Essential oils against bacterial isolates from cystic fibrosis patients by means of antimicrobial and unsupervised machine learning approaches

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Recurrent and chronic respiratory tract infections in cystic fibrosis (CF) patients result in progressive lung damage and represent the primary cause of morbidity and mortality. *Staphylococcus aureus* (*S. aureus*) is one of the earliest bacteria in CF infants and children. Starting from early adolescence, patients become chronically infected with Gram-negative non-fermenting bacteria, and *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most relevant and recurring. Intensive use of antimicrobial drugs to fight lung infections inevitably leads to the onset of antibiotic resistant bacterial strains. New antimicrobial compounds should be identified to overcome antibiotic resistance in these patients. Recently interesting data were reported in literature on the use of natural derived compounds that inhibited *in vitro* *S. aureus* and *P. aeruginosa* bacterial growth. Essential oils, among these, seemed to be the most promising. In this work is reported an extensive study on 61 essential oils (EOs) against a panel of 40 clinical strains isolated from CF patients. To reduce the *in vitro* procedure and render the investigation as convergent as possible, machine learning clusterization algorithms were firstly applied to pick-up a fewer number of representative strains among the panel of 40. This approach allowed us to easily identify three EOs able to strongly inhibit bacterial growth of all bacterial strains. Interestingly, the EOs antibacterial activity is completely unrelated to the antibiotic resistance profile of each strain. Taking into account the results obtained, a clinical use of EOs could be suggested.

Cystic fibrosis (CF), one of the most common lethal genetic disorders in Caucasian population, is inherited as an autosomal recessive disease and affects 70,000 persons worldwide (Cystic Fibrosis Foundation, CFF). The defective gene, identified in 1989, is the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) that is carried by 4% of persons (among Caucasians). Since CFTR encodes for a chloride channel of the epithelial cell surface, CF patients manifest a variety of multi-organ problems due to the alteration of sodium and chloride secretion across cell membranes and the subsequent luminal dehydration. The impairment of mucociliary clearance, which should remove all microbes entering the airways, leads to the production of a thick and dehydrated mucus in the CF lung, which promotes the airway chronic bacterial colonization.

The microbiology of CF respiratory tract is peculiar. In the early stage of life, it is characterized by the prevalence of the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*). Overall, in 2017 more than half of affected individuals had at least one culture positive for methicillin sensitive *S. aureus* (MSSA). The highest prevalence of methicillin resistant *S. aureus* (MRSA) occurs in individuals between the ages of 10 and 30, while MSSA reaches the peak among patients younger than 10 (Cystic Fibrosis Foundation. 2017. Patient Registry Annual Report).
In early adolescence, CF patients' lung becomes chronically infected with Gram-negative non-fermenting bacteria. Among these, \textit{Pseudomonas aeruginosa} (\textit{P}. aeruginosa) is the most relevant and recurring, so that 30% of CF children and up to 80% of CF adults (25 years old and older) have lungs chronically colonized by this pathogen\textsuperscript{3}.

\textit{P}. aeruginosa isolated from respiratory secretions demonstrates great phenotypic diversity and develops genetic mutations over time to adapt and survive in the complex environment of the CF airway\textsuperscript{4}.

\textit{P}. aeruginosa mucoid phenotype, defined by the exopolysaccharide alginate overproduction within lungs of CF patients, is a hallmark of chronic infection and predictive of poor prognosis. Indeed, mucoid \textit{P}. aeruginosa has also been associated with failure of eradication and, compared to non-mucoid counterpart, exhibits enhanced resistance to multiple antibiotics and host immune effectors\textsuperscript{5}.

Due to current therapeutic treatments, life expectancy for CF patients has consistently grown, reaching a median life of 40 years. Assuming a positive trend of clinical care improvements at the actual rate, CF patients born in 2010 are expected to live up to 50 years of age\textsuperscript{6}.

The intensive use of antimicrobial drugs to fight lung infections inevitably leads to the onset of antibiotic resistant bacterial strains. New antimicrobial compounds should be identified to overcome antibiotic resistance during the treatment of CF lung infections.

