Study on inhibitory effect of cinnamaldehyde on Salmonella based on iTRAQ technology

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Abstract

Background

Salmonella is one of the most concerned pathogenic bacterium worldwide. In our previous experiments, cinnamaldehyde showed prominent antibacterial effects on Salmonella Typhimurium (S. Typhimurium), indicating that it could be developed as a novel therapeutic drugs for Salmonella.

Results

In this assay, the mechanism of cinnamaldehyde inhibiting S. Typhimurium was investigated. We studied the antibacterial mechanism of cinnamaldehyde using isobaric tags for relative and absolute quantification (iTRAQ) with two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS), combining with bioinformatics. There were 2,212 proteins detected by iTRAQ, of which 73 proteins were up-regulated, and 82 of proteins were down-regulated. The differently expressed proteins were connected with 10 cellular components, 9 molecular functions and 13 biological processes as well as 57 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. There were some differently expressed proteins involved into biosynthesis and metabolism of amino, redox reaction, energy metabolism, toxins and drug-resistance. Cinnamaldehyde may have the potential to become a novel drug for Salmonella infection.

Conclusions

Based on the iTRAQ technology, this paper explores the mechanism of cinnamaldehyde on Salmonella typhimurium at the protein level, digs the target of action, lays a theoretical foundation for further exploration, and provides new ideas for the clinical use and development of cinnamaldehyde.Therefore, cinnamaldehyde may have the potential to be used combined with antibiotics.

Background

Salmonella is a significant pathogenic bacterium, which can be related with septicemia, gastroenteritis and local infection both in human and animals[1]. As the most prevalent source of food poisoning, Salmonella could contaminate multiple foods, such as raw meat, eggs, raw milk, water and vegetables. People mainly were infected by eating contaminated foods. In the United States, Salmonella infection is a leading cause of bacteria foodborne disease outbreaks, with an estimated 1 million infections occurring annually[2]. In France, Salmonella was also a major cause of foodborne pathogen−associated deaths, ranked first in 2008−2013[3]. It was reported that there were 88715 bromatoxism cases in 28 members of the European Union during the past 15 years[4]. There were 1,300 million people suffering severe Salmonella poisoning and 3 million died worldwide[5]. Salmonella can induce abortion of pregnant female animals[6]. Besides, hens with bacteria in the body would lay contaminated eggs, which would hatch into infected chicks[7]. There are more than 2,500 serotypes of Salmonella in the world[8]. Salmonella infections have made great economic loss and treatment burden in the world. To treat

Salmonella infections, antibiotics are generally used in modern clinical treatment. However, with the overuse of antibiotics, bacteria had developed resistance against almost all kind of drugs[9]. Therefore, new therapeutic drugs are urgently needed for clinical treatment.

Cinnamaldehyde, an aldehyde compound, exists in spice plant cinnamon. Cinnamon essential oil showed good antibacterial effect in food preservation[10]. It was reported that cinnamon essential oil extended the shelf-life of vacuum-packaged common carp fillets by about 2 days, and decreased the relative abundance of Macrococcus compared with control samples[11]. Solart. et al found cinnamon essential oil could efficiently decrease the MIC of enrofloxacin from 2 to 0.031 μg/mL[12]. Gadotti and colleagues found that cinnamaldehyde treatment can reduce Salmonella populations in queso fresco by inhibiting Salmonella growth[13]. Cinnamaldehyde is the major compound in cinnamon essential oil, approximately 60%-70%[14]. Cinnamaldehyde is extensively used in the fields of medicine, food and cosmetics, and has properties of anti-inflammation, anti-oxidant, anti-bacteria and lowering blood pressure[15, 16]. Hancı et al. found that cinnamaldehyde alleviated vascular congestion, and reduced plasma cell, eosinophil, as well as inflammatory cell infiltration in rat allergic rhinitis model[17]. It was reported that cinnamaldehyde could exhibit antimicrobial properties in vitro against yeasts and bacteria, such as filamentous molds, Clostridium perfringens, Escherichia coli, Listeria monocytogenes, Salmonella enterica and Bacillus cereus[18, 19]. After fumigation with trans-cinnamaldehyde, the Salmonella enteritidis on embryonated egg shells was detected negative, indicating the potential of becoming an effective fumigation treatment for eggs[20]. Cinnamaldehyde has a good antibacterial effect. However, the antibacterial mechanism and targets of cinnamaldehyde are still unclear.

Proteomic techniques, such as isobaric tags for relative and absolute quantification (iTRAQ), liquid chromatography tandem mass spectrometry (LC-MS/MS), and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), have paved new pathways toward finding drug mechanism, exploring disease process, evaluating toxicological effect of drug, and discovering new action site. Currently, studies have been conducted on essential oils and their constituents on bacteria by proteomic methods. It was reported that 1, 8-cineole changed energy metabolism of Salmonella both in transcription level and translation level. This result may indicate the potential of 1,8-cineole of becoming a novel therapeutic drugs for Salmonella infections[21]. Xu and colleagues studied the potential antifungal mechanism of tea tree oil by means of proteomics[22].They demonstrated that tea tree oil inhibited glycolysis, disrupted the TCA cycle, and induced mitochondrial dysfunction that disrupted energy metabolism of Botrytis cinerea. Therefore, proteomics technology provides a new research direction for the antibacterial mechanism of essential oils.

In this assay, we explored the mechanism of cinnamaldehyde against Salmonella using iTRAQ technology. And differently expressed proteins between control group and drug-treated group were analyzed to find relative pathways and functions according to bioinformatics.

Results

The global proteomic profile of Salmonella

The MIC of cinnamaldehyde against S. Typhimurium was 128 μg/mL, and the MBC was 512 μg/mL. Cinnamaldehyde was used to observe the effect in iTRAQ analysis at a concentration of 64 μg/mL. There were 2,212 proteins detected by iTRAQ, and 2,057 of them had no remarkable difference on expressional level. 73 of them had an up-regulated expression while 82 of them had a down-regulated expression.

GO function analysis of proteins

All proteins detected by iTRAQ analysis were annotated to three function classifications (GO) as followed: biological process (BP), cellular component (CC) and molecular function (MF). The data was shown in Fig. 1. The proteins were annotated to 13 BP terms, 10 CC terms and 9 MF terms. Differently expressed proteins were also annotated to three function classifications (GO). GO enrichment bubble diagram was shown in Fig. 2. In these data, most of differently expressed proteins were classified to several terms, such as cellular process and metabolic process in BP, cell and cell part in CC, and binding and catalytic activity in MF.

KEGG pathway analysis of differently expressed proteins

As shown in Fig. 3, there were 73 up-regulated and 68 down-regulated proteins, and all differently expressed proteins were divided into 57 KEGG pathways. In Fig. 4, proteins were enriched to 20 KEGG pathway terms. The up- or down-regulated proteins were mainly connected with two-component system, ABC transporters, amino acids, pantothenate and CoA biosynthesis. Other pathways were not significantly affected by cinnamaldehyde: cationic antimicrobial peptide (CAMP) resistance, 2-oxocarboxylic, carbon, glycine, alanine, aspartate glutamate serine and threonine metabolism, valine, leucine and isoleucine biosynthesis, bacterial chemotaxis, for example.

