Linalool's Multifaceted Antimicrobial Potential: Unveiling its Antimicrobial Efficacy and Immunomodulatory Role Against *Saprolegnia parasitica*

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Abstract

Saprolegnia parasitica (*S. parasitica*) is one of the most virulent oomycete species in freshwater aquatic environments, causing severe saprolegniasis and leading to significant economic losses in the aquaculture industry. Thus far, the prevention and control of saprolegniasis face a shortage of medications. Linalool, a natural antibiotic alternative found in various essential oils, exhibits promising antimicrobial activity against a wide range of pathogens. In this study, the specific role of linalool in protecting *S. parasitica* infection at both *in vitro* and *in vivo* levels was investigated. Linalool showed multifaceted anti-oomycetes potential by both of antimicrobial efficacy and immunomodulatory efficacy. For *in vitro* test, linalool exhibited strong anti-oomycetes activity and mode of action included: (1) Linalool disrupted the cell membrane of the mycelium, causing the intracellular components leak out; (2) Linalool prohibited ribosome function, thereby inhibiting protein synthesis and ultimately affecting mycelium growth. Surprisingly, meanwhile we found the potential immune protective mechanism of linalool in the *in vivo* test: (1) Linalool enhanced the complement and coagulation system which in turn activated host immune defense and lysate *S. parasitica* cells; (2) Linalool promoted wound healing, tissue repair, and phagocytosis to cope with *S. parasitica* infection; (3) Linalool positively modulated the immune response by increasing the abundance of beneficial Actinobacteriota; (4) Linalool stimulated the production of inflammatory cytokines and chemokines to lyse *S. parasitica* cells. In all, our findings showed that linalool possessed multifaceted anti-oomycetes potential which would be a promising natural antibiotic alternative to cope with *S. parasitica* infection in the aquaculture industry.

Keywords: *Saprolegnia parasitica*, linalool, anti-oomycetes mechanism, immune protective mechanism, antibiotic alternative

1. Introduction

Saprolegniasis is one of the most serious oomycete diseases in the aquaculture industry all over the world, infecting a wide host range (fish, crustaceans, amphibians, and their eggs et al.) with a conspicuous symptom (grayish-white cotton wool-like covering on the surface of host)[1, 2]. Oomycete infections lead to significant economic losses and serious damage to natural ecosystems[1]. As the infection intensifies, saprolegniasis can cause muscle ulceration, compromised immune system, respiratory problems, secondary infection, and finally result in host death[3]. *Saprolegnia*, *Achlya*, and *Leptolegnia* are key pathogens responsible for Saprolegniasis outbreaks[4], among them, *Saprolegnia* is proven to be the most virulent[5, 6]. *S. parasitica*, *S. ferax*, *S. delica*, and *S. diclina* are the main pathogens causing saprolegniasis[7], as a highly virulent group belonging to the phylum Oomycota, *S. parasitica* serves as the primary species in fish infections[8].

In the past, malachite green was highly effective in controlling *saprolegnia* infections, however, it has been banned worldwide for its carcinogenic and toxicological effects, which led to a sharp re-emergence of *Saprolegnia* infections in aquaculture industry[9]. Recently, various methods have been established to deal with *S. parasitica* infections, mainly including physical (water quality management, temperature control, and so on), chemical (hydrogen peroxide, natural antimicrobial compounds, and so on), and biological (probiotics, antagonistic microbe, and so on) methods[10]. Among these, antibiotics have served as the primary therapeutic and prophylactic tools for Saprolegniasis management in aquaculture. However, their overuse and abuse have resulted in antibiotic resistance among various aquatic pathogens[11]. Therefore, the increasing need for sustainable and eco-friendly strategies to combat *saprolegnia* infections has driven research into natural antimicrobial agents, due to their advantages in terms of avoiding antibiotic resistance issues, health safety, and ecological sustainability[12].

In our earlier studies, the anti-oomycetes activities of 12 plant essential oils (EOs) and 5 of their major compounds (linalool, pinene, limonene, myrcene, and terpinene)

were evaluated, and we found that linalool was one of the strongest anti-oomycetes compounds and selected for further study. Linalool, a monoterpene alcohol found in various EOs, has shown promising antimicrobial activity against a wide range of pathogens[13]. Our groups and other research teams have reported that linalool exhibits antimicrobial activity against bacteria such as *Escherichia coli*[12], *Listeria monocytogenes*[14], *Elizabethkingia miricola*, *Streptococcus pyogenes*[15], *Pseudomonas fragi*[16], *staphylococcus aureus*[17], *Brochothrix thermosphacta*[18] and *Aeromonas hydrophila*[19], as well as against oomycetes like *Colletotrichum lagenarium*[20] and *Phytophthora capsici*[21], and fungi including *Aspergillus flavus*[22], *Penicillium citrinum*, *Chaetomium globosum*[23], *Colletotrichum gloesporioides*, *Fusarium oxysporum*[24], *Botrytis cinerea*[25] and *Candida albicans*[26]. Although the antimicrobial activity of linalool has been extensively studied *in vitro*, the potential immune mechanism *in vivo* remains poorly understood.

Therefore, in this study, for the first time, we aimed to investigate the specific role of linalool in protecting *S. parasitica* infection at both of *in vitro* and *in vivo* level, and hope to unravel its potential molecular mechanisms through comprehensive analyses, including *in situ* fluorescence observation, microstructure observation, transcriptome profiling, histological examinations, gut microbiota analysis, and deep bioinformatics analysis. We believe this study will shed light on the comprehensive of anti-oomycete of linalool and contribute to the development of novel antibiotic alternative strategies to cope with *S. parasitica* infection in aquaculture industry.

2. Results

2.1 The *in vitro* anti-oomycete activity of linalool against *S. parasitica* CQT2

Figure 1A illustrated the anti-oomycetes activities of EOs and their major components against *S. parasitica* CQT2. Notably, linalool and the EO extracted from daidai flowers were particularly effective in restraining mycelium growth.

Linalool exhibited the MIC and MFC of 0.025% and 0.025% (v/v, 0.216 mg/mL) against spores, respectively (Figure 1B). Besides, both the MIC and MFC against mycelium growth were 0.05% (v/v, 0.432 mg/mL) and 0.1% (v/v, 0.864 mg/mL),

respectively. Various linalool doses were examined against *S. parasitica* CQT2 on linalool-containing plates to evaluate their impact on inhibiting mycelium growth. As shown in Figure 1C-D, the findings indicated the anti-oomycetes activity of linalool against *S. parasitica* CQT2 was dose-dependent. Figure 1E displayed mycelium growth on PDA plates under varying linalool concentrations. The viability of mycelium following linalool treatment was assessed using a PI staining assay, where non-viable (dead) mycelium appeared red after PI uptake. Untreated mycelium showed no detectable red fluorescent, indicating 100% viability. In contrast, distinct red fluorescent mycelium was observed after linalool treatment at both MIC and 2×MIC levels (Figure 1F).

2.2 Effects of linalool on cell membrane damage of *S. parasitica* CQT2

The *in situ* fluorescence observations were displayed in Figure 2A, with linalool treatment for 0 minutes, the mycelium structure remained undamaged, and the red and green fluorescence showed an even distribution. After 2 min, red fluorescence started appearing within the mycelium, accompanied by the entry of the styryl dye FM4-64, indicating damage to the cell membrane. As the duration of linalool treatment increased, the intensity of red fluorescence within the mycelium also increased, indicating a rise in the number of mycelial cell deaths along with severe damage to the cell membrane. When examined by SEM, the mycelium exhibited a smooth and intact surface in the control group (Figure 2B), while in the treated groups (Figure 2C), it appeared wrinkled, deformed, and shrunken (Red dashed quadrilateral). When examined by TEM, the untreated mycelium cell exhibited clear and intact ultrastructure in the cell membrane, cell wall, mitochondria, and rough endoplasmic reticulum (Figure 2D). However, after treatment with linalool, the cell membrane was wrinkled and seriously damaged, and mitochondria began to dissolve (Figure 2E). As shown in Figure 2F-G, the cell membrane structure disappeared, the cell wall became thinner, and some exhibited severe deformation and bending. Vacuoles appeared in the cytoplasm, and mitochondria and rough endoplasmic reticulum ruptured and dissolved (red circles). No intact organelles were present within the cytoplasm.

