Antibiofilm Activity of Cajeput (*Melaleuca Leucadendron*) Eessential Oil against *Stenotrophomonas maltophilia*

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Abstract

Stenotrophomonas maltophilia is the causative agent of nosocomial infections and is characterized by biofilm formation. Primary cell adhesion is crucial in biofilm formation and the type of contact surface has a significant effect. The aim of this work was to assess the antibiofilm activity of *Melaleuca leucadendron* essential oil. The changes in the biofilm profile of *Stenotrophomonas maltophilia* were studied using MALDI-TOF MS Biotyper on glass and wooden surfaces. The molecular differences of biofilms in different days were observed as well. The analysis of the mass spectra of *S. maltophilia* experimental group with *Melaleuca leucadendron* essential oil showed similarity of the experimental spectra and the control planktonic spectrum on the third day. The results of work proved as such MALDI-TOF MS profiling could be a useful technology for the analysis of biofilm properties.

Keywords: *Stenotrophomonas maltophilia*, antibiofilm activity, essential oils, MALDI-TOF MS Biotyper

1. Introduction

In recent decades, studies on bacterial biofilms have been expanding. Biofilms consist of aggregates of microorganisms encased in a polysaccharide extrapolymer matrix called an exopolysaccharide. The association in bacterial biofilms differs significantly from planktonic, as they demonstrate high resistance to antimicrobials, antibiotics and sanitizers [1].

The production of extracellular slime or glycocalyx is a crucial factor in the adherence of bacteria and their protection from host defense mechanisms and effects of antimicrobial agents. It has become clear that biofilm-grown cells express properties distinct from those of planktonic cells, one of which is an increased resistance to antimicrobial agents [2].

Stenotrophomonas maltophilia is an emerging multidrug resistant opportunistic pathogen distributed globally. The increasing incidence of nosocomial and community acquired *S. maltophilia* infections is of particular concern for immunocompromised individuals: this bacterial pathogen is associated with a significant fatality/case ratio. *S. maltophilia* is an environmental bacterium found in aqueous

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habitats, including plant rhizospheres, animals, foods, and water sources. Infections of *S. maltophilia* can occur in a range of organs and tissues; the symptoms commonly are typical for respiratory tract infections [3].

Melaleuca leucadendron is used as antiseptic, antibacterial, antiviral, antifungal, antiprotozoal, antioxidant, anti-inflammatory preparations and oral cleansers. The genus *Melaleuca* is currently represented by about 250 species. One species that occurs in Indonesia and can produce commercial essential oil is *Melaleuca leucadendron*. The leaves and stems of this species produce strongly fragrant essential oils, some of which have useful healing properties. An essential oil of this kind is commonly used in Indonesia as an expectorant, a preparation for sore throats, ointments, stomach upset and mosquito bites. However, there is insufficient scientific knowledge about the biological activity of *M. leucadendron* [4].

M. leucadendron contains 26 compounds, mostly monoterpenes, sesquiterpenes and related alcohols. Analyzes showed the presence of 1,8-cineole, α-terpineol, d-limonene and β-caryophyllene as the main compounds in these oils. Some of the substances contained in this oil are important compounds that are considered to be bioactive substances [5].

The aim of this work was to assess the antibiofilm activity of *Melaleuca leucadendron* essential oil to biofilm profile of *Stenotrophomonas maltophilia.*

2. Materials and methods

Microorganisms

Stenotrophomonas maltophilia with biofilm formation from milk company was acquired.

Essential oil

Melaleuca leucadendron essential oil from Slovak company Hanus was evaluated. *Melaleuca leucadendron* contained eucalyptol (49.23%), α-terpineol (9.92%), limonene (8.12%) and caryophyllene (5.65%).

Biofilm development stages by MALDI-TOF MS

For the analyses by MALDI-TOF MS Biotyper two experiments were performed. The first analysis was evaluated to assess whether or not this method would be able to discriminate the stages of biofilm development as a function of the growth time. The second analysis aimed to use MALDI-TOF MS Biotyper to evaluate if biofilms grown in different substrates and essential oils would exhibit any detectable phenotypical distinction. Growing planktonic cells and biofilms: An amount of 20 ml of Mueller Hinton (MH) broth was transferred to five polypropylene plastic tubes the equal amount to each. A rectangular microscope glass slide and wooden toothpick were placed vertically inside the tube, with 0.1% EOs added to experimental group. A pre-inoculum of *S. maltophilia* was incubated in MH culture medium at 37 °C during 24 h and 10 ml of the pre-inoculum and EOs were added to the tubes content. Tubes were placed in a shaker with an inclination of 45 ° with a shaking speed of 170 rpm at 37 °C. The biofilms formed in each of the tubes were collected at 3, 5, 7, 9, and 12 days after inoculation. The culture medium of the remaining tubes was replaced in the same intervals.

