In vitro anti-clostridial action and potential of the spice herbs essential oils to prevent biofilm formation of hypervirulent Clostridioides difficile strains isolated from hospitalized patients with CDI

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Original Article

In vitro anti-clostridial action and potential of the spice herbs essential oils to prevent biofilm formation of hypervirulent *Clostridioides difficile* strains isolated from hospitalized patients with CDI

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ABSTRACT

Background: *Clostridioides difficile* is the most common causative agent of antibiotic-acquired diarrhea in hospitalized patients associated with substantial morbidity and mortality. The global epidemic of CDI (*Clostridioides difficile* infection) began in the early 20th century with the emergence of the hypervirulent and resistant ribotype 027 strains, and requires an urgent search for new therapeutic agents.
Objective: The aim of this study is to investigate the antibacterial activity of the three essential oils isolated from spice herbs (wild oregano, garlic and black pepper) against *C. difficile* clinical isolates belonging to 6 different PCR ribotypes and their potential inhibitory effect on the biofilm production in *in vitro* conditions.
Results: Wild oregano essential oil showed strong inhibitory activity in concentrations 0.02−1.25 mg/mL and bactericidal activity in concentrations from 0.08 to 10 mg/mL. Garlic essential oil was effective in the concentration range of 0.02−40 mg/mL, and 0.16 - > 40 mg/mL. MIC and MBC for black pepper oil ranged from 0.04 to 40 mg/mL, and 0.08 - > 40 mg/mL, respectively. All the tested oils reduced *in vitro* biofilm production, with the best activity of oregano oil.
Conclusion: Essential oils of wild oregano, black pepper and garlic are candidates for adjunctive therapeutics in the treatment of CDI. Oregano oil should certainly be preferred due to the lack of selectivity of action in relation to the ribotype, the strength of the produced biofilm and/or antibiotic-susceptibility patterns.

1. Introduction

Clostridioides difficile (*C. difficile*) is a spore-forming, Gram-positive, anaerobic bacillus responsible for the most common antibiotic-associated nosocomial infections [1], related with substantial morbidity and mortality [2]. The main risk factors for colonization and subsequent development of infections with toxigenic *C. difficile* strains are considered to be hospitalization, age (>65 years) and prolonged treatment with wide-spectrum antibiotics. Increased prevalence and higher mortality rates are associated with an emergence of hypervirulent strains (NAP1/BI/027), recognized as responsible for severe forms of *Clostridioides difficile* infection (CDI), characterized by a high rate of recurrence and resistance to conventional therapy [2,3]. CDI mainly occurs after antimicrobial therapy applied for other infections. A rise of multidrug-resistant (MDR) clinical isolates and their reduced susceptibility to the most commonly used antibiotics have made the treatment of CDI more complicated, allowing the persistence of *C. difficile* in the intestinal environment [4]. Metronidazole, fidaxomicin and vancomycin are three recommended drugs for the treatment of *C. difficile* infection (CDI) [5]. Treatment failure rates have been reported when metronidazole is used for CDI therapy, most likely in association with increased MIC values, e.g due to the presence of plasmid mediated resistance or changes in the haem

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responsive genes [6–8]. Higher MIC values both to metronidazole and vancomycin have been found for hypervirulent PCR ribotype (RT) 027 strains in Israel, known as cause of outbreaks and severe infection [9]. The metronidazole resistance in *C. difficile* is a multifactorial mechanism and one additional mechanism could be protective barriers provided by biofilm that partially explain the CDI treatment failures caused by strains with an *in vitro* susceptible phenotype [10].

Several works reported ability of *C. difficile* to form a monospecies *in vivo* biofilm [11] or biofilm associated with the host gut mucosa microbial community [12,13]. In this form, due to the compact nature and the surrounding extracellular matrix of biofilms, permeation of antimicrobials into the biofilm could be reduced, which affect their efficacy. Mixed biofilms formation in the gut can act as a reservoir for *C. difficile* with the potential to cause recurrent disease [14]. Biofilm related infections are hard to treat due to 10- to 1000-fold higher antibiotic resistance in comparison to planktonic bacteria. *C. difficile* strains belonging to the PCR ribotype (RT) 027 showed that biofilm-growing cells are more resistant to high concentrations of vancomycin than planktonic cells [15] and also could tolerate metronidazole concentrations 100 times higher (100 μg/mL) compared with liquid cultures of the same strain, which were inhibited by antibiotic concentrations above 1 μg/mL [16].

Prevention of resistance spreading and ensuring an effective therapy against CDI are limited when conventional antibiotics are in question. Therefore, the development of alternative therapeutic approaches, including traditional plant-based treatments might be helpful in control of CDI. The antibacterial activity based on essential oils and their derivatives has been recognized for a long time. In comparison with antibiotics, herbal extracts and essential oils contain different antibacterial compounds that could employ a number of inhibitory mechanisms, making it difficult for pathogens to develop resistance [17]. A number of medicinal and spice plants or their components could potentially find use in prevention and combating CDI as an adjunctive therapeutic agent. Essential oils (EOs) can interfere with the mechanisms of biofilm formation, thus disrupting and controlling this process by inhibiting the EPS matrix, suppressing of the cell adhesion (by inhibiting flagella gene transcription or disrupting bacterial motility), or by blocking the quorum sensing system [18,19]. Comparison of the antibiofilm activity of essential oils with the activity of its main components demonstrated higher efficacy of the essential oils, which suggests that minor compounds may have a synergistic effect with major bioactive compounds [19].

Previous *in vitro* studies have shown efficacy of medium-chain fatty acids derived from virgin coconut oil for the growth inhibition of *C. difficile* [20]. Pomegranate (*Punica granatum* L.) extract inhibited toxigenic *C. difficile* strains in minimum inhibitory concentrations (MICs) from 12.5 to 25 mg/mL [21]. Aljarallah [22] suggests that *Nigella sativa* seeds water extract and *Commiphora myrrha* (Myrrh) oil might be considered as potential alternative and adjuvant therapy options for the treatment of human CDI. These natural preparations inhibited *C. difficile* growth in similar active concentrations (2%) [22]. Antimicrobial activity of purified hop (*Humulus lupulus* L.) constituents humulone, lupulone and xanthohumol against toxigenic and clinically relevant gut anaerobic bacteria *B. fragilis*, *C. perfringens* and *C. difficile* was demonstrated, and the strongest activity was observed for xanthohumol (15–107 μg/mL), which is close to the active concentrations of conventional antibiotics in the strains with increased antibiotic resistance [23]. Efficacy of numerous natural raw and processed products against *C. difficile* was demonstrated in the study of Roshan et al. [24], with the highest activity of garlic juice, peppermint oil and the four pure plant-derived compounds *trans*-cinnamaldehyde, allicin, menthol and zingerone.