As shown in the GO classification analysis (Figure 2H), the cellular components were distributed across 8 groups, with "cell part" and "membrane part" being the most prevalent. The DEPs related to intrinsic component of membrane were screened out, categorizing them into 4 classes: related to ribosome function, related to DNA/RNA function, related to signal transduction and regulation, and related to cell metabolism (Figure 2I). Therefore, the cell membrane may be a potential target for linalool.

2.3 The *in vitro* anti-oomycete mechanisms revealed by transcriptome analysis

As shown in Figure S1A-B, a total of 4142 differentially expressed genes (DEGs) were discovered between the control and treatment groups, with 2016 showing upregulation and 2108 downregulation. Figure 2H presented the Gene Ontology (GO) analysis, providing a comprehensive evaluation of the functional aspects of genes and their products. These DEGs were categorized into 20 GO categories, with 6 associated with biological activities, where cellular and metabolic functions had the most DEGs. There were also 6 molecular function categories, with catalytic activity and binding containing the majority of DEGs. Additionally, Figure S1C demonstrated the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, revealing 10 metabolic (M) pathways, 2 environmental information processing (EIP) pathways, 2 cellular processes (CP), and 5 genetic information processing (GIP).

2.3.1 The ribosome biogenesis and protein synthesis of *S. parasitica* were prohibited by linalool

Figure 3A-B displayed the results of GO enrichment analysis, revealing the top 20 biological processes that differed significantly between the control and treated groups. Notably, in Figure 3B, the downregulated biological processes are highlighted, and remarkably, 19 of them were associated with "ribosome", such as ribosome biogenesis, ribonucleoprotein complex biogenesis, pre ribosome, and small-subunit process some. In addition, KEGG enrichment analysis in Figure 3C revealed that "Ribosome biogenesis in eukaryotes" was the most enriched pathway, aligning with the GO enrichment analysis results mentioned earlier. The pathway of ribosome biogenesis in eukaryotes (Figure 3D) showed that linalool influenced DNA

174 transcription, tRNA transport, rRNA processing and maturation (5.8S, 18S, and 25S),
175 and the biogenesis and assembly of ribosome subunits (40S and 60S) in the cell,
176 which might lead to the reduction of *S. parasitica* growth. We found that many DEGs
177 related to ribosome biogenesis and RNA polymerase (Figure 3E) are down expressed.
178 Figure 3A highlighted the upregulated biological processes, and notably, 11 of them
179 were linked to "amino acids", such as the sulfur amino acid, L-phenylalanine, alpha-
180 amino acid, tyrosine, and homocysteine. Amino acids serve essential roles within the
181 cell, acting as both the foundational components for synthesizing new proteins and as
182 precursors for various metabolic processes[27].

183 Molecular docking was carried out to explore the specific binding protein of
184 linalool with ribosome. The binding energy of linalool was -6.2 kcal/mol with NOP, -
185 4.8 kcal/mol with SNU13, and -5.0 kcal/mol with DKC1 (Table S1). Consequently,
186 NOP1 was selected as the receptor protein for subsequent molecular docking. Figure
187 3F illustrated the tertiary structure of ribosomal protein NOP1. Linalool formed
188 hydrogen bonds with glutamic acid (Glu155) and phenylalanine (Phe156) of NOP1
189 while exhibiting hydrophobic interactions with alanine (Ala181), arginine (Arg182),
190 valine (Val201), and isoleucine (Ile209) (Figure 3G). In summary, this interference
191 may hinder the ribosome's ability to execute normal biological processes, such as
192 protein synthesis, thereby impacting the growth and reproduction of oomycetes.

193 **2.3.2 The global metabolisms of *S. parasitica* were regulated by linalool**

194 In KEGG enrichment analysis, we found that 6 processes were associated with
195 metabolic pathways, such as cysteine and methionine metabolism, starch and sucrose
196 metabolism, and sulfur metabolism. Moreover, metabolic pathways were the most
197 annotated pathway which was in line with the results of KEGG annotation analysis.
198 As many changes were linked to metabolism, the Interactive Pathways Explorer
199 (iPath) was employed to comprehend the global differential metabolic response in *S.*
200 *parasitica* CQT2 (Figure 3I) which identified that linalool affects the global metabolic
201 regulation of *S. parasitica* CQT2.

202 **2.3.3 ABC transporters of *S. parasitica* were affected by linalool**

Some other important pathways were significantly enriched in our study, such as ABC transporter and sulfur relay system. The DEGs involved in ABC transporters (ABCA3, ABCB1, ABCC1, ABCG2, and PDR5) were shown in Figure 3H. In brief, these results demonstrated that cell membrane, protein synthesis, functions related to metabolism, and ribosome were significantly impacted by linalool and were potential drug targets of linalool.

2.4 The *in vivo* protective effect of linalool against *Saprolegniosis*

To study the protective effects of linalool on *S. parasitica* infection in fish, linalool was added to grass carp after *S. parasitica* infection (linalool therapeutic group, LT) or 48 h before infection (linalool prophylactic group, LP), and a positive control group (PC) without linalool and a negative control group (NC) without infection and linalool were set up (Figure 4A). The results showed that the survival rate of *S. parasitica* infected grass carp was significantly improved after treatment with linalool (Figure 4C), which proved greatly anti-*Saprolegnia* activity of linalool. In Figure 4B, there was no observable mycelium in both LT and LP groups, while the PC group displayed significant mycelium growth along with inflammation on the dorsum. The LZM activity was significantly increased in LP and LT group on the seventh day (Figure 4E), yet the AKP activity (Figure 4D) was reduced. However, there was no significant difference in the SOD activity (Figure 4F) between LP and LT groups during the whole feeding trial.

To explore the histomorphology alterations caused by *S. parasitica* infection and linalool treatment, we performed histomorphology evaluations through HE staining (Figure 4G). The findings revealed significant pathological changes in the kidney, liver, spleen, and skin of grass carp due to *S. parasitica* infection. In PC group, severe kidney damage was evident, characterized by enlarged glomeruli, glomerular cysts, interstitial bleeding, and infiltration of inflammatory cells. In addition, extensive vacuolation and expansion of intercellular space was observed in hepatocytes, with nuclei displaced toward one side. Cytoplasmic pyknosis and structural disintegration were observed in the pancreatic acinar cells. Meanwhile, the red and white pulp of the

232 spleen was poorly demarcated, the splenic corpuscle was irregularly shaped and
233 arranged, and the white pulp was structurally disrupted. Furthermore, there was a
234 notable increase in the number of macrophages and an expansion in the size of
235 melanoma-macrophage centers. *S. parasitica* not only caused damage to both the
236 dermal and epidermal layers but also induced muscle cell degeneration. Continuous
237 treatment of linalool for 7 days significantly improved kidney, liver, spleen, and skin
238 morphological integrity.

239 In the LP and LT groups, linalool alleviated tissue damage caused by *S. parasitica*
240 infection on the dorsal surface of grass carp, enhancing the healing capacity (Figure
241 4B). Histomorphology demonstrated that linalool could improve the integrity of organ
242 morphology in grass carp (Figure 4G). Transcriptomics of the spleen revealed that
243 linalool promoted wound healing, tissue repair, and phagocytosis to cope with *S.*
244 *parasitica* infection (Figure 5E). In all, our results indicated that linalool promoted
245 wound healing in grass carp.