Recent investigation has disclosed a few small molecules, such as peptides or mannosides, showing promising efficacy in prevention and treatment of both bacterial and fungal biofilm infection \textit{in vivo}\textsuperscript{7}. Nevertheless, due to their mechanism of action based on a specific binding to a main target, the use of small molecules is known to select more and more resistant strains\textsuperscript{8}. Interestingly in the recent literature appeared some reports on the use of natural derived compounds that showed \textit{in vitro} the potentiality to inhibit the development of CF associated infections\textsuperscript{9–12}. In particular essential oils seemed to be the most promising agents among tested natural compounds\textsuperscript{10,11}. In this study is reported an extensive study on 61 essential oils (EOs) against a panel of 40 bacterial strains isolated from CF patients (see Table 1).

To reduce the \textit{in vitro} procedure and to render the investigation as convergent as possible the following workflow was followed. Unsupervised machine learning algorithms and techniques, as implemented in python language\textsuperscript{13}, were firstly applied to pick-up a fewer number of representative strains (RS) among the panel of 40. To this aim, a number of categorical descriptors were collected and used to cluster the CF isolated strains. The clusters' centroids indicated the RS to be investigated for their susceptibility to a list of commercial EOs at fixed doses. Three EOs showed a great efficacy to reduce the microrganisms growth and were therefore promptly assayed against all the available clinical isolates. The three EOs confirmed the initial assumption demonstrating their ability to inhibit bacterial growth. Gas chromatography coupled with mass spectrometry (GC/MS) was then performed on the three EOs to investigate on the likely chemical components mainly responsible for the antibacterial activity.

| Bacterial strains | Biofilm producer | Bacterial strains | Biofilm producer |
|-------------------|-----------------|-----------------|-----------------|
| 6538P*            | STRONG          | PA01*           | STRONG          |
| 25923*            | STRONG          | PA14*           | STRONG          |
| 1S                | WEAK            | 21P             | STRONG          |
| 2S                | MODERATE        | 22P             | NP              |
| 3S                | WEAK            | 23P             | MODERATE        |
| 4S                | WEAK            | 24P             | NP              |
| 5S                | WEAK            | 25P             | WEAK            |
| 6S                | WEAK            | 26P             | NP              |
| 7S                | MODERATE        | 27P             | NP              |
| 8S                | WEAK            | 28P             | NP              |
| 9S                | WEAK            | 29P             | NP              |
| 10S               | MODERATE        | 30P             | WEAK            |
| 11S               | WEAK            | 31P             | MODERATE        |
| 12S               | WEAK            | 32P             | WEAK            |
| 13S               | WEAK            | 33P             | NP              |
| 14S               | WEAK            | 34P             | WEAK            |
| 15S               | MODERATE        | 35P             | WEAK            |
| 16S               | WEAK            | 36P             | WEAK            |
| 17S               | MODERATE        | 37P             | STRONG          |
| 18S               | WEAK            | 38P             | MODERATE        |
| 19S               | WEAK            | 39P             | WEAK            |
| 20S               | MODERATE        | 40P             | WEAK            |

Table 1. Classification of bacterial strains based on their biofilm formation ability. For \textit{S. aureus}, results were analysed according to Cafiso \textit{et al.}\textsuperscript{14}; for \textit{P. aeruginosa} classification was based on Perez \textit{et al.}\textsuperscript{15}. NP: non biofilm producer. *Reference strains.
Results

Characterization of biofilm formation of clinical bacterial strains. Clinical bacterial strains were investigated for their ability to produce biofilm (Fig. 1). Biofilm formation was evaluated at 37 °C in BHI for 18 h as described in Material and Methods section.

Biofilm formation was also evaluated for four reference strains included in the experimental plan. Figure 1A reports biofilm formation of bacterial strains belonging to *S. aureus* species. Clinical strains, named from 1S to 20S, were classified as “weak” or “moderate” biofilm producers according to Cafiso and coworker, 2007\(^{14}\). Both reference strains for *S. aureus* species are strong biofilm producers. Figure 1B reports biofilm formation of bacterial strains belonging to *P. aeruginosa* species. Clinical strains, named from 21P to 40P, were classified as: “non producer”, “weak”, “moderate” and “strong” biofilm producers according to Perez and Barth, 2011\(^{15}\) (Table 1).

