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## Original Article

**Biofilm of *Cutibacterium acnes*: a target of different active substances**Louise Ruffier d'Epenoux,<sup>1,2</sup> Erwan Fayoux,<sup>1</sup> Joëlle Veziers,<sup>3</sup> Marie-Ange Dagnelie,<sup>2</sup> Amir Khammari,<sup>2</sup> Brigitte Dréno,<sup>2</sup> and Stéphane Corvec<sup>1,2</sup> 

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**Keywords**

*Cutibacterium acnes*; biofilm; Myrtus communis extract; acne vulgaris; erythromycin; clindamycin; doxycycline.

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**Introduction**

Acne vulgaris is one of the most prevalent skin diseases. It is often caused by an unbalanced microbiota (dysbiosis linked to an overrepresentation of phylotype IA<sub>1</sub> and not an increase of *Cutibacterium acnes* species), affecting up to 80% of the global population.<sup>1</sup> Four elements are relevant at the pathophysiological level: hyperseborrhea, aberrant follicular keratinization, the growth of *C. acnes* (formerly *Propionibacterium acnes*) in the pilosebaceous unit,<sup>2</sup> and the stimulation of local innate immune reaction.<sup>2,3</sup>

*Cutibacterium acnes* is a Gram-positive bacillus that is part of the human microbiota.<sup>4</sup> The *C. acnes* population strains are

**Abstract**

**Background** Acne vulgaris is a chronic inflammatory dermatosis. *Cutibacterium acnes* plays a crucial role in the acne pathophysiology. Recent works present evidence of *C. acnes* growing as a biofilm in cutaneous follicles. This development is currently considered one of the leading causes of *C. acnes in vivo* persistence and resistance to antimicrobials used to treat acne.

**Objective** Our objective was to evaluate the effects of various active compounds (clindamycin, erythromycin, doxycycline, and myrtle extract) on eight distinct, well-characterized strains of *C. acnes* following their growth in biofilm mode.

**Methods/Results** *Cutibacterium acnes* isolates from phylotypes IA<sub>1</sub> and IA<sub>2</sub> produce more biofilm than other phylotypes. No antibiotic effect was observed either during the curative test or preventive test. Myrtle extract at 0.01% (w/v) showed significant efficacy on the biofilm for *C. acnes* strains (curative assays). Furthermore, it appears that myrtle extract and doxycycline together reduce the overall biomass of the biofilm. A significant dose-dependent effect was observed during the preventive test, greater than the one observed under curative conditions, with an important loss of activity of the myrtle extract observed from 0.001% (w/v) concentration onwards. Transmission electron microscopy showed that bacteria treated with myrtle extract grew biofilms much less frequently than untreated bacteria. Additionally, when the quantity of myrtle extract grew, the overall number of bacteria dropped, indicating an additional antibacterial action.

**Conclusion** These findings support the hypothesis that the different *C. acnes* phylotypes have various aptitudes in forming biofilms. They also suggest that myrtle extract is a promising alternative as an anti-biofilm and antibacterial agent in fighting diseases caused by planktonic and biofilm *C. acnes*.

divided into six main phylotypes: IA<sub>1</sub>, IA<sub>2</sub>, IB, IC, II, and III.<sup>5</sup> *C. acnes* dwells in sebaceous glands rich in lipids, which are colonized by one type of *C. acnes*. *C. acnes* is a cornerstone of acne pathophysiology, stimulating the innate immune system, inducing chronic inflammation,<sup>6–8</sup> and participating in lipogenesis<sup>9–11</sup> and comedogenesis.<sup>12,13</sup> But *C. acnes* is becoming more widely acknowledged as a pathogen, not just in foreign-body infections like those related to spinal instrumentation or arthroplasty<sup>14,15</sup> but also in lung abscesses,<sup>16</sup> prostate cancer,<sup>17</sup> and sarcoidosis.<sup>18</sup>

Initially, various antibiotics, such as clindamycin, erythromycin, β-lactams, and quinolones, were thought to be effective against *C. acnes* strains.<sup>19</sup>

Nevertheless, epidemiological research has demonstrated a significant rise in the prevalence of topical antibiotic resistance in individuals with acne, from 20% in 1979 to 64% in 2000. This resistance is particularly noticeable for erythromycin and clindamycin.<sup>20,21</sup> The production of biofilms,<sup>22,23</sup> which can limit the entry of antibiotics and antibodies,<sup>21</sup> is a possible contributing factor to *C. acnes* resistance. The reason behind treatment failures and the lack of a defined effective regimen for treating *C. acnes* biofilm in acne could be attributed to the diminishing susceptibility of microorganisms in a biofilm phase to main antimicrobials.<sup>24</sup>

With the increase of *C. acnes* antibiotic resistance, new therapeutic approaches have been studied. An isopropyl acetate extracted from *Myrtus communis* leaves (myrtle extract, ME)<sup>25,26</sup> has been studied for its anti-biofilm activity on a reduced number of *C. acnes* strains of unknown phylotypes.<sup>27</sup> Feuillolay *et al.*<sup>27</sup> showed, *in vitro*, that this molecule decreased *C. acnes* biofilm formation and had a noticeable impact on a mature biofilm in just 5 minutes after contact. In addition, the authors showed that its anti-biofilm effect was amplified when combined with antibiotics (erythromycin and clindamycin).

