

Integration of Metabolomics and Transcriptomics Indicates Changes in MRSA Exposed to Terpinen-4-ol

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Abstract

Background: This study investigated the effects of terpinen-4-ol on *methicillin-resistant Staphylococcus aureus* (MRSA) and its biofilm, and the possible mechanisms governing this effect.

Results: We observed that terpinen-4-ol has good antibacterial activity and inhibits the formation of MRSA biofilm. The MIC and MBC values for terpinen-4-ol against *S. aureus* were 0.08%~0.32%. And terpinen-4-ol at 0.32% could kill all bacteria and clear all biofilms. Untargeted metabolomic and transcriptomic analyses showed that terpinen-4-ol strongly inhibited DNA and RNA biosynthesis in MRSA at 2 h after treatment by affecting genes and metabolites related to purine and pyrimidine metabolic pathways. Some differential genes which play important roles in DNA synthesis and the production of eDNA from biofilm exposed to terpinen-4-ol was also significantly decreased compared with that of the control.

Conclusions: Terpinen-4-ol has good antibacterial activity and significantly inhibits the formation of MRSA biofilm by inhibiting purine and pyrimidine metabolism.

1 Background

In recent years, essential oils have become widely used in the biomedical, cosmetics and food industries and other fields due to their broad antibacterial activity and few side effects[1]. Numerous essential oils can not only effectively inhibit the growth of microorganisms but also clear microbial biofilms; such as TTO[2], litsea cubeba oil[3], oregano oil[4] and clove oil[5]. TTO is a colorless to light yellow liquid extracted from the fresh branches and leaves of *Melaleuca alterniflora* in Australia by steam distillation, with the smell of camphor and cooling sensation of menthol[6]. TTO is known for its antiseptic, antimicrobial, and anti-inflammatory properties[7].

Terpinen-4-ol was considered to be the main active ingredient of TTO[6], and ISO 4730-2017 stipulates that the content of terpinen-4-ol should not be less than 30%[8]. But there were few studies about the strong antibiofilm activity[9] of terpinen-4-ol, and its mechanism was not yet fully understood.

Staphylococcus aureus (*S. aureus*) is one of the earliest described pathogens in humans and one of the most common causes of infection[10]. Due to the overuse of antibiotics in treating human and animal diseases, drug resistance in *S. aureus* has become increasingly serious and has led to the MRSA pandemic[11]. MRSA causes many diseases and conditions, such as nosocomial infections, pneumonia, sepsis, and skin infections. Studies have shown that the formation of MRSA biofilm is a main factor contributing to the increased drug resistance and toxicity of this pathogen[12]. Biofilm is a kind of polymeric membrane formed by many bacteria that allows them to adhere to a surface, and it can serve as a barrier for bacteria against host immune cells[13]. The bacteria in biofilm are reportedly approximately 1,000 times more resistant to antibacterial agents than are planktonic bacteria[14]. Few drugs are available that can clear the biofilm of MRSA[15, 16]. Therefore, additional drugs that can effectively remove MRSA biofilm are urgently needed.

2 Results And Discussion

2.1 Antibacterial activity

TTO is a potential anti-bacterial[7, 17] and anti-fungal product[18, 19], but there were few studies about the anti-MRSA activity of terpinen-4-ol, a main ingredient of TTO. Our research focused on the antibacterial activity of terpinen-4-ol against MRSA. The MIC and MBC values for terpinen-4-ol against *S. aureus* were 0.08%~0.32% (Table 2), which is similar to Loughlin`s result (0.25%)[20]. In Table 1, we could see the MBC value was two times the MIC value, which leads us to know that terpinen-4-ol exhibited bactericidal properties against MRSA and other strains in this study [21]. In addition, a time-kill curve of MRSA exposed to terpinen-4-ol was generated by performing plate counts (Fig. 1 A). We found that 0.32% terpinen-4-ol atkilled all the bacteria within two hours and that terpinen-4-ol at 0.16% or 0.08% could obviously inhibit the growth of MRSA, which also showed terpinen-4-ol exhibited bactericidal properties against MRSA.

Table 1 Antimicrobial activities of terpinen-4ol against 13 *S. aureus* strains.

Bacteria	MIC(v/v)	MBC(v/v)
ATCC 43300	0.16%	0.32%
S-1	0.08%	0.16%
S-2	0.08%	0.16%
S-3	0.16%	0.32%
S-4	0.08%	0.16%
S-5	0.32%	0.32%
S-6	0.08%	0.16%
S-7	0.16%	0.16%
S-8	0.08%	0.16%
S-9	0.16%	0.16%
S-10	0.32%	0.32%
S-11	0.16%	0.32%
S-12	0.16%	0.32%

2.2 Antibiofilm activity

The antibiofilm activity of terpinen-4-ol was also assessed by the crystal violet staining. As shown in Fig. 1 B, there was almost no MRSA biofilm formation under treatment with terpinene-4-ol at concentrations of 0.64% and 0.32%. Terpinene-4-ol at 0.16% also had a significant effect on the formation of MRSA biofilm, and the inhibition rates at 24 h and 48 h were $48.09\% \pm 0.97\%$ ($P < 0.01$) and $31.20\% \pm 2.13\%$ ($P < 0.01$). It can be seen that higher concentrations of terpinen-4-ol effectively inhibited the formation of biofilm.

In this study, terpinen-4-ol behaved its inhibition in MRSA biofilm formation. It also confirmed other authors' results that terpinen-4-ol could destroy the biofilm of many oral pathogens such as, *Streptococcus mutans*, and *Lactobacillus acidophilus*[22] *Porphyromonas gingivalis*, *Fusobacterium nucleatum*[23]. We could see that terpinen-4-ol could inhibit biofilm formation of MRSA in a concentration-dependent manner. With the decrease of concentration of terpinen-4-ol, the inhibition rates gradually down.

The destruction of biofilms by terpinen-4-ol was also evaluated. As shown in Fig. 1 C, 0.16% terpinene-4-ol achieved a clearance rate of $93.90\% \pm 3.33\%$ ($P < 0.01$) for MRSA mature biofilm. As the concentration of terpinen-4-ol decreased, the clearance rate gradually decreased. The viability of the preformed MRSA biofilm exposed to terpinen-4-ol was also verified by CLSM analysis. As shown in Fig. 2, as the concentration of terpinen-4-ol increased, the amount of red fluorescence (indicating dead bacteria) increased while that of green fluorescence (indicating live bacteria) decreased.

Terpinen-4-ol showed its activity in clearing MRSA biofilm. Generally, it is more difficult to remove mature biofilm than to inhibit its formation. Thus, terpinen-4-ol could consider to be a potential antibiofilm agent, for it can not only inhibit the formation of biofilms, but also destroy mature biofilm. Therefore, it is necessary to further study the anti-biofilm activity of terpinene-4-ol and explain its mechanism of action.

2.3 Antibiofilm mechanism

2.3.1 Transcriptomics

To understand the changes in MRSA due to treatment with terpinen-4-ol, we measured the transcriptome of MRSA exposed to terpinen-4-ol and screened 304 DEGs as defined by $FC > 2$ and $Q < 0.05$. Among these DEGs, 159 genes were downregulated and 145 genes were upregulated in terpinen-4-ol-treated relative to control (Fig. 3). The RNA-seq data were submitted to Gene Expression Omnibus (GEO) under accession number GSE157638.