Selection of representative microorganisms by machine learning. The 40 selected strains were divided accordingly to the main strains families into *S. aureus* and *P. aeruginosa* dataset and imported into a python pandas dataframe. The principal components analysis (PCA) indicated that 90% of the variance is explained by the first 10\(^{th}\) principal components (PCs) (Fig. 1S). Nevertheless graphical inspection of the PC1 versus PC2 scores and loadings plots indicated the PCs as potential new variables to cluster the datasets (Fig. 2S).

As a matter of fact, application of the Silhouette Analysis\(^ {16}\) coupled with the k-means clustering\(^ {17}\) to the first 2 PCs indicated the optimal number of clusters to be 6 and 3 for the *P. aeruginosa* and *S. aureus* strains, respectively (Figs. 3S and 4S). For each cluster, the nearest datapoint to cluster centroid was selected yielding to a selection of representative strains to be screened with the commercial EOs. Analysis of data revealed the six samples, precisely 22P, 25P, 26P, 27P, 37P and 39P as the representatives for *P. aeruginosa*, whereas samples 4S, 5S and 19S were selected for *S. aureus*.

Figure 1. Biofilm formation of *S. aureus* clinical and reference strains (A) and *P. aeruginosa* clinical and reference strains (B). The biofilm formation was evaluated after 18 h incubation in polystyrene plates at 37 °C. The data are reported as OD 590 nm after crystal violet staining. Each data point represents the mean ± SD of four independent samples.
Antimicrobial activity of EOs on *P. aeruginosa* and *S. aureus* clinical strains from cystic fibrosis patients. Essential oils were tested for their ability to inhibit bacterial growth of *P. aeruginosa* and *S. aureus* clinical and reference strains. Analysis was performed on three representative *S. aureus* strains and six representative *P. aeruginosa* strains, previously selected by machine learning analysis. EOs were tested at a concentration of 1% v/v (Table 2). Several EOs have shown antimicrobial activity on many bacterial strains. It is worthy to note that the *P. aeruginosa* reference strain PAO1 is the most resistant to the action of EOs, since it was inhibited by only four EOs.

This analysis allowed to identify three EOs active against all the representative strains used, namely cade essential oil (22 in Table 2S, CEO), birch essential oil (32 in Table 2S, BEO) and Ceylon cinnamon peel essential oil (39 in Table 2S, CCPEO). Thus, these 22, 32 and 39 EOs were tested on all clinical bacterial strains. Results summarized in Table 3 confirmed that CEO, BEO and CCPEO exerted a strong and effective bactericidal potency on all tested clinical strains.

Chemical composition analysis of active selected essential oils. The results of GC and GC-MS analyses of the essential oils are reported in Table 3S–5S. In the BEO, 21 components were identified and the major constituents were δ-cadinene, calamenene and creosol (22.2%, 15.2% and 12.8% respectively) (Table 3S). The chemical composition of CCPEO was characterized by the presence of 19 compounds and by a high amount of cinnamaldehyde (49.4%) followed by eugenol (21.2%) (Table 4S). The chemical composition of the CEO indicated 21 components and the most abundant were delta-cadinene (27.7%), calamenene (14.8%) and creosol (12.6%) (Table 5S). At first glance the chemical composition of the CEO seems very similar to that of BEO as the main compounds showed comparable percentages. Among the minor components of CEO, α-selinene (2.2%), aromadendrene (1.1%) and gleenol (1.1%) were found, whereas isoleden (5.7%) was found in BEO. At a deeper analysis the chemical qualitative profiles were compared and a 0.62 tanimoto index was calculated, thus indicating that although displaying a similar chromatogram the two EOs are indeed different. EOs producer was also inquired and their technical staff confirmed that the two oils sharing high similarity quantitative profile in the main constituents.

**Discussion**

Long-term administration of antibiotics to prevent and treat airway infections in CF patients has been shown to be associated with the emergence of multi-drug (MDR) antimicrobial resistant microorganisms15,8.

In particular, mecA/mecC genes acquisition in *S. aureus* and accumulation of resistance mechanisms after antibiotic exposure in *P. aeruginosa*, both key pathogens in CF lung, are a concern in this context9,26.

Multidrug resistance significantly limits effective therapeutic options, affecting clinical outcome and prognosis of patients. For this reason, the identification and development of new antibacterial agents is fundamental to improve survival and quality of life of individuals with CF. Therefore the development of antimicrobial agents provided with novel molecular mechanisms that may allow to control bacterial infectious diseases without diffusing antibacterial resistance is desirable27.