The current study's objective was to investigate whether the sensitivity of *C. acnes* clinical strains to different active substances against the biofilm depends on the *C. acnes* phylotype. This is especially relevant considering that acne is mainly associated with phylotype IA<sub>1</sub>.

## Materials and methods

### Selection of *C. acnes* strains

Eight *C. acnes* strains were used for this investigation, comprising six clinical strains collected on patients at the Department of Dermatology and two reference strains (ATCC6919 and ATCC11827). The latter two, originating from the collection of the Bacteriology Department, came from a previous clinical study<sup>28</sup> conducted at the Department of Dermatology. The study was authorized by the Ethics Committee (Comité de Protection des Personnes (CPP)) and the health authorities (Agence Nationale de Sécurité du Médicament et des produits de Santé (ANSM); number 151141B-42). Using multiplex touchdown PCR rapid typing, all strains were classified into the primary phylotypes IA<sub>1</sub> (mainly detected in acne patients), IA<sub>2</sub>, IB, II, and III (mainly detected during progressive macular hypomelanosis).<sup>5,29</sup> In accordance with Scholz *et al.*, all isolates were further subclassified into SLST categories.<sup>30</sup> Table 1 provides a summary of the isolates' characteristics. Strains were preserved in Mueller-Hinton broth (bioMérieux, Marcy l'Etoile, France) complemented with glycerol at -20 °C. Before each experiment, one subculture on Schaedler sheep blood agar (bioMérieux) was performed for 48 hours at 36 °C under anaerobic conditions (Becton Dickinson, Dublin, Ireland).

### Active substances tested

Erythromycin, clindamycin, and doxycycline were obtained from the Department of Pharmacy of our hospital and were all dissolved using sterile water. Myrtle extract, which is an extract of myrtle leaves, was provided by Ducray Dermatological Laboratories; Pierre Fabre Dermo-Cosmétique. The solvent used to prepare the stock solutions was DMSO (dimethyl sulfoxide).

### Bacterial cultures for biofilm experiments

For *in vitro* biofilm experiments, micro-organisms were cultured in brain heart infusion (BHI), adjusted to a density of 0.5 McFarland units. These suspensions were then used to inoculate the 96-well plates (200 µl per well) for each test. Each experiment included the biofilm-forming *C. acnes* ATCC6919 and ATCC11827 strains<sup>15,31</sup> as positive control and wells containing only the culture medium as negative control. Each approach was used to test every strain in a minimum of three different tests.

### *Cutibacterium acnes* minimum inhibitory concentration of antibiotics and myrtle extract determination

Antibiotics minimum inhibitory concentrations (MICs) were determined by using antibiotic gradient strip Etest<sup>®</sup> (bioMérieux). The reported procedure was followed while performing the ME MIC experiment.<sup>32</sup> In short, nutrient broth medium was inoculated with *C. acnes* in its logarithmic growth phase (bacteria suspension concentration is  $1 \times 10^7$  CFU/ml). After adding ME, the nutrient broth medium's final concentrations ranged from 0.025 to 32 mg/ml. *C. acnes* culture without ME was used for control. Subsequently, 100 µl of bacterial suspension were gathered and arranged onto the nutrient agar plates (Schaedler sheep blood agar, bioMérieux) and incubated for 48 hours in anaerobic condition. The MIC was calculated for the lowest dilution of ME at which bacteria did not grow in nutrient plates after bacterial growth was observed.

**Table 1** Characteristics of patients (acne severity) and *Cutibacterium acnes* clinical strains selected for the study

Isolate name	Phylotype	Clonal complex MLST <sup>a</sup>	SLST type	Global acne evaluation (GEA) scale
ATCC6919	IA <sub>1</sub>	CC18	A1	
ATCC11827	IA <sub>1</sub>	CC18	A1	
A47	IA <sub>1</sub>	CC18	A1	4
A56	IA <sub>1</sub>	CC28	D1	3
A85	IA <sub>2</sub>	CC2	F1	2
A132	IB	CC36	H1	1
A104	II	CC53	K1	2
A48	III	CC43	L7	4

<sup>a</sup>Aarhus scheme, Kilian *et al.*, 2012. MLST, Multi-Locus Sequence Typing; SLST, Single-Locus Sequence Typing.

