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Use of MALDI-TOF mass spectrometry to analyze the molecular profile of *Pseudomonas aeruginosa* biofilms grown on glass and plastic surfaces

Flávio D.E.S. Pereira [a, b], Cínthia C. Bonatto [a, c], Cláudio A.P. Lopes [d], Alex L. Pereira [b], Luciano P. Silva [a, c, e, *]

[a] *Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil*
[b] *Faculdade de Ceilândia, Universidade de Brasília (UnB), Brasília, DF, Brazil*
[c] *Programa de Pós-Graduação em Biologia Animal, UnB, Brasília, DF, Brazil*
[d] *Laboratório de Microscopia Eletrônica, Departamento de Biologia Celular, Universidade de Brasília (UnB), Brasília, DF, Brazil*
[e] *Programa de Pós-Graduação em Nanociência e Nanobiotecnologia, UnB, Brasília, DF, Brazil*

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ABSTRACT

Biofilms are microbial sessile communities attached to surfaces that are known for causing many medical problems. A bacterial biofilm of clinical relevance is formed by the gram-negative bacteria *Pseudomonas aeruginosa*. During the formation of a biofilm, the initial adhesion of the cells is of crucial importance, and the characteristics of the contact surface have great influence on this step. In the present study, we aimed to use matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling as a new methodology to monitor *P. aeruginosa* biofilm development. Biofilms were grown within polypropylene tubes containing a glass slide, and were harvested after 3, 5, 7, 9, or 12 days of inoculation. Planktonic cells were obtained separately by centrifugation as control. Two independent MALDI-TOF experiments were performed, one by collecting biofilms from both the glass slide and the polypropylene tube internal surface, and the other by acquiring biofilms from these surfaces separately. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to evaluate the morphological progression of the biofilm. The molecular results showed that MALDI profiling is able not only to distinguish between different biofilm stages, but it is also appropriate to indicate when the biofilm cells are released at the dispersion stage, which occurred first on polypropylene surface. Finally, the present study pointed out that MALDI profiling may emerge as a promising tool for the clinical diagnostic and prognostic workup of biofilms formation and control.

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1. Introduction

Bacteria generally possess two possible distinct modes of life: the planktonic, by which the cells are able to move freely; and the sessile, by which the cells may form organized communities known as biofilms. Bacterial biofilms are commonly found in nature, being formed by colonies adhered to biotic or abiotic surfaces [1]. The adherence and growth of a bacterial biofilm involves a number of phenotypical changes, such as motility alterations, production of quorum sensing signals, and synthesis of a matrix of extracellular polymeric substances (EPS) [2].

The formation of bacterial biofilms can cause serious medical problems since they represent a reservoir of bacteria that can be shed to the body, leading to chronic infections [3]. Besides, this mode of life protects the microbial cells against antibiotics action and other physical or chemical challenges, making them difficult to eliminate with conventional therapies *in vivo* and sterilization procedures in objects [4]. The proximity of the constituent cells of a biofilm also favors the exchange of genetic materials, responsible for antibiotic resistance and consequently increases pathogen virulence and endurance [5]. Ultimately, host exacerbated responses may cause chronic inflammation and extensive tissue damage, without being able to remove the bacteria [3].

* Corresponding author. Embrapa Recursos Genéticos e Biotecnologia, Brasília, 70770-917, DF, Brazil.
E-mail addresses: luciano.paulino@embrapa.br, paulinoluciano@unb.br, lucianopaulinosilva@gmail.com (L.P. Silva).

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The bacterial adhesion is one of the most important steps during biofilm formation and finally establishment of a chronic infection. This process is influenced by several factors, such as temperature, pH, bacterial concentrations, flow conditions, and surface characteristics. Among surface attributes, it is important to highlight the chemical composition, hydrophobicity, electrostatic charge, and surface roughness [3]. Whereas the biofilm undergoes several morphological (e.g. thickness and organization) and molecular changes (e.g. protein expression levels, phenotypic changes, and quorum sensing), it is interesting the use of complementary methodologies for effective understanding of the whole process. Microscopy techniques have been used to characterize the morphology and structure of bacterial biofilms [4–6]. More recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully used to investigate the molecular profile of bacteria [9,10], but, from our knowledge, there is no report that use the molecular profiling data obtained by MALDI-TOF MS in order to discriminate the different stages of bacterial biofilms formation.