Unsupervised Machine Learning algorithms13 applied to a panel of 40 strains of *S. aureus* and *P. aeruginosa* isolated from CF patients, led to select fewer representative strains using phenotypical and genotypical characteristics as categorical descriptors. Therefore, the antibacterial activity of all tested EOs was initially assessed on 9 selected bacterial strains: six representative strains for *P. aeruginosa* and 3 representative strains for *S. aureus*. The activity of all 61 EOs was also assessed on reference strains. Antimicrobial assays led to identify 3 EOs (CEO, BEO and CCPEO) out of the tested 61, that exhibited the highest antibacterial activity on the previously selected bacterial strains and reference ones. The antibacterial activity of the 3 selected EOs was then extended to all strains of both species. Interestingly all three EOs showed an utmost antimicrobial potency on all studied strains. Nothing can be yet ruled out on the chemical compounds’ role. Future studies involving machine learning application14,21, will be devoted to investigate the importance of chemical constituent either on biofilm modulation or in antibacterial capabilities.

Several papers aimed at elucidating the antimicrobial mechanism of action of EOs. For example, cinnamaldehyde, the major component of cinnamon, is able to disrupt the transmembrane potential of *P. aeruginosa*22.

Furthermore, EOs of different origin (lavender, lemongrass, marjoram, peppermint, tea tree and rosewood) show antimicrobial activity against *Burkholderia cepacia* complex by inducing changes in membrane fatty acid composition, followed by membrane disruption23. Also, EO from Alluaudia procera was active against *S. aureus* ATCC25923, a multi-resistant strain24. Reported data confirmed the possibility to use EOs as therapeutic strategies in multi-resistant strains probably due to the heterogeneous composition of the oils themselves.

Notably, in this work we found EOs antibacterial activity unrelated to the antibiotic resistance profile of each strain. This observation is of particular relevance as it suggests the EOs potential uses by topical administration without taking into account the complexity of drug resistance profile of the microbiota in each single patient.

In conclusion the approach herein applied allowed to minimize the experimental steps and it was possible to identify the most promising EOs on the basis of probabilistic evaluations that confirmed their wide spectra of antibacterial potency with a reduced set of experiments.

From a literature survey (www.scopus.com, accessed 2019 December 13, keywords: essential oil, antibacterial activity and resistance) no evidence of resistance to EOs antibacterial activity has yet been reported. This is a characteristic particularly relevant for antibacterial candidates to be administered for a chronic disease such as CF.

Indeed some papers report an increase of susceptibility to antibiotics after treatment with essential oils25,26. Although a plethora of publications did not show development of resistance to EOs, a very recent publication suggested the induction of efflux pumps and multidrug resistance in *P. aeruginosa* by Cinnamaldehyde, the main component of cinnamon27. Therefore, in light of the recent reports, much still needs to be clarified on the effect of essential oils on bacterial multi-drug resistance.
| Eos ID | 6538P | 25923 | 4S | 5S | 19S | PaO1 | Pa14 | 22P | 25P | 26P | 27P | 37P | 39P |
|--------|-------|-------|----|----|-----|------|------|-----|-----|-----|-----|-----|-----|
| 1      | 1%    | 1%    | 1% |
| 2      |       |       |    |
| 3      |       | 1%    | 1% |
| 4      | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   |     |
| 5      |       | 1%    | 1% |
| 6      | 1%    | 1%    | 1% |
| 7      |       |       |    |
| 8      |       |       |    |
| 9      | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 10     |       | 1%    |
| 11     |       |       |    |
| 12     |       |       |    |
| 13     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 14     |       | 1%    |
| 15     |       |       |    |
| 16     |       |       |    |
| 17     |       |       |    |
| 18     |       |       |    |
| 19     |       |       |    |
| 20     |       |       |    |
| 21     |       |       |    |
| 22     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 23     |       | 1%    |
| 24     |       |       |    |
| 25     |       |       |    |
| 26     |       |       |    |
| 27     |       |       |    |
| 28     |       |       |    |
| 29     |       |       |    |
| 30     |       |       |    |
| 31     |       |       |    |
| 32     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 33     |       | 1%    |
| 34     |       |       |    |
| 35     |       |       |    |
| 36     |       |       |    |
| 37     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 38     |       | 1%    |
| 39     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 40     |       | 1%    |
| 41     |       |       |    |
| 42     |       |       |    |
| 43     |       |       |    |
| 44     |       |       |    |
| 45     |       |       |    |
| 46     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 47     |       | 1%    |
| 48     | 1%    | 1%    | 1% | 1% | 1%  | 1%   | 1%   | 1%  |
| 49     |       | 1%    |
| 50     |       |       |    |
| 51     |       |       |    |
| 52     |       | 1%    | 1% |
| 53     |       |       |    |
| 54     | 1%    |       |    |
| 55     |       |       |    |

