A Carvacrol-Rich Essential Oil Extracted From Oregano (*Origanum vulgare* “Hot & Spicy”) Exerts Potent Antibacterial Effects Against *Staphylococcus aureus*

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Oregano essential oil (OEO), as a natural antimicrobial, has gained increased interest from food researchers and manufacturers. However, a few studies have investigated its possible antibacterial effects against *Staphylococcus aureus* using the proteomic tool. The present study aimed to explore the antibacterial effect and mechanism of a carvacrol-rich OEO extracted from *Origanum vulgare* “Hot & Spicy” on the inactivation of *S. aureus*. The gas chromatography–mass spectrometry analysis of the OEO allowed the detection of 27 compounds; the major constituent was carvacrol (84.38% of total compounds). The average diameter of the inhibitory zone (DIZ) value was 29.10 mm, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of OEO against *S. aureus* were 0.125 and 0.25 mg/mL, respectively. The growth curve assay indicated that the OEO prolonged the lag phase of *S. aureus*. The decrease in cell viability, changes in the integrity of cell membrane, and abnormal cell morphology further reflected the cell damage of *S. aureus* caused by the OEO. In addition, a label-free proteomic analysis was applied to analyze the regulatory networks of *S. aureus* in response to 1/2 MIC OEO-treatment stress. Of the 56 differentially expressed proteins (DEPs) identified, 26 were significantly upregulated and 30 downregulated. The Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis indicated that the DEPs were mainly involved in pathways of ribosomes; valine, leucine, and isoleucine biosynthesis; and phenylalanine, tyrosine, and tryptophan biosynthesis, which suggested that the growth inhibition of *S. aureus* might be due to the disordered effect of the OEO on protein synthesis and amino acid metabolism. These findings deepened our understanding regarding *S. aureus* survival and metabolism responses to the OEO treatment and suggested that the carvacrol-rich OEO could be used in food production environments to effectively control *S. aureus*.

**Keywords:** carvacrol, cell membrane, oregano essential oil, proteomics, *Staphylococcus aureus*
INTRODUCTION

*Staphylococcus aureus* is a facultative anaerobic Gram-positive foodborne pathogen involved in human outbreaks regarded as one of the world’s leading causes of disease outbreaks related to food consumption (Jamali et al., 2015; Kang et al., 2019). *S. aureus* produces a wide variety of toxins, including staphylococcal enterotoxins, which commonly lead to staphylococcal food poisoning during the massive growth of *S. aureus* in various foods (Hait et al., 2021; Umeda et al., 2021). Moreover, *S. aureus* is ubiquitous and widely distributed in foods and food-processing environments, such as in raw meat and meat products as well as raw milk and dairy products (Wang et al., 2013; Titouche et al., 2019). The management of *S. aureus* poisoning has become increasingly difficult because of the emergence of multi-resistant strains caused by the widespread and often inappropriate use of antibiotics in livestock (Mehli et al., 2017; Zhang et al., 2020).

In the face of increasing *S. aureus* contamination, several emerging strategies for preventing and treating the contamination have been proposed, including the use of natural antimicrobial agents. Essential oils (EOs), aromatic and volatile secondary metabolites, have the potency to combat significant pathogens due to their broad-spectrum antimicrobial effects, which have been extensively evaluated in numerous food matrices, such as fruits, vegetables, meat, and milk products (Hyldgaard et al., 2012; Kang et al., 2018, 2019; Rao et al., 2019). EOs not only exert highly effective in inhibiting multidrug-resistant bacteria (Lu et al., 2018; Cui et al., 2019) but also can greatly reduce the resistance of microbes through complex mixtures of natural compounds (Lu et al., 2018). Food-grade delivery systems, including microemulsions, nanoemulsions, and liposomes, have been widely used to enhance the antimicrobial efficacy and utilization of EOs in foods (Rao et al., 2019).

Oregano, a bushy, herbaceous perennial plant native to Europe and Central Asia, is a flavoring herb widely used throughout the world to flavor various foods and processed meals, such as salads, pizza, and sausages (Morsheedlo et al., 2018). EOs extracted from the leaves and inflorescence of oregano have been claimed to have numerous useful biological activities, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and cancer-suppressing effects (Leyva-Lopez et al., 2017). Carvacrol-rich chemotypes of oregano cover most of its natural range of biological activities, which have a vast range of applications in food industries (Emrahi et al., 2021). Carvacrol [2-methyl-5-(1-methylethyl) phenol], a monoterpenic phenol, is one of the main essential oil compounds produced by oregano plants, which exhibits significant antimicrobial activity against foodborne pathogens (Miladi et al., 2016). Cui et al. (2019) reported that the antibacterial consequences of the action of oregano essential oil (OEO) could be summarized in the following ways: changes in the permeability of the cell membrane and irreversible damage to the cell membrane; inhibition of respiratory metabolism; carvacrol, as the main component of OEO, forming chimeras with DNA; and reducing the production of a Panton–Valentine Leukocidin (PVL) toxin.

