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RESEARCH ARTICLE

Comparative Proteome Profiles of Methicillin-Resistant *Staphylococcus aureus* in Response to Vanillic Acid and 2-Hydroxycinnamic Acid

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Abstract: ***Background*:** The ability of *Staphylococcus aureus* to cause severe infections and the difficulty of the treatments due to the multiple antibiotic resistance make this bacterium a life-threatening human pathogen. This situation necessitates the exploration of novel antimicrobial compounds with known targets on bacteria. Phenolic acids naturally produced in plants as secondary metabolites are good candidates for being alternative antimicrobials for antibiotic-resistant bacteria.

***Objective*:** Investigation of protein profile of Methicillin-resistant *S. aureus* (MRSA) in the presence of subinhibitory concentrations of phenolic acids.

***Methods*:** MRSA was subjected to subinhibitory concentrations of vanillic acid (VA) and 2-hydroxycinnamic acid (2-HCA), separately, and the proteomic analyses were carried out by using liquid chromatography coupled to mass spectrometry.

***Results*:** Both phenolic acids elicited identification of differently expressed proteins that have roles in DNA replication, repair, RNA processing and transcription, protein synthesis, maintenance of cell homeostasis, several metabolic reactions in energy, carbohydrate and lipid metabolisms and also proteins related with the virulence and the pathogenicity of MRSA when compared with the control group. The numbers of the proteins identified were 444, 375, and 426 for control, VA-treated MRSA, and 2-HCA-treated MRSA, respectively, from which 256 were shared. While VA treatment resulted in 149 unidentified MRSA proteins produced in control, 2-HCA treatment resulted in 126 unidentified proteins. Data are available via ProteomeXchange with identifier PXD016922.

***Conclusion*:** The results obtained from this study might indicate the potential targets on bacteria and the effective use of phenolic acids in the battle with antibiotic-resistant pathogens.

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1. INTRODUCTION

Staphylococcus aureus is a well-recognized bacteria, mainly due to its rate of nosocomial infections and development into drug resistant strains [1]. As being one of the most serious superbugs methicillin-resistant S. aureus (MRSA) is notorious for being the most commonly acquired pathogen with multiple drug-resistance. This hospital- and community-acquired bacterium is a major source of bacterial infections and causes several diseases including soft tissue infections and necrotizing pneumonia. Although it is a commensal organism highly prevalent in the human population, it has an emerging role in secondary infections [3]. Antibiotics are commonly used for the treatments of the diseases caused by this bacterium. However, overuse and misuse of antibiotics often result in the generation of bacterial strains resistant to multiple drugs [1, 4, 5]. Increased resistance to antibiotics not only aggravates the treatments of Staphylococcal infections but also increases the costs of the treatments [2, 6]. In 2011, the number of reported MRSA infections in the US was about 80,461 [7]. In 2017, the World Health Organization reported *S. aureus* among high priority antibiotic-resistant bacteria that require research and development of novel antimicrobial compounds [8]. Unless precautions are taken, untreatable bacterial infections will be responsible for the death of 10 million people each year by 2050 [5].

The rising resistance to multiple antibiotics requires the investigation of effective antimicrobial compounds. The therapeutic properties of plants and antimicrobial effects of plant-derived compounds are curiously being investigated worldwide due to their potentials to combat drug-resistant pathogens [9]. Phenolic acids are mostly plant-based secondary metabolites, and they can be found in all vegetative parts of the plants [4]. Their antioxidant, antimicrobial, anticarcino-

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gen, and anti-inflammatory properties provide benefits to human health [4] and make them promising candidates to be considered as alternative antimicrobials in the battle against human pathogens. Lima *et al*. has shown that when compared with a larger structure phenolic compounds, the antimicrobial effect of phenolic acids is stronger. The antibacterial activities of phenolic acids have been shown in many studies [4, 6, 10]. However, the exploration of novel antimicrobial compounds requires the knowledge of their mode of action [11]. The investigation of global protein expression to enlighten the action mechanisms of antimicrobial compounds is a commonly used approach [5, 11-14]. Proteins are responsible for reflecting the gene expression levels in relation to translational control and regulation. Investigation of proteomic profiles would be accepted as one of the most relevant approaches for understanding biological processes [15], and these protein levels are directly related to drug resistance [16].

In this study, two phenolic acids belonging to two different subclasses were used to discover the potential target sites on MRSA. The number and the position of the hydroxyl groups, as well as the presence of other prosthetic groups, affect the antimicrobial properties of the phenolic acids [10]. For comparison, vanillic acid (VA), a hydroxybenzoic acid derivative, and 2-hydroxycinnamic acid (2-HCA), a hydroxycinnamic acid derivative, were tested against MRSA. The present work aimed to investigate the proteome profiles of MRSA treated with the subinhibitory concentrations of two different types of phenolic acids to assess the potential targets for alternative antimicrobial development.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Culture Conditions

In this study, MRSA strain N315 carrying type II SCC*mecA* was used. MRSA was grown in tryptic soy broth (TSB) (Sigma-Aldrich 22092) and on tryptic soy agar (TSA). Long-term storage of bacteria was maintained by storing at -80 ºC in TSB media containing 20% glycerol.

2.2. Determination of Subinhibitory Concentrations of Phenolic Acids

Vanillic acid (Sigma-Aldrich 94770) and 2-hydroxycinnamic acid (Sigma-Aldrich H228009) were purchased commercially and prepared freshly before each experiment by the addition of 0.6% dimethyl sulfoxide (DMSO) (Sigma-Aldrich D5879) into the TSB media. Subinhibitory VA and 2-HCA concentrations were determined in our previous study [17]. Accordingly, VA at the concentration of 0.35 MIC (1.3 mg/ml), and 2-HCA at the concentration of 0.45 MIC (1.2 mg/ml) were used as subinhibitory concentrations.

2.3. Total Protein Isolation

Total protein isolation was carried out after 18-hour treatment of MRSA with subinhibitory concentrations of either VA or 2-HCA. For protein preparation, steps of the isolation protocol carried out by Sianglum *et al*. [18] were followed with minor modifications. Briefly, phenolic acid-treated and untreated MRSA cells were collected by 20 minute-centrifugation at 10,000x*g* at 4ºC. Then, harvested cells were washed twice with 0.85% (w/v) NaCl solution and resuspended in phosphate-buffered saline solution. The disruption of cells was provided by agitation with an ultrasonic processor. The process was completed at 30% amplitude with 9 seconds on and off intervals for 15 minutes on ice. For the removal of cellular debris, the lyzed cells were centrifuged at 20,000x*g* at 4 ºC for 20 minutes. The obtained supernatant was subjected to acetone precipitation with ice-cold acetone (1:4, v/v), and the pellet was dissolved in resuspension buffer (7 M urea, 2 M thiourea, 0.1 M Tris-HCl pH: 7.8). The protein concentrations were determined via Bradford protein assay [19] and adjusted to 400 µg. The protein isolations were performed with two independent biological samples.

2.4. Preparation of the Peptide Samples

Tryptic digestion of the extracted protein samples was carried out via in-solution digestion [20]. All incubation processes were maintained at room temperature unless indicated otherwise. Briefly, 0.2 M dithiothreitol (DTT) in 50 mM Tris-HCl (pH: 7.8) was added into each protein sample to a final concentration of 10 mM and incubated for 50 minutes. Then, 0.2 M iodoacetamide (IAA) in 50 mM Tris-HCl (pH: 7.8) was added onto the solutions to a final concentration of 20 mM and incubated in the dark for 50 minutes. Following incubation with IAA, DTT was added to a final concentration of 20 mM, and the samples were incubated for 50 minutes. Urea concentration was decreased with the use of 10K cutoff filters (Amicon). The protein samples were enzymatically digested with trypsin (1:50 trypsin to protein ratio) at 37 ºC overnight incubation. Trypsin digested samples were kept in Lobind tubes (Eppendorf) in -20 ºC and vacuum concentrated before fractionation.

2.5. Fractionation of the Peptides

Fractionation of the peptides was conducted using the LC20AD HPLC system (SHIMADZU Prominence UFLC) with a reverse-phase column (C18-Teknokroma Mediterraneasea 18; 25 cm x 0,46 cm; 5µm). The volumes of the samples were brought to 100 µl with the mobile phase A (ddH_2O pH: 10 adjusted with ammonium hydroxide). Mobile phase B was 90% acetonitrile (prepared with pH: 10 adjusted ddH_2O). The column temperature was 40 °C, and the flow rate was 0.5 ml/minute. Fractionation of peptides was analyzed with the LCsolution program, and the eluted peptides were collected with the MALDI-Spotter instrument (SunCollect program). The obtained eluents were concatenated into 12 fractions and vacuum concentrated.

2.6. LC-ESI-MS/MS Analysis

Each fraction was resuspended in 2% acetonitrile and 0.1% formic acid (FA) solution and then purified and concentrated with ZipTip pipette tips (ZipTip C18, Millipore). Prior to LC-MS/MS, the samples were acidified with 0.1% FA addition. Chromatographic peptide separation was car-

ried out with the Ultimate 3000 (Dionex) HPLC system coupled to LTQ Orbitrap XL mass spectrometer (Thermo Fischer). The peptides were separated with a C18 HPLC column (Sigma Supelco Ascentis; 15 cm x 500 µm; 2,7 µm). Two technical runs were performed with 10 µl injection volume. The elution program was started with 98% phase A (0.1% FA in ddH_2O) and 2% phase B (0.1% FA in acetonitrile). During the 46-minute elution process, the percentage of phase B was increased up to 90%. The flow rate was 5 µl/minute, and the collision gas was helium. During the ionization, spray voltage was adjusted to 5 kV and the capillary temperature to 200 °C.

2.7. Mass Spectrometry Data Processing

The analysis of the spectra was carried out with LTQ tune software. The raw spectral data were converted to MGF format by using Proteome Discoverer 1.4 (Thermo Scientific). The identification of the proteins was performed via the Mascot Search engine (Version 2.3, Matrix Science) with comparing the obtained spectra with the dataset of *Staphylococcus aureus* strain N315 proteome (UniProt proteome ID: UP000000751). The search parameters were as follows: enzyme: Trypsin; fixed modifications: carbamidomethylation (C); variable modifications: oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: +/- 1.2 Da; fragment mass tolerance: +/- 0.6 Da; maximum missed cleavages: 1; instrument type: ESI-trap. The percolator algorithm was applied to Mascot results to improve the identifications. The false discovery rate (FDR) was kept below 1%. The assignment of the molecular functions of each protein was achieved by searching the corresponding accession number (protein ID) on UniProt. Proteins with at least two-peptide spectrum matches were included in the list. Two biological repeats were performed.