KEGG pathway analysis was used to obtain insights into the biological functions of the DEGs (Fig. 4). Compared with the control group, there were some pathways different, including valine, leucine, and isoleucine (branched chain amino acids, BCAAs) biosynthesis. nitrogen metabolism, ABC transporters, 2-oxocarboxylic acid metabolism and etc., were significantly enriched ($P < 0.05$) in terpinen-4-ol-treated group. Some of those pathways related to the formation of MRSA biofilm. For example, BCAAs, synthesized by 2-oxocarboxylic acid, regulate the biosynthesis of bacterial amino acids[24] and

nucleotide metabolism[25] by combining with CodY, and they can also regulate the biofilm of *S.aureus*[26-28]. There were 9 genes related to 2-oxocarboxylic acid and BCAAs have been down in MRSA treated with terpinen-4-ol, which showed us the BCAA metabolic pathway of MRSA may be affected by terpinen-4-ol.

Furthermore, there were other pathways that warrant our attention, such as purine metabolism, quorum sensing (QS), and β -Lactam resistance. Most of these pathways are related to the synthesis of nucleic acids or ATP, which are important to biofilm formation of MRSA. We then used metabolomics to verify the results of the transcriptomics analysis.

3.3.2 Metabolomics

Twelve sets of data from the six biological replicates were analyzed to determine the changes in metabolites that occur in MRSA following treatment with terpinen-4-ol. and 215 differential metabolites were screened based on $FC > 1.5$ or $FC < 0.67$, $P < 0.05$, and VIP value > 1.0 . Among these differential metabolites, 125 were upregulated and 90 were downregulated in terpinen-4-ol-treated MRSA relative to control MRSA (Fig. 5). 18 enriched pathways (Fig. 6) were identified by KEGG enrichment analysis, including caffeine metabolism ($P < 0.05$). Caffeine can be converted to purine, and we found that many intermediate products of purine metabolism and the contents of its constituents have decreased, including xanthine and xanthosin, which are intermediate products of purine metabolism, guanosine, and adenosine, which are components of purine metabolism, 2'-deoxyadenosine, and deoxyadenosine, which are components of DNA.

In addition, there were also some metabolites related to pyrimidine metabolism have decreased. The content of these substances is closely related to DNA synthesis, and the reduction of their content may lead to a reduction in the amount of bacterial DNA. Thus, we conjectured that terpinen-4-ol achieves its anti-MRSA activity by inhibiting the synthesis of DNA.

2.3.3 eDNA Quantification

eDNA an important component of the EPS released by the lysis of some bacteria during the formation of the biofilm[29], and it plays a vital role in all stages of biofilm formation, including initial bacterial adhesion, aggregation, microcolony formation, and determining the structure of biofilm[30]. The amount of eDNA in the medium of the biofilm was measured by spectrophotometer and reported as the eDNA per relative biomass to account for the number of bacteria in the biofilm. The production of eDNA from biofilm exposed to terpinen-4-ol was significantly decreased compared with that of the control (Fig. 1 D). In particular, the eDNA content of the biofilm treated with 0.16% terpinen-4-ol was decreased relative to the control content by $61.88\% \pm 0.58\%$. The decrease in the content of eDNA reduces the adhesion ability between bacteria, leading to the destruction of the integrity of its biofilm. Thus, we inferred terpinen-4-ol inhibited biofilm formation of MRSA by inhibiting its release of eDNA.

2.3.4 q-RT-PCR

To verify our results, we also performed qRT-PCR verification of some of the differential genes, which play important roles in DNA synthesis (Table 2). In terpinen-4-ol-treated MRSA relative to control MRSA, the expression of *deoD* was upregulated by 12.378 ± 0.541 times, and the expression of *pyrB* was downregulated by -3.049 ± 0.147 times. Those results were largely consistent with the transcriptome results that terpinen-4-ol inhibit purine and pyrimidine metabolism.

Table 2 Selected MRSA genes that displayed altered expression after terpinene-4-ol treatment of biofilm as determined by RNA-seq and real-time RT-PCR.

Gene	Description	Fold Change \pm SD	
		qRT-PCR	RNA-seq
<i>carB</i>	carbamoyl phosphate synthase large subunit	-2.054 ± 0.304	-6.36
<i>arcC</i>	carbamate kinase	$+2.371 \pm 0.051$	3.38
<i>deoD</i>	purine nucleoside phosphorylase	$+12.378 \pm 0.541$	24.723
<i>pyrF</i>	orotidine 5'-phosphate decarboxylase	-1.297 ± 0.697	-5.07
<i>pyrB</i>	aspartate carbamoyltransferase catalytic subunit	-3.049 ± 0.147	-5.45

2.3.5 Conjoint analysis

The differential genes identified from the transcriptomics analysis and the differential metabolites identified from metabolomics analysis were represented in the KEGG pathway database (Fig. 7). After the enriched pathways were identified for the differential genes and metabolites separately, it was found that the differential genes and metabolites were mainly involved in the synthesis of MRSA nucleic acid. In the transcriptome results, 16 genes related to nucleic acid synthesis were altered, including *nrdF*, *arcC*, *deoD*, *pyrF*, *pyrB* and *carB*. In the metabolome results, the contents of 11 metabolites related to nucleic acid synthesis, including xanthine, 2'-deoxyadenosine, inosine, cytosine, thymidine, and deoxyadenosine, were significantly reduced in terpinen-4-ol-treated MRSA relative to control MRSA. Based on some of the differential genes and metabolites, we constructed a diagram of the pathways through which terpinen-4-ol affects the synthesis of MRSA nucleic acid. It can be seen that terpinen-4-ol significantly affects the salvage synthesis pathway of MRSA DNA and RNA.

The biosynthesis of pyrimidine nucleotides can affect biofilm by influencing the expression of the genes *csgDEFG*[31]. The interplay between the rescue synthesis pathway of nucleotides and Curli amyloid fibers' generation may be closely related to cellulose and other EPS responses to environmental pressure[32-34]. *pyrB* and *carB* are the key enzymes of the nucleotide rescue pathway, being very important for the rescue synthesis of nucleotides. In our research, the expression of these two genes was inhibited by terpinen-4-ol treatment. Furthermore, the levels of four nucleotides, the raw material for UMP

synthesis, declined significantly after terpinen-4-ol treatment, indicating that the salvage synthesis of pyrimidine nucleotides was inhibited by terpinen-4-ol. The inhibition of pyrimidine nucleotides might block the production of modified nucleotides acting as signal molecules for biofilm formation, such as c-di-GMP[35], which is an important signal molecule in biofilm regulation. In addition, the inhibition of pyrimidine nucleotides biosynthesis might lead to the inhibition of eDNA synthesis, which could lead to the destruction of biofilm. The observed decrease in eDNA production in terpinene-4-ol-treated MRSA relative to control MRSA supports this possibility.