For the analysis of the biofilm, the slide and toothpick were washed two times with ultrapure water and bacteria from the glass slide and wooden toothpick were collected with a sterile rod. Planktonic cells were collected by centrifuging 300 µl of the medium of the 5-day tube at 3000 rpm for 3 min. The supernatant was removed and the pellet was resuspended in ultrapure water and centrifuged again. This procedure was repeated and the resulting pellet was resuspended in 25 ml of ultrapure water (one ml of this suspension was used for each well of the MALDI target plate).

For analysis with MALDI-TOF MS Biotyper the intact materials (biofilm and planktonic cells) were spread in 24 wells for each sample of a polished 96-well MALDI target plate (Bruker Daltonics, Germany). One µL of a-cyano-4-hydroxycinnamic acid saturated matrix solution (10 mg/ml) was used to cover every sample and the plate was dried at room temperature prior to MALDI-TOF MS Biotyper analysis. After crystallization, the samples were analyzed onto a commercial MALDI-TOF mass spectrometer MicroFlex (Bruker Daltonics, Germany) in the linear and positive mode for a range of m/z 2000-20000. The spectra were acquired automatically using a standard procedure. The similarities among the acquired spectra of the same sample were used for generating a standardized global spectrum (MSP), so all stages of biofilm

development were represented by 40 spectra, using the software MALDI Biotyper 3.0 (Bruker Daltonics). From the MSPs of samples, it was generated a dendrogram by the MALDI Biotyper method following standard procedures [6].

Evaluation of bacterial biofilm growth on glass and on toothpick surfaces with MALDI-TOF MS

Eighteen propylene tubes of 50 ml were prepared in the same way as described in previously. Bacteria materials of three tubes were gathered in intervals of 3, 5, 7, 9, 12, and 14 days. The culture media of the remaining tubes was replaced in the same intervals. However, in this experiment, the biological materials grown on glass and toothpick were collected separately with a sterile rod and spread over 12 wells of the MALDI-TOF target plate. Furthermore, from the tubes of the 14th day of cultivation, only planktonic cells were collected in the same way as described in the previous section, and one ml of the material was spread over each of the 36 wells. All MALDI target plate wells were covered with 1 µl of a-cyano-4-hydroxycinnamic acid and analyzed within 24 h. In each group 36 spectra were acquired with one the most representative based on its common features, which was chosen for comparison among the experimental groups using FlexAnalysis 3.0 software (Bruker Daltonics). With the MALDI Biotyper 3.0 software, 11 MSPs were created, and subsequently clustered by a dendrogram using Euclidean distances [6].

3. Results and discussion

Figures 1 show the developmental spectra *of S. maltophilia* biofilm stages throughout the experiment. Cajeput essential oil was added to the experimental groups. The spectra were arranged in pairs according to their degree of growth on different surfaces with the exception of the spectra of planktonic cells, which were obtained from the culture medium.

Analysis of the mass spectra of *S. maltophilia*, where the experimental group contained cajeput essential oil (Figure 1), shows that the similarity of the experimental spectra and the control planktonic spectrum was preserved for 3 days (Figure 1A). At day 5, decrease in the number of detected peaks was observed in the experimental group compared to planktonic cells (Figure 1B), but there were no differences in the similarity of the spectra. The loss of detected peaks was the result of a weak inhibitory effect of cajeput essential oil on the development of *S. maltophilia* biofilm. In the following days, the detected peaks in the experimental spectra increased again. During the 7 to 14 days of the experiment, the effect of essential oil was not demonstrated compared to the control (Figure 1C-1F).

The similar results with *S. maltophilia* were found with *Mentha piperita*, *Pimpinella anisum* and *Coriandrum sativum* essential oils [7,8].

Bacteria have two lifestyles: planktonic, where the cells live freely and are able to move, and an attached way of life, where cells form organized communities known as biofilms. Bacterial biofilms are commonly found in nature on both living and non-living surfaces [9].