The MS proteomics data was uploaded to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE [21] partner repository with the data set identifier PXD016922.

3. RESULTS

The proteomic analyses allowed the identification of 444 proteins in control, 375 proteins in VA-treated MRSA, and 426 proteins in 2-HCA-treated MRSA. The distribution of the identified proteins indicated the presence of 597 unique proteins, of which 256 were mutually expressed under all conditions (Fig. **S1**). The partial list of the identified proteins in MRSA after VA treatment was provided in Table **1** (The data were not limited to mutual ones) (Complete list of identified proteins for control, VA-treated, and 2-HCA-treated MRSA were given in supplementary material as Table **S1**, Table **S2,** and Table **S3**, respectively). As shown in Table **1**, the treatment of MRSA with VA affected the expression of many proteins taking roles in several biological processes such as DNA structure and repair, RNA processing and transcription, ribosome assembly and translation, cell wall biosynthesis, metabolic pathways, cell homeostasis, and pathogenicity.

Similar categorization was performed for the proteins identified in MRSA when treated with 2-HCA. 2-HCA treatment resulted in the identification of a higher number of proteins when compared with VA treatment. While 318 of the identified proteins were mutual for control and 2-HCA-treated MRSA, 126 of them were identified only in the control group, and 108 of them were identified as proteins that were expressed in the presence of 2-HCA stress (Fig. **S1**). The proteins listed in Table **2** were comparative proteins of control and 2-HCA-treated MRSA.

The proteins' function categorie for control, VA-treated, and 2-HCA-treated MRSA were attributed according to the gene ontologies (GO) given in the UniProt database and shown in Fig. (**1**). The uncharacterized and predicted proteins with unknown functions were categorized under "Others". Moreover, visualizations of the interactions between proteins were shown via gene names by using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (http://string-db.org/).

Among the identified proteins that were related to DNA structure, replication, and repair, some proteins are unique to the MRSA control cells but were not identified in VA-treated cells (Table **1**). However, the VA treatment allowed the identification of three proteins that were belong to DNA related proteins. . The identification of these proteins indicated the differences in DNA structure, recombination, and repair. Table **2** showed the partial list for the identified proteins of control and 2-HCA-treated MRSA. Among four proteins related to DNA that were unidentified after 2-HCA treatment, DNA repair protein RecN was responsible for recombinational repair of the damaged DNA. The interactions between DNA-related proteins identified in the presence and absence of VA and 2-HCA were shown in Fig. (**2**).

When differently identified proteins related to RNA and transcription processes were examined (Table **1**), it was observed that VA treatment affected protein expressions that were responsible for the regular progress of transcription and the assembly and attachment of RNA polymerase. Similarly, some RNA structure, assembly, and transcription process-related proteins were not identified under 2-HCA treatment. When the control MRSA RNA-related proteins in Table **2** were examined, the 10 proteins were notably absent. These proteins have roles in normal progress of RNA attachment and transcription, as well as regulation. Representations of interactions between RNA related proteins identified in the presence and absence of VA and 2-HCA were shown in Fig. (**3**).

The clustered proteins related to ribosome and protein synthesis (Table **1**) determined the determination of essential differences in the VA-treated cells. VA treatment resulted in non-detection of three ribosomal proteins that were structural constituents of the ribosome. Moreover, the aminoacylation of tRNAs for protein synthesis was also inhibited for alanine, phenylalanine, leucine, and tyrosine. Differential expression of the ribosome and translation-related proteins upon 2-HCA treatment was remarkable in terms of high numbers of unidentified proteins with functions in

Table 1. Partial[a] list of identified proteins in MRSA after vanillic acid (VA) treatment.

Accession Number	Name of Protein	Gene	Function	VA-Treated MRSA	Control MRSA
DNA Related Proteins					
Q7A5K4	3'-5' exonuclease DinG	dinG	DNA replication	NI	Identified
Q7A4Q5	DNA ligase	ligA	DNA replication and repair	NI	Identified
Q7A5S6	Nuclease SbcCD subunit C	sbcC	DNA replication, repair, recombination	NI	Identified
Q7A6H4	ATP-dependent helicase/nuclease subunit A	addA	Double-strand break repair	NI	Identified
Q93KF4	DNA topoisomerase 4 subunit A	parC	Chromosome segregation	NI	Identified
P66939	DNA topoisomerase 4 subunit B	parE	Chromosome segregation	NI	Identified
P66937	DNA gyrase subunit B	gyrA	DNA topological change	Identified	NI
Q99XG5	DNA gyrase subunit A	gyrB	DNA topological change	Identified	NI
P65496	Endonuclease MutS2	mutS2	DNA mismatch repair	Identified	NI
RNA Related Proteins					
P66726	DNA-directed RNA polymerase subunit omega	rpoZ	RNA polymerase assembly	NI	Identified
Q99TT5	RNA polymerase sigma factor SigA	rpoD	Attachment of RNA polymerase to initiation sites	NI	Identified
P65578	Transcription antitermination protein NusB	nusB	Transcription of ribosomal RNA genes	NI	Identified
P0A096	Transcription termination/antitermination protein NusG	nusG	Involved in transcription elongation, termination, and antitermination	NI	Identified
P99156	Transcription elongation factor GreA	greA	Regulation of elongation	NI	Identified
A0A0H3JM44	DEAD-box ATP-dependent RNA helicase CshB	cshB	Proper initiation of transcription	NI	Identified
A0A0H3JTG9	Cold-shock protein C	cspC	Regulation of transcription	NI	Identified
Q7A5P3	Cold shock protein CspA	cspA	Regulation of transcription in cold stress response	NI	Identified
A0A0H3JNE2	Transcription termination factor Rho	rho	Transcription termination by binding to the nascent RNA	Identified	NI
P67182	Probable transcriptional regulatory protein SA0624	SA0624	Regulation of transcription	Identified	NI
P65439	Transcriptional regulator MraZ	mraZ	Transcription regulation	Identified	NI
P65944	Bifunctional protein PyrR	pyrR	Regulates transcriptional attenuation of the pyrimidine operon	Identified	NI
Ribosome and Protein Synthesis Related Proteins					
P66153	50S ribosomal protein L28	rpmB	Translation	NI	Identified
P66299	50S ribosomal protein L36	rpmJ	Translation	NI	Identified
P66494	30S ribosomal protein S19	rpsS	Translation	NI	Identified
P99130	Ribosome-recycling factor	frr	Releases ribosomes from mRNA	NI	Identified
Q7A682	Ribonuclease J 1	mj1	rRNA processing	NI	Identified
P67011	Alanine-tRNA ligase	alaS	Catalyzes the attachment of alanine to tRNA	NI	Identified
A0A0H3JME4	Phenylalanine-tRNA ligase beta subunit	SA1563	Catalyzes the attachment of phenylalanine to tRNA	NI	Identified
P67513	Leucine-tRNA ligase	leuS	Catalyzes the attachment of leucine to tRNA	NI	Identified
Q7A537	Tyrosine-tRNA ligase	tyrS	Catalyzes the attachment of tyrosine to tRNA	NI	Identified

(Table 1) contd....

Accession Number	Name of Protein	Gene	Function	VA-Treated MRSA	Control MRSA
P66019	Peptide chain release factor 1	prfA	Termination of translation in codons UAG and UAA	NI	Identified
P99082	33 kDa chaperonin	hslO	Protein folding	NI	Identified
P60748	Foldase protein PrsA	prsA	Protein secretion	NI	Identified
Q7A468	Protein translocase subunit SecY	secY	Protein transport	NI	Identified
Q7A6Q1	Probable protein-export membrane protein SecG	secG	Protein transport	NI	Identified
A0A0H3JTY9	Signal recognition particle protein	ffh	SRP-dependent protein targeting to membrane	NI	Identified
P67137	Endoribonuclease YbeY	ybeY	70S ribosome quality control	Identified	NI
P65286	Lipoyl synthase	lipA	Protein lipoylation	Identified	NI
Cell Wall and Cell Membrane Related Proteins					
P65463	UDP-N-acetylenolpyruvoylglucosamine reductase	murB	Peptidoglycan biosynthesis	NI	Identified
A0A0H3JMW3	UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	murF	Cell wall formation	NI	Identified
Q7A7B4	Bifunctional protein GlmU	glmU	Cell wall organization	NI	Identified
A0A0H3JNI1	Ribulose-5-phosphate reductase	tarJ	Cell wall organization	NI	Identified
P63665	ATP synthase epsilon chain	atpC	Production of ATP	NI	Identified
Q7A4E8	ATP synthase gamma chain	atpG	Production of ATP	NI	Identified
Q7A3J9	UTP--glucose-1-phosphate uridylyltransferase	gtaB	Catalysis of the formation of UDP-glucose	Identified	NI
A0A0H3JLN8	SA1035 protein	SA1035	Cell cycle, cell division	Identified	NI
Metabolism Related Proteins					
Q7A715	Xanthine phosphoribosyltransferase	xpt	Purine metabolism	NI	Identified
P99085	Hypoxanthine-guanine phosphoribosyltransferase	hpt	Purine ribonucleotide salvage	NI	Identified
A0A0H3JM64	Purine nucleoside phosphorylase DeoD-type	deoD	Purine metabolic process	NI	Identified
P99099	Adenylosuccinate synthetase	purA	AMP biosynthesis	NI	Identified
P99068	Nucleoside diphosphate kinase	ndk	CTP, GTP, UTP biosynthetic processes	NI	Identified
P65936	Uridylate kinase	pyrH	Pyrimidine metabolism	NI	Identified
Q7A6F8	3-oxoacyl-synthase 2		Fatty acid biosynthesis	NI	Identified
P65739	Phosphate acyltransferase	plsX	Fatty acid and phospholipid biosynthetic processes	NI	Identified
Q7A5Z3	Malonyl CoA-acyl carrier protein transacylase	fabD	Fatty acid biosynthesis	NI	Identified
Q7A557	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	accD	Fatty acid biosynthesis	NI	Identified
P99148	Aconitate hydratase A	acnA	TCA. Isomerization of citrate to isocitrate	NI	Identified
P99070	Succinate--CoA ligase subunit alpha	sucD	TCA. Couples the succinyl--CoA hydrolysis to synthesis of ATP/GTP	NI	Identified
A0A0H3JP54	Tagatose-6-phosphate kinase	fruB	D-tagatose 6-phosphate catabolic process	NI	Identified
Q7A535	Formate-tetrahydrofolate ligase	fhs	Tetrahydrofolate interconversion	NI	Identified
P99079	Dihydrofolate reductase	folA	Tetrahydrofolate biosynthesis	NI	Identified
P99144	Orotate phosphoribosyltransferase	pyrE	UMP biosynthesis	Identified	NI
P67544	Bifunctional purine biosynthesis protein PurH	purH	IMP biosynthesis	Identified	NI
P60298	Ornithine aminotransferase 2	rocD2 (AID39393.1)	Conversion of ornithine to glutamate	Identified	NI

(Table 1) contd....

