ANÁLISIS PROTEÓMICO DE *Staphylococcus aureus* RESISTENTE A METICILINA (SARM) FRENTE A LA ACCIÓN DEL PÉPTIDO SINTÉTICO ANTIMICROBIANO GIBIM-P5F8W

ORLANDO BURGOS MORALES

UNIVERSIDAD INDUSTRIAL DE SANTANDER
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Trabajo de grado presentado como requisito para optar al título de Químico

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DEDICATORIA

A mi madre, quien me ha enseñado a afrontar la vida tal como es, cruda a veces, pero siempre justa. Quien dibuja en mí el sol, aunque pareciera no amanecer.

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A Dios gracias porque cada día me permite ver su infinito amor y bendiciones en mi vida. Él ha sido mi roca, mi fuerza y fortaleza.

A mis padres, por su esfuerzo y dedicación en mi formación integral con principios y valores sólidos. A pesar de que las situaciones entre nosotros cambien, siempre les guardaré mi respeto, cariño y admiración.

A mi familia Morales, porque su apoyo incondicional ha sido pieza clave en mis ultima etapas de formación profesional y en mi lucha por cumplir mis sueños.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

RESUMEN

TÍTULO: ANÁLISIS PROTEÓMICO DE *Staphylococcus aureus* RESISTENTE A METICILINA (SARM) FRENTE A LA ACCIÓN DEL PÉPTIDO SINTÉTICO ANTIMICROBIANO GIBIM-P5F8W*

AUTOR: ORLANDO BURGOS MORALES**

PALABRAS CLAVES: *Staphylococcus aureus* Resistente a Meticilina*;* Antimicrobiano; Péptido; proteoma; estrés oxidativo.

DESCRIPCIÓN: La resistencia a los antimicrobianos es un problema emergente y una de las razones del fracaso del tratamiento antiestafilocócico. Los péptidos antimicrobianos catiónicos se han explorado recientemente para el desarrollo de nuevos antibióticos por su alta afinidad por las membranas bacterianas. En el presente trabajo, hemos sintetizado y evaluado un nuevo péptido (GIBIM-P5F8W) con actividad contra *Staphylococcus aureus* Resistente a la Meticilina (SARM). El péptido se sintetizó mediante el método F-moc; se purificó mediante cromatografía de fase reversa, y se caracterizó mediante espectrometría de masas y dicroísmo circular. La inhibición del crecimiento in vitro se determinó mediante el método de microdilución en caldo. Además, el mecanismo bactericida y la respuesta celular se estudiaron mediante análisis proteómico, evaluación del consumo de oxígeno y evaluación de la enzima antioxidante superóxido dismutasa. GIBIM-P5F8W presentó una Concentración Mínima Inhibitoria (CMI_{90}) de 12.5μM a las 8 horas de tratamiento y 14 *spots* de proteínas diferencialmente expresadas se anotaron funcionalmente. La homeostasis de energía alterada se observó con la regulación positiva de las proteínas relacionadas con el catabolismo, mientras que los procesos que consumían energía se inhibieron, posiblemente como una estrategia de adaptación a la presión antibiótica ejercida por el péptido. Sin embargo, el efecto bactericida de GIBIM-P5F8W puede atribuirse a la represión de los mecanismos de eliminación de ROS debido a la inhibición de los transportadores de manganeso y la inhibición del superóxido dismutasa. También se observó una posible adaptación temprana de SARM expuesto a concentración subinhibitoria mediante la modificación de la envoltura celular bacteriana. Sumado a lo anterior, se observó la inhibición del consumo de oxígeno, lo que podría disminuir la generación de ATP por las vías aeróbicas. Estos hallazgos pueden respaldar futuras investigaciones sobre este nuevo compuesto como un candidato para el tratamiento antiestafilocócico

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*Trabajo de Grado

** Facultad de Ciencias, Escuela de Química. Director: Stelia Carolina Méndez Sánchez, *PhD*. Codirector: Claudia Cristina Ortiz López, *PhD*

ABSTRACT

TITLE: PROTEOMIC ANALYSIS OF METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) AGAINST THE ACTION OF THE SYNTHETIC ANTIMICROBIAL PEPTIDE GIBIM-P5F8W*

AUTHOR: ORLANDO BURGOS MORALES**

KEYWORDS: Methicillin Resistant *Staphylococcus aureus;* Antimicrobial; Peptide; proteome; oxidative stress.

DESCRIPTION: Antimicrobial resistance is an emerging problem and one of the reasons for anti-staphylococcal treatment failure. Cationic antimicrobial peptides have been recently explored for the development of new antibiotics by their high affinity for bacterial membranes. In the present work, we synthesized and evaluated a new peptide (GIBIM-P5F8W) with activity against Methicillin Resistant *Staphylococcus aureus* (MRSA). The peptide was synthesized by F-moc method; it was purified by reverse phase chromatography, characterized by mass spectrometry and circular dichroism. *In vitro* growth inhibition was determined by broth microdilution method. Additionally, the bactericidal mechanism and cellular response were studied by proteomic analysis, evaluation of consumption of oxygen and assessment of antioxidant enzyme superoxide dismutase. GIBIM-P5F8W presented a Minimal Inhibitory Concentration (MIC_{90}) of 12.5μM at 8 hours of treatment and 14 differentially expressed protein spots were functionally annotated. Altered energy homeostasis was spotted with the upregulation of catabolism related proteins while energy consuming processes were inhibited, conceivably as an adaptive strategy to the antibiotic pressure exerted by the peptide. However, the bactericidal effect of GIBIM-P5F8W, may be attributed to the repression of ROS scavenging mechanisms due to downregulation of manganese transporters and the inhibition of superoxide dismutase. It was also observed a possible early adaptation of MRSA at sub-inhibitory concentration by modification of the bacterial leaflet. Additionally, inhibition of consumption of oxygen was observed, which could decrease the generation of ATP by aerobic pathways. These findings may support further research on this novel compound as an anti-staphylococcal treatment candidate.

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*Bachelor Thesis

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INTRODUCTION

Spread of multidrug-resistant bacteria (MDB) and untreatable infections is an increasing life-threatening phenomenon of public health concern worldwide(World Health Organization, 2017). The emergence of antibiotic resistant microorganisms has been associated with inappropriate antibiotic use that induces expression of antibiotic resistance genes under such selective pressure (Davies & Davies, 2010; Kola & Urba, 2001).

S. aureus (*Staphylococcus aureus)* has been classified by the World Health Organization as a potential MDB which induces nosocomial and community-acquired infections(World Health Organization, 2014). The Gram-positive pathogen is commonly involved in skin and soft tissues infections, and even in some acute infections such as bacteremia, pneumonia, endocarditis, meningitis, osteomyelitis, toxic shock syndrome, among others(Klevens et al., 2007). *S aureus* was first documented as an epidemiological burden in the 1960s with the outbreak of methicillin-resistant isolates in the clinical environment(Chambers & DeLeo, 2009). Currently, Methicillin-resistant *Staphylococcus aureus* (MRSA) has developed resistance to most antibiotics available for clinical use including last resource antibiotics such as vancomycin (Centers for Disease Control and Prevention, 2002; Hiramatsu et al., 1997; Weigel et al., 2003), linezolid(Gales et al., 2006; Sánchez García et al., 2010; Tsiodras et al., 2001) and daptomycin (Mangili, Bica, Snydman, & Hamer, 2005; van Hal, Paterson, & Gosbell, 2011).