ATP, which is composed of adenine, ribose and 3 molecules of phosphate[36], is the most direct source of energy in living organisms. The decrease of inosine in terpinene-4-ol-treated MRSA affected the synthesis of ATP in MRSA, resulting in the inhibition of energy metabolism and the membrane transport system to varying degrees. In the analyses of the transcriptome and metabolome, we found many ATP-related genes and small-molecule metabolites affected to varying extents by terpinen-4-ol treatment, especially the ABC transporter and genes related to energy metabolism. The ABC transporter is the main transporter of the MRSA efflux pump[37, 38] and is closely related to the formation of MRSA biofilm[39] and the quorum sensing system. A reduction in efflux pump activity can inhibit the formation of bacterial biofilm. The ABC transporter is also involved in one of the main mechanisms of MRSA drug resistance. A study has shown that terpinen-4-ol and β -lactam antibiotics applied in combination have a significant synergistic effect against MRSA[9]. In addition, TTO can inhibit the growth of *Botrytis cinerea* by inhibiting its energy metabolism[40]. Therefore, we speculate that terpinen-4-ol can inhibit the growth of MRSA by inhibiting its ATP synthesis.

3 Conclusion

Terpinen-4-ol has good antibacterial activity and significantly inhibits the formation of MRSA biofilm. It demonstrated the excellent potential in anti-biofilm drug. The results of the transcriptomics and metabolomics analyses suggest that terpinen-4-ol inhibits the growth of MRSA and biofilm by inhibiting purine and pyrimidine metabolism. These findings provide new insights into the antibacterial mechanisms of terpinen-4-ol and may promote the study of the pharmacological activity of terpinen-4-ol as a natural antibacterial product.

4 Materials And Methods

4.1 Materials

MRSA (ATCC 43300) was obtained from Beina Biotechnology (Beijing, China). Twelve isolates of *S. aureus* were obtained from the manure of the dairy farm in Gansu China, and were labeled S-1 through S-12. MHA and MHB were obtained from Aobox Biotechnology (Beijing, China). TSB and BHI were obtained from Oxoid (Fisher, UK), and terpinen-4-ol was obtained from Sigma Aldrich (Shanghai, China).

4.2 Testing the Susceptibility of Planktonic Bacteria

The MIC of terpinen-4-ol was determined according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. 1 standard strain and 12 clinical strains of *S. aureus* were tested in this study.

4.3 Time-kill Curve

Time-kill curve of terpinen-4-ol against MRSA strain ATCC 43300 was generated. MRSA was cultivated in MHB with terpinen-4-ol and incubated at 37°C. The number of MRSA was measured by the plate counting method at regular time intervals.

4.4 Assays the Effect on Biofilm

The adhesion of biofilm was measured by crystal violet staining and CLSM. The crystal violet staining method was performed according to a previous report and used to determine the inhibition and clearance of MRSA biofilm by terpinen-4-ol at different concentrations. The biofilm was cultured and measured following Srdjan Stepanović's report (Stepanovic et al., 2007).

CLSM was performed by staining a pre-established biofilm with the LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen Molecular Probes, Carlsbad, USA) according to the manufacturer's instructions. CLSM images were captured using an LSM 800 (ZEISS, LSM 800) with a 40× objective lens. ZEN 2.3 was used to analysis and exporting images of CLSM.

4.5 RNA-seq and Enrichment Analysis

MRSA was exposed to terpinen-4-ol at a concentration of $1/2 \times \text{MIC}$ for 2 h, and 3 treatment samples and 3 control samples were analyzed. Total RNA was extracted from MRSA using an RNAPrep Pure Bacteria Kit (Tiangen, Beijing, China). The transcriptome sequencing and analysis were conducted by Novogene (Tianjin, China) using the Illumina sequencing platform (Illumina NovaSeq). DEGs screening and KEGG enrichment analysis respectively used Bowtie v2.2.3 and KOBAS.

4.6 Metabolite Extraction and Analysis

The MRSA was ground with liquid nitrogen and its metabolites analysis was carried out to analyze using a Vanquish UHPLC system coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher, USA). The analytical conditions and quantified as a previously described study. Metabolites were relatively quantified by calculating the area of each individual peak with significant differences in content were set with thresholds of variable importance in projection (VIP) ≥ 1 and fold change ≥ 1.5 or ≤ 0.67 . Six replicates were performed in metabolomics analysis, which was carried out by Novogene.

4.7 eDNA Quantification

The extraction of eDNA in MRSA was conducted as previously reported (Rice et al., 2007). First, biofilm was cultured in a 6-well plate, the OD_{600} was measured by enzyme-labeled instrument, and the eDNA was

extracted. The concentration of eDNA was measured with an DNA/Protein Analyzer (Pulstton, USA). The relative content of eDNA was measured in terms of eDNA content per unit of biofilm.

4.8 qRT-PCR

The extraction of total RNA was performed using the Simply P Total RNA Extraction Kit (Bioflux, Hangzhou, China). The cDNAs were synthesized by the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan). The quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using QuantStudio (Thermo Fisher, USA) with TB Green® Premix Ex Taq™ II (Takara, Japan). The primers and genes are listed in Table 3.

Table 3 q-RT-PCR Primers

Primer	Sequence (5'→3')	Length (bp)
pyrB-F	AGGCATGGGCTTGCAGAAGAAAC	23
pyrB-R	CTCTATTCACAGGTGCCGGATGC	23
pyrF-F	ATGGCGTTGTTTGTTCACCTCTTG	24
pyrF-R	CGGTGTCGTAATACGGTGTTGGTC	24
carB-F	TTAACGTGCCACAGCCACAAGG	22
carB-R	TTCCATTGCGCGACCACCTAATAC	24
arcC-F	CATGCGGTGGTGGCGGTATTC	21
arcC-R	GGTATCTGCTTCAATCAGCGTTGC	24
deoD-F	TTACGCTGGTGTCTGAAGCATTAGG	24
deoD-R	CCCTTTCCTCAGGTGTTGTTGACG	24
16S-F	GCTCGTGTCGTGAGATGTTGG	21
16S-R	TTTCGCTGCCCTTTGTATTGT	21

4.9 Statistical Analyses

All data except RNA-seq and metabolomics were reported as means ± SD. The significance of differences was determined by t-test using SPSS 23.0 (SPSS Inc.). A value of $P < 0.05$ was considered to be significant.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of Data and Materials

The datasets generated during or analysed during the current study are available from the corresponding author on reasonable request. The datasets generated and/or analysed during the current study are available in the GEO repository, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157638> and GSE157638.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FC and YL designed the experiments. FC and YM conducted the experiments, FC and YM drafted the manuscript. KC, XS, ZY, BH, RS and JL revised the manuscript. All authors read and approved the manuscript.

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References

1. Firenzuoli F, Jaitak V, Horvath G, Bassolé IHN, Setzer WN, Gori L: **Essential Oils: New Perspectives in Human Health and Wellness**. *Evidence-Based Complementary and Alternative Medicine* 2014, **2014**:1-2.
2. Zhao X, Liu Z, Liu Z, Meng R, Shi C, Chen X, Bu X, Guo N: **Phenotype and RNA-seq-Based transcriptome profiling of Staphylococcus aureus biofilms in response to tea tree oil**. *Microbial Pathogenesis* 2018, **123**:304-313.

