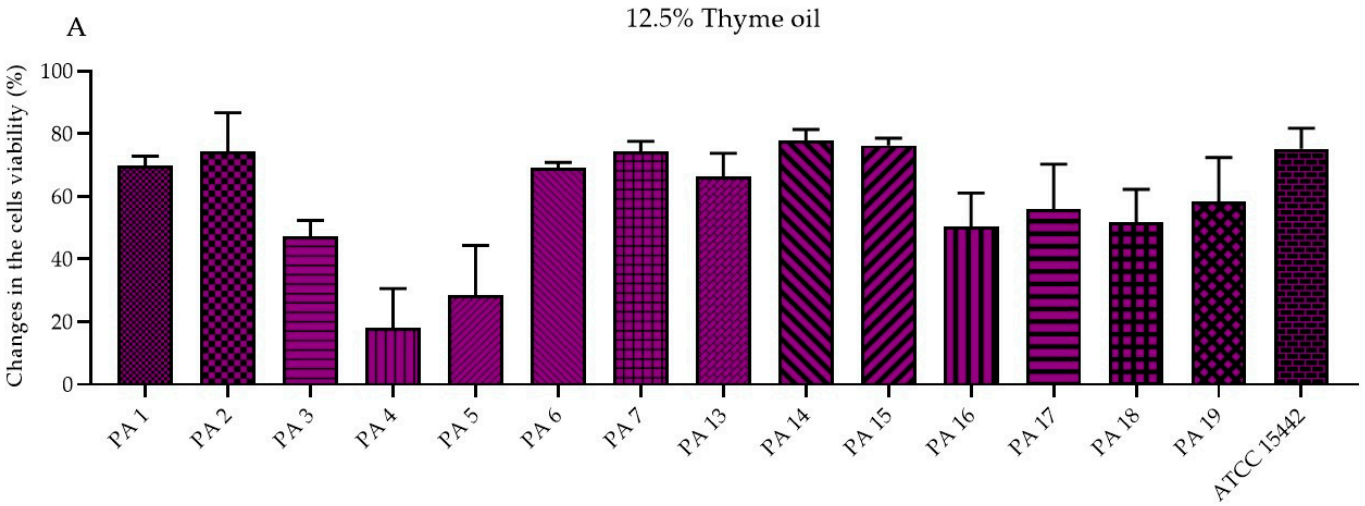
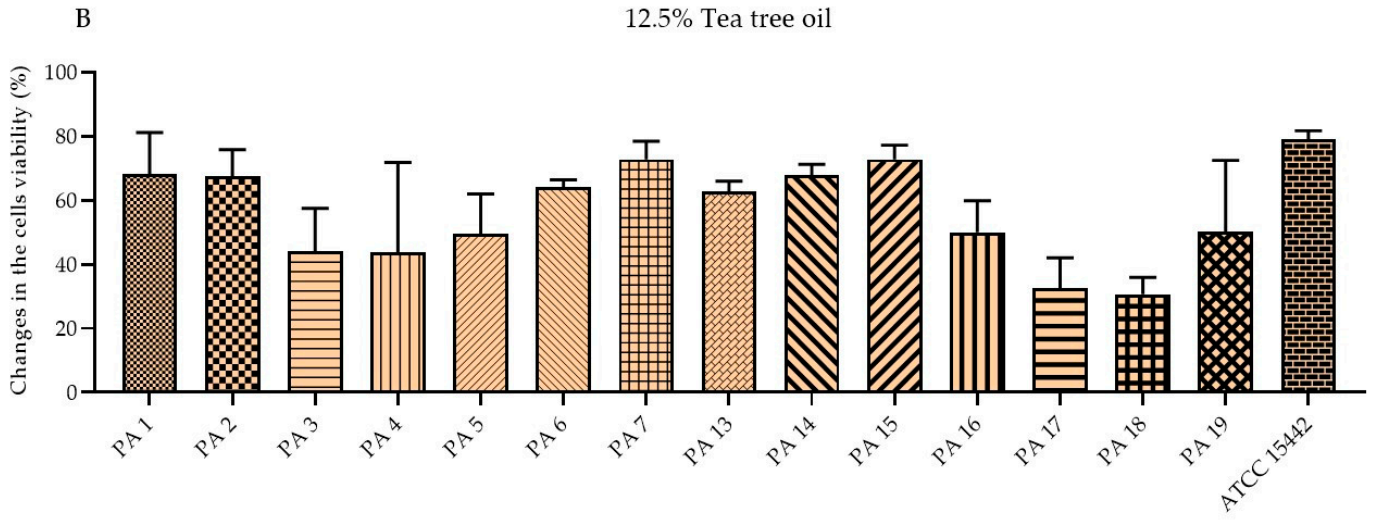
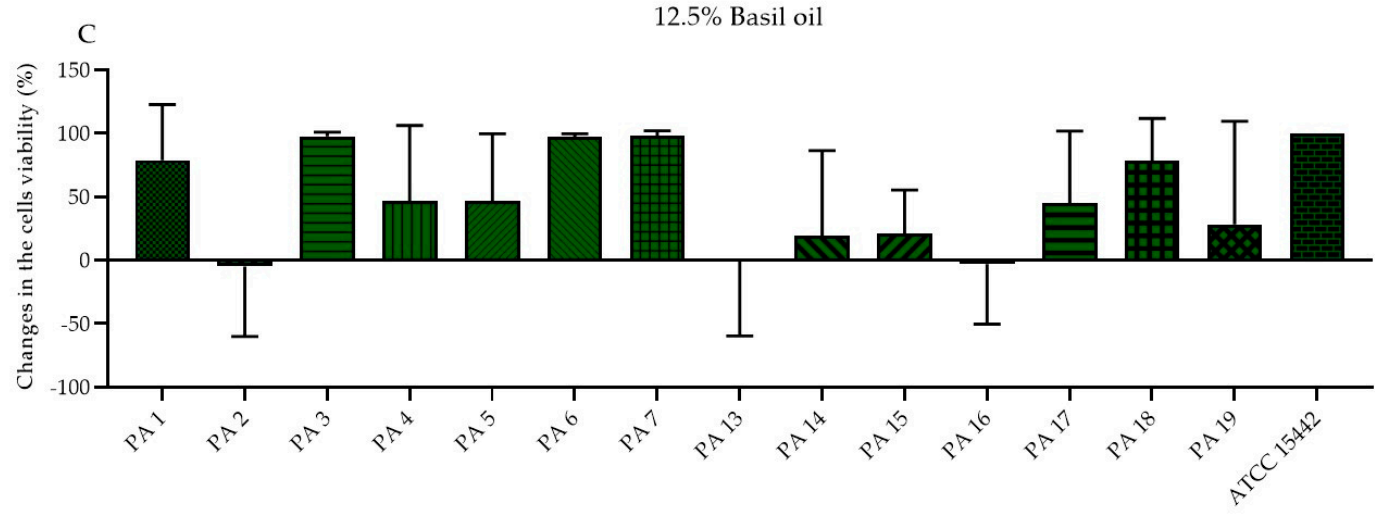
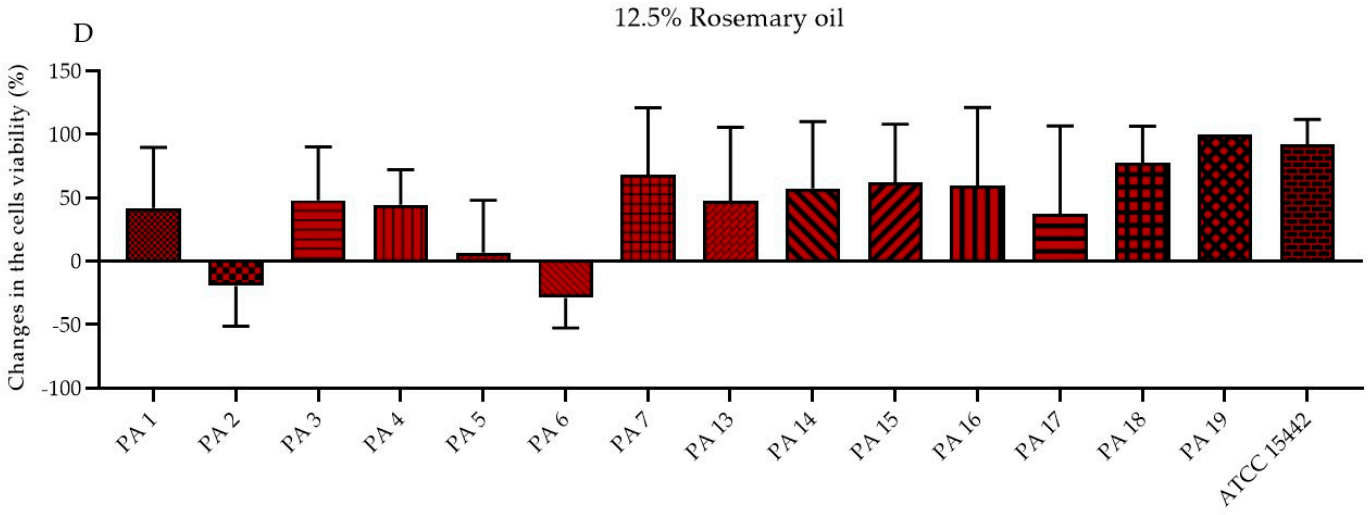


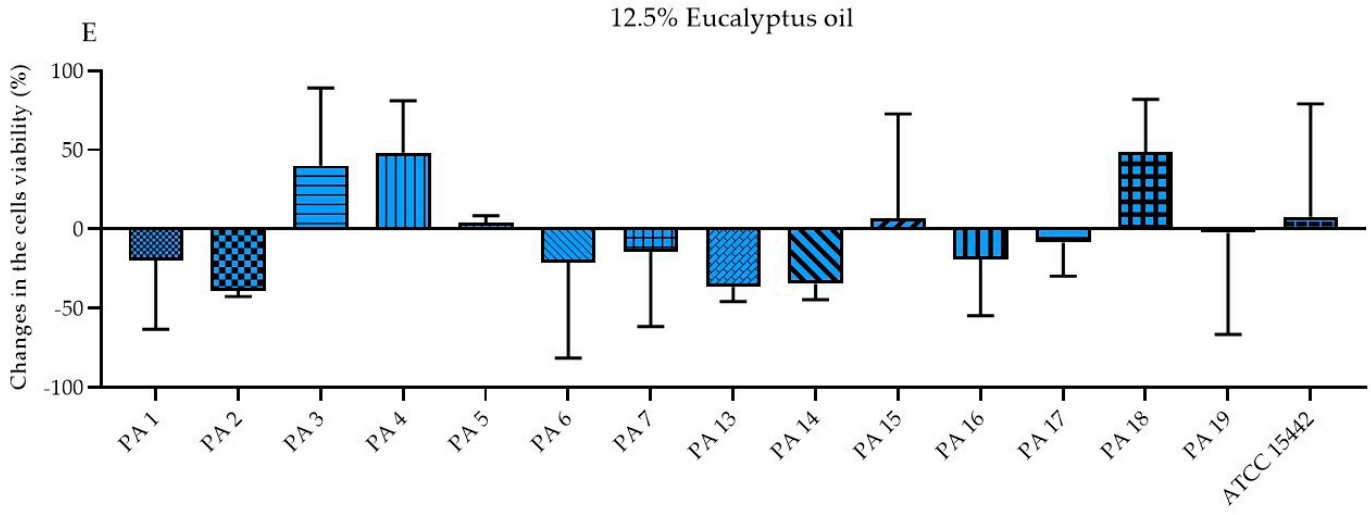
Figure S2. The antimicrobial activity of different concentrations (%) (*v*/*v*) of Tween 20 against planktonic forms of *P. aeruginosa* ATCC 15442 strain. Ab- absorbance after 24 h incubation, C+- untreated cells. The absorbance of untreated cells is marked with a red line.




B
12.5% Tea tree oil
Changes in the cells viability (%)
100
80
60
40
20
0
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


C
12.5% Basil oil
Changes in the cells viability (%)
150
100
50
0
-50
-100
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


D
12.5% Rosemary oil
Changes in the cells viability (%)
150
100
50
0
-50
-100
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


E
12.5% Eucalyptus oil
Changes in the cells viability (%)
100
50
0
-50
-100
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442

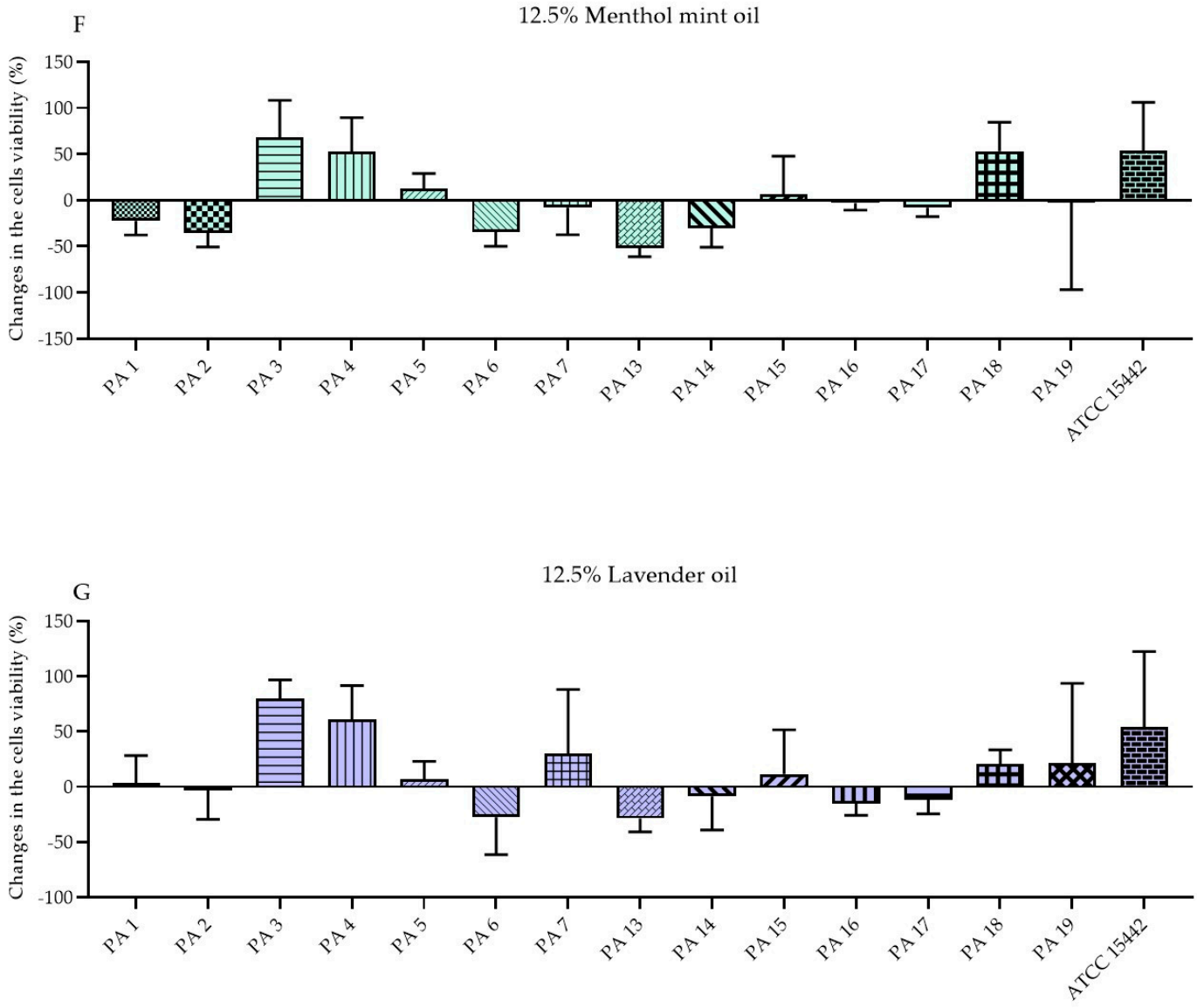
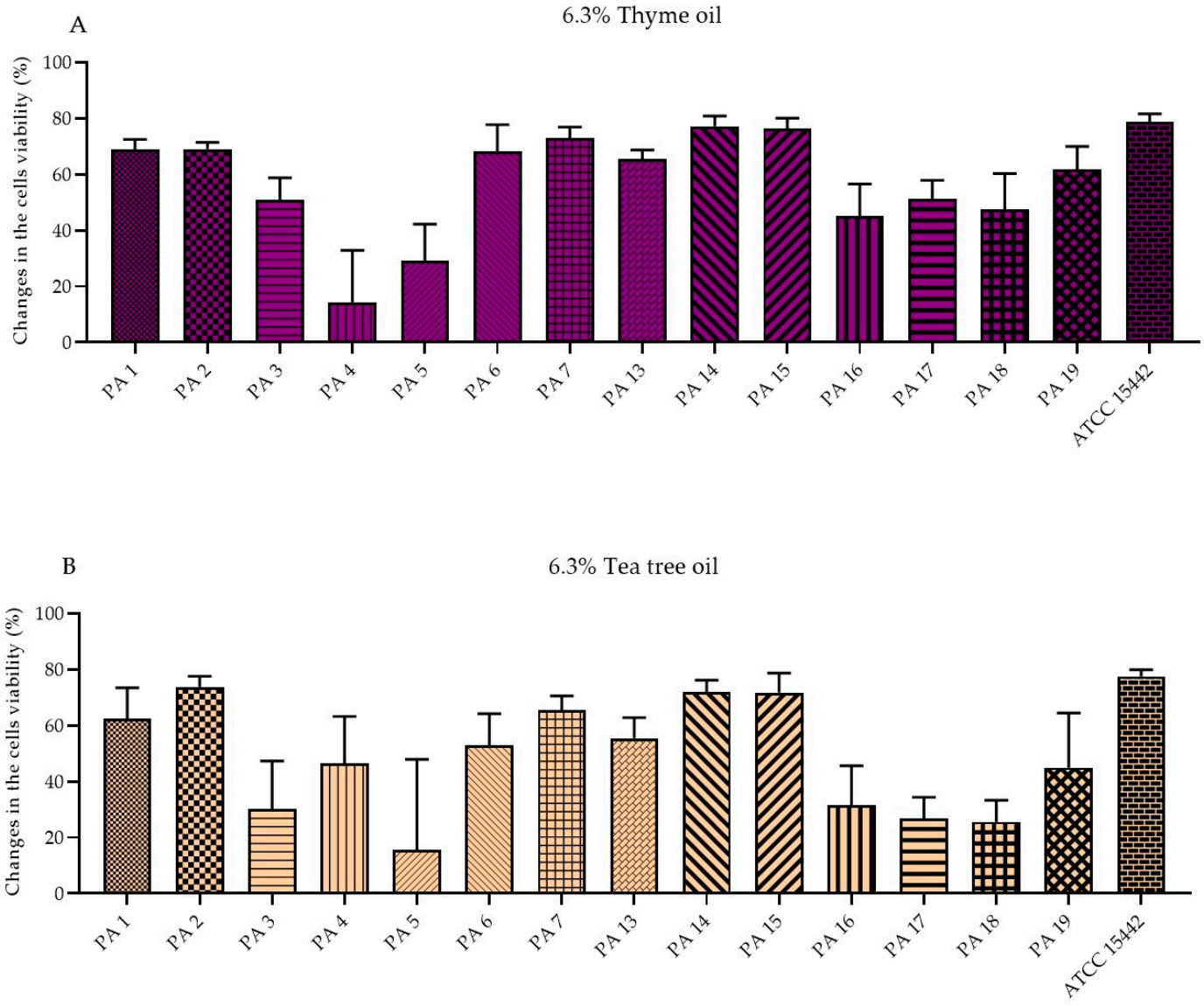
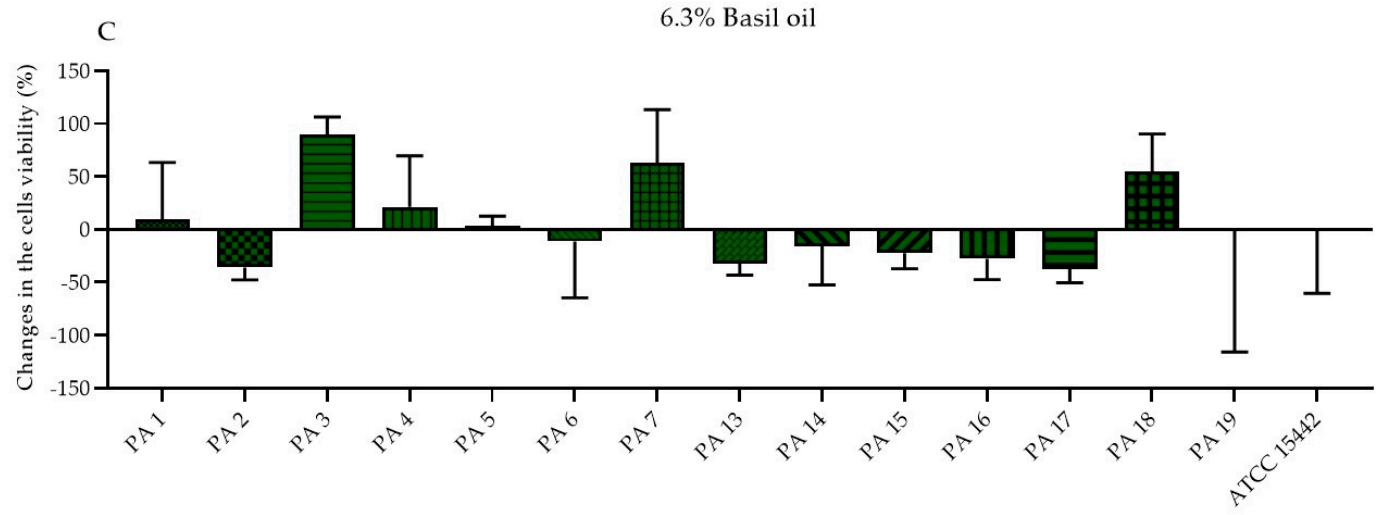
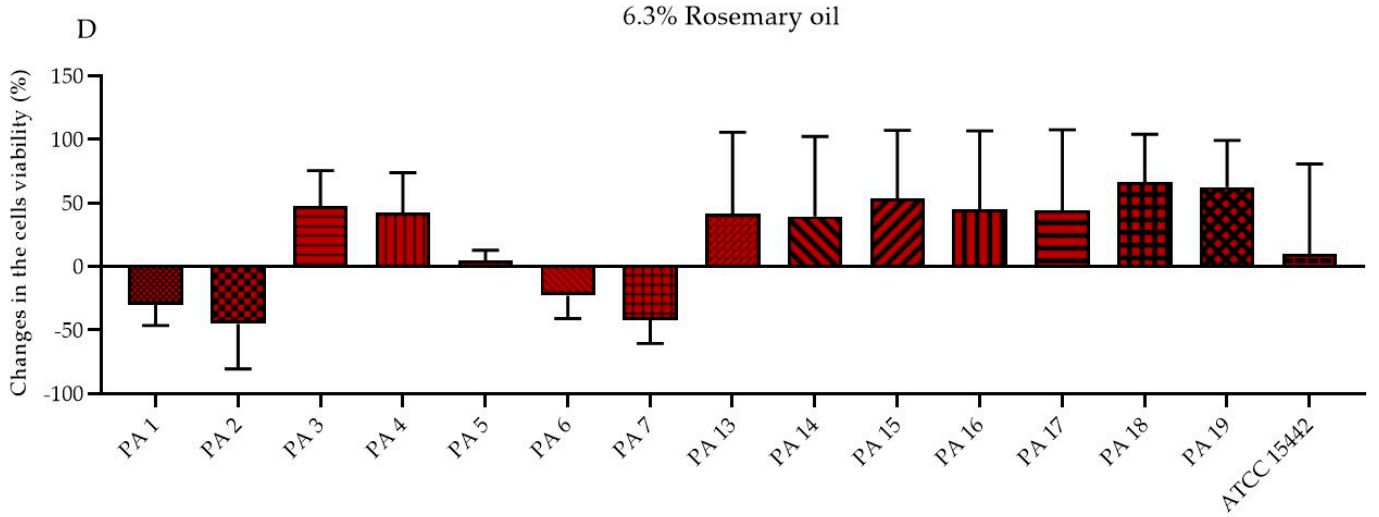
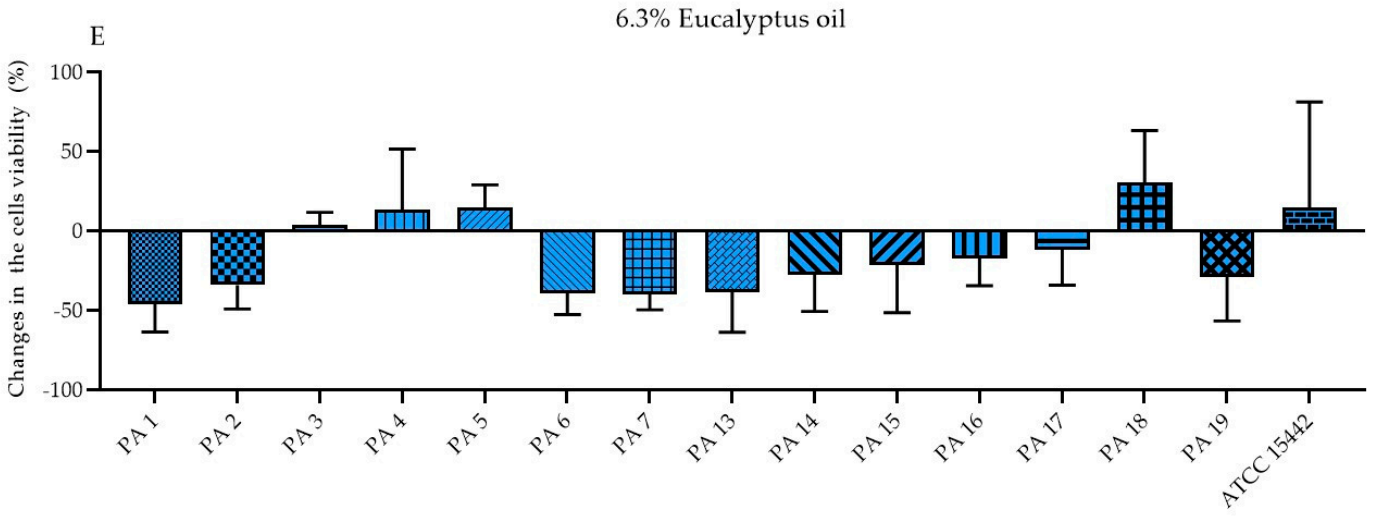


Figure S3. Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1- 7, PA 13- 19) and the reference (ATCC 15442) strains after treatment with emulsified essential oils in their liquid forms in the concentration of 12.5% (*v*/*v*). Results of microdilution methodology with (**A**-**B**) TTC and (**C**-**G**) resazurin staining. Standard deviations are marked. The negative values indicate an increase of biofilm-forming cells' viability after their treatment with EOs in comparison to the growth control (untreated cells).