Essential oils as potential agents for control of CDI and *C. difficile* biofilms have been poorly studied. In previous work of Justin & Antony, antibacterial activity of the clove, nutmeg and ginger essential oils against 40 isolates of *C. difficile* (20 toxigenic and 20 non toxigenic) were investigated *in vitro*, and the results signaled for the first time essential oils as a new therapeutic option for *C. difficile* [25]. Antibacterial and antibiofilm activity of plant-derived compounds, e.g. asiatic acid [26], Manuka honey [27,28] and berberine chloride [29] were investigated in the recently published studies. Asiatic acid displayed an inhibitory effect on 19 *C. difficile* isolates collected from different sources, but did not interfere with biofilm formation [26]. Manuka honey could inhibit biofilm formation *in vitro* in clinically significant strains of *C. difficile*, especially those belonging to PCR RT (ribotype) 027 [27,28]. Berberine chloride decreased the MIC of vancomycin against the planktonic *C. difficile* growth, but decrease in biofilm formation has been observed for only one strain treated with a sub-inhibitory concentration of berberine chloride-vancomycin. In contrast to this, some *C. difficile* strains demonstrated promoted biofilm formation when sub-MIC of berberine chloride in combination with vancomycin was applied [29].

Oregano, garlic and black pepper are spice herbs known worldwide by their culinary use and many biological activities including antimicrobial action [30,31]. Among many other, these plants are classified as Generally Recognized as Safe (GRAS) by FDA standards [32].

Oregano contains carvacrol and thymol as the major compounds, known as strong antimicrobials capable to interact with cell membrane, owing to their lypophilic and amphipatic properties [33–36] and significantly inhibit the formation of biofilm *in vitro* [37] most likely through inducing QS (Quorum sensing) disorders in bacteria [38]. Garlic also represents very important component of folk medicinal prescriptions. Among the antimicrobial organosulfur compounds allicin is the most representative one, found to possess antimicrobial, antiviral, antioxidant and anticancer effects [39–41]. Other compounds contained in garlic EO [40,42,43] exhibits significant antimicrobial action as pure compounds [44]. These organosulfur molecules react with bacterial enzymes, inhibit DNA, RNA and proteins synthesis and also compromise the integrity of the bacterial membrane and down regulate QS and biofilm-associated genes [41,45]. Various extracts and essential oil of black pepper and its active compounds exhibits a wide spectrum of antimicrobial and antibiofilm actions [46–51] in the similar principle as described above for garlic and oregano EOs [52–55].

Since biofilms are difficult to control due to the enhanced resistance of the bacteria within, preventive inhibition of their formation seems a promising approach. This can be achieved by inhibiting the initial colonization step of the biofilm lifecycle [14].

To our knowledge, to date there is no research on the effect of essential oils on the biofilm forming ability in clinically significant *C. difficile* strains. In this study, we investigated the antibacterial activity of the three spice herbs essential oils (oregano, garlic and black pepper) against the *C. difficile* strains isolated from hospitalized patients with confirmed CDI and their potential inhibitory effect on the biofilm production in *in vitro* conditions. The existence of a relationship between the efficacy of essential oils and the characteristics of isolates, such as the strength of the biofilm produced, the ribotype or the antibiotic sensitivity patterns was also considered.

2. Material and methods

2.1. Bacterial strains

A total of 42 toxigenic clinical isolates (designated as CD_1 – CD_{42}) of *C. difficile* were isolated from stool specimens of hospitalized patients with diarrhea and CDI. Reference strains, *C. difficile* ATCC 9689 ($A^+B^+CDT^-$), *C. difficile* ATCC 43593 ($A^-B^-CDT^-$) and *C. difficile* ATCC-BAA 1870 ($A^+B^+CDT^+$) were used as control strains.

All strains were analyzed with multiplex PCR to determine genes encoding *C. difficile* toxins (*tcdA*, *tcdB*, *cdtA* and *cdtB*), 16S rDNA genes and *GluD* genes (GDH, glutamate dehydrogenase), according to the previously described methodology [56]. Ribotyping of selected toxigenic isolates was performed by capillary gel-based electrophoresis (CE-ribotyping) according to methodology of Fawley et al. [57]. Molecular analyses were done at the National Reference Laboratory for *Clostridioides difficile* (Leiden University Medical Centre, Leiden, Netherlands).

2.2. Essential oils

Wild oregano oil (*Origanum minutiflorum* O. Schwarz & P. H. Davis) (Probotanic, Serbia), garlic oil (*Allium sativum* L.) (Zdrava priča, Serbia) and black pepper oil (*Piper nigrum* L.) (AlekPharm, Serbia) were purchased at a local health food market.

2.3. Antibiotic susceptibility testing

The susceptibilities of the 42 toxigenic *C. difficile* clinical isolates and the three reference strains to six antibiotics were determined by the E-test gradient strips, according to manufacturer's instructions, for metronidazole (0.016–256 μg/mL), vancomycin (0.016–256 μg/mL), tigecycline (0.016–256 μg/mL), fusidic acid (0.016–256 μg/mL), rifampicin (0.002–32 μg/mL) and moxifloxacin (0.002–32 μg/mL) (LIOFILCHEM®, Italy). Applied breakpoints were defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [58].

2.4. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils for C. difficile strains

The minimum inhibitory concentrations (MICs) of essential oils were determined using the broth microdilution method in a 96-well plate according to described methodology [29], with slight modifications. Suspensions were made from the 24 h old *C. difficile* culture on Columbia 5% Blood Agar and adjusted to 3 McFarland turbidity by using a densitometer (DEN-1, BioSan). An initial stock solution was prepared by dissolving essential oils in 50% dimethyl sulfoxide (DMSO) in concentration of 200 mg/mL. Serial dilutions were made in Brain Heart Infusion (BHI) broth (HiMedia, Mumbai, India), with starting concentration of 40 mg/mL, which was serially diluted (dilution factor 2) to the final concentration of 0.02 mg/mL. Then, BHI broth (180 μL) containing the decreased concentrations of essential oils was inoculated with suspension of the tested *C. difficile* strains (20 μL, 3 McFarland turbidity, 10-fold dilution of the 3 McFarland in each well) and incubated anaerobically at 37 °C for 48 h. Inoculated BHI broth without essential oil was used as a positive control, while the wells containing sterile BHI broth were used as negative control. The DMSO was tested previously and showed no effect toward the tested strains in used concentrations (10% DMSO and lower concentrations). After incubation, the MICs were determined visually as the lowest concentrations without visible growth in the medium. The minimum bactericidal concentration (MBC) was determined by transferring the suspension from the wells with no visible growth (containing essential oils in MIC and higher concentrations) to 5% Columbia agar plates. Plates inoculated in this way were incubated at 37 °C for 48 h under anaerobic conditions, and after this period the plates with the lowest concentration of oil with no grown colonies were determined as MBCs.

