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ARTICLE

Exploring Bacterial Resistant Metabolism by Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry Imaging

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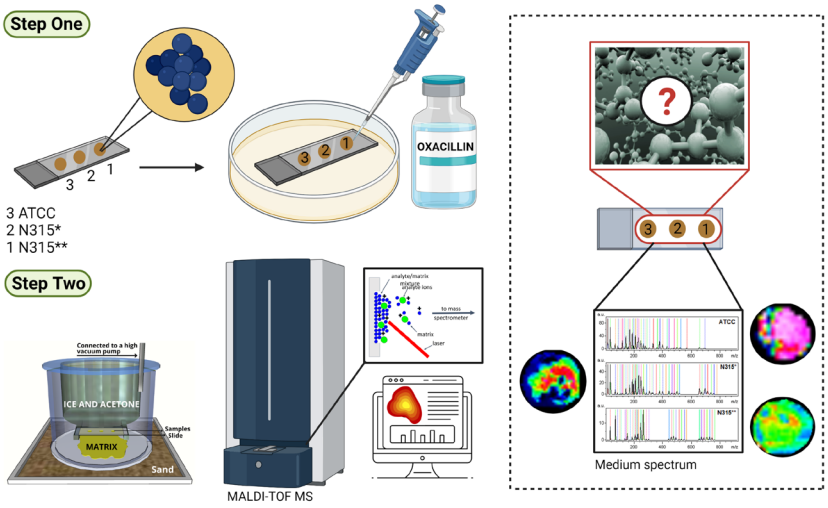

The inappropriate and excessive use of antibiotics for the treatment of bacterial infections has led to the increasing presence of resistant and multidrug-resistant bacteria both in hospital settings and in the community. Thus, understanding the metabolism of resistant bacteria is extremely important to combat them more efficiently. In this scenario, mass spectrometry imaging (MSI) is considered a promising technique for understanding the resistant characteristics of such bacteria and how they can potentially be treated. This process consists of the identification of different ions on the surface of the colonies and the identification of potential metabolites that characterize antibiotic resistance, upon comparison with susceptible bacteria of the same species. This work presents matrix-assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) study of colonies of Methicillin-resistant *Staphylococcus aureus*, as a proof of concept of the technique for obtaining images of bacteria colonies. Images of methicillin-resistant and susceptible colonies of *Staphylococcus aureus* were obtained by a sublimation process to apply the MALDI matrix on the samples followed by MALDI-MSI analysis. Seventeen (17) potential metabolites were identified and spatially localized, such as N,N-dihydroxy-L-valine, 2-(4-Methylphenyl)ethylamine, 3,4-Dihydroxy-L-phenylalanine, 2-Methyl-hexanoic acid, threonine, Arginine, Aureusimine and Glycyl-D-

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asparagine. Thus, this study reinforces the potential of MALDI-MSI for identification of metabolites synthesized by different strains of *Staphylococcus aureus* bacteria.

Keywords: Resistant bacteria, *Staphylococcus aureus*, Mass spectrometry imaging, MALDI-MSI, metabolomics

INTRODUCTION

When bacteria interact with an antibiotic, due to natural selection guided by selective pressure, resistant strains to that antibiotic may emerge. Upon the increasing and inappropriate use of antibiotics for the treatment of bacterial infections, the emergence of resistant or even multi-resistant strains has been often observed. Therefore, bacterial resistance is currently a problem for global health.[1]

Staphylococcus aureus bacterium, and its different strains, have a long history in human pathology, causing endocarditis, pneumonia, and septicemia. The first reports of Methicillin-resistant *Staphylococcus aureus* (MRSA) date back to 1961, but the first epidemic of MRSA in hospitals occurred in the 1980s, and in the late 1990s, when it spread over communities.[2] Currently, the impact of this bacterium on public health and the economy is evident, causing an increase in mortality, use of health resources with high-cost drugs and medical monitoring for their administration.[3]

The *mecA* gene is always located in a mobile cassette and probably acquired by horizontal gene transfer between cells. One of the main reasons for resistance to the antibiotic methicillin in MRSA is the acquisition of *mecA*, which encodes the low affinity Penicillin Binding protein (responsible for modifications on beta lactam antibiotic receptors). The scope of this manuscript is beyond a thorough biological explanation about the establishment of *S. aureus* antibiotic resistance. Nevertheless, readers may be directed to references[2,4] if they want to learn more about it.