### Crystal violet (CV) straining

This reference method was performed to study bacterial biofilm production (total biomass including viable and dead bacteria).<sup>33</sup> This method was modified in 2011 to increase its accuracy.<sup>34</sup> Finally, as recommended by Peeters *et al.*, a 100% methanol fixation step was added.<sup>35</sup> More details are presented in Data S1.

### BioFilmRing Test<sup>®</sup> (BRT)

This innovative method was used to determine the ability of certain bacteria to adhere to magnetic beads in 96-well microplates and also to assess the kinetics of biofilm formation quickly and easily without washing or staining.<sup>36</sup> More details are provided in Data S2.

### Biofilm treatments

ME concentrations were selected between 0.03 and 0.0001% (w/v).<sup>27</sup> Tests were performed using increasing antibiotics (erythromycin, clindamycin, and doxycycline) with concentrations ranging from 0.25 to 32 mg/l. The impact of these active substances was first evaluated with the BRT. Then, these active substances were studied with the crystal violet (CV) method for both (i) preventive treatment, entailing a product addition at the time of bacteria inoculation, and (ii) curative treatment where product addition intervened 48 hours after the initial formation of the biofilm. We performed a curative test by adding this combination to a 48-hour-old biofilm to assess the relevance of combining ME and doxycycline.

### Sample preparation for TEM (Transmission electron microscopy) work

After incubation for 48 hours, all bacteria contained on a microplate were immediately fixed by adding 1.6% (v/v) glutaraldehyde (GA). GA was added to the cells to stop their activity, maintain their structure during fixation, and reduce changes during embedding, sectioning, or exposure to the TEM's electron beam. The wells were post-fixed with 2% osmium tetroxide after being cleaned three times with 1% phosphate-buffered saline. The fixed samples were dehydrated with acetone (33.3, 66.6, 100%), and then, samples were dried with a mix of acetone and freon (75–25%, 50–50%, 25–75%, 0–100%) then left for 12 hours under a bell jar to finish drying. Finally, the lamellae were metalized with gold/palladium for TEM observation.

### Statistical analysis

Mann-Whitney test was used to compare datasets, and  $P < 0.05$  and  $P < 0.01$  were considered significant differences.

## Results

### Antibiotics and myrtle extract MIC determination

The *C. acnes* strains were sensitive to all antibiotics (erythromycin, clindamycin, doxycycline) tested according to CASFM/EUCAST

guidelines. ME MICs revealed differences ranging from 0.1 to 16 mg/l (Table 2).

### *Cutibacterium acnes* biofilm production assays

#### *Kinetic evaluation of biofilm (adhesion step) formation with BioFilm Ring Test<sup>®</sup>*

Following BRT development and optimization, we identified three distinct groups within the *C. acnes* phylotypes. The first group included strains belonging to phylotypes IA<sub>1</sub> and IA<sub>2</sub>, which appeared to be strong biofilm producers. The second group comprised only one isolate belonging to phylotype II (A104 strain) with moderate biofilm production. Finally, the last group (strains belonging to phylotypes III and IB) was composed of strains with low biofilm production in the early stages (adhesion). Phylotypes IA<sub>1</sub> and IA<sub>2</sub> isolates formed significantly more biofilm, 1.3 to 2 times as much as other phylotypes in almost all cases (Figure 1a).

#### *Crystal violet adherence assay in polystyrene microtiter plates*

Although isolates of a particular phylotype exhibited a distinct heterogeneity in biofilm production ability, there were apparent differences among phylotypes. Phylotypes IA<sub>1</sub> and IA<sub>2</sub> isolates formed significantly more biofilm than other phylotypes. Phylotypes IA<sub>1</sub> and IA<sub>2</sub> displayed a significantly higher ability to form mature biofilm compared to that of phylotypes IB and III ( $P < 0.01$ ) (Figure 1b).