Continued
Methods

Ethics approval and informed consent. The approval for this research was granted by the Ethics Committee of Children's Hospital and Institute Research Bambino Gesù in Rome, Italy (No 1437_OPBG_2017 of July 2017), and it was performed according to the principles of the Helsinki Declaration. Informed consent was obtained from all individual participants and all parents/legal guardians included in the study.

Clinical isolates from CF patients. In this study were used 40 bacterial strains (20 S. aureus, 20 P. aeruginosa) obtained from respiratory specimens of 30 CF patients (13 males, 17 females; medium age 20.5) in follow-up at Pediatric Hospital Bambino Gesù (OPBG) of Rome, Italy. In particular, 27 bacterial strains were isolated from sputum, 11 from hypopharyngeal suction and 2 from throat swabs (Tables 4 and 5). As reference strains were used: S. aureus ATCC 6538P (6538P) and S. aureus ATCC 25923 (25923) commonly recognized as reference strains for antimicrobial testing; P. aeruginosa PA01 (PAO1) and P. aeruginosa PA14 respectively recognized as moderately and highly virulent.

Patients were treated according to current standards of care with at least four microbiological controls per year. Informed consent was obtained from all subjects aged 18 years and older and from parents of all subjects under 18 years of age prior to enrolment.

Microbiological cultures have been performed according to approved Guidelines, using selective media, manual and automatic systems (API20NE, Vitek2, MALDI-TOF mass spectrometry) for isolates identification and 16S rRNA sequencing to assess ambiguous identifications.

The strains were selected from a local bacteria collection including about 10,000 CF bacterial isolates.

| Table 2. Antimicrobial activity of EOs listed in Table 2S on representative clinical strains and reference strains of S. aureus and P. aeruginosa. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial strains | CEO | BEO | CCPEO | Bacterial strains | CEO | BEO | CCPEO |
| ATCC6538P | 1% | 1% | 1% | PAO1 | 1% | 1% | 1% |
| ATCC25923 | 1% | 1% | 1% | PA14 | 1% | 1% | 1% |
| SA01 | 1% | 1% | 1% | PA21 | 1% | 1% | 1% |
| SA02 | 1% | 1% | 1% | PA22 | 1% | 1% | 1% |
| SA03 | 1% | 1% | 1% | PA23 | 1% | 1% | 1% |
| SA04 | 1% | 1% | 1% | PA24 | 1% | 1% | 1% |
| SA05 | 1% | 1% | 0.1% | PA25 | 1% | 1% | 1% |
| SA06 | 1% | 1% | 1% | PA26 | 1% | 1% | 1% |
| SA07 | 1% | 1% | 1% | PA27 | 1% | 1% | 1% |
| SA08 | 1% | 1% | 1% | PA28 | 1% | 1% | 1% |
| SA09 | 1% | 1% | 1% | PA29 | 1% | 1% | 1% |
| SA10 | 1% | 1% | 1% | PA30 | 1% | 1% | 1% |
| SA11 | 1% | 1% | 1% | PA31 | 1% | 1% | 1% |
| SA12 | 1% | 1% | 1% | PA32 | 1% | 1% | 1% |
| SA13 | 1% | 1% | 1% | PA33 | 1% | 1% | 1% |
| SA14 | 1% | 1% | 1% | PA34 | 1% | 1% | 1% |
| SA15 | 1% | 1% | 1% | PA35 | 1% | 1% | 1% |
| SA16 | 1% | 1% | 1% | PA36 | 1% | 1% | 1% |
| SA17 | 1% | 1% | 1% | PA37 | 1% | 1% | 1% |
| SA18 | 1% | 1% | 1% | PA38 | 1% | 1% | 1% |
| SA19 | 1% | 1% | 1% | PA39 | 1% | 1% | 1% |
| SA20 | 1% | 1% | 1% | PA40 | 1% | 1% | 1% |