A biofilm that has a high impact in chronic bacterial infection, being known especially for the damage it causes in lungs of patients with cystic fibrosis, is formed by the Gram-negative bacteria *Pseudomonas aeruginosa* [7]. This is an opportunistic human pathogen that can cause acute infections in hospitalized people, especially those immunocompromised, such as patients with acquired immunodeficiency syndrome (AIDS), and neutropenic patients due to chemotherapy treatments. *P. aeruginosa* also cause deleterious infections in individuals with burns, pneumonia in patients receiving artificial ventilation, and keratitis in contact lens wearers [8,11]. The aim of the present study was to evaluate possible changes in the molecular profile of biofilms from *P. aeruginosa* in varying stages of maturity in two distinct surfaces (glass and polypropylene) using MALDI-TOF MS. In addition, the morphology of such biofilm stages was examined and compared by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

2. Material and methods

2.1. Evaluation of biofilm development stages by MALDI-TOF MS

Two experiments were performed for the analyses by MALDI-TOF MS. The first experiment was performed 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 experiment aimed to use MALDI-TOF MS to evaluate if biofilms grown in different substrates would exhibit any detectable phenotypical distinction.

Growing planktonic cells and biofilms: Five polypropylene plastic tubes of 50 ml capacity received 20 ml each of Mueller Hinton (MH) culture medium. A rectangular microscope glass slide was placed vertically inside the tube. A pre-inoculum of *P. aeruginosa* strain ATCC 27853 was incubated in MH culture medium at 37 °C during 24 h. Ten μl of the pre-inoculum were added to the 50 ml polypropylene tubes, which 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 3, 5, 7, 9, and 12 days after the inoculation. The culture medium of the remaining tubes was replaced in the same intervals. For the analysis of the biofilm, the tube and slide were washed two times with ultrapure water and bacteria from the tube internal surface and glass slide were collected with a sterile toothpick. Planktonic cells were collected by centrifuging 300 μl of the medium of the 5-day tube at 3000 *g* 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 μl of ultrapure water (one μl of this suspension was used for each well of the MALDI target plate).

MALDI-TOF MS: The intact materials (biofilm and planktonic cells) were then spread in 24 wells for each sample in a polished 96-well MALDI target plate (Bruker Daltonics, Germany). One μl of α-cyano-4-hydroxycinnamic acid saturated matrix solution (10 mg/ml) was used to cover every sample and dried at room temperature prior to MALDI-TOF MS 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.

MALDI Biotyper approach: 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 24 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.

2.2. Use of MALDI-TOF MS to evaluate bacterial biofilm growth on glass and on polypropylene surfaces

Eighteen propylene tubes of 50 ml were prepared in the same way as described in the previous section. Bacteria materials of three tubes were gathered in each interval 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 polypropylene were collected separately with a sterile toothpick, and spread over 12 wells of the MALDI-TOF target plate. Furthermore, from the tubes of the 14th day, only planktonic cells were collected in the same way as described in the previous section, and one μl of the material was spread over each of the 36 wells. All MALDI target plate wells were always covered with one μl of α-cyano-4-hydroxycinnamic acid and analyzed within 24 h.

In summary, 36 spectra were acquired in every group, and one was chosen from these as the most representative based on its common features and displayed 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.

2.3. Morphological characterization of the biofilms and planktonic cells

Atomic force microscopy (AFM): For the morphological characterization, polypropylene tubes of 15 ml were filled with 1 ml of MH culture medium and a circular glass coverslip was placed inside each tube. Every tube received a drop from a pre-inoculum, prepared in the same way of the previous sections, with an inoculating loop. They were placed in a shaker at the same conditions as the tubes from the previous sections. The coverslips were collected at 3, 5, 7, 9, and 12 days intervals, and every remaining tube had its medium replaced in these gaps. Two μl of planktonic cells grown for two days were spread over a coverslip and dried at room temperature. *P. aeruginosa* biofilms present in the coverslips were fixed using 1 ml of modified Karnovsky fixative (2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 0.1 M cacodylate buffer, pH 7.2) for 3 h at room temperature, being stored afterwards in cacodylate at 4 °C. Afterwards, samples were mounted on a metal sample support using double-sided adhesive tape being wrapped at the base of the instrument. The analyses were performed at ambient air with a temperature of approximately 22 °C using an atomic force microscope Shimadzu SPM-9600 (Shimadzu, Japan) equipped with

a scanner with maximum scan area of 125 × 125 μm in dynamic phase mode using a rectangular cantilever integrated with conical silicon tip having a spring constant of 10–130 N/m and resonance frequency of 204–497 kHz, and using a sweep frequency of 1 Hz. The images (10 × 10 μm) were acquired at a resolution of 512 × 512 lines and processed using SPM-9600 off-line software. The processing consisted in an automatic plane fit leveling of the surface.