Cinnamaldehyde effected toxins transcription

The transcription of toxins in Salmonella co-cultured with cinnamaldehyde was quantified using real-time PCR. The results of cinnamaldehyde against toxins were shown in Fig. 5. After co-cultured with cinnamaldehyde, transcription levels of sipA, prgI and fimW were up-regulated, while levels of invB, fimA and CR079_01855 were attenuated.

Discussion

Cinnamaldehyde has been proved that is an antimicrobial agent. Previous study in our laboratory indicated that cinnamaldehyde had inhibitory effect on proteins synthesis of Salmonella at a concentration of 64 μg/mL (data not shown) by SDS-PAGE. Therefore we applied a proteomic approach to detect worldwide proteins in S. Typhimurium. And results showed cinnamaldehyde undoubtedly affect multiple functions of S. Typhimurium.

Compared with control group, biosynthesis and metabolism process of amino were obviously influenced by cinnamaldehyde (Table 1, Fig. 6 and Fig. 7). It was reported that branched-chain amino acids (BCAAs) were considered as an indicator of general nutritional status of the bacteria, while Leucine, isoleucine and valine are essential elements of BCAAs[23, 24]. Acetolactate synthase is necessary for biosynthesis of isoleucine and valine, and Salmonella strains lacking acetolactate synthase requires an additional supplement of isoleucine and valine to grow properly in culture[25]. CR079_08520 and ilvH, acetolactate synthase regulatory subunit I and III, were down-regulated co-cultured with cinnamaldehyde. ilvD and ilvC mediates the synthesis of BCAAs in Salmonella, and the latter promotes the synthesis of coenzyme A[26]. rpiA plays a significant role in energy carbohydrate anabolism and catabolism[27]. RpiA interconverts ribose-5-phosphate and ribulose-5-phosohate. In this assay, repressed rpiA would affect the energy metabolism level of the bacteria. carB regulates the synthesis of large chains of aminoacylphosphatase, which catalyzes the synthesis of carbamyl phosphate, a necessary precursor for the synthesis of arginine and pyrimidine[28]. In arginine synthesis pathway, carbamyl phosphate and ornithine are synthesized to obtain citrulline, which is converted to arginine by enzymatic reaction[29]. DD95_13265 is an arginine decarboxylase, which mediates the synthesis of arginine. It is generally believed that Salmonella cannot tolerate an acid condition of pH 2.5, but the arginine-dependent acid-resistance mechanism activated under hypoxia conditions allows Salmonella to survive for a long time at pH2.5[30]. Lacking BCAAs may repress biosynthesis and metabolism of Salmonella, therefore may reduce resistance against adverse environment, for example, antibiotics. In this study, it was found that cinnamaldehyde can affect the synthesis and metabolism of various amino acids, which may be one of the targets of cinnamaldehyde inhibiting bacteria.

Table 1

differently expressed proteins related with metabolism of amino acids

Accession name	Gene name	Function	Fold change	P value
A0A0M2J4K4	asnB	L-asparaginase 2	1.383	0.002
A0A0J6GMN8	aspA	Aspartate ammonia-lyase	1.211	0.039
A0A0D6HJJ8	carB	Carbamoyl-phosphate synthase large chain	0.764	0.005
A0A0D6I697	CR079_19530	Dihydroxy-acid dehydratase	1.263	0.004
A0A0D6GT32	serC	Phosphoserine aminotransferase	1.241	0.009
A0A0D6FTK7	CR079_24500	L-serine ammonia-lyase	1.204	0.011
A0A0D6HM19	thrA	Bifunctional aspartokinase/homoserine dehydrogenase	0.695	0.013
A0A0M2J5W4	dsdA	D-serine dehydratase	0.540	0.0000227
A0A0J6DT19	ilvH	Acetolactate synthase 3 regulatory subunit	0.719	0.004
A0A0J6DTT7	CR079_08520	Acetolactate synthase 1 regulatory subunit	0.718	0.029
A0A0M2IUW2	DD95_13265	Arginine decarboxylase	0.522	0.006
A0A0D6IQZ8	katG	Catalase-peroxidase	1.400	0.009

Differently expressed proteins related with redox reaction were shown in Table 2. Iron-containing sulfur (Fe-S) proteins contribute to a variety of biological processes, including redox reactions or regulation of gene expression, and are key metal cofactors for cellular function. Besides, Fe-S protein IlvD is confirmed as active nitrogen (NO) target. In E. coli, IlvD interacts with NO, forming the dinitrosyl iron complex (DNIC), and inducing BCAA auxotrophy[31]. After cinnamaldehyde treatment, Fe-S protein assemble systems obviously declined including CR079_14145、IscU and sufE. IscU is the core component of iron sulfur cluster in the Fe-S proteins assembling process. During invasion into epithelial cells, wild-type Salmonella is much more efficiently compared with iscU mutant[32]. In E. coli, SufE binds with SurS to stimulate cysteine desulfurase, and accepts sulfane transferred from SufS[33, 34]. Therefore, cinnamaldehyde may have the property of inducing BCAA auxotrophy and NO damage against Salmonella.

Table 2

Information of differently expressed proteins relative to redox reaction

Accession name	Gene name	Function	Fold change	P value
A0A0W5XM88	DD95_14245	Dimethyl sulfoxide reductase	0.723	0.029
A0A0D6HUU0	DD95_13310	Dimethyl sulfoxide reductase	0.713	0.047
A0A0D6GTP6	CR079_22995	Dimethyl sulfoxide reductase	0.688	0.048
A0A0D6GC40	sufE	Cysteine desulfuration protein	0.811	0.030
A0A0L9JQ40	IscU	Iron-sulfur cluster assembly scaffold protein	0.787	0.020
A0A0D6GAI2	CR079_14145	Cysteine desulfurase	0.780	0.047
A0A0M2IZP2	grxB	Glutaredoxin	0.704	0.0001
A0A0D6IN33	wecF	TDP-N-acetylfucosamine:lipid II N-acetyl fucosaminyltransferase	0.697	0.010
A0A0D6I4S1	CR079_08080	LPS 1,2-N-acetylglucosaminetransferase	0.713	0.042
A0A0K6RDY0	CR079_21580	Trehalose-6-phosphate synthase	0.816	0.030
A0A0D6FU80	CR079_24700	Hydrogenase	0.600	0.007
A0A0D6FUX2	CR079_24705	Hydrogenase	0.535	0.009
A0A0D6IM00	CR079_24200	Trimethylamine N-oxide reductase I catalytic subunit	2.228	0.0001
A0A0J5H1R6	CR079_10450	Cytochrome b562	1.331	0.012
A0A0D6IKN9	CR079_24195	Cytochrome c-type protein	2.499	0.010

There are several differently expressed proteins related with energy metabolism (Table 3). In Escherichia coli rpiA is an essential enzyme in the first step of pentose phosphate pathway (PPP), catalyzing the reversible conversion of D-ribose-5-phosphate to D-ribulose-5-phosphate[35]. Meanwhile rpe was reduced to 0.409 (fold change), which catalyzed the reversible conversion of D-ribulose-5-phosphate to D-xylulose-5-phosphate. Rpe-mutunt E. coli lost the capacity of utilizing single pentose sugars, and showed limited growth in complex LB medium, suggesting the importance of rpe in PPP[36]. These results suggested that cinnamaldehyde affected the energy metabolism of Salmonella.