Several instrumental techniques have been used to study the metabolic profile of bacteria, such as liquid chromatography coupled to mass spectrometry (LC-MS)[5] and gas chromatography coupled to mass spectrometry (GC-MS)[6]. On the other hand, mass spectrometry imaging (MSI) is considered a promising technique for comprehending the resistant characteristics of these bacteria and how they can potentially be treated. Since the use of this technique allows the localization of ions on the surface of colonies, analysis of samples with little or no preparation and the identification of potential MRSA metabolites that characterize antibiotic resistance may be achieved.[7]

Analysis by MALDI-MSI requires that a matrix be uniformly deposited on the sample surface. This process consists of the formation of matrix crystals on the sample surface by sublimation.[8] Afterwards, sample is inserted on the mass spectrometer ion source, and the laser hits the sample incorporated into the matrix crystals, which are photoionized producing matrix ions. In this phase, the laser charge is transferred from the ionized matrix to the neutral molecules of the analyte, followed by introduction of ions into the mass analyzer. The matrix is fundamental in this process and must be selected according to the analyte's chemical functions and the laser wavelength, so that optimum energy absorption occurs.[9]

MSI has become particularly popular because of its ability to spatially locate a compound of interest on a surface by investigating ions *m/z* ratios. Briefly, an area of the sample is selected and subdivided into pixels, which is analyzed by MS. Smaller pixels result in higher lateral resolution of the image. The obtained spectra are compiled and the equipment´s software generates images for each *m/z*. Since each pixel of the generated image has a respective spectrum, from which the relative ion abundance information is obtained, compounds identification may be achieved, as well as their respective abundance in each region of the surface.[10]

Therefore, spatial information obtained by MSI of compounds synthesized by *S. aureus* allows locating, identifying, and comparing metabolites synthesized by resistant and susceptible strains, which may contribute to comprehension of antibiotic resistance, as well as on nosocomial infections treatment.

MATERIALS AND METHODS

Growth of bacteria colonies

Imaging was performed on three samples: (i) methicillin-susceptible bacteria; (ii) methicillin-resistant bacteria; and (iii) resistant bacteria after oxacillin antibiotic treatment. Methicillin-resistant (N315) and methicillin-susceptible (ATCC29213) *Staphylococcus aureus* strains were cultivated on ITO slides (indium tin oxide coated slide), which were placed in standard 10 cm Petri dishes, followed by the addition of Mueller Hinton bacterial culture medium (17.5 g L^{-1} casein peptone, 2 g L^{-1} meat peptone, 1.5 g L^{-1} starch, 17 g L^{-1} agar, and deionized water) until a 1 mm thick layer was formed on the slide. After medium solidification, three strains of a pre-seeding of the bacterium were equally spaced sown. The medium with the bacteria was kept in oven with controlled humidity at 30 °C for 4 days.

Investigation of the metabolic composition by MSI of the resistant strain in the presence of the antibiotic oxacillin was performed after the fourth day of cultivation. For this purpose, one of the colonies was covered with a thin layer of 64 μg mL^{-1} aqueous oxacillin solution and the sample was returned to the oven for 30 min.

Sample preparation for MALDI-MSI

Bacteria cultivated with or without antibiotics were prepared for MALDI-MSI analyses by uniform deposition of α-cyano-4-hydroxycinnamic acid (CHCA) matrix over the entire slide using a sublimation process. Sublimation was performed by placing the slide inside the lab-made apparatus (Figure 1) with the surface of the bacteria strain facing downwards and above of the matrix, which was in the lower space (*i.e.,* under the slide). Ice and acetone were placed on the top of the sublimator.

The apparatus was placed on the top of a sand bath heated to 170 °C (Figure 1) and connected to a high vacuum pump, which provided a pressure close to 50 mmTor. A thick vapor cloud of the matrix was formed and, after 10 minutes, the system was cooled and the pump turned off, resulting on a uniform layer of matrix on the blade.