Scanning electron microscopy (SEM): *P. aeruginosa* biofilms and planktonic cell samples were grown and fixed as previously described for AFM. The coverslips were then washed three times with sodium cacodylate buffer (0.1 M, pH 7.2) and post-fixed in 1% osmium tetroxide in the absence of light for 1 h. After this, the samples were washed three times with distilled water and progressively dehydrated in crescent concentrations of acetone baths (30–100%). In the last acetone bath (100%), the critical point drying was done using liquid CO_2 (Balzers - CPD030). After that, the samples were mounted in stubs and sputter coated with a thin gold layer. The samples were analyzed using a Jeol® JSM 840A scanning electron microscope (Jeol, Japan).

3. Results

The images obtained by SEM and AFM (Fig. 1) showed the morphological development of the *P. aeruginosa* biofilm, starting at the point where the bacteria showed planktonic behavior (Fig. 1a). In such stage, the bacteria population has no tridimensional conformation and there is no evidence of any interaction between cells, which can be observed by their well-defined contours. In contrast, the 3-day biofilm already presented a differentiated configuration, with some regions displaying higher thickness and cell borders surrounded by materials that constitute the extracellular matrix (Fig. 1c). The AFM image of the 3-day biofilm (Fig. 1b) further evidenced the contiguity of the cells, and their organization in a plural number of layers. Also, the bacterial borders were not evident due to the adhesion among adjacent bacteria or due to presence of large amounts of surrounding EPS. As the biofilms formation age progressed, its structure became more and more complex, culminating in a well-developed functional microcolony, evidenced in Fig. 1d by SEM.

Fig. 2a presents the dendrogram generated by the first MALDI-TOF MS experiment. These results indicated that the MSPs of the groups were distinguishable by the use of MALDI profiling approach, since they have been separated into different clusters. It can be observed that the planktonic stage of *P. aeruginosa* showed the highest difference, in terms of MSP distance level, from the 3- and 5-day biofilms. Moreover, their divergence seems to decrease as the biofilm grows older, as can be observed by the clustering of the planktonic, 9-day and 12-day groups in the same main cluster.

The dendrogram of the second MALDI-TOF MS experiment, that was carried to verify possible differences shown by biofilms grown on glass slides and on polypropylene internal surfaces, is also displayed in Fig. 2b. In this analysis, the 3-day biofilms clustered closer to the free-living (planktonic) cells, followed by the 12-day biofilm grown on the tube internal surface. It is also noteworthy that the 7- and 9-day biofilms that grew on plastic were clustered in the same branch, whereas the 7- and 9-day biofilms that grew on glass were