A
6.3% Thyme oil
Changes in the cells viability (%)
100
80
60
40
20
0
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442
B
6.3% Tea tree oil
Changes in the cells viability (%)
100
80
60
40
20
0
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


C
6.3% Basil oil
Changes in the cells viability (%)
150
100
50
0
-50
-100
-150
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


D
6.3% Rosemary oil
Changes in the cells viability (%)
150
100
50
0
-50
-100
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442


E
6.3% Eucalyptus oil
Changes in the cells viability (%)
100
50
0
-50
-100
PA 1
PA 2
PA 3
PA 4
PA 5
PA 6
PA 7
PA 13
PA 14
PA 15
PA 16
PA 17
PA 18
PA 19
ATCC 15442

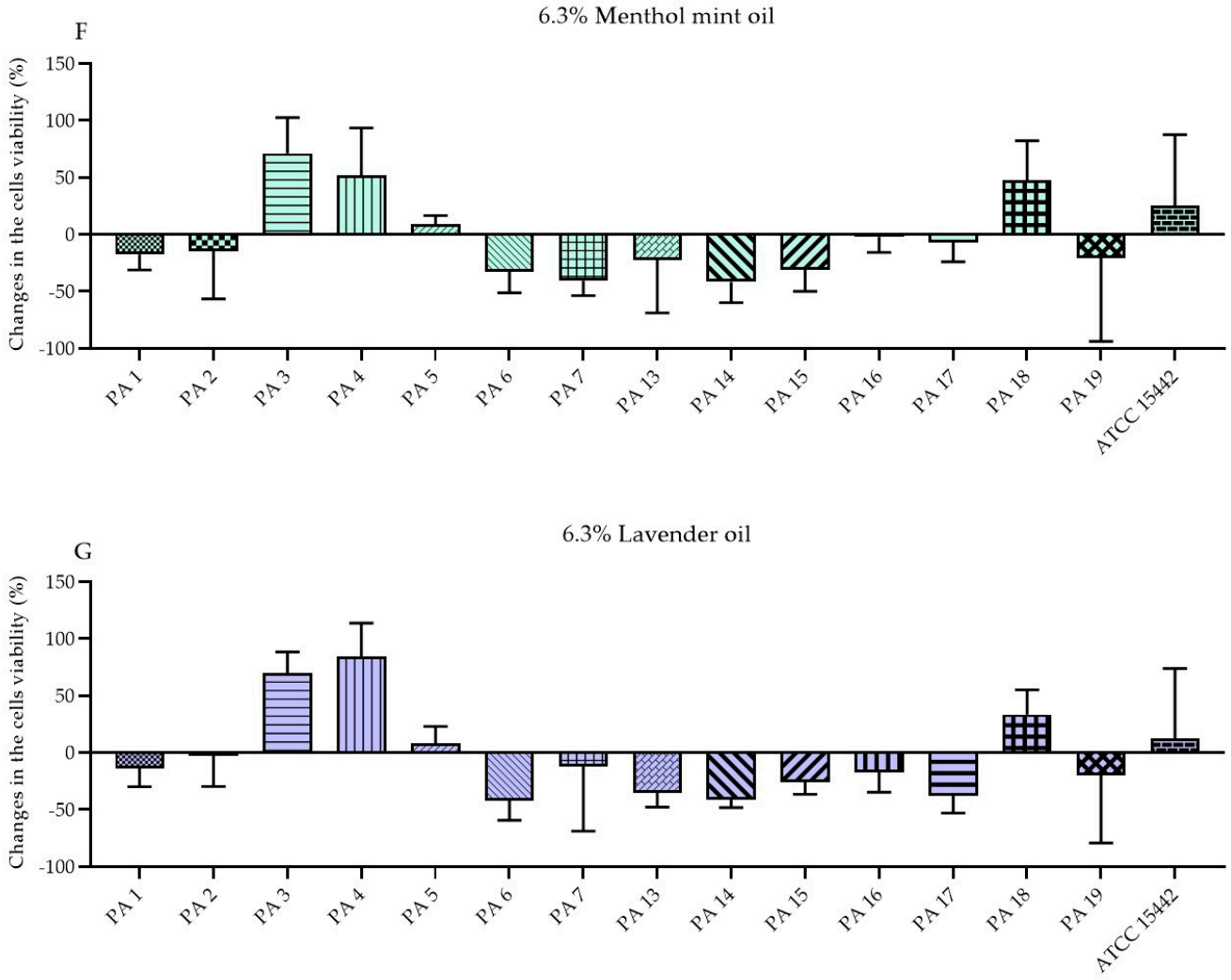


Figure S4. Changes in the biofilm-forming cells viability (%) of *P. aeruginosa* clinical (PA 1- 7, PA 13- 19) and the reference (ATCC 15442) strains after treatment with emulsified essential oils in their liquid forms in the concentration of 6.3% (*v*/*v*). Results of microdilution methodology with (**A**-**B**) TTC and (**C**-**G**) resazurin staining. Standard deviations are marked. The negative values indicate an increase of biofilm-forming cells viability after their treatment with EOs in comparison to the growth control (untreated cells).

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Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity

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Abstract

The treatment of infected non-healing wounds poses a significant challenge to contemporary medicine. Essential oils (EOs) are being increasingly investigated as potential antibiofilm agents for the management of biofilm-related wound infections. However, their *in vitro* antimicrobial activity reported in literature does not necessarily reflect the actual *in vivo* activity due to, among others, methodological imperfections of performed tests. In our study, we cultivated a *Staphylococcus aureus* biofilm in a novel IVWM (In Vitro Wound Milieu) medium designed to simulate the wound environment and compared it with biofilms cultured in the standard microbiological Tryptic Soy Broth (TSB) medium. We examined and compared critical biofilm properties such as morphology, biomass, metabolic activity, cell count, thickness, and spatial distribution of live and dead cells. Subsequently, staphylococcal biofilms and planktonic cells cultured in both media were exposed to the activity of thyme or rosemary EOs (T-EO, R-EO, respectively). We found that morphology of biofilms cultured in IVWM resembled more the morphology of biofilms visualized in the non-healing wounds than biofilms cultured in TSB. The biomass, metabolic activity, cell number, and the ratio of live to dead cells of *S. aureus* biofilms were all lower in IVWM compared to the TSB medium. Additionally, while EOs demonstrated overall significant anti-staphylococcal activity, their efficacy varied depending on the medium used. Generally, EOs displayed lower antimicrobial activity (against planktonic cells) in the IVWM than in the TSB medium. Interestingly, T-EO caused a higher reduction of biofilm cells in

IVWM than in the TSB medium, in contrast to R-EO. Our findings suggest that EOs hold promise as agents for the treatment of biofilm-related wound infections. However, it is crucial to apply *in vitro* conditions that closely reflect the wound infection site to gain an accurate insight into the real-world activity of these antimicrobial/antibiofilm agents.

1 Introduction

The dissemination of antibiotic-resistant microorganisms poses a global threat to public health care. As the efficacy of existing antibiotics decreases and new ones remain undeveloped, the applicability of nonantibiotic therapeutics to combat infections is being thoroughly investigated worldwide (Mühlen and Dersch, 2016).

Non-healing wounds are particularly susceptible to infections caused by antibiotic-resistant microorganisms (Bowler, 2018). These wounds affect 20 million patients annually and require more than 31 billion USD per year for treatment (Leaper et al., 2015). The five-year mortality rate for people with diabetic non-healing wounds is comparable to the five-year mortality rate for patients with cancer (30.5% vs. 31%, respectively) (Armstrong et al., 2020).

Infections, one of the most frequent complications of non-healing wounds, are caused by biofilms – complex microbial communities embedded within an extracellular matrix (ECM) (Percival et al., 2012). This scaffold provides not only a physical framework for the biofilm but also impedes the immune response and penetration of antimicrobial agents. Furthermore, some microbial cells within the biofilm matrix exhibit low cellular activity, rendering them insensitive to antibiotics that target cellular transcription or translation processes (Povolotsky et al., 2021). In general, biofilms exhibit significantly higher tolerance to the immune system and antimicrobials compared to their free-floating (planktonic) counterparts.

Thus, the treatment of infected non-healing wounds is a major challenge for modern medicine. Current strategies to eradicate wound biofilms involve the debridement (removal) of infected tissue, combined with the topical application of dressings and antiseptic agents (Kaiser et al., 2021). Topical administration of antibiotics is no longer recommended due to their low activity against biofilms and the potential to induce microbial resistance, hypersensitivity, or contact allergy (Siddiqui and Bernstein, 2010).

Modern antiseptic agents are considered first-line antimicrobials for non-healing wound management due to their broad spectrum of antimicrobial activity, non-specific mode of action, and low *in vivo* cytotoxicity (Alves et al., 2021). However, several reports indicate the potential for microorganisms to develop tolerance to modern antiseptic agents (compounds obtained through industrial chemical synthesis). The survival of *Burkholderia cepacia* in octenidine dihydrochloride-containing solutions and the increased tolerance of certain strains of *Pseudomonas aeruginosa* to octenidine dihydrochloride exemplify such risks (Becker et al., 2018; Shepherd et al., 2018)

It can be inferred that the effectiveness of systemic antibiotic therapy against wound biofilms is limited, and the topical administration of these antimicrobials is not recommended. The use of modern antiseptic agents (e.g., polyhexanide, povidone-iodine) still correlates with favorable clinical outcomes, but the example of octenidine dihydrochloride raises concerns that the efficacy of these antiseptics may also be reduced due to microorganisms' tendency to develop resistance (Becker et al., 2018; Shepherd et al., 2018). Therefore, not only non-antibiotic but also non-antiseptic approaches are now considered next-generation strategies for treating biofilm-related infections. These methods include the use of natural compounds, enzymes, and other bioactive molecules that can disrupt biofilm formation and/or enhance host immune responses (Silva et al., 2020). In this regard, plant-derived essential oils (EOs) are of great interest as potential antibiofilm agents. These multi-component volatile liquids exhibit a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Agreles et al., 2021). EOs can also interfere with or impede various processes

occurring in biofilms, such as adhesion, quorum-sensing, or modulation of the expression of biofilm-related genes (Reichling, 2020).

The non-specific mode of EOs' antimicrobial action is considered to limit the risk of triggering bacterial resistance (Yap et al., 2014). Thanks to their low cytotoxicity, anti-inflammatory activity, and ability to promote the wound-healing process, the use of EOs can be perceived as an effective future strategy for the treatment of non-healing wound infections (Costa et al., 2019). Although several *in vitro* studies report significant antimicrobial properties of EOs, these likely do not reflect their actual *in vivo* activity (Orchard et al., 2017; Sienkiewicz et al., 2017; Patterson et al., 2019). Major shortcomings of this *in vitro*, research include using standard microbiological media (rather than media reflecting the composition of wound exudate) and the evaluation of EOs' activity primarily against planktonic forms of microbes, not against biofilms. Recent reports indicate that providing a milieu that mimics the wound environment in *in vitro* tests significantly alters key biofilm characteristics such as metabolic activity, three-dimensional structure, and matrix composition, thereby affecting its tolerance to antimicrobial agents (Thaarup and Bjarnsholt, 2021; Vyas et al., 2022). Therefore, in this study, we cultivated a *Staphylococcus aureus* biofilm (one of the main factors of wound infection) in a newly formulated medium known as the IVWM (In Vitro Wound Milieu), which comprises serum, cell-matrix elements, and host factors that reflect the wound environment (Kadam et al., 2021). After conducting a thorough analysis of the key properties of the biofilm grown in IVWM and comparing them to those of the biofilm cultivated in standard microbiological TSB medium, we exposed staphylococcal biofilms to the activity of thyme and rosemary EOs (T-EO, R-EO, respectively). We also carried out control analyses on planktonic *Staphylococcus aureus* cells. To the best of our knowledge, this is the first study that assesses the antimicrobial properties of EOs under conditions that resemble a chronic wound milieu.

2 Materials and methods

2.1 Microorganisms

One reference strain, *Staphylococcus aureus* ATCC 6538 (American Type and Culture Collection), and eleven clinical isolates were tested for research purposes. The clinical strains included four MSSA strains (Methicillin-Susceptible *Staphylococcus aureus*) and seven MRSA strains (Methicillin-Resistant *Staphylococcus aureus*). The MSSA strains were marked S3, S6, S8, S11; MRSA as R1, R8-R13. The strains are part of the Strain and Line Collection of the Pharmaceutical Microbiology and Parasitology Department of the Medical University of Wroclaw.

2.2 Essential oils

The antimicrobial activity of two commercial essential oils (EOs) was tested:

- thyme oil, thyme chemotype (T-EO, obtained from *Thymus vulgaris* L. leaves), produced by Instytut Aromaterapii, Poland;
- rosemary oil, camphor chemotype (R-EO, obtained from *Rosmarinus officinalis* L. leaves), produced by Instytut Aromaterapii, Poland.

2.3 Culture conditions

Bacteria were cultured in two media:

1. Standard microbiological Tryptic Soy Broth (Biomaxima, Poland) marked TSB. The detailed composition of TSB is presented in **Table 1**.

2. Medium prepared according to the formula presented by (Kadam et al., 2021), marked IVWM (In Vitro Wound Milieu).

Sterile Fetal Bovine Serum (Biowest, France, cat. no. S181H) was used as the base component of IVWM. Firstly, stock solutions of the components were prepared as follows:

- fibronectin (Human plasma fibronectin, Sigma-Aldrich, USA, cat. no. FC010) 1 mg/mL solution in autoclaved distilled water,
- fibrinogen (Fibrinogen from human plasma, Sigma-Aldrich, USA, cat. no. F3879) 10 mg/mL solution in saline (Stanlab, Poland),
- lactoferrin (Lactoferrin human, Sigma-Aldrich, USA, cat. no. L4040) 2 mg/mL solution in Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, USA),
- lactic acid (Sigma-Aldrich, USA, cat. no. W261114) 11.4 M solution,

and they were filtered using a 0.22 μm syringe filter (Sungo, Europe). Collagen (Collagen solution from bovine skin, concentration 2.9-3.2 mg/mL, Sigma-Aldrich, USA, cat. no. C4243) was purchased sterile. The IVWM was obtained by combining the ingredients at concentrations presented in **Table 1**. The medium was stored for a maximum of seven days at 2-8°C and was protected from light.

Table 1. Composition of Tryptic Soy Broth (TSB) according to the manufacturer's specification **(A)**. Composition of In Vitro Wound Milieu (IVWM) **(B)**.

(A) TSB	
Pancreatic digest of casein	17 g/L
Peptic digest of soybean	3 g/L
Dipotassium hydrogen phosphate	2.5 g/L
Sodium chloride	5 g/L
Glucose monohydrate	2.5 g/L
(B) IVWM	
Fetal Bovine Serum	70%
Fibronectin	30-60 μg/mL
Fibrinogen	200-400 μg/mL
Lactoferrin	20-30 μg/mL
Lactic acid	11-12 mM
Collagen	10-12 μg/mL
Saline	19.6%

2.4 Assessment of EOs Chemical Composition using Gas Chromatography-Mass Spectrometry (GC-MS)

EOs were diluted 50 times with hexane (JTB, GB), vortexed, and immediately analyzed. The analysis was performed on Agilent 7890B GC system coupled with 7000GC/TQ connected with PAL RSI85 autosampler (Agilent Technologies, USA) and equipped with an HP-5 MS column (30 m × 0.25 mm × 0.25 μm). Helium was used as a carrier gas at a total flow of 1 mL/min, and the injection mode was split in a ratio of 1:100. Analysis conditions were as follows: the initial temperature was 50°C for 1 min, then increased to 4°C/min to 170°C, and then 10°C/min to 280°C which maintained for 2 min. The MS detector settings were as follows: ionization voltage 70 eV, transfer line, source, and quadrupole temperature – 320, 230, and 150°C, respectively. Detection was performed in full scan mode in a range of 30-400 m/z. Identification was based on a comparison of retention index (RI) and

mass spectra with data from the NIST 17.1 library and literature. Linear retention indexes were determined using a mixture of C8-C20 saturated alkanes (Sigma-Aldrich, USA) under the same conditions as for EOs. The relative abundance of each EO component was expressed as percentage content based on peak area normalization. The MassHunter Workstation Software version B.09.00 was used for peak normalization. All analyses were performed in triplicate.

2.5 Evaluation of biofilm biomass, biofilm metabolic activity, and the number of colony-forming units

Biofilm mass and biofilm metabolic activity of one reference and eleven clinical *S. aureus* strains were evaluated in TSB (Tryptic Soy Broth, Biomaxima, Poland) or IVWM (In Vitro Wound Milieu) medium. The number of colony-forming units was assessed for the reference strain. For this purpose, the bacteria were pre-incubated overnight in an appropriate medium (TSB or IVWM) at 37°C. Next, the bacterial suspensions were prepared in saline (Stanlab, Poland) and adjusted to 0.5 MF (McFarland, 1.5 x 10^8 CFU/mL (Colony-Forming Unit) using a densitometer (DEN-1, Biosan SIA, Latvia), and diluted thousand times in TSB or IVWM. 500 µL of this suspension was poured into the wells of a 48-well polystyrene plate (Wuxi Nest Biotechnology, China) and incubated for 24 h under static conditions at 37°C. To assess the total biofilm mass, crystal violet staining was performed. The level of biofilm activity was evaluated using tetrazolium staining. Both tests were performed in two independent experiments in six replicates. Quantitative culturing was performed in one experiment in triplicate to determine the number of colony-forming units.

- Evaluation of biofilm biomass level using crystal violet assay

After the biofilm culturing described above, the medium was removed, and the plates were dried for 10 min (37°C). Subsequently, 500 µL of 20% (v/v) crystal violet (Aqua-med, Poland) solution in water was added to the wells, and the plates were kept at room temperature for 10 min. The stain was gently removed, the biofilm was washed once with 500 µL of saline (Chempur, Poland), and the plates were incubated at 37°C for 10 min. Next, violet crystals were dissolved with 500 µL of 30% (v/v) acetic acid (Stanlab, Poland) water solution, and the plates were shaken for 30 min at 450 rpm (Mini-shaker PSU-2T, Biosan SIA, Latvia) at room temperature. 100 µL of the solution was transferred from one well in four replicates to 96-well plates (Wuxi Nest Biotechnology, China), and the absorbance was measured at 550 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, USA). The average absorbance was calculated for each sample. The absorbance of media without bacteria was also measured, and their average values were subtracted from the absorbance of each sample. Based on the results, the strains were divided into four groups according to their biofilm biomass:

  - high biofilm biomass in TSB: S11, R12, R13;
  - low biofilm biomass in TSB: ATCC 6538, S3, R1;
  - high biofilm biomass in IVWM: S6, S8, R9;
  - low biofilm biomass in IVWM: R8, R10, R11.

- Assessment of biofilm activity level using tetrazolium staining

The biofilm was cultured as presented above, and the medium was removed from above the cells. Next, metabolically active biofilm cells were two-hours stained with 500 µL of 0.1% (w/v) TTC solution (2,3,5-triphenyl-tetrazolium chloride, Sigma-Aldrich, USA) in TSB (Tryptic Soy Broth, Biomaxima, Poland) or IVWM (In Vitro Wound Milieu) medium at 37°C. The medium was pipetted-out, and the plates were dried for 10 min at 37°C. 500 µL of methanol (Stanlab, Poland) and acetic acid (Stanlab, Poland) (9:1 ratio) solution was introduced to the wells, and the plates were shaken (Mini-shaker PSU-2T, Biosan SIA, Latvia) for 30 min at room temperature (400 rpm). 100 µL of the solution was

transferred from one well in four replicates to 96-well plates (Wuxi Nest Biotechnology, China), and the absorbance was measured at 490 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, USA). The average absorbance was calculated for each sample. The absorbance of media without bacteria was also measured, and their average values were subtracted from the absorbance of each sample.

- Assessment of the number of colony-forming units

As described above, the biofilm was cultured in polystyrene plates in TSB or IVWM medium. Subsequently, the medium was removed, and each well was shaken with 500 µL of 0.1% (w/v) saponin (VWR Chemicals, USA) water solution for 30 s at 600 rmp. Solutions from each well were resuspended and transferred to Eppendorf tubes. Plates were shaken again for 30 s/600 rpm with the fresh saponin solution (500 µL), and solutions from both steps were combined. The serial dilutions of the suspension were then prepared in saline solution and cultured onto Mueller–Hinton agar (Biomaxima, Poland) Petri dishes (Noex, Poland) and incubated for 24 h at 37°C. The CFU number was counted using ImageJ (National Institutes of Health, Bethesda, MD, USA, accessed on 1 December 2022).

2.6 Visualization of live and dead biofilm-forming cells using fluorescent dyes and a Confocal Microscopy

The staphylococcal strains were cultured in TSB (Tryptic Soy Broth, Biomaxima, Poland) or IVWM (In Vitro Wound Milieu) medium in 24-well plates (Wuxi Nest Biotechnology, China). The preparation of suspensions and biofilm cultivation conditions were the same as described in the section "Evaluation of biofilm biomass, biofilm metabolic activity, and the number of colony-forming units". Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Thermo Fischer Scientific, USA), prepared according to the manufacturer's instruction, was applied as a dye to assess the membrane integrity and indirectly to measure a relative number of live and dead biofilm-forming cells and also to visualize the morphology/spatial distribution of cells within biofilm structure. The microscopic visualizations were performed using an SP8 MP laser-scanning confocal microscope (Leica, Germany). SYTO-9 showing live bacteria was excited at 488 nm wavelength using a laser line (SP8). The collected emission was within the 500–530 nm range. Propidium iodide (PI) for the visualization of dead bacteria was a 552 nm laser line (SP8). The emission of PI was collected within the 575–625 nm SP8 ranges. The acquisition was performed using 20µm dry objectives in a sequence to avoid a spectral bleed through. For a given set of experimental conditions (untreated biofilm and biofilms with EOs), the same acquisition settings were applied to each system to enable quantitative comparisons between the conditions. The settings and signal intensity were always set on the brightest samples to avoid oversaturation. Next, the pictures were processed using ImageJ (National Institutes of Health, Bethesda, MD, USA) software. The whole captured picture was treated as the Region of Interest (ROI). The mean grey value (MGV) of each ROI was recorded for green and red fluorescence channels and served as an estimator of changes in the number of live and dead cells, respectively. The MGV was considered the sum of gray values of all the pixels in the selection divided by the number of pixels. For RGB images recorded for the purpose of this analysis, the MGV was calculated by converting each pixel to grayscale using the Equation: gray = 0.299red + 0.587green + 0.114blue.

2.7 Visualization of biofilm using a Scanning Electron Microscope

The *S.aureus* ATCC 6538 biofilm strain was visualized with a Scanning Electron Microscope (SEM, Auriga 60, ZEISS, Germany). Firstly, 1 mL of 2% (w/v) Bacteriological Lab Agar (Biomaxima, Poland) was poured into a 24-well plate (Wuxi Nest Biotechnology, China) and left for solidification. Next, 500 µL of the appropriate medium (TSB (Tryptic Soy Broth, Biomaxima, Poland) or IVWM (In

Vitro Wound Milieu)) was poured into the wells with agar, and the plate was kept refrigerated for 24 h. Subsequently, the medium was removed from above the agar, and the bacterial suspension in TSB or IVWM medium was prepared as described in the section "Evaluation of biofilm biomass, biofilm metabolic activity, and the number of colony-forming units", and added in the amount of 500 µL to the agar wells. The plate was incubated for 24 h at 37°C under static conditions. The medium was then removed, and 500 µL of 4.5% (v/v) glutaraldehyde (ChemPur, Poland) was poured. Next, samples were dried in a critical point dryer EM CPD300 (Leica Microsystems, Germany). Subsequently, the samples were subjected to sputtering with Au/Pd (60:40) using EM ACE600, Leica sputter (Leica Microsystems, Germany). The sputtered samples were examined using a scanning electron microscope (SEM, Auriga 60, Zeiss, Germany).

2.8 Evaluation of Minimal Inhibitory and Minimal Biofilm Eradication Concentrations of EOs Emulsions

The antimicrobial activity of T-EO or R-EO was assessed in 96-well plates (Wuxi Nest Biotechnology, China) in TSB (Tryptic Soy Broth, Biomaxima, Poland) or IVWM (In Vitro Wound Milieu) media. Tests were carried out in two independent experiments in three replicates. For both experiments, bacterial suspension was prepared as follows. From overnight cultures in TSB or IVWM medium suspensions were prepared in saline (Stanlab, Polnad) and adjusted to 0.5 MF (McFarland, 1.5 x 10^8 CFU/mL (Colony-Forming Unit)) using a densitometer (DEN-1, Biosan SIA, Latvia), and diluted a thousand times in TSB or IVWM. EOs were tested as emulsions in TSB or IVWM medium with the addition of Tween 20 (Pol-aura, Poland). In the T-EO emulsions stock solution, the oil constituted 5% (v/v) of the emulsion volume, and in R-EO, 10% (v/v). Tween 20 constituted 1% (v/v) of the entire volume on both emulsions. The emulsions were prepared as follows: the EOs were mixed with Tween 20 for 30 min using a magnetic stirrer (IKA RH basic 2, IKA-Werke GmbH & CO. KG, Deutschland). Next, the medium was gradually added and stirred for one hour more at room temperature.

- Minimal Inhibitory Concentration (MIC) evaluation

To evaluate MIC values, geometric concentrations of EOs' stock solutions were prepared in TSB or IVWM medium at concentrations ranging from 5 % to 0.002% (v/v) for T-EO and from 10% to 0.005% (v/v) for R-EO and added in a volume of 100 µL to the wells of the plates. Next, 100 µL of bacterial suspensions in TSB or IVWM medium (prepared as described above) were added to the wells, and the plates were incubated for 24 hours at 37°C with shaking (Mini-shaker PSU-2T, Biosan SIA, Latvia) at 450 rpm. Controls of bacterial growth (bacteria in TSB or IVWM medium) and medium sterility (medium only) were also prepared. After incubation, 20 µL of 1% (w/v) TTC solution (2,3,5-triphenyl-tetrazolium chloride, Sigma-Aldrich, USA) in the medium was added to each well, and plates were incubated for 2 hours under the same conditions. MIC values were evaluated in the first well, where no red color was observed. The influence of five concentrations [(%) (v/v)] of Tween 20 on *S. aureus* ATCC 6538 planktonic forms cultured in TSB medium was also evaluated in the same manner as the test with EOs. In addition, the absorbance of the samples at 580 nm was measured using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, USA) before adding the TTC solution. The percentage of cell viability was calculated in each Tween 20 concentration with respect to the growth control. The test was performed in one experiment in six replicates.

- Minimal Biofilm Eradication Concentration (MBEC) evaluation

To analyze the antibiofilm properties of EOs emulsions, biofilms were first cultivated in polystyrene plates. For this purpose, 100 µL of TSB or IVWM was added to the wells of the plates, and 100 µL of bacterial suspensions in TSB or IVWM medium (prepared as described above) was poured. The plates were incubated at 37°C for 24 hours under static conditions. The geometric concentrations of the EOs'

stock solutions were then prepared in TSB or IVWM medium at concentrations ranging from 5 % to 0.002% (v/v) for T-EO and from 10% to 0.005% (v/v) for R-EO. After the biofilm's incubation, the medium was gently removed, and 200 µL of EOs emulsions was added to the wells. The plates were incubated at 37°C for 24 hours under static conditions. Subsequently, the medium was removed, and 200 µL of 0.1% (w/v) TTC solution in the medium was added to each well for 2 hours at 37°C. Then MBEC values were assessed in the first colorless well. Subsequently, the medium was removed, and 200 µL of a solution of methanol (Stanlab, Poland) and acetic acid (Stanlab, Poland) in a 9:1 ratio was poured into the wells, and the plates were subjected to shaking (Mini-shaker PSU-2T, Biosan SIA, Latvia) for one hour at room temperature at 550 rpm. 100 µL of the solution was transferred to fresh 96-well plates (Wuxi Nest Biotechnology, China), and the absorbance of the solution was spectrophotometrically measured at 490 nm (Multiskan Go, Thermo Fisher Scientific, USA). Controls of bacterial growth (bacteria in TSB or IVWM medium) and medium sterility (medium only) were also prepared. To evaluate the percentage reduction of biofilm cells treated with EOs emulsions, the absorbance value of the sample was compared to the average bacterial growth absorbance.