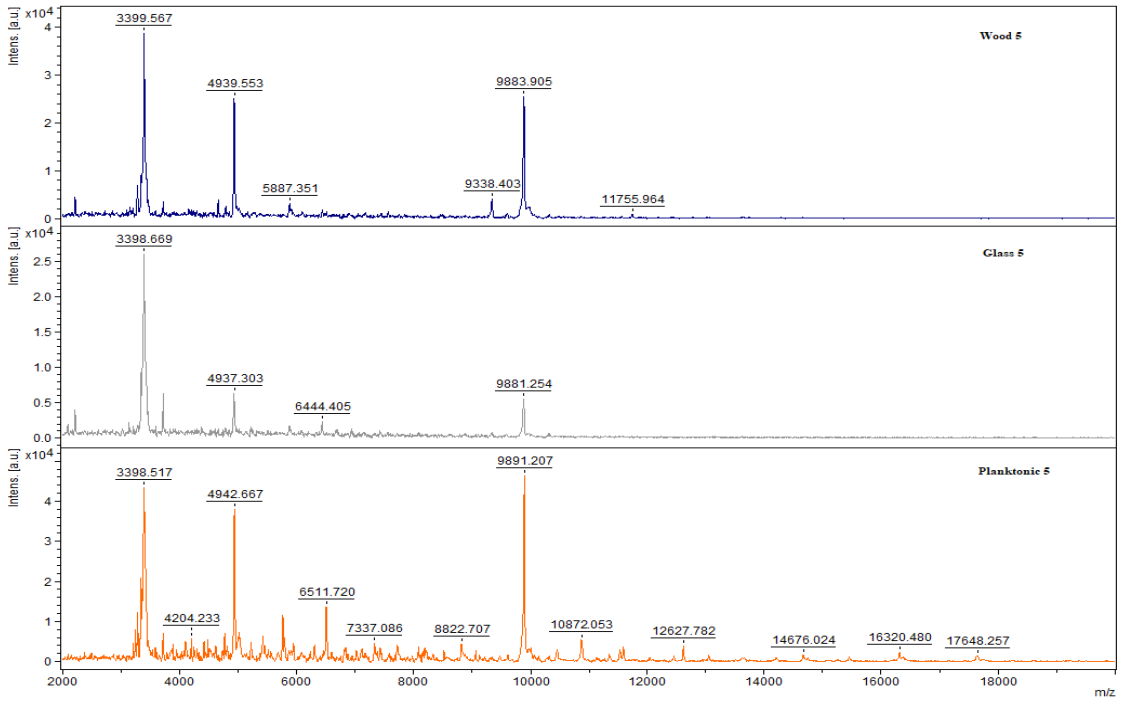
Biofilms are the predominant lifestyle of bacteria in natural environments and have a serious impact on society in many different areas. Therefore, biofilm formation is the subject of growing interest in microbiology, and various bacterial models are currently being studied to better understand the molecular strategies that bacteria undergo in biofilm formation with *Bacillus subtilis* commonly used for this purpose. Biofilms exhibit remarkable architectural features that are the result of sophisticated programs of cell specialization and communication between cells within the community. The biological role of the morphological features of biofilms and investigations of the molecular basis underlying cell differentiation are on the way [10].

To form a biofilm, cells transition from a planktonic to a quiescent state by reducing the expression of flagellar genes while upregulating genes involved in extracellular matrix production [11]. This mechanism is guided by the intensification of external stimuli, for example nutrient depletion, low oxygen levels or adhesion to the surface. Sessile cells begin to form chains by repressing cell wall hydrolases, and the chains enclose themselves in a self-produced extracellular matrix that provides rigidity and is needed to form robust biofilms. Biofilm expansion is mediated by the action of motile cells and the release of surfactant molecules [12].