A shortage of anti-staphylococcal therapy and the current multi-drug resistance trend have stressed the necessity for new antimicrobial agents to be developed. A promising option is afforded by antimicrobial peptides (AMPs) that have been recently studied to treat *S. aureus* strains (Alvarez-Bravo, Kurata, & Natori, 1994; J Cruz et al., 2017; Fan et al., 2011; Mohamed, Hamed, Panitch, & Seleem, 2014; Prada et al., 2016a; Saravolatz et al., 2012). AMPs are effector molecules that

participates in the host defense system of all domains of life, they are usually amphipathic cationic (charge at physiological pH: +2 to +9) peptides of 30 amino acids (<10kDa) and exhibit specific secondary structures (α-helical, β-sheet, cyclic or extended conformation) (Nguyen, Haney, & Vogel, 2011). The latter properties have been associated with selective binding to pathogenic bacteria cell membranes as their main target. However, AMPs seem to additionally target intracellular processes such as DNA replication, transcription and protein biosynthesis (Ageitos, Sánchez-Pérez, Calo-Mata, & Villa, 2017; Krijgsveld et al., 2000; Park, Kim, & Kim, 1998).

Most mechanistic studies on AMPs have been able to suggest several modes of insertion of peptides into the bacterial membrane bilayer (Arouri, Dathe, & Blume, 2009; Arouri, Kiessling, Tamm, Dathe, & Blume, 2011; Brogden, 2005; Dathe et al., 2002; Joanne et al., 2009) and their interaction with lipid domains (Epand & Epand, 2009; Wadhwani et al., 2012). However, it is still not very clear the bactericidal mechanisms induced in this pathogenic microorganism. Proteomics is an alternative strategy to study the antimicrobial effects of AMPs on bacteria. Among the techniques applied to study protein expression, two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) has been used as a robust methodology to reference *S. aureus* proteomes under different growth conditions of biological, pathological and physiological significance (Becher et al., 2009; Jenkins, Burton, & Cooper, 2014; Monteiro et al., 2012; Pompilio et al., 2016; Sianglum, Srimanote, Wonglumsom, Kittiniyom, & Voravuthikunchai, 2011).

Recently, our research group have designed a novel antimicrobial synthetic peptide GIBIM-P5F8W (under patent), which elicits antimicrobial activity at micromolar concentrations against MRSA. In order to provide insights into the antibacterial mechanisms of this promising candidate drug, a proteomic approach was used to investigate the effects of GIBIM-P5F8W on protein expression in treated MRSA cells at sub-inhibitory concentration.

1. MATERIALS AND METHODS

1.1. MATERIALS

Fmoc (fluorenylmethyloxycarbonyl)-protected L-amino acids, TBTU (O-(Benzotriazol-1-yl)-N,N,N$'$,N$'$-tetramethyluronium tetrafluoroborate), HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOAt (1-Hydroxy-7-azabenzotriazole) were Novabiochem® reagents acquired from Merck Millipore. DCC (dicyclohexylcarbodiimide) and DIEA (N,N-diisopropylethylamine) were obtained from Alfa Aesar. Rink amide 4MBHA resin from Merck Millipore. HPLC grade solvents: Acetonitrile (ACN) and methanol (MetOH); and dichloromethane (DCM) were purchased from Merck. Culture media Mueller Hinton (MH) and brain heart infusion (BHI) from OXOID.

1.2. PEPTIDE SYNTHESIS AND CHARACTERIZATION

GIBIM-P5F8W peptide was synthesized by the solid phased approach as described in the “tea-bag” method reported by Houghten (Houghten, 1985). Rink amide 4MBHA resin (100-200 mesh; loading :0.63 mmol g^{-1}) was used as solid support. The peptide cleavage from the solid support was carried out by a 2h treatment with trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/ ethanedithiol/ H_2O in a 92.5/2.5/2.5:2.5 ratio and then precipitated with cold diethyl ether. The peptide was desalted by gel exclusion chromatography using a G-10 column (Amersham, USA) and purified by solid-phase extraction on a Clean-Up C_{18} sorbent (CEC18153 50 mg. mL^{-1}, United Chemical Technologies, USA). Acetonitrile (ACN) eluted fractions (10%-100% v/v) were collected for purity analysis by reverse phase- high performance liquid chromatography (RP-HPLC). R-HPLC was performed on a ZORBAX Eclipse XDB-C18 column (4.6 mm i.d. x 250 mm, 5µm, Agilent Technologies, USA) with an Agilent 110 system (Agilent Technologies, USA), using

buffer A (H_2O with TFA 0.1%(v/v)) and buffer B (acetonitrile (ACN) containing TFA 0.1% (v/v)) under a gradient of 0 to 100% (v/v) of B over 30 min at flow rate 1 mL. min^{-1} and detection at 220 nm. The identity of the purified peptide was confirmed by MALDI-TOF mass spectrometry. The most effectively purified fractions were mixed and subjected to lyophilization.

Circular Dichroism (CD) spectra of peptide was carried out at 25°C in a 1 mm path length cuvette over 190-260 nm in a CD Spectrometer (J-815 Jasco Corporation, Japan) using 0.2 mM peptide dissolved in 70% (v/v) 50 mM sodium phosphate buffer (pH 7.4)/30% (v/v) 2,2,2-trifluoroetanol (TFE) solution. Each spectrum was recorded as an average of four scan repetitions in continuous scanning mode at 50 nm/min scanning speed and a response time of 1 s. The solvent contribution blank was subtracted from each sample spectrum. Molar ellipticity [θ] was calculated for the next equation:

$$[\theta] = \ 3298.2\ \Delta\varepsilon$$; where ε is the molar extinction coefficient

1.3. BACTERIAL STRAIN AND GROWTH CONDITIONS

MRSA strain was acquired from Pontificia Universidad Javeriana microorganisms' collection (CM-PUJ, WDCM857), a member of the World Federation for Culture Collections (WFCC). Bacteria were cultured overnight on Brain-Hear Infusion (BHI) agar. All assays were made from a standardized inoculum where a single colony from the BHI agar plate was grown in Mueller-Hilton broth (MH) overnight at 37^0C with orbital shaking. Then it was diluted in sterile MHB to adjust the bacterial suspension to ~$5x10^5$ CFU according to McFarland scale (O. D_{595nm} 0.07-0.1).

1.4. MINIMUM INHIBITORY CONCENTRATION DETERMINATION

MIC was determined by the broth microdilution method as previously described (J Cruz et al., 2017; Jenniffer Cruz et al., 2014; Prada et al., 2016a) based on the Clinical & Laboratory Standards Institute protocol (Clinical Laboratory Standard Institue [CLS], 2016). Bacterial growth inhibition was evaluated in triplicates (0.5 -30 µM of peptide) in medium MH by O.D readings at 595nm using an iMark microplate absorbance reader (Bio-Rad Laboratories, USA), for 8h treatment with orbital shaking at 37^0C. MIC_{99} was determined as the minimal peptide concentration that inhibits 99% of the bacterial cell population.

1.5. PROTEOMIC STUDY OF MRSA DURING GIBIM-P5F8W PEPTIDE TREATMENT

1.5.1. MRSA treatment with GIBIM-P5F8W peptide. Treatment was made at early exponential growth phase (after 2h incubation) by aseptically adding either PBS buffer (pH 7.4) or GIBIM-P5F8W peptide to final concentration of 0.5 MIC for control and treated samples, respectively.

1.5.2. Protein preparation for proteomic analysis. After peptide exposure, control and treated replicates were harvested by centrifugation (10000 x g, 40C) for 10min, bacterial pellets were washed three times with Tris-HCl buffer (20mM, pH 7.4). Samples were subjected to peptidoglycan enzymatic digestion by resuspending bacterial pellets in buffer A (lysostaphin 100 U/mL, Sigma-Aldrich and lysozyme 10x103 U/mL, Merck) for 2h at 370C. Then, protoplast suspensions were resuspended in buffer B (urea 7M, thiourea 2M, dithiothreitol (DTT) 100mM, Tris 40mM, Phenylmethanesulfonyl fluoride (PMSF) 1mM, Ethylenediaminetetraacetic acid (EDTA) 1mM, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) 4% w/v, Biolyte 3/10 ampholite 1% v/v (Bio-Rad), sodium orthovanadate 1mM). The cell suspensions were lysed in an ice bath by an ultrasonic processor

(130-Watt, Cole-palmer, USA) applying 5 sec short bursts (under 80% intensity) followed by intervals of 30 sec for cooling, over a 1min period. Finally, protein precipitation was carried out by mixing with 10% w/v TFA in acetone in 1:10 ratio and kept at -700C overnight. Total protein bacterial extracts were harvested by centrifugation at 18000 x g and 40C, then washed three times with ice-cold acetone as a clean-up step. Protein was dissolved in buffer B up to minimal solubilization volume. Protein quantification was determined by the Bradford spectrophotometric method (Bradford, 1976).