2.9 Analysis of the Size of EOs Emulsion Droplets

The hydrodynamic diameters of the EOs droplets within emulsions were measured with the dynamic light scattering method. The analysis was performed using the Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., UK) equipped with a laser light source (λ=633 nm) and a detector operating at a scattering angle of 173°. Emulsions of EOs were prepared in TSB medium (Tryptic Soy Broth, Biomaxima, Poland) with 1% (v/v) Tween 20 (Pol-aura, Poland) and diluted in sterile purified water one thousand times prior to the measurement. The oil phase content was 5% (v/v) in the case of T-EO and 10% (v/v) regarding R-EO. The test was run at 25±0.1°C, and the samples were measured at least 5 times. The values of hydrodynamic diameters included in the work are Z-Average values.

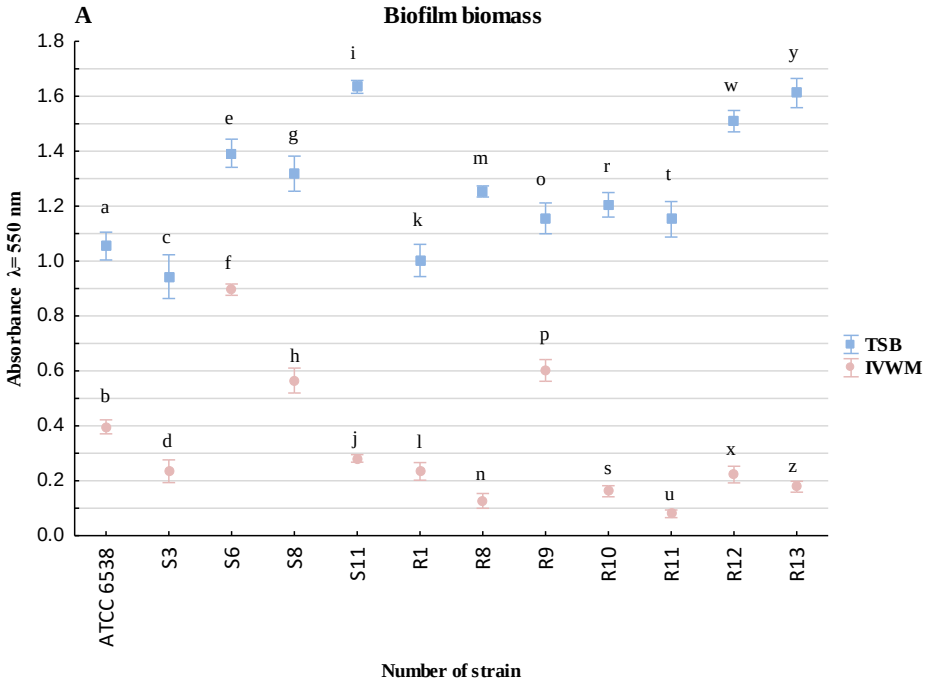
2.10 Statistical analysis

Calculations were performed using Statistica software (Version 13; TIBCO Software Inc, Palo Alto, California, USA). The Hampel test was performed to identify outliers in the results of the biofilm biomass test, the biofilm metabolic activity assay, the biofilm thickness, and the share of live/ dead cells when all strains were analyzed together. The normality distribution and variance homogeneity were calculated with the Shapiro–Wilk and Levene tests, respectively. The t-test and the Mann-Whitney U test were performed to compare differences in biofilm biomass, biofilm metabolic activity, number of colony-forming units, thickness, and live/dead cell ratio between both media. The t-tests were used when normal distribution was determined ($p>0.05$). A Welch's adjusted t-test was used for unequal variances ($p<0.05$). A Mann-Whitney U test was used when the normal distribution was not determined. The Pearson correlation was calculated to assess a linear correlation between biofilm biomass level and biofilm metabolic activity. Multivariate analysis of variance was performed to evaluate the effect of medium, strain, and EOs concentration on the reduction of biofilm cells after treatment with EOs. The results of statistical analyses with a significance level of $p<0.05$ were considered significant. The graphical abstract was created with BioRender.com (BioRender Inc, Switzerland, accessed on 17 May 2023)

3 Results

In the first line of the experiment, a GC-MS analysis was performed to evaluate the percentage composition of the EOs' components. T-EO comprised 50.6% thymol, 19.2% p-cymene, and 9.1% γ-terpinene. Three main components of R-EO were: 21.1% α-pinene, 20.0% 1,8-cineole, and 18.5% camphor. A detailed list of the composition of the EOs is presented in **Supplementary Table 1**.

The ability of *S. aureus* strains to form biofilm biomass in standard TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium was evaluated using the crystal violet method (CV). The biofilms' metabolic activity was assessed using tetrazolium chloride (TTC) staining **(Figure 1).**



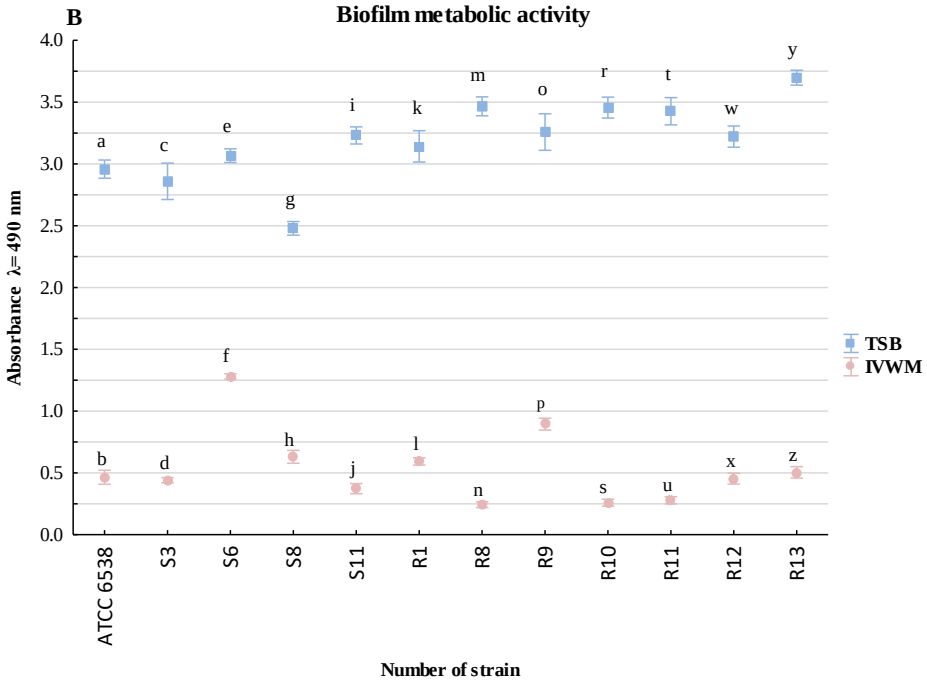


Figure 1. The average biofilm biomass **(A)** and metabolic activity **(B)** in Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) of analyzed reference (ATCC 6538, American Type Culture Collection) and clinical (S3, S6, S8, S11, R1, R8-R13) strains of *S. aureus*. The pairs of letters (a/b, c/d, e/f, g/h, i/j, k/l, m/n, o/p, r/s, t/u, w/x, y/z) refer to the statistically significant differences ($p < 0.05$,

t-test or Welch's t-test for biofilm biomass assay, t-test or Welch's t-test or Mann-Whitney U test for the biofilm metabolic activity assay). The error lines represent the standard error of the mean (n=12).

All strains were able to form a biofilm in TSB or IVWM medium. The biomass and metabolic activity of all biofilms were higher in TSB than in IVWM medium. For all strains, this trend was statistically significant ($p < 0.05$, t-test or Welch's t-test for the biofilm biomass assay, t-test or Welch's t-test or Mann-Whitney U test for the biofilm metabolic activity assay, **Supplementary Tables 3, 4, Supplementary Figures 5, 6**), and it also remained statistically significant when the mean of all strains was calculated ($p<0.05$, Mann-Whitney U test), **Supplementary Figure 7, Supplementary Table 5)**. A significant linear correlation ($p=0.001$) between the level of biofilm biomass and metabolic activity for biofilms cultured in IVWM medium, but not in TSB medium ($p=0.315$), was observed (**Supplementary Table 6, Supplementary Figure 8**). The correlation coefficient was determined as moderate or very strong for TSB ($r=0.32$) or IVWM ($r=0.83$), respectively. To confirm data obtained by semiquantitative CV and TTC methods, the number of biofilm-forming cells of reference strain cultivated in TSB or IVWM was quantitatively cultured **(Table 2)**. The average number of biofilm-forming cells was significantly higher ($p<0.05$, t-test, **Supplementary Table 7, Supplementary Figure 9 A, B**) in TSB than IVWM medium. A macroscopic visualization of representative biofilm stained with crystal violet or tetrazolium chloride confirming differences in the level of biofilm biomass and metabolic activity between bacteria cultured in TSB or IVWM is presented in **Figure 2**. The biofilm in the TSB was highly confluent, i.e., biofilm-forming cells covered essentially the entire surface of the plate's wells **(Figure 2 A, C)**. Biofilm cells cultured in IVWM formed cells aggregates unequally distributed on the well's surface **(Figure 2 B, D)**.

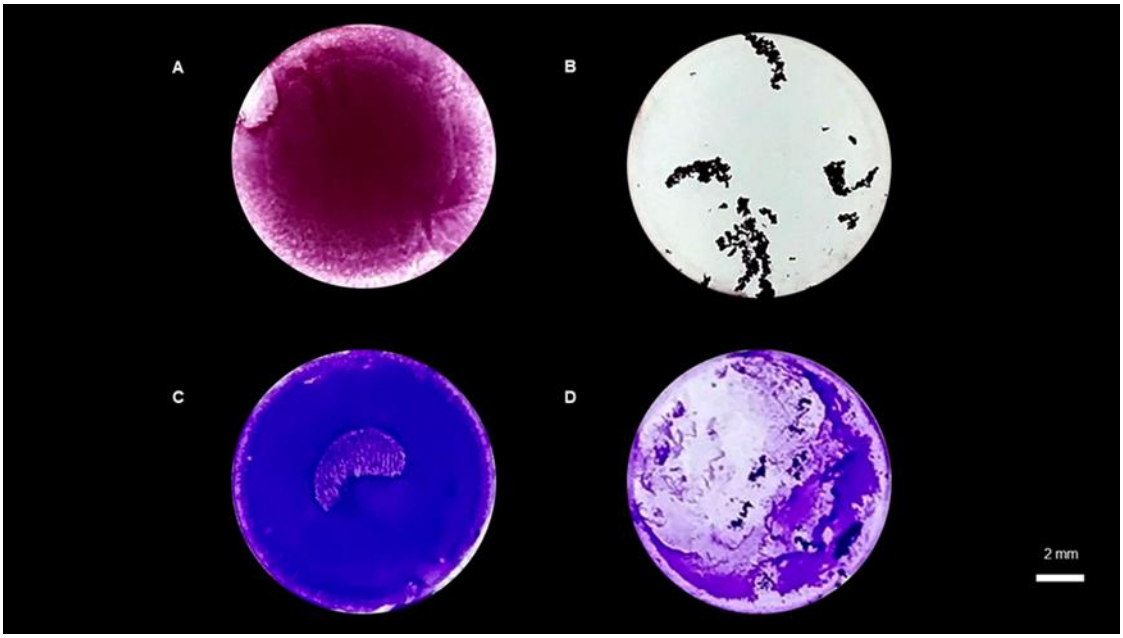


Figure 2. Macroscopic visualization of the *S. aureus* S11 biofilm formed on polystyrene. **A, B**- biofilm cultured in TSB or IVWM, respectively, and stained with tetrazolium chloride; **C, D**- biofilm cultured in TSB or IVWM**,** respectively, and stained with crystal violet. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu. In macroscopic visualizations, the contrast was enhanced using GIMP software (Version 2.20.22, www.gimp.org, assessed 26.11.2020, original images are presented in **Supplementary Figure 1**). The scale bar is 2 mm.

Table 2. A mean number of Colony-Forming Units/mL (CFU/mL) of biofilm of *S. aureus* ATCC 6538 reference strain (American Type Culture Collection) cultured in TSB (Tryptic Soy Broth) or the IVWM (In Vitro Wound Milieu). SEM- standard error of the mean. A statistically significant difference ($p<0.05$, t-test) is marked with the pair of letters a/b.

Number of CFU/mL				
Strain	**TSB**		**IVWM**	
ATCC 6538	**Mean**	**SEM**	**Mean**	**SEM**
	8.98E+14[a]	1.07E+14	1.46E+12[b]	6.65E+11

Next, the analysis of live and dead cells within the Z-axis (thickness) of the 24-hour-old staphylococcal biofilm structure was performed using LIVE/DEAD (L/D) dyeing and confocal microscopy **(Figure 3).** The staphylococcal biofilm cultured in TSB resembled the structure of a dense lawn **(Figure 3 A, C, E).** The biofilm-forming cells evenly covered the whole field of vision. In turn, the biofilm cultivated in IVWM formed mushroom-like structures of diversified sizes **(Figure 3 B, D, F).** The IVWM biofilm contained regions of differential thickness and cell density, including areas not covered with cells (black regions). Structural differences between biofilms cultured in TSB or IVWM medium were also observed in microscopic visualizations performed with the Scanning Electron Microscope (SEM) **(Figure 4).**

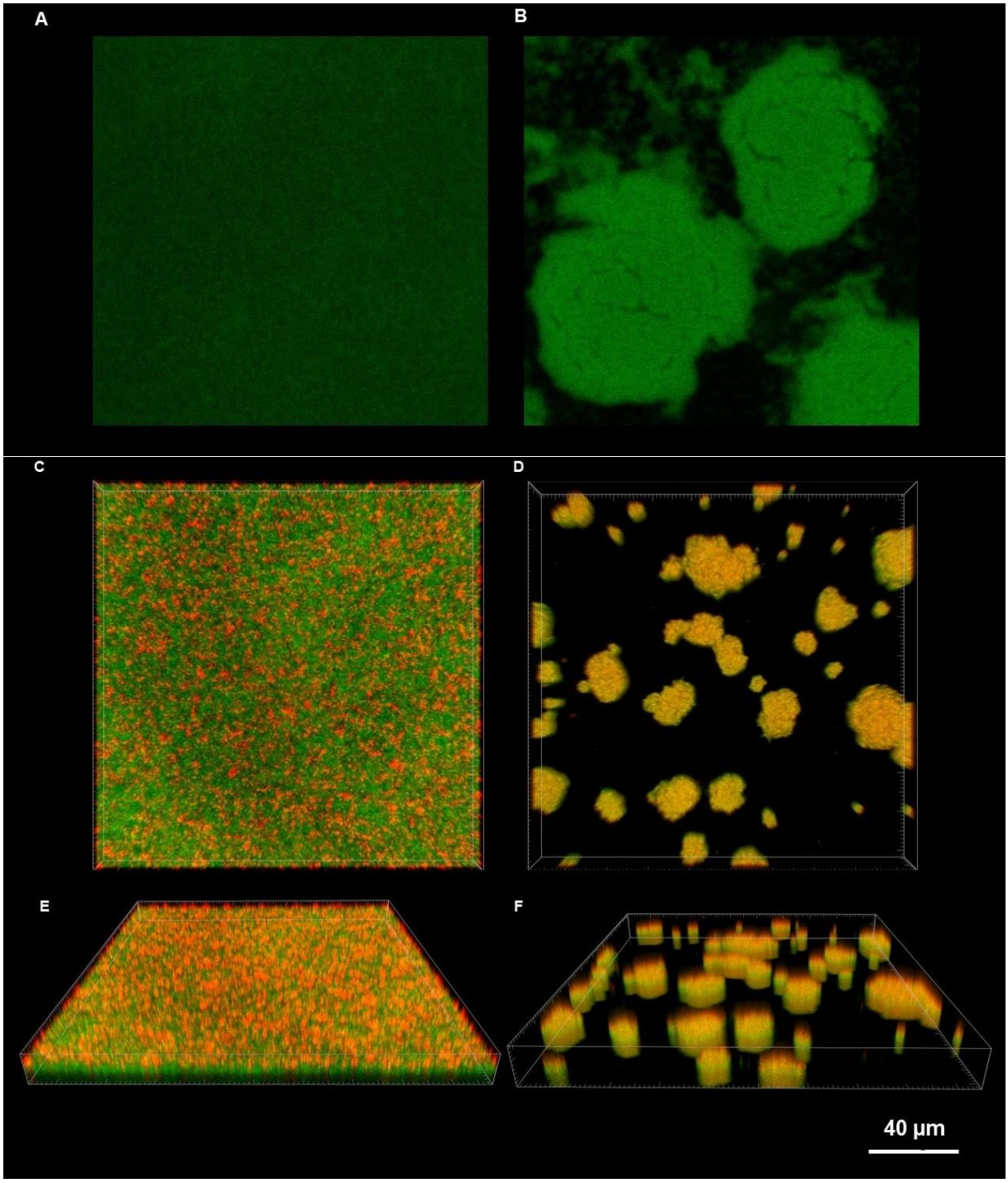


Figure 3. Microscopic visualizations of the *S. aureus* biofilm formed on polystyrene and stained with a LIVE/DEAD dye. **A, B**- an aerial perspective of the S8 biofilm cultured in TSB **(A)** or IVWM **(B)** – **C, D**- an aerial perspective of the S11 biofilm cultured in TSB **(C)** or IVWM **(D)**; **E, F-** the Z- axis image stack visualizing the S11 biofilm cultured in TSB **(E)** or IVWM **(F)** from the side aerial perspective. The red/orange color indicates staphylococcal cells of altered/damaged cell walls, while green-colored shapes show unaltered cell walls. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu. The scale bar is 40µm. The confocal microscope SP8, magnification 25x.

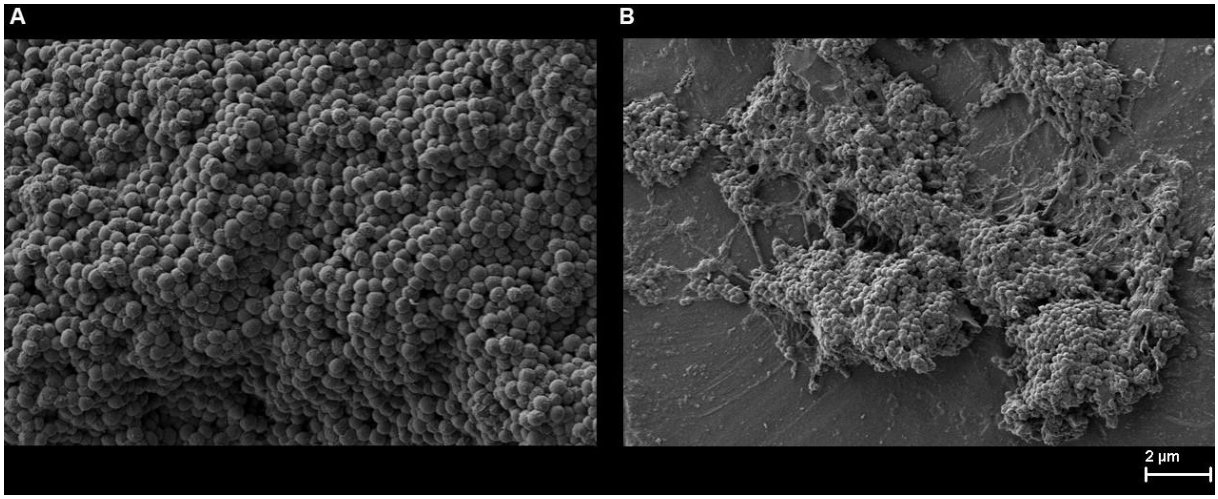


Figure 4. Microscopic visualizations of the *S. aureus* ATCC 6538 biofilm formed on agar and cultured in TSB **(A)** or IVWM **(B)** medium. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu. The scale bar is 2µm. Scanning Electron Microscope Zeiss Auriga 60 (magnification 10000x).

The average thickness of the biofilm formed by all *S. aureus* strains in TSB medium was significantly lower than in IVWM medium ($p<0.05$, Mann-Whitney U test, **Figure 5, Supplementary Table 7, Supplementary Figure 9 C, D**).

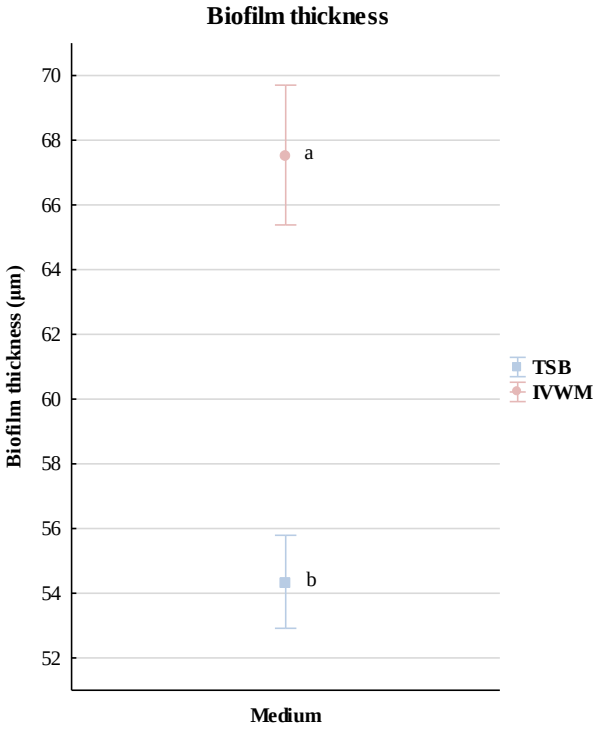


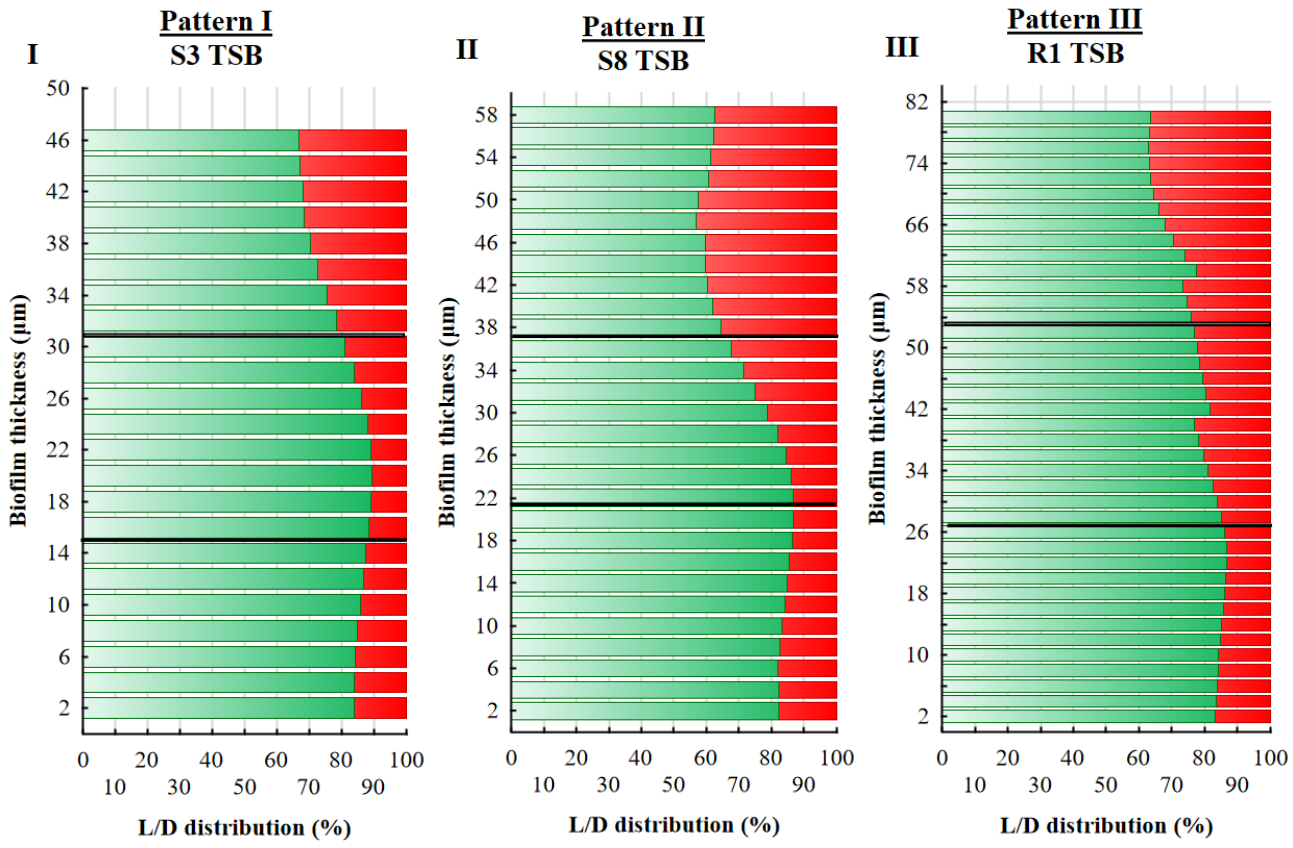
Figure 5. Comparison of the average thickness (µm) of all staphylococcal biofilms cultured in TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium measured with the confocal microscopy. The error lines represent the standard error of the mean (n=36). The statistically significant difference ($p<0.05$, Mann-Whitney U test) is marked with a pair of letters a/b.

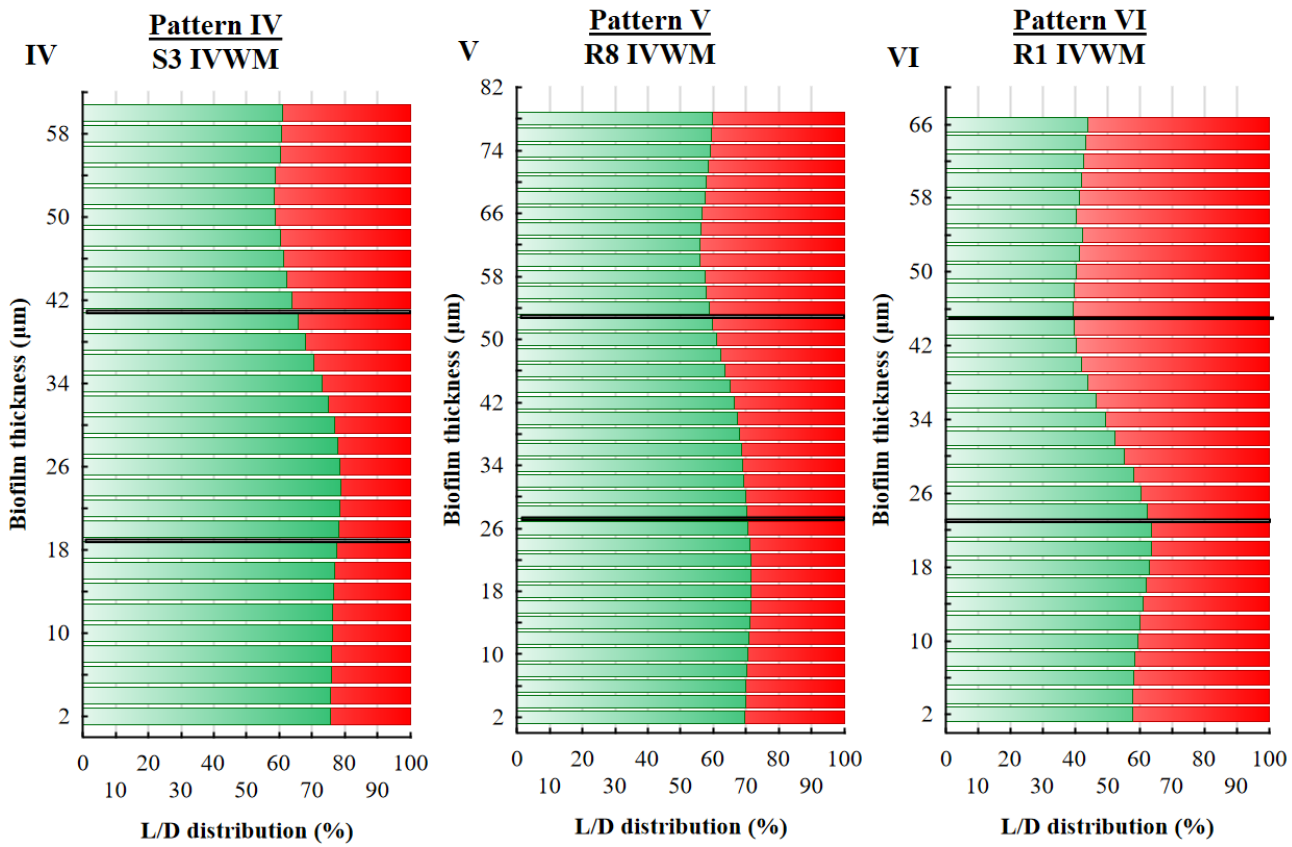
In turn, the share of live cells was higher for ten (83% of strains) strains cultured in TSB medium than in IVWM **(Table 3)**. The average share of live cells was also significantly higher ($p<0.05$, Mann-Whitney U test) in TSB than in IVWM when all strains were analyzed together **(Supplementary Table 7, Supplementary Figure 9 E, F, G**).