### The activity of antibiotics on *C. acnes* biofilm

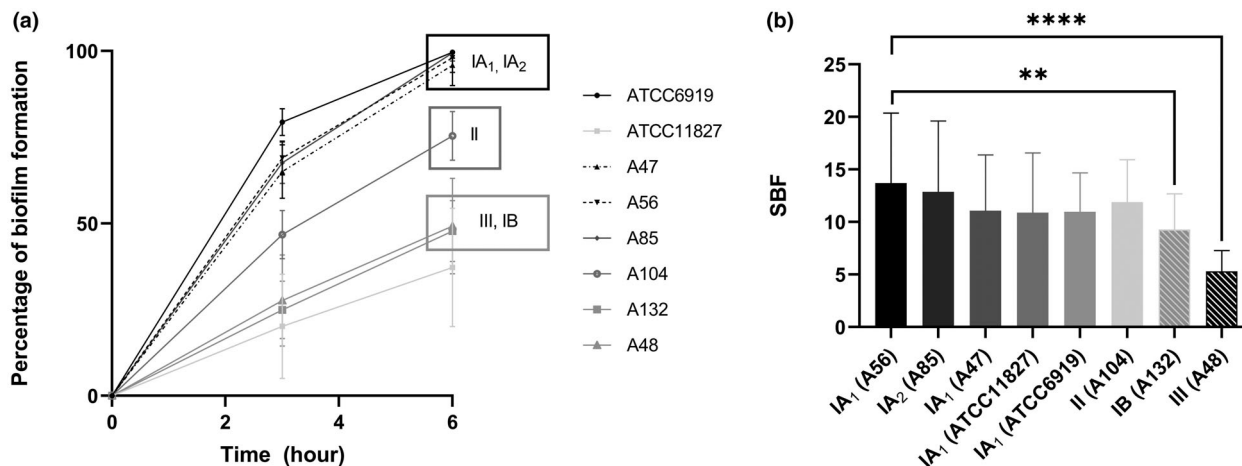
#### *BioFilm Ring Test<sup>®</sup>*

Regarding the early stages of biofilm formation, a slight effectiveness of macrolides at 3 h on biofilm production was observed. Interestingly enough, we noted an overproduction of biofilm for the majority of *C. acnes* strains (except for A56, A85, and A104 at 3 h) in the presence of doxycycline.

**Table 2** MIC (mg/l) of erythromycin, clindamycin, doxycycline, and myrtle extract for the selected strains

	Erythromycin MIC	Clindamycin MIC	Doxycycline MIC	Myrtle extract MIC
ATCC6919	<0.016	<0.016	0.047	8
ATCC11827	<0.016	0.016	0.064	8
A47	<0.016	0.016	0.047	16
A56	<0.016	0.032	0.032	0.5
A85	<0.016	0.016	0.047	4
A104	<0.016	0.023	0.047	2
A132	<0.016	<0.016	0.032	0.1
A48	<0.016	0.016	0.032	0.1

MIC, Minimum Inhibitory Concentration.



**Figure 1** (a) Biofilm formation (first stages of adhesion) by *Cutibacterium acnes* was obtained using Biofilm Control image analysis software. Results are expressed as a percentage of biofilm formation according to the incubation time. Bars represent the standard deviations from three independently performed eight times each. (b) The biofilm formation capacity is shown as the ratio (SBF) obtained after crystal violet staining (mature biofilm) (see Data S1 for details). Bars represent the standard deviations from three independent experiments performed eight times each. Significant differences are indicated as follows: \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ , using the Mann-Whitney test

### Crystal violet method

In curative conditions, a decrease in mature biofilm production by all *C. acnes* phylotypes was observed with the introduction of antibiotics (erythromycin, clindamycin, or doxycycline). No significant difference was observed in the concentration of antibiotics used (Table S1).

In a preventive challenge, we noticed that the addition of doxycycline at a concentration of 2 mg/l induced a significant decrease in the formation of the total biofilm biomass for all the *C. acnes* strains (except for the A104 strain, phylotype II) (Figure S1).

### Myrtle extract activity

#### ME anti-biofilm activity on a mature biofilm

In curative conditions (Figure 2), a significant effect was observed, with a substantial decrease in biofilm formation at a concentration of 0.01% (w/v). In a preventive challenge (Figure 3), a drastic dose-dependent effect was observed, greater than in curative conditions, with a significant increase of the ME activity from a concentration of 0.001% (w/v) onwards.

#### ME anti-biofilm activity when combined with doxycycline

In curative conditions, a medium efficacy of the ME/doxycycline combination on the mature biofilm of *C. acnes* strains was

observed. Moreover, the efficacy of this combination seemed to be higher when a concentration of 0.03% of ME was used (Figure S2).