Table 3. Antimicrobial activity corresponding to minimal bactericidal concentration of previously selected EOs on all 40 clinical isolates.
The species *S. aureus* and *P. aeruginosa* have been chosen for their clinical relevance in the natural history of CF disease, since they are related to a worst prognostic impact compared to other pathogens whose role is still under discussion. In order to represent the complexity of CF lung microbiota population attending OPBG Center, a selection of specific strains with different phenotypic and biochemical features has been performed. The strains’ characteristics are described in Tables 4 and 5.

**Qualitative description of the clinical isolates.** Twenty *S. aureus* strains with a different susceptibility profile, belonging to 20 CF patients, were selected: 10 Methicillin-Sensitive (MSSA) and 10 Methicillin-Resistant (MRSA). Among the MRSA strains, three *S. aureus* with phenotypic "small colony variants" (SCVs) have been chosen, characterized by slow growth of small, unpigmented, non-haemolytic colonies.

Antimicrobial susceptibility profiles of MSSA and MRSA isolates were defined by automatic system Vitek2 (Biomerieux, France) or manual system E-test (Liofilchem, Italy). In particular, susceptibility to quinolones (ciprofloxacin, levofloxacin), trimethoprim/sulfamethoxazole, erythromycin, clindamycin, linezolid was assessed, according to EUCAST criteria. Moreover, the clindamycin-inducing resistance test (40% positive test) was performed to classify *S. aureus* according to EUCAST (www.EUCAST.org) criteria. The colistin MIC values were evaluated by automatic system ComASP (Liofilchem, Italy). Twenty isolates belonging to 11 CF patients were also selected (Table 5). The selected strains had been categorized as first, early and late isolates. In particular, seven strains have been associated to first acquisition of *P. aeruginosa* (first strains), 2 strains have been isolated 1 year after the first acquisition (early strains) and 11 strains have been isolated at least 5 years after the onset of chronic colonization (late strains).

Moreover, different phenotypes (mucoid, wrinkle surface, irregular edges or smooth) and strains with different antibiotic susceptibility patterns, e.g. *P. aeruginosa* producing Metallo-Beta-Lactamases (MBL) or *P. aeruginosa* multi-drug resistant (MDR), have been selected.

Sensitivity testing to carbapenems (imipenem, meropenem), piperacillin/tazobactam, aminoglycosides (tobramycin, amikacin), quinolones (ciprofloxacin, levofloxacin), monobactam (aztreonam), and cephalosporins (cefazidime, cefepime) was carried out by Minimum Inhibitory Concentration (MIC) determined by E-test on Mueller Hinton (MH) agar plates, according to EUCAST criteria. The colistin MIC values were evaluated by Broth Microdilution (ComASP Colistin Liofilchem, Italy); 35% of *P. aeruginosa* isolates were MDR (i.e. resistant

