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Action of carvacrol in *Salmonella* Typhimurium biofilm: A proteomic study

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Abstract
Carvacrol presents action in *Salmonella* Typhimurium biofilms, however the antibiofilm mechanism of this compound has not been fully established yet. In the present study, the aim was to evaluate protein profile changes in *S*. Typhimurium biofilm treated with carvacrol. Proteomic analysis of treated versus untreated biofilm showed several changes in proteins involved with *S*. Typhimurium biofilm and antioxidant activity. The proteins DsbA (thiol: disulfide interchange protein DsbA), LuxS (*S*-ribosylhomocysteine lyase), DksA (RNA polymerase binding transcription factor DksA), and SODs (superoxide dismutases) A, B and C had their synthesis decreased after treatment with carvacrol. These proteins play a key role in *S*. Typhimurium biofilm formation, demonstrating the dynamic antibiofilm action of carvacrol. The differentially expressed proteins identified provide possible action targets for future studies in order to gain more insight into the mechanism of action of carvacrol on *S*. Typhimurium biofilm.

Keywords: Carvacrol; Biofilms; Proteome; *Salmonella* Typhimurium

Highlights:
- The data showed the dynamic action of carvacrol in *S*. Typhimurium biofilm.
- Were identified proteins involved in the oxidative stress and quorum sensing.
- Treatment with carvacrol showed a decrease of proteins related to biofilm.

Introduction

Infections caused by *Salmonella* spp. remain some of the most frequent foodborne diseases and are among the main global public health concerns (WHO, 2018). It is estimated that this bacterium causes 1.2 million cases of foodborne disease each year in the United States, leading to 23,000 cases of hospitalization and 450 deaths (CDC, 2019). According to the Center for Disease Control and Prevention, different *Salmonella* serotypes have been associated with foodborne disease outbreaks involving a wide variety of foods such as vegetables and also products of animal origin (CDC, 2018).

The ability of *Salmonella* spp. to form biofilms on different surfaces contributes to its resistance and persistence in different environments (Steenackers et al., 2012). Several studies have demonstrated that *Salmonella* spp. form biofilms on surfaces that are commonly found in food processing environments (Wang et al., 2016). Bacterial biofilms are recognized as a source of food and surface contamination and can lead to economic losses (Cappitelli et al., 2014). Biofilms are complex communities with a distinct architecture and are more resistant and more difficult to remove than planktonic bacteria (Borges et al., 2016). The resistance of biofilm cells is multifactorial and is related to the barrier promoted by the extracellular polymeric substances, the slow growth rate of sessile cells, the physiological heterogeneity, and the expression of resistance genes (Borges et al., 2016; Cappitelli et al., 2014).

Different strategies have been proposed to control biofilms based on their biological and physical characteristics. Various

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targets have been suggested to prevent biofilm establishment or induce its disruption, and include inhibition of bacterial adhesion, interference with bacterial communication, induction of dispersal, and eradication of sessile cells (Borges et al., 2016). Currently, natural compounds are studied as an alternative for biofilm control in food processing environments (Bridier et al., 2015). Their broad spectrum of biological activity, as well as the natural origin and low toxicity level makes them an attractive alternative to synthetic compounds (Walsh et al., 2019). Carvacrol is the major component of oregano (*Origanum vulgari*) essential oil (Burt, 2004), is classified as a Generally Recognized as Safe (GRAS) by Food and Drug Administration (FDA, 2019), and has been applied to control *Salmonella* spp. biofilm (Amaral et al., 2015; Burt et al., 2014; Trevisan et al., 2018; Uchida et al., 2014). Although the antibiofilm mechanism of carvacrol has not yet been fully established, Knowles et al. (2005) have demonstrated that this compound inhibits matrix formation during the early stages of biofilm development. Other studies showed that anti-biofilm activity of carvacrol appears to be related to disruption of quorum sensing (Burt et al., 2014; Knowles et al., 2005; Tapia-Rodriguez et al., 2017).