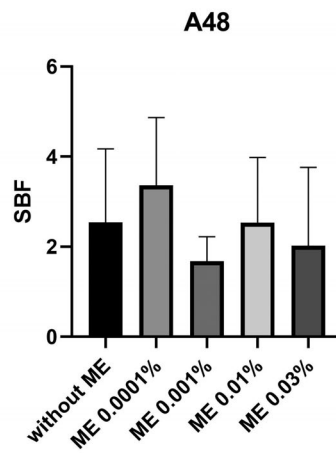
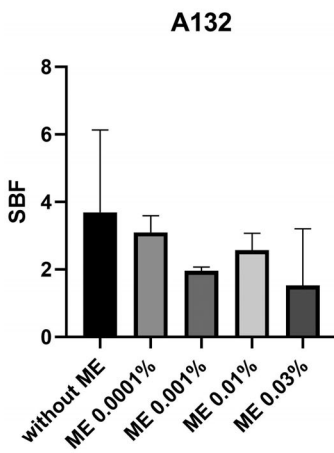
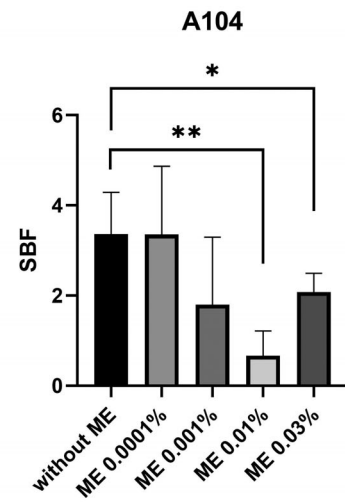
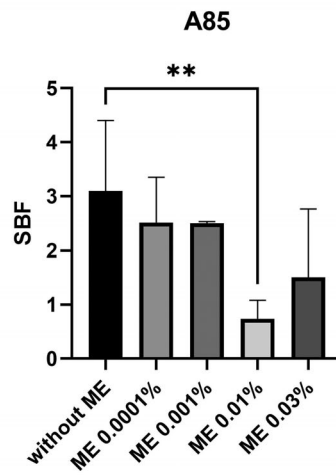
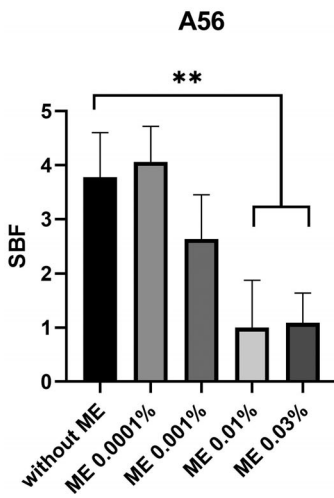
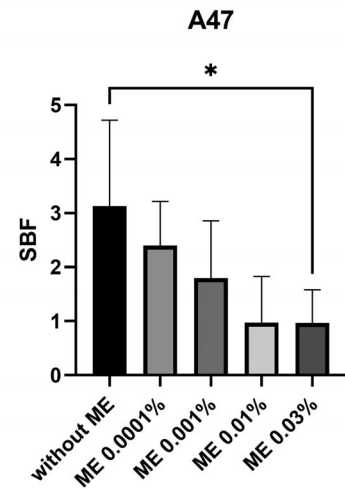
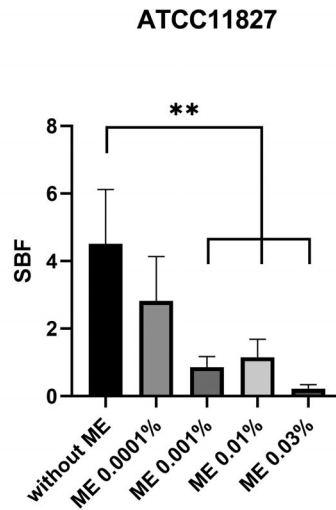
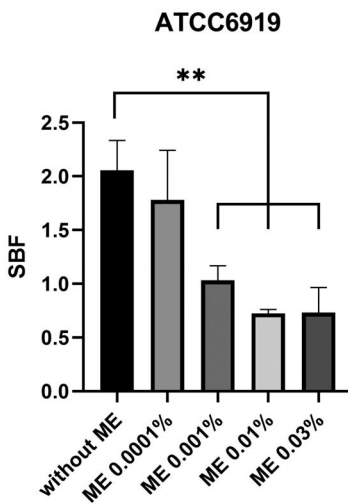
### Characterization of the biofilm microstructure