Table 3. Average percentage (%) share of live and dead cells in biofilms of staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains cultured in TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium measured with the confocal microscope. SEM- standard error of the mean (n=3).

Percentage (%) share of cells in *S. aureus* biofilms						
Strain number	**TSB**			**IVWM**		
	LIVE	**DEAD**	**SEM**	**LIVE**	**DEAD**	**SEM**
ATCC 6538	84	16	0.1	80	20	1.0
S3	83	17	0.5	73	27	0.9
S6	76	24	2.3	61	39	3.1
S8	79	21	3.3	73	27	1.8
S11	74	26	2.4	63	37	1.5
R1	82	18	0.5	54	46	3.2
R8	86	14	1.7	67	33	0.5
R9	86	14	0.4	76	24	1.4
R10	66	34	1.1	68	32	0.9
R11	71	29	2.2	69	31	0.5
R12	70	30	0.5	74	26	1.3
R13	82	18	0.6	47	53	5.4

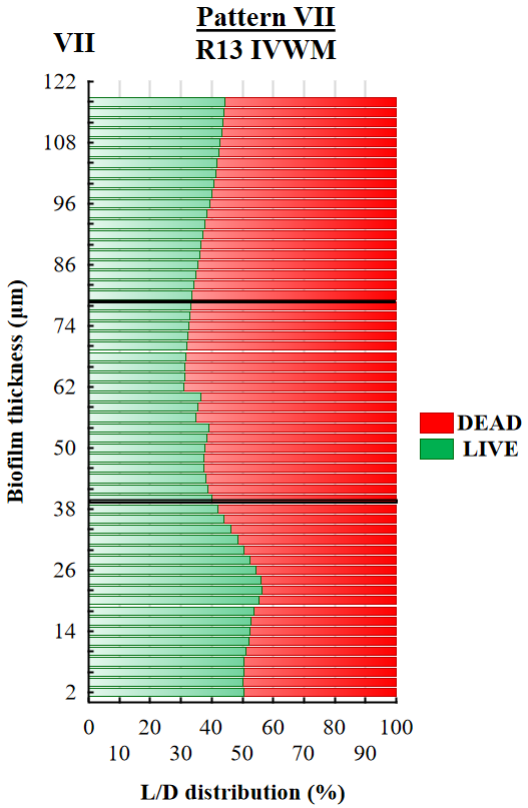
The analysis of the cellular spatial composition of the biofilms allowed us to distinguish their three sections: bottom one (B, the closest to the polystyrene surface), middle (M), and top (T, the farthest from the polystyrene surface) **(Figure 3, 6, Supplementary Figure 2, Supplementary Table 2)**. Different patterns of live (green) and dead (red) share in parts (T, M, and B) of biofilms were revealed in each medium. Three (I, II, III) patterns were distinguished in TSB medium, and four (IV, V, VI, VII) in IVWM **(Figure 6, Supplementary Figure 2)**. The share of dead cells was higher in the T parts than in the M and B parts in 100% or 92% of staphylococcal biofilms grown in TSB or IVWM medium, respectively **(Figure 6, Supplementary Table 2)**. A higher share of live cells was observed in B (for 92% of strains) and M (for 83% of strains) parts of biofilms cultured in TSB medium than in IVWM **(Figure 6, Supplementary Figure 2, Supplementary Table 2)**. For 58% of staphylococcal biofilms, the share of live cells in T parts was higher in TSB medium than in IVWM.




IV
Pattern IV
S3 IVWM
V
Pattern V
R8 IVWM
VI
Pattern VI
R1 IVWM
Biofilm thickness (μm)
L/D distribution (%)

454

455


VII
Pattern VII
R13 IVWM
Biofilm thickness (μm)
L/D distribution (%)
DEAD
LIVE

456

Figure 6. The main patterns of live (L, green) and dead (D, red) cells distribution (%) in staphylococcal biofilms across the Z-axis, cultured in TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium. The thickness of each section was 2 μm. Black lines divide graphs into the top (T), middle (M), and bottom (B) parts. **I, II**, and **III**- strains representing particular patterns of cells share in TSB; **IV, V, VI,** and **VII**- strains representing particular patterns of cells share in IVWM. S3, S8, R1, R8, R13- *S. aureus* clinical strains. Figures of the other strains are presented in **Supplementary Figure 2**. The confocal microscope SP8, magnification 25×.

The antimicrobial activity of the emulsified T- and R-EOs against planktonic *S. aureus* cells was evaluated using a microdilution technique **(Table 4)**. The applied emulsifier, Tween 20, did not affect the viability of staphylococcal cells (**Supplementary Figure 3).** The Minimal Inhibitory Concentrations (MIC) values of applied EOs were higher for 75% (T-EO) or 58% (R-EO) of strains cultivated in IVWM compared to strains cultivated in TSB medium. T-EO acted 2 to 4 times weaker against this 75% of strains in IVWM than in TSB medium. For 50% of strains treated with R-EO, the MIC values were twice higher in IVWM than in TSB, and for 8%, this parameter was sixty-four times higher in IVWM than in TSB.

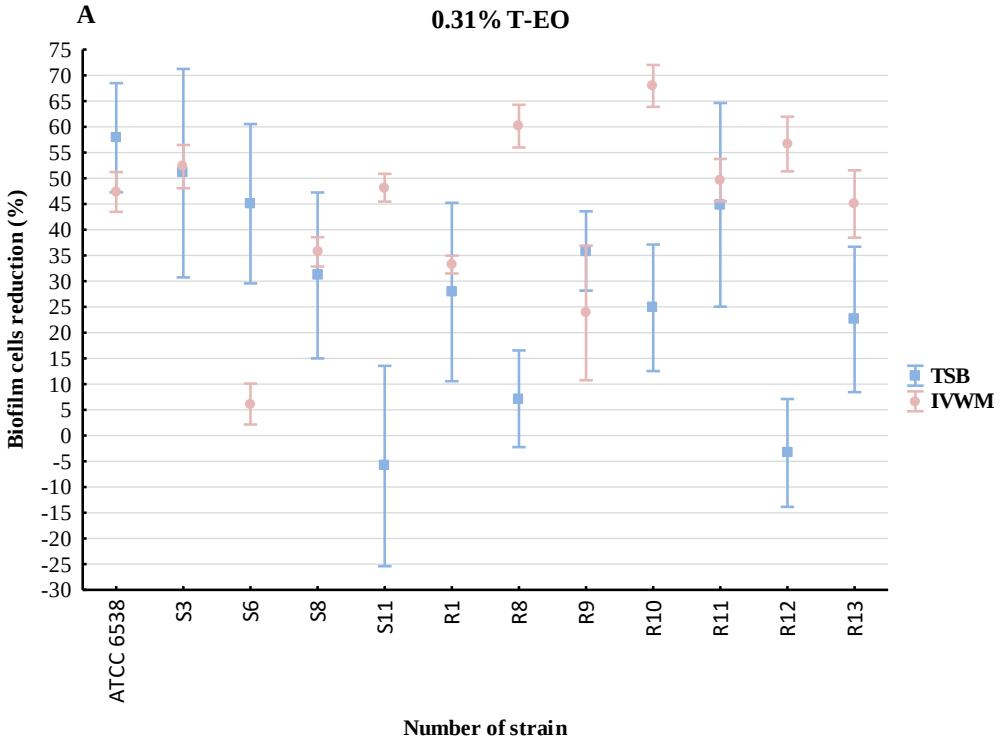
In the case of biofilms, T-EO exhibited higher activity than R-EO regardless of the medium applied **(Table 4)**. The MBEC (Minimal Biofilm Eradication Concentration) values of T-EO were determined for all staphylococcal strains and ranged from 2.5% (v/v) to 0.63% (v/v). The MBEC value of T-EO differed in 50% of strains, depending on the medium applied (TSB or IVWM). For half of these strains, the MBEC value was higher in IVWM than in TSB medium. In the case of R-EO, no MBEC values were achieved, therefore, the assessment of the biofilm cells' reduction (%) was performed in the subsequent analyses.

Table 4. Antimicrobial activity of the tested EOs' emulsions against planktonic (MIC (%) (v/v)) and biofilm cells (MBEC (%) (v/v)) of reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains of *S. aureus*. N/R indicates EOs where MBEC values were not reached at the highest applied concentration (10% (v/v)) of R-EO. Each EO's MIC and MBEC values were compared in both media and marked green in a medium where the difference was higher than one geometric dilution, blue where the difference was one geometric dilution, and yellow where the parameters were equal. T-EO—thyme oil, R-EO—rosemary oil, TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu, MIC- Minimal Inhibitory Concentration, MBEC- Minimal Biofilm Eradication Concentration.

	MIC (%)				MBEC (%)			
	T-EO		R-EO		T-EO		R-EO	
Strain number	TSB	IVWM	TSB	IVWM	TSB	IVWM	TSB	IVWM
ATCC 6538	0.02	0.04	1.25	1.25	0.63	0.63	N/R	N/R
S3	0.16	0.31	1.25	2.5	0.63	0.63	2.5	N/R
S6	0.31	0.31	1.25	2.5	0.63	0.63	N/R	N/R
S8	0.16	0.31	2.5	2.5	0.63	0.63	N/R	N/R
S11	0.08	0.16	1.25	1.25	0.63	0.63	N/R	N/R
R1	0.31	0.31	1.25	2.5	1.25	0.63	N/R	N/R
R8	0.16	0.31	0.04	2.5	1.25	2.5	N/R	N/R
R9	0.16	0.31	1.25	1.25	1.25	0.63	N/R	N/R
R10	0.16	0.16	1.25	2.5	0.63	0.63	N/R	N/R
R11	0.16	0.31	1.25	2.5	0.63	1.25	N/R	N/R
R12	0.04	0.16	2.5	2.5	1.25	0.63	N/R	N/R
R13	0.08	0.31	1.25	2.5	1.25	2.5	N/R	N/R

The level of biofilm cell reduction (%) was above 90% for all strains in TSB or IVWM medium after the treatment with T-EO at concentrations of 2.5% - 0.63% (v/v) (**Supplementary Figure 4 A).** At a T-EO concentration of 0.31%, (v/v) lower reduction was observed for nine of twelve strains cultured in TSB comparing to the IVWM medium (**Figure 7 A**). The three strains with different patterns of reduction were: ATCC 6538, S6, and R9. The reduction of biofilm cells treated with R-EO was reached for all strains at concentrations ranging from 10% (v/v) to 0.63% (v/v) when cultured in TSB medium or from 10% (v/v) to 1.25% (v/v) in IVWM medium (**Figure 7 B, Supplementary Figure 4 B-F**). In concentration ranges of 10% (v/v) to 2.5% (v/v), a higher level of biofilm reduction was evaluated only for 30% of strains (R8-R11) cultured in IVWM medium than in TSB. The percentage reductions of staphylococcal biofilms after treatment with selected EOs concentrations are presented in **Figure 7** and **Supplementary Figure 4**. Multivariate analysis of variance was performed to evaluate the effect of medium, strain and EOs concentrations on the reduction of biofilm cells after treatment with T-EO or R-EO (**Supplementary Tables 8, 9, Supplementary Figures 10, 11**). In the case of both EOs, all of the factors significantly influenced the biofilm cells reduction. Additionally, for T-EO, there were

significant interactions between each factor and all factors together. The biofilm cells reduction was significantly lower in TSB than in IVWM medium for T-EO and significantly higher in TSB than in IVWM for R-RO. In the case of R-EO, significant interactions occurred between medium and strain, strain and the oil concentration, and all three factors together.



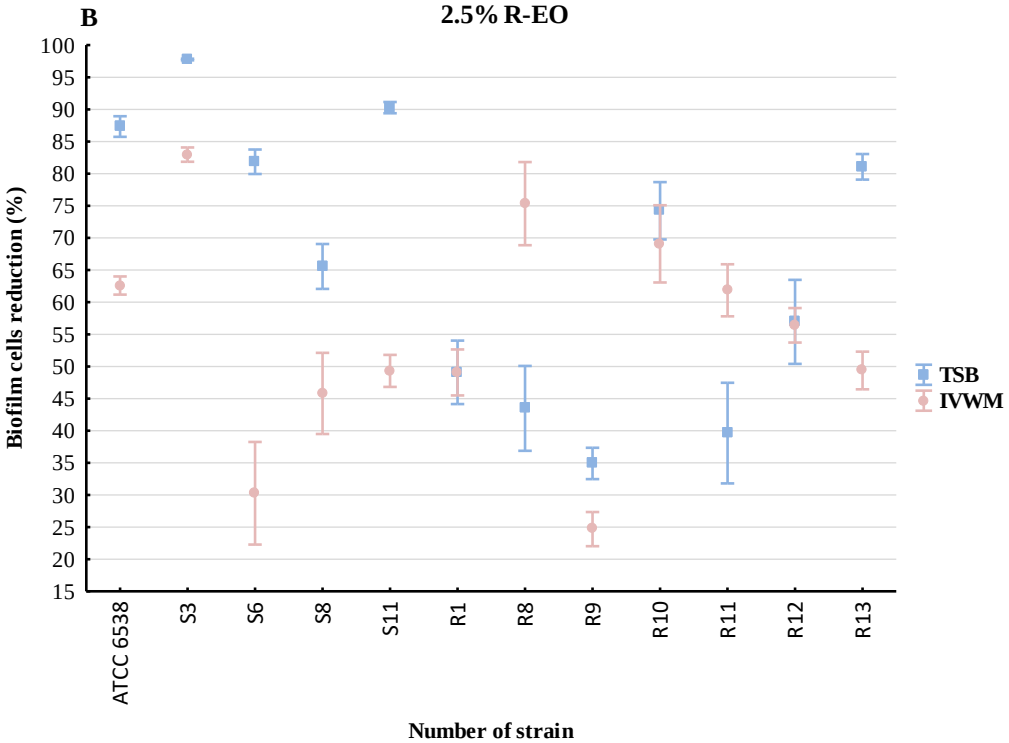


Figure 7. Average reduction (%) of biofilm cells of reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains of *S. aureus* after treatment with selected concentrations (%) (v/v) of thyme oil (**A**, T-EO) and rosemary oil (**B**, R-EO). The error lines represent the standard error of the mean (n=6). TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu.

Finally, the average diameters of the EOs droplets within the prepared formulations were measured. The droplets of T-EO and R-EO were 209±22 nm and 995±341 nm, respectively. The polydispersity index (PDI) of T-EO and R-EO's emulsions was 0.46 and 0.68, respectively.

4 Discussion

Due to the limited usability of antibiotics in non-healing wound management and the emerging microbial resistance to antiseptics, there is a need to develop novel strategies for treating biofilm-related infections, such as utilizing natural compounds, e.g., essential oils (EOs). However, most *in vitro* research evaluates EOs' antimicrobial activity against planktonic forms of microorganisms and in standard microbiological media. In contrast, the influence of EOs on microbial biofilm in a wound's exudate-like medium should be assessed to accurately gauge their effectiveness at the infection site. Therefore, in this study, we applied a novel medium (In Vitro Wound Milieu, IVWM) containing serum, cell-matrix elements, and host factors that mimic the wound environment (Kadam et al., 2021). The biofilm characteristics and eradication following EO treatment were assessed in IVWM and a standard microbiological medium (Tryptic Soy Broth, TSB). Our first line of research involved the use of crystal violet and TTC (2,3,5-triphenyl-tetrazolium chloride) staining to evaluate the strains' ability to form a biofilm (**Figure 1**). The results revealed high intraspecies variability in biofilm biomass and metabolic activity, regardless of the medium used.

The biomass, metabolic activity, and cell number of *S. aureus* biofilms were higher in TSB than in IVWM medium (**Figures 1, 2**). Unlike TSB, which primarily contains proteins, glucose, and sodium chloride, IVWM presents host factors that may impair biofilm formation (**Table 1**). Studies indicate that lactoferrin inhibits bacterial growth by binding iron, which restricts its availability for bacteria, or through direct interaction with negatively charged regions of bacterial membranes, causing cell damage (Aguila et al., 2001; Ammons and Copié, 2013). Lactoferrin can also obstruct biofilm formation by preventing its adhesion, disrupting existing structures, and significantly altering the expression of genes responsible for cell metabolism (Roseanu et al., 2010; Ammons and Copié, 2013). According to Abraham et al., non-protein compounds of bovine serum (which constitutes 70% of IVWM medium) inhibit staphylococcal biofilm formation (Abraham and Jefferson, 2010).

Moreover, the biofilm in TSB was highly confluent (**Figure 2 A, C**), while the biofilm cultured in IVWM formed cellular aggregates unevenly distributed on the well's surface. However, these were covered with an extracellular matrix to a greater extent than the biofilms formed in the TSB (**Figure 2 B, D**). These findings confirm that IVWM medium accurately reflects biofilm structure under *in vivo* conditions because bacterial aggregates were also observed in biopsy materials from chronic wounds (Kirketerp-Møller et al., 2008; Fazli et al., 2009; Bay et al., 2018). *S. aureus* is equipped with surface proteins that bind to fibrinogen and fibronectin, leading to the formation of cell clusters. Aggregation facilitates bacterial evasion from host's immune system, including phagocytosis (Crosby et al., 2016; Pestrak et al., 2020). Nevertheless, cell aggregation has been reported to limit bacterial attachment to surfaces such as steel or hydroxyapatite. This may be explained by the fact that larger particle sizes lead to higher drag forces and lower attachment (Pestrak et al., 2020). This phenomenon presumably accounts for the significantly lower biofilm mass formed in IVWM than in TSB, demonstrated in our study.

A positive high correlation (r=0.83) was observed between the level of biofilm biomass and metabolic activity for biofilms cultured in IVWM medium but not in TSB medium (**Supplementary Table 6,**

Supplementary Figure 8). One explanation for the lack of aforementioned correlation in TSB could be that biofilm structure is metabolically differentiated, and regions with decreased metabolic activity may be present in biofilms where an abundant extracellular matrix hinders the diffusion of nutrients and oxygen (Marcos-Zambrano et al., 2014; Xu et al., 2016). To extensively assess biofilm characteristics in both media, we analyzed the biofilm's 3D structure using LIVE/DEAD staining and confocal microscopy. The biofilm cells in TSB are evenly distributed, thus forming a thinner structure than in IVWM, where the cells are clustered in aggregates with more cell layers, forming mushroom-like structures. However, in IVWM, there are also cell-free areas between these aggregates (**Figures 3, 4**). The different spatial distribution of cells in both media may be the cause of the lower thickness of the biofilm formed in the TSB medium than in the IVWM medium (**Figure 5**). It was indicated that the percentage of live cells was higher in biofilms cultured in TSB medium than in IVWM (**Table 3, Supplementary Figure 89G**). The finding aligns with the results of the metabolic analysis assay, where the cells' metabolic activity was higher in TSB than in IVWM medium (**Figure 1B**). This may be due to less favorable conditions for biofilm formation in IVWM, such as the presence of antibiofilm compounds and a lower concentration of nutrients. When analyzing the cellular spatial composition of the biofilms, we demonstrated that in both media, the share of dead cells was higher in the top (T) than in the middle (M) and bottom (B) parts (**Figure 6**, **Supplementary Figure 2, Supplementary Table 2**). In IVWM medium, this could be a biofilm protection mechanism against antimicrobial substances. Dead cells are preferentially localized and act as a barrier, limiting the diffusion of antimicrobials into deeper layers of the biofilm (Chambless et al., 2006). Subsequently, *S. aureus* planktonic and biofilm cells were cultured in both media and treated with T- and R-EO. EOs showed significant antistaphylococcal activity, though intraspecies variability was observed (**Table 4**, **Figure 7**, **Supplementary Figure 4**). This is consistent with the results presented by our research team and other researchers (Tohidpour et al., 2010; Kot et al., 2018; Brożyna et al., 2021) and indicates the necessity of including a broad spectrum of various strains in the tests. Differences in EOs' MIC and MBEC values were observed between experimental settings using IVWM or TSB medium, and they were higher in the case of planktonic forms than in biofilms. EOs exhibited lower antimicrobial activity against planktonic forms in IVWM than in TSB medium for more than half of the strains. This may be due to the impairment of EOs activity by bovine serum albumin (Juven et al., 1994; Hammer and Carson, 2010). EOs influence bacteria mainly by binding to the cell wall/membrane and leading to the disruption of its integrity. Albumin presumably binds to the hydrophobic components of EOs and hinders the interaction of EOs with bacterial membrane proteins, which decreases their efficacy (Juven et al., 1994; Veldhuizen et al., 2007). In general, a lower level of biofilm reduction was obtained after treatment with R-EO in IVWM than in TSB medium (**Figure 7**, **Supplementary Figure 4**). Regarding the antibiofilm activity of T-EO, higher cell reduction was achieved for 75% of strains cultured in IVWM than in TSB medium.

Previous studies demonstrated that clustered bacterial cells (like those in IVWM medium) exhibit increased tolerance to antimicrobial substances (Pestrak et al., 2020). Thicker biofilms, observed in IVWM medium, are also more difficult to eradicate because antimicrobial agents' penetration through the biofilm structure is reduced. The higher antibiofilm activity of T-EO in IVWM than in TSB medium against specific strains suggests that the EO influences biofilm not only by direct interaction with the cell wall but also through other mechanisms, which are enhanced with IVWM medium components.

In both the present study and our previous research, we demonstrated that key biofilm characteristics differ significantly depending on the medium in which microorganisms are cultivated (Paleczny et al., 2021, 2022). These differences translate to variances in the results of the antimicrobial activity of the tested substances. Therefore, using only standard microbiological media in *in vitro* studies to evaluate the antimicrobial substances' efficacy may lead to over- or underestimation of their effect.

Although intraspecies variability was observed, we demonstrated the high effectiveness of EOs in the eradication of *S. aureus* biofilm and planktonic forms in both TSB and IVWM media. EOs, especially

T-EO, are promising agents for the treatment of biofilm-related wound infections since, in specific cases, their activity in the IVWM medium was higher than in TSB.

The research results underscore the importance of using a medium that reflects the state of the infection site (e.g., wound exudate) and including a high number of tested strains in *in vitro* studies. This understanding will help develop effective treatments for biofilm-related infections and better manage wound healing processes in the future.

Highlights

- Biofilm cultured in IVWM is thicker, and the biomass, metabolic activity, and cell number are higher than in TSB medium.
- T- and R-EO exhibited lower antimicrobial activity against planktonic forms in IVWM than in TSB medium.
- A lower level of biofilm reduction was obtained after treatment with R-EO in IVWM than in TSB medium.
- A higher level of biofilm reduction was obtained after treatment with T-EO in IVWM than in TSB medium.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

Author Contributions

MB, AJ designed the research. MB, JP, WK, KM, and AJ performed the experiments. MB and JD performed the statistical analysis. MB wrote the first draft of the manuscript. MB, JD, and AJ analyzed the data and prepared graphics. WK and KM wrote sections of the manuscript. AJ and KFM revised and edited the manuscript. JD and AJ revised the English. AJ, JD supervised the work. All authors contributed to the article and approved the submitted version.

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References

Abraham, N. M., and Jefferson, K. K. (2010). A low molecular weight component of serum inhibits biofilm formation in staphylococcus aureus. Microb Pathog 49, 388–391. doi: 10.1016/j.micpath.2010.07.005.

Agreles, M. A. A., Cavalcanti, I. D. L., and Cavalcanti, I. M. F. (2021). The Role of Essential Oils in the Inhibition of Efflux Pumps and Reversion of Bacterial Resistance to Antimicrobials. Curr Microbiol 78, 3609–3619. doi: 10.1007/s00284-021-02635-1.

Aguila, A., Herrera, A. G., Morrison, D., Cosgrove, B., Perojo, A., Montesinos, I., et al. (2001). Bacteriostatic activity of human lactoferrin against Staphylococcus aureus is a function of its iron-binding properties and is not influenced by antibiotic resistance. FEMS Immunol Med Microbiol 31, 145–152. doi: 10.1111/j.1574-695x.2001.tb00511.x.

Alves, P. J., Barreto, R. T., Barrois, B. M., Gryson, L. G., Meaume, S., and Monstrey, S. J. (2021). Update on the role of antiseptics in the management of chronic wounds with critical colonisation and/or biofilm. Int Wound J 18, 342–358. doi: 10.1111/iwj.13537.

Ammons, M. C., and Copié, V. (2013). Mini-review: Lactoferrin: a bioinspired, anti-biofilm therapeutic. Biofouling 29, 443–455. doi: 10.1080/08927014.2013.773317.

Armstrong, D. G., Swerdlow, M. A., Armstrong, A. A., Conte, M. S., Padula, W. V., and Bus, S. A. (2020). Five year mortality and direct costs of care for people with diabetic foot complications are comparable to cancer. J Foot Ankle Res 13. doi: 10.1186/s13047-020-00383-2.

Bay, L., Kragh, K. N., Eickhardt, S. R., Poulsen, S. S., Gjerdrum, L. M. R., Ghathian, K., et al. (2018). Bacterial Aggregates Establish at the Edges of Acute Epidermal Wounds. Adv Wound Care (New Rochelle) 7, 105–113. doi: 10.1089/wound.2017.0770.

Becker, S. L., Berger, F. K., Feldner, S. K., Karliova, I., Haber, M., Mellmann, A., et al. (2018). Outbreak of Burkholderia cepacia complex infections associated with contaminated octenidine mouthwash solution, Germany, August to September 2018. Eurosurveillance 23. doi: 10.2807/1560-7917.ES.2018.23.42.1800540.

Bowler, P. G. (2018). Antibiotic resistance and biofilm tolerance: a combined threat in the treatment of chronic infections. J Wound Care 27, 273–277. doi: 10.12968/jowc.2018.27.5.273.