2.5. Crystal-violet biofilm assays

2.5.1. Biofilm formation

The ability of toxigenic isolates *C. dificile* to produce *in vitro* biofilm was tested with crystal violet (CV) biofilm assay, as previously described [28]. 180 μL of the BHI broth was pipetted into each well of a 96-well flat-bottomed microplate (ThermoBioLite, USA). The strains were incubated in a BHI medium supplemented with 0.5% yeast extract (Torlak, Serbia) and 1% glucose (Centrohem, Serbia) for 24 h at 37 °C, and 20 μL was used as inoculum. Plates were incubated at 37 °C for 72 h under anaerobic conditions for biofilm formation. Wells with BHI broth without the inoculum were used as the controls. After 72 h, the supernatant was discarded and the wells were washed twice with 1 x PBS (phosphate buffer saline, pH 7.4) and air-dried at 37 °C for 15 min. Wells were then stained with 1% crystal violet (Centrohem, Serbia) for 15 min. Following the staining procedure, the well content was removed and the wells were washed eight times with 0.85% NaCl. CV stain from the attached cells within the formed biofilm was extracted with 96% ethanol. After incubation time of 30 min, ethanol was transferred to new microtiter plates and the absorbance (OD) was measured at 595 nm using a MultiscanFC Microplate Photometer (ThermoFisher, USA). Experiment was performed in triplicate and the average values were calculated. For classification of strains as positive biofilm producers (strong, moderate or weak) or negative biofilm producers [58], the adherence index (AI) [59] was calculated based on our findings, according to the following formula:

$$\text{ODcontrol} = \text{ODcontrol} + 3\ \text{STDVcontrol}$$
$$\text{ODstrain} = \text{ODstrain (average)} - \text{ODcontrol}$$

and the isolates were grouped as: strong ($AI > 0.80$), moderate ($0.40 < AI \leq 0.80$), weak ($0.20 < AI \leq 0.40$) and negative ($AI < 0.2$).

2.5.2. Effect of (sub)inhibitory concentrations of essential oils on biofilm production

Testing of the potential anti-biofilm producing activity of the essential oils was performed similarly as for testing of the biofilm-producing ability. Biofilm-positive strains were incubated in a BHI medium containing three different inhibitory concentrations of each essential oil (0.5 x MIC, MIC and 2 x MIC). The experiment was performed in triplicate and the inhibition was observed as a decreased absorbance at 595 nm compared to positive controls (wells without essential oils). Differences in the biofilm formation were calculated using two-way ANOVA and Tukey post-hoc analysis (GraphPad PRISM v.6.0.) and considered as statistically significant for p values < 0.05.

3. Results

3.1. Characteristics of the investigated bacterial strains

Based on molecular analysis results (multiplex PCR and CE-ribotyping), among the 42 tested isolates, six different ribotypes were detected and presence of the *GluD* and genes for *C. difficile* toxins was confirmed. Toxin A and toxin B genes were detected in

all strains, while binary toxin encoding genes were present only in strains belonging to RT 027. The most prevalent ribotypes were RT 001 (20/42; 48%) and RT 027 (18/42; 43%), while RTs 012, 015, 020 and 205 were represented by only one isolate (2.4%). Characteristics of the tested strains are given in Table 1.

The MIC values of antibiotics commonly used for the treatment of CDI were determined with E-test strips and calculated MIC_{50} and MIC_{90} values for individual ribotypes are presented in Table 2. The MIC breakpoints for metronidazole (2 μg/mL), vancomycin (2 μg/mL), moxifloxacin (4 μg/mL), fusidic acid (2 μg/mL), tigecycline (0.25 μg/mL) and rifampicin (0.004 μg/mL) are based on the epidemiological cut-off values (ECOFF) defined by EUCAST [57]. All the tested isolates were sensitive to metronidazole, vancomycin and tigecycline with MIC_{50} values of 0.25, 0.38, and 0.016 μg/mL, respectively (Table 2). From 42 strains tested, 36 showed resistance to moxifloxacin, with the same MIC_{50} and MIC_{90} of 32 μg/mL, and average MIC of 24.49 μg/mL. In addition to moxifloxacin resistance, two isolates (CD4 and CD13) were resistant to rifampicin (with MICs 4 and 32 μg/mL, respectively) (data not presented).

3.2. Essential oils antimicrobial susceptibility assay

Minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs) of the three essential oils were determined by broth microdilution method and the results are presented in Table 1. Oregano essential oil showed strong inhibitory activity in concentrations from 0.02 to 1.25 mg/mL, and bactericidal activity in concentrations from 0.08 to 10 mg/mL. Garlic essential oil was effective in concentration range of 0.02−40 mg/mL, and 0.16 - > 40 mg/mL. MIC and MBC for black pepper oil ranged from 0.04 to 40 mg/mL, and 0.08 - > 40 mg/mL, respectively.

3.3. Biofilm production assay

The ability of different *C. difficile* strains to produce *in vitro* biofilm was tested by crystal violet biofilm assay [28] and the results were interpreted on the basis of calculated adherence index (AI) [58,59]. Among the tested isolates, 82.2% (37/45) were biofilm producers, where 29.73% were classified to be strong, 13.51% as

Table 1
Characteristics of the tested *C. difficile* isolates.