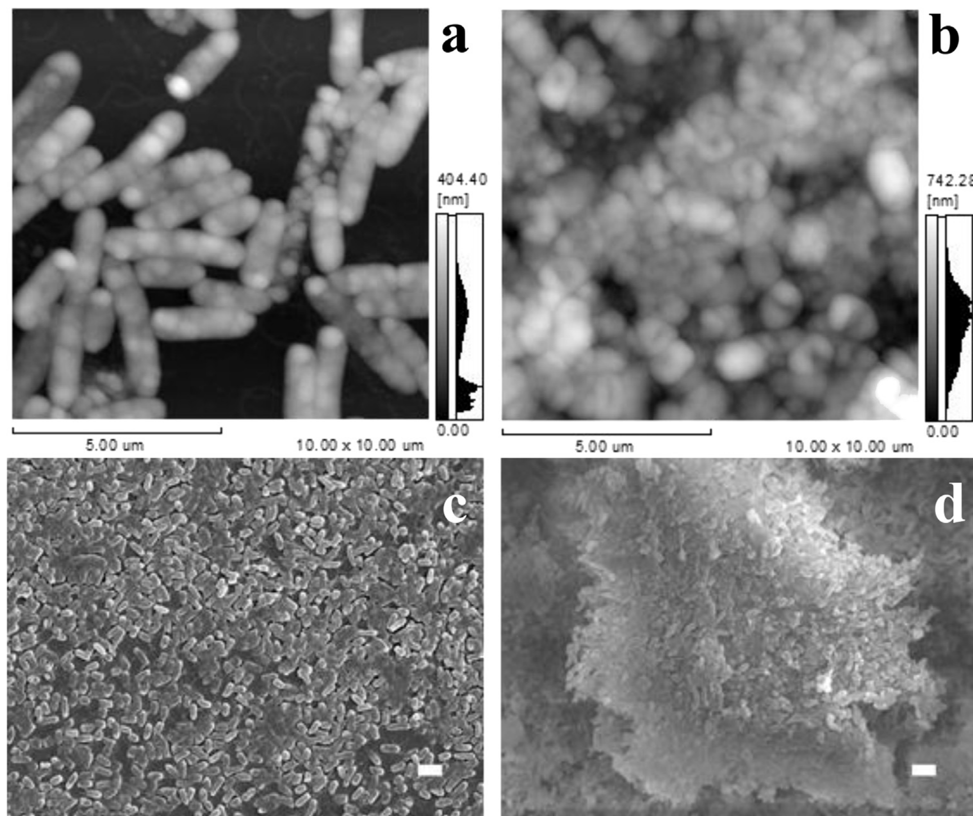

Fig. 1. Atomic force microscopy (AFM) and scanning electron microscopy (SEM) images depicting the most relevant stages in the morphological progression of the *P. aeruginosa* biofilm. a: Planktonic cells observed by AFM; it is possible to note the presence of many filaments, likely flagella. b: 3-day biofilm observed by AFM; note how the cells appear in greater contiguity one with each other, and how difficult is to precise their contours due to cell-to-cell interactions. c: 3-day biofilm observed by SEM (scale bar = 2 μm); the magnification allows better observation of the overall structure of the biofilm, which is more homogeneous than in latter stages. d: 12-day biofilm observed by SEM (scale bar = 2 μm); the image evidences a well-developed microcolony, surrounded by regions of multilayered cells in the relatively flat biofilm.

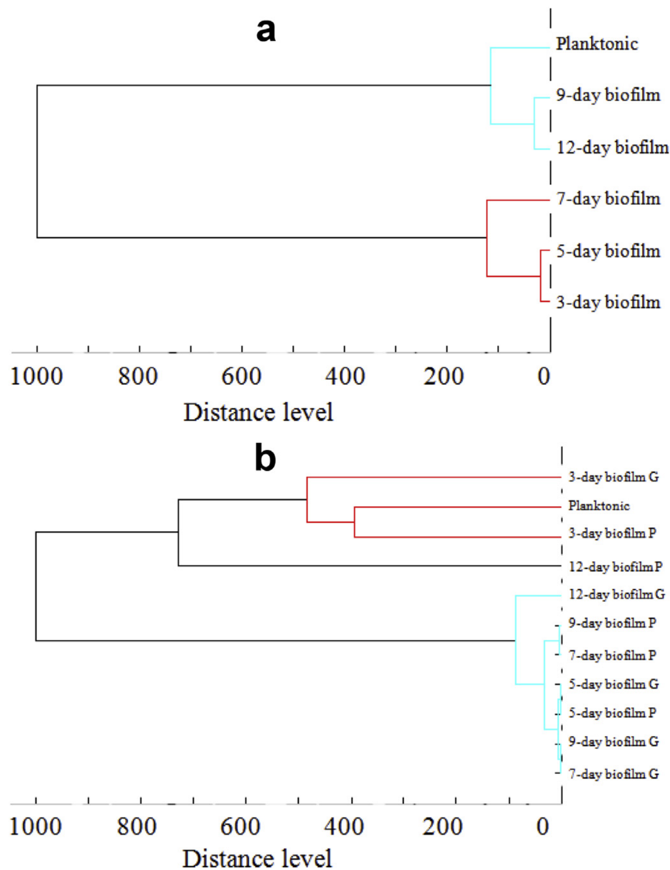

Fig. 2. Dendrograms generated using the MSPs of *P. aeruginosa* biofilms at different developmental stages. a: first experiment, in which samples were collected from both the tube internal surface and glass surface. The separation of MSPs into clades shows that MALDI-TOF MS is able to distinguish between the different stages of biofilm growth. b: second experiment, in which the samples were collected separately from the plastic tube internal surface (P) and from the glass surface (G). The array of MSPs reveals differences of biofilm growth on those different surfaces.