Proteomic analysis has been applied to better understand the complexity of biofilm of different bacteria (Giaouris et al., 2013; Mangalappalli-Illathu et al., 2008; Silva et al., 2018) and to evaluate its adaptation to different conditions (Mangalappalli-Illathu et al., 2008; Silva et al., 2018), profiles of resistance and susceptibility to drugs (Magalhães et al., 2017), and tolerance to bioactive agents (Condell et al., 2012). The proteomic approach provides the opportunity to determine the functional genome, but also it facilitates the identification of proteins that may not have been predicted by analysis of the genome (Sharma et al., 2010; Tomlinson and Holt, 2001). Considering antibiofilm properties of carvacrol, this study evaluated the changes in protein profile of *Salmonella* Typhimurium biofilm treated with this compound.

Materials and methods

Chemical free radical quenching activity

The *in vitro* chemical antioxidant capacities of carvacrol (purity ≥ 98% Sigma-Aldrich) were evaluated using both the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assays (Erel, 2004; Soares et al., 2009). The cationic ABTS form was generated by incubating 3.7 mM ABTS with 1.3 mM potassium persulfate in the dark for 12 h. The assay system comprised the methanolic cationic ABTS (61 μM) containing carvacrol at concentrations ranging from 0.0015 to 150.2 μg/ml. After one hour, the absorbance decrease was measured at 734 nm. The DPPH assay system comprised ethanolic 10.5 mM DPPH to which samples containing carvacrol ranging from 0.0015 to 751.1 μg/ml were added. After one hour in the dark, the absorbance at 515 nm was measured. Computation of the IC_{50} (half-maximal stimulatory concentrations) values was done by numerical interpolation using Stineman's formula (Wagon, 1999). The software used was the Scientist program from MicroMath Scientific Software (Salt Lake City, UT, USA).

Bacterial strain and culture conditions

Salmonella enterica serovar Typhimurium ATCC 14028 was used in this study. The strain was stored at –20 °C in Tryptic Soy Broth (TSB, Difco, Le Pont de Claix, France) containing 20% glycerol. Prior to use an aliquot of the frozen strain was transferred to Brain Heart Infusion Broth (BHI, Difco, Le Pont de Claix, France) and incubated at 35 °C for 24 h. The culture was plated on Hektoen Agar (Difco, Le Pont de Claix, France) and incubated under the same conditions.

Biofilm formation and exposure to carvacrol

Biofilm formation on polypropylene was performed according to Amaral et al. (2015). Briefly, a *S.* Typhimurium culture in TSB (10^7 CFU/ml) was added to polypropylene coupons (90 mm × 15 mm × 1 mm) that were incubated for 48 h at 35 °C. The coupons were washed with saline solution at 0.85% (w/v), incubated with TSB supplemented with carvacrol (624 μg/ml) for one hour at room temperature. Biofilms were removed mechanically using cell scraper with 20 ml of 0.85% saline solution, an aliquot of 1 ml of cell suspension was removed, and the biofilm cultivability was evaluated by the colony forming units (CFU) count. Colony counts were performed in triplicate and the results were expressed as $\log_{10}$ CFU/cm^2. The remaining bacterial suspension (19 ml) was centrifuged at 4500 × g for 5 min, washed with sterile saline solution at 0.85% (w/v) and the obtained pellet was used for the extraction of proteins. Experiments were repeated twice.

Protein extraction

The pellet obtained from two assays, as described above, was suspended in lysis buffer with protease inhibitor and subjected to sonication (Silva et al., 2018). The sample was centrifuged at 14000 × g for ten minutes at 4 °C and the supernatant was used for analysis. Protein concentration was estimated by Bradford method (Bradford, 1976) using bovine serum albumin standard. The 2D Clean-Up Kit (Amersham Biosciences, Piscataway, New Jersey) was used to purify the protein solution according to the manufacturer's instructions.