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| ID pt | ID | SAM | Date   | Str | Ph  | QUIN | B | ER | CLI | LIN | RCLI | CF  | CPA | GEN |
|-------|----|-----|--------|-----|-----|------|---|----|-----|-----|------|-----|-----|-----|
| 1     | 1S | ESP | 10/11/2006 | MRSA | SCV | R   | S  | Nt | R   | S  | N   | Clp | J   |
| 2     | 2S | ESP | 11/22/2007 | MRSA | SCV | R   | S  | Nt | R   | S  | N   | Ca  | X   |
| 3     | 3S | ESP | 11/15/2009 | MRSA | SCV | S   | Nt | S  | S  | N   | X   |
| 4     | 4S | ESP | 2/20/2009 | MRSA | —   | S   | S  | Nt | S   | P  | X   |
| 5     | 5S | ESP | 11/13/2009 | MRSA | —   | R   | S  | Nt | R   | S  | N   | Sp  | C   |
| 6     | 6S | AT  | 1/10/2011  | MRSA | —   | R   | S  | Nt | R   | S  | P   |
| 7     | 7S | AT  | 4/4/2011   | MRSA | —   | R   | S  | Nt | R   | S  | N   | Ca  | D   |
| 8     | 8S | AT  | 7/22/2013  | MRSA | —   | R   | S  | Nt | S   | S  | N   |
| 9     | 9S | ESP | 1/15/2014  | MRSA | —   | S   | Nt | R   | S  | P   | Ca  | X   |
| 10    | 10S| AT  | 1/29/2015  | MRSA | —   | S   | Nt | R   | S  | N   | Ca/Gd/Pb | G |
| 11    | 11S| AT  | 6/15/2017  | MASA | —   | S   | R   | R   | S  | P   |
| 12    | 12S| AT  | 6/15/2017  | MASA | —   | S   | R   | R   | S  | P   |
| 13    | 13S| AT  | 5/23/2017  | MASA | —   | I   | S   | I  | S   | N   | Sa  | B   |
| 14    | 14S| AT  | 5/25/2017  | MASA | —   | S   | S   | S  | S   | S   |
| 15    | 15S| AT  | 5/24/2017  | MASA | —   | S   | R   | R   | S  | N   | X   |
| 16    | 16S| AT  | 5/26/2017  | MASA | —   | S   | R   | R   | S  | N   | H   |
| 17    | 17S| AT  | 5/25/2017  | MASA | —   | S   | R   | R   | S  | N   | A   |
| 18    | 18S| ESP | 5/24/2017  | MASA | —   | S   | R   | R   | S  | P   | Ca  | X   |
| 19    | 19S| ESP | 6/15/2017  | MASA | —   | S   | R   | R   | S  | P   | X   | L   |
| 20    | 20S| ESP | 5/19/2017  | MASA | —   | R   | S   | R   | S  | P   | X   | F   |
to three or more classes of antimicrobials)\(^3\). Table 3. Table 1 of Supplementary Materials reports the percentage of bacterial strains resulted sensitive or resistant to different classes of antibiotics here tested (Table 1S).

Co-infection by bacterial (\(P.\) aeruginosa/\(S.\) aureus) and fungal agents (\(Aspergillus\) \(fumigatus/\(Candida\) \(albicans/\(Candida\) \(parapsilosis/\(Candida\) \(lusitaniae/\(Scedosporium\) \(prolificans/\(Scedosporium\) \(apiospermum/\(Pseudoallescheria\) \(boydii\)) was also evaluated for each patient (Tables 4 and 5).

Table 6 reports letters’ code correspondence for the strains associated genotype reported in Tables 4 and 5.

Biofilm production assay. The quantification of biofilm production was based on microtiter plate biofilm assay (MTP) as reported in literature\(^1\). Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 \(\mu\)L of the appropriate medium. 1/100 dilution of overnight bacterial cultures was added into each well (about 0.5 OD 600 nm). The plates were incubated aerobically for 18 h at 37 °C. Biofilm formation was measured using crystal violet staining. After incubation, planktonic cells were gently removed; each well was washed three times with double-distilled water and patted dry with a piece of paper towel in an inverted position. To quantify biofilm formation, each well was stained with 0.1% crystal violet and incubated for 15 min at room temperature, rinsed twice with double-distilled water, and thoroughly dried. The dye bound to adherent cells was solubilized with 20% (v/v) glacial acetic acid and 80% (v/v) ethanol. After 30 min of incubation at room temperature, OD590 was measured to quantify the total biomass of biofilm formed in each well. Each data point is composed of 4 independent experiments, each performed at least in 6-replicates.

Statistical analysis of biological evaluation. Data reported were statistically validated using Student’s t-test comparing mean absorbance of treated and untreated samples. The significance of differences between mean absorbance values was calculated using a two-tailed Student’s t-test. A p value of <0.05 was considered significant.