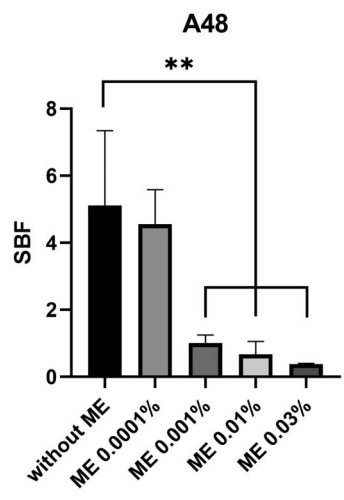
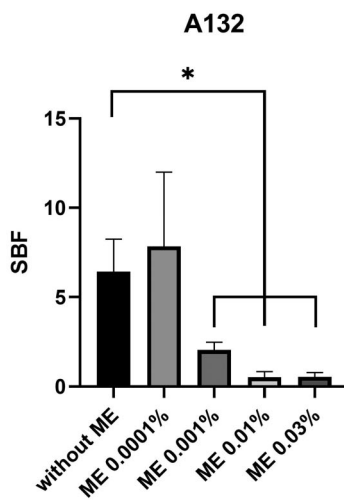
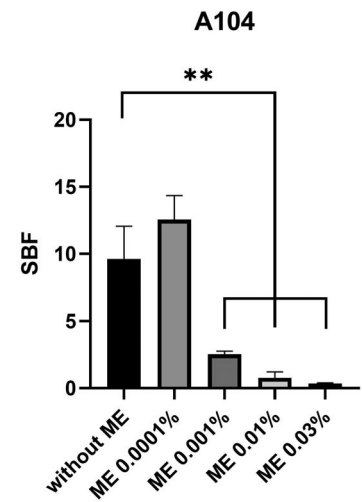
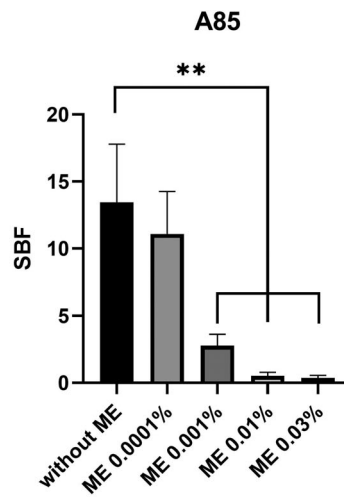
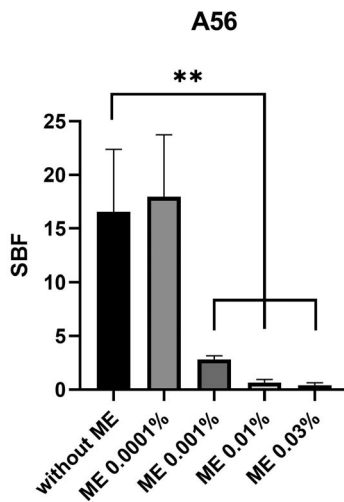
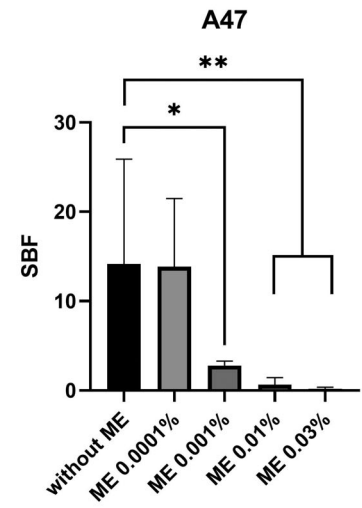
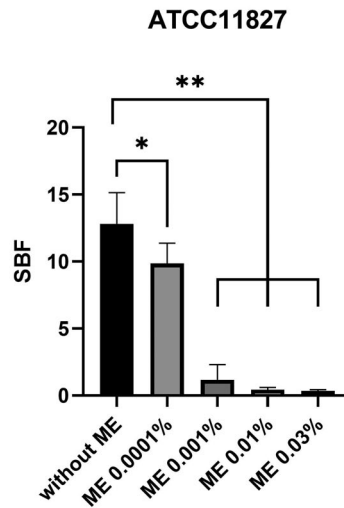
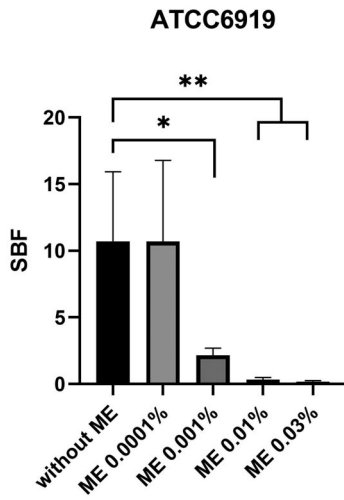
The *C. acnes* biofilm grown (strain ATCC6919) with and without ME was examined by TEM (Figure 41719). The results revealed an important decreased biofilm growth in treated bacteria compared to untreated bacteria. *C. acnes* produced a dense biofilm consisting of aggregates, and the cells were interconnected to form a three-dimensional structure. On the other hand, the biofilm's extracellular matrix and cell count were considerably decreased, and the cells were dispersed in the ME-treated samples. Furthermore, it was evident that when the concentration of ME rose, the overall number of bacteria dwindled.

### Discussion

This study brings new insights into the implication of *C. acnes* biofilm in acne. Phylotypes IA<sub>1</sub> and IA<sub>2</sub> produce more biofilm and are faster than other phylotypes. Macrolides and tetracyclines did not have any effect on a curative challenge and had little influence on a preventive test. However, the ME is quite effective against *C. acnes* biofilm regardless of the phylotype and more potent at a concentration of 0.001% during preventive