Proteomics is the systematic evaluation of all proteins expressed by one particular organism in a given time (Dos Santos et al., 2016). The quantitative proteomics based on chromatographic separations coupled with mass spectrometry improves the identification of pathogenic proteins in response to the treatment of antimicrobial agents. The technology is considered a useful means to identify and characterize the differential proteins of microbes treated with natural agents, which is a key strategy to understand better the antibacterial mechanism (Tang et al., 2020). Despite some reports on the antimicrobial activity of OEO, mainly related to its effects on bacterial phenotype and physiology, the change in the protein profile of foodborne bacteria caused by an OEO is unknown or less studied. Thus, in the present study, we investigated the potential antibacterial mechanism of a carvacrol-rich OEO against *S. aureus*. On the one hand, the changes in the cell membrane of *S. aureus* after exposure to an OEO were detected using a cryo-scanning electron microscope (cryo-SEM), flow cytometry, and laser confocal microscopy. On the other hand, the label-free quantitative proteomic analysis was employed to characterize the differentially expressed proteins (DEPs) between the OEO-treated and untreated groups, which could reveal the potential functions and the biological processes involved in the anti-*S. aureus* action of OEO.

MATERIALS AND METHODS

Plant Materials, Essential Oil Extraction, and Bacterial Strains

The aerial parts of *Origanum vulgare* “Hot & Spicy” were harvested in the full-bloom stage in Nanyang, Henan Province, at the coordinates 32°78’N, 112°57’E, 116 m of altitude, in July 2019. Then, the aerial parts were air dried under the shade for 4 weeks. Dried samples were ground to a powder before extracting the OEO. The OEO was extracted from 100 g of powdered samples in 1000 mL of distilled water by steam distillation (Clevenger apparatus) for 3 h (Baranauksiene et al., 2013). The extracted OEO was dried using anhydrous sodium sulfate and stored in an amber bottle at 4°C until use. The *S. aureus* strain CGMCC 1.4519 was obtained from the China General Microbiological Culture Collection Center (Beijing, China). The strains were stored in the Luria-Bertani (LB) broth with 25% glycerol (v/v) at ~80°C. Before each experiment, the test strain was shake-cultured in LB broth for 12 h at 37°C.

Chemical Characterization

The composition of OEO was analyzed by gas chromatography–mass spectrometry (GC-MS) (7890A-7000B, Agilent Technologies, United States) equipped with a 30 m × 250 μm × 0.25 μm HP-5MS column (Agilent Technologies). Further, 1 μL of the sample was injected at a split mode of 40:1. The injector temperature was 250°C, and the temperature programming was as follows: the temperature remained at 40°C for 2 min and was then ramped up linearly to 77°C at a rate of 8°C/min; the temperature was ramped up to 150°C at a rate of 5°C/min, and then ramped to 185°C at a rate of 3°C/min, followed by ramping to 310°C at 60°C/min. The transfer line temperature was 280°C, and helium was used as
the carrier gas at a flow rate of 1.0 mL/min through the column. The MS operating parameters were as follows: ionization energy, 70 eV; ion source temperature, 230°C; quadrupole temperature, 150°C; and mass range, 40–700 u. The OEO compounds were identified by comparing the National Institute of Standards and Technology 17 library spectra and retention index (RI) values. The RI values were calculated using a series of n-alkane (C7–C40) hydrocarbons under the same conditions. The relative percentage composition of OEO components was determined based on the peak area.

Antibacterial Activity
Diameter of the Inhibitory Zone Assay
The disk diffusion method was used to assess the diameter of the inhibitory zone (DIZ) of OEO on S. aureus. Briefly, 100 μL of S. aureus suspensions (approximately 10^7 CFU/mL) were evenly spread onto LB agar plates. Sterilized antimicrobial disks were placed on test plates, and 6-μL spread onto LB agar plates. Sterilized antimicrobial disks were washed three times with PBS and finally suspended in sterile water. The morphology of S. aureus cells treated or untreated with the OEO was observed using a cryo-SEM (Regulus 8100, Hitachi Co., Ltd., Japan) (Wu et al., 2014).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration
The double broth dilution method described by Kang et al. (2019) was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Briefly, approximately 10^7 CFU/mL of the S. aureus strains were cultured in LB broths and mixed with a range of concentrations of OEO (0.0625–8 mg/mL) in test tubes. The LB without OEO was regarded as a control. Then, these tubes were incubated at 37°C for 24 h. The MIC was considered as the lowest concentration of OEO at which no visible bacterial growth was observed. An S. aureus suspension from each tube with no visible bacterial growth was cultured on LB agar plates. The MBC was the lowest concentration of OEO that prevented the growth of bacterial colonies on the LB agar surface.