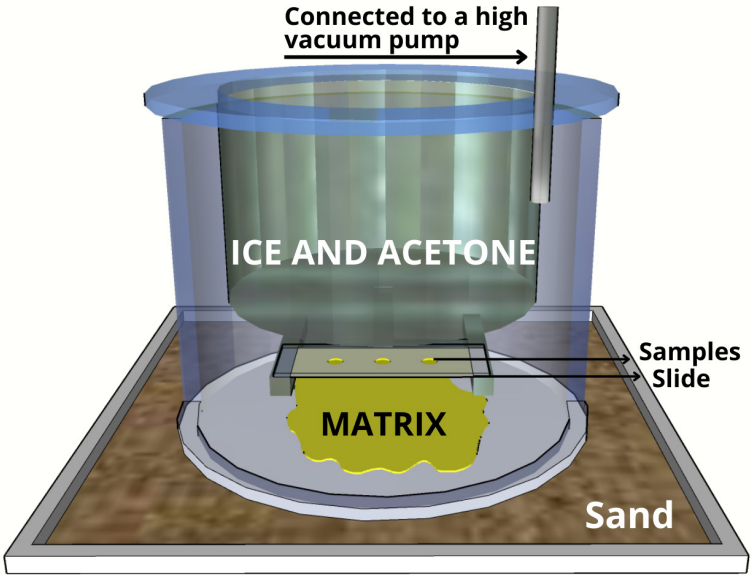

Figure 1. Scheme of the matrix sublimation process on the bacteria colony sample for subsequent analysis by MALDI-MSI.

MSI analysis

Samples were analyzed in a MALDI-TOF MS Bruker Autoflex Smart Beam III equipment containing a nitrogen laser (337 nm) with FlexControl control program (version 3.3) and FlexImaging imaging program (version 3.0). A method previously available on the equipment was used. Data were visualized with the FlexImaging program (version 3.0). Spectra from colonies samples were generated by averaging 500 laser shots for each pixel. The sample was rastered to a resolution of 200 × 200 µm. A summary of the imaging mass spectrometry workflow is presented in Figure 2.

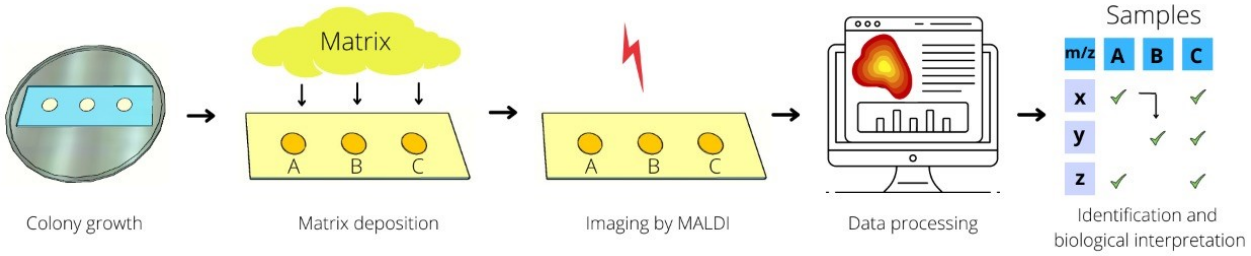

Figure 2. Workflow of MALDI-MSI on bacteria slides culture.

RESULTS AND DISCUSSION

MSI data analysis

Data processing with Flex Imaging software allowed observation of images obtained for the studied bacteria strains. Upon comparison of different strains and different culture medium composition, the potential biomarkers were identified and interpreted according to their precise masses and by free databases, such as Metlin;[11] HMDB;[12] Lipid Maps;[13] MassBank[14] and KEGG[15]. Identification was performed by comparison of the acquired mass spectra with the data available in the databases. For this process, the accuracy of the equipment was considered, and the limit of +/- 5 ppm was established.