Brożyna, M., Paleczny, J., Kozłowska, W., Chodaczek, G., Dudek-Wicher, R., Felińczak, A., et al. (2021). The antimicrobial and antibiofilm in vitro activity of liquid and vapour phases of selected essential oils against staphylococcus aureus. Pathogens 10. doi: 10.3390/pathogens10091207.

Chambless, J. D., Hunt, S. M., and Stewart, P. S. (2006). A three-dimensional computer model of four hypothetical mechanisms protecting biofilms from antimicrobials. Appl Environ Microbiol 72, 2005–2013. doi: 10.1128/AEM.72.3.2005-2013.2006.

Costa, M. F., Durço, A. O., Rabelo, T. K., Barreto, R. de S. S., and Guimarães, A. G. (2019). Effects of Carvacrol, Thymol and essential oils containing such monoterpenes on wound healing: a systematic review. Journal of Pharmacy and Pharmacology 71, 141–155. doi: 10.1111/jphp.13054.

Crosby, H. A., Kwiecinski, J., and Horswill, A. R. (2016). Staphylococcus aureus Aggregation and Coagulation Mechanisms, and Their Function in Host–Pathogen Interactions. Adv Appl Microbiol 96, 1–41. doi: 10.1016/bs.aambs.2016.07.018.

Fazli, M., Bjarnsholt, T., Kirketerp-Møller, K., Jørgensen, B., Andersen, A. S., Krogfelt, K. A., et al. (2009). Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. J Clin Microbiol 47, 4084–4089. doi: 10.1128/JCM.01395-09.

Hammer, K. A., and Carson, C. F. (2010). "Antibacterial and Antifungal Activities of Essential Oils," in Lipids and Essential Oils as Antimicrobial Agents (John Wiley and Sons, Ltd), 255–306. doi: 10.1002/9780470976623.ch11.

Juven, B. J., Kanner, J., Schved, F., and Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. Journal of Applied Bacteriology 76, 626–631. doi: 10.1111/j.1365-2672.1994.tb01661.x.

689 Kadam, S., Madhusoodhanan, V., Dhekane, R., Bhide, D., Ugale, R., Tikhole, U., et al. (2021). Milieu
690 matters: An in vitro wound milieu to recapitulate key features of, and probe new insights into, mixed-
691 species bacterial biofilms. Biofilm 3. doi: 10.1016/j.bioflm.2021.100047.

692 Kaiser, P., Wächter, J., and Windbergs, M. (2021). Therapy of infected wounds: overcoming clinical
693 challenges by advanced drug delivery systems. Drug Deliv Transl Res 11, 1545–1567. doi:
694 10.1007/s13346-021-00932-7.

695 Kirketerp-Møller, K., Jensen, P., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., et al. (2008).
696 Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol 46, 2717–2722.
697 doi: 10.1128/JCM.00501-08.

698 Kot, B., Wierzchowska, K., Grużewska, A., and Lohinau, D. (2018). The effects of selected
699 phytochemicals on biofilm formed by five methicillin-resistant Staphylococcus aureus. Nat Prod Res
700 32, 1299–1302. doi: 10.1080/14786419.2017.1340282.

701 Leaper, D., Assadian, O., and Edmiston, C. E. (2015). Approach to chronic wound infections. British
702 Journal of Dermatology 173, 351–358. doi: 10.1111/bjd.13677.

703 Marcos-Zambrano, L. J., Escribano, P., Bouza, E., and Guinea, J. (2014). Production of biofilm by
704 Candida and non-Candida spp. isolates causing fungemia: Comparison of biomass production and
705 metabolic activity and development of cut-off points. International Journal of Medical Microbiology
706 304, 1192–1198. doi: 10.1016/j.ijmm.2014.08.012.

707 Mühlen, S., and Dersch, P. (2016). Anti-virulence strategies to target bacterial infections. Curr Top
708 Microbiol Immunol 398, 147–183. doi: 10.1007/82_2015_490.

709 Orchard, A., Sandasi, M., Kamatou, G., Viljoen, A., and van Vuuren, S. (2017). The in vitro
710 Antimicrobial Activity and Chemometric Modelling of 59 Commercial Essential Oils against
711 Pathogens of Dermatological Relevance. Chem Biodivers 14. doi: 10.1002/cbdv.201600218.

712 Paleczny, J., Brożyna, M., Dudek-Wicher, R., Dydak, K., Oleksy-Wawrzyniak, M., Madziała, M., et
713 al. (2022). The Medium Composition Impacts Staphylococcus aureus Biofilm Formation and
714 Susceptibility to Antibiotics Applied in the Treatment of Bone Infections. Int J Mol Sci 23. doi:
715 10.3390/ijms231911564.

716 Paleczny, J., Junka, A., Brożyna, M., Dydak, K., Oleksy-Wawrzyniak, M., Ciecholewska-Juśko, D., et
717 al. (2021). The high impact of Staphylococcus aureus biofilm culture medium on in vitro outcomes of
718 antimicrobial activity of wound antiseptics and antibiotic. Pathogens 10. doi:
719 10.3390/pathogens10111385.

720 Patterson, J. E., McElmeel, L., and Wiederhold, N. P. (2019). In vitro activity of essential oils against
721 gram-positive and gram-negative clinical isolates, including carbapenem-resistant Enterobacteriaceae.
722 Open Forum Infect Dis 6. doi: 10.1093/ofid/ofz502.

723 Percival, S. L., Emanuel, C., Cutting, K. F., and Williams, D. W. (2012). Microbiology of the skin and
724 the role of biofilms in infection. Int Wound J 9, 14–32. doi: 10.1111/j.1742-481X.2011.00836.x.

Pestrak, M. J., Gupta, T. T., Dusane, D. H., Guzior, D. V., Staats, A., Harro, J., et al. (2020). Investigation of synovial fluid induced Staphylococcus aureus aggregate development and its impact on surface attachment and biofilm formation. PLoS One 15. doi: 10.1371/journal.pone.0231791.

Povolotsky, T. L., Keren-Paz, A., and Kolodkin-Gal, I. (2021). Metabolic Microenvironments Drive Microbial Differentiation and Antibiotic Resistance. Trends in Genetics 37, 4–8. doi: 10.1016/j.tig.2020.10.007.

Reichling, J. (2020). Anti-biofilm and Virulence Factor-Reducing Activities of Essential Oils and Oil Components as a Possible Option for Bacterial Infection Control. Planta Med 86, 520–537. doi: 10.1055/a-1147-4671.

Roseanu, A., Damian, M., and Evans, R. W. (2010). Mechanisms of the Antibacterial Activity of Lactoferrin and Lactoferrin-Derived Peptides. Romanian Journal of Biochemistry 47, 203–209. Available at: https://www.researchgate.net/publication/268421952.

Shepherd, M. J., Moore, G., Wand, M. E., Sutton, J. M., and Bock, L. J. (2018). Pseudomonas aeruginosa adapts to octenidine in the laboratory and a simulated clinical setting, leading to increased tolerance to chlorhexidine and other biocides. Journal of Hospital Infection 100, e23–e29. doi: 10.1016/j.jhin.2018.03.037.

Siddiqui, A. R., and Bernstein, J. M. (2010). Chronic wound infection: Facts and controversies. Clin Dermatol 28, 519–526. doi: 10.1016/j.clindermatol.2010.03.009.

Sienkiewicz, M., Łysakowska, M., Kowalczyk, E., Szymańska, G., Kochan, E., Krukowska, J., et al. (2017). The ability of selected plant essential oils to enhance the action of recommended antibiotics against pathogenic wound bacteria. Burns 43, 310–317. doi: 10.1016/j.burns.2016.08.032.

Silva, N. B. S., Marques, L. de A., and Röder, D. von D. de B. (2020). Antibiofilm Activity of Natural Products: Promising Strategies for Combating Microbial Biofilms. Annals of Public Health Reports 4, 92–99. doi: 10.36959/856/502.

Thaarup, I. C., and Bjarnsholt, T. (2021). Current in vitro biofilm-infected chronic wound models for developing new treatment possibilities. Advances in Wound Care (New Rochelle) 10, 91–102. doi: 10.1089/wound.2020.1176.

Tohidpour, A., Sattari, M., Omidbaigi, R., Yadegar, A., and Nazemi, J. (2010). Antibacterial effect of essential oils from two medicinal plants against Methicillin-resistant Staphylococcus aureus (MRSA). Phytomedicine 17, 142–145. doi: 10.1016/j.phymed.2009.05.007.

Veldhuizen, E. J. A., Creutzberg, T. O., Burt, S. A., and Haagsman, H. P. (2007). Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against Listeria monocytogenes in steak tartare. J Food Prot 70, 2127–2132. doi: 10.4315/0362-028X-70.9.2127.

Vyas, H. K. N., Xia, B., and Mai-Prochnow, A. (2022). Clinically relevant in vitro biofilm models: A need to mimic and recapitulate the host environment. Biofilm 4. doi: 10.1016/j.bioflm.2022.100069.

Xu, Z., Liang, Y., Lin, S., Chen, D., Li, B., Li, L., et al. (2016). Crystal Violet and XTT Assays on Staphylococcus aureus Biofilm Quantification. Curr Microbiol 73, 474–482. doi: 10.1007/s00284-016-1081-1.

763 Yap, P. S. X., Yiap, B. C., Ping, H. C., and Lim, S. H. E. (2014). Essential Oils, A New Horizon in
764 Combating Bacterial Antibiotic Resistance. Open Microbiol J 8, 6–14. doi:
765 10.2174/1874285801408010006.

Supplementary Material

Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity

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Supplementary Table 1. Ingredients of T-EO **(A)** and R-EO **(B)** measured with GC-MS (Gas Chromatography-Mass Spectrometry). Components in line with Polish Pharmakopea XI standards are marked green; the ones not in line are marked red. RI - retention index, RT – retention time, T-EO- thyme essential oil, R-RO- rosemary essential oil.

(A) T-EO			
RI	**RT**	**Compound**	**T-EO**
925	8.94	α-thujene	0.38
932	9.19	α-pinene	0.91
948	9.77	camphene	1.06
976	10.75	β-pinene	0.31
988	11.19	myrcene	1.11
1016	12.19	α-terpinene	1.81
1024	**12.50**	**p-cymene**	**19.20**
1028	12.65	limonene	0.37
1030	12.70	eucalyptol	0.28

1058	**13.74**	**γ-terpinene**	**9.06**
1099	15.28	linalool	3.21
1147	17.01	camphor	0.62
1172	17.92	borneol	1.98
1180	18.23	terpinen-4-ol	1.02
1195	18.76	α-terpineol	0.42
1238	20.27	thymol methyl ether	0.46
1291	**22.15**	**thymol**	**50.59**
1297	22.36	carvacrol	5.65
1426	26.37	caryophyllene	1.66

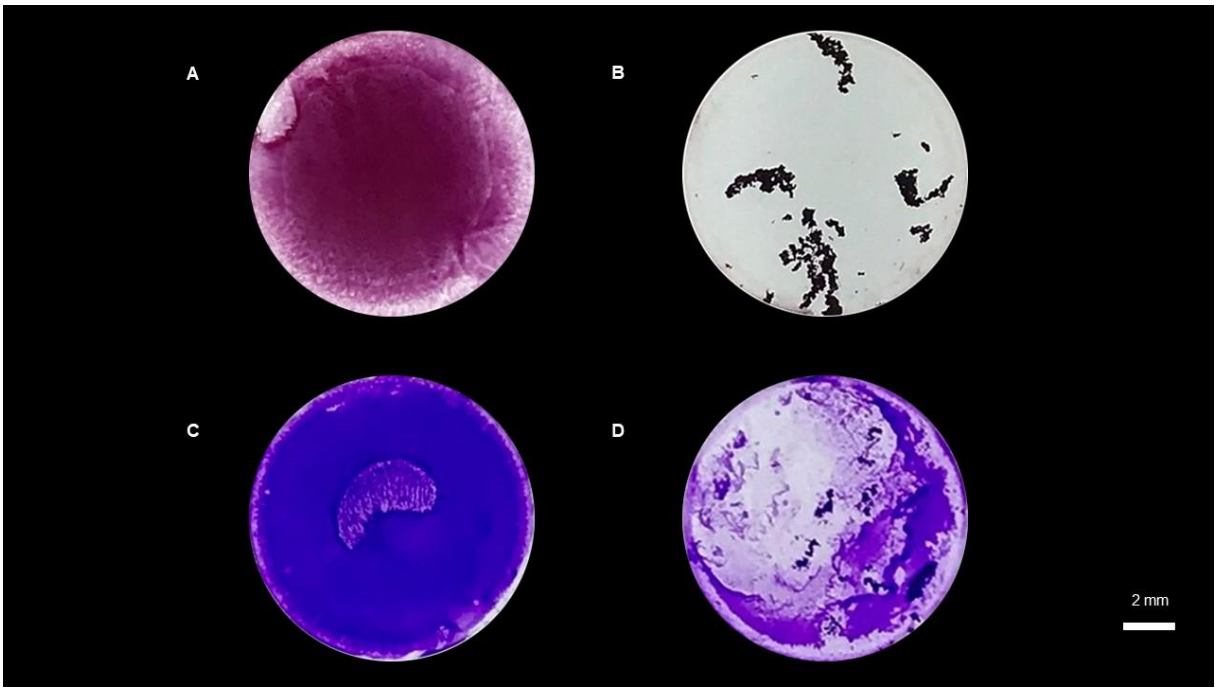
Polish Pharmakopea XI ranges for thyme oil, thymol chemotype:
α- thujene: 0.2 percent to 1.5 percent
β-myrcene: 1.0 percent to 3.0 percent
α-terpinene: 0.9 percent to 2.6 percent
p-cymene: 14.0 percent to 28.0 percent
γ-terpinene: 4.0 percent to 12.0 percent
linalool: 1.5 percent to 6.5 percent
terpinen-4-ol: 0.1 percent to 2.5 percent
carvacrol methyl ether: 0.05 percent to 1.5 percent
thymol: 37.0 percent to 55.0 percent
carvacrol: 0.5 percent to 5.5 percent

(B) R-EO			
RI	**RT**	**Compound**	**R-EO**
921	8.81	tricyclene	0.41
925	8.94	α-thujene	0.11
933	**9.22**	**α-pinene**	**21.07**
948	9.77	camphene	8.81
952	9.90	thuja-2,4(10)-diene	0.30
976	10.76	β-pinene	3.78
984	11.04	octen-2-ol	0.20
989	11.20	myrcene	2.70
1005	11.80	α-phellandrene	0.52
1008	11.89	3-carene	0.61
1016	12.19	α-terpinene	0.65
1024	12.48	p-cymene	2.46
1029	12.66	limonene	3.88
1032	**12.79**	**1,8-cineole**	**19.98**
1035	12.91	trans-β-ocimene	0.16
1057	13.73	γ-terpinene	1.01
1084	14.73	terpinolene	0.68

1099	15.28	linalool	0.71
1148	**17.05**	**camphor**	**18.52**
1172	17.92	borneol	3.54
1180	18.23	terpinen-4-ol	0.68
1194	18.76	α-terpineol	2.32
1206	19.18	verbenone	1.91
1284	21.88	bornyl acetate	1.23
1369	24.74	copaene	0.15
1419	26.37	β-caryophyllene	2.76
1456	27.50	α-humulene	0.50
1479	28.23	γ-muurolene	0.11
1517	29.41	δ-cadinene	0.12

For rosemary oil, Spanish type**,** the percentages are within the following ranges:

α-pinene: 18 percent to 26 percent
camphene: 8.0 percent to 12.0 percent
β-pinene: 2.0 percent to 6.0 percent
β-myrcene: 1.5 percent to 5.0 percent
limonene: 2.5 percent to 5.0 percent
cineole: 16.0 percent to 25.0 percent
p-cymene: 1.0 percent to 2.2 percent
camphor: 13.0 percent to 21.0 percent
bornyl acetate: 0.5 percent to 2.5 percent
α-terpineol: 1.0 percent to 3.5 percent
borneol: 2.0 percent to 4.5 percent
verbenone: 0.7 percent to 2.5 percent



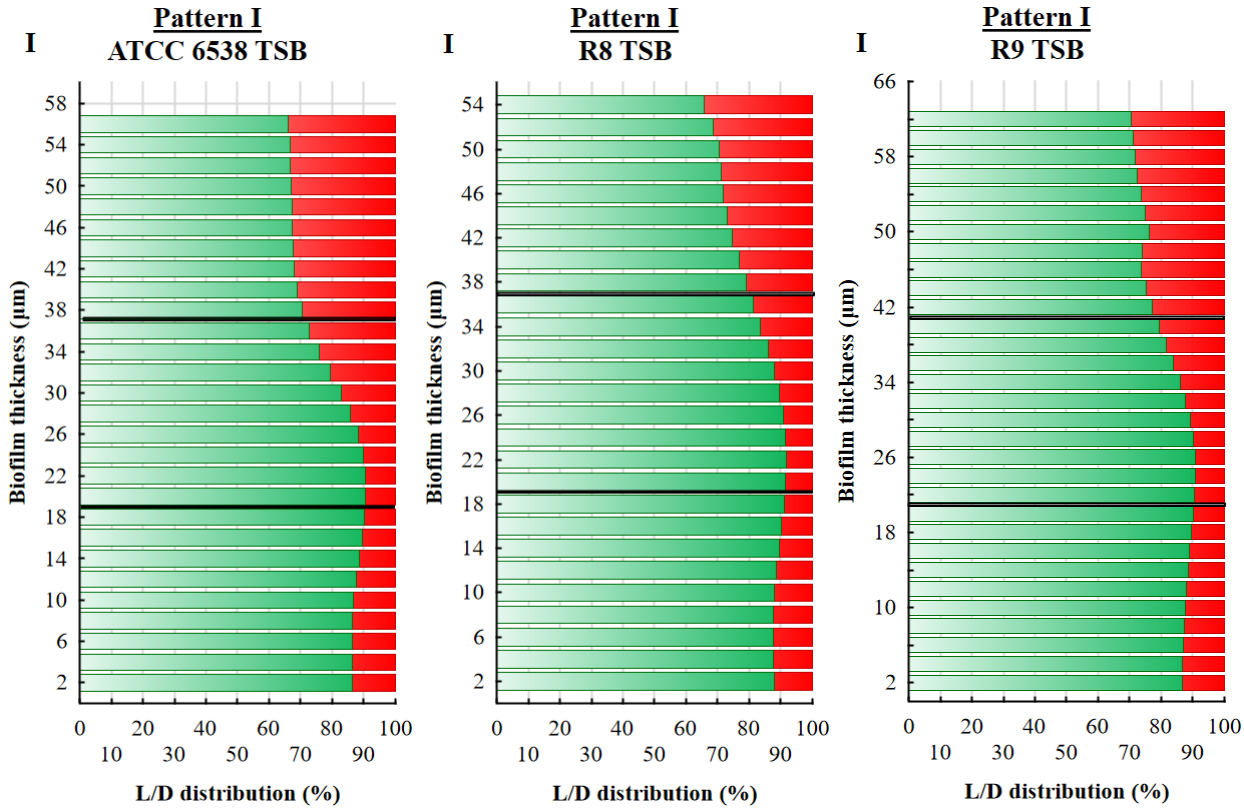
Supplementary Figure 1. Original images of *S. aureus* S11 biofilm formed on polystyrene. **A, B**- biofilm cultured in TSB (**A**) or IVWM (**B**) and stained with tetrazolium chloride; **C, D**- biofilm cultured in TSB (**C**) or IVWM (**D**) and stained with crystal violet. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu. The scale bar is 2 mm.

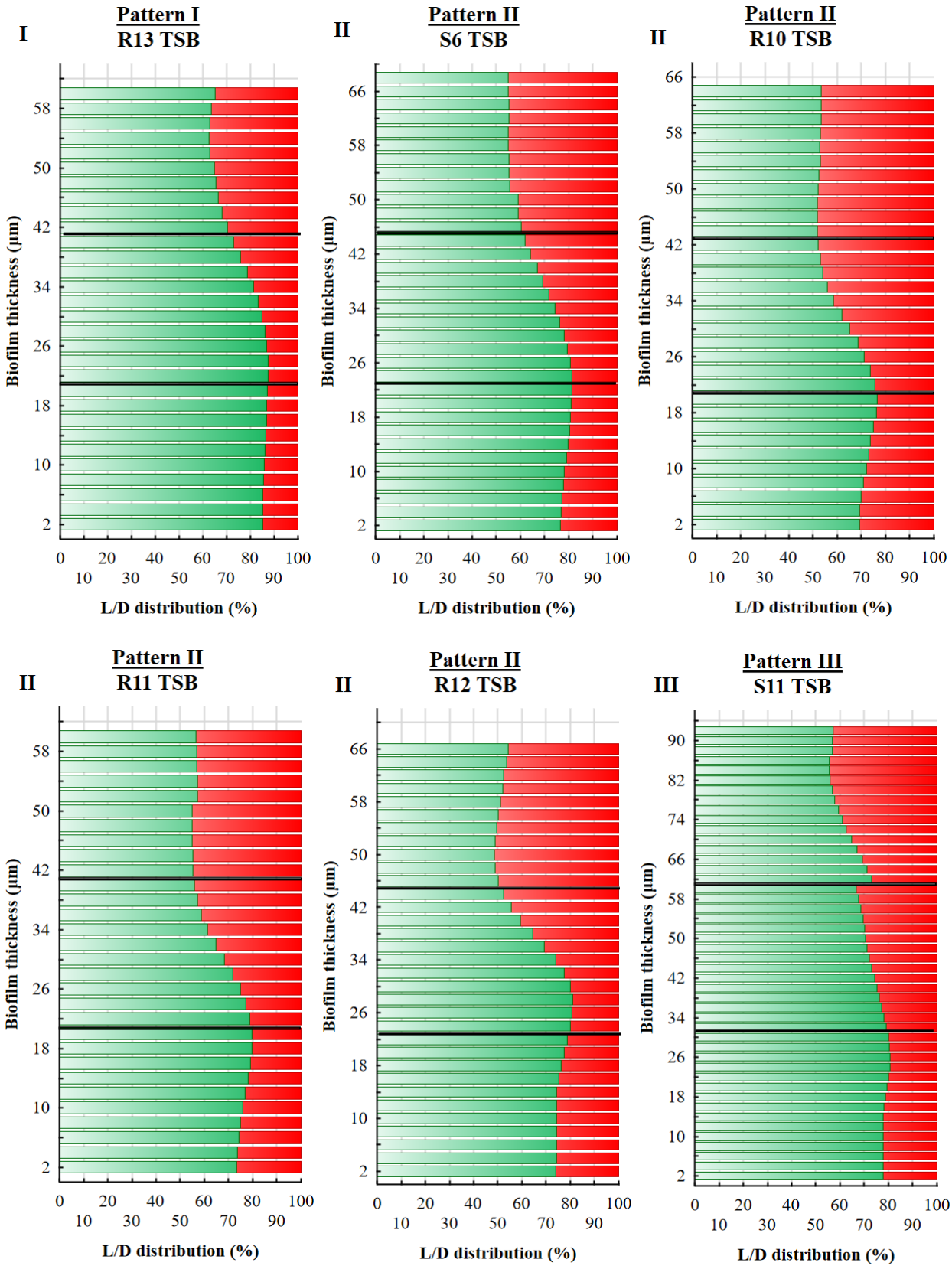
Supplementary Table 2. Average percentage share of live and dead cells in biofilms of staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains cultured in TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium measured with a confocal microscope. Biofilms were divided into three parts along the Z-axis: top (T), middle (M), and bottom (B). The share of cells in each part was compared in both media and marked green in medium where the parameter was higher and red where it was lower.