246 **2.5 The *in vivo* Saprolegniasis protective mechanism of linalool revealed by**
247 **comparative transcriptome analysis**

248 To analyze the contrasting gene-level responses in grass carp spleens, a vital
249 immune organ, following *S. parasitica* infection and linalool treatment, spleen
250 samples were collected for transcriptome analysis. Compared to PC group, 2029
251 genes in LT group exhibited significant difference, with 809 DEGs up regulated and
252 1220 down regulated. Meanwhile, 671 genes were identified as significantly different,
253 with 609 DEGs up-regulated and 618 downregulated in LP group relative to PC group
254 (Figure S2A-B).

255 Based on the GO annotation analysis, a total of 2219 unigenes in the LT group and
256 520 unigenes in the LP group were classified into 3 primary functional categories
257 (Figure S2C). Within the biological process category, "cellular process," "metabolic
258 process," and "biological regulation" were the most prominent subcategories. In terms
259 of cellular components, "membrane part" and "cell part" were highly represented.
260 "Binding" and "catalytic activity" were the most prevalent subcategories within

261 molecular functions. Regarding KEGG annotations, the largest cluster within
262 organismal systems was the immune system, encompassing 209 genes (42.47%) in
263 the LT group (Figure S2D) and 38 genes (50.00%) in the LP group (Figure S2E). In
264 the environmental information processing category, most unigenes were associated
265 with signal transduction, with 227 (65.60%) in the LT group and 38 genes (52.00%) in
266 the LP group. These results suggested that the immune response and signal
267 transduction pathways were crucial for grass carp in dealing with *S. parasitica*
268 infections.

269 The GO enrichment analysis of the spleens illustrated in Figure 5A-B to reveal
270 distinct biological process. Those down regulated biological process were shown in
271 Figure 5A, compared to PC group, the “positive regulation of immune system
272 process” term (GO:0002684) and “regulation of body fluid levels” term
273 (GO:0050878) were enriched with the greatest number of DEGs in LT group. It is
274 worth noting that there are 9 terms related to "body fluids (especially blood)", such as
275 regulation of coagulation (GO:0050818), blood coagulation (GO:0007596), and
276 regulation of hemostasis (GO:1900046). In addition, those up regulated biological
277 process, the “ion binding” term (GO:0043167) was enriched with the greatest number
278 of DEGs in LT group. Figure 5B illustrated the GO terms with the largest number of
279 down regulated DEGs in LP group was “small molecule binding” (GO:0036094).

280 KEGG enrichment analysis was used to explore the functional pathways involving
281 DEGs. The 20 significantly enriched up regulated and down regulated pathways of
282 each group are shown in Figure 5C-D. Multiple pathways associated with immune
283 response were enriched in down regulated genes after linalool treatment in LT group,
284 including complement and coagulation cascades, Toll-like receptor signaling pathway,
285 IL-17 signaling pathway, C-type lectin receptor signaling pathway, T cell receptor
286 signaling pathway, B cell receptor signaling pathway, and so on (Figure 5C). Toll-like
287 receptor signaling pathway, RIG-I-like receptor signaling pathway, Complement and
288 coagulation cascades, and Chemokine signaling pathway were enriched with mostly
289 up-regulated genes. Furthermore, metabolic pathways were enriched with mostly

290 down-regulated genes in LP group. Some pathways associated with immune response
291 were significantly enriched, such as Antigen processing and presentation, Fc epsilon
292 RI signaling pathway, Intestinal immune network for IgA production, and so on
293 (Figure 5D). GO and KEGG enrichment analyses revealed the presence of numerous
294 immune-related genes within corresponding functional processes and pathways. The
295 expression of *fb*, *c1q*, *c2*, *c3*, *c4*, *c6*, *c7*, *c8A*, *c8b*, and *c9* genes were up expressed.
296 Additionally, there was a significant up expressed in the gene expression levels of
297 coagulation factors II (thrombin), VII, VIIi, VIII, X, coagulation factor IXb and IXa,
298 serpin peptidase inhibitor clade C/D/G/F, *fga*, *fgb*, *fgg*, protein C, plasminogen, and
299 plasminogen activator (Figure 5E). The differential expression of immune-related
300 DEGs implied their role in host defense against *S. parasitica* infection.

301 To ensure the regulation of immune-related DEGs by linalool, the expression of
302 several vital immune related genes (*cfh*, *masp2*, *c1r*, *c3*, *cfb*, *ap-1*, *il-1β*, and *il-6*) in
303 different groups were tested by RT-qPCR assay. The results showed that the
304 expression of these genes in the LP and LT groups significantly exceeded those in the
305 PC group ($P < 0.05$) (Figure 5H). Further exploration of immune-related genes
306 indicated that linalool treatment promoted the expression of genes linked to the
307 complement system and inflammatory factors. Additionally, analysis of 8 genes
308 revealed that the real-time fluorescence quantitative analysis results were consistent
309 with the expression trend of transcriptome analysis genes.

310 **2.6 Linalool regulated gut microbiota composition**

311 In our study, 862 OTUs were obtained from 12 samples through 16S sequencing
312 analysis, with 48 common OTUs shared across all samples (Figure 6E). Alpha
313 diversity analysis (Figure 6A-D) showed that the Simpson, Shannon, Chao1, and Ace
314 index of gut microbiota decreased after linalool treatment which indicated that
315 linalool reduced the abundance and diversity of gut microbes. PCoA analysis showed
316 a significant difference in microbial composition among the PC, LP, and LT groups
317 (Figure 6F).

318 The composition of gut microbiota in Figure 6G showed that the main dominant
319 bacteria in the intestinal tract are Proteobacteria, Actinobacteriota, Verrucomicrobiota,
320 Firmicutes, and Bacteroidota at the phylum level. Among them, compared with PC
321 group, an obvious tendency of greater relative abundances of phyla Proteobacteria and
322 Actinobacteriota was evidenced in LP and LT groups, the relative abundance of
323 Verrucomicrobiota was decreased. From the composition of the genus level, the
324 *Neochlamydia* decreased, while *Achromobacter* increased. It is worth noting that the
325 genus *Aurantimicrobium* (classified as Actinobacteriota) was increased in linalool
326 prophylactic and linalool therapeutic groups. Notably, among biomarkers with an
327 LDA SCORE exceeding 3, the genus *Actinomycetales* bacterium held the highest
328 number of positions, which contributed to the major differences between the positive
329 control and linalool treatment groups. Moreover, the potential metabolic pathways of
330 gut microbiota primarily showed enrichment in functions related to metabolism,
331 including carbohydrate, amino acid, and cofactors and vitamins metabolism (Figure
332 6I).

333 **2.7 Correlation analysis of potential related immune genes, specific microbial**
334 **species, and physical characteristics**

335 Correlation analysis showed that complement system were significantly positively
336 correlated to Actinobacteriota, AKP, and SR (survival rate), which, in turn, a
337 significantly negatively correlated with Verrucomicrobiota. Additionally, there was a
338 significant positive correlation between PPRs and AKP. Notably, IgA related immune
339 genes were correlated significantly with Proteobacteria (Figure 6J). In summary, our
340 findings suggested that linalool effectively modulated gut microbiota during *S.*
341 *parasitica* infection which was associated with immune response.