Table 3

Information of differently expressed proteins relative to energy metabolism

Accession name	Gene name	Function	Fold change	P value
A0A0J0XAS3	rpiA	Ribose-5-phosphate isomerase A	1.242	0.017
A0A0D6I769	rpe	Ribulose-phosphate 3-epimerase	0.409	0.011
A0A0D6I3W9	dlgD	2,3-diketo-L-gulonate reductase	1.303	0.020
A0A0L9GCU5	acpP	Acyl carrier protein	1.307	0.007

Until now, the clinical treatment strategy for bacterial infections has been antibiotics, but the abuse of antibiotics has accelerated bacterial resistance. Drug-resistance has become a global problem in recent years. In our results, we found some drug-resistance proteins (Table 4) were down-regulated compared with control group. arnA mediated polymyxin resistance by catalyzing UDP-glucuronic acid to pentose sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N), which can interact with lipid A to reduce the magnetism between lipid A and polymyxin. D-alanyl-D-alanine dipeptidase pcgL catalyzes the hydrolysis of D-Ala-D-Ala, essential for resistance to vancomycin[37]. In Mycobacterium abscessus, D-alanyl-D-alanine dipeptidase MAB1843 potentially stimulated the DC maturation via toll-like receptor 4 to regulate host immune response[38]. blaSHV-12 can be found in a wide variety of bacteria, such as Salmonella, escherichia coli and enterobacter cloacae[39]. Beta-lactamase blaSHV-12, mediating resistance to beta-lactam drug, was reduced to 0.771 (fold change) by cinnamaldehyde. Drug resistance has been a great problem worldwide, but the resistance may be reversed with the application of cinnamaldehyde.

Table 4

differently expressed proteins relative to drug-resistance

Accession name	Gene name	Function	Fold change	P value
A0A0D6FBV2	arnA	Bifunctional polymyxin resistance protein	0.810	0.031
C4NZW3	blaSHV-12	Beta-lactamase	0.771	0.003
A0A2D0MNI5	OppA	Oligopeptide ABC transporter substrate-binding protein	1.231	0.007
Q9 × 523	pcgL	D-alanyl-D-alanine dipeptidase	0.754	0.006
A0A0D6IPG0	CR079_25305	Thiol:disulfide interchange protein	1.258	0.025
A0A0J5IUL5	CR079_20285	Transcriptional regulator	0.829	0.030
A0A0M2IQW1	CR080_14695	Copper homeostasis protein	0.677	0.008

Type I fimbria (Table 5) is one of the down-regulated cell parts. It was reported that fimbria is extracellular structure, and plays a crucial role in adhesion. Anna et al. demonstrated that type I fimbriae could adhere

to host epithelial cells, and regulate biofilm formation according to fimH gene[40]. It was found that type I fimbria of S. enterica could bind to host membrane plasminogen and activate plasminogen to plasmin[41]. Type I fimbria is a vital component in early infection stage for binding to epithelial cells and invading into host.

Table 5
differently expressed proteins related to toxins

Accession name	Gene name	Function	Fold change	p. value
A0A0C5PU36	invB	SPI-1molecular chaperone	0.66	0.03
A0A0C5PPT0	sipA	SPI-1effector	0.61	0.0002
A0A0C5Q2B9	prgI	SPI-1needle protein	0.55	0.001
A0A0D6H7T1	fimA	Fim subunit	0.80	0.02
A0A0M2IX42	CR079_01855	Fim subunit	0.72	0.01
A0A0D6H693	fimW	Fim regulator	0.73	0.02

Type III secretion system (Table 5) is another down-regulated system. Under the stress of cinnamaldehyde, needle structure protein and effector proteins were decreased in protein level. Needle protein PrgI plays a significant role in delivery toxins into host cells. PrgI is not only the needle structure protein, also can induce host inflammation in gut. It was reported that YscF could activate NAIP inflammasome in human macrophages[42]. InvB is defined as chaperone protein of SipA, and mediate the translocation of SipA from bacteria to host cells. InvB-mutant strain can only secret less than 50% of SipA compared with wild type[43]. SipA is one of effectors of SPI-1, which could induce membrane ruffling of host cell and have pro-inflammatory activity[44]. After endocytosis, SipA can also activate caspase-3 to promote infection in early phase. As a conclusion, cinnamaldehyde may have the property of inhibiting SPI-1 of Salmonella[45]. From the above results, we may come to a conclusion that cinnamaldehyde could attenuate the pathogenicity of S. typhimurium. In RT-PCR assay, however, the transcription levels of toxic proteins were up-regulated. This may because translational level is influenced negatively, or protein degradation is accelerated.

Conclusion

In this study, we detected the effect of cinnamaldehyde against S. typhimurium at a concentration of 64 μg/mL using iTRAQ technology and proteomic analysis. After co-cultured with cinnamaldehyde, biosynthesis and metabolisvm of amino of Salmonella was seriously disrupted especially BCAAs, inducing BCAA auxotrophy, and then the resistance against active nitrogen was damaged. Redox reaction of S. typhimurium was also inhibited. Moreover, pentose phosphate pathway was affected via down-regulated rpe. And some drug-resistance proteins as well as toxins were suppressed by cinnamaldehyde.

Therefore, cinnamaldehyde may have the potential to be used combined with antibiotics. However, further studies are needed to ascertain the mechanism of cinnamaldehyde at different targets.

Methods

Bacterial strain and growth conditions

Salmonella enterica serotype Typhimurium (ATCC14028) was purchased from the American type culture collection (ATCC). The bacteria were cultured overnight at 37 °C in TSB broth medium with gentle shaking. Then bacteria cultures were diluted to OD_{600} = 0.3 for standby. Cinnamaldehyde (Fig. 1, purity ≥ 99.5%) was purchased from the Chengdu herbpurify CO., LTD (Chengdu, China). The compound was diluted in dimethyl sulfoxide (DMSO, ≥ 99.5%; Sigma) to get a drug stockpile (40.96 mg/mL).