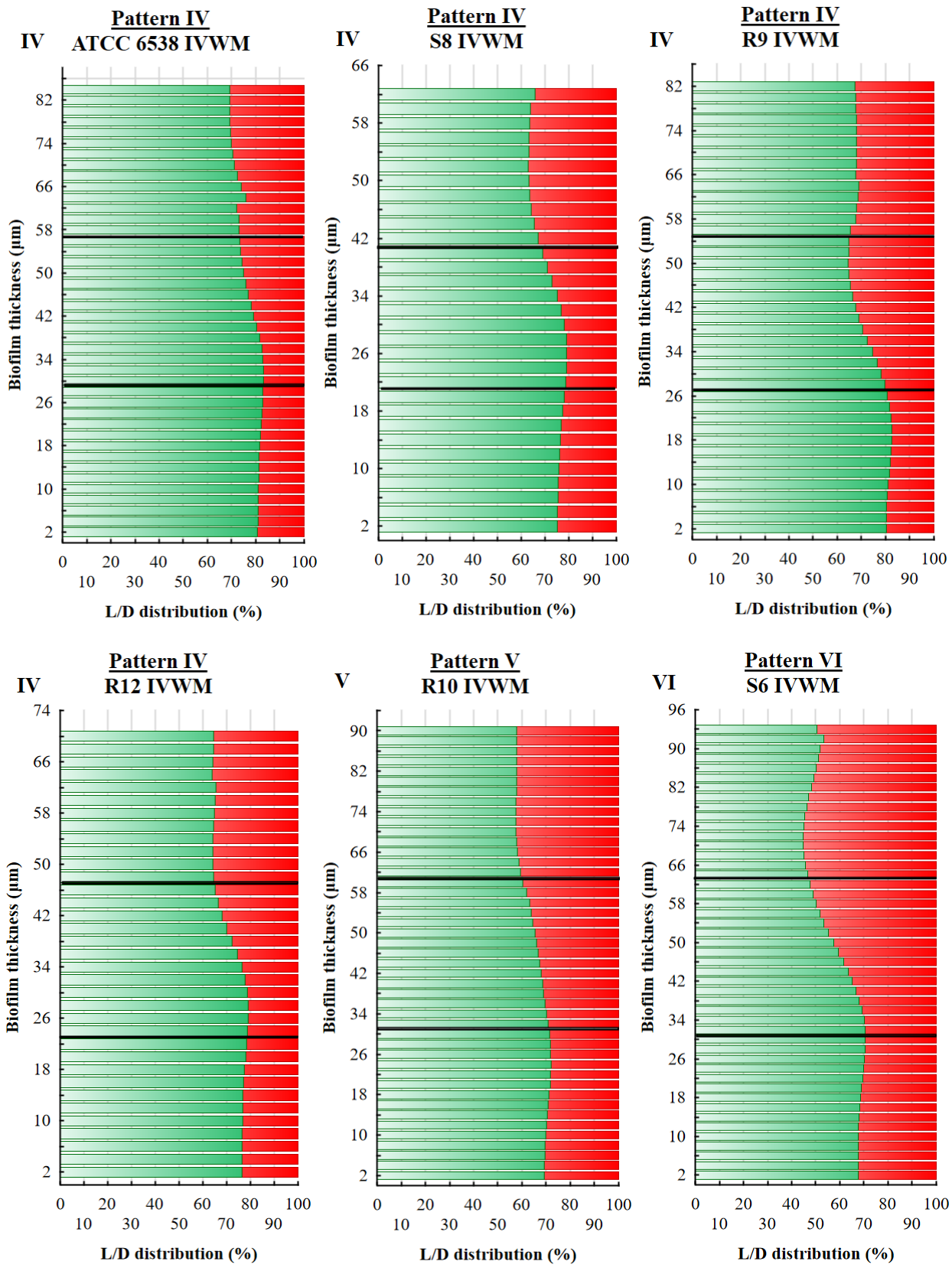
			Share (%) of cells in *S. aureus* biofilms	
Strain number	**Part of biofilm**	**Type of cells**	**TSB**	**IVWM**
ATCC 6538	T	Live	68	71
		Dead	32	29
	M	Live	84	79
		Dead	16	21
	B	Live	87	82
		Dead	13	18
S3	T	Live	71	61
		Dead	29	39
	M	Live	87	74
		Dead	13	26

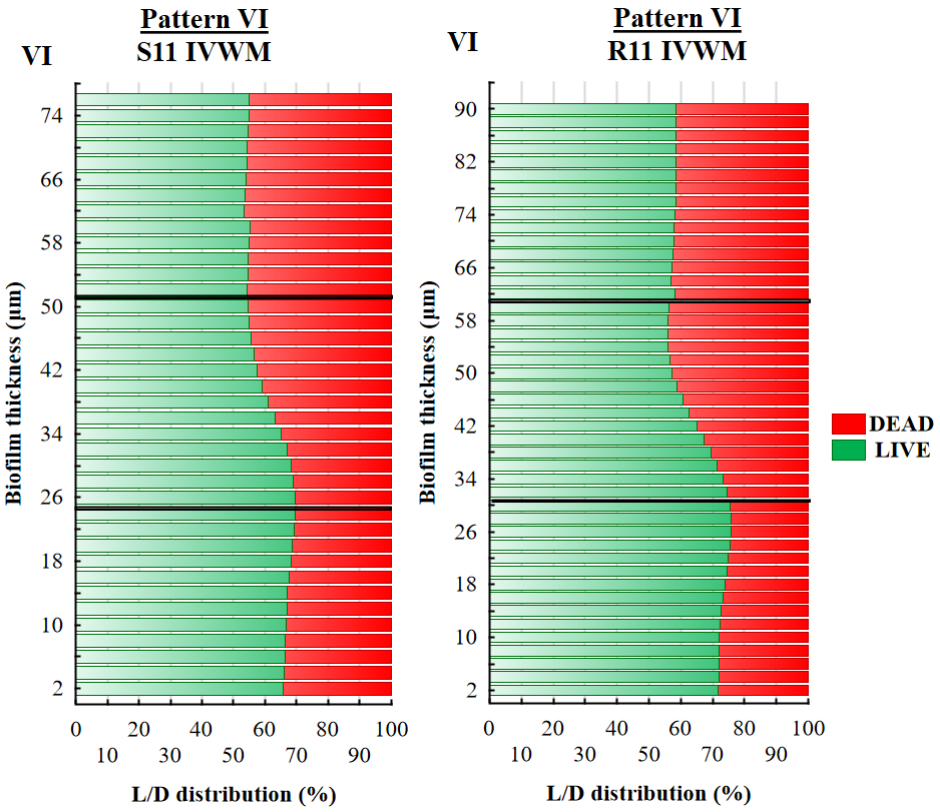
	B	Live	85	76
		Dead	15	24
S6	T	Live	56	48
		Dead	44	52
	M	Live	73	60
		Dead	27	40
	B	Live	79	69
		Dead	21	31
S8	T	Live	60	64
		Dead	40	36
	M	Live	78	76
		Dead	22	24
	B	Live	84	76
		Dead	16	24
S11	T	Live	61	54
		Dead	39	46
	M	Live	73	62
		Dead	27	38
	B	Live	79	67
		Dead	21	33
R1	T	Live	69	41
		Dead	31	59
	M	Live	80	50
		Dead	20	50
	B	Live	85	60
		Dead	15	40
R8	T	Live	72	58
		Dead	28	42
	M	Live	88	66
		Dead	12	34
	B	Live	89	71
		Dead	11	29
R9	T	Live	74	68
		Dead	26	32
	M	Live	87	70
		Dead	13	30
	B	Live	88	81
		Dead	12	19
R10	T	Live	53	58
		Dead	47	42
	M	Live	63	66
		Dead	37	34
	B	Live	73	71
		Dead	27	29
R11	T	Live	56	58
		Dead	44	42

	M	Live	67	63
		Dead	33	37
	B	Live	77	74
		Dead	23	26
R12	T	Live	51	65
		Dead	49	35
	M	Live	70	74
		Dead	30	26
	B	Live	75	77
		Dead	25	23
R13	T	Live	65	39
		Dead	35	61
	M	Live	82	35
		Dead	18	65
	B	Live	86	51
		Dead	14	49

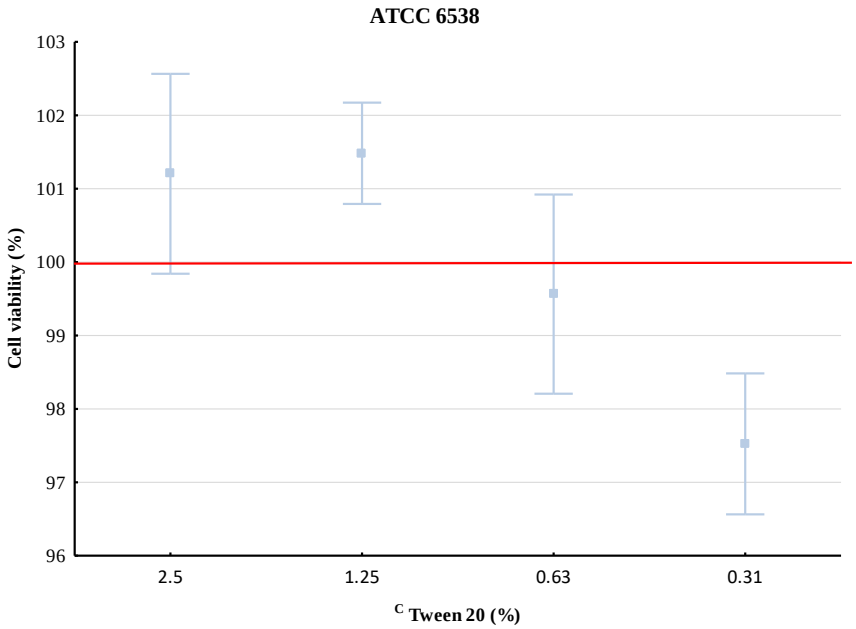



I
Pattern I
R13 TSB
II
Pattern II
S6 TSB
II
Pattern II
R10 TSB
II
Pattern II
R11 TSB
II
Pattern II
R12 TSB
III
Pattern III
S11 TSB
Biofilm thickness (µm)
L/D distribution (%)

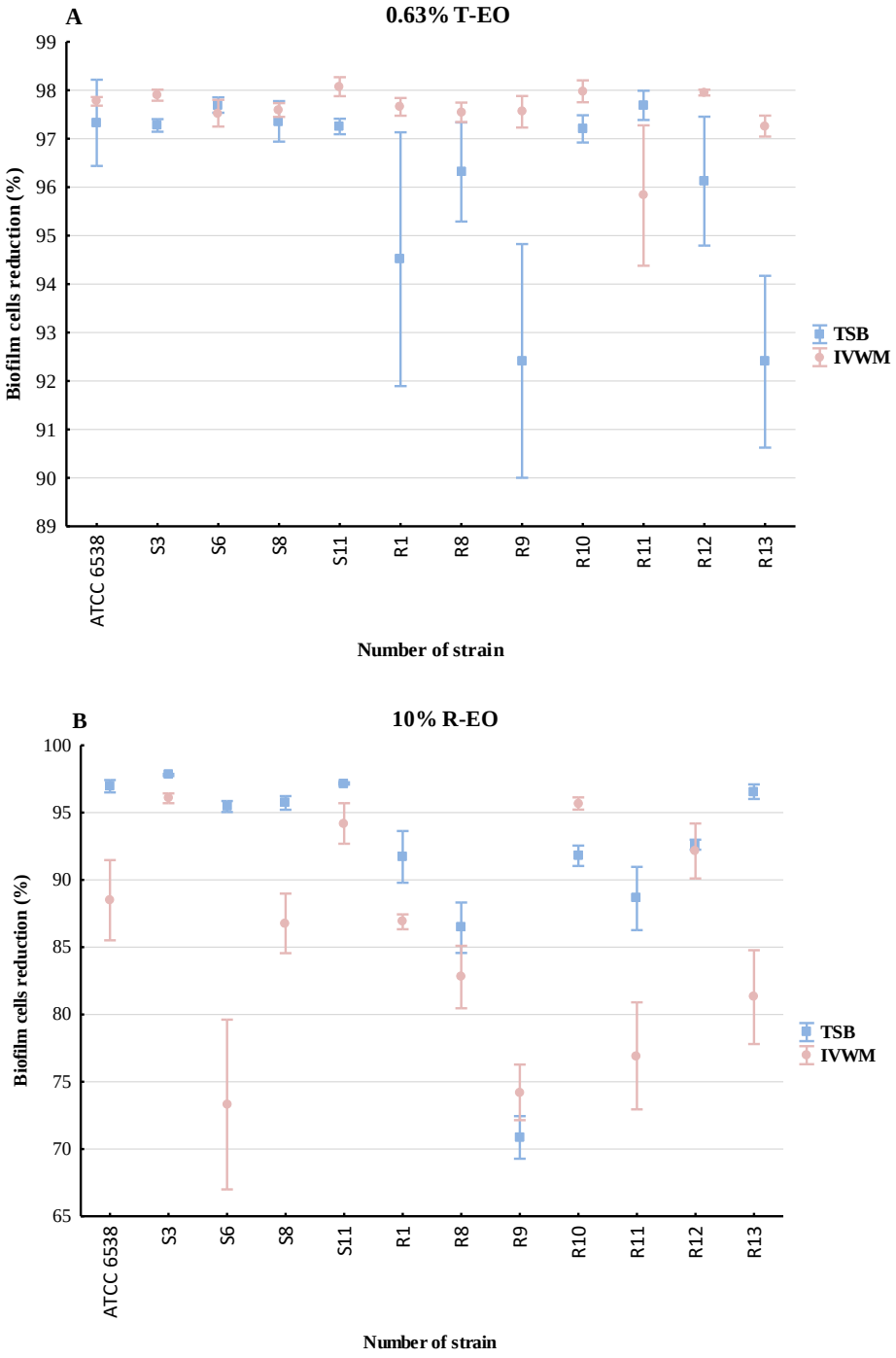

Pattern IV
IV ATCC 6538 IVWM
Pattern IV
IV S8 IVWM
Pattern IV
IV R9 IVWM
Pattern IV
IV R12 IVWM
Pattern V
V R10 IVWM
Pattern VI
VI S6 IVWM
Biofilm thickness (µm)
L/D distribution (%)

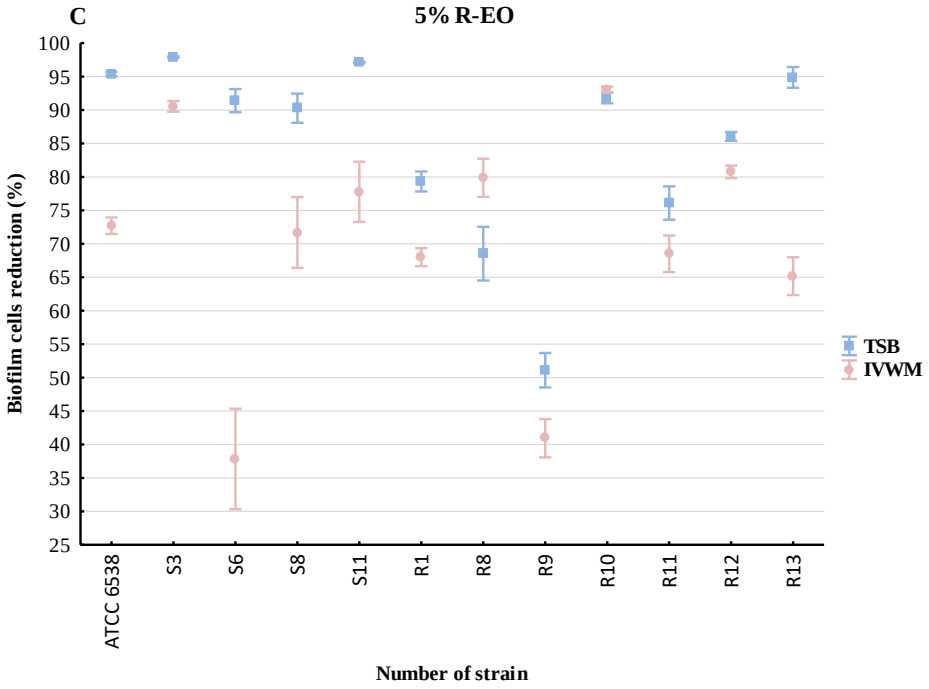


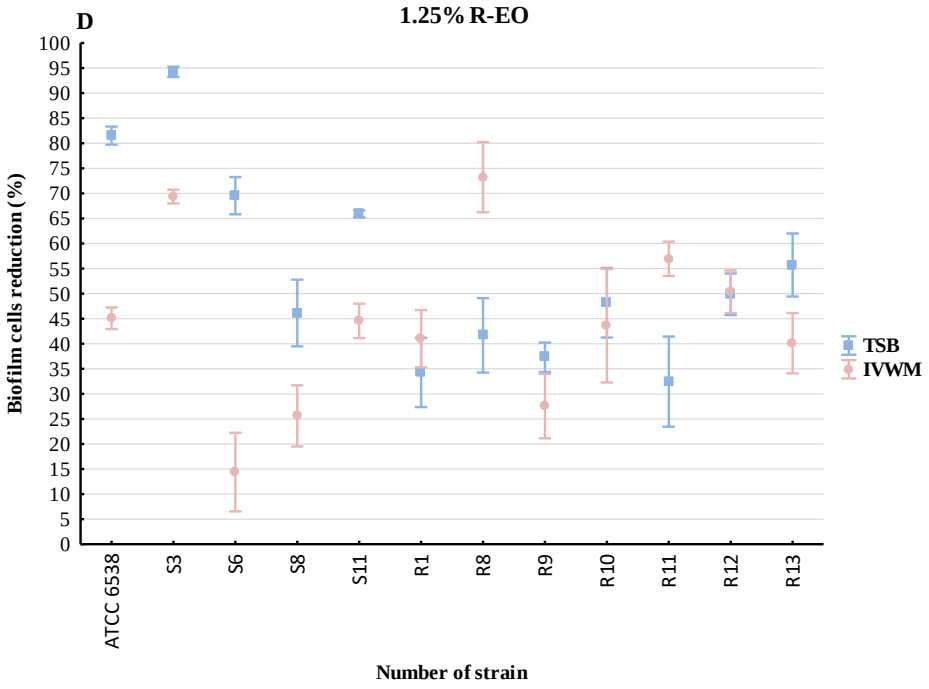
Supplementary Figure 2. The main patterns of live (L, green) and dead (D, red) cells distribution (%) in staphylococcal biofilms across the Z-axis, cultured in TSB (Tryptic Soy Broth) or IVWM (In Vitro Wound Milieu) medium. The thickness of each section was 2 µm. Black lines divide graphs into the top (T), middle (M), and bottom (B) parts. **I, II, III**- strains of particular patterns of cells share in TSB; **IV, V, VI**-strains of particular patterns of cells share in IVWM. ATCC 6538- *S. aureus* reference strain (American Type Culture Collection), S6, S8, S11, R8-R13- clinical strains of *S. aureus*. The confocal microscope SP8, magnification 25×.



Supplementary Figure 3. Average viability (%) of *S. aureus* ATCC 6538 (American Type Culture Collection) planktonic forms treated with different concentrations of Tween 20 [(%)(v/v)] in TSB (Tryptic Soy Broth) medium with regard to untreated cells. Error lines represent the the standard error of the mean (n=6). A red line marks untreated cells.




C
5% R-EO
Biofilm cells reduction (%)
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
Number of strain
TSB
IVWM


D
1.25% R-EO
Biofilm cells reduction (%)
100
95
90
85
80
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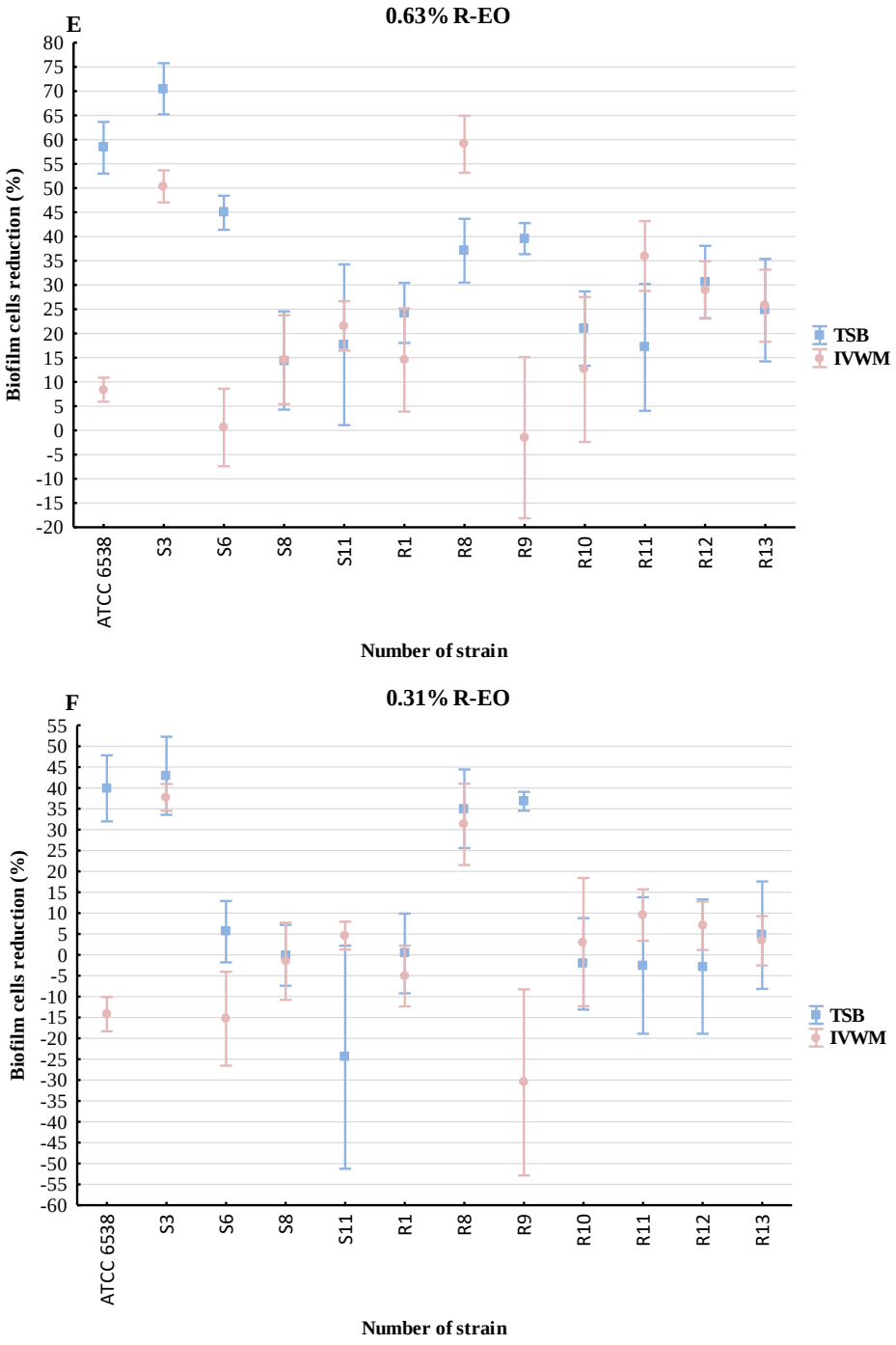
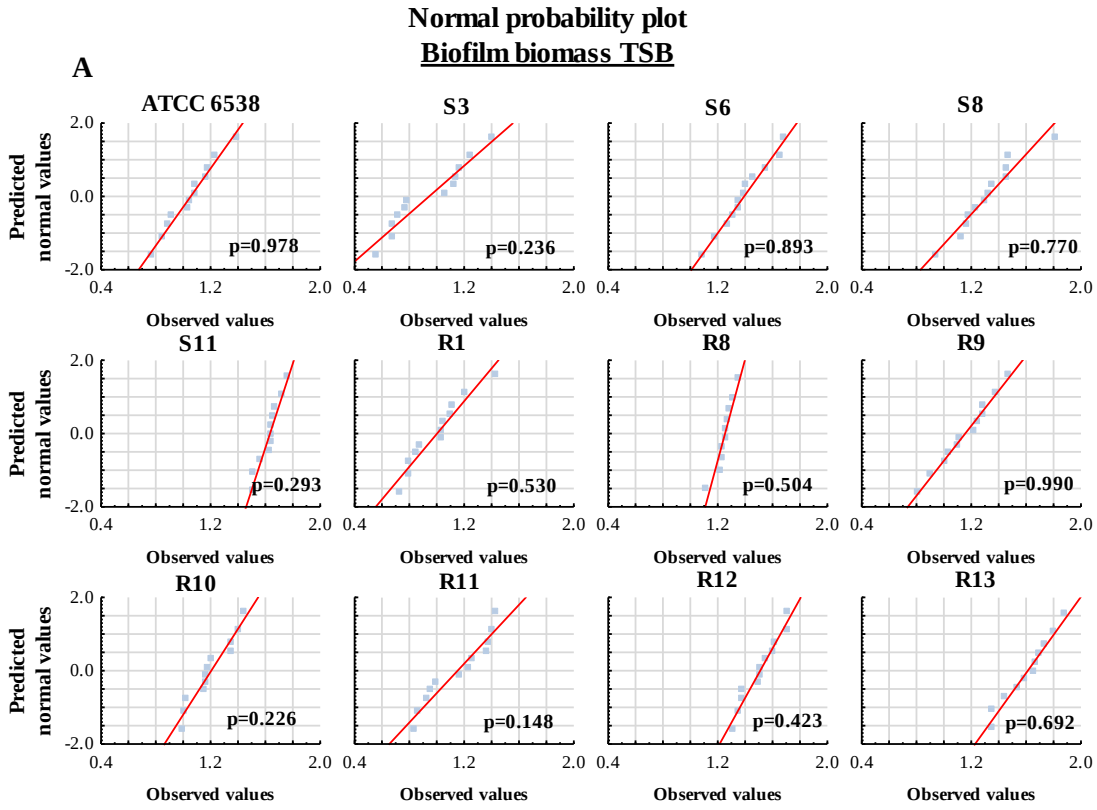
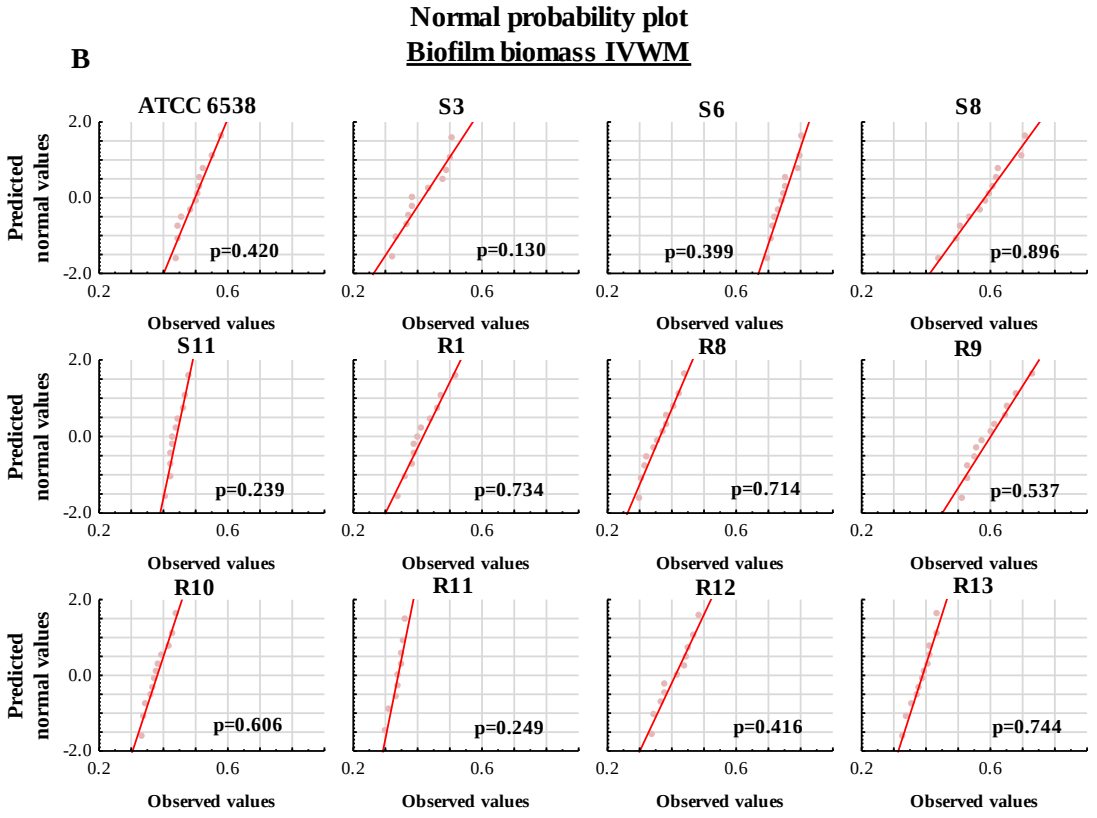
20
15
10
5
0
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
Number of strain
TSB
IVWM



Supplementary Figure 4. Average reduction (%) of biofilm cells of reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains of *S. aureus* after treatment with selected concentrations (%) (v/v) of thyme oil (**A**, T-EO) or rosemary oil (**B-F**, R-EO). Error lines represent the standard error of the mean (n=6). TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu.