Two-dimensional (2-D) gel electrophoresis

The electrophoresis was performed according to Giaouris et al. (2013) with some modifications. Samples containing 400 μg/ml of proteins were suspended with rehydration solution with 0.6% IPG buffer and applied to the strips (performed in Immobiline DryStrip gels of 13-cm length and pH gradient 4–7 – GE Healthcare Life Science, Uppsala, Sweden). Rehydration and isoelectric focusing of the strips was performed using the Ettan IPGphor II Isoelectric Focusing system according to the following parameters: 50 volts (V) for rehydration overnight, 500 V for 1 h, 1000 V for 1 h and 8000 V for 3.5 h. Then, the strips were incubated for 30 minutes with equilibration solution containing 1% DTT and then another 30 min with equilibration solution containing 2.5% iodoacetamide. Separation in the second dimension was performed in 12.5% SDS-polyacrylamide gels in a vertical electrophoretic unit 600 Ruby (GE Healthcare, USA) in two steps: (a) 16.4 mA per gel during the stacking period and (b) 40 mA in the separation period. The proteins were fixed (1.3% orthophosphoric acid in 20% methanol solution) and the gels were stained overnight with Coomassie Blue G250 (Neuhoff et al., 1988). Two-dimensional (2-D) gel electrophoresis was repeated twice.

Image analysis

Image analysis of 2-D gels was performed using an Image Scanner II system. The spot detection, quantification, and analysis were realized by Image Master Software. Duplicates obtained from 2-D protein patterns in the treatment were separately compared with untreated biofilm. The differentiation in spot intensity was carried out by Image Master Software using a cutoff value ≥ 2.0-fold changes.

Mass spectrometry analysis

Spots of interest were subjected to tryptic in-gel digestion (Shevchenko et al., 1996). An aliquot of 4.5 µl (for protein analysis) of peptides resulting from proteins digestion were separated using a C18 (100–6100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-Tof Premier mass spectrometer (Waters) with nanoelectrospraysource at a flow rate of 0.6 ml/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 45 min. The nanoelectrospray voltage was set to 3.5 kV, with a cone voltage of 30 V and source temperature of 100 ºC being used. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on the exclusion list for 60 s and for the analysis of endogenous cleavage peptides, a real-time exclusion was used (Aragão et al., 2012).

Data analysis

Spectra were acquired using MassLynx v.4.1 software and raw data files were converted to a peak list format (mgf) without summing the scans using Mascot Distiller software v.2.3.2.0, 2009 (Matrix Science Ldt.). Peptide mass fingerprint data were searched using Mascot engine v.2.3.01 (Matrix Science Ltd.), *Salmonella* spp. Uniprot 2016 protein database, with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions.

Search Tool for Retrieval of Interacting Genes/Proteins analysis (STRING)

The protein interaction network of differentially regulated proteins was obtained using the dataset from the STRING database (Jensen et al., 2009). The obtained protein-protein interaction network was visualized using Cytoscape v 3.6.0 software (Cline et al., 2007).

Results

Antioxidant activity

The antioxidant activity of carvacrol was assessed by two different methodologies, DPPH radical scavenging and $ABTS^+$ assay. The IC_{50} value of carvacrol on DPPH and $ABTS^+$ was 228.12 ± 2.3 and 36.34 ± 3.15 µg/ml, respectively, indicating antioxidant activity of the compound.

Proteins changes in S. Typhimurium biofilm exposed to carvacrol

Carvacrol (624 µg/ml) was able to reduce bacterial counts from 8.1 $\log_{10}$ CFU/cm^2 to 4.1 $\log_{10}$ CFU/cm^2 on *S.* Typhimurium biofilms. In a previous study, Trevisan et al. (2018) also observed that carvacrol at 624 µg/ml was able to reduce about 50% of *S.* Typhimurium biofilm in polypropylene. Based on these results, this concentration was used to evaluate the protein changes in the present study.

The protein profile of *S.* Typhimurium biofilms treated and untreated biofilms were compared and differences confirmed in all comparisons were accepted. Using mass spectrometry, 48 protein spots were successfully identified. Among the differentially expressed protein spots in response to carvacrol treatment, 6 were up-regulated, 7 spots were down-regulated, 3 were expressed only after treatment, and 32 were observed only in the control gel (Table 1). The proteins were classified into the following categories based on their functions: carbon metabolism, protein metabolism, nitrogen metabolism, transmembrane transport, metabolism of nucleotides, oxidative stress, amino acid metabolism and quorum sensing.