Accession Number	Name of Protein	Gene	Function	VA-Treated MRSA	Control MRSA
P99076	1-pyrroline-5-carboxylate dehydrogenase	rocA	Proline catabolic process to glutamate	Identified	NI
A0A0H3JMB8	Citrate synthase	citZ	TCA	Identified	NI
P99167	Isocitrate dehydrogenase	icd	TCA	Identified	NI
A0A0H3JS20	Acetate-CoA ligase	SA2402	Acetyl-CoA ligase activity	Identified	NI
Q7A7L2	Probable acetyl-CoA acyltransferase	SA0342	Acetyl CoA C-acetyl transferase activity	Identified	NI
A0A0H3JVC9	Iron-sulfur cluster carrier protein	SA1969	Binds and transfers iron-sulfur clusters to target apoproteins	Identified	NI
A0A0H3JU75	Ferredoxin	fer	Iron ion binding	Identified	NI
Cell Homeostasis Related Proteins					
P99118	Alkyl hydroperoxide reductase subunit F	ahpF	Cell redox homeostasis	NI	Identified
P60386	Redox-sensing transcriptional repressor Rex	rex	Response to redox state	NI	Identified
Q7A759	Putative heme-dependent peroxidase SA0544	SA0544	Peroxidase activity	NI	Identified
Q7A4R2	Bacterial non-heme ferritin	ftnA	Cellular iron ion homeostasis	NI	Identified
Q7A6M9	Organic hydroperoxide resistance protein-like	SA0755	Response to oxidative stress	NI	Identified
Q7A417	Putative 2-hydroxyacid dehydrogenase SA2098	SA2098	Oxidoreductase activity	NI	Identified
Q7A5T2	Catalase	katA	Protect cells from the toxic effects of H_2O_2	Identified	NI
Q7A782	FMN-dependent NADPH-azoreductase	azo1	Oxidoreductase activity	Identified	NI
A0A0H3JMU4	Alkyl hydroperoxide reductase AhpD	SA2262	Antioxidant protein with alkyl hydroperoxidase activity	Identified	NI
Virulence and Pathogenicity Related Proteins					
P65330	S-ribosylhomocysteine lyase	luxS	Quorum sensing	NI	Identified
Q7A5Q1	Conserved virulence factor B	cvfB	Expression of virulence factors & pathogenicity in a cell density-dependent manner	NI	Identified
P67278	Ribonuclease Y (Conserved virulence factor A)	rny	Pathogenesis. mRNA catabolic process	NI	Identified
Q7A872	HTH-type transcriptional regulator SarS	sarS	Expression of virulence factors in a cell density-dependent manner	NI	Identified
Q7A514	HTH-type transcriptional regulator rot	rot	Global regulator of several genes involved in virulence	NI	Identified
Q99SU9	Staphylococcal complement inhibitor	scn	Inhibition of phagocytosis and killing of *S. aureus* by neutrophils	NI	Identified
A0A0H3JPQ1	SA1000 protein	SA1000	Complement binding	NI	Identified
Q7A7R8	Type VII secretion system extracellular protein B	esxB	Required for the infection in the host	NI	Identified
Q7A4R9	Response regulator protein VraR	vraR	Promotes resistance to beta-lactams, glycopeptide, methicillin and teicoplanin	NI	Identified
A0A0H3JLY5	PBP2	pbp2	Penicillin-binding	NI	Identified
Q7A5H7	Sensor protein SrrB	srrB	Regulates virulence factors in response to oxygen levels	Identified	NI
Q99VJ4	Clumping factor A (Fibrinogen-binding protein A)	clfA	Promotes attachment of bacteria to fibrinogen	Identified	NI
Q99U54	Extracellular matrix-binding protein EbhA	ebhA	Pathogenesis	Identified	NI

[a]Full list was included in the supplementary data in Table S1 and Table S2 for control and VA-treated MRSA, respectively. NI: Not identified.

Table 2. Partial[a] list of identified proteins in MRSA after 2-hydroxycinnamic acid (2-HCA) treatment.

Accession Number	Name of Protein	Gene	Function	2-HCA Treated MRSA	Control MRSA
DNA Related Proteins					
Q7A5K4	3'-5' exonuclease DinG	dinG	DNA replication	NI	Identified
Q7A6H4	ATP-dependent helicase/nuclease subunit A	addA	Double-strand break repair	NI	Identified
A0A0H3JM17	DNA repair protein RecN	recN	Recombinational repair of damaged DNA	NI	Identified
Q7A5S6	Nuclease SbcCD subunit C	sbcC	DNA repair, recombination, replication	NI	Identified
Q99XG5	DNA gyrase subunit A	gyrA	Negative supercoiling of closed circular double-stranded DNA	Identified	NI
A0A0H3JW98	Anaerobic ribonucleoside-triphosphate reductase	nrdD	DNA replication	Identified	NI
P67425	UvrABC system protein B	uvrB	Nucleotide excision repair. SOS response	Identified	NI
P68844	Protein RecA	recA	DNA repair. SOS response	Identified	NI
RNA Related Proteins					
P60392	Ribosomal RNA small subunit methyltransferase H	rsmH	Methylation of 16S rRNA	NI	Identified
P0A0N7	Ribosomal RNA large subunit methyltransferase H	rlmH	Methylation of 23S rRNA	NI	Identified
Q7A5X7	Polyribonucleotide nucleotidyltransferase	pnp	RNA processing. Involved in mRNA degradation	NI	Identified
A0A0H3JM44	DEAD-box ATP-dependent RNA helicase CshB	cshB	RNA catabolic process	NI	Identified
P66726	DNA-directed RNA polymerase subunit omega	rpoZ	Promotes RNA polymerase assembly	NI	Identified
Q99TT5	RNA polymerase sigma factor SigA	rpoD	Promotes the attachment of RNA polymerase to specific initiation sites	NI	Identified
P99156	Transcription elongation factor GreA	greA	Transcription elongation	NI	Identified
P65578	Transcription antitermination protein NusB	nusB	Transcription of rRNA genes. Antitermination.	NI	Identified
A0A0H3JTG9	Cold-shock protein C	cspC	Regulation of transcription	NI	Identified
Q7A5P3	Cold shock protein CspA	cspA	Involved in cold stress response	NI	Identified
P64230	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	gidA	tRNA wobble uridine modification	Identified	NI
A0A0H3JKA9	Ribonuclease R	rnr	3'-5' exoribonuclease. Maturation of structured RNAs	Identified	NI
P60379	Regulatory protein Spx	spxA	Reducing the transcription of genes involved in growth-promoting processes	Identified	NI
Ribosome and Protein Synthesis Related Proteins					
A0A0H3JKT3	Ribosome biogenesis GTPase A	AID3985.1	Assembly of 50S ribosomal subunit	NI	Identified
A0A0H3JKS3	Ribosome-binding ATPase YchF	ychF	Binds to 70S ribosome and the 50S ribosomal subunit	NI	Identified
P66440	30S ribosomal protein S16	rpsP	Translation	NI	Identified
P66133	50S ribosomal protein L27	rpmA	Translation	NI	Identified
P66153	50S ribosomal protein L28	rpmB	Translation	NI	Identified
P66231	50S ribosomal protein L33 2	rpmG2	Translation	NI	Identified
P67015	Aspartate--tRNA ligase	aspS	Catalyzes the attachment of aspartate to tRNA	NI	Identified
P67513	Leucine--tRNA ligase	leuS	Catalyzes the attachment of leucine to tRNA	NI	Identified
Q7A537	Tyrosine--tRNA ligase	tyrS	Catalyzes the attachment of tyrosine to tRNA	NI	Identified
P68808	Aspartyl/glutamyl-tRNA amidotransferase subunit C	gatC	Translational fidelity. Formation of correctly charged Asn/Gln-tRNA	NI	Identified

(Table 2) contd....