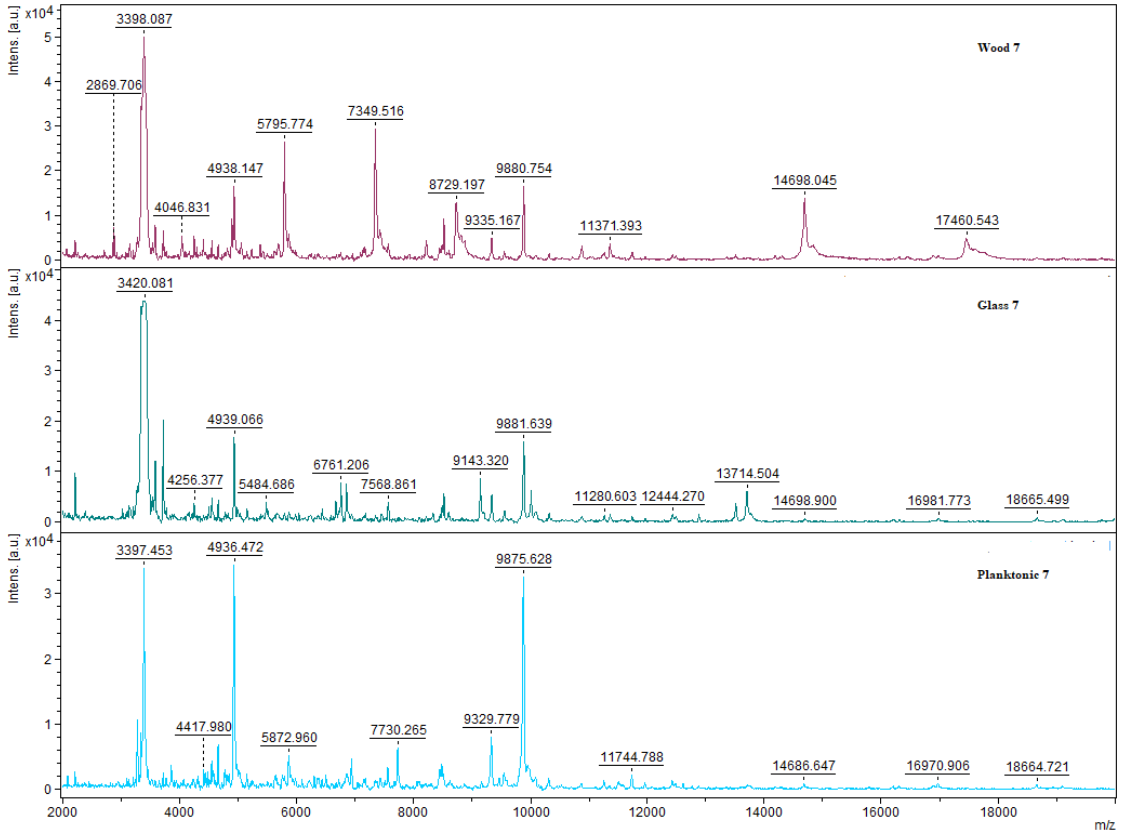
A


Wood 3
3398.364
4257.794
4940.500
6768.487
8522.563
9885.778
11377.183
13538.171
14704.832
Glass 3
3420.397
4938.639
6944.273
6444.595
9881.194
4256.707
5483.816
5881.204
7752.703
9143.079
11276.348
12891.163
13537.653
14698.345
17648.531
19112.398
Planktonic 3
3398.517
4942.667
9891.207
6511.720
4204.233
7337.086
8822.707
10872.053
12627.782
14676.024
16320.480
17648.257
Intens. [a.u.]
x10^4
m/z

B


Wood 5
3399.567
4939.553
9883.905
5887.351
9338.403
11755.964
Glass 5
3398.669
4937.303
9881.254
6444.405
Planktonic 5
3398.517
4942.667
9891.207
6511.720
4204.233
7337.086
8822.707
10872.053
12627.782
14676.024
16320.480
17648.257
Intens. [a.u.]
x10^4
m/z