1.5.3. Two-dimensional (2D) electrophoresis. MRSA proteomic expression analysis under GIBIM-P5F8W peptide treatment was conducted by 2-DE as described by O'Farrell (O'Farrell, 1975) and modified by Görg et al (Görg, Weiss, & Dunn, 2004). First dimension (isoelectric focusing, IEF) was carried out on immobilized pH gradient (IPG) strips (Ready Strip, 17cm, pH 4–7, Bio-Rad Laboratories) rehydrated passively for 12h with 300μL of sample (5μg protein/μL) diluted in rehydration buffer: urea 8M, CHAPS 2%, DTT 50Mm, Biolyte 3/10 ampholite 0.2% v/v and bromophenol blue 0.001% w/v. IEF was performed in a Protean IEF cell (1654001, Bio-Rad) until 80000 Vhrs was reached. Subsequently, 2-step (30 min each) equilibration treatment in the dark was applied to IPG strips, where they were first incubated with 2% DTT in equilibration buffer (urea 6M, tris-HCl 0.375M pH 8.8, 2%w/v SDS, 20% v/v glycerol) to reduce disulfide bonds, and then with 2% iodoacetamide as an alkylating agent. Second dimension was conducted in a SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) system (large format PROTEAN® II xi cell, Bio-Rad). Hand-casted SDS-PAGE gels(Laemmli, 1970) T=12% were run at 150v until bromophenol blue tracking dye reached the lower edge of the gel.

1.5.4. Imaging and analysis. Control and treatment 2D gels were fixed in 50% v/v methanol and 2% v/v orthophosphoric acid overnight and then transferred to staining solution A (33% methanol, 3% orthophosphoric acid, 17% ammonium sulfate). After

2 h incubation in solution A, solution B was added to a final concentration of 0.6 mg/mL Coomassie brilliant blue G-250 (Bio-Rad) dissolved in methanol and stained for 1h. Gels were incubated in destaining solution (5% methanol, 10% acetic acid) until background removal. Stained gels images were acquired with a Bio-Rad Gel Doc™ XR imaging system and image analysis was performed using PDQuest Advanced Software (version 8.0.1, Bio-Rad) for spot detection, matching and quantification. Protein expression of MRSA under peptide treatment was subjected to statistical assessment with the control replicates to detect spots that are differentially expressed using unpaired two-tailed Student's t-test. Differentially expressed spots were stablished as those that exhibit statistical significance (p-value < 0.05) and relevant biological variation (fold-change: $2 \geq x \leq 0.5$).

1.5.5. Differentially expressed protein identification. Differentially expressed protein spots from 2D gels were functionally characterized by their peptide mass fingerprint (PMF) using a matrix-assisted laser desorption ionization tandem time of flight mass spectrometer (MALDI-TOF/TOF MS). Spots were manually picked and destained with 50% v/v ACN and 20 mM NH_4HCO_3. Then, gel plugs were dehydrated with 100% ACN and completely air dried. In-gel protein digestion was accomplished by 10ng/µL trypsin treatment (Sequencing Grade Modified, Promega, USA) at 37^{o}C overnight. Tryptic peptides were isolated from gel plugs with extracting buffer (50% ACN in 1% TFA) after a sonication bath for 15 min. Peptides were spotted with α-cyano-4- hydroxy-cinnamic acid matrix solution (20 mg/mL in buffer: 50% ACN in 1% TFA) onto a ground steel target in a 1:1 ratio (sample: matrix).

Tryptic digests were analyzed with MALDI-TOF/TOF Ultraflextreme mass spectrometer (Bruker Daltonics, Bremen, Germany) operated by FlexControl v.33 software and equipped with a 355nm Nd:YAG laser. The whole mass spectra set were recorded in the positive reflectron mode and averaged from approximately 1000 laser shots to improve the S/N ratio. External calibration was performed using a carbamidomethylated tryptic digest of bovine albumin serum. Additionally,

identified protein samples by PMF were further analyzed using LIFT MS/MS approach, where most abundant ions per sample were chosen for tandem mass spectrometry analysis. Precursor ion selector range was 0.8% of parent ion mass. The voltage parameters were: IS1 7.5 kV, IS2 6.8 kV, lens 3.6kV, reflector1 29.5kV, reflector 2 14.0kV, LIFT1 19kV, LIFT2 3.3Kv. PMF and MS/MS mass spectra were submitted to the Mascot search engine (http://www.matrixscience.com) and compared to the Swiss-Prot database under the Firmicutes taxonomy filter. Protein identification was performed on the basis of statistically significant Mowse score ($p<0.005$) (Pappin, Hojrup, & Bleasby, 1993).

1.5.6. Bioinformatic analysis. Identified proteins were mapped to a functional protein association network by using *Staphylococcus aureus* COL as a methicillin-resistant and a biofilm-producing reference strain. Interactome was designed using the STRING software (https://string-db.org/) to statistically evaluate highly represented biological functions and pathways associated with the protein list. Differentially expressed proteins were classified according to their biological and molecular function gene ontology (GO) terms by importing gene list to PANTHER (Protein Analysis Through Evolutionary Relationships) (http://pantherdb.org/software).

1.6. SUPEROXIDE SCAVENGING ACTIVITY ASSAY

Peptide treated and control lysates were prepared following the procedure described in section 2.5.2 by omitting buffer B addition and the TFA: Acetone precipitation step. The scavenging activity of superoxide radical of cell lysates was determined by the method described by Nishimiki et al.(Nishikimi, Rao, & Yagi, 1972), with some modifications. The reaction buffer consisted of 10 mM Tris–HCl (pH 8.0), 340 μM NADH, 72 μM NBT and 30 μM PMS. The reaction was followed at 560 nm for 1 min and the scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = 1 - \frac{\text{Abs sample}}{\text{Abs no scavenging control}} \times 100$$

1.7. BACTERIAL CELL RESPIRATION

Oxygen consumption was polarographically determined in a 1.3 mL closed thermostatically controlled water-jacketed chamber under magnetic stirring, using a Clark-type electrode connected to a Oxygraph Plus system (Hansatech Instruments Ltd., England). MRSA culture triplicates (standardized inoculum as described in section 2.3) were incubated for 1h at 37ºC under aeration conditions prior to the assay. Bacterial oxygen uptake was directly measured in the broth suspension at 37ºC for about 10 min. Peptide treatment was carried out 3 minutes after the measurement started.

2. RESULTS

2.1. SYNTHESIS AND CHARACTERIZATION OF GIBIM-P5F8W

GIBIM-P5F8W was synthetized by the solid phase approach according to the F-moc synthesis method and purified by preparative reverse phase chromatography. In silico design of the peptide was done following the methodology developed in our lab previously described (J Cruz et al., 2017; Prada et al., 2016b). Briefly, the primary structure was generated using a genetic algorithm optimization strategy where physicochemical and biological properties were used as descriptors such as high probability to be antimicrobial (>95%), low instability, high helix index and positive charge; as well as the CAMP database of antimicrobial peptides (http://www.camp.bicnirrh.res.in) as the parent sequences. HPLC and MALDI-TOF analysis (Table 1) indicates that a pure synthetic product was obtained with 1.89 min retention time (Figure 1a) and 1802.20 ± 1 m/z (Figure 1b). The molecular ion monoisotopic mass $[M+H]^+$ agrees well with the theoretical molecular weight (MW).