Table 5. The 20 \(Pseudomonas\) \(aeruginosa\) clinical isolates and their characterization by several properties. ID pt: patient identification; ID: strain code; SAM: Sample; Date: Date of collection; Str: Strain; Ph: Phenotype; CAR: Carbapenems; MP: Meropenem; IP: Imipenem; PTC: Piperacillin/tazobactam; AM: Aminoglycosides; QUIN: Quinolones; CI: Ciprofloxacin; LE: Levofloxacin; MB: Monobactam; CEF: Cephalosporins; COL: Colistin; 1 St: \(P.\) aeruginosa first isolate; E: \(P.\) aeruginosa early isolate; L: \(P.\) aeruginosa late isolate; CF: Fungal co-infection; CSA: \(S.\) \(aureus\) co-infection; Gen: pts genotype; BP: Biofilm Producer; Esp:sputum; AT: hypopharyngeal suction; TF: throat swabs; PA: \(P.\) aeruginosa; PA MDR: \(P.\) aeruginosa multi-drug resistant; PA MBL+: \(P.\) aeruginosa Metallo-Beta-Lactamases producing; s: small colony phenotype; w- wrinkled colony surface; m: mucoid colony; i: irregular colony edges; sm: smooth phenotype; R: Resistant; S: Susceptible; I: Intermediate; CA: \(Candida\) \(albicans\); CL: \(Candida\) \(lusitaniae\); X: denotes positive for the feature. See Table 6 showing the correlation between letter code, CFTR gene mutation of the patient and bacterial strain isolated from the same patient.
Chemical composition analysis of active selected essential oils. EOs were purchased from Farmalabor srl (Assago, Italy) and analyzed to characterize their composition as following.

Chemical analyses of EOs were performed by a Turbomass Clarus 500 GC-MS/GC-FID from Perkin Elmer instruments (Waltham, MA, USA) equipped with a Stabilwax fused-silica capillary column (Restek, Bellefonte, PA, USA) (60 m × 0.25 mm, 0.25 mm film thickness). The operating conditions used were as follows: GC oven temperature was kept at 40 °C for 5 min and programmed to 220 °C at a rate of 6 °C/min, and kept constant at 220 °C for 20 min. Helium was used as carrier gas (1.0 mL/min). Solvent delay 0–2 min and scan time 0.2 s. Mass range was from 30 to 350 m/z using electron-impact at 70 eV mode. 1 μL of each essential oil was diluted in 1 mL of methanol and 1 μL of the solution was injected into the GC injector at the temperature of 280 °C. Relative percentages for quantification of the components were calculated by electronic integration of the GC-FID peak areas. The identification of the constituents was achieved by comparing the obtained mass spectra for each component with those reported in mass spectra Nist and Wiley libraries. Linear retention indices (LRI) of each compound were calculated using a mixture of aliphatic hydrocarbons (C8–C30, Ultrasci) injected directly into GC injector at the same temperature program reported above.

Table 6. Table shows the correlation between letter code, CFTR gene mutation of the patient and bacterial strain isolated from the same patient.
Determination of EOs minimal inhibitory concentration (MIC). The MIC was determined as the lowest concentration at which the observable bacterial growth was inhibited. MICs were determined according to the guidelines of Clinical Laboratory Standards Institute (CLSI20). Each EO was solubilized by adding DMSO, to generate a mother stock solution of 1 g/mL. Appropriate dilution (10^6 cfu/mL) of bacterial culture in exponential phase was used. Antimicrobial activity of each EO was evaluated at a concentration of 1 mg/mL range. Experiments were performed in triplicate.

Unsupervised machine learning clustering of clinical isolates
The cluster analysis was implemented in the Python (version 3.6) programming language11. The S. aureus and P. aeruginosa datasets were imported in a jupyter-notebook (version 5.7)34 and the categorical variables loaded into a Pandas35 dataframe were transformed into dummy indicator variables for the subsequent Principal Component Analysis (PCA) using the utilities available in the Pandas (version 0.23) library. The PCA analysis was performed using the scikit-learn library (version 0.20)36 to extract the first 20 principal components (PCs, Fig. 1S). The scores and loadings were graphically inspected on plots generated using the matplotlib library (version 3.0)37 (Fig. 2S). The PCs were used as features for the k-means clustering. Silhouette analysis16 was performed to evaluate the separation distance between the resulting clusters and choose an optimal value for the number of clusters. Optimal number of clusters was identified by the maximum silhouette scores as graphically reported in Fig. 3S. Through k-means, the centroid of each cluster was calculated and the closest datapoint directly indicated the RS (Fig. 4S).

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**Competing interests**
The authors declare no competing interests.

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