Ions showed different spatial distributions in the investigated samples, and the characterization proposed in Table I corresponded to the range of *m/z* 102.6 to 779.1. Additionally, the average spectra (resulting from the sum of all spectra of all pixels in the sample) are shown in Figure 3. Some ions were identified only on the *S. aureus* resistant strain cultivated with culture medium containing antibiotic, such as *m/z* 174.8, *m/z* 219.5, *m/z* 229.5, and *m/z* 493.9. On the other hand, only ion *m/z* 130.7 was identified in the resistant bacteria strain cultivated without antibiotic. Ions *m/z* 136, *m/z* 161.9, *m/z* 226.4, and *m/z* 291.5 were identified only in the methicillin-susceptible strain. In addition, some common ions for both strains showed different intensities. Thus, such characteristics suggest the existence of exclusive metabolites for each studied condition.

Table I. Observed ions in the average spectrum by MALDI-MSI and respective putative identifications

n°	*m/z*			Putative identification	MW	Protonated /Adduct	Ref.
	ATCC	N315*	N315**				
1		102.6	102.7	-	-	-	-
2	110.1	110.7		-	-	-	-
3			111.8	-	-	-	-
4	120.9	120.8		Threonine	119.1	H	14
5		130.7		2-Methyl-hexanoic acid	130.0	H	5
6	136.0			2-(4-Methylphenyl)ethylamine	135.1	H	14

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Table I. Observed ions in the average spectrum by MALDI-MSI and respective putative identifications (continuation)

n°	*m/z*			Putative identification	MW	Protonated /Adduct	Ref.
	ATCC	N315*	N315**				
7			138.8	-	-	-	-
8	145.2	145.5		-	-	-	-
9			146.7	-	-	-	-
10	155.3	155.8	155.1	-	-	-	-
11	161.9			Indole-3-carboxylic acid	161.0	H	14
12		163.8		-	-	-	-
13	172.7	172.1		N,N-dihydroxy-L-valine	149.0	Na	11
14			174.8	L-Arginine	174.1	H	12
15			188.8	-	-	-	-
16	189.7	190.2		Glycyl-D-asparagine	189.0	H	5
17	197.8	198.4	197.9	3,4-Dihydroxy-L-phenylalanine	197.0	H	14
18	205.6	206.2	206.3	-	-	-	-
19	212.7	212.7	212.1	-	-	-	-
20			219.5	2-dimethylaminoethyl cinnamate	219.2	H	14
21		221.1	222.2	-	-	-	-
22	226.4			3,6-bis(2-methylpropyl)-2,5-piperazinedione	226.2	H	5
23			229.5	Aureusimine B	228.1	H	16
24	233.4	234.4		-	-	-	-
25	250.4	250.6	249.9	Alprenolol	249.1	H	14
26	267.5	266.9	266.3	-	-	-	-
27	279.3			-	-	-	-
28			287.0	-	-	-	-
29		289.5		-	-	-	-
30	291.5			12,15-Octadecadinoic acid, methyl ester	290.2	H	5
31			304.4	-	-	-	-
32	310.1	309.8		-	-	-	-
33		326.9		-	-	-	-
34	336.0	335.6		-	-	-	-
35		355.9		-	-	-	-
36	363.7			-	-	-	-
37	380.4	379.9		1-Amino-2,4-dibromoanthraquinone	378.9	H	15

(continues on the next page)

Table I. Observed ions in the average spectrum by MALDI-MSI and respective putative identifications (continuation)

n°	*m/z*			Putative identification	MW	Protonated /Adduct	Ref.
	ATCC	N315*	N315**				
38	402.3	402.1		-	-	-	-
39		424.2		-	-	-	-
40	432.6			-	-	-	-
41	446.0	446.1	445.7	-	-	-	-
42			461.5	-	-	-	-
43		466.0		-	-	-	-
44			477.3	-	-	-	-
45	481.5	482.8		-	-	-	-
46			493.9	N,N-bis(4-acetamidobutyl)-15-methylhexadec-6E-enamide	493.4	H	13
47	502.0	501.4		-	-	-	-
48			516.7	-	-	-	-
49		519.5		-	-	-	-
50	525.8			-	-	-	-
51			533.8	-	-	-	-
52			550.9	-	-	-	-
53			567.6	-	-	-	-
54	570.4			-	-	-	-
55	658.5	658.5	657.1	Sphingolipids	657.5	H	7,9
56		674.6	673.3	-	-	-	-
57			690.0	-	-	-	-
58	696.2	696.8		Polyketides	695.2	H	9,11
59			710.6	-	-	-	-
60		715.2		-	-	-	-
61			729.0	-	-	-	-
62		740.3		-	-	-	-
63			746.5	-	-	-	-
64		759.7		-	-	-	-
65			763.6	-	-	-	-
66		779.1		-	-	-	-

*Without addition of antibiotic in the culture medium and the colony; **With addition of antibiotic in the culture medium and the colony.