Effects of Oregano Essential Oil on the S. aureus
Antibacterial Curve Assay
The growth curve determination was performed as described by Kang et al. (2020), with slight modification. The OEO was dissolved in a sterile LB broth to obtain the final concentrations of 1/8MIC, 1/4MIC, 1/2MIC, MIC, and MBC. S. aureus CGMCC 1.4519 growth curve was determined by measuring OD_{600nm} for 0 to 24 h at 1-h intervals using a Bioscreen C Automated Microbiology Growth Curve Analysis System (Oy, Finland) at 37°C in LB broth. The OD_{600nm} value of LB broth without OEO and S. aureus was regarded as a blank control.

Cell Membrane Integrity Assay
An S. aureus suspension (approximately 10^7 CFU/mL) was treated with or without OEO at final concentrations of 0, 1/2 MIC, MIC, and MBC at 37°C for 8 h. The collected proteins (20 g) for each bacterial sample were mixed with 5 × loading buffer and further boiled for 5 min. The proteins were separated on 12.5% SDS-PAGE gel (constant current 14 mA, 90 min). Protein bands were visualized by Coomassie Blue R-250 staining (Kang et al., 2018).

SDS-PAGE Analysis
The collected proteins (20 μg) for each bacterial sample were mixed with 5 × loading buffer and further boiled for 5 min. The proteins were separated on 12.5% SDS-PAGE gel (constant current 14 mA, 90 min). Protein bands were visualized by Coomassie Blue R-250 staining (Kang et al., 2018).

LC-MS/MS Analysis
An LC-MS/MS analysis was performed on a Thermo Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled to Easy nLC-1200 nanoLC system (Thermo Fisher Scientific, Waltham, MA, United States) for 90 min. The peptides were loaded onto a reverse phase trap column (manually packed
reverse phase C18 column, 100 µm × 2.5 cm, 1.9 µm particle size; 120 Å pore diameter; Dr. Maisch GmbH Inc., Germany) connected to a C18-reversed-phase analytical column (manually packed reverse phase C18 column, 150 µm × 25 cm in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 500 nL/min. The mass spectrometer was operated in the positive ion mode with the following parameters: the resolution set to 120 K for MS1. In MS1, the scan range was 350–1550 m/z. The automatic gain control was 4E5, and the charge state was 2–7. In MS2, the normalized collision energy was set to 32%. Ions were broken by higher collision dissociation and then analyzed by Orbitrap with AGC targets set at 5E4.

The MS raw data for each sample were combined and searched using the MaxQuant 1.5.3.17 software for identification and quantitation analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD028357.

Bioinformatics Analysis
Cluster 3.01 and Java Treeview software2 were used to carry out a hierarchical clustering analysis. A heatmap was often presented as a visual aid in addition to the dendrogram. The protein sequences of the selected DEPs were locally searched using the NCBI BLAST+ client software and InterProScan to find homolog sequences. Then, Gene Ontology (GO) terms were mapped, and sequences were annotated using the software program Blast2GO. The GO annotation results were plotted using R scripts. Following annotation steps, the studied proteins were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database3 to retrieve their KEGG orthology identifications and were subsequently mapped to pathways in KEGG. The enrichment analysis was applied based on the Fisher exact test, considering the whole quantified proteins as the background dataset. Benjamini–Hochberg correction for multiple testing was further applied to adjust derived P-values. Only functional categories and pathways with P-values under a threshold of 0.05 were considered significant.

Statistical Analysis
All experiments were carried out in triplicate independently. All data were presented as the mean ± standard deviation. Differences between means were tested using one-way analysis of variance and LSD test and analyzed using IBM SPSS software (version 19.0; SPSS Inc., Chicago, IL, United States). A P-value ≤ 0.05 indicated a significant difference.

RESULTS

Chemical Composition of Oregano Essential Oil
The chemical composition analysis of the OEO via GC-MS resulted in identifying 27 chemical compounds (Figure 1)

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1http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm  
2http://jtreeview.sourceforge.net  
3http://geneontology.org/
and Table 1). The most abundant bioactive components were monoterpenes and sesquiterpenes. Monoterpene carvacrol was the main compound, which comprised 84.38% of the identified compounds. In addition, several other natural compounds were reported, including p-cymene, γ-terpinene, β-caryophyllene, and terpinen-4-ol. These compounds comprised 5.07%, 1.7%, 1.56%, and 1.06% of all compounds identified in the OEO, respectively.

**Antibacterial Effects of Oregano Essential Oil Against S. aureus**

Table 2 shows the DIZ, MIC, and MBC values of the OEO against S. aureus. As shown in Table 2, the carvacrol-rich OEO had a potent inhibitory effect against S. aureus. The average DIZ value was 29.1 (±0.6) mm, and the MIC and MBC of OEO against S. aureus were 0.125 and 0.25 mg/mL, respectively.