Some other less abundant signals observed in the spectrum (Figure1b) correspond to positively charged adducts formed in the ionization process by interaction with the matrix, salts and metallic ions (Na^+, K^+).

Table 1 Purification parameters of GIBIM-P5F8W peptide

MW (Da)	$[MH]^+$ (m/z)	Retention time (min)	Predicted secondary structure
1802.24	1802.20 ± 1	1.89	α-helix

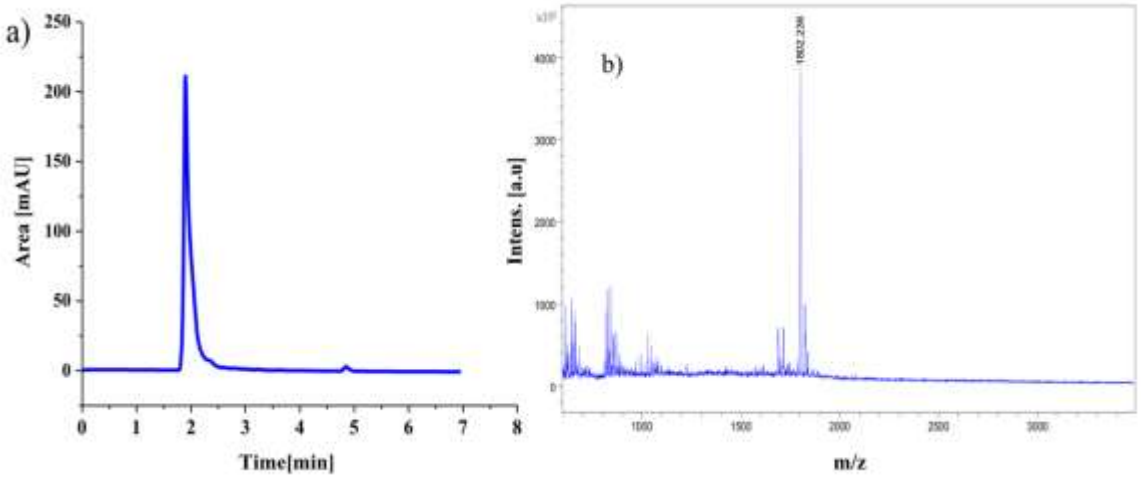

Figure 1 Chromatography and Mass spectrometry analysis. a) RP-HPLC chromatogram. b) MALDI-TOF spectra.

Furthermore, GIBIM-P5F8W peptide presented an α-helical conformation, which was characterized in 30% TFE by CD, and identified by minima at 202 and 210 nm and the maximum at 190 nm (Figure 2).

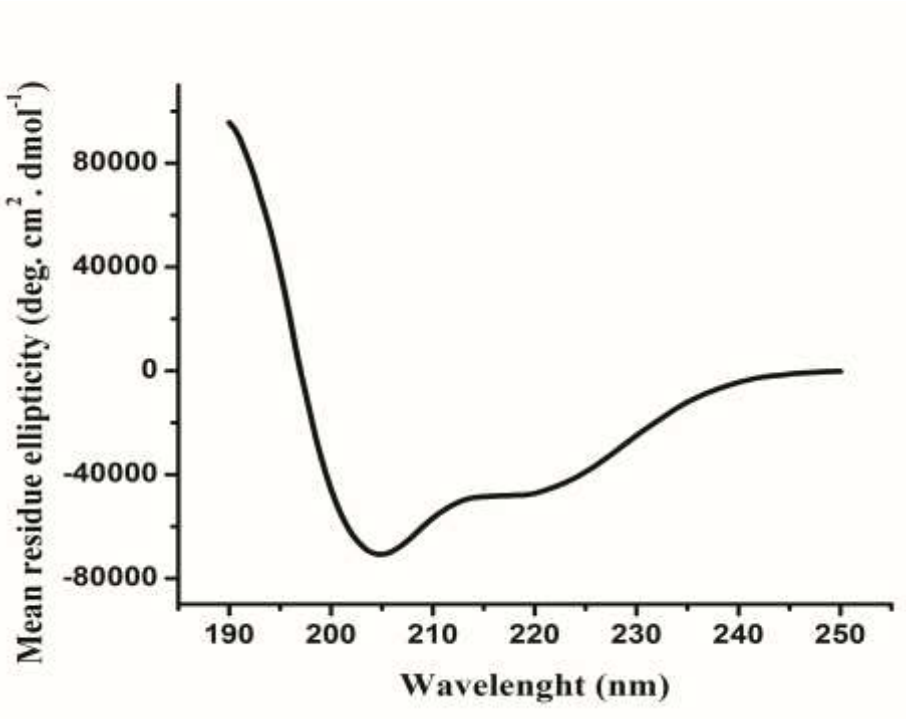

Figure 2 CD spectra of the peptide GIBIM-P5F8W. Peptide was dissolved in 30% TFE at 100 μM. The mean residue ellipticity was plotted against wavelength. The values from three scans per sample were averaged.

2.2. GIBIM-P5F8W ANTIMICROBIAL ACTIVITY

Antimicrobial susceptibility assay of MRSA under GIBIM-P5F8W peptide treatment in a concentration range between 0.2 μM and 100 μM was conducted by the broth microdilution method. Inhibition curve (Figure 3) shows that the synthetic peptide disrupts bacterial growth in a dose-dependent manner, where MIC_{99} was reached at 12.5 ± 0.2 μM.

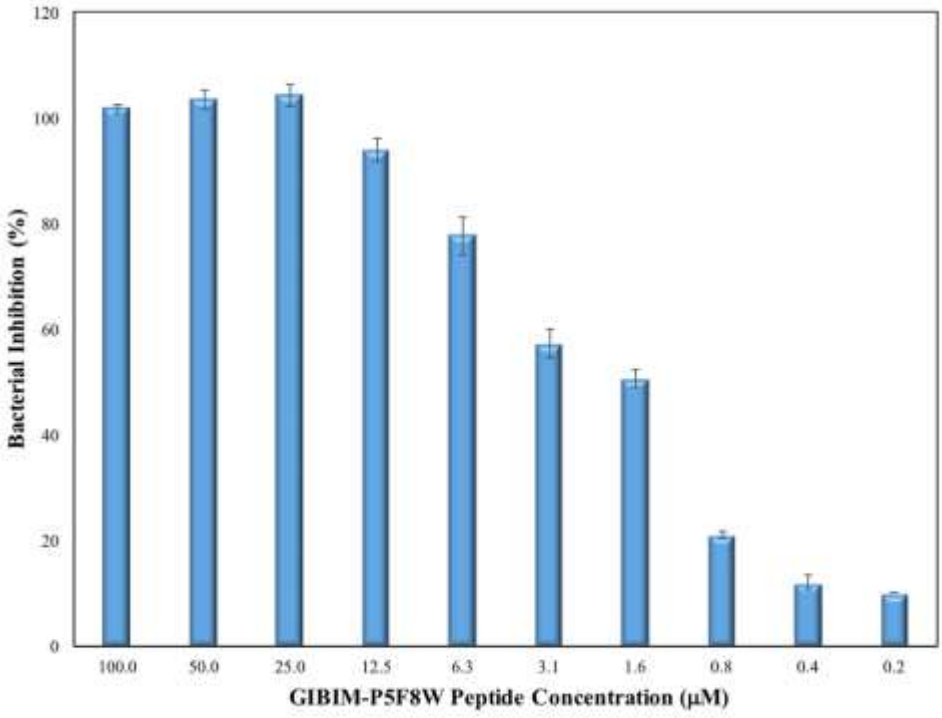

Figure 3 Antimicrobial activity of the synthetic peptide GIBIM-P5F8W against Methicillin-resistant Staphylococcus aureus. Experiments were performed until 8h of microorganism exposition to the peptide in a concentration range (0.2-100 µM).