3. Li W-R, Shi Q-S, Liang Q, Xie X-B, Huang X-M, Chen Y-B: **Antibacterial Activity and Kinetics of Litsea cubeba Oil on Escherichia coli.** *PLoS ONE* 2014, **9**(11).
4. Rodriguez-Garcia I, Silva-Espinoza BA, Ortega-Ramirez LA, Leyva JM, Siddiqui MW, Cruz-Valenzuela MR, Gonzalez-Aguilar GA, Ayala-Zavala JF: **Oregano Essential Oil as an Antimicrobial and Antioxidant Additive in Food Products.** *Critical Reviews in Food Science and Nutrition* 2015, **56**(10):1717-1727.
5. Rajkowska K, Nowicka-Krawczyk P, Kunicka-Styczyńska A: **Effect of Clove and Thyme Essential Oils on Candida Biofilm Formation and the Oil Distribution in Yeast Cells.** *Molecules* 2019, **24**(10).
6. Carson CF, Hammer KA, Riley TV: **Melaleuca alternifolia (tea tree) oil: a review of antimicrobial and other medicinal properties.** *Clinical Microbiology Reviews* 2006, **19**(1):50+.
7. Yadav E, Kumar S, Mahant S, Khatkar S, Rao R: **Tea tree oil: a promising essential oil.** *J Essent Oil Res* 2016, **29**(3):201-213.
8. Organisation IS: **Essential oil of Melaleuca, terpinen-4-ol type (Tea Tree oil).** In: *Huile essentielle de Melaleuca, terpinen-4-ol type (Huile essentielle de Tea tree)* Geneva, Switzerland; 2017.
9. Cordeiro L, Figueiredo P, Souza H, Sousa A, Andrade-Júnior F, Medeiros D, Nóbrega J, Silva D, Martins E, Barbosa-Filho J *et al.*: **Terpinen-4-ol as an Antibacterial and Antibiofilm Agent against Staphylococcus aureus.** *International Journal of Molecular Sciences* 2020, **21**(12).
10. Lakhundi S, Zhang K: **Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology.** *Clinical Microbiology Reviews* 2018, **31**(4).
11. Otto M: **Community-associated MRSA: What makes them special?** *International Journal of Medical Microbiology* 2013, **303**(6-7):324-330.
12. Frieri M, Kumar K, Boutin A: **Antibiotic resistance.** *Journal of Infection and Public Health* 2017, **10**(4):369-378.
13. Del Pozo JL: **Biofilm-related disease.** *Expert Review of Anti-infective Therapy* 2017, **16**(1):51-65.
14. Mah TF: **Biofilm-specific antibiotic resistance.** *Future Microbiol* 2012, **7**(9):1061-1072.
15. Max V, Ranall MSB, Mark A, Blaskovich and Matthew A. Cooper*: **Resolving Biofilm Infections: Current Therapy and Drug Discovery Strategies.** *Current Drug Targets* 2012, **13**:1375-1385.
16. Bjarnsholt T, Ciofu O, Molin S, Givskov M, Høiby N: **Applying insights from biofilm biology to drug development – can a new approach be developed?** *Nature Reviews Drug Discovery* 2013, **12**(10):791-808.
17. Brun P, Bernabe G, Filippini R, Piovan A: **In Vitro Antimicrobial Activities of Commercially Available Tea Tree (Melaleuca alternifolia) Essential Oils.** *Curr Microbiol* 2019, **76**(1):108-116.
18. Li Z, Wang N, Wei Y, Zou X, Jiang S, Xu F, Wang H, Shao X: **Terpinen-4-ol Enhances Disease Resistance of Postharvest Strawberry Fruit More Effectively than Tea Tree Oil by Activating the Phenylpropanoid Metabolism Pathway.** *Journal of Agricultural and Food Chemistry* 2020, **68**(24):6739-6747.

19. Li Y, Shao X, Xu J, Wei Y, Xu F, Wang H: **Tea tree oil exhibits antifungal activity against *Botrytis cinerea* by affecting mitochondria.** *Food Chem* 2017, **234**:62-67.
20. Loughlin R, Gilmore BF, McCarron PA, Tunney MM: **Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells.** *Letters in Applied Microbiology* 2008, **46**(4):428-433.
21. G. A. Pankey LDS: **Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections.** *Clinical Infectious Diseases* 2004, **38**(6):864-870.
22. Bordini EAF, Tonon CC, Francisconi RS, Magalhaes FAC, Huacho PMM, Bedran TL, Pratavieira S, Spolidorio LC, Spolidorio DP: **Antimicrobial effects of terpinen-4-ol against oral pathogens and its capacity for the modulation of gene expression.** *Biofouling* 2018, **34**(7):815-825.
23. Maquera-Huacho PM, Tonon CC, Correia MF, Francisconi RS, Bordini EAF, Marcantonio E, Spolidorio DMP: **In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces.** *Biofouling* 2018, **34**(6):699-709.
24. Majerczyk CD, Dunman PM, Luong TT, Lee CY, Sadykov MR, Somerville GA, Bodi K, Sonenshein AL: **Direct targets of CodY in *Staphylococcus aureus*.** *J Bacteriol* 2010, **192**(11):2861-2877.
25. Pohl K, Francois P, Stenz L, Schlink F, Geiger T, Herbert S, Goerke C, Schrenzel J, Wolz C: **CodY in *Staphylococcus aureus*: a Regulatory Link between Metabolism and Virulence Gene Expression (vol 191, pg 2953, 2009).** *Journal of Bacteriology* 2009, **191**(14):4695-4695.
26. Rivera FE, Miller HK, Kolar SL, Stevens SM, Jr., Shaw LN: **The impact of CodY on virulence determinant production in community-associated methicillin-resistant *Staphylococcus aureus*.** *Proteomics* 2012, **12**(2):263-268.
27. Montgomery CP, Boyle-Vavra S, Roux A, Ebine K, Sonenshein AL, Daum RS: **CodY deletion enhances in vivo virulence of community-associated methicillin-resistant *Staphylococcus aureus* clone USA300.** *Infect Immun* 2012, **80**(7):2382-2389.
28. Majerczyk CD, Sadykov MR, Luong TT, Lee C, Somerville GA, Sonenshein AL: ***Staphylococcus aureus* CodY negatively regulates virulence gene expression.** *J Bacteriol* 2008, **190**(7):2257-2265.
29. Desvaux M, Das T, Sehar S, Koop L, Wong YK, Ahmed S, Siddiqui KS, Manefield M: **Influence of Calcium in Extracellular DNA Mediated Bacterial Aggregation and Biofilm Formation.** *PLoS ONE* 2014, **9**(3).
30. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D: **Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*.** *Microbiology* 2007, **153**(7):2083-2092.
31. Marinus MG, Garavaglia M, Rossi E, Landini P: **The Pyrimidine Nucleotide Biosynthetic Pathway Modulates Production of Biofilm Determinants in *Escherichia coli*.** *PLoS ONE* 2012, **7**(2).
32. White AP, Gibson DL, Kim W, Kay WW, Surette MG: **Thin Aggregative Fimbriae and Cellulose Enhance Long-Term Survival and Persistence of *Salmonella*.** *Journal of Bacteriology* 2006, **188**(9):3219-3227.
33. Gualdi L, Tagliabue L, Bertagnoli S, Ieranò T, De Castro C, Landini P: **Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*.**

Microbiology 2008, **154**(7):2017-2024.