Table 1. List of identified proteins in biofilm of *S.* Typhimurium ATCC 14028 treated with 624 µg ml^{-1} of carvacrol compared to untreated biofilm

Protein	Protein name	Protein function (www.uniprot.org)	Fold change	Nominal mass/pI	Sequence coverage %	Mascot score
	UP-REGULATED					
	Protein metabolism					
Tsf	Elongation factor Ts	Associates with the EF-Tu.GDP complex and induces the exchange of GDP to GTP	Present	30453/5.13	47	1422
SerS	Serine-tRNA ligase	Catalyzes the attachment of serine to tRNA(Ser)	Present	48835/5.39	7	191
	Carbon metabolism					
Mdh	Malate dehydrogenase	Catalyzes the reversible oxidation of malate to oxaloacetate	2.43	32626/6.01	3	48
TalB	Transaldolase B	Important for the balance of metabolites in the pentose-phosphate pathway	14.66	35320/5.09	12	180
SucC	Succinyl-CoA ligase [ADP-forming] subunit beta	This protein is involved in step 1 of the subpathway that synthesizes succinate from succinyl-CoA (ligase route)	Present	41740/5.29	25	396
	Transmembrane transport					
OmpD	Outer membrane porin protein OmpD	Forms pores that allow passive diffusion of small molecules across the outer membrane (By similarity)	2.55	39655/4.66	57	4222
OmpA	Outer membran protein A	Required for the action of colicins K and L and for the stabilization of mating aggregates in conjugation	2.81	37568/5.60	44	2286
	Oxidative stress					
AhpC	Alkyl hydroperoxide redutase c	Directly reduces alkyl hydroperoxides with the use of electrons donated by the 57 kDa flavoprotein alkyl hydroperoxide reductase	11.93	20848/5.03	22	85

Table 1. *(Continued)*

Protein	Protein name	Protein function (www.uniprot.org)	Fold change	Nominal mass/pI	Sequence coverage %	Mascot score
	Nitrogen metabolism					
RpsF	30S ribosomal protein S6	Binds together with S18 to 16S ribosomal RNA	6.44	15163/5.26	59	390
	DOWN-REGULATED					
	Protein metabolism					
FusA	Elongation factor G	Catalyzes the GTP-dependent ribosomal translocation step during translation elongation	2.48	77722/5.17	54	4521
PheT	Phenylalanine-tRNA ligase beta subunit	ATP binding , magnesium ion binding, phenylalanine-tRNA ligase activity, tRNA binding	Absent	87968/5.13	2	30
ProS	Proline-tRNA ligase	Catalyzes the attachment of proline to tRNA(Pro) in a two-step reaction: proline is first activated by ATP to form Pro-AMP and then transferred to the acceptor end of tRNA(Pro)	Absent	63557/5.18	53	1722
Def	Peptide deformylase	Removes the formyl group from the N-terminal Met of newly synthesized proteins	Absent	19384/5.02	27	194
GreA	Transcription elongation factor GreA	Necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites	Absent	17702/4.75	87	876
DsbA	Thiol:disulfide interchange protein DsbA	Required for disulfide bond formation in some periplasmic proteins such as PhoA or OmpA	Absent	23011/5.65	12	51
SelD	Selenide, water dikinase	Synthesizes selenophosphate from selenide and ATP	Absent	36902/5.15	23	656
	Carbon metabolism					
Eno	Enolase	Catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate	2.20	45627/5.25	61	2674
TpiA	Triosephosphate isomerase	Involved in the gluconeogenesis	9.46	27071/5.68	87	3640
Ppa	Inorganic pyrophosphatase	Catalyzes the hydrolysis of inorganic pyrophosphate (PPi) forming two phosphate ions.	5.48	19778/5.01	34	504
AcnA	Aconitate hydratase A	Involved in the catabolism of short chain fatty acids (SCFA) via the tricarboxylic acid (TCA)(acetyl degradation route) and the 2-methylcitrate cycle I (propionate degradation route)	Absent	97840/5.30	3	67
RhmA	2-keto-3-deoxy-L-rhamnonate aldolase	Catalyzes the reversible retro-aldol cleavage of 2-keto-3-deoxy-L-rhamnonate (KDR) to pyruvate and lactaldehyde	Absent	28880/5.27	5	43
Acs	Acetyl-coenzyme A synthetase	Catalyzes the conversion of acetate into acetyl-CoA (AcCoA), an essential intermediate at the junction of anabolic and catabolic pathways	Absent	72563/5.56	12	160
PckA	Phosphoenolpyruvate carboxykinase [ATP]	Involved in the gluconeogenesis	Absent	59895/5.67	64	2721
GlpK	Glycerol kinase	Catalyzes the phosphorylation of glycerol to yield sn-glycerol 3-phosphate	Absent	56301/5.42	24	653
PykF	Pyruvate kinase I	This protein is involved in step 5 of the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate	Absent	509675.66	23	428
SdhA	Succinate dehydrogenase flavoprotein subunit	Two distinct, membrane-bound, FAD-containing enzymes are responsible for the catalysis of fumarate and succinate interconversion	Absent	65048/5.91	3	82
NuoE	NADH-quinone oxidoreductase subunit E	NDH-1 shuttles electrons from NADH, via FMN and iron-sulfur (Fe-S) centers, to quinones in the respiratory chain. The immediate electron acceptor for the enzyme in this species is believed to be ubiquinone	Absent	18875/5.29	12	103
STM 1119	NAD (P)H dehydrogenase (quinone)	Is na enzyme that catalyse the chemical reaction: NAD(P)H + a quinone =NAD(P)+ + a hydroquinone	Absent	20854/5.78	27	77
GapA	Glyceraldehyde-3-phosphate dehydrogenase	Catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (BPG) using the cofactor NAD	Absent	35735/6.33	48	2607