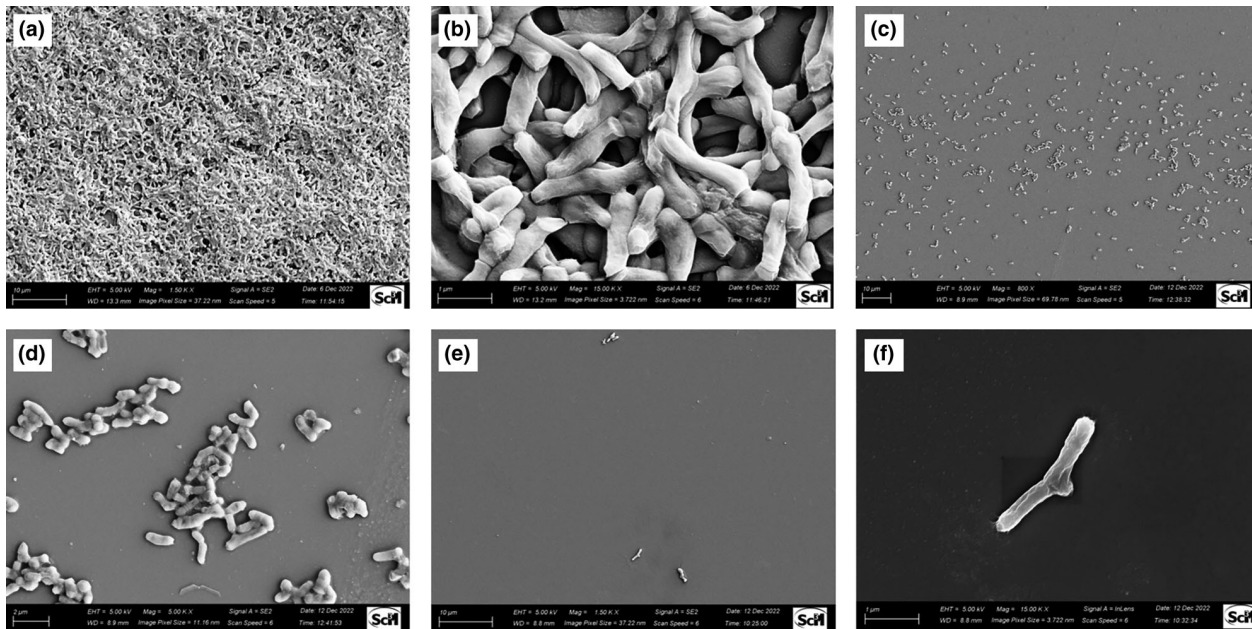
**Figure 2** Impact of the addition of myrtle extract (ME) on different strains of *Cutibacterium acnes*: curative test. The impact of ME was studied with the crystal violet method for curative treatment, which consisted of product addition on a 48-hour preformed biofilm. The biofilm formation capacity is shown as the ratio (SBF) obtained after crystal violet staining (mature biofilm) (see Data S1 for details). Bars represent the standard deviations from three independent experiments. Significant differences are indicated as follows: \* $P < 0.05$ ; \*\* $P < 0.01$ , using the Mann-Whitney test







**Figure 3** Impact of the addition of myrtle extract (ME) on different strains of *Cutibacterium acnes*: preventive challenge assay. The impact of ME was studied with the crystal violet method for preventive treatment, which consisted of product addition at the time of the bacteria inoculation. The biofilm formation capacity is shown as the ratio (SBF) obtained after crystal violet staining (mature biofilm) (see Data S1 for details). Bars represent the standard derivations from three independent experiments. Significant differences are indicated as follows: \* $P < 0.05$ ; \*\* $P < 0.01$ , using the Mann–Whitney test



**Figure 4** Transmission electron microscopy observations of ATCC6919 strain biofilm formation. (a) and (b) Control images without any substance; (c) and (d) images with 0.001% myrtle extract; (e) and (f) images with 0.03% myrtle extract

tests. Finally, TEM experimentation also revealed a bacterial effect with low ME MICs.

Thus, using two distinct biofilm research techniques (BRT and CV), we present a comparative investigation of the *in vitro* biofilm production capacity of various *C. acnes* isolates. Phylotypes IA<sub>1</sub> and IA<sub>2</sub> isolates (phylotypes mostly found on the skin and, in the case of phylotype IA<sub>1</sub>, mainly involved in the development of acne) formed significantly more biofilm than other phylotypes in almost all cases. We also demonstrated the anti-biofilm activity of ME as a preventive or curative agent against eight strains of different *C. acnes* phylotypes. A strain-dependent effect was observed. These findings support the findings of Kuenast *et al.*<sup>16</sup> about the various capacities of the different *C. acnes* phylotypes to produce biofilm.

Bacterial biofilms are heterogeneous structures consisting of aggregates of bacterial populations embedded in a matrix. They constitute a reservoir of bacteria that can colonize other surfaces. The pathogenic role of biofilms is now well established not only in the development and exacerbation of chronic infections<sup>37–41</sup> but also in chronic inflammatory skin diseases such as acne.<sup>42,43</sup> The intricate composition of the biofilm protects bacteria from antibiotics<sup>24,46–49</sup> and the immune system's

activity.<sup>44,45</sup> Thus, it is challenging to eradicate a bacterial biofilm once set.