Strain	PCR-RT	Toxins	BFC	EOs MIC/MBC		
				Oregano	Black pepper	Garlic
[1]CD+	001	$A^+ B^+ CDT^-$	STRONG	0.04/0.32	10.00/40.00	2.50/10.00
[2]CD-	060	$A^+ B^+ CDT^-$	NEGATIVE	0.04/0.16	0.63/0.63	1.25/1.25
[3]CD027	027	$A^+ B^+ CDT^+$	NEGATIVE	0.08/0.08	0.63/0.63	0.32/1.25
CD1	027	$A^+ B^+ CDT^+$	STRONG	1.25/1.25	40.00/>40.00	1.25/>40.00
CD2	027	$A^+ B^+ CDT^+$	STRONG	0.63/0.63	40.00/40.00	40.00/>40.00
CD3	027	$A^+ B^+ CDT^+$	STRONG	0.32/0.63	40.00/40.00	40.00/>40.00
CD4	027	$A^+ B^+ CDT^+$	WEAK	0.63/0.63	20.00/40.00	40.00/40.00
CD5	001	$A^+ B^+ CDT^-$	MODERATE	0.16/5.00	10.00/20.00	2.50/5.00
CD6	027	$A^+ B^+ CDT^+$	STRONG	0.08/5.00	20.00/>40.00	5.00/10.00
CD7	001	$A^+ B^+ CDT^-$	STRONG	0.63/5.00	40.00/>40.00	5.00/10.00
CD8	001	$A^+ B^+ CDT^-$	STRONG	0.08/5.00	10.00/20.00	0.60/10.00
CD9	001	$A^+ B^+ CDT^-$	STRONG	0.63/2.50	20.00/>40.00	40.00/>40.00
CD10	001	$A^+ B^+ CDT^-$	STRONG	0.63/2.50	20.00/>40.00	40.00/>40.00
CD11	001	$A^+ B^+ CDT^-$	STRONG	0.16/1.25	40.00/>40.00	40.00/>40.00
CD12	205	$A^+ B^+ CDT^-$	STRONG	0.08/1.25	5.00/20.00	0.60/5.00
CD13	001	$A^+ B^+ CDT^-$	MODERATE	0.63/10.00	10.00/10.00	2.50/5.00
CD14	001	$A^+ B^+ CDT^-$	WEAK	0.32/5.00	1.25/5.00	0.63/1.25
CD15	027	$A^+ B^+ CDT^+$	WEAK	0.32/2.50	2.50/10.00	2.50/5.00
CD16	012	$A^+ B^+ CDT^-$	NEGATIVE	0.08/0.63	5.00/10.00	0.32/0.63
CD17	001	$A^+ B^+ CDT^-$	NEGATIVE	0.08/5.00	5.00/10.00	0.63/1.25
CD18	027	$A^+ B^+ CDT^+$	NEGATIVE	0.08/2.50	1.25/2.50	1.25/2.50
CD19	001	$A^+ B^+ CDT^-$	NEGATIVE	0.08/5.00	1.25/5.00	0.63/1.25
CD20	027	$A^+ B^+ CDT^+$	WEAK	0.08/5.00	1.25/5.00	0.63/1.25
CD21	027	$A^+ B^+ CDT^+$	WEAK	0.04/0.08	0.08/0.32	0.08/0.32
CD22	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.32	2.50/5.00	0.63/1.25
CD23	001	$A^+ B^+ CDT^-$	WEAK	0.08/0.08	2.5/500	2.5/500
CD24	027	$A^+ B^+ CDT^+$	WEAK	0.08/0.16	0.04/0.16	0.16/0.32
CD25	001	$A^+ B^+ CDT^-$	NEGATIVE	0.16/0.16	0.32/0.63	0.63/1.25
CD26	001	$A^+ B^+ CDT^-$	NEGATIVE	0.63/1.25	0.63/0.63	0.32/0.32
CD27	027	$A^+ B^+ CDT^+$	MODERATE	0.16/0.16	0.32/0.32	0.32/0.63
CD28	001	$A^+ B^+ CDT^-$	WEAK	0.08/0.08	0.16/0.16	0.32/0.63
CD29	027	$A^+ B^+ CDT^+$	WEAK	0.32/0.32	0.16/1.25	0.32/0.32
CD30	027	$A^+ B^+ CDT^+$	WEAK	0.08/0.08	0.32/0.63	0.16/0.16
CD31	027	$A^+ B^+ CDT^+$	WEAK	0.02/0.63	0.63/0.63	0.02/0.16
CD32	027	$A^+ B^+ CDT^+$	MODERATE	0.16/0.16	0.08/0.08	0.32/0.32
CD33	015	$A^+ B^+ CDT^-$	WEAK	0.16/0.63	0.16/0.63	0.63/5.00
CD34	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.63	0.16/2.50	0.16/0.16
CD35	027	$A^+ B^+ CDT^+$	WEAK	0.63/1.25	0.63/1.25	0.63/1.25
CD36	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.32	0.16/0.32	0.32/0.63
CD37	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.32	1.25/2.50	0.63/1.25
CD38	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.32	0.32/0.63	0.63/1.25
CD39	001	$A^+ B^+ CDT^-$	WEAK	0.16/0.32	0.63/1.25	0.32/1.25
CD40	027	$A^+ B^+ CDT^+$	WEAK	0.63/0.63	0.63/1.25	0.63/2.50
CD41	027	$A^+ B^+ CDT^+$	WEAK	0.16/0.32	0.63/2.50	0.08/0.32
CD42	020	$A^+ B^+ CDT^-$	MODERATE	0.16/0.32	0.63/1.25	1.25/5.00

[1]*C. difficile* ATCC 9689; [2]*C. difficile* ATCC 43593; [3]*C. difficile* ATCC-BAA 1870.
EOs MICs/MBCs (Essential Oils Minimum Inhibitory/Bactericidal Concentrations) given in mg/mL; PCR-RT-Polymerase Chain Reaction-Ribotype; BFC- Biofilm Category.

Table 2
MICs of tested antibiotics on *Clostridium difficile* isolates presented as MIC_{50}, MIC_{90}, mean and geometric mean for all tested strains (n = 45) and separately for ribotypes.

	Antibiotic	MIC_{50}	MIC_{90}	Mean	Geometric mean
All strains	Metronidazole	0.25	0.50	0.28	0.24
	Vancomycin	0.38	0.85	0.43	0.36
	Moxifloxacin	32.00	32.00	24.49	15.88
	Fusidic acid	0.38	1.00	0.49	0.39
	Tigecycline	0.016	0.023	0.02	0.019
	Rifampicin	0.002	0.012	0.80	0.003
RT 027	Metronidazole	0.25	0.50	0.26	0.23
	Vancomycin	0.38	0.75	0.37	0.32
	Moxifloxacin	32.00	32.00	27.05	20.66
	Fusidic acid	0.25	1.00	0.38	0.32
	Tigecycline	0.016	0.023	0.019	0.018
	Rifampicin	0.002	0.003	0.21	0.003
RT 001	Metronidazole	0.25	0.70	0.31	0.26
	Vancomycin	0.38	0.95	0.49	0.39
	Moxifloxacin	32.00	32.00	26.15	19.08
	Fusidic acid	0.38	1.00	0.50	0.39
	Tigecycline	0.016	0.02	0.02	0.019
	Rifampicin	0.002	0.015	1.53	0.004
Other RTs	Metronidazole	0.19	0.50	0.23	0.19
	Vancomycin	0.25	1.00	0.43	0.36
	Moxifloxacin	2.00	32.00	7.75	2.70
	Fusidic acid	1.00	1.50	0.89	0.73
	Tigecycline	0.016	0.05	0.02	0.021
	Rifampicin	0.002	0.002	0.002	0.002

MIC_{50}/MIC_{90}. minimum inhibitory concentration at which 50%/90% of isolates are inhibited given in μg/mL.

moderate, while 56.75% produced weak form of biofilm. The remaining isolates (17.8%, 8/45) showed no biofilm producing ability. Weak correlation between isolates belonging to different ribotypes and produced biofilm biomass was observed (Spearman r = 0.046; p = 0.765). Average OD_{595nm} for strong, moderate and weak biofilm producers belonging to the ribotypes 001 and 027 are shown in Fig. 1. The difference in the biomass of the produced biofilm between the tested ribotypes (RT 001 vs. RT 027) for weak and moderate producers is not evident, while for strong biofilm producers this difference is notable (OD_{595} for RT 027 = 1.97 and for RT 001 = 1.39). Statistical test showed no significant differences for any biofilm category tested (Fig. 1.).