Table 1. *(Continued)*

Protein	Protein name	Protein function (www.uniprot.org)	Fold change	Nominal mass/pI	Sequence coverage %	Mascot score
	Transmembrane transport					
MalE	Maltose-binding periplamic protein	Involved in the high-affinity maltose membrane transport system	7.60	43153/6.27	18	435
FruB	Multiphosphoryl transfer protein	This system is involved in fructose transport (By similarity)	Absent	39569/4.87	68	4570
HisJ	Histidine-binding periplasmic protein	Part of the histidine permease ABC transporter. Binds histidine	Absent	28476/6.03	61	1387
	Nitrogen metabolism					
GshB	Glutathione synthetase	This protein is involved in step 2 of the subpathway that synthesizes glutathione from L-cysteine and L-glutamate	5.56	35638/5.07	29	441
DnaK	Chaperone protein Dnak	Acts as a chaperone	Absent	69246/4.83	50	3567
HscB	Co-chaperone protein HscB	Co-chaperone involved in the maturation of iron-sulfur cluster-containing proteins	Absent	19996/5.08	6	45
GroL	60 kDa chaperonin	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions	Absent	57421/4.85	57	4996
RplE	50S ribosomal protein L5	This is 1 of the proteins that binds and probably mediates the attachment of the 5S RNA into the large ribosomal subunit, where it forms part of the central protuberance	Absent	20362/4.74	20	26
	Nucleotides metabolism					
Adk	Adenylate kinase	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP	8.56	23530/5.53	49	1189
DeoA	Thymidine phosphorylase	The enzymes which catalyze the reversible phosphorolysis of pyrimidine nucleosides are involved in the degradation of these compounds and in their utilization as carbon and energy sources, or in the rescue of pyrimidine bases for nucleotide synthesis	Absent	47086/4.96	41	860
Upp	Uracil phosphoribosyltransferase	Catalyzes the conversion of uracil and 5-phospho-alpha-D-ribose 1-diphosphate (PRPP) to UMP and diphosphate	Absent	22576/5.33	7	42
DeoD	Purine nucleoside phosphorylase DeoD type	Cleavage of guanosine or inosine to respective bases and sugar-1-phosphate molecules	Absent	26190/5.42	9	22
RpoA	DNA-directed RNA polymerase subunit alpha	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	Absent	36717	66	2351
	Oxidative stress					
SodA	Superoxide dismutase [Mn]	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems	Absent	23093/6.23	5	66
SodB	Superoxide dismutase [Fe]	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems	Absent	16000/6.12	52	2171
SodC	Superoxide dismutase [Cu-Zn] 2	Destroys radicals which are normally produced within the cells and which are toxic to biological systems	Absent	17897/6.03	16	65
	Amino acid metabolism					
ArgI	Ornithine carbamoyltransferase	Reversibly catalyzes the transfer of the carbamoyl group from carbamoyl phosphate (CP) to the N(epsilon) atom of ornithine (ORN) to produce L-citrulline	Absent	36917/5.39	17	453
DksA	RNA polymerase-binding transcription factor DksA	Required for negative regulation of rRNA expression and positive regulation of several amino acid biosynthesis promoters	Absent	17733/5.06	29	778
	Quorum sensing					
LuxS	S-ribosylhomocysteine lyase	Involved in the synthesis of autoinducer 2 (AI-2) which is secreted by bacteria and is used to communicate both the cell density and the metabolic potential of the environment	Absent	19467/5.71	15	73