Working on these complex structures allows us to understand them better and find ways to circumvent the acquired antibiotic tolerance. Additionally, this study shed light on the interactions among members of the cutaneous microbiome within the biofilm. This understanding is instrumental for healthy skin homeostasis where the commensal bacterium *C. acnes* is prevalent.<sup>50</sup> During dysbiosis in acne, this bacterium plays a primary role in the development of comedones<sup>51</sup> and in inducing chronic inflammation.<sup>6</sup>

Recent studies have shown that acne is not related to a difference in bacterial species abundance<sup>52</sup> but rather to a loss of *C. acnes* phylotype diversity with a predominance of the IA<sub>1</sub> phylotype (change in phylotype distribution).<sup>28</sup> To the best of our knowledge, this subtype is mainly detected after acne lesion sampling.<sup>28</sup> This bacterial subtype exhibits a wide range of virulence traits. Additionally, it has the ability to produce biofilm, which is linked to inflammation<sup>53</sup> and has lately been proposed as a primary cause of the loss of antibiotic resistance.<sup>39</sup> Jahns *et al.*<sup>43</sup> suggested that this bacterium could form biofilm within the pilosebaceous follicles, allowing it to protect itself from the



immune system but also from antibiotics. Moreover, the authors suggest that biofilm production is more important with acne patients than it is with healthy patients. Bacterial biofilm formation is an important factor in antibiotic resistance but clearly not the only contributing factor.

Systemic and topical antibiotics have long been the core of the therapeutic arsenal for acne. The primary topical antimicrobial treatment for acne vulgaris includes erythromycin and clindamycin, with a high risk of resistance selection. Doxycyclines are prescribed in 50% of their antibiotic dose for their anti-inflammatory properties, modulating effect on innate immunity and anti-seborrhea. However, increased resistance has gradually surfaced over the years, becoming a global issue. Nowadays, high rates for topical erythromycin (macrolides) and clindamycin (lincosamides) (between 21 and 70%) are reported.<sup>54</sup> This work confirms the limited potency of erythromycin, clindamycin, and doxycycline on the biofilm of *C. acnes* in both preventive and curative treatments, even at high concentrations. These results are in accordance with previous experiments performed by Coenye *et al.*<sup>24</sup> using a microtiter plate crystal violet assay. The authors demonstrated that these antibiotics had little effect while measuring a significant decrease in the total biofilm biomass.

ME was also evaluated for its antibacterial and antibiofilm properties. The results indicated that ME had an intrinsic antibacterial activity against *C. acnes* and that its MICs were dependent on the phylotype of *C. acnes* but were relatively low. In the bacterial growth curve, all the groups treated with ME showed apparent signs of growth inhibition compared to the control. As previously mentioned by Pécastaings *et al.*,<sup>55</sup> we demonstrated for the first time the potential antibiofilm and antimicrobial properties of ME.

Subsequently, it seems appropriate to test the efficacy of new active substances such as ME, a lipophilic extract of *M. communis* leaves six times richer in myrtucommulones and ursolic acid than ethanolic ME.<sup>26</sup> Recent research by Mias *et al.*<sup>56</sup> showed that ME dramatically reduced the production of proinflammatory cytokines (IL-6, IL-8, IL-12p40, and TNF- $\alpha$ ) by monocyte-derived dendritic cells in response to *C. acnes* phylotype IA<sub>1</sub> and a reduction in the severity of acne.<sup>57</sup>

Finally, it would be interesting to explore the antimicrobial and antibiofilm mechanism(s) of ME further. Antimicrobial mechanisms are extensively studied using scanning electron microscopy (SEM). Flow cytometric analysis may reveal the cell membrane's integrity and permeability.

The limitations of this study are the limited number of *C. acnes* strains, even if each main phylotype was included, and the risk of classification bias inherent with these methods, but the results confirmed the data performed on uncharacterized strains. The data were obtained by the same principal investigator, which made it possible to homogenize the data collection method.

The primary significance of our study reveals that ME is a promising candidate for decreasing the biofilm production of all tested *C. acnes* phylotypes in both curative and preventive conditions. To regulate and rebalance *C. acnes* populations in acne patients, the effectiveness of ME on *C. acnes* biofilm alone or in combination with antibiotics must be taken into consideration, given the rising percentages of *C. acnes* strains resistant to both erythromycin and clindamycin. In conclusion, based on the ME's antibacterial, antibiofilm, and anti-inflammatory qualities, this molecule would seem to be a great ally in treating acne.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Impact of the addition of doxycycline (DO) on different strains of *Cutibacterium acnes*: preventive test.

**Figure S2.** Impact of the addition of doxycycline (DO) and myrtle extract (ME) combination on different strains of *Cutibacterium acnes*: curative test.

**Data S1.** Crystal violet (CV) straining.

**Data S2.** BioFilm Ring Test®.

**Table S1.** Impact of the addition of antibiotics (erythromycin, clindamycin, doxycycline) on the total biomass of *C. acnes* strains: curative challenge assay.