342 **3. Discussion**

343 **3.1 *In vitro* mode of action of linalool against *S. parasitica***

344 **3.1.1 Membrane disruption**

345 The cell membrane maintains cell integrity, regulates substance entry and exit,
346 while the cell wall provides structural support, protection, and promotes growth and

347 cell-to-cell connections[28]. The mycelium forms a dense structure to protect *S.*
348 *parasitica* spores from external factors. CLSM, SEM, and TEM observations
349 suggested that the cell membrane of the mycelium treated with linalool was damaged,
350 causing the intracellular components to leak out, and could not develop completely
351 (Figure 2A-G). Meanwhile, 678 genes related to the "intrinsic component of
352 membrane" showed significant expressed in GO enrichment analysis, suggesting that
353 linalool may enhance the expression of intracellular membrane components, reflecting
354 its impact on cell membrane structure or function. Gao et al. illustrated that linalool
355 caused significant damage to the cell wall/membrane of *E. coli*, resulting in the lysis
356 and death of bacterial cells[12]. Gao et al. demonstrated that linalool[14], *Changshan-*
357 *huyou* Y.B. Chang EO[29], and Fingered Citron EO[30] can disrupt the cell membrane of
358 *L. monocytogenes*. Lemon essential oil nanoemulsions altered the permeability of *E.*
359 *coli* cell membrane, affecting membrane potential, integrity, and efflux function[31].
360 Cruz et al suggested that alkamides in *Echinacea* disrupted the cell wall/membrane
361 complex, making it an ideal target for specifically inhibiting pathogens[32]. Hinokitiol
362 caused mycelium malformation by inducing cell component leakage and influencing
363 chitinase activity[33]. Therefore, linalool may act anti-oomycete effects against *S.*
364 *parasitica* by targeting the cell membrane.

365 ABC transporters utilize ATP hydrolysis energy to facilitate the export of harmful
366 compounds or essential lipids across bacterial and eukaryotic membranes[34]. The
367 DEGs involved in ABC transporters (ABCA3, ABCB1, ABCC1, ABCG2, and PDR5)
368 in our study, almost all of which have been linked to illness states usually involving
369 loss of function[35]. PDR5-related proteins in fungi played a role in excreting
370 xenobiotics, including antifungal agents[36]. Multidrug resistance-associated protein
371 (MRP) transporters were typically associated with multidrug resistance, cellular
372 detoxification, and drug metabolism[37]. To sum up, linalool may greatly inhibit
373 mycelium growth by interfering with protein synthesis from the “inside” and
374 damaging the integrity and permeability of the cell membrane and wall from the
375 “outside.”

376 **3.1.2 Reduction of ribosome biogenesis and RNA polymerase**

377 To further investigate the mechanism of linalool against *S. parasitica*, the gene
378 expression profiles of *S. parasitica* were compared (with or without exposed to
379 linalool). Many DEGs related to ribosome biogenesis and RNA polymerase were
380 down expressed, indicating that the linalool may influence translation, replication, and
381 repair processes, leading to increased protein diversity and potential disruption of
382 corresponding functions[38]. Linalool influenced DNA transcription, tRNA transport,
383 rRNA processing and maturation (5.8S, 18S, and 25S), and the biogenesis and
384 assembly of ribosome subunits (40S and 60S) in the cell, which might lead to the
385 reduction of *S. parasitica* growth. Ribosomes are assembled logistically by more than
386 200 ribosomal proteins (such as NOP, RIOK, and UTP) and RNA factors in a highly
387 coordinated manner[39]. Depletion of Nop-7-associated 2 (NSA2) led to decreased
388 nascent rRNA synthesis, reduced protein synthesis, and impacted cell proliferation
389 due to its critical role in ribosome biogenesis regulation[40]. The inhibitory effect of
390 myriocin on *Fusarium oxysporum* includes both membrane damage and interactions
391 with intracellular targets, such as proteins like RIOK2, within the ribosome biogenesis
392 pathway[41]. Innate immunity in *Caenorhabditis elegans* has been discovered to be
393 negatively regulated by NOL-6 (also named UTP22)[42]. The growth of *Aspergillus*
394 *niger* may be inhibited by cyclosporin A because it may prevent the transcription and
395 translation of the RNA polymerase III complex[43]. Rifampicin can prevent RNA
396 transcription in bacteria by interacting with the β-subunit of DNA-dependent RNA
397 polymerase, which can then inhibit protein biosynthesis[44]. These results indicated
398 that the down-regulation genes may limit the proteins necessary for normal cell
399 biogenesis and growth. Therefore, ribosome is a promising potential target of linalool
400 against *S. parasitica*.

401 **3.1.3 The influence on the metabolism function of *S. parasitica***

402 Metabolism function is crucial for cells to maintain homeostasis, survive, and
403 proliferate[45]. Linalool caused the activation of energy metabolism pathways,
404 compensating for the insufficient energy needed for cell survival[46]. In our study,

405 linalool was found to enhance amino acid metabolism, carbohydrate metabolism,
406 energy metabolism, and so on in mycelial cells, which suggested that cells may boost
407 functions related to energy metabolism to acquire additional energy for repairing
408 damage caused by linalool. Hence, linalool treatment caused intracellular energy
409 metabolism disruption, resulting in impediments to protein synthesis, cellular signal
410 transduction, DNA synthesis, and ultimately affecting cell viability and proliferation.

411 **3.2 *In vivo* mode of action of linalool against *S. parasitica* infection**

412 Recent studies demonstrated that the spleen serves antibacterial functions in various
413 fish species, with a high quantity of melano-macrophages that assist in phagocytosis
414 during the immune response[47]. The rise of macrophages and the change in the
415 pathological and physiological circumstances in fish are correlated[48]. Similarly,
416 histopathological analysis demonstrated that linalool effectively reduced *S. parasitica*
417 infection-induced damage in the spleen, indicating its potential immunomodulatory
418 effects on immune organs. Here we used comparative transcriptome analysis to
419 investigate potential genes and signaling pathways regulated by linalool in the spleen
420 of grass carp. Several genes associated with both innate and adaptive immunity, such
421 as complement molecules, pattern recognition receptors, inflammatory cytokines,
422 immunoglobulins, and chemokines, were differentially expressed as shown in Figure
423 5E-G.

424 **3.2.1 Enhancement of complement and coagulation system**

425 It is widely acknowledged that the complement and coagulation system in fish is an
426 important immune defense mechanism for fighting pathogens and maintaining
427 immune homeostasis[49]. The complement system is made up of over 30 different
428 proteins (complement receptors, internal components, and regulatory proteins) and 3
429 activation pathways (the classic pathway, the lectin pathway, and the alternative
430 pathway)[50]. In our study, the expression of *fb*, *c1q*, *c2*, *c3*, *c4*, *c6*, *c7*, *c8a*, *c8b*, and
431 *c9* genes were all up regulated after linalool therapeutic. Meanwhile, the c3a
432 anaphylatoxin chemotactic receptor (*c3aar1*) and mannan-binding lectin serine
433 protease 2 (*masp2*) genes were up expressed. During complement activation, *c3* and

434 *c5* split into molecules such as *c3a*, *c3b*, *c5a*, and *c5b*, of which *c3a* and *c5a* are
435 considered strong inflammatory mediators because of their ability to attract
436 leukocytes and promote inflammation and pathogen clearance[51]. *masp2* activates
437 complement components such as *c4* and *c2*, triggering a complement cascade reaction
438 that ultimately leads to pathogen rupture[52]. *c8b* is a subunit of the complement
439 component 8 (*c8*) protein, which helps form the membrane attack complex (MAC) for
440 cell lysis and immune defense against microbes[53]. In addition, high expression of
441 complement factor H like 3 (*chfl3*) prevents overactivation of the complement system
442 to avoid damage to their own tissues[54]. The effective antimicrobial role of the *c5a-*
443 *c5ar1* signaling pathway in the complement system has been identified. It functions
444 by enhancing neutrophil phagocytosis of fungi and macrophage-mediated fungicidal
445 activity to combat fungal infections[55]. In all, our findings suggested that linalool may
446 enhance the complement system which in turn activated host immune defense and
447 lysate *S. parasitica* cells.