Search Tool for Retrieval of Interacting Genes/Proteins analysis (STRING)

Analysis of identified proteins was performed using protein interaction information from the STRING database and visualized using Cytoscape (Fig. 1). STRING analyses revealed that up-regulated proteins involved in carbon and protein metabolism, transmembrane transport and nitrogen metabolism showed interaction with down-regulated proteins of the same categories. Proteins involved with oxidative stress and nucleotides metabolism showed no interaction with others. These results suggest that carvacrol could promote a multisite disturbance in *S*. Typhimurium biofilm.

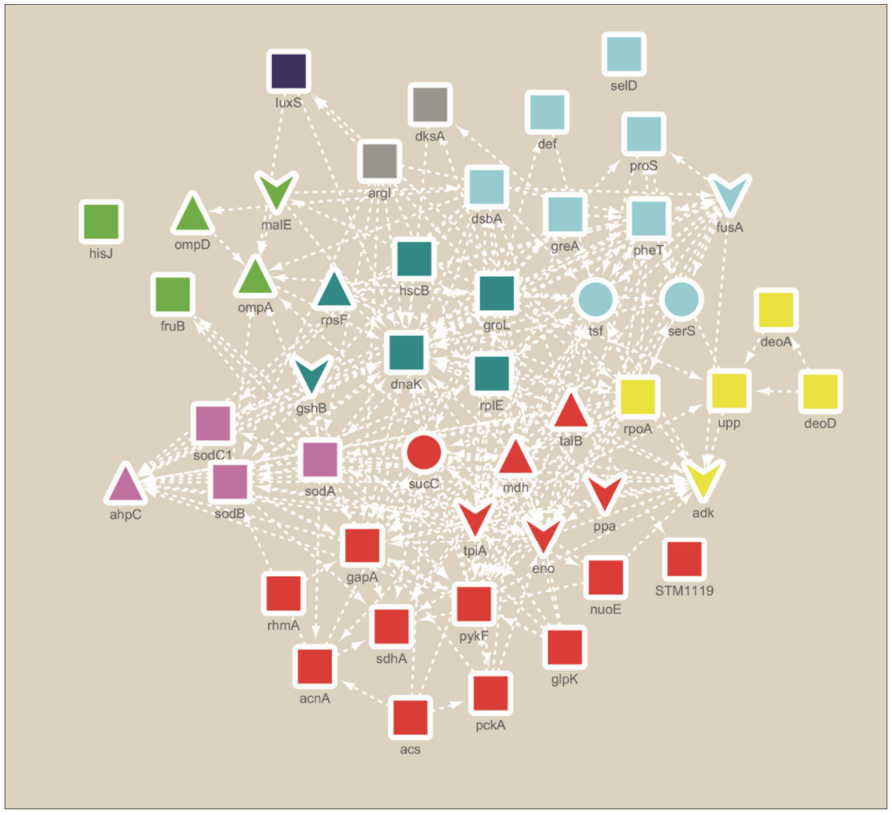

Fig. 1. A network of functional interactions between proteins altered in *S*. Typhimurium biofilm treatment with carvacrol. The nodes represent the proteins, and the edges represent the predicted functional associations. The proteins expressed only after treatment represented as circle; proteins that were observed only in the control gel was represented as square; proteins up-regulated after treatment represented as triangle and down-regulated proteins as represented as inverted triangle. Nodes are colored in red represent the proteins of the carbon metabolism; in aqua the protein metabolism; in teal the nitrogen metabolism; in lawn green the transmembrane transport; in yellow the metabolism of nucleotides; in magenta the oxidative stress; in gray the amino acid metabolism and in dark blue the quorum sensing.