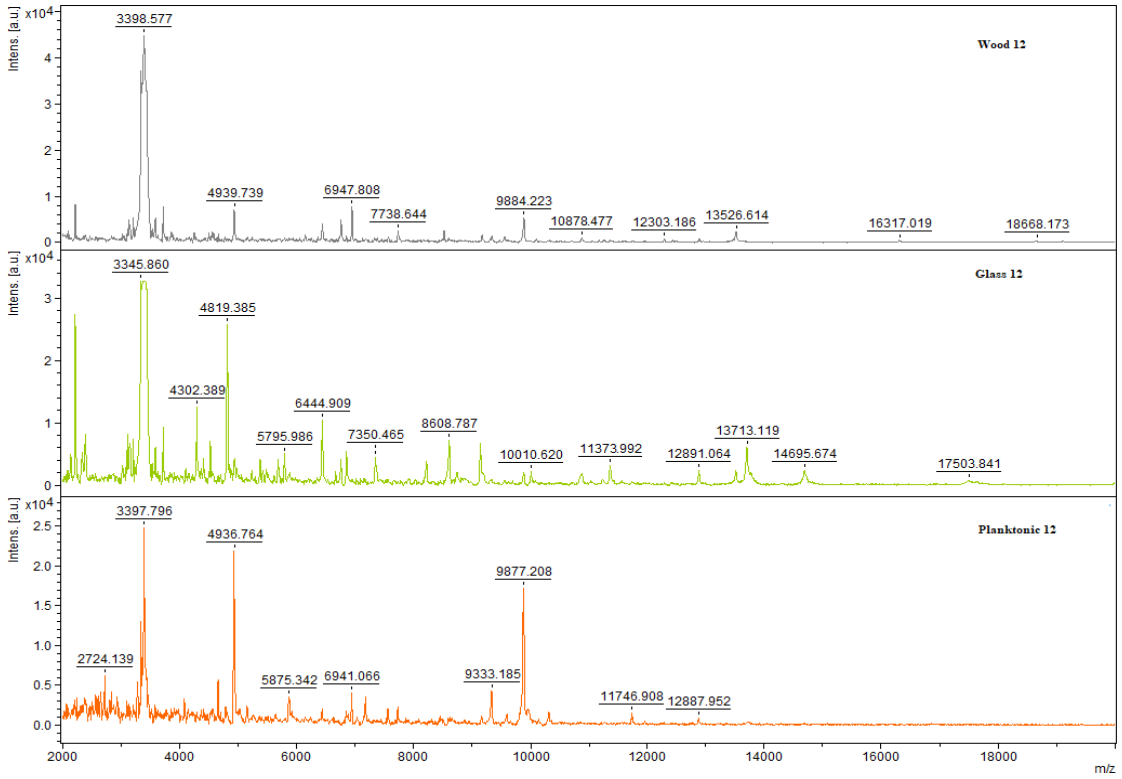
C


Wood 7
3398.087
2869.706
5795.774
7349.516
4938.147
8729.197
9880.754
14698.045
4046.831
9335.167
11371.393
17460.543
Glass 7
3420.081
4939.066
9881.639
4256.377
5484.686
6761.206
7568.861
9143.320
13714.504
11280.603
12444.270
14698.900
16981.773
18665.499
Planktonic 7
3397.453
4936.472
9875.628
4417.980
5872.960
7730.265
9329.779
11744.788
14686.647
16970.906
18664.721
Intens. [a.u.]
x10⁴
m/z

D


Wood 9
3400.003
4941.145
9885.792
4258.407
5797.253
7351.720
8522.290
10882.293
12449.819
14703.460
16320.181
18676.462
Glass 9
3399.399
4820.333
9882.303
4302.211
5685.466
8221.258
9335.590
11372.604
14698.180
Planktonic 9
4559.127
9885.958
6732.477
7738.502
3398.970
9338.160
10883.881
3905.279
5876.345
12454.263
16329.025
14703.639
16984.534
18686.214
Intens. [a.u.]
x10⁴
m/z