The effects of OEO at 1/8MIC, 1/4MIC, 1/2MIC, MIC, and MBC on the growth of S. aureus are shown in Figure 2. All the OEO concentrations showed inhibition against S. aureus but with different degrees. The growth of S. aureus was completely inhibited in the presence of OEO at the MIC and MBC in the LB broth. The OEO at 1/2 MIC decreased the maximal cell populations of S. aureus compared with the control group within 24 h. The OEO concentrations within 1/4 MIC or 1/8 MIC showed slightly inhibitory effects against the S. aureus growth. These results demonstrated that the concentration and treatment time of OEO had great influences on antibacterial effects.

**Effects of Oregano Essential Oil on the Cell Membrane of S. aureus**

Cryo-SEM observation allows one to study the alterations in several compartments in the cell membrane; it has been used to offer direct evidence of the ability of EOs to disrupt the structure of microbial cells (Rao et al., 2019). Figure 3A shows the changes in the cell morphology of S. aureus. The untreated S. aureus cells had a regular and smooth surface with no visible fractures and a typically spherical or elliptical Staphylococcus morphology. When the S. aureus cells were treated with OEO at 0.0625 (1/2MIC), 0.125 (MIC), and 0.25 (MBC) mg/mL, the bacterial cell surfaces showed more shrinkage compared with normal S. aureus cells and the number of S. aureus cells decreased with the increasing OEO concentration.

Furthermore, we used flow cytometry to determine the cell membrane integrity and cell viability of S. aureus treated with the OEO. Figure 3B shows the changes in cell viability; in this figure, R1, R2, and R4 regions correspond to dead, injured, and intact S. aureus cells, respectively. The control group showed approximately 98.6% S. aureus cells and the number of S. aureus cells decreased with the increasing OEO concentration. When the S. aureus cells were treated with the OEO at 1/2 MIC and MIC levels, the percentage of injured cells decreased. These results indicate that the OEO could significantly alter the cell membrane integrity and cell viability of S. aureus

**Table 1 | Chemical composition (%) of OEO.**

| CAS      | Compound                        | Empirical formula | Rref | RT    | RI   | Percentage (%) |
|----------|---------------------------------|-------------------|------|-------|------|----------------|
| 2987-05-2| α-Thujene                       | C10H16            | 929  | 7.551 | 932  | 0.42 ± 0.07    |
| 123-35-3 | β-Myrcene                       | C10H16            | 991  | 8.884 | 991  | 0.42 ± 0.03    |
| 99-83-2  | α-Pheillandrene                 | C10H16            | 1005 | 9.219 | 1006 | 0.09 ± 0.01    |
| 99-86-5  | β-Terpine                       | C10H18O           | 1017 | 9.526 | 1018 | 0.61 ± 0.03    |
| 99-87-6  | α-Cymene                        | C10H14            | 1025 | 9.722 | 1026 | 5.07 ± 0.22    |
| 555-10-2 | β-Pheillandrene                 | C10H16            | 1031 | 9.835 | 1030 | 0.22 ± 0.01    |
| 99-85-4  | γ-Terpine                       | C10H16            | 1050 | 10.595| 1060 | 1.7 ± 0.05     |
| 17699-16-0| Trans-Sabinene hydrate          | C10H18O           | 1070 | 10.812| 1069 | 0.31 ± 0.08    |
| 586-62-9 | Terpinolene                     | C10H16            | 1088 | 11.372| 1091 | 0.13 ± 0.01    |
| 29803-82-5| Cis-2-p-Menthene-1-ol            | C10H18O           | 1126 | 12.234| 1124 | 0.07 ± 0.02    |
| 507-70-0 | Endo-Borneol                    | C10H18O           | 1167 | 13.439| 1170 | 0.17 ± 0.01    |
| 562-74-3 | Terpin-4-ol                     | C10H18O           | 1177 | 13.738| 1181 | 1.06 ± 0.1     |
| 98-55-5  | α-Terpineol                     | C10H18O           | 1189 | 14.083| 1194 | 0.16 ± 0.01    |
| 5948-04-9| Trans-Dihydrocarvone            | C10H18O           | 1201 | 14.269| 1201 | 0.25 ± 0.03    |
| 6379-73-3| Carvacrol methyl ether          | C11H16O           | 1244 | 15.488| 1248 | 0.78 ± 0.02    |
| 89-83-8  | Thymol                          | C10H14O           | 1291 | 16.714| 1294 | 0.33 ± 0.05    |
| 499-75-2 | Carvacrol                       | C10H14O           | 1299 | 16.97 | 1304 | 84.38 ± 0.4    |
| 3856-25-5| Copaene                         | C15H24            | 1376 | 18.96 | 1382 | 0.1 ± 0.05     |
| 5208-59-3| β-Bourbonese                    | C15H24            | 1384 | 19.232| 1393 | 0.07 ± 0.01    |
| 87-44-5  | β-Caryophyllene                 | C15H24            | 1419 | 20.106| 1428 | 1.56 ± 0.08    |
| 6753-98-6| Humulene                        | C15H24            | 1454 | 20.946| 1462 | 0.18 ± 0.01    |
| 23986-74-5| Germacrene D                   | C15H24            | 1481 | 21.485| 1484 | 0.38 ± 0.02    |
| 3691-11-0| α-Bulnesese                     | C15H24            | 1505 | 21.942| 1502 | 0.07 ± 0.07    |
| 10208-80-7| α-Murolene                     | C15H24            | 1499 | 22.076| 1507 | 0.07 ± 0.01    |
| 495-61-4 | β-Bisabolene                    | C15H24            | 1509 | 22.248| 1514 | 0.2 ± 0.01     |
| 483-76-1 | α-Cadinene                      | C15H24            | 1524 | 22.658| 1530 | 0.33 ± 0.02    |
| 1139-30-6| Caryophyllene oxide             | C15H24O           | 1581 | 24.272| 1592 | 0.33 ± 0.01    |
**TABLE 2** | Diameter of the inhibition zone (DIZ), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of OEO against *S. aureus*.