Accession Number	Name of Protein	Gene	Function	2-HCA Treated MRSA	Control MRSA
P60748	Foldase protein PrsA	prsA	Required in protein secretion	NI	Identified
P99104	10 kDa chaperonin	groS	Protein folding	NI	Identified
P99082	33 kDa chaperonin	hslO	Protein folding	NI	Identified
A0A0H3JTY9	Signal recognition particle protein	ffh	Protein transport	NI	Identified
Q7A468	Protein translocase subunit SecY	secY	Protein transport	NI	Identified
Q7A6Q1	Probable protein-export membrane protein SecG	secG	Protein transport	NI	Identified
P64085	GTPase Era	era	16S rRNA processing and 30S ribosomal subunit biogenesis	Identified	NI
P65967	Ribosome-binding factor A	rbfA	rRNA processing. Assists the maturation steps of the 30S ribosomal subunit	Identified	NI
A0A0H3JMR4	Octanoyltransferase LipM	lipM	Protein lipoylation	Identified	NI
A0A0H3JKZ6	Octanoyl-[GcvH]: protein N-octanoyltransferase	lipL	Protein lipoylation	Identified	NI
P63971	Chaperone protein DnaJ	dnaJ	Protein folding	Identified	NI
Q7A366	Protein translocase subunit SecA 2	secA2	Protein import. Part of the Sec protein translocase complex	Identified	NI
A0A0H3JNG3	Glycine betaine/carnitine/choline ABC transporter opuCA	opuCA	Glycine betaine transport	Identified	NI
P65797	ATP-dependent protease subunit HslV	hslV	Proteolysis	Identified	NI
Cell Wall and Cell Membrane Related Proteins					
Q7A7B4	Bifunctional protein GlmU	glmU	Cell wall organization. LPS biosynthesis	NI	Identified
P65463	UDP-N-acetylenolpyruvoylglucosamine reductase	murF	Cell wall formation. Peptidoglycan biosynthesis	NI	Identified
Q7A615	Cell division protein SepF	sepF	Cell septum assembly	NI	Identified
Q7A5I6	Elastin-binding protein EbpS	ebpS	Promotes binding of soluble elastin peptides and tropoelastin to *S.aureus* cells	NI	Identified
P63665	ATP synthase epsilon chain	atpC	Production of ATP	NI	Identified
Q7A4E8	ATP synthase gamma chain	atpG	Production of ATP	NI	Identified
Q99TF4	Alanine dehydrogenase 2	ald2	Cell wall biosynthesis	Identified	NI
Q7A4F2	Probable transglycosylase SceD	sceD	Cleaves peptidoglycan. Affects clumping and separation of bacterial cells	Identified	NI
P99109	ATP synthase subunit delta	atpH	Production of ATP	Identified	NI
Metabolism Related Proteins					
P66695	Ribose-5-phosphate isomerase A	rpiA	Pentose-phosphate shunt	NI	Identified
A0A0H3JMN5	Glucose-6-phosphate 1-dehydrogenase	zwf	Pentose-phosphate shunt	NI	Identified
P68779	Adenine phosphoribosyltransferase	apt	Purine ribonucleoside salvage	NI	Identified
P65936	Uridylate kinase	pyrH	CTP biosynthesis	NI	Identified
A0A0H3JP54	Tagatose-6-phosphate kinase	fruB	D-tagatose catabolism	NI	Identified
Q7A699	Probable quinol oxidase subunit 1	qoxB	Oxidative phosphorylation pathway	NI	Identified
P99148	Aconitate hydratase A	acnH	TCA. Isomerization of citrate to isocitrate	NI	Identified
P67420	Uroporphyrinogen decarboxylase	hemA	Protoporphyrinogen IX biosynthesis	NI	Identified
P99096	Glutamate-1-semialdehyde 2,1-aminomutase 1	hemL1	Protoporphyrinogen IX biosynthesis	NI	Identified
A0A0H3JUQ8	Protoporphyrinogen oxidase	hemY	Protoporphyrin-IX biosynthesis	NI	Identified
P64334	Delta-aminolevulinic acid dehydratase	hemB	Protoporphyrin-IX biosynthesis	NI	Identified
Q7A428	Urease accessory protein UreF	ureF	Nitrogen metabolic process	NI	Identified
P67404	Urease subunit alpha	ureC	Urea degradation	NI	Identified

(Table 2) contd....

Accession Number	Name of Protein	Gene	Function	2-HCA Treated MRSA	Control MRSA
Q99TL8	GTP pyrophosphokinase (ppGpp synthase I)	relA	Guanosine tetraphosphate (ppGpp) biosynthetic process. Stringent response	Identified	NI
Q7A584	GTPase Obg	obg	Binds GTP, GDP and ppGpp with moderate affinity	Identified	NI
P99128	Phosphoenolpyruvate carboxykinase	pckA	Gluconeogenesis	Identified	NI
A0A0H3JRW0	SA2318 protein	SA2318	Gluconeogenesis	Identified	NI
A0A0H3JMA3	Pyruvate carboxylase	pycA	Gluconeogenesis	Identified	NI
A0A0H3JMB8	Citrate synthase	citZ	TCA	Identified	NI
P99167	Isocitrate dehydrogenase	icd	TCA	Identified	NI
P60298	Ornithine aminotransferase 2	rocD2 (AID39393.1)	Interconversion of ornithine to glutamate semialdehyde	Identified	NI
P99076	1-pyrroline-5-carboxylate dehydrogenase	rocA	Proline catabolic process to glutamate	Identified	NI
A0A0H3JU75	Ferredoxin	fer	Iron ion binding. Electron transfer activity	Identified	NI
Cell Homeostasis Related Proteins					
P99101	Thioredoxin reductase	trxB	Removal of superoxide radicals	NI	Identified
Q7A5T2	Catalase	katA	Protect cells from the toxic effects of H2O2	Identified	NI
P99097	Glutathione peroxidase homolog BsaA	bsaA	Response to oxidative stress. Glutathione peroxidase activity	Identified	NI
Q7A4T8	Peroxide-responsive repressor PerR	perR	Controls oxidative stress resistance and iron-storage proteins	Identified	NI
A0A0H3JMU4	Alkyl hydroperoxide reductase AhpD	AID41169.1	Antioxidant protein with alkyl hydroperoxidase activity	Identified	NI
Virulence and Pathogenicity Related Proteins					
P65330	S-ribosylhomocysteine lyase	luxS	Quorum sensing	NI	Identified
Q7A5Q1	Conserved virulence factor B	cvfB	Expression of virulence factors & pathogenicity in a cell density-dependent manner	NI	Identified
P67278	Ribonuclease Y (Conserved virulence factor A)	rny	Pathogenesis. mRNA catabolic process	NI	Identified
Q7A514	HTH-type transcriptional regulator rot	rot	Global regulator of several genes involved in virulence	NI	Identified
Q99SU9	Staphylococcal complement inhibitor	scn	Inhibition of phagocytosis and killing of S. aureus by neutrophils	NI	Identified
Q7A7R8	Type VII secretion system extracellular protein B	esxB	Required for the infection in the host	NI	Identified
Q7A4R9	Response regulator protein VraR	vraR	Promotes resistance to beta-lactams, glycopeptide, methicillin and teicoplanin	NI	Identified
A0A0H3JLY5	PBP2	pbp2	Penicillin-binding	NI	Identified
Q7A5H7	Sensor protein SrrB	srrB	Regulates virulence factors in response to oxygen levels	Identified	NI
Q99VJ4	Clumping factor A (Fibrinogen-binding protein A)	clfA	Promotes attachment of bacteria to the fibrinogen	Identified	NI
Q7A781	Serine-aspartate repeat-containing protein C	sdrC	Mediates the interactions of *S. aureus* with the extracellular matrix of higher eukaryotes	Identified	NI
Q99VW2	Response regulator protein GraR	graR	Involved in resistance against cationic antimicrobial peptides	Identified	NI
Q7A447	Lipid II: glycine glycyltransferase	femX	Response to antibiotic	Identified	NI

[a]Full list was included in the supplementary material in Table S1 and Table S3 for control and 2-HCA-treated MRSA, respectively. NI: Not identified.

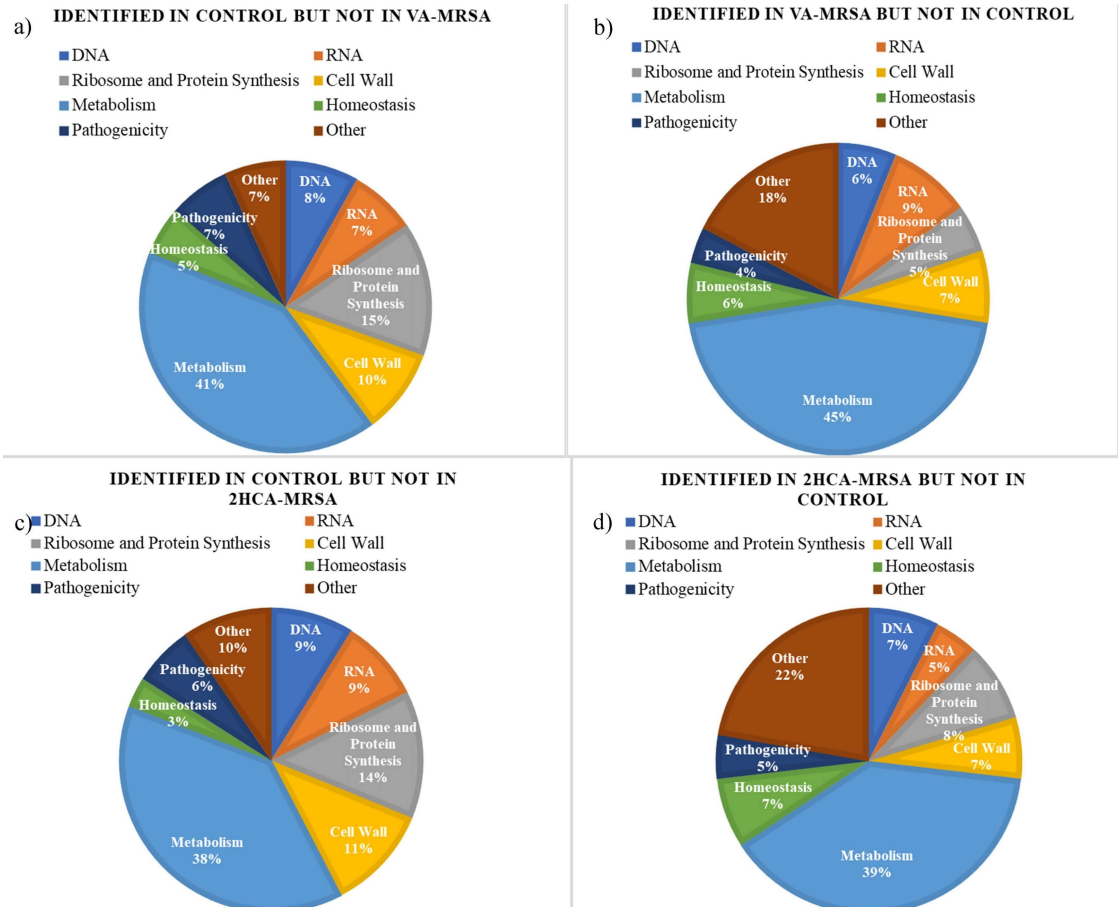

Fig. (1). Summary of percentages of functional categories that identified specificity to corresponding conditions **(a)** control MRSA compared with **(b)**VA-treated MRSA, **(c)** control MRSA compared with **(d)** 2-HCA treated MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