Susceptibility testing

The minimum inhibitory concentration (MIC) of cinnamaldehyde was confirmed by microdilution method in 96-well polystyrene plates[46]. The MIC was determined as the lowest concentration of cinnamaldehyde with which bacterial growth was inhibited[47].

Proteomic sample preparation

Bacteria (OD_{600} = 0.3) were cultured with or without cinnamaldehyde (1/2 MIC) at 37 °C for 6 h until logarithmic phase with gentle shaken (150r/min). Bacteria cultures were centrifuged to gather bacterial pellets, and the pellets were washed with PBS (0.1M, pH 7.4) for three times to remove residual culture. Then pellets were re-suspended in buffer solution containing 1% dithiothreitol (DTT) and a protease inhibitor. The cells were broken using sonic oscillator (Biosafer150-93, Chengdu, China) at 20 °C for total protein extraction. After cell breaken, the mixture was centrifuged at 6,000 r/min for 10 min at 4 °C. The supernatant was harvested for iTRAQ analysis. Each group was repeated for three times.

Enzymolysis and peptide fragment labeling

400 μg of each sample was taken for enzymolysis with 100 mM/L DTT, and then boiled for 5 min. The cooling samples were separated by several of centrifugal velocities, and divided to different length of peptides. Peptide fragment labeling was conducted according to iTRAQ Reagent-6plex Multiplex Kit (AB SCIEX, USA).Samples were dissolved in labeling reagent containing 50 μL isopropanol at room temperature for 1 h. All labeled peptides were pre-classified by AKTA Purifier 100 (GE Healthcare), then freeze-dried and desalted (66872-U sigma).

Protein Identification 2D-LC-MSMS

Labeled samples were re-suspended with HPLC buffer A (98% H_2O, pH10) and loaded. Then samples were washed by buffer B (98% acetonitrile, pH10) at a flow rate of 700 μL/min, and the elution gradient was shown in Table 6. The process was conducted with the monitoring of absorbance value (OD_{214nm}).

And the elution products were collected every 1.5 min starting with the fifth minute. After elution, products were vacuum freeze-dried, re-dissolved with 0.5% formic acid (FA), and combined into several products.

Table 6
the high pH reversed-phase chromatography gradient during elution

Time (min)	A (98% H_2O, pH10)	B (98%ACN, pH10)	Flow speed (µL/min)
3	97%	3%	700
5.1	97%	0%	700
10	95%	5%	700
35	82%	18%	700
45	66%	34%	700
53	5%	95%	700
58	5%	95%	700

Each product was separated using NanoDrop 2000C (Thermo Scientific, China). Chromatographic column was balanced by 95% buffer A (0.1%FA, H_2O) in advance. Samples were loaded into column and washed through analytical column. Liquid phase and flow speed were shown in Table 7.

Table 7
the gradient of liquid Chromatogram during elution

Time (min)	A (0.1%FA, H_2O)	B (0.08%FA, 80%ACN)	Flow speed (nL/min)
0	93%	7%	600
14	87%	13%	600
51	77%	23%	600
68	64%	36%	600
69	0%	100%	600
75	0%	100%	600

Q-Exactive mass spectrograph (Thermo Finnigan) was used to perform the LC-MS/MS assay and analyze the results. The mass spectrometer was set to positive ion mode. Sweep range was from 300 to 1800 m/z. automatic gain control (AGC) target was settled to 3e6 with 10 ms of maximum inject time, and Dynamic exclusion duration was settled to 40.0 s. number of scan ranges was 1, and the resolution of mass spectra survey scans is 70,000 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

LC-MSMS date analysis

The results were handled and defined by Mascot 2.2 and Proteome Discoverer 1.4 according to the uniprot. All dates were screened with at least 95% confidence for analysis of protein samples. The ionic peak strength of the peptides and proteins was extracted, and the quantitative values were normalized. Differential expression proteins were defined as those with > 1.2 (up-regulation) or 0.8 (down-regulation) fold change and with P < 0.05.

Proteomic data analyses

The protein information was identified according to UniProt online database (http://www.uniprot.org/). Gene Ontology GO was used to define the function of proteins as biological process, cellular component or molecular function. The metabolic pathways or signal paths which proteins participate were analyzed according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/). Differentially expressed proteins were mapped to certain terms in Gene Ontology database (http://www.geneontology.org/) and were analyzed by hypergeometric test ($P=1-\sum_{i=0}^{m-1}\frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$). N is the number of all proteins which were annotated in GO; n is the number of differently expressed proteins in N; M is the number of all proteins which were annotated to certain term; m is the number of differently expressed proteins in certain term. Pathway enrichment analysis was conducted the same as GO enrichment. Enrichment analysis were used to decide the most significant GO or KEGG pathway of differently expressed proteins.

Real-time quantitive PCR

The expression levels of differently expressed virulence factors were detected via real-time quantitive PCR. Salmonella was grown with or without cinnamaldehyde for 6 h. Total RNA was collected according to the instruction of RNAprep Pure Cell/Bacteria Kit. And total RNA was reversely transcribed according to the instruction of Primix ScriptTM RT reagent Kit with gDNA Eraser in 20 μL volume. PCR was carried out using SYBR® Premix Ex Taq™ II in a Bio-Rad CFX Connect™ Real-Time PCR System[48]. All experiments were repeated three times. The primer sequences of target genes and housekeeping gene 16 s RNA are shown in Table 8.

Table 8
primer sequences with their corresponding PCR product length

Gene	Primer sequence(5'-3')	Tm(℃)	Product length
16 s-F	AGAGTTTGATCCTGGCTCAG	56	1506 bp
16 s-R	TACGGCTACCTTGTTACGACTT		
fimA-F	TGACCTCTACTATTGCGAGTCTG	57	526 bp
fimA-R	ATAAAGGTGGCGTCGGCATTAG		
fimW-F	CATCATGACGCGTCTGGCGAATC	60	462 bp
fimW-R	ATAATGCTCCGGCGGAGTGAC		
invB-F	TCATCTCATTAGCGACCGAC	56	383 bp
invB-R	TAGTTCGTTCCGCACTGGAAG		
prgI-F	CAACACCTTGGTCAGGCTATC	55	141 bp
prgI-R	TCTGATACGCCGCCAGTAG		
sipA-F	TTCAAATAATGTCGCCGGTA	55	108 bp
sipA-R	TTCATCAGTAGCGTCTTCGC		
CR079_01855-F	CGCAACGCTGGAACAACA	56	307 bp
CR079_01855-R	CCGCTGGAAGAGGTAGGATTA		

Statistics

The results of real-time PCR were analyzed using GraphPad Prism6.0 for significance. $P < 0.05$ was considered significant, and $P < 0.01$ was considered extremely significant.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Abbreviations

AGC: Automatic gain control

ATCC: American type culture collection

BCAAs: Branched-chain amino acids

BP: Biological process

CAMP: Cationic antimicrobial peptide

CC: Cellular component

DMSO: Dimethyl sulfoxide

DNIC: Dinitrosyl iron complex

DTT: Dithiothreitol

Fe-S: Iron-containing sulfur

GO: Gene Ontology

iTRAQ: Isobaric tags for relative and absolute quantification

KEGG: Kyoto Encyclopedia of Genes and Genomes

L-Ara4N: 4-amino-4-deoxy-L-arabinose

LC-MS/MS: Liquid chromatography tandem mass spectrometry

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight

MF: Molecular function

MIC: Minimum inhibitory concentration

NO: Nitrogen

PCR: Polymerase Chain Reaction

PPP: Pentose phosphate pathway

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Contributions

Conceptualization, Y.L., H.X and C.H.; methodology, Y.Z.; software, F.H.; validation, L.J., G.Y., H.C and S.G.;

formal analysis, L.X.; data curation, S.X.; visualization, L.L.; supervision, Z.Y.; project administration, O.P.; funding acquisition, Y.L.All of the authors reviewed the manuscript.