2.3. DETECTION AND ANALYSIS OF MRSA PROTEINS DIFFERENTIALLY EXPRESSED FOLLOWING TREATMENT WITH GIBIM-P5F8W

To investigate the changes in *MRSA* protein expression induced by GIBIM-P5F8W, *MRSA* cultures were grown both without treatment and in the presence of the peptide at 0.5 MIC and subjected to 2DE analysis. Figure 4 shows representative 2D maps obtained for peptide treatment and control samples. Most protein spots were observed in the acidic range in both samples. A total of 114 spots were matched in both sample groups replicates. 33 protein spots were found to be differentially expressed ($p < 0.05$, 2x fold change): 26 downregulated spots and 7 upregulated spots (Figure 5). Differential spots showing statistical and biological relevance were

functionally annotated by MS analysis. Successfully characterized protein spots are listed in Tables 2 and 3.

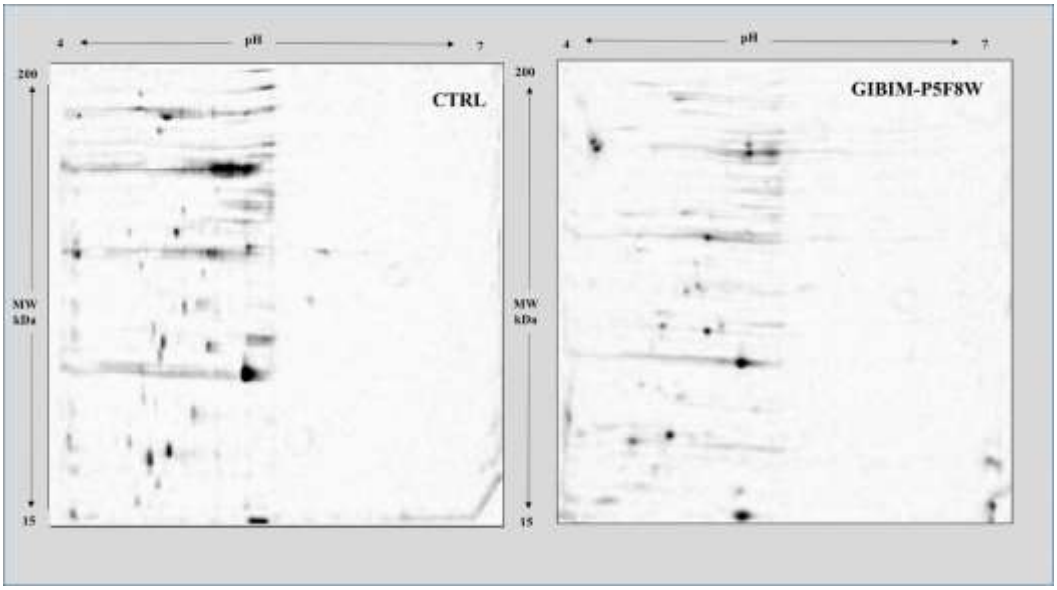

Figure 4 Representative 2DE maps from subinhibitory MRSA treatment and control samples. CTRL: proteome without treatment; GIBIM-P5F8W: proteome on presence of peptide (0.5 MIC) and 2 hours of treatment.

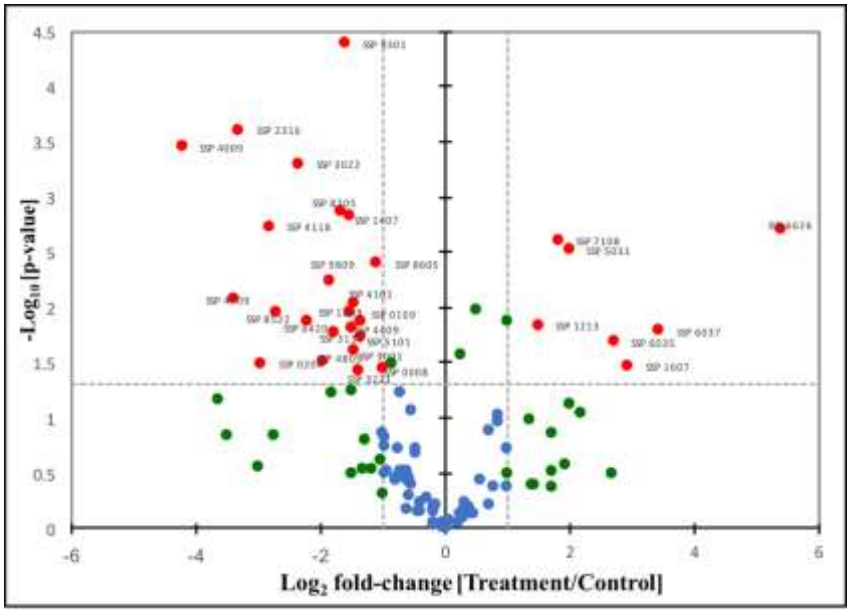

Figure 5 Volcano plot of 2D-E protein spots expressed during MRSA treatment with the synthetic peptide GIBIM-P5F8W. Horizontal and vertical dash lines represent the $p < 0.05$ and 2x fold change boundaries respectively.

Table 2 Differentially expressed upregulated proteins of MRSA with GIBIM-P5F8W treatment

Spot	Protein	SwissProt AC	Gene	Molecular function	Biological Process	pI/Mw	Fold change	p-value	Mascot Score
SSP 1608	Enolase	Q5HHP1	eno	Magnesium ion binding, phosphopyruvate hydratase activity	Glycolysis, Virulence	4.6/45	2	1.3E-2	90
SSP 1213	UDP-N-acetylmuramyl-tripeptide synthetase	Q1G8B5	murE	amino-acid acid ligase activity	Peptidoglycan synthesis	5.4/57	2.8	1.5E-2	64
SSP 6037	Transcriptional regulator MntR	A8FF36	mntR	bacterial-type RNA polymerase transcriptional repressor activity, sequence-specific DNA binding	Transcription	5.1/17	10.8	1.6E-2	62
SSP 6035	UTP-glucose-1-phosphate uridylyltransferase	S4CSE4	gtaB	Nucleotidyltransferase, Transferase	Carbohydrate metabolism, Stress response	5.0/33	6.5	2.1E-2	94

SSP 6626	Dihydrolipoyl dehydrogenase	Q5HGY8	pdhD	Oxidoreductase	Glycolysis, cell redox homeostasis	5/49.5	42.4	2.E-3	66

Table 3 Differentially expressed downregulated proteins of MRSA during GIBIM-P5F8W treatment

Spot	Protein	SwissProt AC	Gene	Molecular function	Biological Process	pI/Mw	Fold change	p-value	Mascot Score
SSP 2316	Adenylosuccinate synthetase	B9MLK0	purA	adenylosuccinate synthase activity, GTP binding, metal ion binding	Purine biosynthesis	6.1/48	0.1	2.5E-4	69
SSP 1407	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	Q5XB05	murG	Glycosyltransferase, Transferase	Cell cycle, Peptidoglycan synthesis	6.4/40	0.3	1.5E-3	66
SSP 4118	Peptide chain release factor 1	B2UZI5	prfA	translation release factor activity, codon specific	Protein biosynthesis	5.0/41	0.1	1.9E-3	70

SSP 9809	Adenylate kinase	B7HJ69	adk	Kinase, Transferase	Nucleotide biosynthesis, cellular energy homeostasis	4.9/24	0.3	5.8E-3	67
SSP 1209	GTPase Obg	Q2YT86	obg	Hydrolase	control of the cell cycle, stress response, ribosome biogenesis and morphogenesis control	5.0/47	0.3	1.1E-2	60
SSP 5101	Chromosomal replication initiator protein DnaA	A9KPP1	dnaA	DNA-binding	regulation of DNA replication	5.9/52	0.4	1.9E-2	63
SSP 0008	DNA-binding protein HU	Q5HFV0	hup	DNA-binding	DNA condensation	9.5/10	0.5	3.6E-2	116
SSP 8205	Protein GrpE	Q04EE0	grpE	Chaperone	Stress response	6.6/22	0.3	1.3E-3	64
SSP 3022	50S ribosomal protein L6	Q5HDX3	rplF	rRNA binding	translation	9.5/20	0.2	5.0E-4	40

2.4. BIOINFORMATIC ANALYSIS

Gene ontology analysis (Figure 6) shows that differentially expressed proteins of MRSA during GIBIM-P5F8W peptide treatment at subinhibitory concentration are involved in both cellular processes 40% (mainly related to cellular division, morphogenesis and sporulation) and metabolic processes required for energy production, 60%. From these metabolic processes, associated proteins with biosynthetic processes such as nitrogen compounds metabolism, phosphate-containing compounds metabolism and primary metabolism were highly represented.