| Bacteria | DIZ (mm) | Concentrations of OEO (mg/mL) |
|----------|----------|-----------------------------|
|          |          | 0 | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 |
| *S. aureus* | 29.1 ± 0.6 | ++ | ++ | + | – | – | – | – | – | – |

“++” means observed growth of bacteria, “+” means no visible growth of bacteria, and “–” no cell colonies on the surface of LB agar.

Distribution of Identified Differentially Expressed Proteins

A Venn diagram showed the details of all 1268 proteins between the two groups, including 1178 shared DEPs (Figure 4C). Furthermore, the volcano plot shown in Figure 4C depicts $\log_2$ (treatment/control) against $-\log_{10}$ (P-value) representing the possibility for DEPs. In this figure, DEPs were regarded as proteins with $\geq 2/3$ or $\leq 2/3$ fold change and P-value less than 0.05. As shown in Figure 4B, the red points and blue dots indicate upregulated and downregulated DEPs, respectively. The gray dots indicate unchanged proteins. Based on this, 56 DEPs were confirmed, of which 26 were significantly upregulated and 30 downregulated. All the DEPs were transformed into a heat map. A hierarchical clustering analysis with the Euclidean distance algorithm was employed to analyze DEP expression patterns between control and OEO-treatment groups. As shown in Figure 4D, blue represents downregulated DEPs, red represents upregulated DEPs, and white represents no detectable expression change.

The DEPs in the control and OEO-treatment groups are listed in Table 3. DEPs with a fold change (FC) value of more than 2 included A5IV11 (50S ribosomal protein L36), A0A380DL04 (ribitol-5-phosphate cytidylyltransferase), A0A380DI91 (ribokinase), A0A033UKY8 (50S ribosomal protein L7/L12), A0A0D1EWN0 (phosphoribosylformylglycinamidine synthase subunit PurS), A5IPU8 (30S ribosomal protein S18), A8Z216 (50S ribosomal protein L33 2), and A0A1Q8DDL3 (cysteine synthase). The protein encoded by cysM in *S. aureus* was sequentially and functionally homologous to the O-acetylserine (thiol)-lyase B family of cysteine synthase proteins. A mutation of cysM caused increased *S. aureus* sensitivity to tellurite, hydrogen peroxide, acid, and diamide but decreased the...
ability of *S. aureus* to recover from starvation under amino acid- or phosphate-limiting conditions (Lithgow et al., 2004). Among these DEPs, A0A1Q8DDL3 protein was downregulated, suggesting that the OEO might inhibit the growth of *S. aureus* by decreasing the activity of cysteine synthase. Thus, GO and KEGG enrichment analyses were further carried to understand the biological functions regarding the inhibition of *S. aureus* by the treatment of 1/2 MIC OEO.

### Gene Ontology Functional Classification and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis of Differentially Expressed Proteins

A GO enrichment analysis was carried out to identify the biological processes, cellular components, and molecular functions of identified proteins. The number of proteins involved in each of these function terms is listed in Figure 5A. As illustrated in Figure 5A, metabolic process (GO:0008152) and cellular process (GO:0009987) were the main distributed terms in the biological process ontology. For the ontology of molecular function, most DEPs were mainly gathered at the following terms: catalytic activity (GO:0003824) and binding (GO:0005488). In the cellular component ontology, the main terms were cell (GO:0005623) and cell part (GO:0044464).