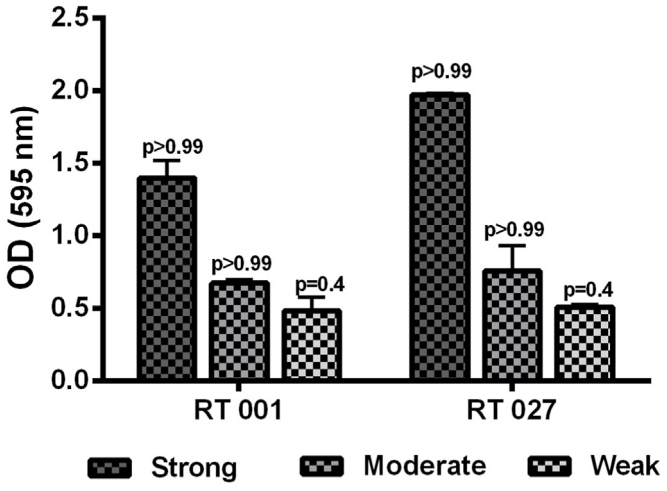

Fig. 1. Ability of *C difficile* ribotypes 001 and 027 to form *in vitro* biofilm presented as average OD_{595} values for the three defined categories of biofilm formed (strong, weak and moderate). The bars show the average values. The error bars show the standard deviations. Differences in biofilm formation were assessed using the Mann–Whitney *U* test.

3.4. Effect of essential oils on biofilm formation

Oregano essential oil reduced biofilm production in 100% of the tested toxigenic *C. difficile* strains in all tested concentrations (Fig. 2). Black pepper and garlic essential oil exhibited weaker anti-biofilm producing activity (Figs. 3 and 4). If we compare produced control biofilm and biofilm produced under the EOs treatment, for oregano EO we could observe reduced biofilm biomass for all tested ribotypes and all tested concentrations, and this non-selective effect highlights oregano in comparison to garlic and black pepper essential oils. Percentages of biofilm biomass produced under the treatment with 0.5 x MIC of oregano is in range 12.4–97.2% for RT001, 26–82.5% for RT 027 and 29–90% for other tested RTs. Following the treatment with oregano MICs RT001 isolates produced 11–81.7%, RT 027 14.6–79.6%, and other RTs 31.7–69.8% of control biofilm. In 2 x MIC concentrations of oregano EO, biofilm production was 11–92%, 5.5–68% and 25–77%, for RT001, RT027 and other RTs, respectively.

When treated with garlic EO, strains belonging to RT001 produced 12.42–172.2%, 16.8–200.4% and 14–184.7% of biofilm, while RT027 strains produced 15.9–104.5%, 20–154.5% and 15.6–90.2%, and other RTs 41–56.3%, 16.1–99.6% and 17.8–56.7%, in 0.5 x MIC, MIC and 2 x MIC, respectively. In strains stimulated to produce stronger biofilms than in control, higher increases have been observed for RT001 in comparison to RT027.

When treated with black pepper EO in 0.5 x MIC, MIC and 2 x MIC, in comparison with untreated control, RT 001 isolates produced 15.3–238.01%, 12.55–192.55% and 12.88–153.8% of biofilm, RT027 14.1–108.7%, 12.9–91.8% and 11.32–58% of biofilm, and other RTs 15.9–52.1%, 15–44.4% and 10.52–28.5%, respectively. Stronger promotional effect could be observed for RT001 in comparison to RT027. Other RTs biofilms were inhibited with black pepper EO in all tested concentrations. More detailed information about precise absorbances obtained in anti-biofilm producing experiments is provided in Supplementary Table 1.

4. Discussion

Clostridioides difficile is the most common causative agent of antibiotic-acquired diarrhea in hospitalized patients. A reason for high concern is the limited number of antibiotics for the CDI therapy and frequent post-treatment recurrences. For this purpose, three conventional antimicrobial agents, metronidazole, vancomycin and fidaxomicin are recommended. Tigecycline and rifaximin are effective in the treatment of a severe CDI forms or in prevention of CDI relapse [60]. Many clinical isolates, including those belonging to PCR RTs 001, 012, 017, 018, 027 or 078, are considered to be multi-drug resistant, mostly to the MLSB (macrolide-lincosamide-streptogramin B) antibiotics family, recognized as high-risk agents for the development of CDI. Resistance to metronidazole and vancomycin is reported rarely, but decreased sensitivity to multiple antibiotics occurs more frequently among epidemic and emergent *C. difficile* strains [3,8,61–63]. Moxifloxacin is not an option for antibiotic treatment of CDI, but moxifloxacin resistance is an important marker for the spread of *C. difficile* in the hospital settings. Reduced susceptibility to moxifloxacin in *C. difficile* isolates was identified as a risk factor for fatal outcome of the patients, so it can be considered as a factor of pathogenicity *per se* and as an important marker in surveillance of CDI [64]. In addition, fluoroquinolones resistance caused by Thr82Ile, the most prevalent mutation, plays a significant role in the global spread of RT 027 [65]. According to the recent published data, moxifloxacin resistance among *C. difficile* strains was 49% based on the of EUCAST breakpoints [63].