Discussion

Recently, the effectiveness of carvacrol against bacterial biofilms has been investigated including those formed by *Salmonella* spp. (Amaral et al., 2015; Burt et al., 2014; Soni et al., 2013; Trevisan et al., 2018). Nevertheless, the mechanism of action of carvacrol against biofilms has not been completely elucidated. Burt et al. (2014) suggested that the mechanism by which carvacrol inhibits biofilm formation may be linked to the disruption of quorum sensing. Nostro et al. (2009), hypothesized that carvacrol, due to their amphipathic nature, could diffuse through the polysaccharide matrix of the biofilm and destabilize it. According to our knowledge, this is the first study reporting the changes in the protein profile of *S*. Typhimurium biofilm exposed to carvacrol, formed on a polypropylene surface.

Several changes in protein profile of biofilm cells were observed after treatment with carvacrol and main protein functions were showed in Table 1. Proteins that show differentially expression were searched in Uniprot and in scientific literature aiming to know their function. We decided to discuss only proteins that were directly related to bacterial biofilms.

DsbA (thiol: disulfide interchange protein DsbA; protein metabolism) was decreased after treatment. This protein is part of the DSB (DiSulfide Bond) system that was first characterized in *Escherichia coli* K-12 (Jarrott et al., 2010). Proteins of this system are essential for the oxidative folding of secreted proteins in Gram-negative bacteria (Heras et al., 2010).

The DsbA protein inserts a reversible covalent disulfide bond in proteins exported from the cytoplasm to the periplasm. The disulfide bond formation adds stability to the proteins, allowing them to resist hostile extracellular environments (Shouldice et al., 2011). The oxidative folding system of DsbA and DsbB proteins has an important role in bacterial virulence. For instance, DsbA catalyzes the correct folding of proteins involved in bacterial pathogenicity such as proteins associated with adhesion, toxin production, and cellular spread (Heras et al., 2009). The role of DsbA has also been related to the virulence and biofilm formation of others pathogens including *Salmonella* spp. (Anwar et al., 2014; Lee et al., 2008; 2011).

Anwar et al. (2014) studied the contribution of DsbA to the biofilm formation of S. Typhimurium using DsbA- or DsbB-deficient strains and observed that the mutant strains exhibited a loss of biofilm formation, showing that DsbA is important in biofilm formation. In our study, the synthesis of the DsbA protein was decreased after treatment with 624 μg/ml of carvacrol, suggesting that the antibiofilm property of this compound may be associated with the reduction of the relative quantity of this protein. Interestingly, Mangalappalli-Illathu and Korber (2006) observed that DsbA was up-regulated in the biofilm of *Salmonella* Enteritidis after adaptation with benzalkonium chloride. However, this study aimed to understand the mechanisms of development of adaptive resistance in *S*. Enteritidis biofilms after sublethal exposure to benzalkonium chloride.

Another protein that was also decreased after treatment of *S*. Typhimurium biofilm with carvacrol was S-ribosylhomocysteine lyase (LuxS; quorum sensing). This protein is involved in quorum sensing, a process of intercellular communication important to bacterial virulence and biofilm formation (Borges et al., 2016; Jiang and Li, 2013). This process occurs by releasing, sensing, and responding to small diffusible signal molecules termed autoinducers (Borges et al., 2016; Jiang and Li, 2013; Li and Tian, 2012). *S*. Typhimurium produces and releases autoinducer-2 (AI-2) as a signaling molecule and the *luxS* gene is required for its synthesis (Surette et al., 1999).

It has been shown that the *luxS* gene is necessary for virulence and biofilm formation in *S*. Typhimurium (Choi et al., 2007; Jesudhasan et al., 2010). Jesudhasan et al. (2010) studied the effects of deletion of luxS on the formation of S. Typhimurium biofilm and observed that mutant strains formed significantly less biofilm than the wild strain, indicating that luxS is involved in the biofilm formation process.