34. Kevin A. Hughes IWSaMVJ: **Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase.** *Microbiology* 1998, **144**(11):3039-3047.
35. Karaolis DKR, Rashid MH, Chythanya R, Luo W, Hyodo M, Hayakawa Y: **c-di-GMP (3'-5'-Cyclic Diguanilylic Acid) Inhibits Staphylococcus aureus Cell-Cell Interactions and Biofilm Formation.** *Antimicrobial Agents and Chemotherapy* 2005, **49**(3):1029-1038.
36. Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, Giorgi C, Marchi S, Missiroli S, Poletti F *et al.*: **ATP synthesis and storage.** *Purinergic Signalling* 2012, **8**(3):343-357.
37. McCarthy H, Rudkin JK, Black NS, Gallagher L, O'Neill E, O'Gara JP: **Methicillin resistance and the biofilm phenotype in Staphylococcus aureus.** *Frontiers in Cellular and Infection Microbiology* 2015, **5**.
38. Fernandez L, Hancock REW: **Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance.** *Clinical Microbiology Reviews* 2012, **25**(4):661-681.
39. Abouelhassan Y, Zhang Y, Jin S, Huigens RW: **Transcript Profiling of MRSA Biofilms Treated with a Halogenated Phenazine Eradicating Agent: A Platform for Defining Cellular Targets and Pathways Critical to Biofilm Survival.** *Angewandte Chemie International Edition* 2018, **57**(47):15523-15528.
40. Xu J, Shao X, Wei Y, Xu F, Wang H: **iTRAQ Proteomic Analysis Reveals That Metabolic Pathways Involving Energy Metabolism Are Affected by Tea Tree Oil in Botrytis cinerea.** *Frontiers in Microbiology* 2017, **8**.

Figures

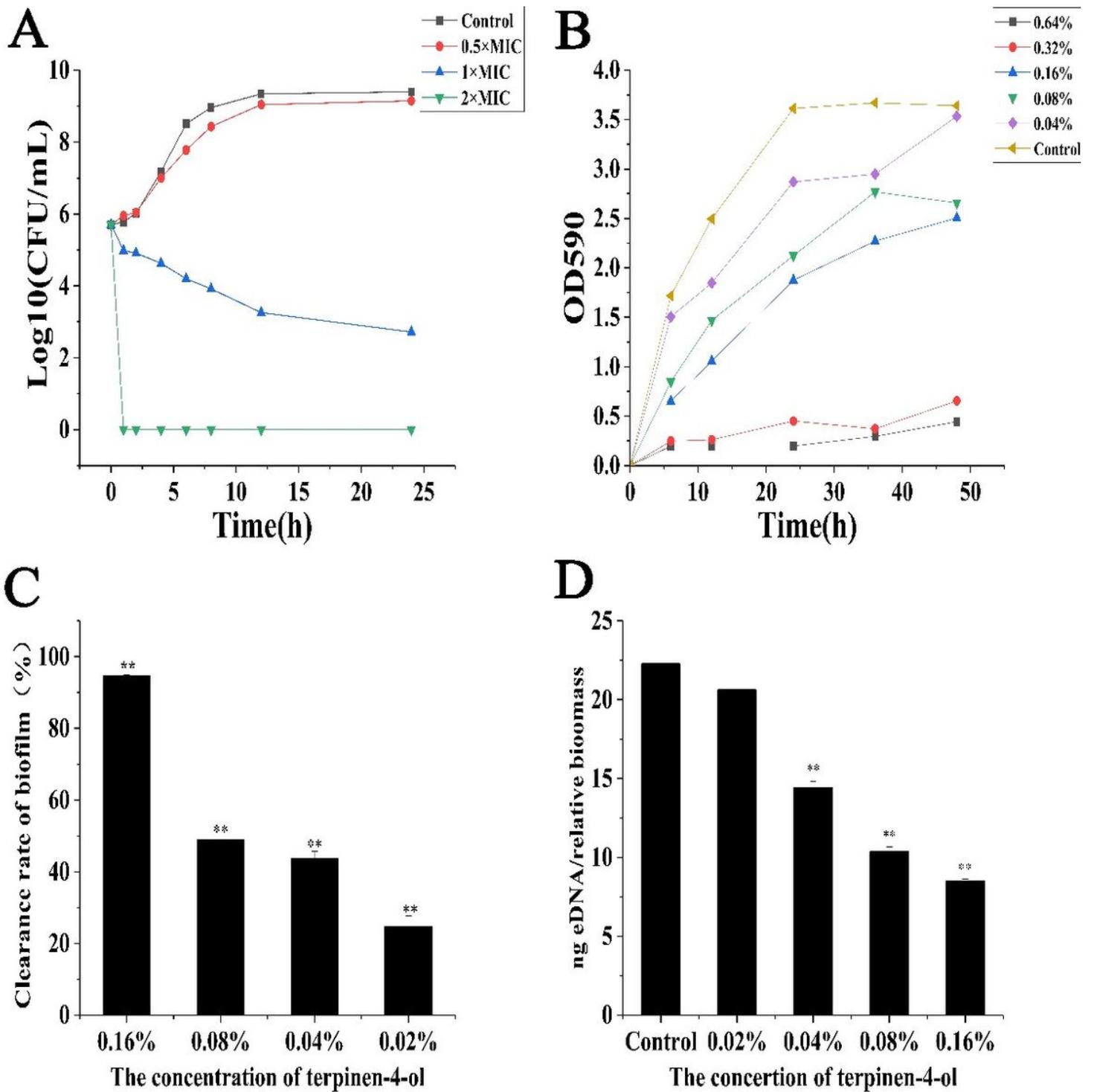


Figure 1

The effect of terpinen-4-ol on MRSA. (A) Time-kill curve (B) Effect of terpinen-4-ol on biofilm formation. (C) Effect of terpinen-4-ol on mature biofilm. (D) Effect of terpinen-4-ol on eDNA in biofilm. **: Compare with blank control group, $P < 0.01$, t test.