A KEGG pathway analysis was used to collect information regarding protein functions in the metabolic processes to explore the specific biological events of the DEPs. The distribution of the enriched KEGG pathway is shown in Figure 5B. As shown in the figure, the Rich factor represented the ratio of the number of DEPs annotated to the KEGG pathway category to the number of all identified proteins annotated to the category. The ordinate means the description of the main KEGG pathway; the bubble color indicates the significance of
the enriched KEGG pathway, that is, the closer the color is to red, the smaller the P-value and the higher the significance level of the enrichment of the corresponding metabolic pathway. Based on this, the ribosome (ko03010) pathway enriched the largest number of DEPs and exhibited a higher significance level. Among these enriched pathways, chlorocyclohexane and chlorobenzene degradation (ko00361), styrene degradation (ko00643), ribosome biogenesis in eukaryotes (ko03008), and AMPK signaling pathway (ko04152) had a high value of the Rich factor. In addition, amino acid metabolism, including valine, leucine, and isoleucine biosynthesis (ko00290) and phenylalanine, tyrosine, and tryptophan biosynthesis (ko00400), was identified as the important enriched pathway, suggesting that the OEO might interfere with the amino acid metabolism of S. aureus.

**DISCUSSION**

Oregano essential oils are considered as the highly complex mixtures of bioactive compounds, in which the preponderant constituents are terpenes, generally mono- and sesquiterpenes. The main terpenes identified in the different species of oregano are carvacrol, thymol, γ-terpinene, and p-cymene, followed by
### TABLE 3 | Some bacterial response-related DEPs of *S. aureus* after 1/2-OEO treatment.

| Protein ID | Ratio | P-value | Change | Protein description |
|------------|-------|---------|--------|---------------------|
| A0A033UKY8 | 2.33  | 0.019   | Up     | 50S ribosomal protein L7/L12 |
| A0A033UL39 | 0.62  | 0.006   | Down   | Glutamate dehydrogenase |
| A0A033LZSR | 0.62  | 0.004   | Down   | Probable tRNA sulfurtransferase |
| A0A033VST2 | 1.94  | 0.022   | Up     | Elongation factor Ts |
| A0A033VH0  | 0.61  | 0.001   | Down   | Pyruvate dehydrogenase E1 component subunit beta |
| A0A03OZ4  | 0.51  | 0.049   | Down   | 3-Deoxy-7-phosphohexulonate synthase |
| A0A01ENN0 | 2.26  | 0.042   | Up     | Phosphoribosylformylglycinamidine synthase subunit PurS |
| A0A016LJ9 | 0.54  | 0.010   | Down   | Rqc2 homolog RqcH |
| A0A016GV2 | 1.82  | 0.049   | Up     | DM13 domain-containing protein |
| A0A011H92 | 0.55  | 0.003   | Down   | Putative tRNA (cytidine(34)-2-O)-methyltransferase |
| A0A011HM04 | 1.52  | 0.016   | Up     | Molybdopterin molybdenumtransferase |
| A0A060EKS | 1.72  | 0.049   | Up     | Esterase Yl1 |
| A0A060VLR7 | 0.63  | 0.022   | Down   | N-Acyl-l-amino acid amidohydrolase |
| A0A060MRR0 | 0.65  | 0.003   | Down   | Transcriptional regulator, MarR family |
| A0A060NV93 | 0.60  | 0.029   | Down   | Coenzyme A biosynthesis bifunctional protein CoaBC |
| A0A060QY23 | 0.65  | 0.011   | Down   | NifU domain protein |
| A0A060Q743 | 0.59  | 0.034   | Down   | Glycine cleavage system H-like protein |
| A0A172PCR7 | 0.64  | 0.014   | Down   | Triacylglycerol lipase |
| A0A1Q8DC67 | 0.67  | 0.016   | Down   | TRAM domain-containing protein |
| A0A1Q8DL3 | 0.31  | 0.018   | Down   | Cysteine synthase |
| A0A1Q8DHF4 | 0.66  | 0.026   | Down   | Molybdenum cofactor biosynthesis protein B |
| A0A1Q8QH2 | 1.86  | 0.020   | Up     | Ferrichrome ABC transporter substrate-binding protein |