Ethics approval and consent to participate

Consent for publication

Competing interests

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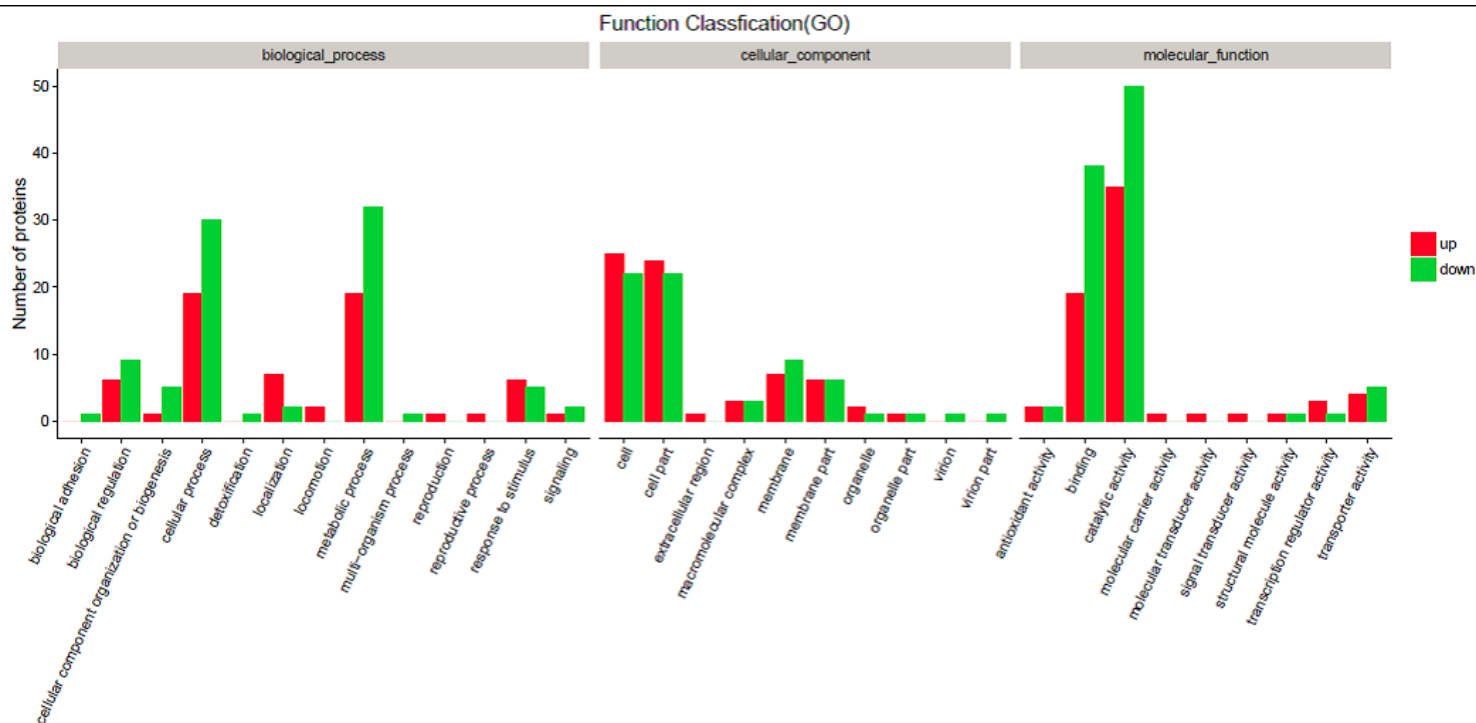
Figures
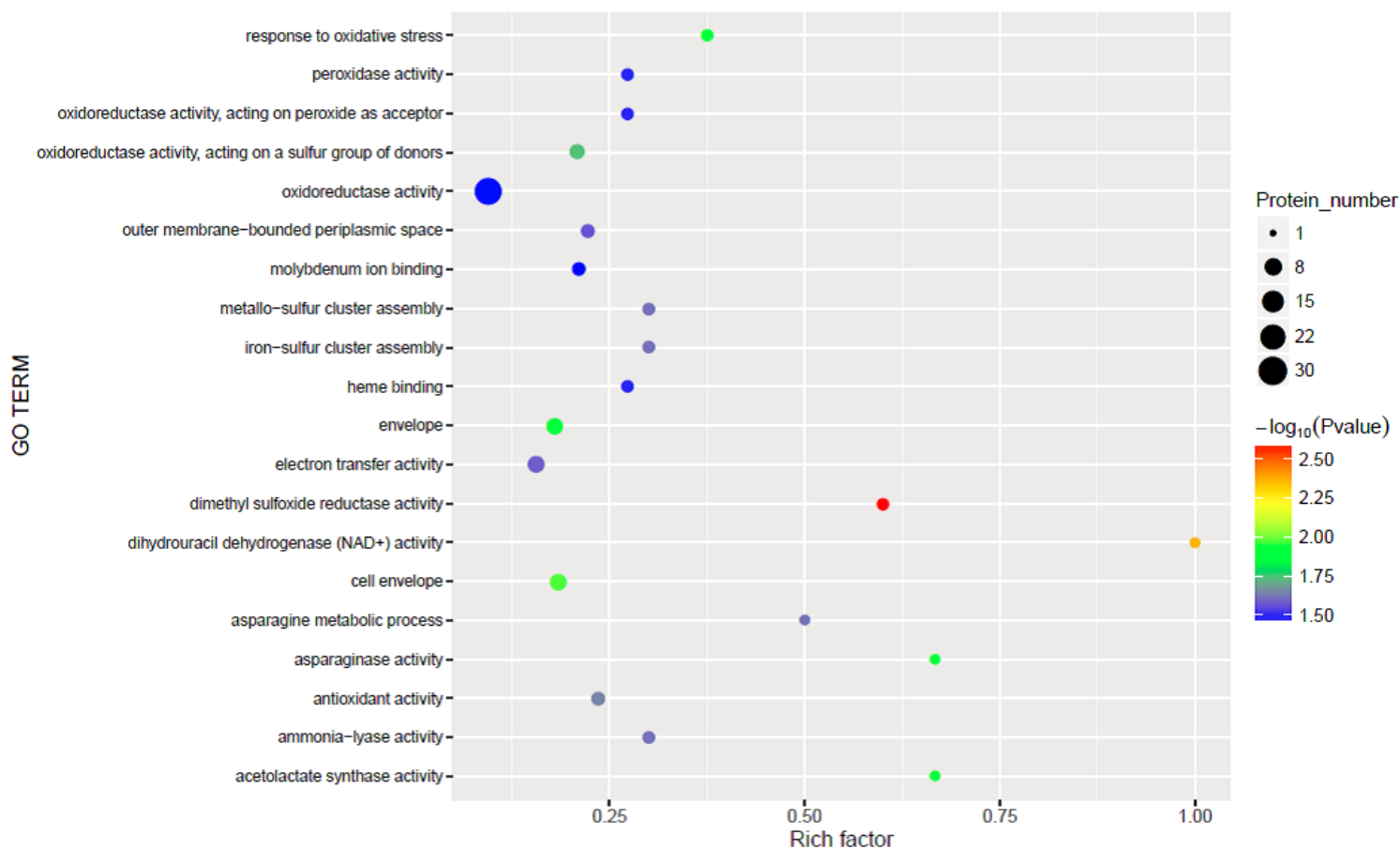
Figure 1