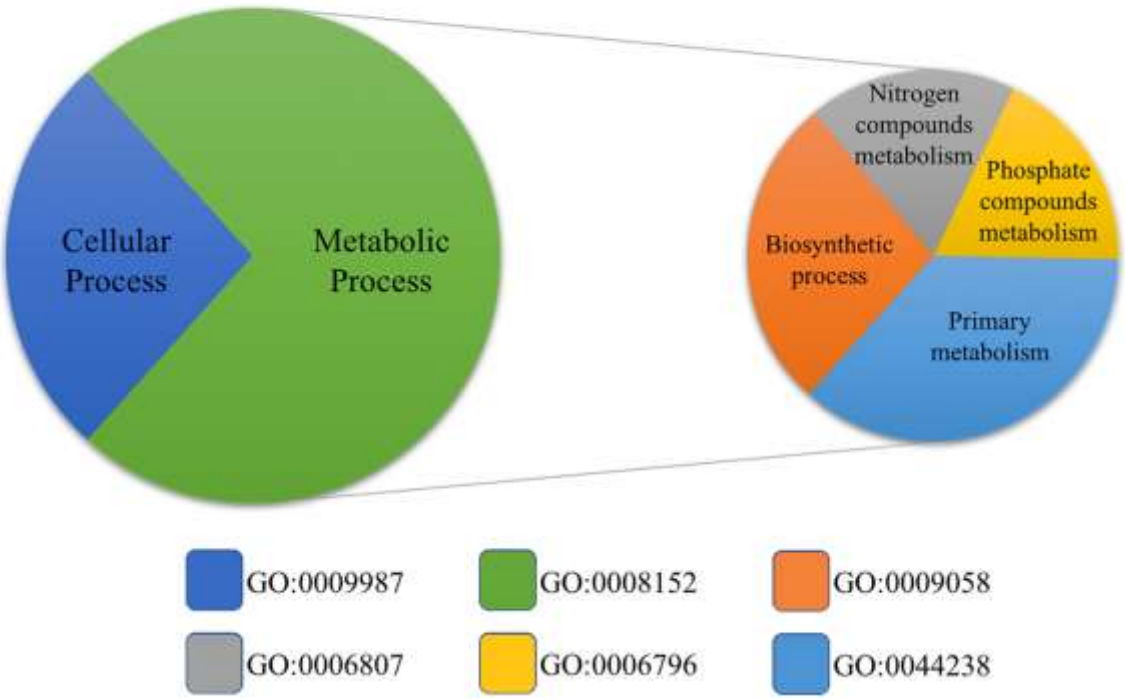

Figure 6 Functional categories of proteins differentially expressed by MRSA in response to GIBIM-P5F8W at 0.5 MIC.

Interactome network was built with the differentially expressed proteins during peptide treatment using the STRING online tool (Figure 7). Possible functional

association among the set of proteins allowed to identify some interesting pathways in response to the antibiotic stress. Predominantly, most proteins clustered into pathway 1 related to regulation of biological processes such as cell division, DNA replication and cell wall synthesis were downregulated. Similarly, inhibited pathway 2 cluster was associated with the translation process. Pathway 1 and 2 exhibited strong functional interaction with the glycolytic enzyme enolase (eno) and the AMP biosynthesis related enzyme adenylate kinase(adk). These two enzymes were 31clustered into pathway 3, that is involved in purine ribonucleotide metabolism, which in turn could be associated with ATP regeneration. Upregulation of proteins in catabolic pathway 3 and inhibition of energy consuming pathway 1 and 2, as observed in the proteomic analysis, would reveal a destabilization of energy balance induced by the peptide treatment.

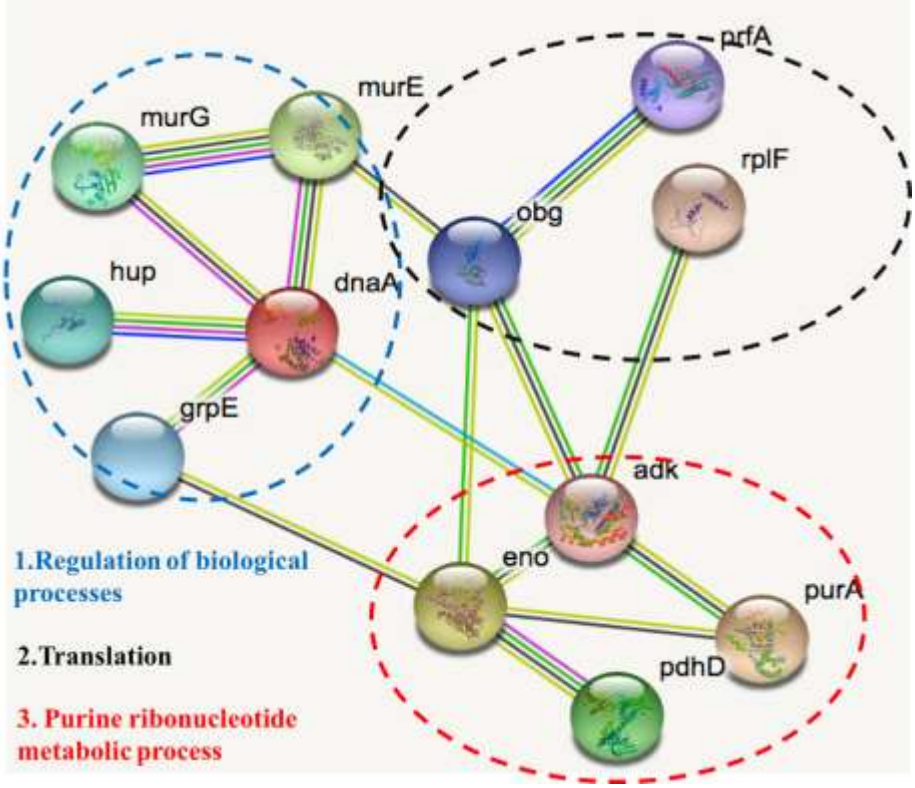

Figure 7 Interactome of differentially expressed proteins by Methicillin Resistant Staphylococcus aureus in response to GIBIM-P5F8W built by STRING v10.0. In the predicted network, proteins are represented as nodes. Experimentally determined interactions are represented with pink and light blue predicted from curated databases. Predicted interactions are represented with blue (co-occurrence) and green (neighborhood). Textmining interactions are represented with light green lines and co-expression is represented with black lines.

2.5. SUPEROXIDE SCAVENGING ACTIVITY

The activity of the superoxide dismutase enzymes was analyzed, after two hours of treatment. In this test, the ability of *S aureus* protein extract to scavenge 50% of the generated superoxide radical was evaluated, evidencing that it is necessary more protein of the treated bacteria to generate the same activity as the control. GIBIM-P5F8W inhibits the activity of this enzyme by approximately 50% (Figure 8).