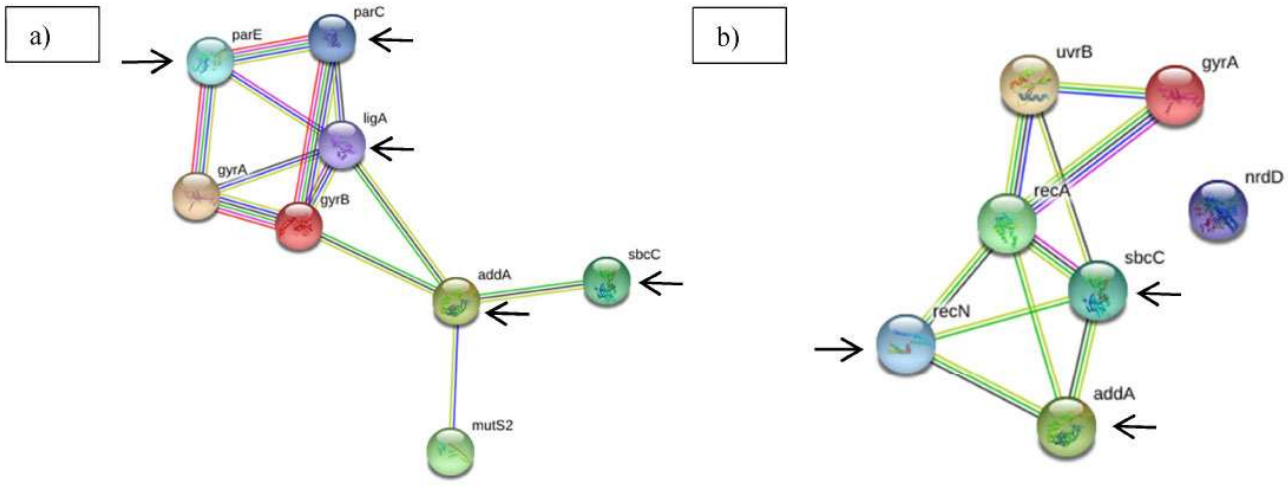

Fig. (2). Visualization of protein interactions in MRSA for the proteins related to DNA. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

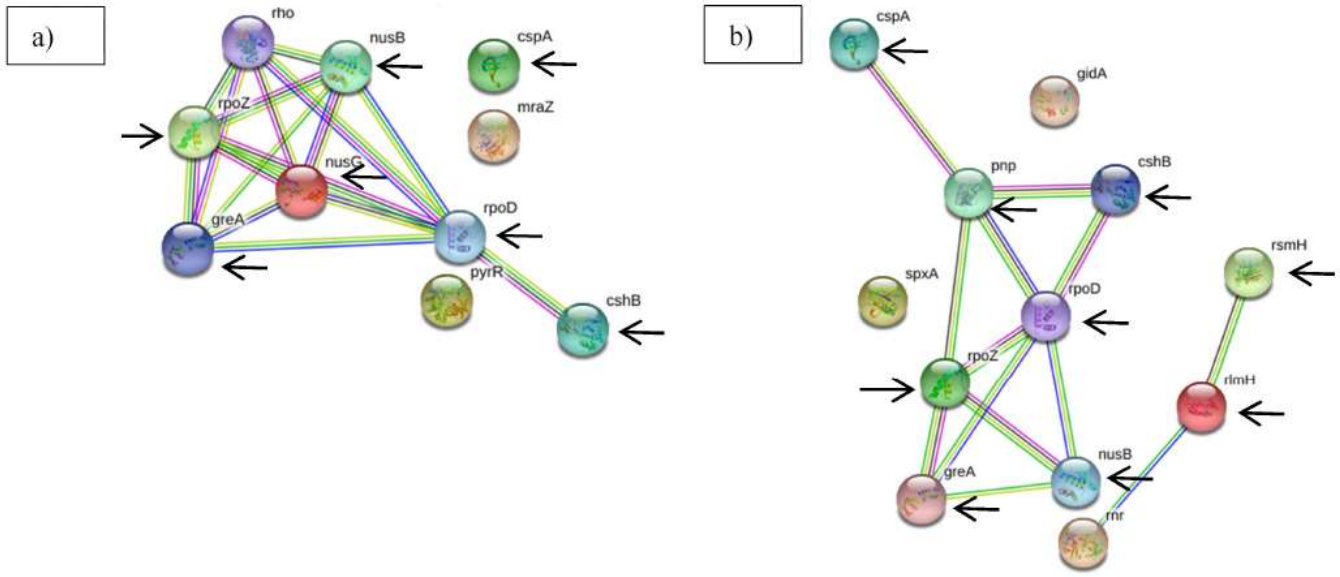

Fig. (3). Visualization of protein interactions in MRSA for the proteins related to RNA. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

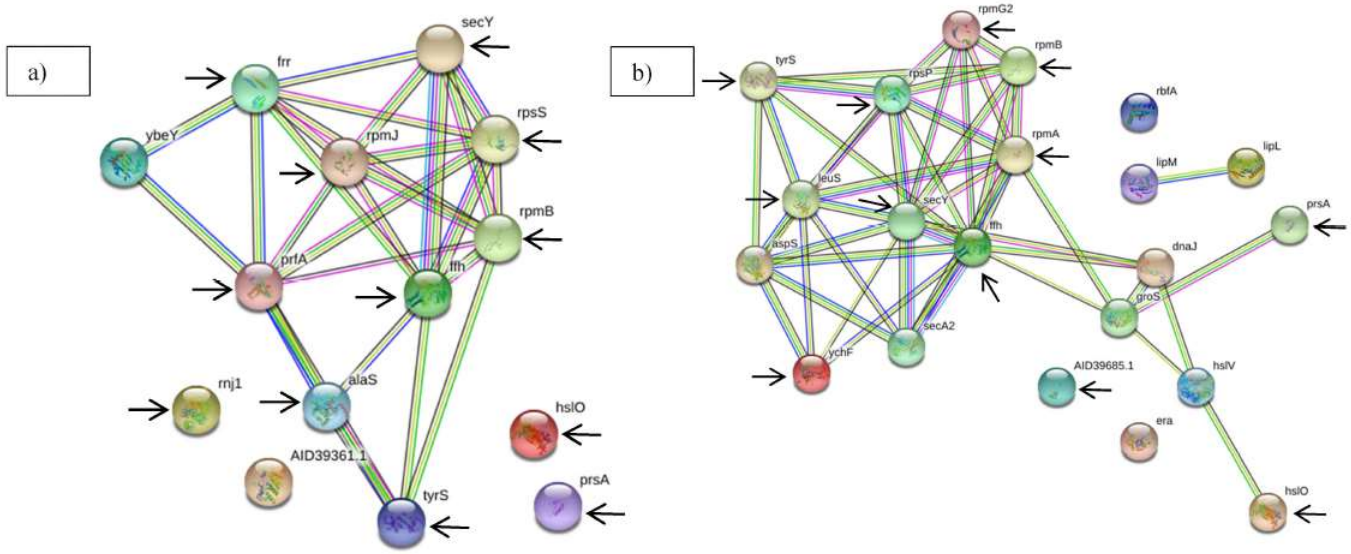

Fig. (4). Visualization of protein interactions in MRSA for the proteins related to ribosome and protein synthesis. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

structural constituents of the ribosome, correct assembly of the ribosome, aminoacylation of tRNAs, protein folding and protein transport (Table **2**). Presence of these proteins in control but not in 2-HCA-treated MRSA showed defects in protein synthesis, correct folding of proteins, and proper export of them. The interactions between these proteins were shown in Fig. (**4**).

The identified proteins having roles in the formation and stability of cell wall and membrane showed that VA treatment affected the formation and the organization of the cell wall as well as the ATP production (Table **1**). Similar observation for ATP production-related proteins occurred for 2-HCA-treated cells; differently, 2-HCA-treated MRSA allowed the identification of ATP synthase subunit delta

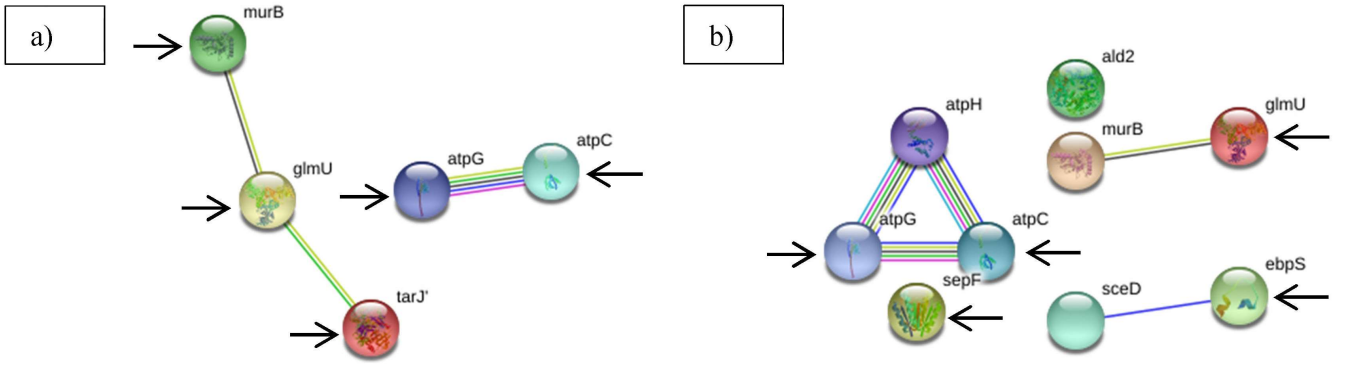

Fig. (5). Visualization of protein interactions in MRSA for the proteins related to cell wall and membrane. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