448 The coagulation cascades are connected with the complement system. Linalool
449 significantly increased the expression of fibrinogen alpha (*fga*), beta (*fgb*), and
450 gamma (*fgg*) chain, which together encoded fibrinogen[56], fibrinogen is a crucial
451 component of blood clots to prevent blood loss, and also important in the initial stages
452 of wound healing[57]. Secondly, activated prothrombin *f2* is central in clot formation,
453 platelet aggregation, and activating other clotting factors. It also supports tissue repair,
454 cell growth, angiogenesis, and vascular integrity[53]. Thirdly, an increase in
455 plasminogen (PLG) levels can cause an immune response[58], and serve as a regulator
456 of the natural immune system by promoting the phagocytosis of phagocytes[59]. Taken
457 together, the upregulation of fibrinogen, prothrombin, and plasminogen in the
458 coagulation system suggested that linalool may promote wound healing, tissue repair,
459 and phagocytosis to cope with *S. parasitica* infection.

460 **3.2.2 Regulation of gut microbiota and increase the abundance of**
461 **Actinobacteriota**

Gut microbiota controls host growth and health status through various pathways[60]. Firstly, in our study, the function of gut microbiota is mainly enriched in metabolic-related pathways linalool increased the abundance of the beneficial phylum Actinobacteriota and decreased the abundance of the harmful phylum Verrucomicrobiota. Actinobacteriota plays a crucial role because Actinobacteria can produce enzymes, antibiotics, Short-chain fatty acids (SCFAs), signaling molecules, and immunomodulators[61]. Meanwhile, Actinobacteriota correlated positive with the complement and coagulation system according to the correlation analysis. Secondly, intestinal IgA production was correlated significantly with Proteobacteria which are the primary inducers of IgA production by B cells[62]. In summary, our findings suggested that linalool may positively modulate the immune response by increasing the abundance of beneficial phylum Actinobacteriota to *S. parasitica* infection.

3.2.3 Regulation of Inflammatory Factors

Pattern Recognition Receptors (PPRs) detect and initiate multiple signaling pathways upon recognizing Pathogen-Associated Molecular Patterns (PAMPs) on the surface of pathogens (bacteria, viruses, fungi, etc.), inducing subsequent host immune responses[63]. Myeloid differentiation primary-response protein 88 (MyD88) serves as the crucial signaling adaptor protein for Toll-like and interleukin-1 receptors, which is responsible for the conduction of multiple downstream signaling pathways[64]. The expression of *ap-1*, *p-38*, and *pI3k* were up regulate in downstream signaling pathways, causing higher expression of inflammatory factors and enhanced cellular oxidation and antioxidant levels[65]. Meanwhile, in our study, the gene expression levels of *il-1β*, *il-6*, *ccl19*, and *ccl5* which are closely related to the inflammasome, were up expression. Inflammasomes are protein complexes capable of detecting diverse inflammatory stimuli and are vital for eliminating pathogens or injured cells[66]. Therefore, linalool might have influenced the immune system response in grass carp, regulating the inflammatory processes.

4. Conclusions

In summary, our study proved that linalool is an effective natural antimicrobial agent against *S. parasitica*. Linalool disrupted the cell membrane, resulting in the leakage of cellular components and restricting ribosomal function, thereby inhibiting protein synthesis. Comprehensive analyses revealed the specific role of linalool in protecting *S. parasitica* infection skin inflammation in grass carp. Meanwhile, the complement and coagulation systems, inflammatory factors, along with Actinobacteriota, significantly involved in this protective effect (Figure 7). Further investigation would be carried out to explore and refine how linalool affects the inflammatory effects of *S. parasitica* on fish through signaling pathways and mechanisms. We believe that this study will contribute to developing an innovative alternative strategy, distinct from antibiotics, to cope with *S. parasitica* infection in the aquaculture industry.

5. Materials and methods

5.1 Oomycete strains and materials

S. parasitica used in this study was from our lab, which was isolated from grass carp (*Ctenopharyngodon idella*). Linalool (0.862g/mL), pinene (0.874g/mL), limonene (0.842g/mL), myrcene (≥95%), terpinene (≥95%), resazurin dye, and Prodium iodide (PI) were purchased from Sigma-Aldrich (USA). SYTO 9 and FM4-64 were purchased from ThermoFisher (USA). Potato dextrose agar (PDA) and glucose yeast extract (GY) were purchased from Guangdong Huankai Microbial Sci. and Tech. Co., Ltd, China.

5.2 Agar diffusion assay

The anti-oomycetes effects of EOs and 5 components (linalool, terpinene, limonene, myrcene, and pinene) were assessed by using the agar diffusion method. Initially, a 6 mm mycelium plug was inoculated at the center of the plates. Subsequently, 6 mm sterile filter paper discs were soaked in EOs and components for more than 12 h and placed symmetrically at a distance of 2.5 cm from the target mycelium plug. Sterile distilled water was used as negative control. Finally, the width of the colonies was measured once the mycelium in the control group was completely covered the plate.

Inhibition rate (%) = [(P1 − P2)/P1] × 100%, where, P1 = colony growth diameter (mm) of control group, P2 = colony growth diameter (mm) of treated group.

5.3 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) assays.

To determine the MIC and MFC of linalool on mycelium, 6 mm mycelium plugs were inoculated into a 24 well cell culture plate. The wells contained linalool at concentrations of 0.8%, 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, 0.0125%, and 0.00625%, dissolved in 1% Tween 20 and potato dextrose broth (PDB). Wells without mycelium plugs were served as negative controls. After incubating at 25°C for 48 h, the lowest concentration of linalool with no visible mycelium growth was recorded as MIC. Mycelium plugs showing no growth at this MIC concentration were then inoculated onto PDA plates and incubated for 7 days at 25°C to determine MFC.

The method for spores induction is similar as previously described by our previous research[67]. In a 96-well cell culture plate, 100 μL of linalool at concentrations of 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, 0.0125%, 0.00625%, and 0.003125% were dissolved in 1% Tween 20 and GY were added initially to each well, followed by the addition of 100 μL of spore suspension at a concentration of 1×10^6 spores/mL to each well. Malachite green and Tween 20 served as positive and negative controls, respectively. Following a 24-hour incubation at 20°C, 5 μL of resazurin dye was added. When the color of the wells turned to pink, the linalool concentration in the first non-pink well corresponded to the MIC. The culture liquid from wells with no visible mycelium growth was streaked onto new plates. After incubating at 25°C for 7 days, the well with the lowest linalool concentration with no visible mycelium growth was determined as MFC.

5.4 Effect of linalool on the mycelium radial growth rate of *S. parasitica*

Different concentrations of linalool were added to molten PDA plates at 50°C for quantitative assessment. A similarly sized mycelium plug was placed at the center of each plate. Once the mycelium in the control group without linalool reached the edge of the plate (approximately 60 h), the radial diameter of the mycelium was measured.

548 The calculation of growth inhibition was determined by assessing the percentage
549 inhibition of radial growth, as below:
550 PIRG % = [(R1− R2)/R1] × 100%, where, R1 = radial growth of control plate
551 (mm), R2 = radial growth of treatment plate (mm)

552 **5.5 Confocal laser scanning microscopy (CLSM) assay**

553 2.5.1 Analysis of viability of *S. parasitica*

554 The mycelium was treated with linalool at MIC (0.05%) and 2×MIC (0.1%) for 2 h,
555 and untreated control group was prepared simultaneously. The mycelium resuspended
556 in PBS was further treated with PI at a final concentration of 2.5 g/mL for 15 minutes.
557 PI is excited at a wavelength of 488 nm and emits light at 620 nm. Fluorescence
558 images were observed using CLSM (LSM880, Zeiss, Germany) at 40× magnification.