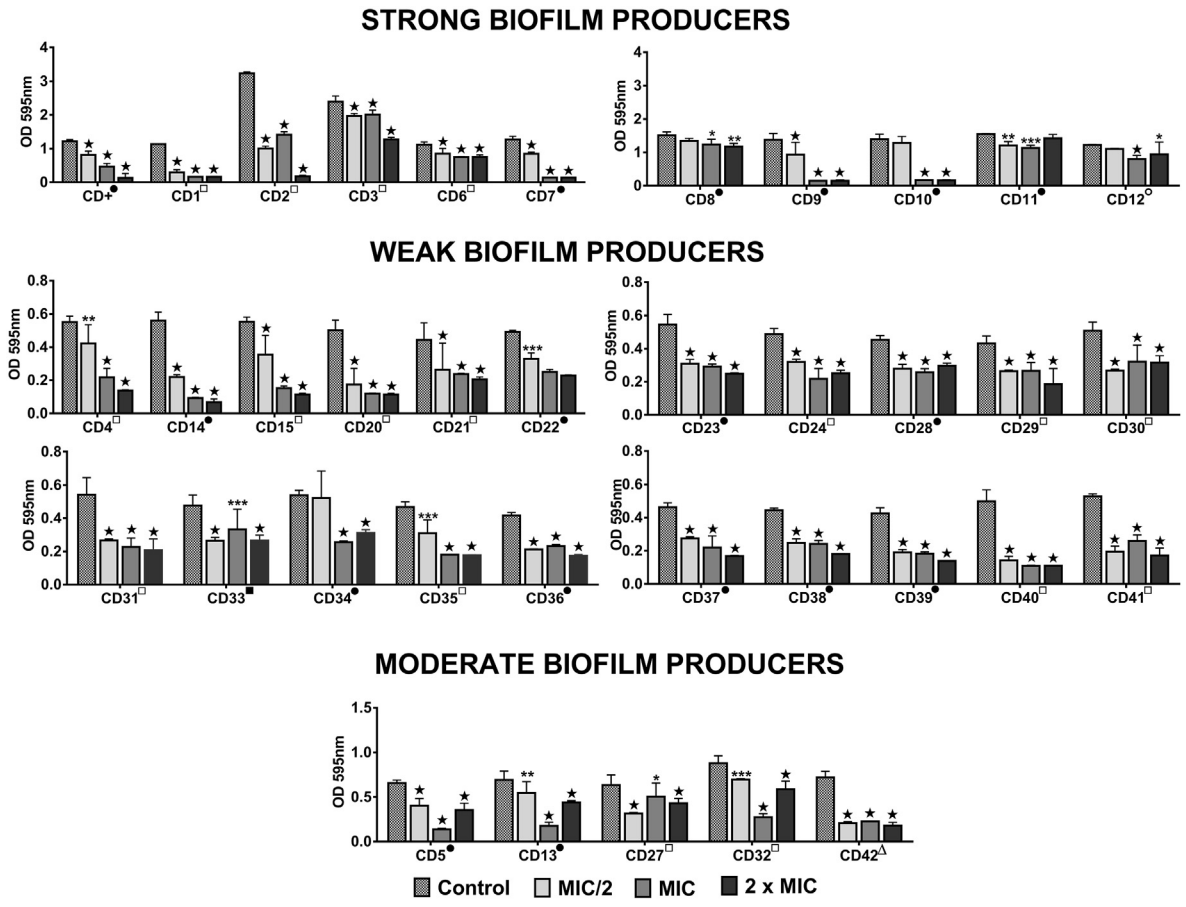

Fig. 2. Biofilm formation by the tested *C difficile* strains treated with 0.5 x MIC, MIC and 2xMIC of oregano essential oil for the three defined categories of biofilm formed (strong. weak and moderate). The bars show the average values from the three measurements. The error bars show the standard deviations. An asterisks shows a statistically significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p < 0.0001$) in the average OD_{595} values when the same strain was grown in presence or absence of the essential oils; $p < 005$). ● RT 001; □ RT 027; ○RT 205; ■ RT 015; ΔRT 020.

In the present study, out of the 42 clinical isolates tested, 86% (36/42) showed resistance to moxifloxacin (Table 2). Proportion of resistant isolates for RT 027, RT 001 and other tested ribotypes were 94%, 90% and 25%, respectively, indicating higher resistance rate among epidemic strains (RT 027 and RT 001). The other tested antibiotics did not exhibit notable differences in mean MIC values between the different ribotypes. However, in the study of Isidro et al. [61], decreased sensitivity to metronidazole has been observed in some of the most common *C. difficile* RTs, including RT 027, RT 001, and RT 010. Decreased susceptibility to vancomycin was also observed in two RTs (RT 018 and RT 356) and in the epidemic RT027. Rifampicin resistance is mainly associated with frequent RTs (027, 018 and 356). Strains with reduced susceptibility could be a potentially serious problem for the first treatment of CDI in the future [3,61,66].

The paradox related to CDIs is the fact that the main risk factor is at the same time the most common treatment for these infections, which is antibiotic-based therapy [67]. The increasing frequency of highly virulent *C. difficile* strains, hospital outbreaks, patients with severe complications, high recurrence rates and reports of reduced susceptibility to antibiotics [63,68] among the epidemic ribotypes highlighted need for new agents [68,69]. Plant-derived compounds are considered as a safer, less toxic and more environment-friendly option compared to conventional therapies [68]. Antimicrobial properties of spice plants oregano, black pepper and garlic are well investigated [24,50,52,70–73]. Wild oregano is a well-known folk remedy with confirmed antimicrobial properties but to date, there is no scientific data on the efficacy of oregano oil against clinically relevant isolates *C. difficile*. Due to the high content of carvacrol, oregano essential oil is considered to be one of the strongest natural antiseptics, with no documented evidence of resistance development in the presence of its sublethal doses [73]. In this study, the most of the isolates were inhibited at oregano oil concentration of 0.16 mg/mL (67%), and no selectivity was observed against specific ribotype or antibiotic sensitivity patterns. Two-antibiotic resistant strains (CD4 and CD13) were inhibited with oil concentration of 0.63 mg/mL (both MIC/MBC) and 0.63/10 mg/mL (MIC/MBC), respectively. Earlier studies have shown that carvacrol substantially reduced *C. difficile* toxin production, sporulation and cytotoxicity on Vero cells and significantly down-regulated toxin production genes [74], as well as critical genes involved in spore production [75]. It can be concluded that the oregano essential oil and its compounds (primarily carvacrol) may be useful as an alternative or complementary treatment for CDI. It has been effective against all *C. difficile* pathogenicity aspects without negative impact on normal intestinal microflora [75]. In the future, it is necessary to examine *in vivo* toxicity and possible side effects of the oil and its compounds.