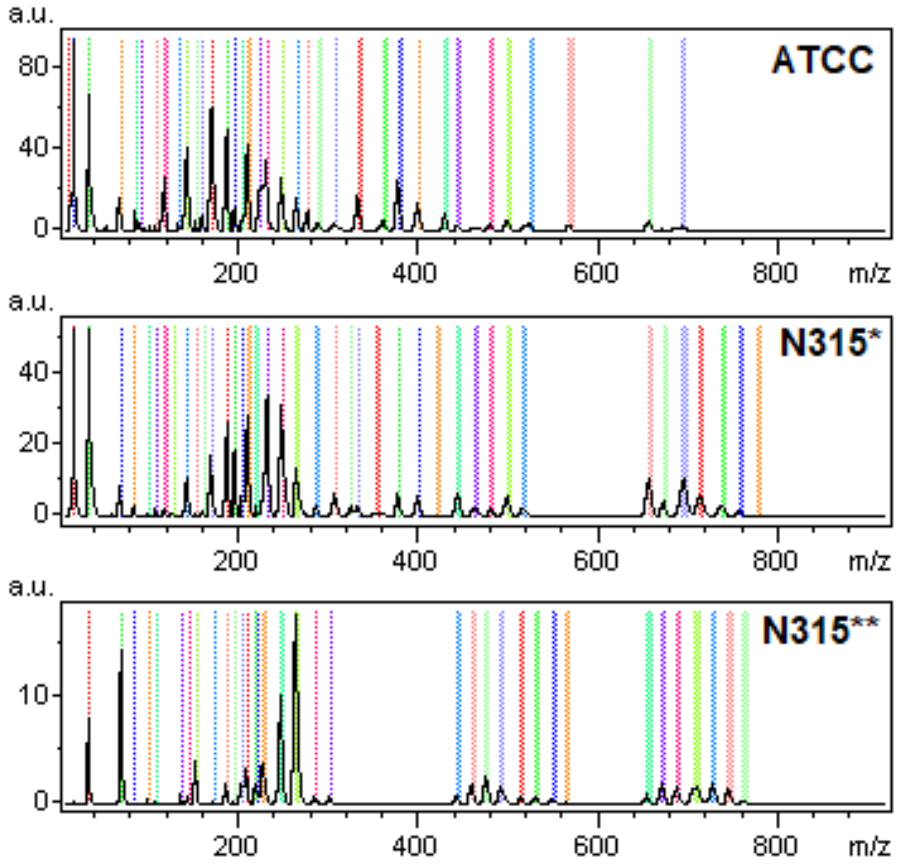

Figure 3. Average spectrum of different *Staphylococcus aureus* strains. ATCC: susceptible type strain. N315: resistant type strain. *Without addition of antibiotic in the culture medium and the colony; **With addition of antibiotic in the culture medium and the colony.

The different ions intensities are presented in Figure 4. Ions *m/z* 172.7 and *m/z* 172.1 were assigned to the same molecule (N,N-dihydroxy-L-valine), based on the equipment's precision and the similarity index furnished by Metlin database. Figure 4 shows higher concentrations of N,N-dihydroxy-L-valine in the *S. aureus* wild strain (ATCC) than in the resistant one (N315), cultured in the absence of antibiotic. Interestingly, N,N-dihydroxy-L-valine has already been reported to be derived from L-valine, which in turn is related to bacterial resistance.[14]