GO analysis of differently expressed proteins

Figure 2

GO enrichment bubble diagram of differently expressed proteins
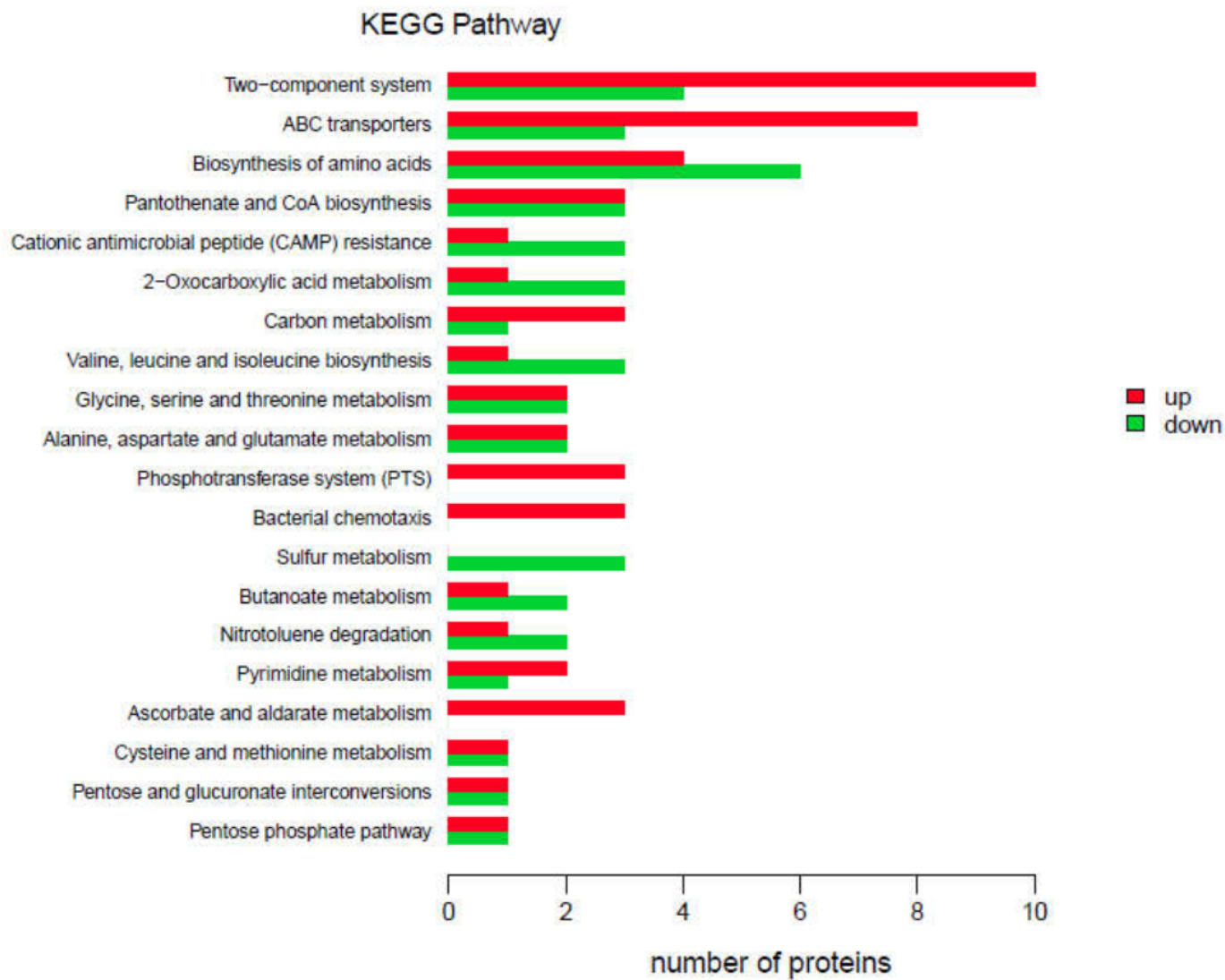