E



F

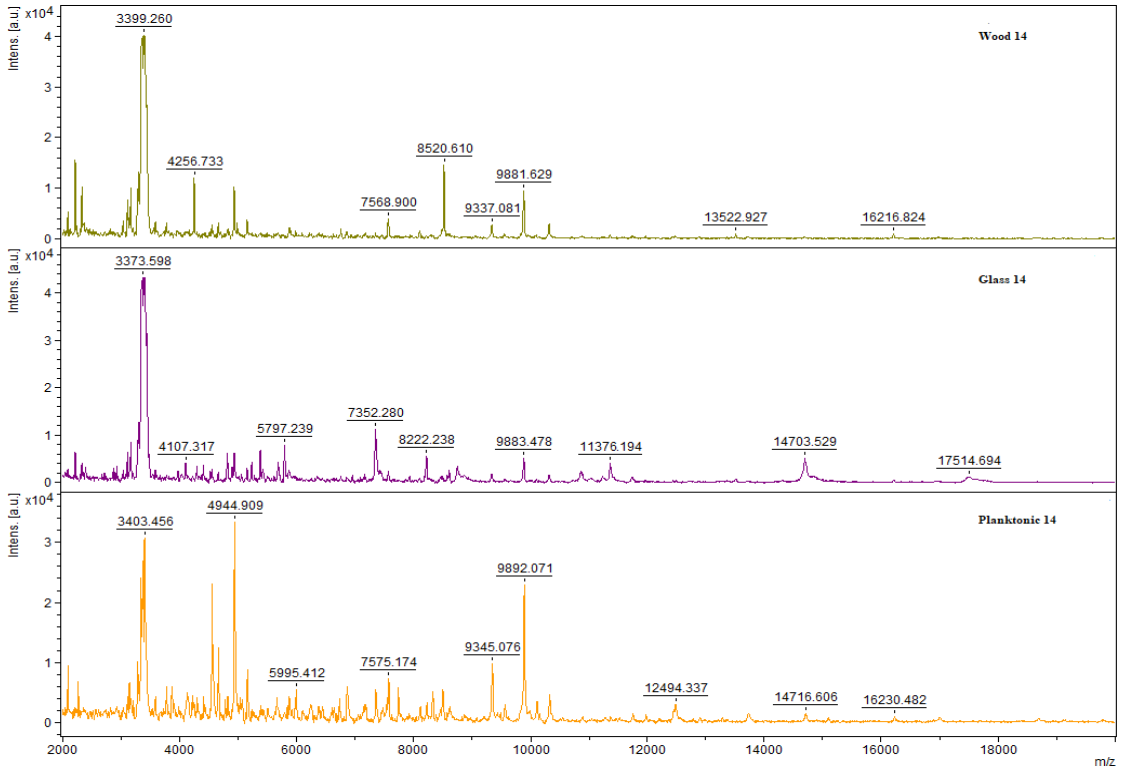


Figure 1. Developmental stages of *S. maltophilia* biofilm with the addition of cajeput (*Melaleuca leucadendron*) essential oil to the experimental groups in the following order: wood, glass, planktonic cells (A - 3rd day, B - 5th day, C - 7th day, D - 9th day, E - 12th day, F - 14th day)

As the biofilm expands and matures, the production of the extracellular matrix continues and the biofilm forms extensive wrinkles. This is due to spatially limited cell death, which dictates the lateral compressive forces induced by the stiffness of the biofilm matrix, followed by mechanical struts of the matrix. Overall, wrinkling for microcolonies has several advantages [13]. It increases the surface-to-volume ratio to ensure better cell access to oxygen, promotes the formation of a complex network of fluid channels in the biofilm, which facilitates the circulation of fluids and 3D structure of the surface serve as preferred sites for sporulation and spreading of spores [14].

The solid extracellular matrix that forms biofilms contains exopolysaccharides (EPS) and proteins. The functions of EPS are not yet clear, but EPS-defective mutants were known to develop flat colonies and extremely fragile thin coatings, however, they were still able to grow and contained the remaining extracellular material due to the presence of other components of the protein matrix [14]. TasA and TapA are two secreted proteins, which provide structural integrity to the matrix. Of those, TasA is a functional amyloid protein secreted into the extracellular space, where it assembles into fibers that are anchored to the cell wall by TapA. These mutants also produce cell chains that are not held together [15].

One of the most important challenges in the study of biofilm formation is to find new methodologies for dispersing biofilms. Biofilm dispersion could be beneficial, for example, in the eradication of chronic biofilm-mediated infections [16]. For this reason, many scientists are actively looking for small molecules that can effectively induce biofilm dispersion. However, the molecules must meet certain criteria that need to be considered in other applications, including that they are harmless to humans and capable of reproducing phenotypes defective in the biofilm. Nevertheless, genetic phenotypes are stable, reproducible, and often difficult to replicate by chemical inhibition, a feature that complicates the search for biofilm dispersants [17].

4. Conclusions

The results of the mass spectra show that *Melaleuca leucadendron* essential oil had very weak effect on the inhibition of the biofilm, which was manifested by decrease in the detected peaks on 5th of the experiment while maintaining the similarity of the spectra with the control planktonic spectrum. The dendrogram could not be constructed due to the high affinity of the control and experimental groups, which confirms the weak to no effect of this essential oil on the inhibition of *S. maltophilia* biofilm.