559 2.5.2 Analysis of membrane damage of *S. parasitica*

560 Mycelium cultured for 18 h was collected and sequentially treated with SYTO 9 at
561 a concentration of 20 μmol/mL, FM4-64 at a concentration of 10 μmol/mL, and
562 linalool at a concentration of 3%. Record observations at 0, 2, 8, and 20 min. Both
563 SYTO 9 and FM4-64 have an excitation wavelength of 488 nm and emit light at 620
564 nm and 520 nm, respectively. Fluorescence images were observed using CLSM
565 (LSM880, Zeiss, Germany) at 40× magnification.

566 **5.6 Scanning Electron Microscope (SEM) assay and transmission electron**
567 **microscopy (TEM) assay**

568 Mycelium exposed to MIC (0.05%) linalool for 2 h underwent fixation with 2.5%
569 glutaraldehyde at 4°C for 4 h to examine the impact of linalool on membrane damage.
570 Following a rinse with phosphate-buffered saline (PBS; pH 7.2), the fixed samples
571 experienced dehydration for 15 min using a graded series of ethanol concentrations
572 (10%, 30%, 50%, 70%, 90%, and 100%), followed by an additional 20 min in 100%
573 ethanol. Post-dehydration, ethanol was exchanged twice for 20 min each with tertiary
574 butanol. Subsequently, the samples were subjected to vacuum freeze-drying, gold-
575 spraying, and examination with SEM (Hitachi, SU8010). After fixation, the samples

576 were ultrathin-sectioned, subjected to negative staining using 1% phosphotungstic
577 acid for 5 min and observed by TEM (Hitachi, HT-7700).

578 **5.7 RNA-Seq and bioinformatics analysis**

579 Total RNA was isolated from linalool treated (exposed to 0.5×MIC linalool for 10
580 minutes) and untreated control groups using the TRIzol method. The messenger RNA
581 (mRNA) was enriched and fragmented into 200 bp fragments. These mRNA
582 fragments were reverse-transcribed into cDNA using random primers and reverse
583 transcriptase. The cDNA ends were modified and ligated to an adaptor. After PCR
584 amplification and purification, Illumina sequencing was performed. Quality control
585 removed low-quality reads. The mapped reads were assembled using StringTie. Gene
586 expression was quantified with the transcripts-per-million-reads method to identify
587 differentially expressed genes (DEGs) using a threshold of |log twofold change| ≥ 2
588 and FDR < 0.05. Afterward, functional enrichment analysis was performed using the
589 Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KEGG pathway
590 analysis was conducted using Goatools. The Majorbio cloud platform was used for
591 data analysis[68].

592 **5.8 Molecular docking**

593 Linalool may interact with specific domains of the ribosome, potentially
594 influencing its structure and function. Therefore, our investigation aimed to determine
595 whether linalool could bind to the ribosome-associated upstream proteins of *S.*
596 *parasitica*. As no crystal structure of *S. parasitica* ribosome proteins existed in the
597 Protein Data Bank (PDB), we performed homology modeling. The protein sequences
598 NOP1 (XP_012204452.1), SNU13 (XP_012198008.1), and DKC1
599 (XP_012193765.1) were obtained from GenBank, and homology modeling was
600 conducted using AlphaFold2. The most optimal configuration identified from the
601 homologous modeling results was chosen as the receptor protein for subsequent
602 molecular docking. The model was then evaluated using ProSA-web and
603 PROCHECK server. The secondary structure of linalool was then loaded on
604 PubChem. Both the ligand (linalool) and target proteins underwent processing with

AutoDockTools 1.5.6, including energy minimization, dehydration, and hydrogenation. The conformation exhibiting the lowest energy was selected as the most likely binding state. The ultimate docking outcome was visualized utilizing the open-source software PyMOLv2 and Discovery Studio Visualizer V2021.

5.9 Animal Experiments

5.9.1 Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, China (Approval No. 430516).

5.9.2 Linalool therapeutic and prophylactic assays

In this study, 4 groups were established: (1) Positive control (10 fish infected with *S. parasitica*). (2) Linalool therapeutic group (10 fish infected with *S. parasitica*, soaked in 0.00039% linalool in a 20L tank for 7 days). (3) Linalool prophylactic group (10 uninfected fish soaked in 0.00039% linalool in a 20L tank for 2 days, followed by the addition of 1×10^6 spores/mL secondary zoospores). (4) Negative control (10 uninfected fish without linalool treatment). Each group had 3 replicate tanks. The number of fish without visible mycelium on day 7 and the cumulative survival rate were recorded. The fish samples from the negative control, positive control, linalool therapeutic, and linalool prophylactic groups were collected for histopathological examinations. Tissue samples underwent fixation, sectioning, hematoxylin and eosin (H&E) staining, and microscopic examination using a microscope (BX53, Olympus).

5.9.3 16S rRNA and transcriptome sequencing and analysis

Genomic DNA was extracted from grass carp intestinal content samples and stored at -20°C following the instructions of the 16S rRNA gene pyrosequencing DNA kit. We conducted paired-end sequencing on the Illumina MiSeq platform, amplifying the V3-V4 regions with specific barcoded primers using a thermocycler PCR system. To unveil the intricate microbial diversity within the samples, we employed Quantitative Insights Into Microbial Ecology (QIIME) which can compute and visualize α-

634 diversity, β-diversity, and principal coordinate analysis (PCoA). Each sample was
635 measured 3 times to ensure robust results.

636 Spleen tissue was obtained from 3 fish in each group on day 7. These collected
637 spleen tissues were promptly flash-frozen in liquid nitrogen and then stored at -80°C
638 until they were ready for RNA isolation. RNA-Seq and bioinformatics analysis was
639 carried out as shown in Section 2.6.

640 In each group, 8 fish were utilized for immunological assays, and on day 7, blood
641 samples were collected from the tail veins using heparinized syringes and left to
642 coagulate overnight at 4°C. After centrifugation at 3500×g for 10 minutes at 4°C,
643 serum was obtained from each fish and stored at -80°C for subsequent analysis. Kits
644 from Nanjing Jiancheng Institute (Nanjing, China) were used to measure lysozyme
645 (LZY) activity, superoxide dismutase (SOD) activity, and alkaline phosphatase (AKP)
646 activity.

647 **5.10 Detection of immune-related gene expression**

648 Spleen tissues RNA extraction utilized the EZNA Total RNA Kit II (OMEGA
649 R6934-02). RNA concentrations were quantified using a spectrophotometer
650 (Eppendorf BioSpectrometer basic, Germany), and quality assessment was conducted
651 through 1% gel electrophoresis. Subsequent cDNA synthesis employed reverse
652 transcription with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher
653 Scientific, USA). RNA concentrations were quantified via spectrophotometry
654 (Eppendorf BioSpectrometer basic, Germany). Subsequently, cDNA was synthesized
655 via reverse transcription using the RevertAid First Strand cDNA Synthesis Kit
656 (Thermo Fisher Scientific, USA). Expression levels of complement factor H (*cfh*),
657 mannan-binding lectin serine protease 2 (*masp2*), complement component 1, r
658 subcomponent (*c1r*), complement component 3 (*c3*), complement factor B (*cfb*),
659 transcription factor AP-1 (*ap-1*), interleukin 1 beta (*il-1β*) and interleukin 6 (*il-6*) were
660 detected. The PCR procedure was described as follows: 95 °C for 30 s, followed by
661 40 cycles of 95 °C for 5 s and 60 °C for 40 s. Employing the CFX96 Touch Real-time
662 PCR Detection System (Bio-rad, USA), gene expression levels were assessed using

the $2^{-\Delta\Delta Ct}$ method, with β-actin serving as the reference gene. The primers in qPCR analysis are listed in Table 1.