also grouped together, but in a different branch. The spectra chosen as representative for each biofilm development stage in this experiment are shown in Fig. 3. With the exception of the spectrum representing planktonic cells, which were obtained from the culture medium, the remaining spectra were grouped in pairs according to their time of growth, so that biofilms obtained from different surfaces could be viewed together.

4. Discussion

The sessile bacterial mode of life as biofilm differs greatly from the planktonic behavior. The distinction does not lie solely on the changes regarding motility and adhesion, but also in the differential expression of genes and consequent production of proteins that allows the transition from free-living cells to a biofilm community [12–14]. Some authors have divided the process of biofilm formation roughly into three important steps: attachment, maturation, and detachment [15]. Sauer and collaborators (2002) have further divided the progress of the *P. aeruginosa* biofilm taking into account different phenotypes displayed along its development [2].

The aim of the present study was to observe some possible differences in the molecular profiles of *P. aeruginosa* in several time intervals using MALDI-TOF MS. In addition, in order to make sure the bacterial biofilm acquired an aspect of maturity, SEM and AFM were used to visually inspect its maturation stage. The planktonic cells images obtained from both microscopies evidenced that the bacteria were not organized in multiple cell layers and had no interaction one with each other, even though they appeared close to their neighbor cells. In the AFM images, it was even possible to see some filaments, most likely flagella, which have great importance in free-living motility as well as in the initial attachment to surfaces, such as glass [2].

The images of the 12-day biofilm showed the formation of microcolonies (Fig. 1d), a type of organization observed in mature biofilms, which has great importance in its dispersion phase [2,16,17]. Although SEM is a method that allows the visualization of the biofilm structure, it is not possible to observe details of the extracellular matrix due to the dehydration of the prepared samples. However, it is possible that the elements such as fibers seen in the images are condensed matrix components or polymers [18].

Regarding the MSP dendrogram of the first MALDI-TOF MS experiment, it is possible to observe two main clades: one containing only biofilms (3-, 5-, and 7-day) and one containing the planktonic cells along with the oldest stages of biofilms development (9- and 12-day). The reason by which the latter groups were displayed closer to the planktonic MSP is most likely because those biofilms probably followed-up the dispersion stage. Sauer and colleagues have previously observed that after 9 days, cell clusters undergo modifications, in which bacteria from biofilm core reacquire motility in order to disperse. This reversion back to the planktonic condition was supported by the fact that the protein patterns obtained for dispersion stage biofilms were closer to the patterns observed for planktonic bacteria than for the previous stage of biofilm maturation [2].

The second MALDI-TOF MS experiment was performed to investigate whether biofilms grown on plastic (polypropylene) or glass had a different progression over time. The MSP dendrogram presented three main clusters. In one of them, the planktonic cells were clustered with both of the 3-day biofilms. This suggests that the biofilms at this stage had little maturity, and most likely the bacteria took longer to colonize the surface than in the previous experiment.

Another cluster of the dendrogram contained exclusively the 12-day biofilm grown on plastic, and its proximity to the planktonic cell and 3-day biofilms suggests that this biofilm had also reached the dispersal stage, therefore becoming phenotypically more similar to free-living cells, in a similar way that occurred in the studies by Sauer and collaborators (2002) [2]. The third cluster contained all remaining biofilm groups. In its organization, it is interesting to note the formation of three grouped pairs. One of the pairs consisted of both 5-day biofilms, the second of the 7- and 9-day biofilms grown on glass and the third of the 7- and 9-day biofilms grown on the plastic tubes. The 12-days biofilm grown on glass was in a singular secondary branch. This fact suggests that after 5 days of maturation, the biofilms display specific phenotypes according to the type of surface where they are attached to, a reasonable assumption, considering how surface interactions affect molecular expressions in *P. aeruginosa* [19,20].