Allicin (present in the garlic cloves) and piperine (bioactive alkaloid in the black pepper fruit) exhibits broad spectrum antimicrobial activity [32,50]. So far, only one study investigated the effect of garlic raw preparations against *C. difficile*, while black

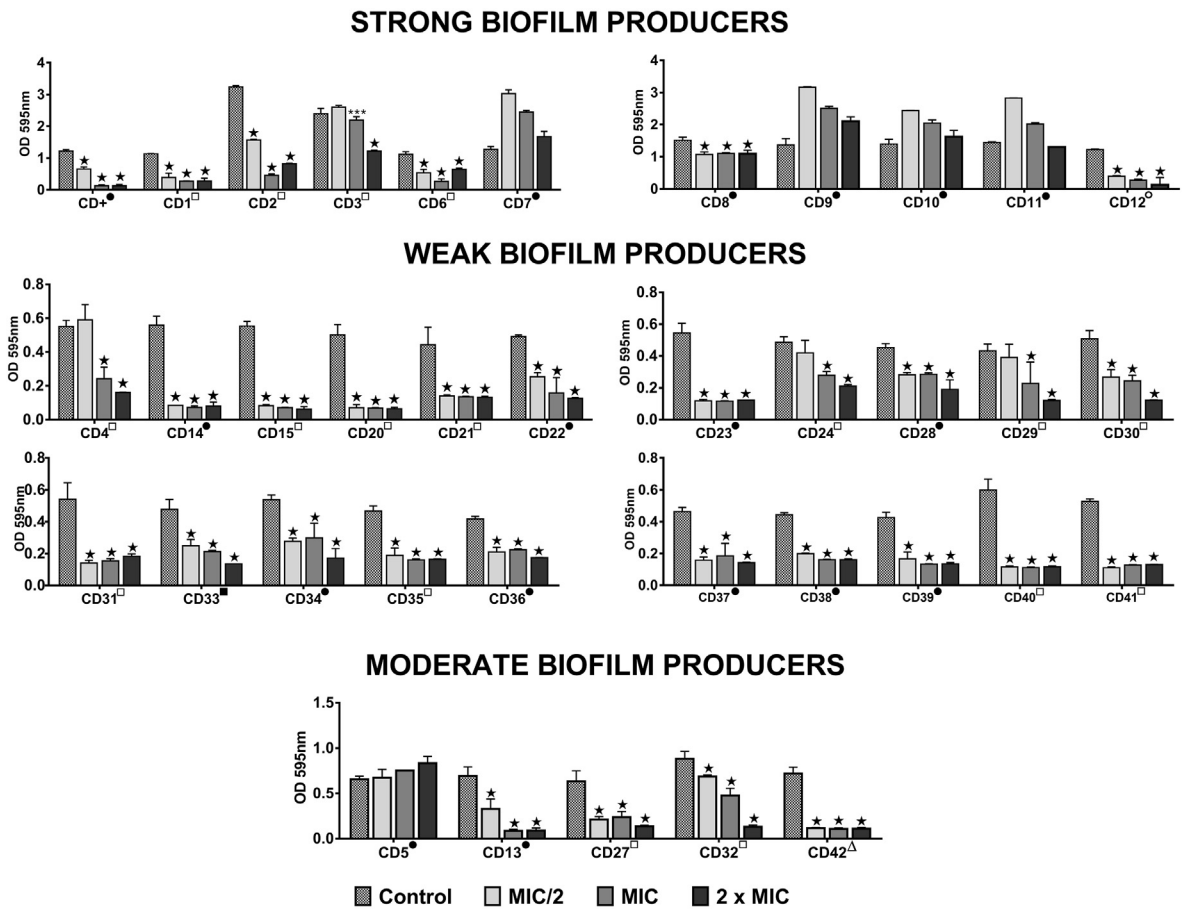

Fig. 3. Biofilm formation by the tested *C difficile* strains treated with 0.5 x MIC, MIC and 2xMIC of black pepper essential oil for the three defined categories of biofilm formed (strong. weak and moderate). The bars show the average values from the three measurements. The error bars show the standard deviations. An asterisks shows a statistically significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p < 0.0001$) in the average OD_{595} values when the same strain was grown in presence or absence of the essential oils; $p <$ 005). ● RT 001; □ RT 027; ○RT 205; ■ RT 015; ΔRT 020.

pepper was not studied at all. Antimicrobial potential of numerous natural (raw and processed) products, including garlic juice and garlic tablets against four *C. difficile* strains were investigated in the study Roshan et al. [24]. Among the raw products, garlic juice and powder showed the highest inhibitory activity, while bactericidal effect was not observed. Allicin inhibited growth of *C. difficile* in concentrations from 2.3 to 4.7 mg/mL, while microbicidal action was not observed in the tested range (up to 37.5 mg/mL) for all tested strains [24]. In the present study, essential oils of garlic and black pepper exhibited good anti-clostridial activity, with higher MIC_{90} and MBC values, compared to oregano essential oil. For both oils, the ribotype-dependent selectivity of action is evident with significantly higher MIC_{90} values for RT 027 and RT 001 in comparison to other RTs (Table 3). The observed difference among garlic tablets and juice, allicin and the herein tested garlic essential oil activities might be a consequence of the presence of allicin in the mentioned raw products, which is not present as a compound in essential oil (due to degradation during distillation procedure).

Persistence of bacterial cells and enhanced protection within the biofilm in the human intestine during infections could have an important clinical relevance in the treatment failure and recurrence of CDI [10]. While relatively well investigated for aerobic medically important bacteria, in the case of anaerobic pathogens such as *C. dificile*, biofilm production as well as the potential of various plants products to reduce the biofilm production has been poorly studied [28]. Earlier studies [28,76] demonstrated the biofilm producing ability of toxigenic *C. difficile* isolates. *Clostridioides difficile* strains belonging to RT 027 showed the highest potential to form biofilms, but these studies were performed on only three strains (ATCC 9689, RT 027 and RT 106) [76] and 20 clinical isolates belonging to four different ribotypes (017, 023, 027 and 046) [28]. In our research, the ability to produce biofilm in a large number of isolates (82.2%; 37/45) has been proven, which can be related to problems that occurs in CDI therapy as well as to frequent recurrences of the disease. The difference in the biomass of the produced biofilm between the ribotypes 001 and 027 for strong, weak and moderate producers is presented on Fig. 1.

Our results indicate that the tested oils revealed high efficacy in reducing the biofilm production. Certain dependence between biofilm production ability and antimicrobial action of the black pepper and garlic essential oil was observed. In isolates CD1, CD2, CD3, CD7 and CD11, the minimum inhibitory concentration of black pepper essential oil is very high (40 mg/mL), while garlic essential oil had a weaker effect on isolates CD2, CD3, CD4, CD9, CD10 and CD11, which are all (except CD4) strong biofilm producers (Table 1). Oregano essential oil showed non-selective (in regard to RT, antibiotic-susceptibility patterns or biofilm producing ability) strong inhibitory, bactericidal and anti-biofilm producing activity against all tested strains, which certainly highlights the advantage of this essential oil compared to the others tested. It has been shown that sub-inhibitory concentrations of antimicrobial agents might promote biofilm production. In the study of Vuotto et al.