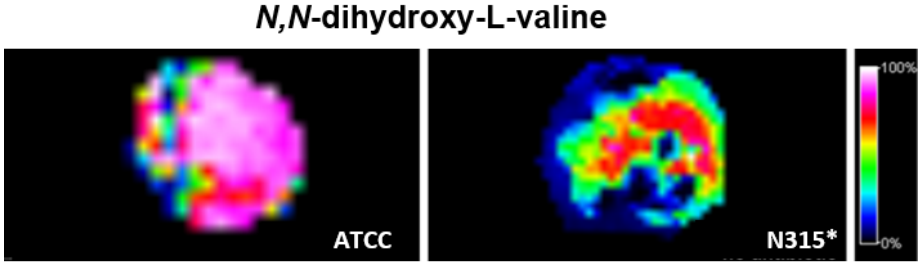

Figure 4. MALDI-MSI of different *S. aureus* strains (ATCC and N315*). Images obtained for the ion *m/z* 172.7, identified as N,N-Dihydroxy-L-valine. The right scale shows 100% for maximum intensity, and 0% for the absence of the ion. *Without addition of antibiotic in the culture medium and the colony.

Ion *m/z* 120.9 was observed in both samples containing the resistant strain cultivated without antibiotic, and the susceptible one, and was attributed to Threonine. According to Ohlsen and Donat (2010), this amino acid together with serine plays an important role in central metabolic processes through phosphorylation and dephosphorylation in *S. aureus* cell wall, making it susceptible to the action of antibiotics, which generally acts on this part of the cell.[18] The observation of *m/z* 136.0 ion in the susceptible bacterium strain cultivated without antibiotic was assigned to 2-(4-Methylphenyl)ethylamine - a primary amine. The presence of such compound in this strain suggests the occurrence of decarboxylation through enzymatic processes of free amino acids, which produces primary or secondary biogenic amines, indicating the occurrence of deterioration caused by the presence of *S. aureus*.[19] 3,4-Dihydroxy-L-phenylalanine (DOPA) was assigned to the ion *m/z* 197.8, which was observed in the susceptible strain at higher concentrations than in the antibiotic-resistant one (Figure 5). This compound is related to highly efficient protein synthesis by cross-linking of specific proteins.[20] These interactions are considered weak and/or transient and facilitate cells to turn signals on and off.[21] In addition, high concentrations of DOPA in cell culture have already been reported to be toxic.[22]

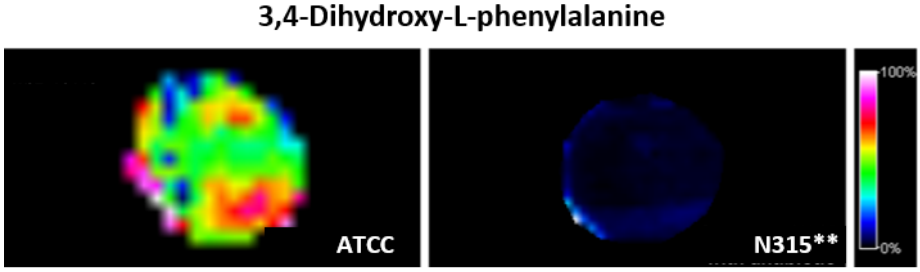

Figure 5. MALDI-MSI of 3,4-Dihydroxy-L-phenylalanine on different *S. aureus* strains (ATCC and N315**). 100% indicates the maximum intensity and 0% the absence of the ion, and **with addition of antibiotic in the culture medium and the colony.

The literature reports the identification of ions *m/z* 130.7 and *m/z* 190.2 by gas chromatography coupled to mass spectrometry (GC-MS) in the analysis of volatile metabolites from methanolic extracts of *S. aureus*. 2-Methyl-hexanoic acid and Glycyl-D-asparagine metabolites were assigned to these ions, respectively, both secreted from the cell membrane.[5] In MALDI-MSI analysis, *m/z* 130.7 ion was observed only in the resistant strain cultivated without antibiotic, while *m/z* 190.2 ion was observed in both susceptible and resistant strains cultivated without antibiotic, and ions *m/z* 226.4 and *m/z* 291.5 were observed only in the susceptible strain. Such observations suggest that only these metabolites are present in common in both bacteria strains, indicating that a larger variety of metabolites can be observed exclusively by different analysis processes. Thus, both GC-MS and MALDI-MSI can be complementary in the metabolomics investigation of *S. aureus*. Furthermore, *m/z* 658.5 and *m/z* 696.2 (observed in this study) were characterized according to the category of compounds indicated in the databases, since many possible metabolites have the same *m/z*.