5.11 Statistical analysis

The data was expressed as mean ± standard error, and all experiments were conducted in triplicate. Pearson correlation analysis was employed to investigate the correlation between potential immune-related genes in grass carp and their characteristics, the method was implemented in R Programming language. Statistical analysis was performed using GraphPad Prism 9 software, and the student t-test was conducted.

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Author contributions

Tao Tang was in charge of conceptualization, methodology, writing-original draft, writing-review & editing.

Weiming Zhong was in charge of data curation.

Puyu Tang was in charge of methodology.

Rongsi Dai was in charge of data curation.

Jiajing Guo was in charge of writing - review & editing.

Zhipeng Gao was in charge of funding acquisition, supervision, writing-original draft, writing-review & editing.

Declaration of interests

The authors declare no competing interests.

Data and code availability

The data that support the findings of this study are available in the supplementary material of this article and are available from the corresponding author upon reasonable request.

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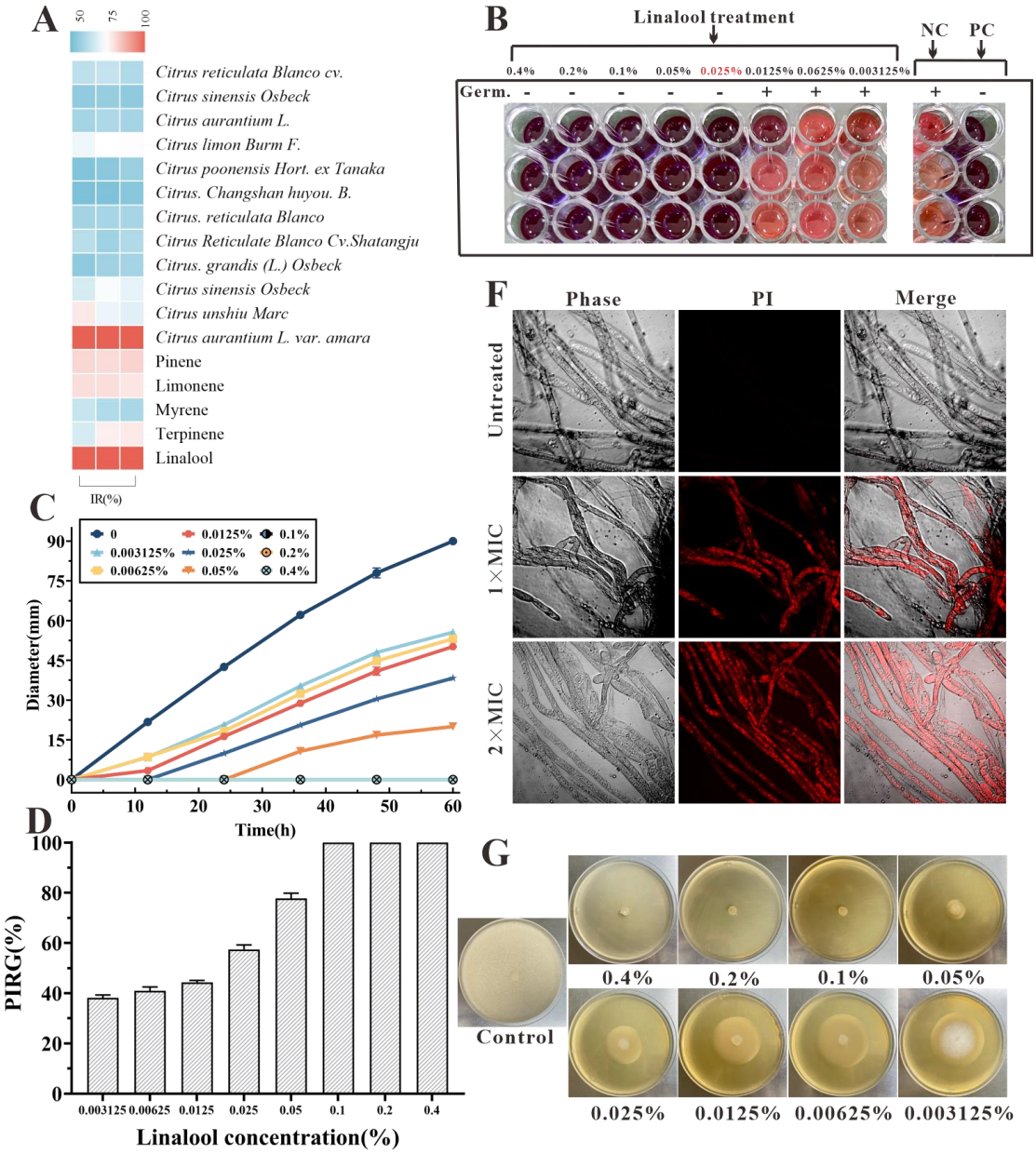
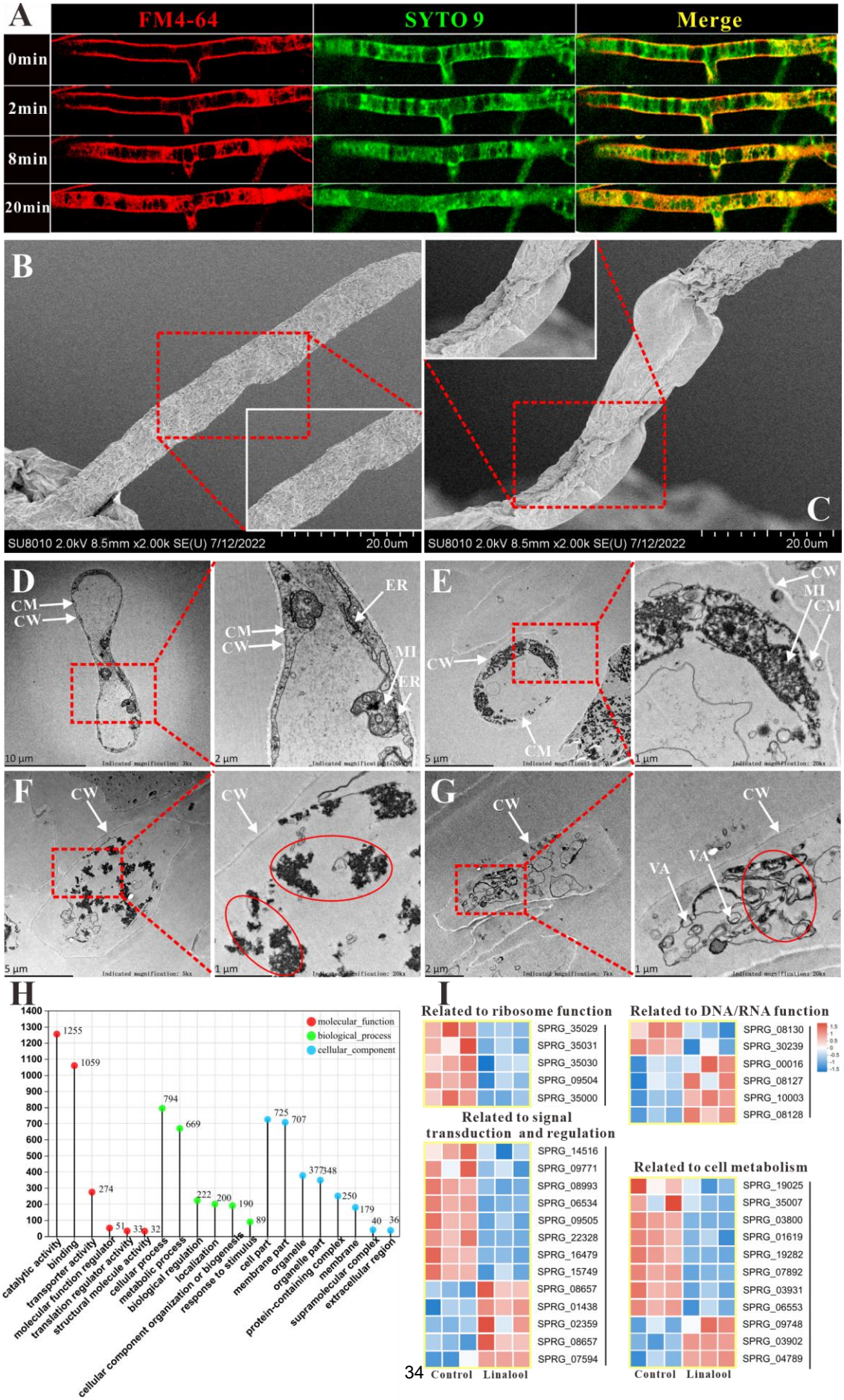