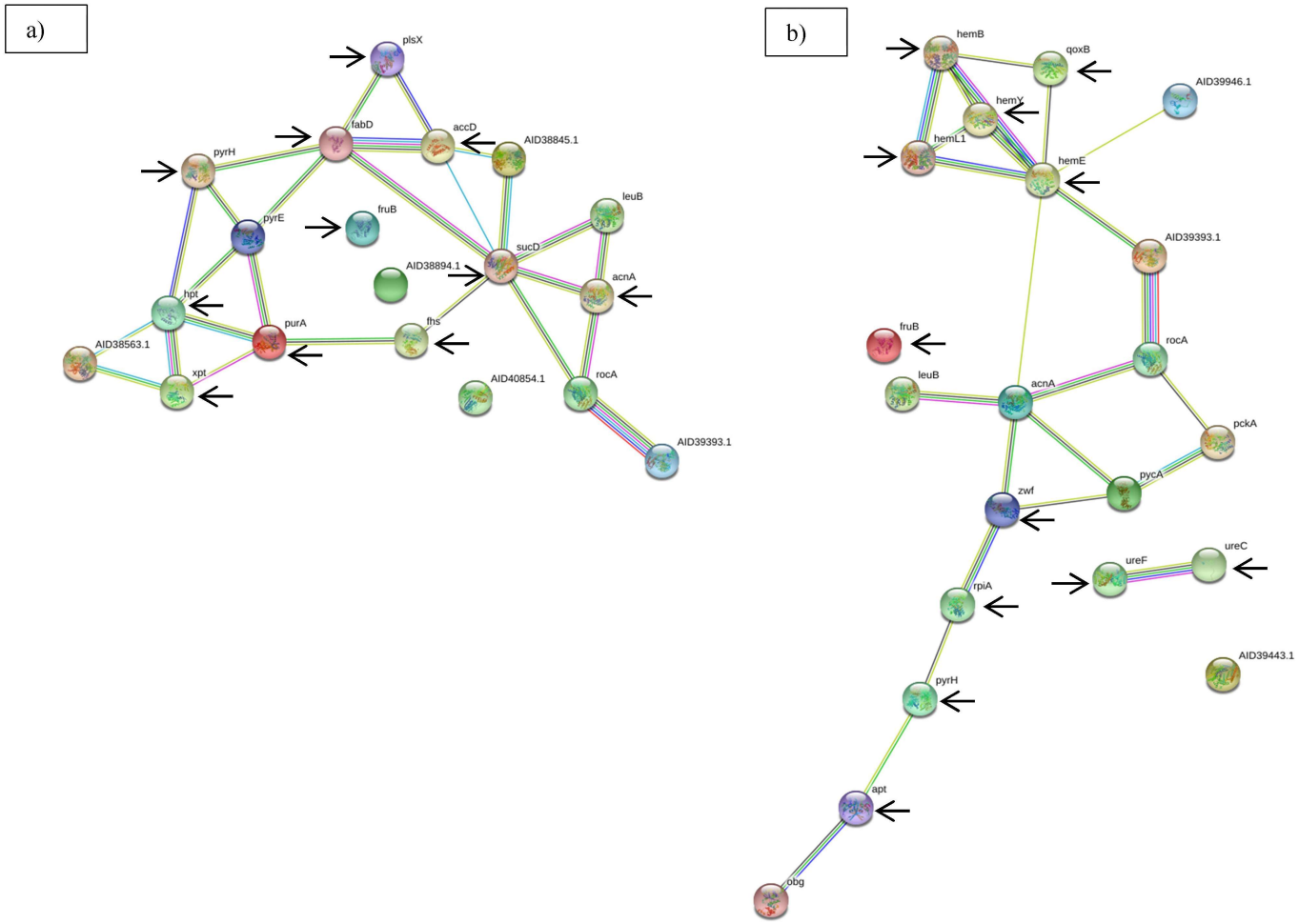

Fig. (6). Visualization of protein interactions in MRSA for the proteins related to metabolism. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

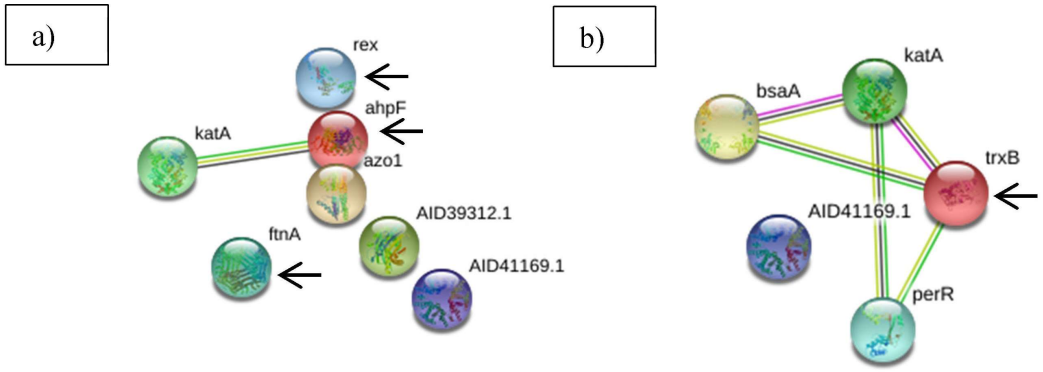

Fig. (7). Visualization of protein interactions in MRSA for the proteins related to cell homeostasis. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Table **2**). The interactions between identified proteins related to the cell wall and membrane structure in VA-treated MRSA and its control as well as 2-HCA-treated and control MRSA were represented in Fig. (**5**).

Most of the proteins detected in the control and VA-treated MRSA were related to metabolic reactions. VA affected the proteins having roles in purine and pyrimidine metabolism, energy, carbohydrate, lipid, amino acid, and iron metabolisms. Among the listed proteins (Table **1**), the absence of 15 and presence of 10 different metabolism-related proteins in VA-treated MRSA showed the changes in metabolic pathways under treatment. The network of protein interactions having roles in several metabolic pathways for VA-treated and untreated MRSA was complex (Fig. **6a**). When Table **2** was examined, changes in the preferences of metabolic pathways could be seen for 2-HCA-treated MRSA. While 13 proteins were listed corresponding to metabolism-related proteins for control, seven proteins were identified in 2-HCA-treated cells. Most of these proteins identified in the presence and absence of 2-HCA had interactions with at least one protein (Fig. **6b**).

When the proteins functioning in the maintenance of cell homeostasis were clustered together, the effect of VA was obvious (Table **1**). Six proteins responsible for oxidoreductase and peroxidase activities were not identified in MRSA; in contrast, three proteins in VA-treated MRSA were responsible for the protection of cells from oxidative stress. The proteins having roles in cell homeostasis for VA-treated and untreated MRSA had weaker interactions among all (Fig. **7a**). While only thioredoxin reductase was identified in control as differently expressed protein, four proteins were identified in 2-HCA-treated cells. Interaction of thioredoxin reductase with other identified proteins was shown in Fig. **7b**.

Most significant changes in the expression of proteins upon VA treatment were obtained for the proteins related to the pathogenicity and the virulence properties of MRSA. VA treatment hindered the identification of S-ribosylhomocysteine lyase, conserved virulence factor B, ribonuclease A, HTH-type transcriptional regulator SarS, HTH-type transcriptional regulator rot, staphylococcal complement inhibitor, SA1000 protein, Type VII secretion system extracellular protein B, response regulator protein VraR and PBP2. These changes indicated the decrease in the pathogenicity and virulence of MRSA in the presence of VA. Likewise, 2-HCA hindered the identification of eight proteins. The network of virulence and pathogenicity related proteins for 2-HCA-treated and untreated MRSA showed more interactions with each other, in contrast to VA-treated MRSA and its control (Fig. **8**).

4. DISCUSSION

4.1. Action on DNA

Differences in the identified proteins indicated the effects of VA and 2-HCA on DNA replication and repair. VA inhibited the identification of DNA topoisomerase 4 subunit A and subunit B, which are responsible for daughter chromosome segregation during the replication. This inhibition is promising on revealing the action mechanism of VA, as this is similar to the action mechanism of quinolone antibiotics targeting topoisomerases [22]. Moreover, identification of DNA gyrases in phenolic acid-treated MRSA (Tables **2** and **2**) might indicate the requirement for more DNA strand separation for replication and repair. This idea was supported by the identification of endonuclease MutS2 in VA-treated MRSA (Table **1**) and UvrABC system protein B and protein RecA in 2-HCA-treated MRSA (Table **2**). These proteins identified in 2-HCA-treated MRSA might indicate the inducement of SOS response, which might cause error-prone DNA synthesis [16]. The increased SOS response would result in the generation of mismatches due to the lack of homologous recombination [23]. The proteins having roles in DNA recombination, replication, and repair had direct interactions with each other. The observed changes in the expression of one protein possibly affected the expression of the others, either positively or negatively (Fig. **2**).

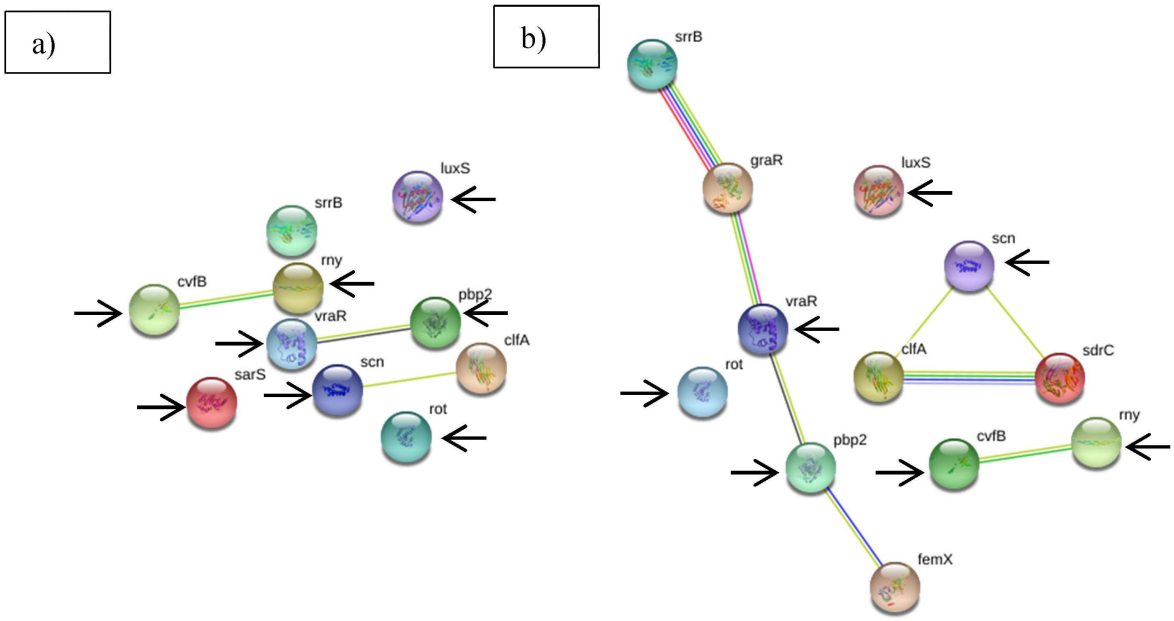

Fig. (8). Visualization of protein interactions in MRSA for the proteins related to virulence and pathogenicity. Networks represent the identified proteins in the absence and presence of VA **(a)** and 2-HCA **(b)**. Arrows indicate the proteins identified only in control MRSA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.2. Action on RNA

Inhibition of RNA polymerase sigma factor SigA could be another mechanism for the action of phenolic acids on MRSA. Well-known antibiotic rifampicin shows its antibacterial effect by affecting RNA polymerase and causing reduced affinity for magnesium ions (Mg^{2+}) [24]. Similarly, treatment of MRSA with VA and 2-HCA might induce such allosteric changes on RNA polymerases and slow down the transcription.