According to Li, several bacteria, including *S. aureus*, may survive under stress by using bacterial arginine repressor (ArgR) to accelerate arginine metabolism in the presence of arginine.[23] According to Aharonowitz, the *arcR* gene enables arginine to be an energy source for growth under anaerobic conditions. Thus, *S. aureus* growth would be subject to catabolic repression of glucose, which is enhanced by arginine.[24] Indeed, all these findings corroborate with the data obtained herein, since arginine was detected only in the resistant strains colonies cultivated with antibiotics. Possibly, the stress condition imposed by the presence of the antibiotic led to a higher production of arginine.

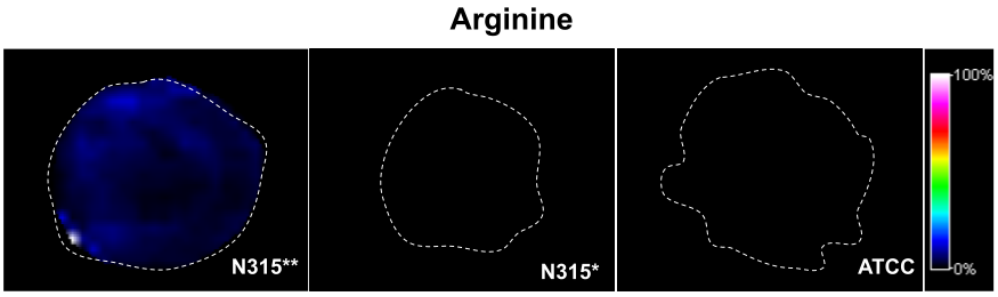

Figure 6. MALDI-MSI of Arginine on different *S. aureus* strains (ATCC, N315* and N315**). 100% indicates the maximum intensity and 0% the absence of the ion, *without addition of antibiotic in the culture medium and the colony, and **with addition of antibiotic in the culture medium and the colony.

According to Magarvey, Aureusimine A and B are secondary metabolites of previously unidentified non-ribosomal peptides with a fundamental role in the ability of *S. aureus* to act as an infectious agent, controlling various virulence factors.[25] In our study, only the resistant strains cultivated with antibiotic presented these metabolites, which reinforces the participation of Aureusimine in the virulence metabolism of this *S. aureus*.

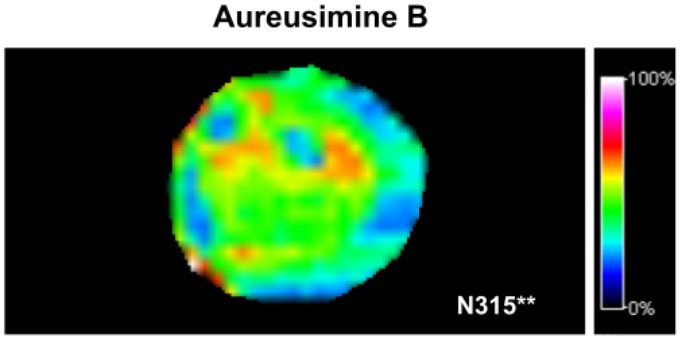

Figure 7. MALDI-MSI of Aureusimine on *S. aureus* strains (N315**). 100% indicates the maximum intensity and 0% the absence of the ion, and **with addition of antibiotic in the culture medium and the colony.

CONCLUSIONS

MALDI is a well-established technique for the identification of bacterial strains. Herein, the authors intended to show a proof of concept that MALDI-MSI can be a useful tool for mass spectrometry imaging of laboratory-grown bacterial colonies for metabolomics investigation. The obtained images allowed to observe different metabolic profiles of resistant bacteria upon antibiotic treatment. Several metabolites were observed in both susceptible and resistant *S. aureus* strains, and seventeen of them were putatively identified with the aid of scientific literature and databases, such as N,N-dihydroxy-L-valine, 2-(4-Methylphenyl) ethylamine, 3,4-Dihydroxy-L-phenylalanine, 2-Methyl-hexanoic acid, threonine, Aureusimine, Arginine and Glycyl-D-asparagine. Further studies are underway for additional characterization and identification of other metabolites.

Conflicts of interest

The authors declare there are no conflicts of interest.

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