| A0A380D91 | 2.51  | 0.005   | Up     | Ribokinase |
| A0A380D4H0 | 0.57  | 0.006   | Down   | Exotoxin 15 |
| A0A380DL4 | 2.52  | 0.008   | Up     | Ribitol-5-phosphate cytidylyltransferase |
| A0A380E7 | 0.58  | 0.004   | Down   | Similar to hydrolase (HAD superfamily) |
| A0A39WPE3 | 1.50  | 0.026   | Up     | Dihydroxyacetone kinase subunit L |
| A0A47999Y | 0.65  | 0.002   | Down   | Dihydrolipoamide-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex |
| A0A5C8  | 0.57  | 0.031   | Down   | Iron-hydroxamate ABC transporter substrate-binding protein |
| A0A5C8  | 0.56  | 0.010   | Down   | Hyperosmolarity resistance protein Ebh (fragment) |
| A0A5PHPL4 | 0.53  | 0.025   | Down   | Autoexportin |
| A0A635Y3 | 1.51  | 0.046   | Up     | ATP-binding cassette domain-containing protein |
| A0A635X6H | 0.63  | 0.020   | Down   | Recombinase RecT (fragment) |
| A0A73WVJ7 | 1.54  | 0.006   | Up     | Phosphoglycerate kinase |
| A5IPU8 | 2.22  | 0.033   | Up     | 30S ribosomal protein S18 |
| A5IR11 | 0.56  | 0.007   | Down   | Glycine cleavage system H protein |
| A5IRV6 | 0.66  | 0.013   | Down   | Phosphoribosylformylglycinamidine synthase subunit PurL |
| A5IS9 | 1.63  | 0.021   | Up     | DNA-directed RNA polymerase subunit omega |
| A5ITD6 | 0.56  | 0.036   | Down   | Uridine kinase |
| A5ITM2 | 1.57  | 0.033   | Up     | ATP-dependent 6-phosphofructokinase |
| A5IV11 | 5.36  | 0.015   | Up     | 50S ribosomal protein L36 |
| A5IV16 | 1.97  | 0.001   | Up     | 50S ribosomal protein L30 |
| A7×6P6 | 0.64  | 0.034   | Down   | UDP-N-acetylmuramoyl-l-alanyl-d-glutamate-lysine ligase |
| A8Z216 | 2.13  | 0.017   | Up     | 50S ribosomal protein L33 2 |
| A2FGA1 | 2.00  | 0.009   | Up     | UPF0337 protein SAUSA300_1582 |
| A2G009 | 1.69  | 0.004   | Up     | Cold shock protein CspA |
| A2YXZ9 | 1.59  | 0.025   | Up     | Probable CtpA-like serine protease |
| A5HTFO | 1.58  | 0.005   | Up     | Transcriptional regulatory protein SrrA |
| A6G7C8 | 1.68  | 0.003   | Up     | Zinc-type alcohol dehydrogenase-like protein SAS2087 |
| A6GL7 | 0.64  | 0.004   | Down   | Hydroxyethylthiazole kinase |
| A7A078 | 1.55  | 0.016   | Up     | 50S ribosomal protein L23 |
terpinen-4-ol, linalool, β-myrcene, trans-sabinene hydrate, and β-caryophyllene (Leyva-Lopez et al., 2017), which was consistent with the chemical composition of OEO identified in this study. Carvacrol was present in the essential oil of O. vulgare subspecies: subsp. hirtum and subsp. gracile as the main components. These two subspecies were considered to be among the richest sources of EOs and carvacrol in the so-called oregano world (Emrahi et al., 2021). They suggested that under purposive or regulated water deficiency stresses, the subsp. hirtum yielded the higher EO content (70% higher than subsp. gracile), carvacrol content (20% higher), and dry material (15% higher) (Emrahi et al., 2021). Lambert et al. (2001) reported that the mixtures of carvacrol and thymol gave an additive effect, and the overall inhibition by the OEO could be attributed mainly to the additive antimicrobial action of these two compounds against Pseudomonas aeruginosa and S. aureus. Miladi et al. (2016) found that carvacrol and thymol served as the potential sources of the efflux pump inhibitor in foodborne pathogens. In addition, carvacrol can be regarded as an effective quorum-sensing inhibitor to combat the virulence and the biofilm formation of pathogenic bacteria on the surface of stainless steel (Tapia-Rodriguez et al., 2017). Therefore, exploring the antibacterial mechanism of carvacrol-rich OEO was of great significance for the further development of this natural product resource.