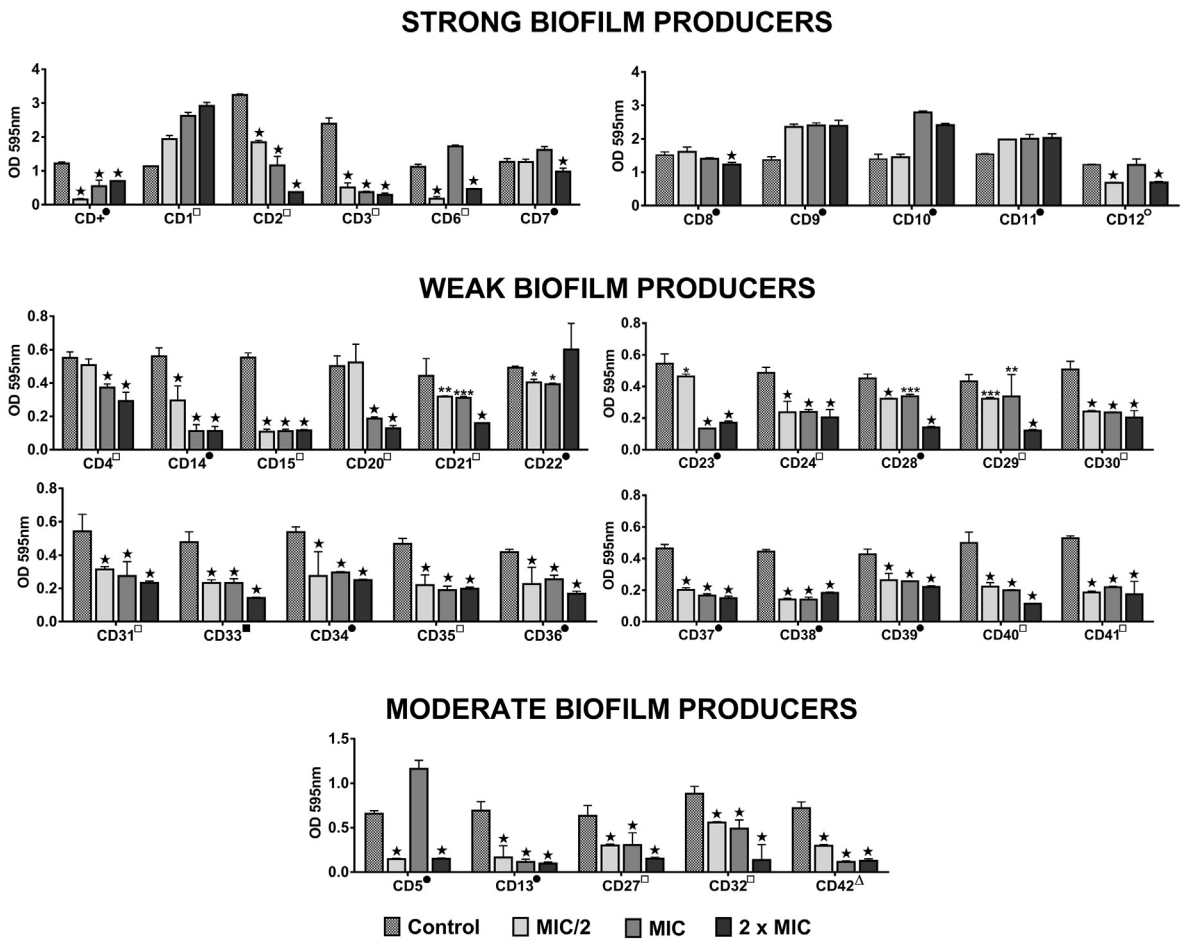

Fig. 4. Biofilm formation by the tested *C difficile* strains treated with 0.5 x MIC, MIC and 2xMIC of garlic essential oil for the three defined categories of biofilm formed (strong. weak and moderate). The bars show the average values from the three measurements. The error bars show the standard deviations. An asterisks shows a statistically significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p < 0.0001$) in the average OD_{595} values when the same strain was grown in presence or absence of the essential oils; $p < 005$).● RT 001; □ RT 027; ○RT 205; ■ RT 015; ΔRT 020.

Table 3
MICs of tested essential oils on *Clostridium difficile* isolates presented as MIC_{50}. MIC_{90}. mean and geometric mean for all tested strains (n = 45) and separately for ribotypes.

	Essential oil	MIC_{50}	MIC_{90}	Mean	Geometric mean
All strains	Oregano	0.16	0.63	0.26	0.17
	Black pepper	1.25	40.00	7.92	1.64
	Garlic	0.63	40.00	6.20	1.00
RT 027	Oregano	0.16	0.63	0.30	0.18
	Black pepper	0.63	40.00	8.90	1.22
	Garlic	0.63	40.00	7.04	0.79
RT 001	Oregano	0.16	0.63	0.25	0.18
	Black pepper	2.50	36.00	8.39	2.36
	Garlic	0.63	40.00	6.74	1.34
Other RTs	Oregano	0.16	0.63	0.23	0.16
	Black pepper	0.63	5.00	1.55	0.86
	Garlic	0.63	1.25	0.74	0.58

MIC_{50}/MIC_{90}. minimum inhibitory concentration at which 50%/90% of isolates are inhibited given in mg/mL.

(2016), increased biofilm production was reported among metronidazole-sensitive isolates *C. difficile* when metronidazole was applied in sub-inhibitory concentrations [10]. Oregano oil showed no promotional effect even at these concentrations, which makes it a principal candidate for future studies of clostridial biofilms inhibition. The future studies should also be focused on the principal compounds carvacrol and thymol alone or in combination with antibiotics used for therapy of CDI, which would be tested against mature biofilms *in vitro* and also on *in vivo* models.

5. Conclusion

The global epidemic of nosocomial diarrhea caused by *C. difficile* and the increasing frequency of highly virulent strains with reduced susceptibility to multiple antibiotics highlighted need for new agents against CDI. In the present study, the antimicrobial and anti-biofilm producing activity of the three essential oils isolated from oregano, black pepper and garlic showed potent anti-clostridial and anti-biofilm producing activity against the tested toxigenic *C. difficile* isolates. The highest activity was observed for oregano oil, which makes it a principal candidate for future studies of clostridial biofilms inhibition. The *in vivo* efficacy of these essential oils, their major compounds and their effect on the other CDI pathogenesis factors, such as toxin production and sporulation, should certainly be examined in the future.

Declaration of competing Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2022.102604.

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