KEGG Pathway

Figure 3

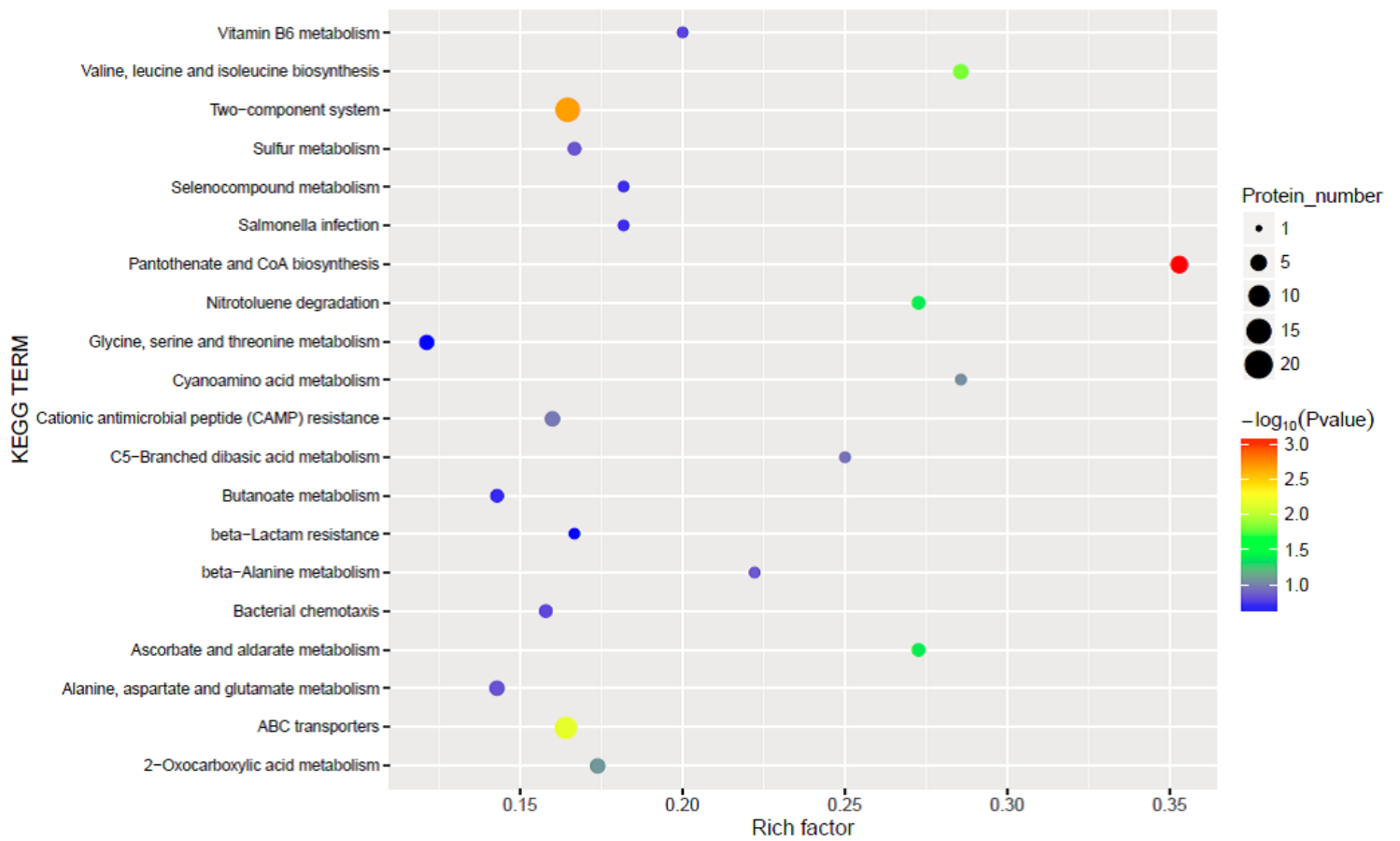KEGG pathway analysis of differently expressed proteins

Figure 4

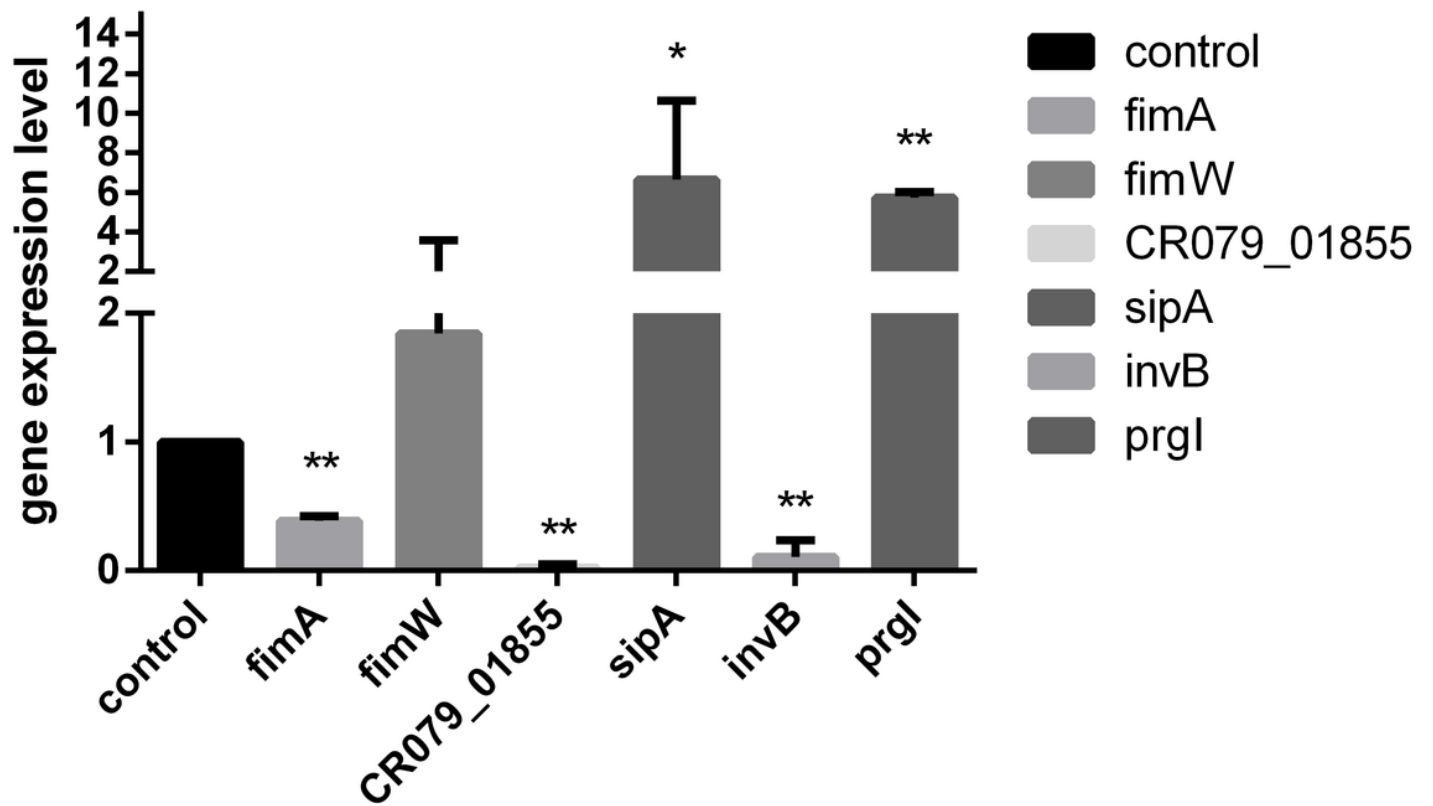KEGG pathway enrichment bubble diagram of differently expressed proteins