Furthermore, the disposition of the groups in the dendrogram suggests that the biofilms that grew on plastic tube surfaces matured more rapidly towards the dispersal phase, since the 12-day biofilm grown in plastic was most likely already in dispersion phase, whilst the 12-day biofilm grown in glass was more similar to immature biofilms.

It was expected that the ease with which the bacteria cells adhered to a support would have a huge impact on the time necessary for a biofilm to reach the mature state. However, the *P. aeruginosa* biofilms seem to have colonized both types of surfaces at quite similar (but not identical) rates. In fact, the dendrogram even suggests that the 3-day biofilm that grew on glass was slightly

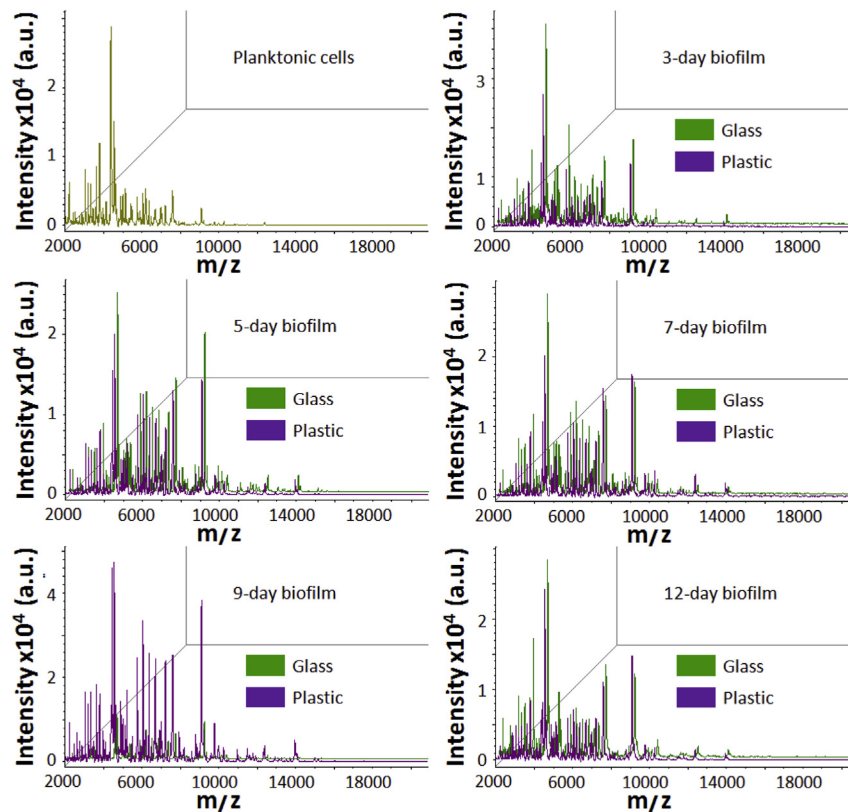

Fig. 3. Representative MALDI-TOF mass spectra from the second experiment chosen for the different *P. aeruginosa* biofilm formation stages. Each stage, with the exception of the planktonic cells, shows two spectra, one of them representing biofilms grown on glass surface (green); the other representing biofilms grown on plastic/polypropylene tube internal surface (purple). The *y* axes represent the signal intensity in arbitrary units and *x* axes represent the mass-to-charge ratios (*m*/*z*) of the ions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

more developed. In that way, it is possible that the characteristics of the surface, such as charge, hydrophobicity, and roughness, as well as the bacterial strain [21,22], have dictated the phenotypical adaptations of the bacteria, causing them to develop on different surfaces.

5. Conclusion

MALDI-TOF MS profiling is a very useful tool aiming the identification of microorganisms. In the present study, instead of focusing on different bacteria species, we decided to apply a MALDI-TOF profiling method to evaluate different stages of *P. aeruginosa* biofilm development and compare such results with the morphological behavior of this bacteria biofilm. The results showed not only that the approach is sensitive enough to detect phenotypical changes in the biofilm progression, but also is able to detect some distinct characteristics related to the surface on which the bacteria were grown. It was also observed that the biofilms that grew on plastic had a quicker maturation, reaching earlier the dispersal stage.

Ethical statement

All ethical principles were considered when performing the experiments.

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