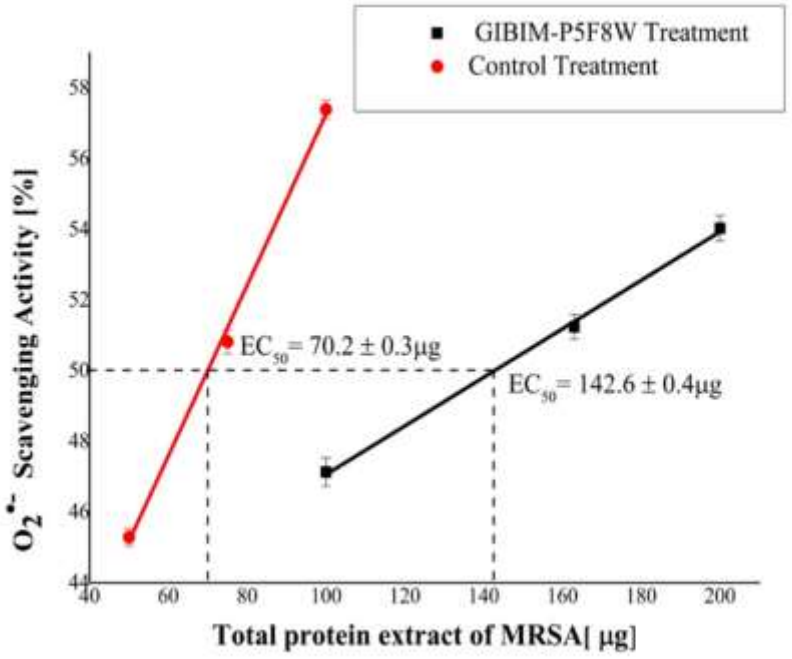

Figure 8 Scavenging activity of superoxide radicals of GIBIM-P5F8W peptide treatment and control treatment (absence of peptide) MRSA lysates. Each value represents the mean ± S.D. of three independent experiments. EC_{50} values were obtained from the linear regression model.

2.6. BACTERIAL CELL RESPIRATION

Given the assumption that the identified overexpressed proteins could promote cell catabolism, the effect of GIIBIM-P5F8W on the rate of oxygen consumption was analyzed (Figure 9). However, MRSA oxygen consumption before treatment (18.2 ±

4.2 nmol.mL^{-1}.min^{-1}) dramatically dropped after peptide exposure (9.6 ± 2.2 nmol.mL^{-1}.min^{-1}), showing that the electrons transport from the respiratory chain of the bacteria would be affected around 50% and therefore the ability to produce ATP by this way.

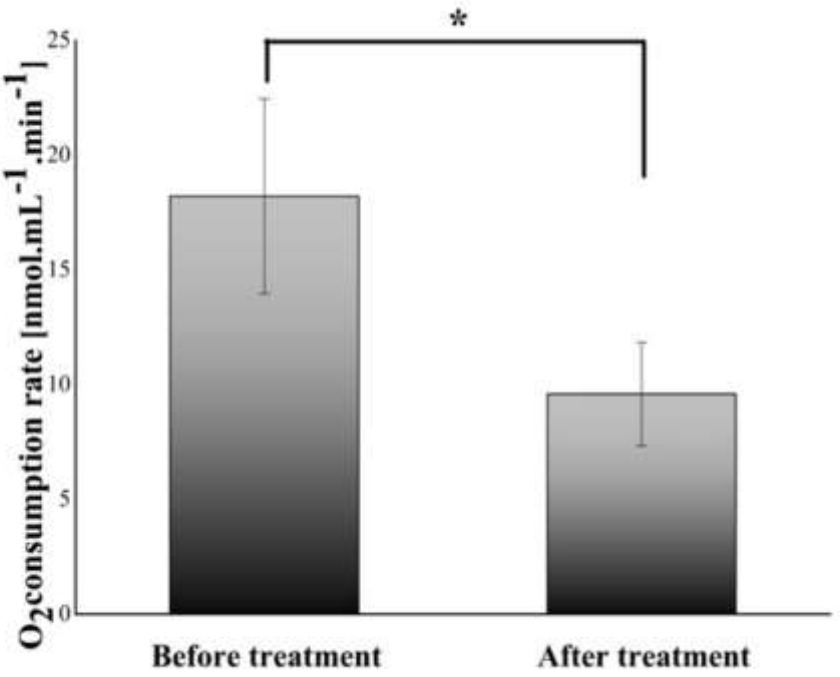

Figure 9 Bacterial oxygen uptake under GIBIM-P5F8W antimicrobial peptide treatment. Oxygen consumption rate of both basal bacterial respiration and under peptide treatment (0.5MIC). The results represent the mean ± S.D of three experiments. Statistical comparison was performed using one-way analysis of variance (ANOVA) and Student *t*-test. *$p < 0.05$ when compared to the respective control.

3. DISCUSSION

Antimicrobial effectiveness of GIBIM-P5F8W peptide against MRSA at low micro molar level draw a great deal of interest among the antimicrobial peptides strategies to fight this multi-drug resistant pathogen. A large number of biosynthetic and synthetic anti-MRSA peptides (*ca.* 118 peptides) have been characterized, from which approximately 70% exhibit high activity against MRSA isolates with MIC

values ranging from 0.5 μM to 50 μM(Zouhir et al., 2017; Zouhir, Jridi, Nefzi, Ben Hamida, & Sebei, 2016). The low inhibitory concentration of GIBIM-P5F8W against MRSA (MIC 12.5 μM) clearly stands within the range of the most bioactive peptides including the most recently reported compounds: the synthetic peptide based on the human antimicrobial peptide LL-37 , SAAP-148 (MIC 1.6μM) ; the 2-hydroxyethyl-NH125 analogue 11 (MIC 0.39-1.17 μM)(Basak et al., 2017); persulcatusin (IP) found in the *Ixodes persulcatus* midgut (MIC 0.6 μM) and medusin-PT analog from the skin secretion peptide of the *Phyllomedusa tarsius* (MIC 8.7-17.4 μM)(Gao et al., 2017). Therefore, exploring the mode of action of the promising GIBIM-P5F8W peptide and the effects on the bacterial physiology of MRSA by a proteomic approach may support further research in novel antimicrobial.

A major proteomic signature of the antimicrobial effects of the peptide might be associated with cell viability reduction through energetic imbalance. One of the fundamental requirements of all cells is to balance ATP consumption and ATP generation. Consequently, There are metabolic enzymes that sense cellular (AMP+ADP): ATP ratios to modulate catabolic processes in the cell and maintain energy homeostasis (Are, Daddaoua, Krell, & Ramos, 2009; Hardie, 2011).

Two proteins from AMP biosynthesis and related to purine metabolism were downregulated (Table 3): adenylosuccinate synthetase (purA), which catalyzes the first step in the biosynthesis of AMP from IMP and adenylate kinase (adk), that catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Inhibition of adk may indicate an energetic imbalance exerted by the peptide treatment inducing accumulation of high amounts of AMP that signals stimulation of catabolic pathways including glycolysis and TCA cycle in order to restore ATP levels (Dzeja & Terzic, 2009; Sanwal, 1970). Additionally, high AMP levels could have repressed purA expression in a feedback inhibition related mechanism.

AMP-induced upregulation of catabolic pathways during peptide treatment was evidenced in the overexpression of the glycolytic enzyme enolase (Figure 7, Table 2). According to this, it might suggest a higher rate of phosphoenol pyruvate formation from 2-phospho-D-glycerate which in turn may well promotes regeneration of ATP levels from the downstream glycolysis steps mediated by pyruvate kinase and the TCA cycle. However, enolase has also been found participating in some other relevant processes in *S. aureus* in both the cell surface and the cytoplasm. Enolase has been described as a staphylococcal virulence factor which enables adherence to host cells by binding to laminin and plasminogen (Carneiro, Postol, Nomizo, Reis, & Brentani, 2004; Mölkänen, Tyynelä, Helin, Kalkkinen, & Kuusela, 2002) and it has been found included in the *S. aureus* mRNA degradosome holoenzyme complex (Roux, DeMuth, & Dunman, 2011), possibly acting as a sensor of the cell energetic state or phosphosugar stress (Bernstein, Lin, Cohen, & Lin-Chao, 2004).