Burt et al. (2014), using other approaches, reported that carvacrol inhibited *S*. Typhimurium biofilm formation, probably by disrupting quorum sensing signaling. Similar to findings by Burt et al. (2014), our previous study also showed the ability of carvacrol to decrease *S*. Typhimurium biofilm (Trevisan et al., 2018), corroborating with the hypothesis that these results may be a consequence of reduction of relative quantity of luxS.

Carvacrol also decreased relative quantity of DksA (RNA polymerase binding transcription factor DksA; amino acid metabolism) which was previously related to biofilm formation and pathogenicity of *S*. Typhimurium (Azriel et al., 2016). The latter authors characterized the role of DksA in *S*. Typhimurium biofilm using a *S*. Typhimurium *dksA* mutant strain and observed that the ability of these bacteria to form biofilms was significantly decreased in the absence of *dksA*. In our study, after treatment with carvacrol, the DksA was decreased, suggesting that the antibiofilm property of this compound may also be associated with the reduction in relative quantity of this protein.

Proteins involved in the generation of reducing agents were also affected by carvacrol treatment. The increased in the relative quantity of protein transaldolase B (TalB; carbon metabolism) involved in the pentose shunt process deviates the substrate from one of the main mechanisms of generation of antioxidant agents, the pentose phosphate pathway. The diminished quantity of proteins NAD(P)H dehydrogenase (STM1119; carbon metabolism), superoxide dismutase [Mn] (SodA; oxidative stress), superoxide dismutase [Fe] (SodB; oxidative stress), superoxide dismutase [Cu-Zn] (SodC; oxidative stress), selenite water dikinase (SelD; protein metabolism), and glutathione synthetase (GshB; nitrogen metabolism), all involved in reduction reactions of oxidized components in the cell, supports the aforementioned hypothesis.

Carvacrol presents reducing activity as shown by the antioxidant assays. In this way, we hypothesize that the cell mechanism for production of reducing agents would not be so required. Therefore, this mechanism may have been suppressed due to carvacrol treatment. The fact that a reducing substance is high in the cell would certainly cause an imbalance of the oxidation-reduction reaction system, which is one of the mechanisms controlling the cellular process. This would justify why so many proteins from different mechanisms were found to be down regulated. In an effort to maintain cellular homeostasis, the protein alkyl hydroperoxide reductase subunit c (AhpC; oxidative stress) that is involved in maintenance of cell oxidation-reduction homeostasisis was present in high quantity (Parsonage et al., 2008). In addition, the antioxidant superoxide dismutase (SOD) protein as previously been found to be associated with bacterial virulence and biofilm formation (Kim et al., 2006; Krishnakumar et al., 2004; Suo et al., 2012). The SODs are metalloproteins that catalyze the toxic superoxide radicals to oxygen and hydrogen peroxide as protective reactions to remove cytotoxic free radicals from cells (McCord and Fridovich, 1969). Proteomic analyses performed by Kim et al. (2006) examined the role of SOD in the biofilm formation of *Escherichia coli* O157:H7. They showed that SodC had a significantly higher relative quantity in biofilms than under planktonic conditions. In the same study, it was observed that the deletion of *sodC* in *E. coli* O157:H7 reduced the attachment to abiotic surfaces during static growth. Trémoulet et al. (2002) compared differences between protein patterns of *Listeria monocytogenes* biofilm and planktonic cells and found that SOD protein was increased in the biofilm state. In another study, Suo et al. (2012) evaluated the role of SOD in *L. monocytogenes* formation. The authors observed that the mutant of *sod* presented reduced ability to form biofilm (Suo et al., 2012). These studies suggest that the *sod* gene plays a significant role in the development of biofilms. In our study, relative quantities of SodA, SodB, and SodC proteins were decreased after treatment with carvacrol. These results support the idea that this treatment affects the system of response to oxidative stress and that decreased synthesis of SOD can disestablish biofilm.

Conclusions

The proteomic analysis evidenced the action of carvacrol in proteins that can play a key role in *S*. Typhimurium biofilm formation. We highlight the decrease in the relative quantity of DsbA, LuxS, DksA and SODs proteins, important in bacterial biofilm, after treatment with carvacrol. Others tools beyond proteomics analysis can be applied to gain more insight into the mechanism of action of carvacrol on biofilm of *S*. Typhimurium.

Conflict of interests

The authors declare that they have no conflict of interests.

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