The absence of DEAD-box ATP-dependent RNA helicase CshB in phenolic acid-treated might indicate the lack of energy metabolism or the direct effect of phenolic acids on the protein. Since this protein plays a role in many processes related to RNA metabolism [25], its lack might signal the apparent changes in RNA structure and transcription. The decreased amount of transcription might indicate the reduced energy within the cells, resulting in a lower metabolic rate.

Methylation of RNA is part of the defense mechanism of bacteria against drug stress, and overexpression of RNA-methylation proteins might contribute to antimicrobial resistance [16]. The absence of RNA-methylation proteins among the identified proteins of 2-HCA-treated MRSA might point out to the lack of resistance against that phenolic acid. The inability of MRSA to develop resistance to subinhibitory concentrations of phenolic acids may prove that phenolic acids are suitable alternative treatments to this superbug [17].

4.3. Action on ribosome and protein synthesis

The bacterial ribosome is a complex molecular machine that consists of more than 50 proteins, three ribosomal RNAs, and many receptors. Its association with other cell functions makes all translation related steps excellent targets for antimicrobial compounds. Many antibiotics target different steps in protein synthesis [24]. Based on this knowledge, the possible inhibition of some of the 30S and 50S ribosomal subunits by both VA (Table **1**) and 2-HCA (Table **2**) might be responsible for their antimicrobial actions.

Signal recognition particle (SRP) protein is required for proper secretion of proteins [26]. Accordingly, the possible change in the expression of SRP might affect the proteins having roles in translocation of membrane proteins, translation, protein transport, and tRNA aminoacylation (Fig. **4**). Other proteins identified only in control MRSA were protein translocase subunit SecY and probable protein-export membrane protein SecG. According to the literature [27], the general secretory (Sec) pathway is required for the export of most of the *S. aureus* expo-proteins. Thus, the unidentifiability of these proteins in phenolic acid-treated cells might indicate the defects in the transport of several proteins. Both VA and 2-HCA resulted in non-detection of four enzymes required for the aminoacylation of tRNAs. The presence of tRNAs without the addition of correct amino acids would negatively affect protein synthesis.

4.4. Action on Cell Wall and Membrane

The ability of phenolic acids to enter the cell by passive diffusion and disrupt the cell membrane was well documented [10, 28]. Consistently, our results showed the changes in the expression of the proteins taking roles in the synthesis of peptidoglycan structure, which then would affect the cell wall composition and stability. The unidentifiability of ATP synthase epsilon chain and gamma chain in the presence of VA (Table **1**) and 2-HCA (Table **2**) indicated the changes in energy metabolism coupled to proton gradient across the membrane [29, 30].

4.5. Action on Metabolism

Changes in the proteins of carbohydrate metabolism might indicate MRSA's preferences of other metabolic pathways for ATP generation, to compensate for the decreased levels of energy produced from oxidative pathways [29]. The loss of proton gradient across the membrane, the unidentifiability of proteins taking roles in oxidative phosphorylation, and the pentose phosphate pathway indicated the decreased ATP generation. Another difference was observed for the proteins taking roles in the tricarboxylic acid cycle (TCA). The proper function of TCA is essential for the production of reducing equivalents and ATP production [31]. Moreover, alterations in the proteins of TCA might be related to the attempt to enhance the oxoglutarate levels [26]. The identification of citrate synthase and isocitrate dehydrogenase in phenolic acid-treated cells indicated the contribution to glutamate forming pathways. Supportively, ornithine aminotransferase 2 and 1-pyrrolline-5-carboxylate dehydrogenase proteins were identified in the presence of phenolic acids. These two proteins take roles in the conversion of other amino acids to glutamate that functions as a nitrogen donor in the cell for molecules [32]. It might indicate that MRSA under phenolic acid stress attempts to increase the nitrogen metabolism for the production of all nitrogen-containing biological molecules.

Unidentifiability of these fatty acid metabolism-related proteins indicated the reduced fatty acid production under the VA stress (Table **1**). It might signal the struggle of the cells to maintain the membrane fluidity [29] due to the cytoplasmic membrane disrupting the effects of phenolic acids [28]. Affecting the fatty acid metabolism might be an effective action mechanism for VA because inhibition of biosynthetic pathway of fatty acids is considered an important target during the development of new drugs [33]. Complex networks of metabolism-related protein interactions for VA-treated MRSA and 2-HCA-treated MRSA (Fig. **6**) indicates the strong regulation between the metabolism-related proteins and their compensation for each other.

4.6. Action on Cell Homeostasis

MRSA cells allowed the identification of catalase enzyme in the presence of phenolic acid stress as well as other redox sensor proteins. The increased identification of stress-responsive proteins strongly indicates the induction of oxidative stress [29, 34].

4.7. Action on Pathogenicity and Virulence

Treatment of bacteria with subinhibitory concentrations of VA and 2-HCA resulted in a decrease in the identification of quorum sensing and virulence-related proteins. This finding contradicts what is known about the treatment of bacteria with subinhibitory antibiotic concentrations [35]. Therefore, the use of phenolic acids instead of antibiotics might provide benefits in terms of reducing the cell density-dependent behavior of bacteria, which directly affect the virulence. The ability of phenolic compounds to decrease virulence-related proteins was also indicated by the literature [4]. The production of cell surface proteins and extracellular proteins is highly regulated by SarA protein [36]. The identification of staphylococcal complement inhibitor and SA1000 in control indicated the use of these proteins by bacteria to protect themselves from destruction by the host immune system [37]. Therefore, the unidentifiability of these proteins in the presence of VA and 2-HCA might indicate the increased susceptibility of MRSA against host defense mechanisms. When the networks of virulence-related proteins were examined (Fig. **8**), it could be seen that even the directly interacted proteins might not be identified within the cells when they were treated with phenolic acids. Reduced virulence of MRSA achieved by both phenolic acids may help the decreased formation of diseases related to the acquisition of this most commonly acquired human pathogen and may contribute to the protection of public health.

CONCLUSION

In conclusion, understanding the complex action mechanism of phenolic acids on pathogenic bacteria may enlighten the production of alternative antimicrobial compounds to combat human pathogens. The comprehensive proteomic data obtained in our study enrich the knowledge about the action mechanisms of phenolic acids by showing the potential targets on bacteria to be used in the development of novel drugs. Separate from the antibiotics that commonly target for a single site within the cells, phenolic acids seem to display their inhibitory effects via versatile action mechanisms. When we examine the proteins identified either in treated or untreated MRSA, the proteins that act on ribosome and protein synthesis take the lead in terms of being a target for bacterial growth inhibition. Since correct ribosome assembly is vital for protein synthesis, any ribosome assembly problem may result in ribosomal damage, and in problems in protein synthesis. This kind of problem directly affects almost all biological processes. Thus, we contend that, the starting point for further investigation in drug development for MRSA inhibition is the ribosome assembly and protein synthesis related proteins. Apart from the ribosomal proteins, topoisomerases, cell wall proteins, and virulence and pathogenicity proteins are absent in humans and are good candidates for the development of novel antimicrobials. However, further data is required to determine the specific target sites to develop novel antimicrobial compounds.

AUTHORS' CONTRIBUTIONS

Both authors contributed to the design of the study. DK conducted experiments. DK and FS analyzed data. DK wrote the manuscript. DK and FS contributed to the correction of the manuscript. All authors read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The mass spectrometry proteomics data supporting the findings of the article is available in the ProteomeXchange Consortium via the PRIDE partner repository with the reference number PXD016922.

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CONFLICT OF INTEREST

The authors have no conflicts of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on publishers' website.

REFERENCES

[1] Pasberg-Gauhl, C. A need for new generation antibiotics against MRSA resistant bacteria. *Drug Discov. Today Technol.,* **2014**, *11*(), 109-116. http://dx.doi.org/10.1016/j.ddtec.2014.04.001 PMID: 24847660

[2] Davies, J; Davies, D Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.,* **2010**, *74*(3), 417-433. http://dx.doi.org/10.1128/MMBR.00016-10 PMID: 20805405

[3] Lakhundi, S.; Zhang, K. Methicillin-Resistant *Staphylococcus aureus*: Molecular Characterization, Evolution, and Epidemiology. *Clin. Microbiol. Rev.,* **2018**, *31*(4), e00020-e18. http://dx.doi.org/10.1128/CMR.00020-18 PMID: 30209034

[4] Lima, M.C.; Paiva de Sousa, C.; Fernandez-Prada, C.; Harel, J.; Dubreuil, J.D.; de Souza, E.L. A review of the current evidence of fruit phenolic compounds as potential antimicrobials against pathogenic bacteria. *Microb. Pathog.,* **2019**, *130*, 259-270. http://dx.doi.org/10.1016/j.micpath.2019.03.025 PMID: 30917922

[5] Maldonado-Carmona, N.; Vázquez-Hernández, M.; Patiño Chávez, O.J.; Rodríguez-Luna, S.D.; Jiménez Rodríguez, O.; Sanchez, S.; Ceapă, C.D. Impact of omics in the detection and validation of potential anti-infective drugs. *Curr. Opin. Pharmacol.,* **2019**, *48*, 1-7. http://dx.doi.org/10.1016/j.coph.2019.02.008 PMID: 30921690

[6] Alves, M.J.; Ferreira, I.C.F.R.; Froufe, H.J.C.; Abreu, R.M.V.; Martins, A.; Pintado, M. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *J. Appl. Microbiol.,* **2013**, *115*(2), 346-357. http://dx.doi.org/10.1111/jam.12196 PMID: 23510516

[7] Dantes, R.; Mu, Y.; Belflower, R.; Aragon, D.; Dumyati, G.; Harrison, L.H.; Lessa, F.C.; Lynfield, R.; Nadle, J.; Petit, S.; Ray, S.M.; Schaffner, W.; Townes, J.; Fridkin, S. Emerging Infections Program–Active Bacterial Core Surveillance MRSA Surveillance Investigators. National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011. *JAMA Intern. Med.,* **2013**, *173*(21), 1970-1978. http://dx.doi.org/10.1001/jamainternmed.2013.10423 PMID: 24043270

[8] World Health Organization. *Prioritization of Pathogens to Guide Discovery, Research and Development of New Antibiotics for Drug-Resistant Bacterial Infections, Including Tuberculosis*; WHO: Geneva, **2017**.