Figure 5

Gene transcription of differently expressed toxins of salmonella treated with cinnamaldehyde
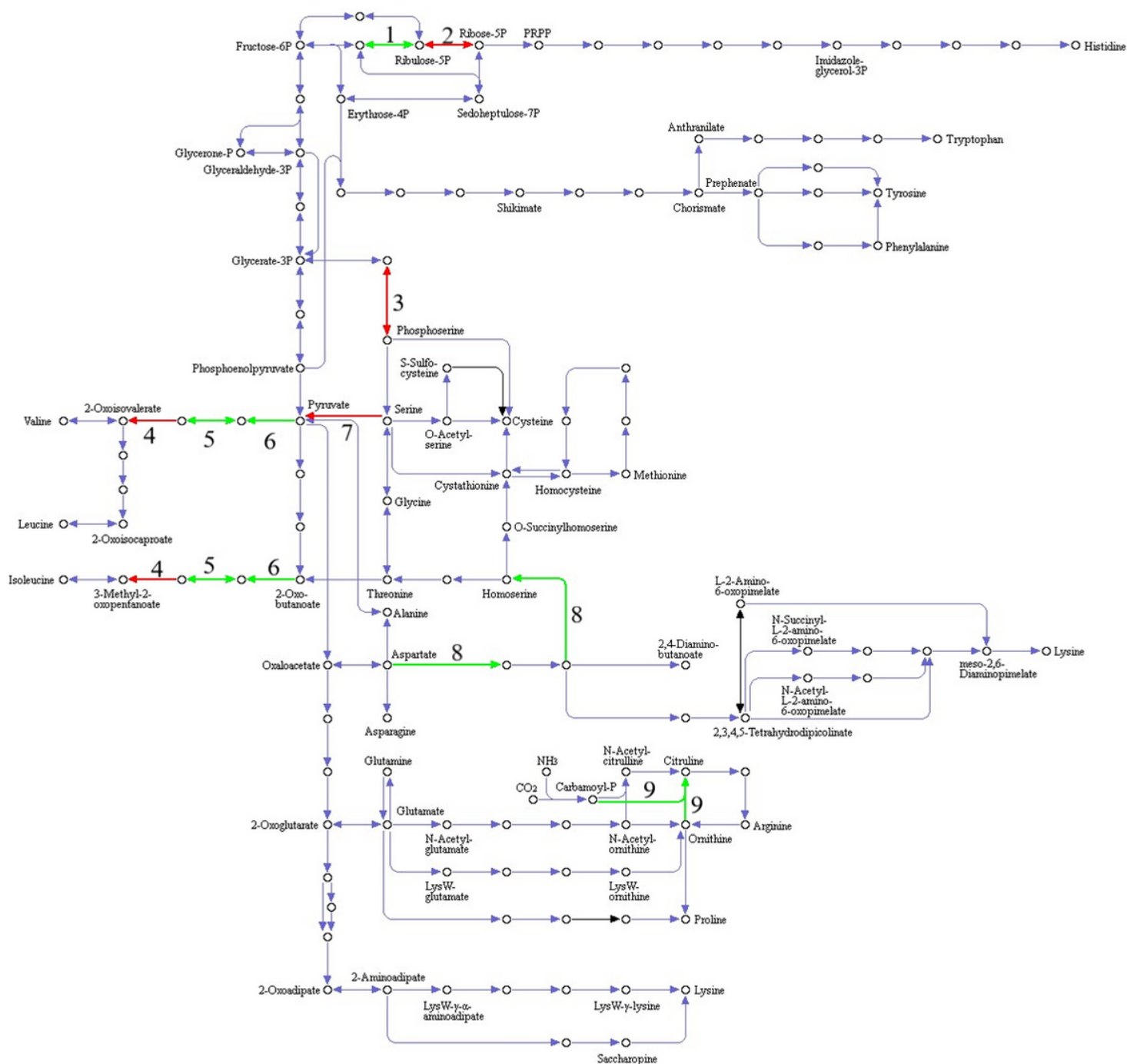
Figure 6

Biosynthesis of amino acids Note: 1, rpe; 2, rpiA; 3, serC; 4, ilvD; 5, ilvC; 6, CR079_08520, ilvH; 7, CR079_24500; 8, thrA; 9, argF; red means up-regulated proteins, green means down-regulated proteins
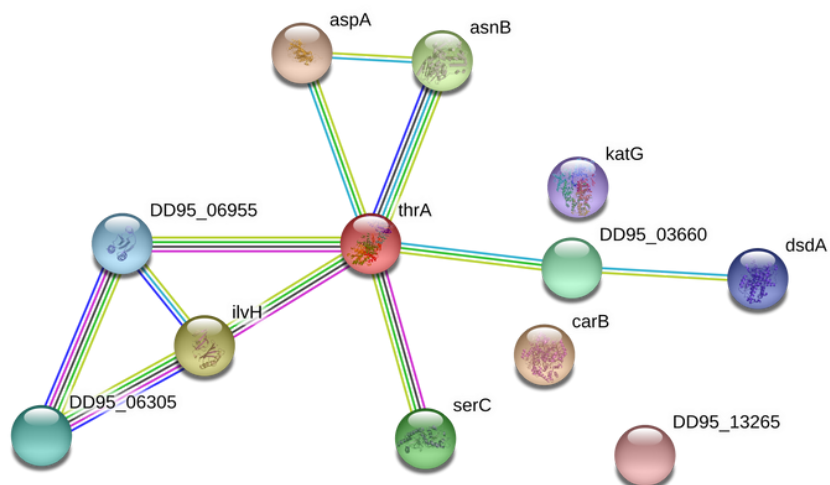
Figure 7

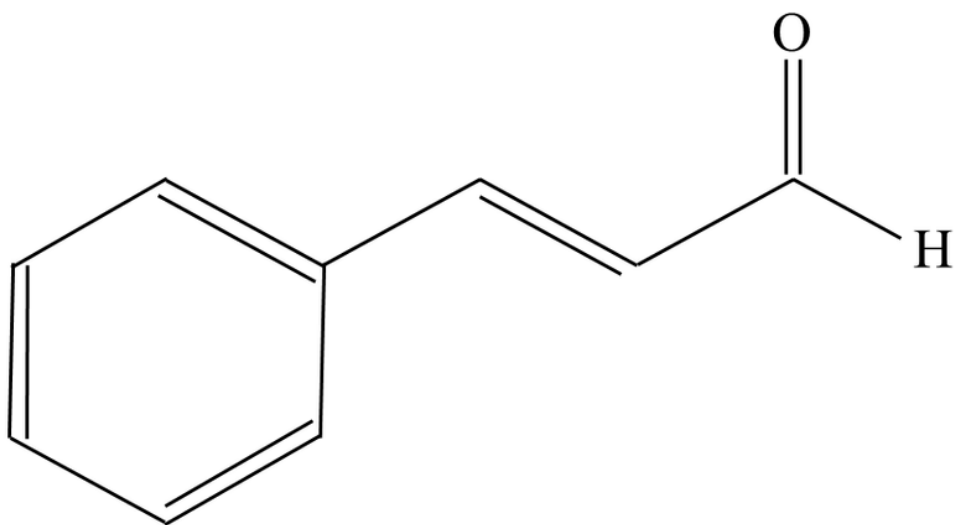protein-protein interaction networks for amino biosynthesis and metabolism

Figure 8

Chemical structure of cinnamaldehyde (CAS No. 104-55-2)