Previous reports confirmed the antimicrobial effect of OEO against foodborne pathogens and spoilage organisms, such as Bacillus coagulans, Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, Listeria monocytogenes, S. aureus, Salmonella typhimurium, and Alicyclobacillus spp. (Haberbeck et al., 2012; Gonçalves Cattelan et al., 2013; Dutra et al., 2019; Dogruyol et al., 2020; Das et al., 2021). These results suggested that the OEO had potential spectral antibacterial properties. Other studies suggested that the OEO exhibited the inhibition of multiple targets against drug-resistant strains and multi-drug-resistant strains, especially methicillin-resistant S. aureus (MRSA) (Nostro et al., 2004; Cui et al., 2019). Carvacrol at sub-inhibitory concentrations significantly inhibited the formation of monom- or dual-species biofilms by P. fluorescens and S. aureus by reducing quorum-sensing autoinducer-2 (Wang et al., 2020). Obviously, the OEO showed great advantages as an effective natural antibacterial agent to combat the antibiotic resistance and capacity for biofilm formation of foodborne pathogens.

The bacterial cell membrane is an essential active barrier structure between the cytoplasm and the extracellular medium that is important for maintaining energy transduction and cellular metabolism (Sanchez et al., 2010). Many natural agents that kill bacteria are mediated by a membrane damage mechanism. We studied the integrity of the cell membrane with the help of fluorescent probe labeling to further investigate the action mechanism of OEO on the cell membranes of S. aureus. PI is a fluorescent probe impermeable to cell membranes, while SYTO9 is a fluorescent probe permeable to cell membranes. The former binds to injured or dead cells and presents red fluorescence, while the latter binds to intact cells and presents green fluorescence. The two are often used in combination to assess bacterial cell membrane integrity and cell viability (Shi et al., 2016; Kang et al., 2020). Our results demonstrated that the OEO disrupted the

![FIGURE 5](https://example.com/figure5.png)
membrane integrity of *S. aureus*. Shi et al. (2016) reported that syringic acid caused cell membrane hyperpolarization and changes in the cellular morphology of *Cronobacter sakazakii*. Kang et al. (2019) reported that the peppermint essential oil damaged the cell membranes of *S. aureus*. These finds supported the integrity results of the cell membrane damage in present study.

Proteomics analysis is a powerful technique for the identification of DEPs. Here, the label-free proteomic analysis revealed that OEO-inhibited growth of *S. aureus* might be due to the disordered effect of protein synthesis and amino acid metabolism. Based on a KEGG pathway analysis, the ribosome (ko03010) pathway enriched the largest number of DEPs. Ribosomes are organelles playing a vital role in protein synthesis. Other studies also suggest that interference with the ribosome pathway is an important means for natural products to exhibit effective antibacterial effects (Kang et al., 2020, 2021). In addition, the expression of A0A380DJH0 (exotoxin 15) protein was significantly inhibited by OEO treatment. Staphylococcal food poisoning caused by the ingestion of staphylococcal enterotoxins produced by enterotoxigenic strains of *S. aureus* has become a serious concern worldwide (Hennekinne et al., 2012). Thus, the findings on the inhibition of exotoxins by the OEO were obviously exciting. The OEO exhibited multiple targets for the inhibition of *S. aureus* according to the number and functional distribution of DEPs. Hua et al. (2018) reported that the inhibition of ribosome formation was the main mechanism of aspidinol killing *S. aureus*; the inhibition of amino acid synthesis and the reduction of virulence factors played a secondary role. Disordering the amino acid metabolism was considered a main antibacterial mechanism of OEO against *S. aureus*. Tang et al. (2020) indicated that the *Amomum villosum* Lour. essential oil affected the carbohydrate and amino acid metabolism in MRSA. The *Blumea balsamifera* (L.) essential oil disordered amino acid metabolism, physiological function and inhibited the synthesis of nucleic acids and proteins, of *S. aureus* (Yang et al., 2021). Overall, the present study provided some exciting results regarding the potent bactericidal effect of a carvacrol-rich OEO and its possible mechanisms of action. The OEO showed great potential in causing metabolic disorders, especially affecting bacterial protein synthesis and amino acid metabolism.

Sarengaowa et al. (2019) found that the DEPs generated from *L. monocytogenes* treated without and with thyme essential oil were mainly related to cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, organismal systems. In accordance with the treated and untreated *Salmonella Enteritidis*, Barbosa et al. (2020) pointed out that the DEPs belonged to four distinct categories by GO (molecular function, biological process, cellular component, and protein class) and regulatory activity with greater change in expression in the *Oregano vulgare* essential oil, carvacrol, and thymol treatments. Tang et al. (2020) found that 48% of proteins were related to catalytic activity and 34% were related to binding; also, these DEPs were ranked from high to low and were related to the cellular process (32%), metabolic process (30%), and localization (18%), by the GO enrichment analysis of DEGs generated by control and *Amomum villosum* Lour. essential oil-treated MRSA groups. These results were similar to the result obtained in this study, indicating that the inhibitory effect of OEO on *S. aureus* might be closely related to the terms such as catalytic activity and binding.

**CONCLUSION**

In conclusion, a carvacrol-rich OEO extracted from *O. vulgare* “Hot & Spicy” demonstrated potent antimicrobial activity against *S. aureus*. The OEO appeared to some intuitive mechanisms of action: decreased maximal bacterial populations, decreased bacterial viability, and damage to the cell membrane. The label-free quantitative proteome analysis was used as a powerful technique to explore further the changes in the protein expression of *S. aureus* induced by the OEO treatment. Several identified DEPs were associated with antibacterial mechanisms related to interference on the pathway of ribosome and amino acid metabolism, which broadened our understanding of the molecular mechanisms underlying the response of *S. aureus* under the carvacrol-rich OEO stress. Future studies should investigate the bactericidal effect of the OEO on *S. aureus* in different food matrices and make it more applicable through nanotechnology.

**DATA AVAILABILITY STATEMENT**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028357 (http://www.ebi.ac.uk/pride; username: reviewer_pxd028357@ebi.ac.uk; password: uqrrfEud).

**AUTHOR CONTRIBUTIONS**

YH analyzed the data, wrote the manuscript, and performed the experiments. JL proofread the revised manuscripts. LS supervised the experiments and provided financial support of this study. All authors contributed to manuscript revision and approved the submitted version.

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