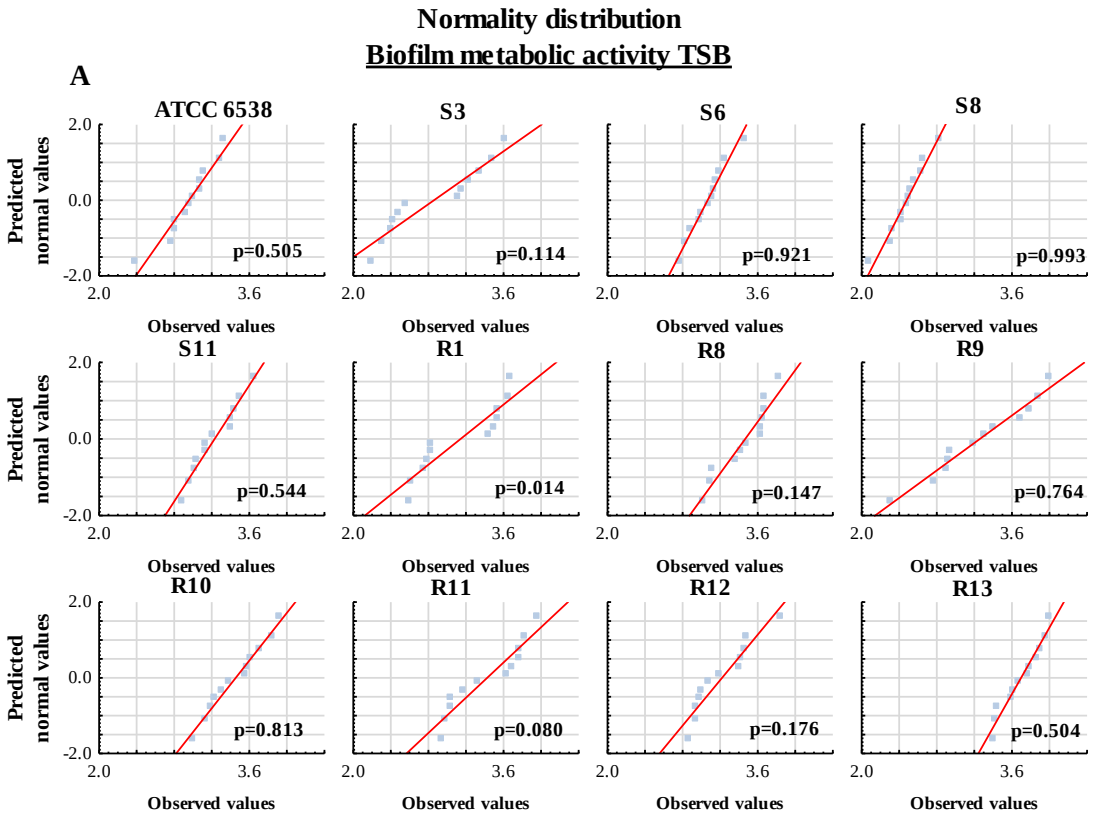

Normal probability plot
Biofilm biomass TSB
A
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
p=0.978
p=0.236
p=0.893
p=0.770
p=0.293
p=0.530
p=0.504
p=0.990
p=0.226
p=0.148
p=0.423
p=0.692
Predicted normal values
Observed values
2.0
0.0
-2.0
0.4
1.2
2.0

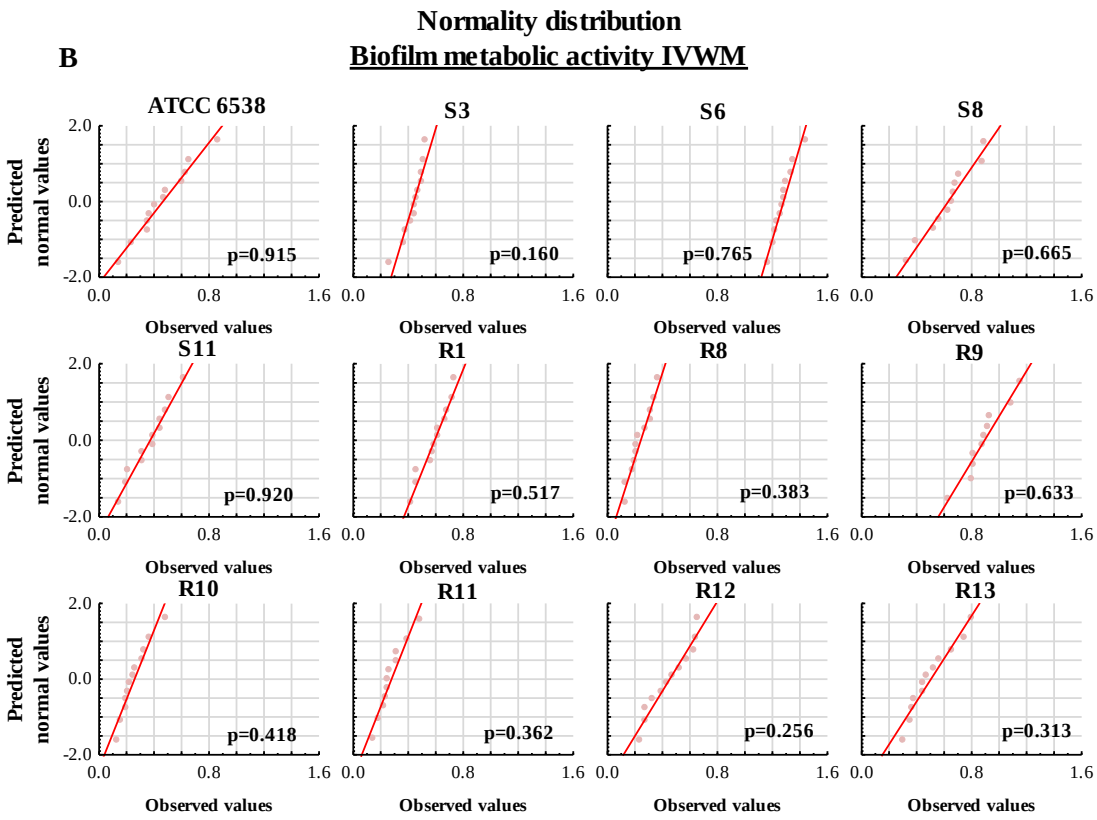

Normal probability plot
Biofilm biomass IVWM
B
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
p=0.420
p=0.130
p=0.399
p=0.896
p=0.239
p=0.734
p=0.714
p=0.537
p=0.606
p=0.249
p=0.416
p=0.744
Predicted normal values
Observed values
2.0
0.0
-2.0
0.2
0.6

Supplementary Figure 5. Normal probability plots for analysis of differences in particular staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains' ability to form biofilm biomass between Tryptic Soy Broth (**A**, TSB) or In Vitro Wound Milieu (**B**, IVWM) medium. Red curves indicate the matching of the values to the normal distribution. P- probability level calculated with Shapiro-Wilk test. Normal distribution was considered for values of $p>0.05$.

Supplementary Table 3. Parameters of the tested statistic of the differences in particular staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains' ability to form biofilm biomass between Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. The normal distribution of the absorbance values was determined in each group (Shapiro-Wilk test, $p<0.05$). Depending on the variance homogeneity (Levene's test, $p<0.05$), t-test (for homogeneous variances, ATCC 6538, S8, S11, R1, R8, R9, R12 strains), or Welch's t-test (for non-homogeneous variances, S3, S6, R10, R11, R13 strains) were performed. T- values of t-test, df- degrees of freedom, p- probability level (values of $p<0.05$ were considered significant).

Biofilm biomass					
Number of strain	**Average TSB**	**Average IVWM**	**t**	**df**	**p**
ATCC 6538	1.1	0.4	11.6	22	0.000000
S3	0.9	0.2	7.9	16	0.000001
S6	1.4	0.9	9.0	15	0.000000
S8	1.3	0.6	9.6	22	0.000000
S11	1.6	0.3	50.0	20	0.000000
R1	1.0	0.2	11.2	21	0.000000
R8	1.3	0.1	32.0	20	0.000000
R9	1.2	0.6	8.1	22	0.000000
R10	1.2	0.2	21.3	15	0.000000
R11	1.2	0.1	16.2	12	0.000000
R12	1.5	0.2	25.7	21	0.000000
R13	1.6	0.2	25.3	13	0.000000

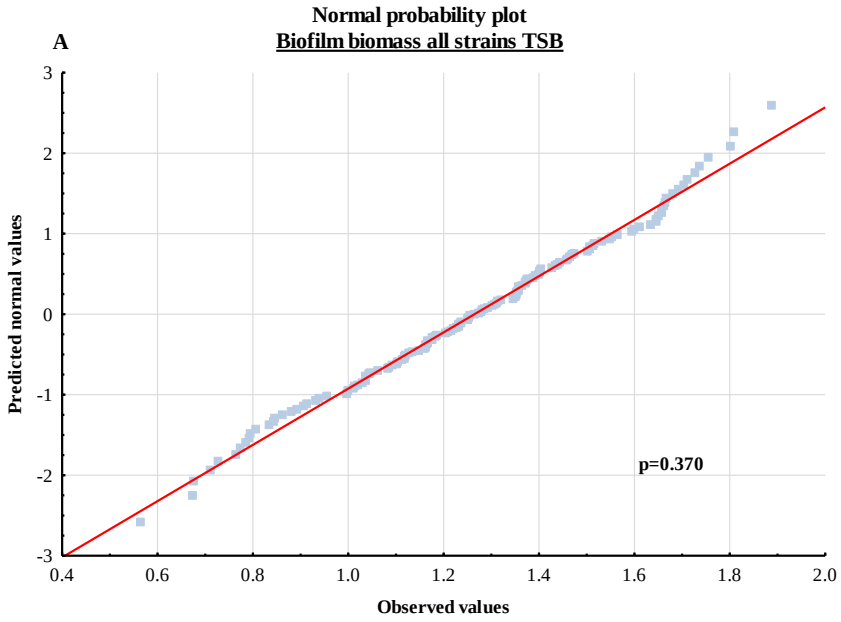

Normality distribution
Biofilm metabolic activity TSB
A
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
p=0.505
p=0.114
p=0.921
p=0.993
p=0.544
p=0.014
p=0.147
p=0.764
p=0.813
p=0.080
p=0.176
p=0.504
Predicted normal values
Observed values
2.0
0.0
-2.0
2.0
3.6

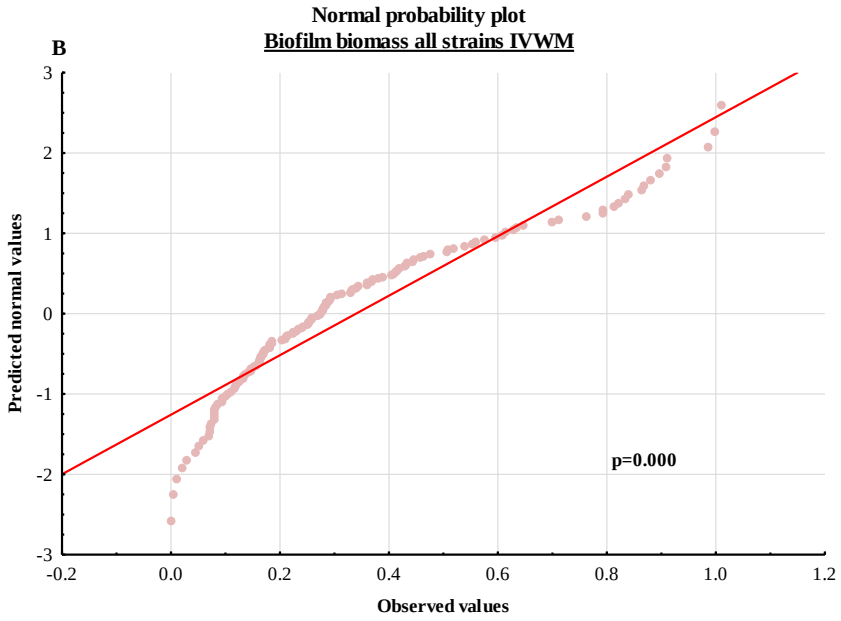

Normality distribution
Biofilm metabolic activity IVWM
B
ATCC 6538
S3
S6
S8
S11
R1
R8
R9
R10
R11
R12
R13
p=0.915
p=0.160
p=0.765
p=0.665
p=0.920
p=0.517
p=0.383
p=0.633
p=0.418
p=0.362
p=0.256
p=0.313
Predicted normal values
Observed values
2.0
0.0
-2.0
0.0
0.8
1.6

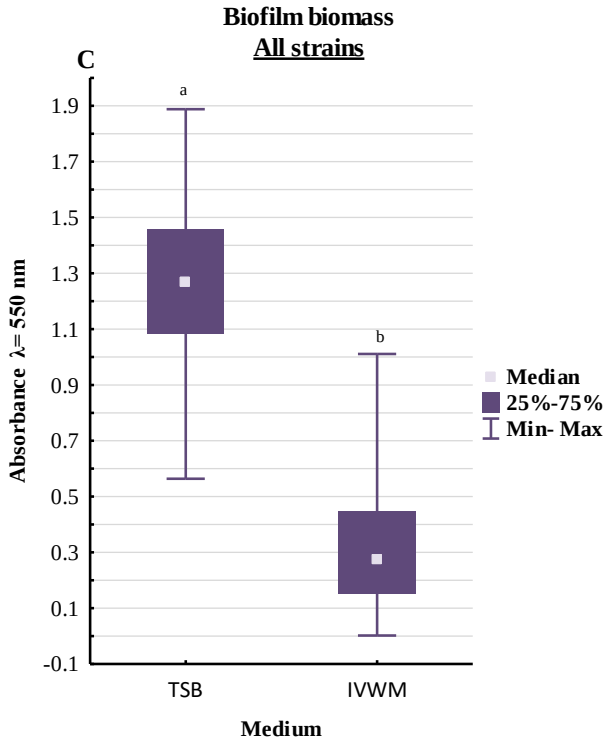
Supplementary Figure 6. Normal probability plots for analysis of differences in particular staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains' metabolic activity of biofilms between Tryptic Soy Broth (**A**, TSB) or In Vitro Wound Milieu (**B**, IVWM) medium. Red curves indicate the fitting of the values to the normal distribution. P- probability level calculated with Shapiro-Wilk test. Normal distribution was considered for values of $p>0.05$.

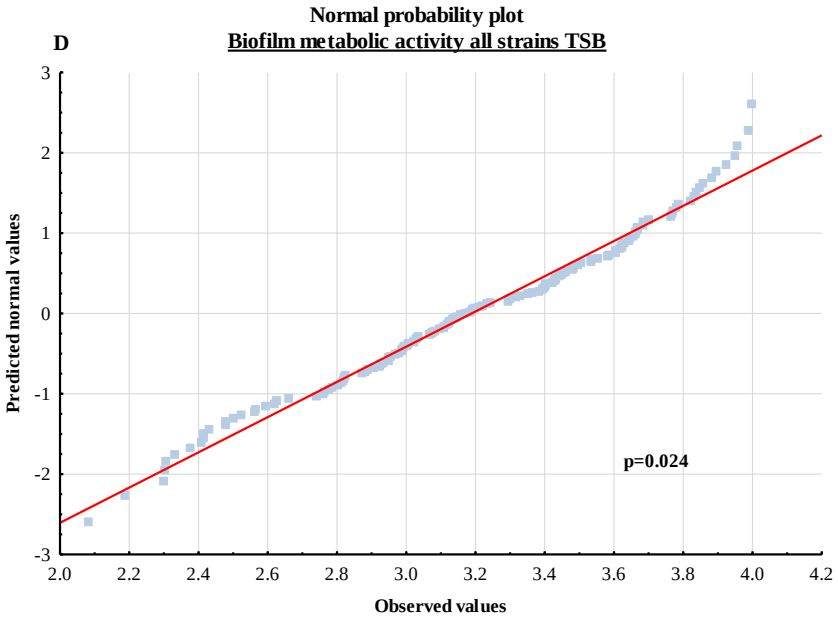
Supplementary Table 4. Parameters of the tested statistic of the differences in particular staphylococcal reference (ATCC 6538, American Type Culture Collection) or clinical (S3, S6, S8, S11, R1, R8-R13) strains' metabolic activity of biofilms between Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Normal distribution of the values was determined in each group except the R1 strain (Shapiro-Wilk test, $p<0.05$). Depending on the variance homogeneity (Levene's test, $p<0.05$), t-test (for homogeneous variances, ATCC 6538, S8, R13 strains) or Welch's t-test (for non-homogeneous variances, S3, S6, S11, R8, R9, R10, R11, R12 strains) were performed in groups with the normal distribution of values. For the R1 strain, Mann-Whitney U test was performed. T-values of t-test, df- degrees of freedom, p- probability level (values of $p<0.05$ were considered significant), U and Z- values of U-test.

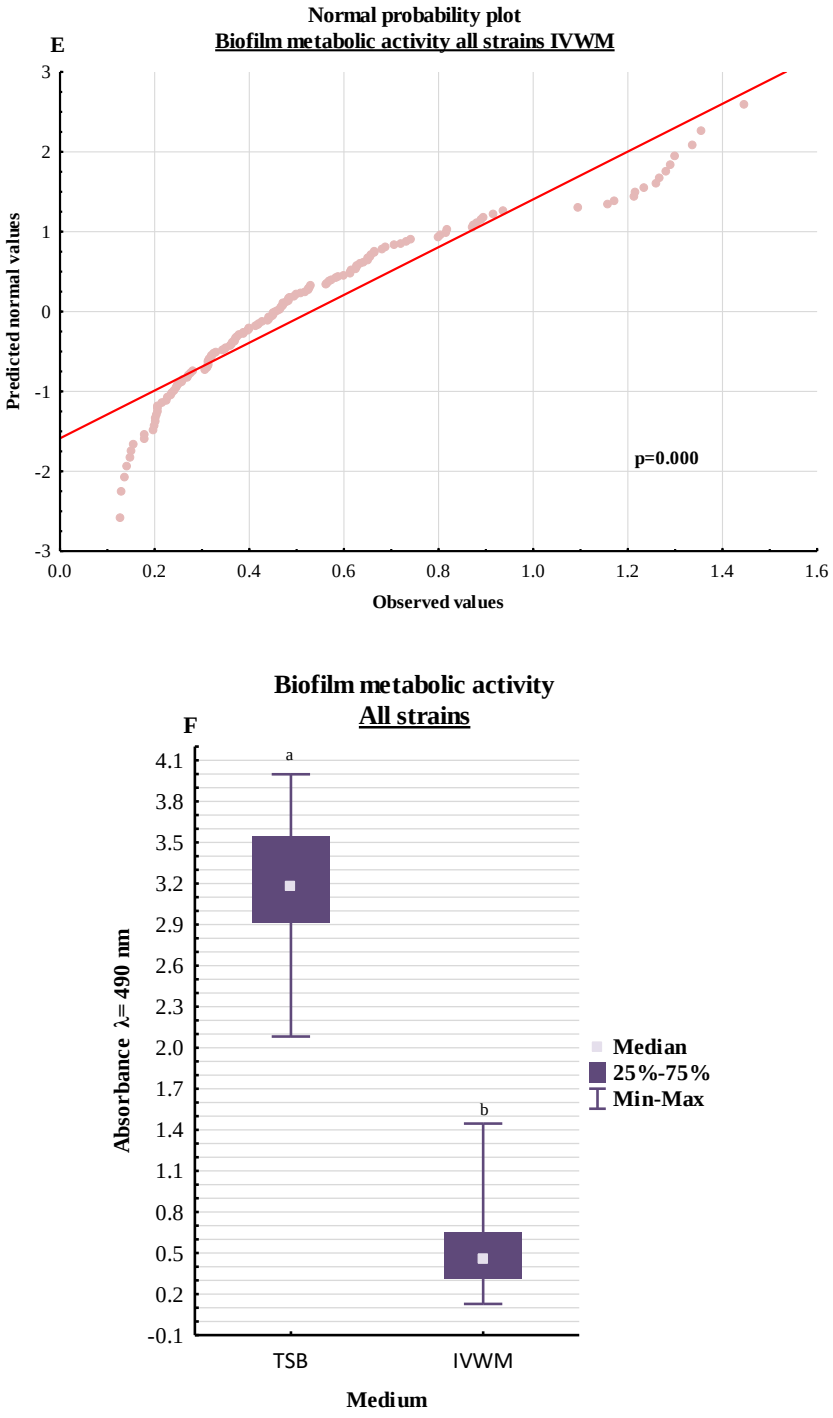
Biofilm metabolic activity					
Number of strain	**Average TSB**	**Average IVWM**	**t**	**df**	**p**
ATCC 6538	3.0	0.5	26.7	22	0.000000
S3	2.9	0.4	16.2	11	0.000000
S6	3.1	1.3	30.2	14	0.000000
S8	2.5	0.6	24.3	21	0.000000
S11	3.2	0.4	35.4	18	0.000000
R8	3.5	0.2	40.4	13	0.000000
R9	3.3	0.9	15.2	13	0.000000
R10	3.5	0.3	35.9	13	0.000000
R11	3.4	0.3	27.7	13	0.000000
R12	3.2	0.5	28.7	16	0.000000
R13	3.7	0.5	42.0	22	0.000000
	Rank sum TSB	**Rank sum IVWM**	**U**	**Z**	**p**
R1	222	78	0	4	0.000037


Normal probability plot
Biofilm biomass all strains TSB
A
Predicted normal values
Observed values
p=0.370
3
2
1
0
-1
-2
-3
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0


Normal probability plot
Biofilm biomass all strains IVWM
B
Predicted normal values
Observed values
p=0.000
3
2
1
0
-1
-2
-3
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2


Biofilm biomass
All strains
C
a
b
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
Absorbance λ= 550 nm
TSB
IVWM
Medium
Median
25%-75%
Min- Max


Normal probability plot
Biofilm metabolic activity all strains TSB
D
3
2
1
0
-1
-2
-3
Predicted normal values
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
Observed values
p=0.024

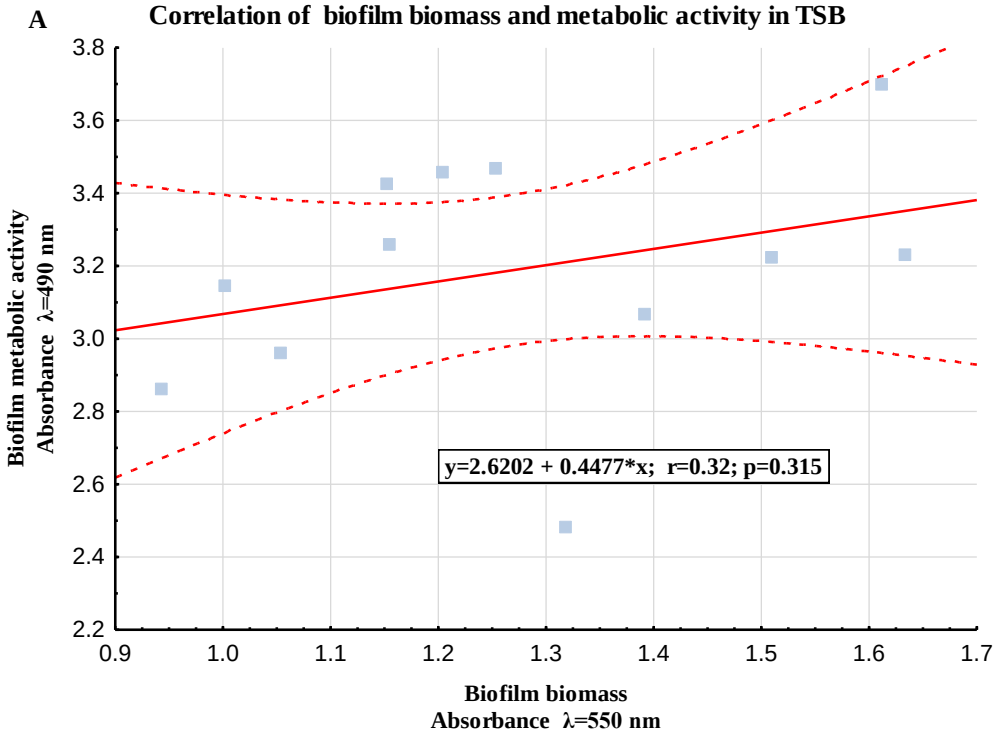


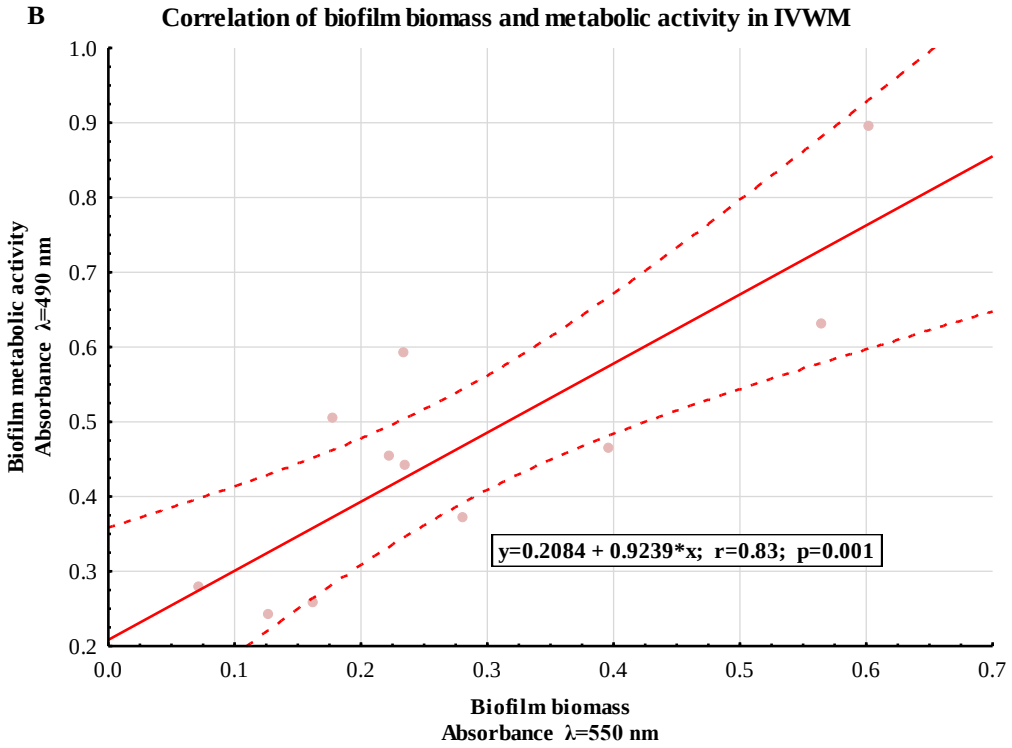
Supplementary Figure 7. Detailed analysis of differences in all (as a group) staphylococcal strains' ability to form biofilm biomass or their metabolic activity between Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Normal probability plots for biofilm biomass in TSB (**A**) or IVWM (**B**) medium. Box and whisker plot of the absorbance values of biofilm biomass (**C**). Normal probability plots for biofilm metabolic activity in TSB (**D**) or IVWM (**E**) medium. Box and whisker plot of the absorbance values of biofilm metabolic activity (**F**). 25%-75%- interquartile range, Min-Max- a range between minimal and maximum values. Red curves indicate the fitting of the values to the normal distribution. The statistically significant differences are marked with pairs of letters a/b. P-

probability level calculated with Shapiro-Wilk test. Normal distribution was considered for values of p>0.05.

Supplementary Table 5. Parameters of the tested statistics of the differences in all (as a group) staphylococcal strains' ability to form biofilm biomass or their metabolic activity between Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Non-parametric Mann-Whitney U test was performed because the distributions were non-normal strain (Shapiro-Wilk test, p<0.05). P- probability level (values of p<0.05 were considered significant), U and Z- values of U-test.