[9] Akiyama, H.; Fujii, K.; Yamasaki, O.; Oono, T.; Iwatsuki, K. Antibacterial action of several tannins against *Staphylococcus aureus. J. Antimicrob. Chemother.,* **2001**, *48*(4), 487-491. http://dx.doi.org/10.1093/jac/48.4.487 PMID: 11581226

[10] Borges, A.; Ferreira, C.; Saavedra, M.J.; Simões, M. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb. Drug Resist.,* **2013**, *19*(4), 256-265. http://dx.doi.org/10.1089/mdr.2012.0244 PMID: 23480526

[11] Bandow, J.E.; Brötz, H.; Leichert, L.I.O.; Labischinski, H.; Hecker, M. Proteomic approach to understanding antibiotic action. *Antimicrob. Agents Chemother.,* **2003**, *47*(3), 948-955. http://dx.doi.org/10.1128/AAC.47.3.948-955.2003 PMID: 12604526

[12] Brötz-Oesterhelt, H.; Bandow, J.E.; Labischinski, H. Bacterial proteomics and its role in antibacterial drug discovery. *Mass Spectrom. Rev.,* **2005**, *24*(4), 549-565. http://dx.doi.org/10.1002/mas.20030 PMID: 15389844

[13] Hesketh, A.; Deery, M.J.; Hong, H-J. High-Resolution Mass Spectrometry Based Proteomic Analysis of the Response to Vancomycin-Induced Cell Wall Stress in Streptomyces coelicolor A3(2). *J. Proteome Res.,* **2015**, *14*(7), 2915-2928. http://dx.doi.org/10.1021/acs.jproteome.5b00242 PMID: 25965010

[14] Li, W.; Zhang, S.; Wang, X.; Yu, J.; Li, Z.; Lin, W.; Lin, X. Systematically integrated metabonomic-proteomic studies of *Escherichia coli* under ciprofloxacin stress. *J. Proteomics,* **2018**, *179*, 61-70. http://dx.doi.org/10.1016/j.jprot.2018.03.002 PMID: 29522880

[15] Aslam, B.; Basit, M.; Nisar, M.A.; Khurshid, M.; Rasool, M.H. Proteomics: Technologies and Their Applications. *J. Chromatogr. Sci.,* **2017**, *55*(2), 182-196. http://dx.doi.org/10.1093/chromsci/bmw167 PMID: 28087761

[16] Sharma, D.; Garg, A.; Kumar, M.; Khan, A.U. Proteome profiling of carbapenem-resistant *K. pneumoniae* clinical isolate (NDM-4): Exploring the mechanism of resistance and potential drug targets. *J. Proteomics,* **2019**, *200*, 102-110. http://dx.doi.org/10.1016/j.jprot.2019.04.003 PMID: 30953729

[17] Keman, D.; Soyer, F. Antibiotic-Resistant *Staphylococcus aureus* Does Not Develop Resistance to Vanillic Acid and 2-Hydroxycinnamic Acid after Continuous Exposure *in Vitro*. *ACS Omega,* **2019**, *4*(13), 15393-15400. http://dx.doi.org/10.1021/acsomega.9b01336 PMID: 31572838

[18] Sianglum, W.; Srimanote, P.; Wonglumsom, W.; Kittiniyom, K.; Voravuthikunchai, S.P. Proteome analyses of cellular proteins in methicillin-resistant Staphylococcus aureus treated with rhodomyr-

tone, a novel antibiotic candidate. *PLoS One,* **2011**, *6*(2)e16628 http://dx.doi.org/10.1371/journal.pone.0016628 PMID: 21326597

[19] Walker, J.M. *The Protein Protocols Handbook,* 2nd ed; Humana Press Inc: New Jersey, USA, **2002**. http://dx.doi.org/10.1385/1592591698

[20] Dinç, M. *Proteomic Analyses of Biological Samples by Using Different Mass Spectrometric Strategies.,* **2018**,

[21] Perez-Riverol, Y.; Csordas, A.; Bai, J.; Bernal-Llineras, M.; Hewapathirana, S.; Kundu, D.J.; Inuganti, A.; Griss, J.; Mayer, G.; Eisenacher, M.; Perez, E.; Uszkoreit, J.; Pfeuffer, J.; Sachsenberg, T.; Yilmaz, S.; Tiwary, S.; Cox, J.; Audain, E.; Walzer, M.; Jarnuczak, A.F.; Ternent, T.; Brazma, A.; Vizcaimo, J.A. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.,* **2019**, *47*(1), D442-450.

[22] Hawkey, P.M. Mechanisms of quinolone action and microbial response. *J. Antimicrob. Chemother.,* **2003**, *51*(Suppl. 1), 29-35. http://dx.doi.org/10.1093/jac/dkg207 PMID: 12702701

[23] Cirz, R.T.; Jones, M.B.; Gingles, N.A.; Minogue, T.D.; Jarrahi, B.; Peterson, S.N.; Romesberg, F.E. Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. *J. Bacteriol.,* **2007**, *189*(2), 531-539. http://dx.doi.org/10.1128/JB.01464-06 PMID: 17085555

[24] Davies, J.; Spiegelman, G.B.; Yim, G. The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.,* **2006**, *9*(5), 445-453. http://dx.doi.org/10.1016/j.mib.2006.08.006 PMID: 16942902

[25] Linder, P.; Jankowsky, E. From unwinding to clamping - the DEAD box RNA helicase family. *Nat. Rev. Mol. Cell Biol.,* **2011**, *12*(8), 505-516. http://dx.doi.org/10.1038/nrm3154 PMID: 21779027

[26] Fernández-Reyes, M.; Rodríguez-Falcón, M.; Chiva, C.; Pachón, J.; Andreu, D.; Rivas, L. The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics,* **2009**, *9*(6), 1632-1645. http://dx.doi.org/10.1002/pmic.200800434 PMID: 19253303

[27] Enany, S.; Yoshida, Y.; Yamamoto, T. Exploring extra-cellular proteins in methicillin susceptible and methicillin resistant *Staphylococcus aureus* by liquid chromatography-tandem mass spectrometry. *World J. Microbiol. Biotechnol.,* **2014**, *30*(4), 1269-1283. http://dx.doi.org/10.1007/s11274-013-1550-7 PMID: 24214678

[28] Rivas-Sendra, A.; Landete, J.M.; Alcántara, C.; Zúñiga, M. Response of *Lactobacillus casei* BL23 to phenolic compounds. *J. Appl. Microbiol.,* **2011**, *111*(6), 1473-1481. http://dx.doi.org/10.1111/j.1365-2672.2011.05160.x PMID: 21951613

[29] Fu, F.; Cheng, V.W.T.; Wu, Y.; Tang, Y.; Weiner, J.H.; Li, L. Comparative proteomic and metabolomic analysis of *Staphylococcus warneri* SG1 cultured in the presence and absence of butanol. *J. Proteome Res.,* **2013**, *12*(10), 4478-4489. http://dx.doi.org/10.1021/pr400533m PMID: 23961999

[30] Guan, N.; Liu, L. Microbial response to acid stress: mechanisms and applications. *Appl. Microbiol. Biotechnol.,* **2020**, *104*(1), 51-65. http://dx.doi.org/10.1007/s00253-019-10226-1 PMID: 31773206

[31] Reddy, P.J.; Ray, S.; Sathe, G.J.; Prasad, T.S.K.; Rapole, S.; Panda, D.; Srivastava, S. Proteomics analyses of Bacillus subtilis after treatment with plumbagin, a plant-derived naphthoquinone. *OMICS,* **2015**, *19*(1), 12-23. http://dx.doi.org/10.1089/omi.2014.0099 PMID: 25562197

[32] Somerville, G.A.; Proctor, R.A. At the crossroads of bacterial metabolism and virulence factor synthesis in Staphylococci. *Microbiol. Mol. Biol. Rev.,* **2009**, *73*(2), 233-248. http://dx.doi.org/10.1128/MMBR.00005-09 PMID: 19487727

[33] Foster, T.J. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiol. Rev.,* **2017**, *41*(3), 430-449. http://dx.doi.org/10.1093/femsre/fux007 PMID: 28419231

[34] Blair, S.E.; Cokcetin, N.N.; Harry, E.J.; Carter, D.A. The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis. *Eur. J. Clin. Microbiol. Infect. Dis.,* **2009**, *28*(10), 1199-1208. http://dx.doi.org/10.1007/s10096-009-0763-z PMID: 19513768

[35] Liu, Z.; Wang, W.; Zhu, Y.; Gong, Q.; Yu, W.; Lu, X. Antibiotics at subinhibitory concentrations improve the quorum sensing behavior of *Chromobacterium violaceum*. *FEMS Microbiol. Lett.,* **2013**, *341*(1), 37-44. http://dx.doi.org/10.1111/1574-6968.12086 PMID: 23330731

[36] Cordwell, S.J.; Larsen, M.R.; Cole, R.T.; Walsh, B.J. Comparative proteomics of *Staphylococcus aureus* and the response of methicillin-resistant and methicillin-sensitive strains to Triton X-100. *Microbiology (Reading),* **2002**, *148*(Pt 9), 2765-2781. http://dx.doi.org/10.1099/00221287-148-9-2765 PMID: 12213923

[37] Boisset, S.; Geissmann, T.; Huntzinger, E.; Fechter, P.; Bendridi, N.; Possedko, M.; Chevalier, C.; Helfer, A.C.; Benito, Y.; Jacquier, A.; Gaspin, C.; Vandenesch, F.; Romby, P. Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev.,* **2007**, *21*(11), 1353-1366. http://dx.doi.org/10.1101/gad.423507 PMID: 17545468