Another signature of catabolism stimulation is observed by increased expression of dihydrolipoyl dehydrogenase or lipoamide dehydrogenase (LPD). LPD is an ubiquitous flavoprotein, which catalyzes the NAD^+-dependent oxidation of dihydrolipoamide in several multienzyme complexes: pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched chain 2-oxo acid dehydrogenase (BCDH) (Carothers, Pons, & Patel, 1989). Induced high levels of LPD in treated *S. aureus* (Table 2) could increase the amount of available reducing equivalents NAD^+ /NADH from TCA.

This reduction capacity could not be used by the electron transport chain (Figure 9), promoting the generation of free radical species as side products and oxidative stress in the bacterial cell if detoxifying mechanisms cannot withstand the stress applied (Bunik, 2003; Tretter & Adam-Vizi, 2005).

Induced oxidative stress by upregulation of the aerobic metabolism may be one of the main consequences of MRSA susceptibility to GIBIM-P5F8W, as ROS (Reactive Oxygen Species) scavenging mechanisms seem to be suppressed. The latter assumption may be confirmed by the inhibition of superoxide dismutase activity (figure 8) and the overexpression of the transcriptional regulator MntR during peptide treatment (Table 2) which may indicate a significant decrease in manganese uptake, since expression of the two Mn uptake systems in *S. aureus* (the transporter MntABC and MntH) (Horsburgh et al., 2002) are regulated by this DtxR-like metalloregulatory protein (O'Halloran, 1993; Que & Helmann, 2000).

Availability of manganese in the bacterial cell is of great importance due to the fact that it plays an essential role as cofactor in diverse cellular processes such as sugar metabolism, signal transduction, the stringent response, and oxidative stress (Kehres & Maguire, 2003; Papp-Wallace & Maguire, 2006). Furthermore, being Mn^{2+} a very strong electron donor (E_H -1.18V), it plays an important role in detoxification of ROS in bacteria as seen in superoxide dismutase SodA and SodM which requires Mn^{2+} for the catalytic degradation of superoxide radical into peroxide and molecular oxygen ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) (Clements, Watson, & Foster, 1999; Valderas & Hart, 2001).

Additionally, in order to maintain energy homeostasis bacterial cell could also adopt a strategy to reduce ATP consumption by modulating the cell cycle. The hydrolase GTPase Obg was found downregulated during peptide exposure (Table 3) which is an enzyme that convert GTP into GDP allowing the transfer of the GTP terminal phosphate group to a target protein(Bourne, Sanders, & McCormick, 1991). Its low rate of GTP hydrolysis, binding constants at micromolar levels for GTP and GDP, and rapid dissociation constants for either GTP or GDP, suggest they act as intracellular sensors where binding is modulated by GTP/GDP ratio according to the energy balance in the cell(Kint, Verstraeten, Hofkens, Fauvart, & Michiels, 2014). Obg homologs are essential for the survival of both Gram-positive and Gram-

negative bacteria(Arigoni et al., 1998; Okamoto & Ochi, 1998; Shah, Das, & Bhadra, 2008; Trach & Hoch, 1989; Zielke, Wierzbicki, Baarda, & Sikora, 2015). Bacterial Obg proteins have been associated with a wide range of cellular processes comprising ribosome assembly, DNA repair, sporulation, and morphological development(Bonventre, Zielke, Korotkov, & Sikora, 2016). In V. *cholerae*, it has been proven that depletion of the Obg homologue CgtA increased levels of the alarmone nucleotide ppGpp that correlates with induction of the global stress response and cell growth inhibition(Raskin, Judson, & Mekalanos, 2007). Decresed levels of GTPase Obg in MRSA under antibiotic pressure prompted by GIBIM-P5F8W peptide may have as well induced an stringement response making the bacterial cell, as a survival strategy, to divert gene expression away from cell division processes and towards metabolic pathways to restore energy levels.

On the other hand, cell division may be tightly regulated by other biosynthetic pathways. For instance, glucolipid biosynthesis has been found to act as a metabolic sensor to synchronize cell size with growth rate in a Gram-positive model, *Bacillus subtilis* (*B. subtilis*)(Weart et al., 2007). In *B. subtilis*, not only UDP (Uridine diphosphate)-glucose works as a precursor for the biosynthesis of the lipoteicoic acids (LPs) membrane anchor diglucosyl-diacylglycerol (Glc2-DAG), catalyzed by diacylglycerol β-glucosyltransferase (UgtP); but when bound to UgtP, it can inhibit FtsZ (tubulin-like protein) assembly which delays cell division until cells reach the appropriate mass under a certain growth rate(Haeusser & Margolin, 2016). The proteomics analysis (Table 2) showed that *MRSA* under exposure to the synthetic peptide enhances expression of the UTP-glucose-1-phosphate uridylyltransferase which means higher levels of UDP-glucose that eventually could help to signal a slow cell division rate due to energetic imbalance.

In addition to the above, further evidence was found related to the suppression of cell division as a survival strategy during treatment with the peptide. Proteins involved in replication such as chromosomal replication initiator protein DnaA and

the histone-like DNA-binding protein HU were inhibited (Table 3). It was found inhibition of peptidoglycan synthesis by downregulation of the glycosyltransferase MurG that participates in lipid II formation (Mohammadi et al., 2007). Moreover, an equally energetically costly process like translation could have been inhibited by downregulation of the peptide chain release factor 1, 50S ribosomal protein L6 and ATP-dependent chaperone activity of Protein GrpE that works synergistically with DnaJ and DnaK proteins (Liberek, Marszalek, Ang, Georgopoulos, & Zylicz, 1991).

During proteomic analysis, another strategy to cope with the antimicrobial agent GIBIM-P5F8W induced stress was profiled, which is related to the bacterial leaflet modification. Peptidoglycan is a major part of the *S. aureus* that preserves cell integrity by withstanding inner osmotic pressure(Vollmer, Blanot, & De Pedro, 2008). The long glycan chains crosslinked by short peptides are assembled by a ATP-dependent amide-bond ligases superfamily, the Mur ligases(Eveland, Pompliano, & Anderson, 1997). Amino acid addition by MurE differs considerably with species(Barreteau et al., 2008; Schleifer & Kandler, 1972). MurE orthologue gene in S. aureus codes for a ligase protein that catalyzes the addition of L-lysine to the nucleotide precursor UDP-N-acetylmuramoyl-L-alanyl-D-glutamate (UMAG)(Patin et al., 2010). Upregulation of MurE induced during peptide treatment (Table 2) could result in a stimulated incorporation of lysine to the bacterial cell wall that might increase the overall positive charged of the cell envelope due to the protonated amine residues exhibited by lysine under physiological pH. Consequently, it may bring about depletion of electrostatic interaction of the bacterial membrane with the cationic synthetic peptide GIBIM-P5F8W.

4. CONCLUSIONS

The GIBIM-P5F8W is presented as a promising peptide against *S. aureus*. The bactericidal effect of the peptide may be attributed by the repression of manganese transporters spotted at the protein level, by the inhibition of superoxide dismutase and the decrease of energy by inhibition of oxygen consumption. As a survival strategy, the pathogen seems to activate key control steps of catabolism for energy recovery, while energy consuming processes are hindered, principally those related to cell growth and division.

These findings present an opportunity to further considerate this novel compound as an anti-staphylococcal treatment candidate, and to explore these less common bactericidal mechanisms as well as some others molecular targets for the development of new antimicrobial strategies.

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