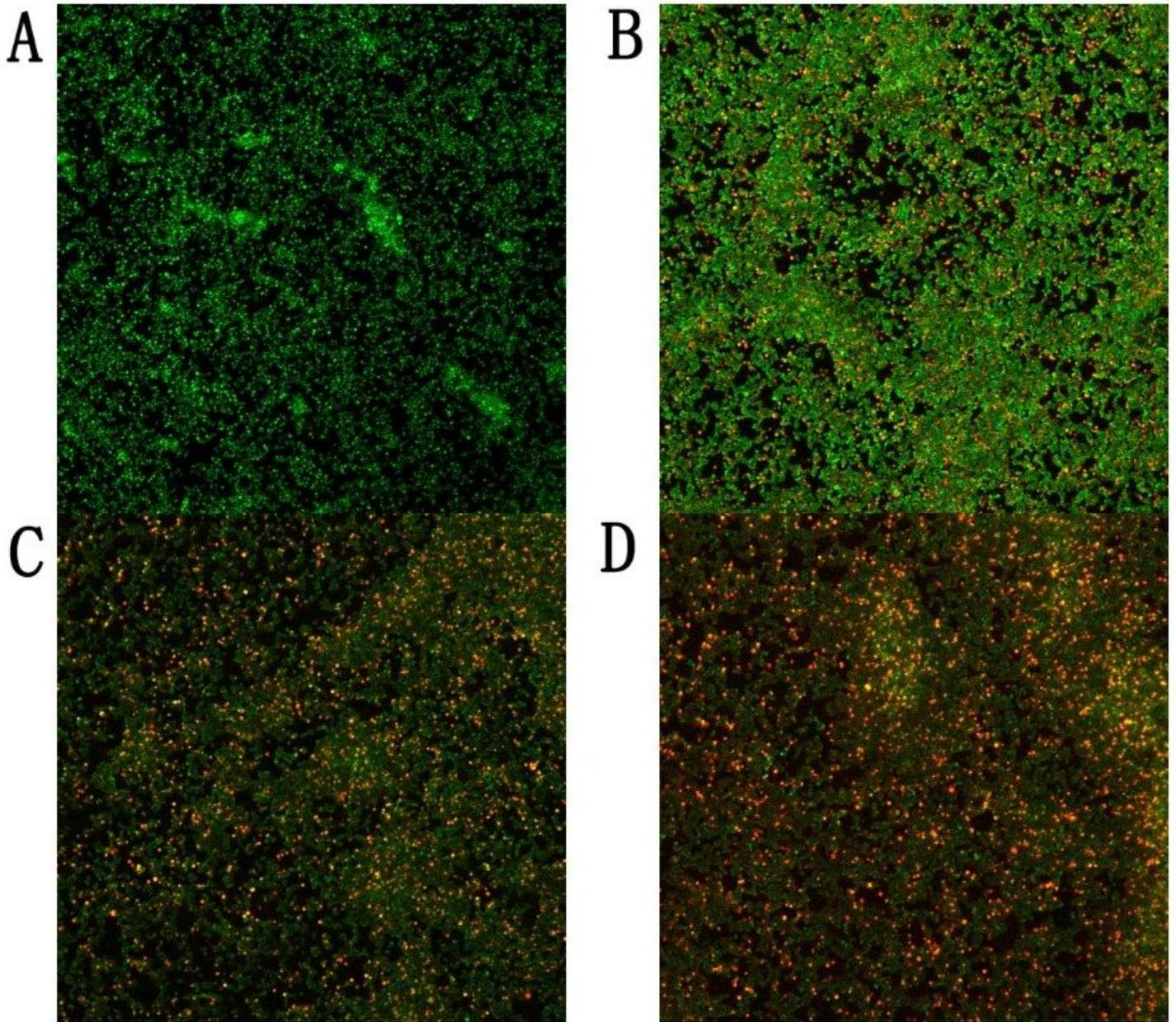


Figure 2

CLSM image of LIVE/DEAD stained MRSA biofilm grown on cell slide. Green (viable cells) and red (dead cells). (A) Control cells (no treated). (B) Cells treated with terpinen-4-ol (0.04%). (C) Cells treated with terpinen-4-ol (0.08%). (D) Cells treated with terpinen-4-ol (0.16%).