Analysis of all strains as a group					
Type of compared biofilm characteristic	**Rank sum TSB**	**Rank sum IVWM**	**U**	**Z**	**p**
Biofilm biomass	28802	9702	249	14	0.000000
Biofilm metabolic activity	30600	9870	0	15	0.000000

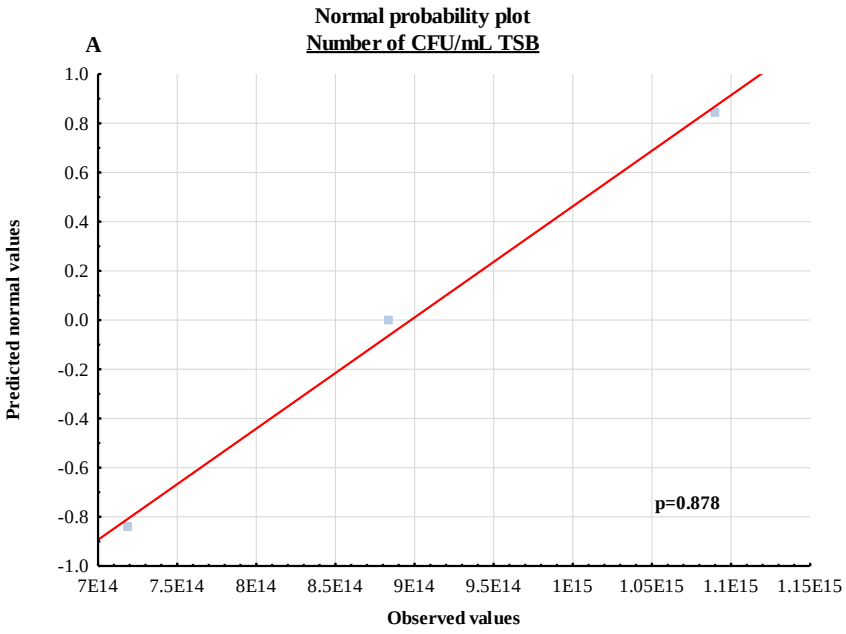


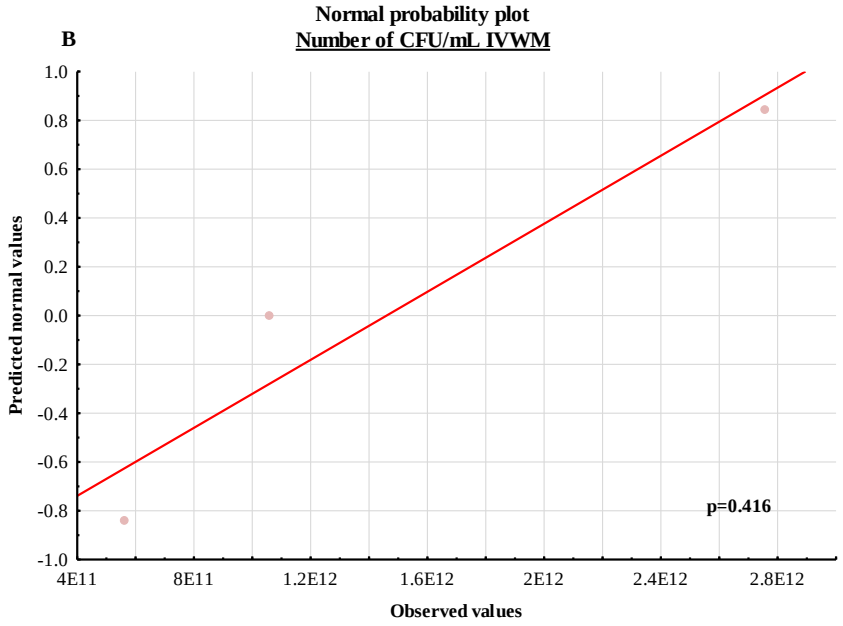


Supplementary Figure 8. Scatter plots of correlations of average staphylococcal strains' ability to form biofilm biomass and average metabolic activity for strains cultured in Tryptic Soy Broth (TSB, **A**) or In Vitro Wound Milieu (IVWM, **B**) medium. R- correlation coefficient, p- probability level (values of $p<0.05$ were considered significant). Red lines indicate 95% confidence intervals.

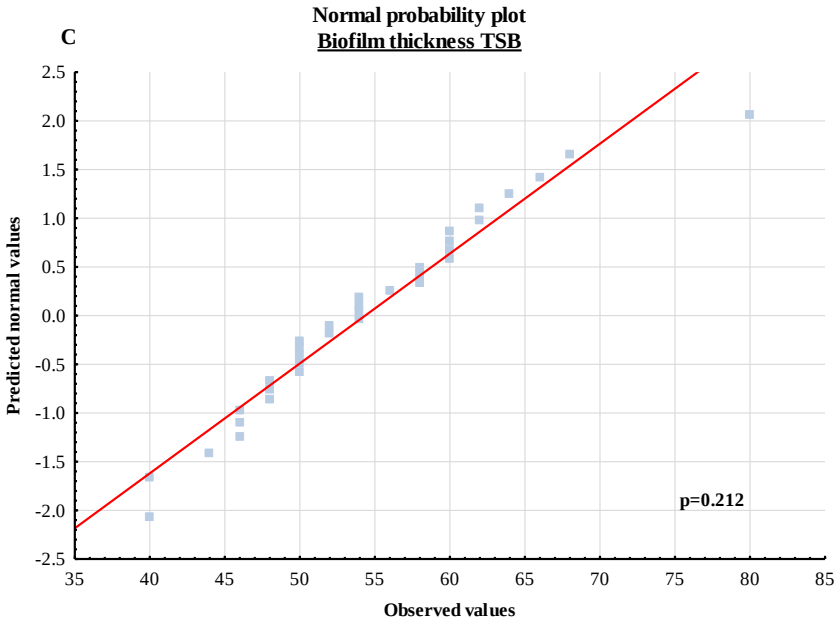
Supplementary Table 6. Results of the correlation analysis of average staphylococcal strains' ability to form biofilm biomass and average metabolic activity for strains cultured in Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Avr- average, SD- standard deviation, r- correlation coefficient, t- values of statistics, p- probability level (values of $p<0.05$ were considered significant).

	Correlation of biofilm biomass and metabolic activity						
	Biofilm biomass		**Metabolic activity**				
Medium	**Avr**	**SD**	**Avr**	**SD**	**r**	**t**	**p**
TSB	1.3	0.2	3.2	0.3	0.32	1.1	0.314698
IVWM	0.3	0.2	0.5	0.2	0.83	4.5	0.001463

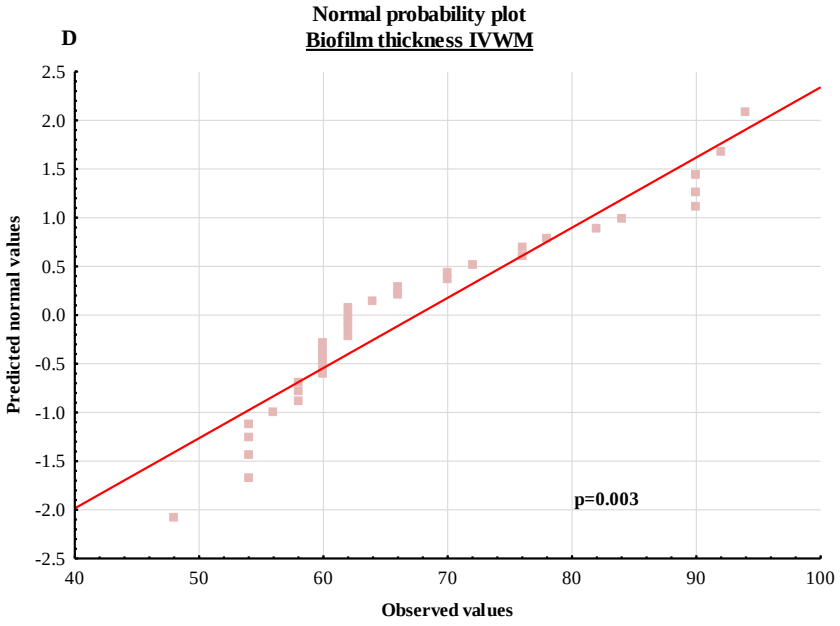

A
Normal probability plot
Number of CFU/mL TSB
Predicted normal values
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
p=0.878
7E14
7.5E14
8E14
8.5E14
9E14
9.5E14
1E15
1.05E15
1.1E15
1.15E15
Observed values

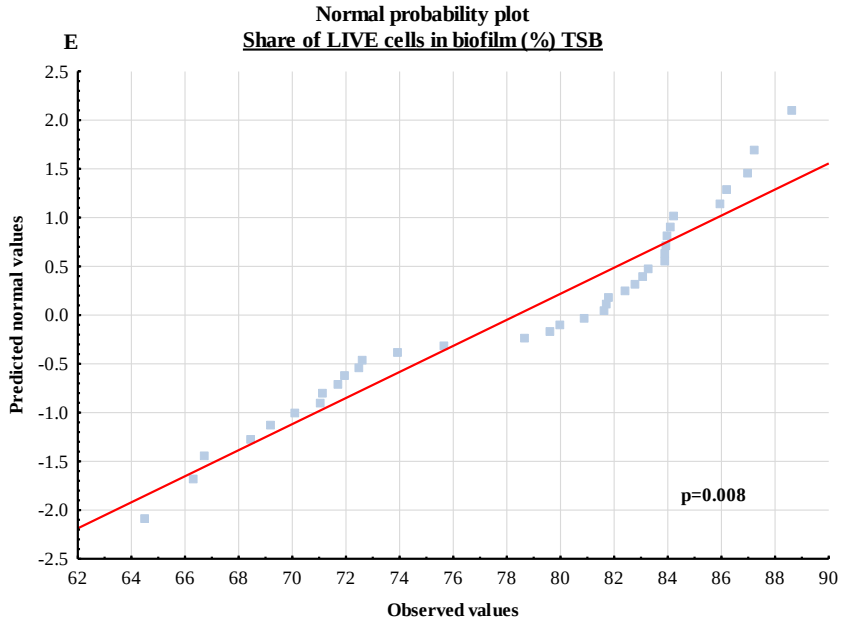

B
Normal probability plot
Number of CFU/mL IVWM
Predicted normal values
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
p=0.416
4E11
8E11
1.2E12
1.6E12
2E12
2.4E12
2.8E12
Observed values

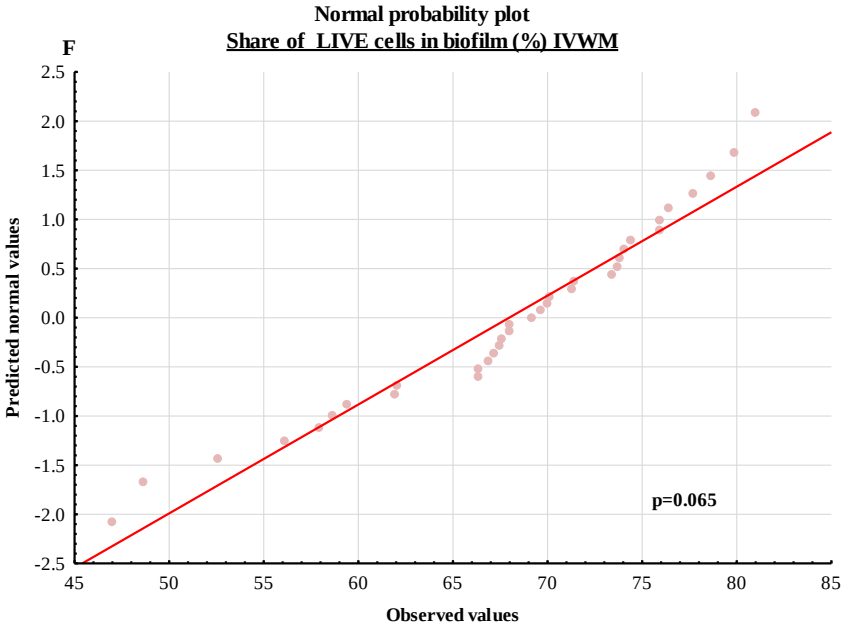
C

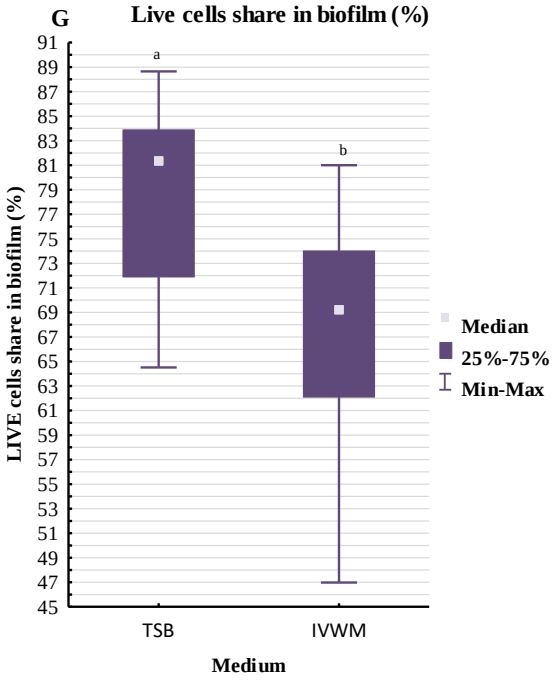

Normal probability plot
Biofilm thickness TSB
Predicted normal values
Observed values
p=0.212

D


Normal probability plot
Biofilm thickness IVWM
Predicted normal values
Observed values
p=0.003


Normal probability plot
E
Share of LIVE cells in biofilm (%) TSB
Predicted normal values
Observed values
p=0.008


Normal probability plot
F
Share of LIVE cells in biofilm (%) IVWM
Predicted normal values
Observed values
p=0.065



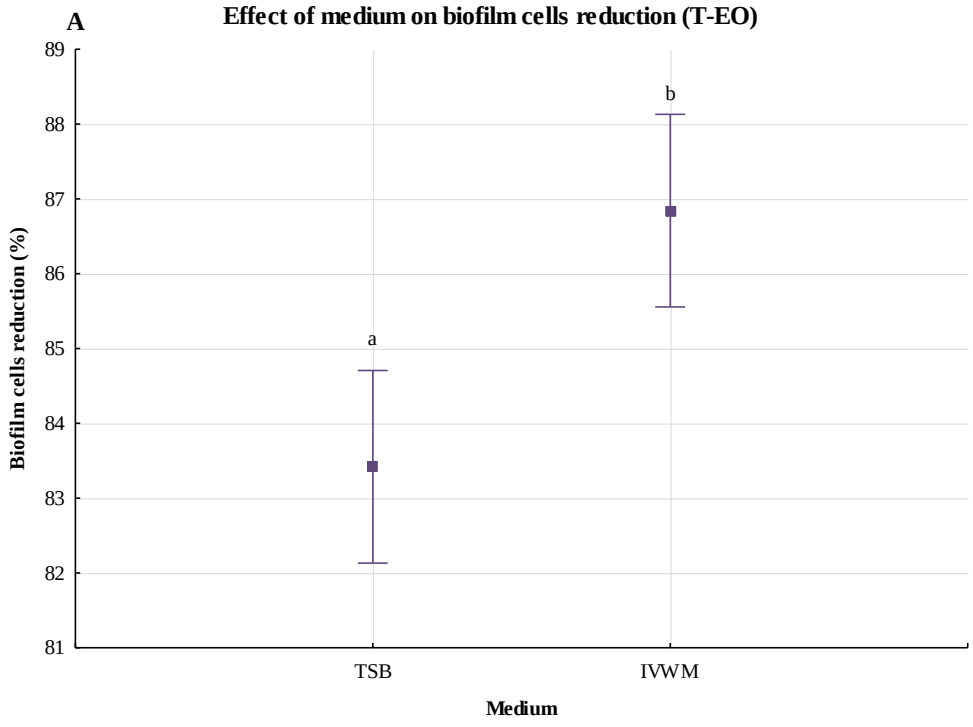
Supplementary Figure 9. Detailed analysis of the differences in the number of CFU/mL (Colony-Forming Units), biofilm thickness, and percentage share of LIVE cells between staphylococcal biofilms cultured in Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Normal probability plots for the number of CFU/mL in TSB (**A**) or IVWM (**B**) medium. Normal probability plots for biofilm thickness in TSB (**C**) or IVWM (**D**) medium. Normal probability plots for a share of LIVE cells in biofilm in TSB (**E**) or IVWM (**F**) medium. Box and whisker plot of share of LIVE cells in biofilm (**G**). 25%-75%- interquartile range, Min-Max- a range between minimal and maximum values. Red curves indicate the fitting of the values to the normal distribution. The statistically significant difference is marked with a pair of letters a/b. P- probability level calculated with Shapiro-Wilk test. Normal distribution was considered for values of p>0.05.

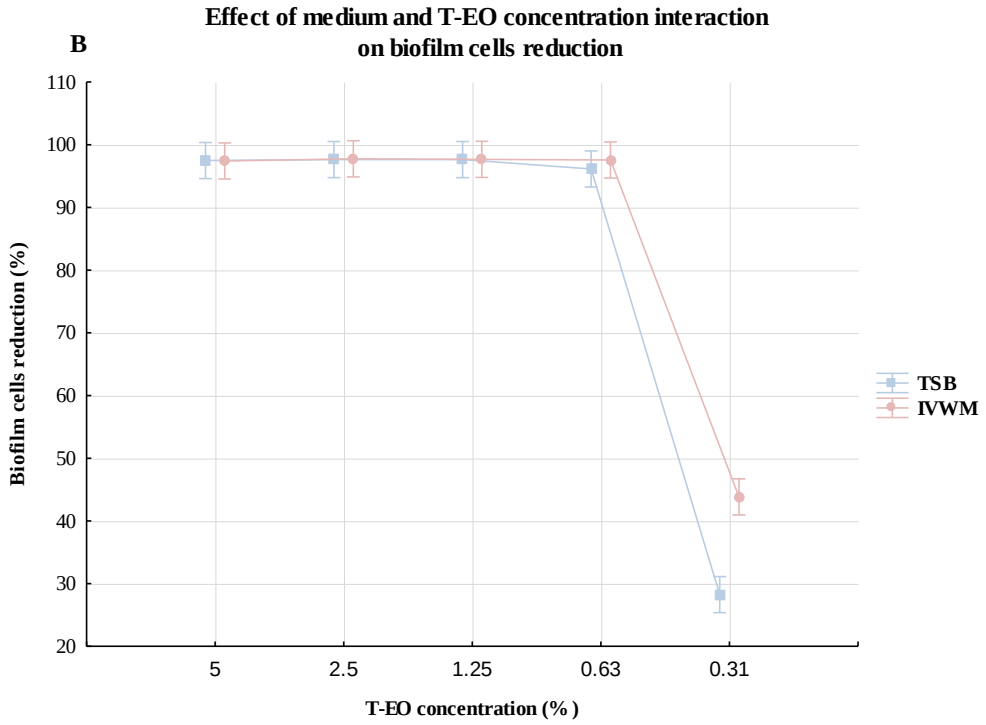
Supplementary Table 7. Parameters of the tested statistics of the differences in the number of CFU/mL (Colony-Forming Units), biofilm thickness, and percentage share of LIVE cells between staphylococcal strains cultured in Tryptic Soy Broth (TSB) or In Vitro Wound Milieu (IVWM) medium. Normal distribution of the values and homogeneity of variances were determined in the number of CFU/mL (Shapiro-Wilk test, Levene's test, respectively p<0.05), therefore t-test was performed. Mann-Whitney U test was performed for biofilm thickness and share of LIVE cells because the distributions were non-normal. T- values of t-test, df- degrees of freedom, p- probability level (values of p<0.05 were considered significant), U and Z- values of U-test.

Analysis of other biofilm characteristics					
Type of compared characteristic	**Average TSB**	**Average IVWM**	**t**	**df**	**p**
Number of CFU/mL	9.0E+14	1.5E+12	8.4	4	0.001124
	Rank sum TSB	**Rank sum IVWM**	**U**	**Z**	**p**
Biofilm thickness	813	1602	218	-5	0.000006
Share of LIVE cells	1702	854	224	5	0.000003

Supplementary Table 8. Results of the multivariate analysis of variance for biofilm cells reduction after T-EO treatment. F- values of the test, p- probability level (values of $p<0.05$ were considered significant).

Effect	f	p
Medium	13.7	0.000239
Strain	1.9	0.036016
T-EO concentration (%)	701.9	0.000000
Medium and Strain	4.0	0.000013
Medium and T-EO concentration (%)	10.9	0.000000
Strain and T-EO concentration (%)	1.8	0.002131
Medium and Strain and T-EO concentration (%)	3.7	0.000000

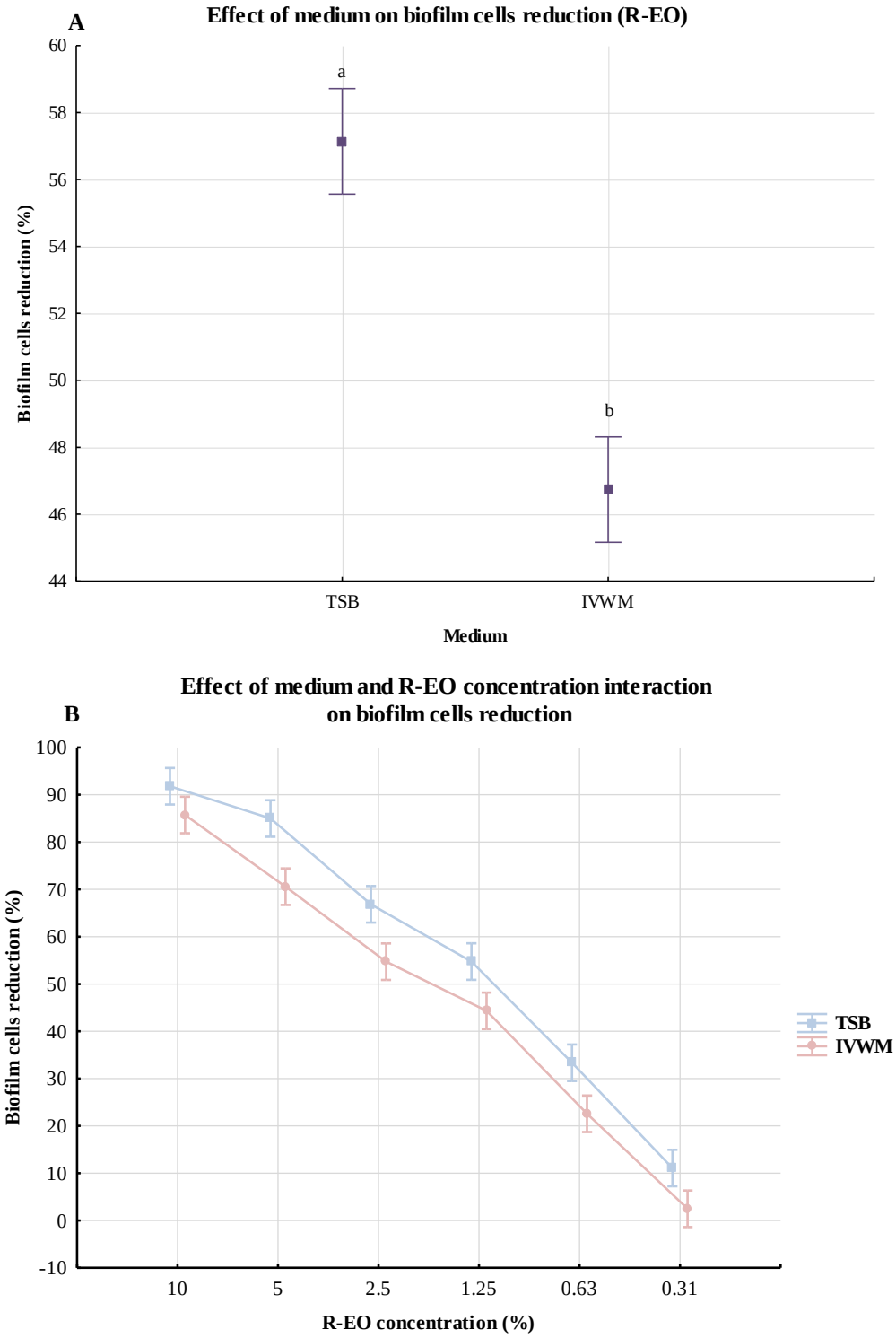




Supplementary Figure 10. Effect of medium (**A**) or medium and EO concentration (%) (v/v) interaction (**B**) on biofilm cells reduction after treatment with T-EO (thyme oil). Results of the multivariate analysis of variance. The statistically significant difference is marked with pairs of letters a/b. Tags denote means, and vertical bars denote 0.95 confidence intervals. Tags are dislocated on the X-axis values to gain higher visibility. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu.

Supplementary Table 9. Results of the multivariate analysis of variance for biofilm cells reduction after R-EO treatment. F- values of the test, p- probability level (values of $p<0.05$ were considered significant).

Effect	f	p
Medium	83.9	0.000000
Strain	30.0	0.000000
R-EO concentration (%)	488.2	0.000000
Medium and Strain	17.1	0.000000
Medium and R-EO concentration (%)	1.1	0.379590
Strain and R-EO concentration (%)	3.1	0.000000
Medium and Strain and R-EO concentration (%)	2.6	0.000000



Supplementary Figure 11. Effect of medium (**A**) or medium and EO concentration (%) (v/v) interaction (**B**) on biofilm cells reduction after treatment with R-EO (rosemary oil). Results of the multivariate analysis of variance. Tags denote means, and vertical bars denote 0.95 confidence intervals. The statistically significant difference is marked with pairs of letters a/b. Tags are dislocated on the X-axis values to gain higher visibility. TSB- Tryptic Soy Broth, IVWM- In Vitro Wound Milieu.

OŚWIADCZENIA AUTORÓW

Szczecin, 13.06.23

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miejsce zatrudnienia

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Oświadczam, że w pracy

Malwina Brożyna, Anna Żywicka, Karol Fijałkowski, Damian Gorczyca, Monika Oleksy-Wawrzyniak, Karolina Dydak, Paweł Migdał, Bartłomiej Dudek, Marzenna Bartoszewicz, Adam Junka, 2020, „The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol)", Applied Sciences-Basel, 10, art. 7343

(autorzy, rok wydania, tytuł, czasopismo lub wydawca, tom, strony)

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(autorzy, rok wydania, tytuł, czasopismo lub wydawca, tom, strony)

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- Malwina Brożyna, Justyna Paleczny, Weronika Kozłowska, Daria Ciecholewska-Juśko, Adam Parfieńczyk, Grzegorz Chodaczek, Adam Junka, 2022, „Chemical composition and antibacterial activity of liquid and volatile phase of essential oils against planktonic and biofilm-forming cells of *Pseudomonas aeruginosa*", Molecules, 27, art. 4096

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- Malwina Brożyna, Justyna Paleczny, Weronika Kozłowska, Daria Ciecholewska-Juśko, Adam Parfieńczyk, Grzegorz Chodaczek, Adam Junka, 2022, „Chemical composition and antibacterial activity of liquid and volatile phase of essential oils against planktonic and biofilm-forming cells of *Pseudomonas aeruginosa*", Molecules, 27, art. 4096

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- Malwina Brożyna, Weronika Kozłowska, Katarzyna Malec, Justyna Paleczny, Jerzy Detyna, Krystyna Fabianowska-Majewska, Adam Junka, „Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity", przedstawionej jako preprint

  (autorzy, rok wydania, tytuł, czasopismo lub wydawca, tom, strony)

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Malwina Brożyna, Weronika Kozłowska, Katarzyna Malec, Justyna Paleczny, Jerzy Detyna, Krystyna Fabianowska-Majewska, Adam Junka, „Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity", przedstawionej jako preprint

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- Malwina Brożyna, Anna Żywicka, Karol Fijałkowski, Damian Gorczyca, Monika Oleksy-Wawrzyniak, Karolina Dydak, Paweł Migdał, Bartłomiej Dudek, Marzenna Bartoszewicz, Adam Junka, 2020, „The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol)", Applied Sciences-Basel, 10, art. 7343

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mój udział polegał na współprzygotowaniu koncepcji badań, współprzygotowaniu oraz walidacji metodologii, przeprowadzeniu części eksperymentów kontrolnych, pozyskaniu finansowania, nadzorowaniu projektu oraz współudziale w przygotowaniu i recenzji szkicu manuskryptu.

- Malwina Brożyna, Justyna Paleczny, Weronika Kozłowska, Grzegorz Chodaczek, Ruth Dudek-Wicher, Anna Felińczak, Joanna Gołębiewska, Agata Górniak, Adam Junka, 2021, „The antimicrobial and antibiofilm in vitro activity of liquid and vapour phases of selected essential oils against *Staphylococcus aureus*", Pathogens, 10, art. 1207

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- Malwina Brożyna, Justyna Paleczny, Weronika Kozłowska, Daria Ciecholewska-Juśko, Adam Parfieńczyk, Grzegorz Chodaczek, Adam Junka, 2022, „Chemical composition and antibacterial activity of liquid and volatile phase of essential oils against planktonic and biofilm-forming cells of *Pseudomonas aeruginosa*", Molecules, 27, art. 4096

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- Malwina Brożyna, Weronika Kozłowska, Katarzyna Malec, Justyna Paleczny, Jerzy Detyna, Krystyna Fabianowska-Majewska, Adam Junka, „Chronic Wound Milieu Challenges Essential Oils' Antibiofilm Activity", przedstawionej jako preprint

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