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References

1. Donlan, R. M., Costerton, J. W., Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Revision, 2002, 15, 167-193.
2. Elvers, K. T., Leeming, K., Lappin-Scott, H. M., Binary culture biofilm formation by *Stenotrophomonas maltophilia* and *Fusarium oxysporum*, 2001, Journal of Industrial Microbiology and Biotechnology, 26, 178–183.
3. Brooke, J. S., *Stenotrophomonas maltophilia*: an Emerging Global Opportunistic Pathogen, Clinical Microbiological Review, 2012, 25 (1), 2–41.
4. Pujiarti, R., Ohtani, Y., Ichiura, H., Physicochemical properties and chemical compositions of Melaleuca leucadendron leaf oils taken from the plantations in Java, Indonesia, Journal of Wood Science, 2011, 57(1), 446-451.
5. Cleber, J. S., Luiz, C. A. B., Celia, R. A. M., Antonio, L. P., Fraz, M. D. I., Comparative study of the essential oils of seven Melaleuca *(Myrtaceae)* species grown in Brazil, Flavour and Fragrance Journal, 2007, 22, 474–478.
6. Pereira, F.D.E.S., Bonatto, C.C., Lopes, C.A.P., Pereira, A.L., Silva, L.P., Use of MALDI-TOF mass spectrometry to analyze the molecular profile of *Pseudomonas aeruginosa* biofilms grown on glass and plastic surfaces, Microbial Pathogenesis, 2015, 86, 32-37.
7. Kačániová, M., Galovičová, L., Ivanišová, E., Kántor, A., Kunová, S., Terentjeva, M., Anti-biofilm and Antiradical Activity of Different Essential Oils Scientific Papers: Animal Science and Biotechnologies, 2019, 52(1), 82-88.
8. Kačániová, M., Galovičová, L, Ivanišová, E., Vukovic, N.L., Štefániková, J., Valková, V., Borotová, P., Žiarovská, J., Terentjeva, M., Felšöciová, S., Tvrdá,

E., Antioxidant, Antimicrobial and Antibiofilm Activity of Coriander (*Coriandrum sativum* L.) Essential Oil for Its Application in Foods, Foods, 2020, 9, 282.
9. Cassagne, C., Normand, A. C., Bonzon, L., L'ollivier, C., Gautier, M., Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., Marrie, T. J., Bacterial biofilms in nature and disease, Annual Review of Microbiology, 1987, 41(1), 435-464.
10. Mielich-Süss, B., Lopez, D., Molecular mechanisms involved in Bacillus subtilis biofilm formation, Environmental Microbiology, 2015, 17(3), 555–565.
11. Cairns, L. S., Marlow, V. L., Bissett, E., Ostrowski, A., Stanley-Wall, N. R. A mechanical signal transmitted by the flagellum controls signalling in *Bacillus subtilis*, Molecular Microbiology, 2013, 90(1), 6–21.
12. Angelini, T. E., Roper, M., Kolter, R., Weitz, D. A., Brenner, M. P., Bacillus subtilis spreads by surfing on waves of surfactant, Proceedings of the National Academy of Sciences of the USA, 2009, 106(4)3, 18109–18113.
13. Kolodkin-Gal, I., Elsholz, A. K., Muth, C., Girguis, P. R., Kolter, R., Losick, R., Respiration control of multicellularity in Bacillus subtilis by a complex of the cytochrome chain with a membrane-embedded histidine kinase, Genes and development, 2013, 27(8), 887–899
14. Branda, S. S., Chu, F., Kearns, D. B., Losick, R., Kolter, R., A major protein component of the Bacillus subtilis biofilm matrix, Molecular Microbiology, 2006, 59(4), 1229–1238.
15. Romero, D., Vlamakis, H., Losick, R., Kolter, R., An accessory protein required for anchoring and assembly of amyloid fibres in B. subtilis biofilms, Molecular Microbiology, 2011, 80(5), 1155–1168.
16. Mirani, Z. A., Aziz, M., Khan, M. N., Lal, I., Hassan, N. U., Khan, S. I., Biofilm formation and dispersal of Staphylococcus aureus under the influence of oxacillin, Microbial Pathogenesis, 2013, 61(6), 66–72.
17. Hobley, L., Kim, S. H., Maezato, Y., Wyllie, S., Fairlamb, A. H., Stanley-Wall, N. R., Michael, A. J., Norspermidine Is Not a Self-Produced Trigger for Biofilm Disassembly, Cell, 2014, 156(4), 844–854.