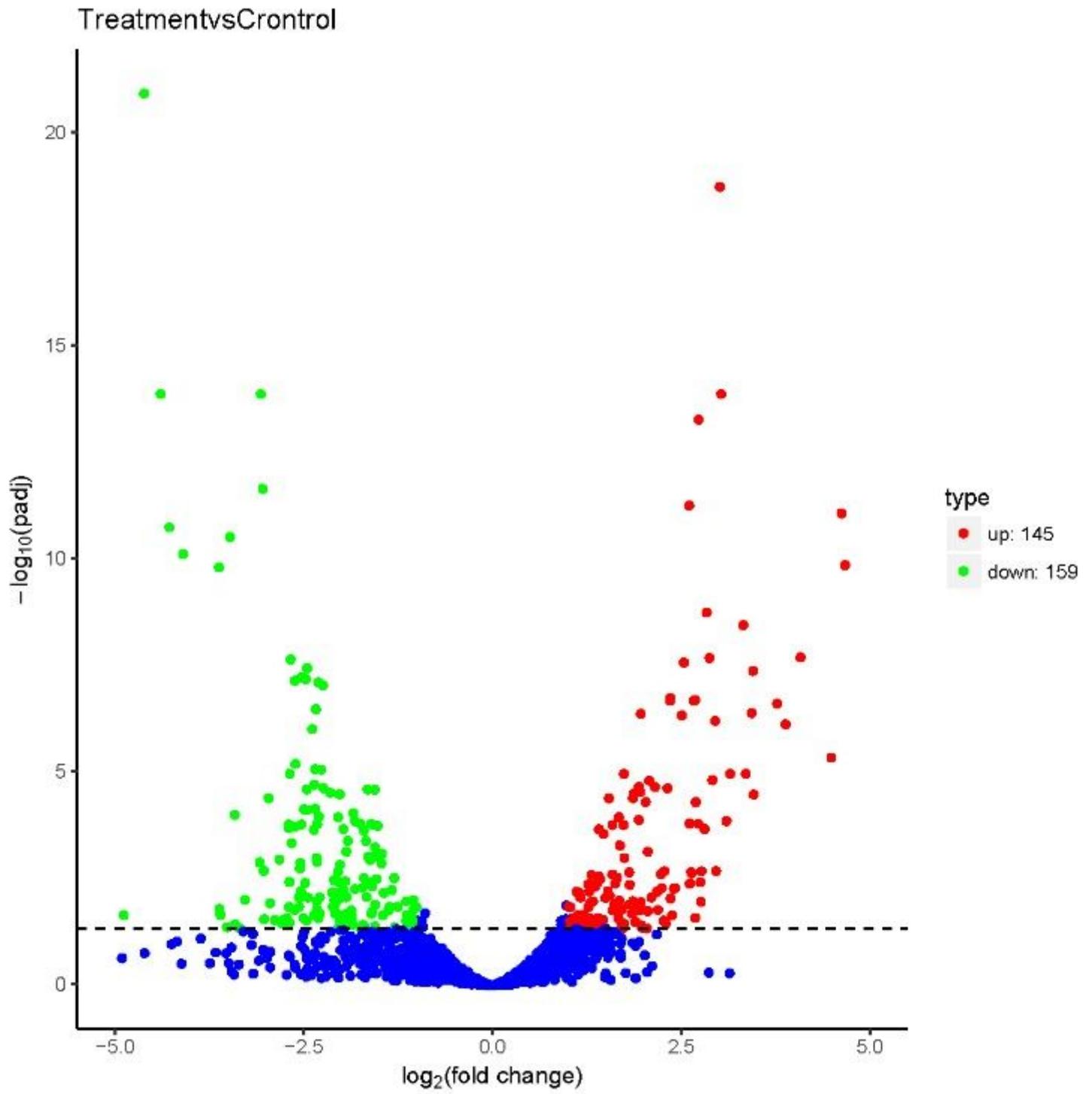


Figure 3

Volcano plot of DEGs in MRSA treated with terpinen-4-ol

Statistics of Pathway Enrichment

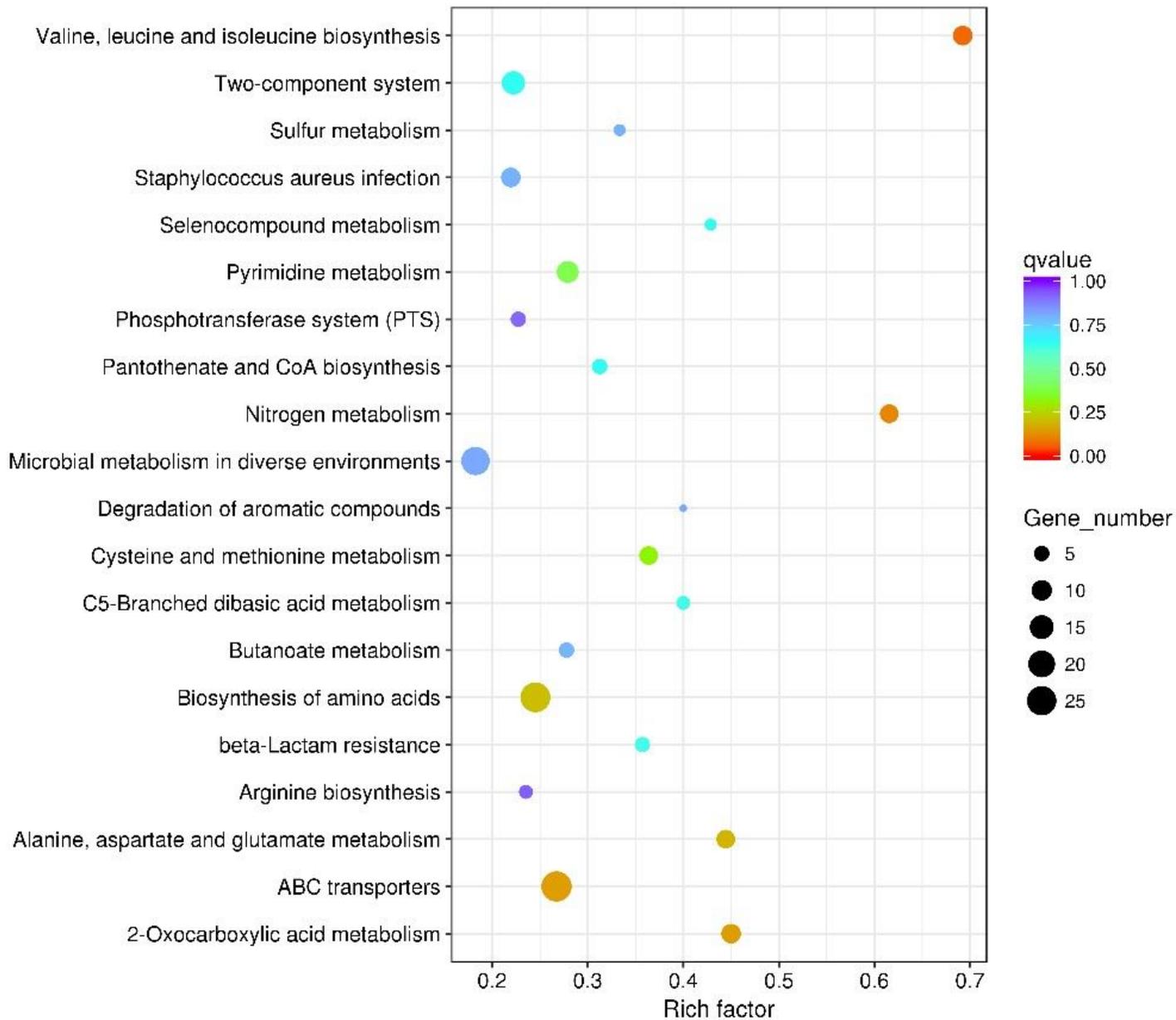


Figure 4

Bubble chart of KEGG enrichment results of transcriptome

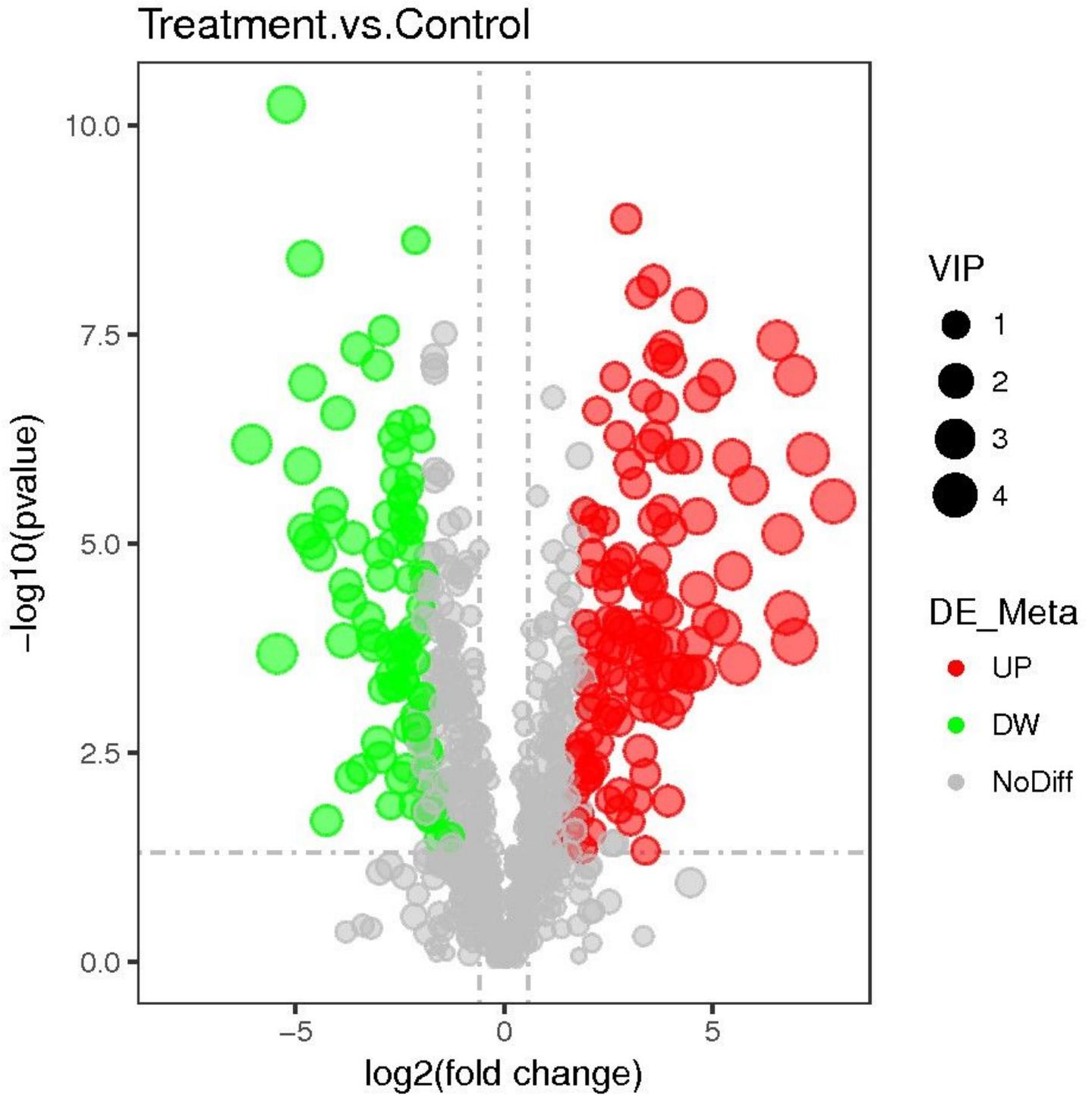


Figure 5

Volcano plot of different metabolites in MRSA treated with terpinen-4-ol.

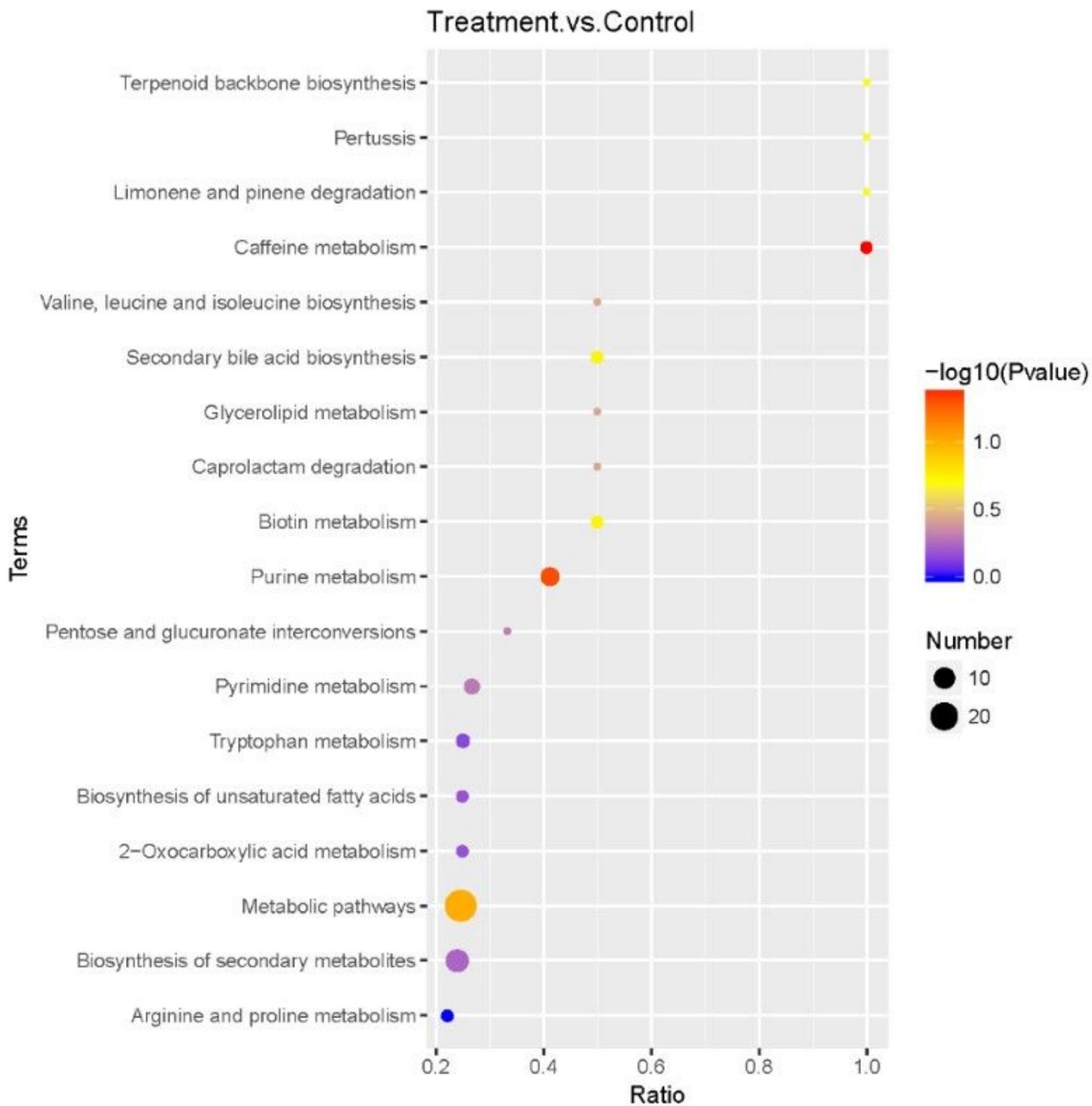


Figure 6

Bubble chart of KEGG enrichment results.

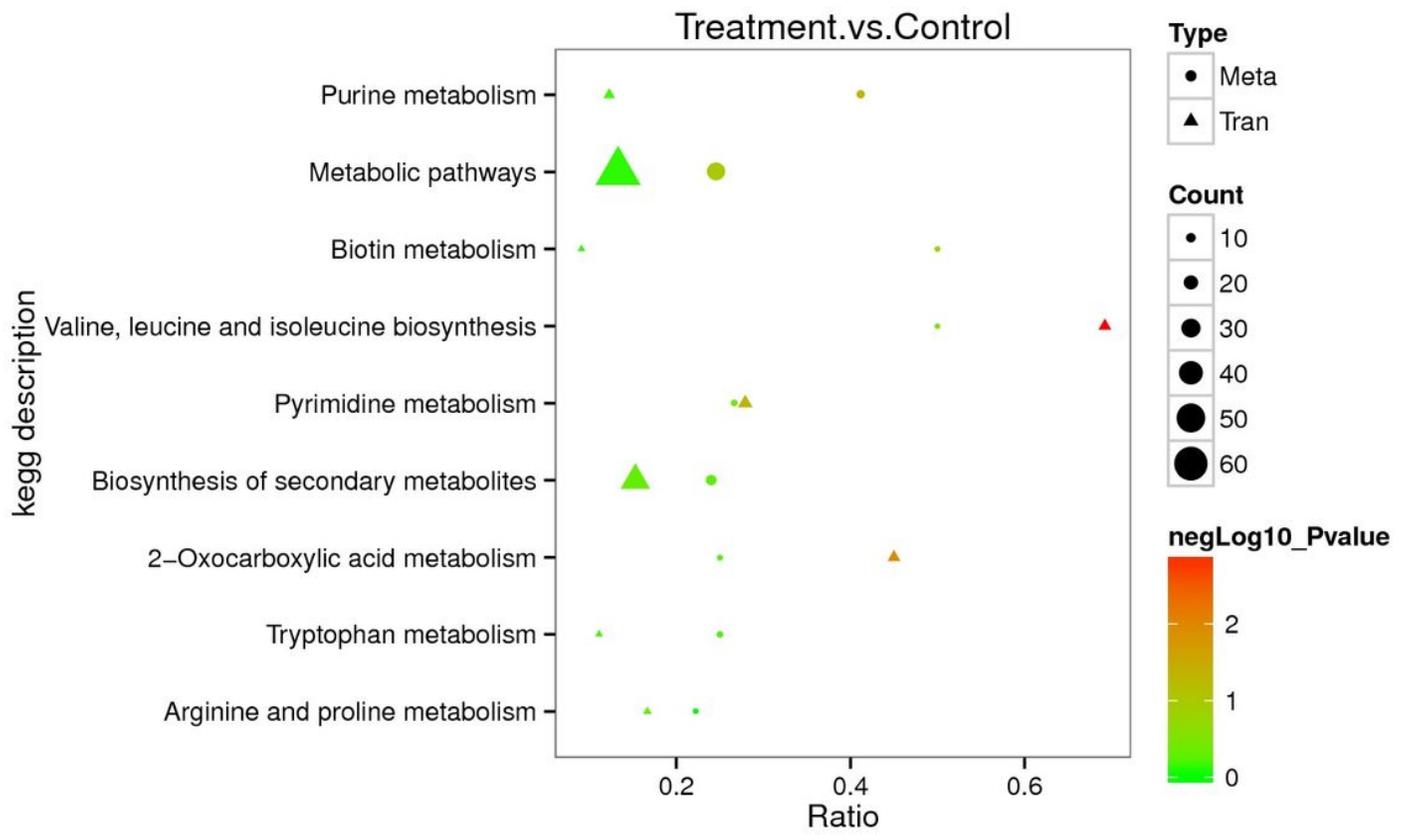


Figure 7

KEGG enrichment of conjoint analysis