Figure 1. The *in vitro* anti-oomycetes activity of linalool against *S. parasitica* CQT2. A) Inhibition rate (IR) of EOs and their major components against *S. parasitica* CQT2. B) Determination of MIC of linalool against *S. parasitica* spores, employing malachite green (2.5 mg/mL) as a positive control (PC) and Tween 20 as a negative control (NC). C) Effects of linalool on mycelium growth inhibition over a 6 h period at varying concentrations. D) Examination of PIRG % in mycelium treated with linalool after 60 hours. E) Effects of different concentrations of linalool on mycelium growth on PDA plates. F) Effects of linalool on the viability of *S. parasitica* CQT2

966 through a PI staining assay was conducted under 3 conditions: Control (no linalool),
967 1×MIC (0.05%) linalool treatment, and 2×MIC (0.1%) linalool treatment.


A
FM4-64
SYTO 9
Merge
0min
2min
8min
20min
B
C
SU8010 2.0kV 8.5mm x2.00k SE(U) 7/12/2022
20.0um
D
CM
CW
ER
MI
10 μm
2 μm
E
CW
CM
MI
5 μm
1 μm
F
CW
5 μm
1 μm
G
CW
VA
2 μm
1 μm
H
molecular_function
biological_process
cellular_component
catalytic activity
binding
transporter activity
molecular function regulator
translation regulator activity
structural molecule activity
cellular process
metabolic process
biological regulation
localization
cellular component organization or biogenesis
response to stimulus
cell part
membrane part
organelle
organelle part
protein-containing complex
supramolecular complex
membrane
extracellular region
I
Related to ribosome function
SPRG_35029
SPRG_35031
SPRG_35030
SPRG_09504
SPRG_35000
Related to DNA/RNA function
SPRG_08130
SPRG_30239
SPRG_00016
SPRG_08127
SPRG_10003
SPRG_08128
Related to signal transduction and regulation
SPRG_14516
SPRG_09771
SPRG_08993
SPRG_06534
SPRG_09505
SPRG_22328
SPRG_16479
SPRG_15749
SPRG_08657
SPRG_01438
SPRG_02359
SPRG_08657
SPRG_07594
Related to cell metabolism
SPRG_19025
SPRG_35007
SPRG_03800
SPRG_01619
SPRG_19282
SPRG_07892
SPRG_03931
SPRG_06553
SPRG_09748
SPRG_03902
SPRG_04789
Control
Linalool

968

969 **Figure 2**. Effects of linalool on the cell membrane integrity of *S. parasitica* CQT2. A)
970 Observation of the effect of linalool on the cell membrane of *S. parasitica* CQT2
971 using the FM4-64 and SYTO 9 staining assay. B-C) SEM images of *S. parasitica*
972 CQT2 mycelium without (B) and with (C) linalool treatment. D-G) TEM images of *S.*
973 *parasitica* CQT2 mycelium without (D) and with (E-G) linalool treatment. CM: cell
974 membrane; CW: cell wall; ER: rough endoplasmic reticulum; MI: mitochondria; VA:
975 vacuoles. H) The GO classification of DEGs. I) DEPs involved in intrinsic component
976 of membrane.

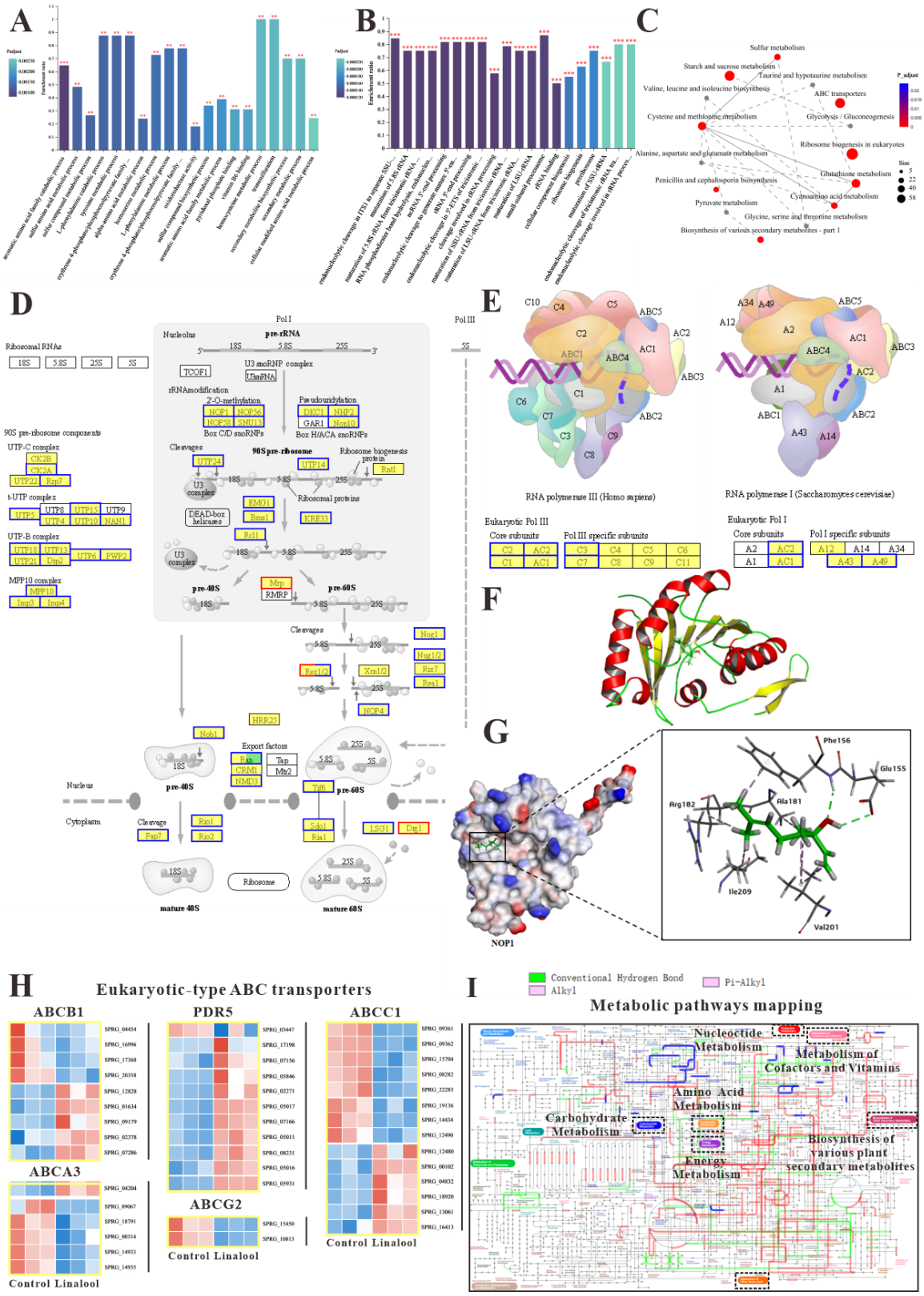
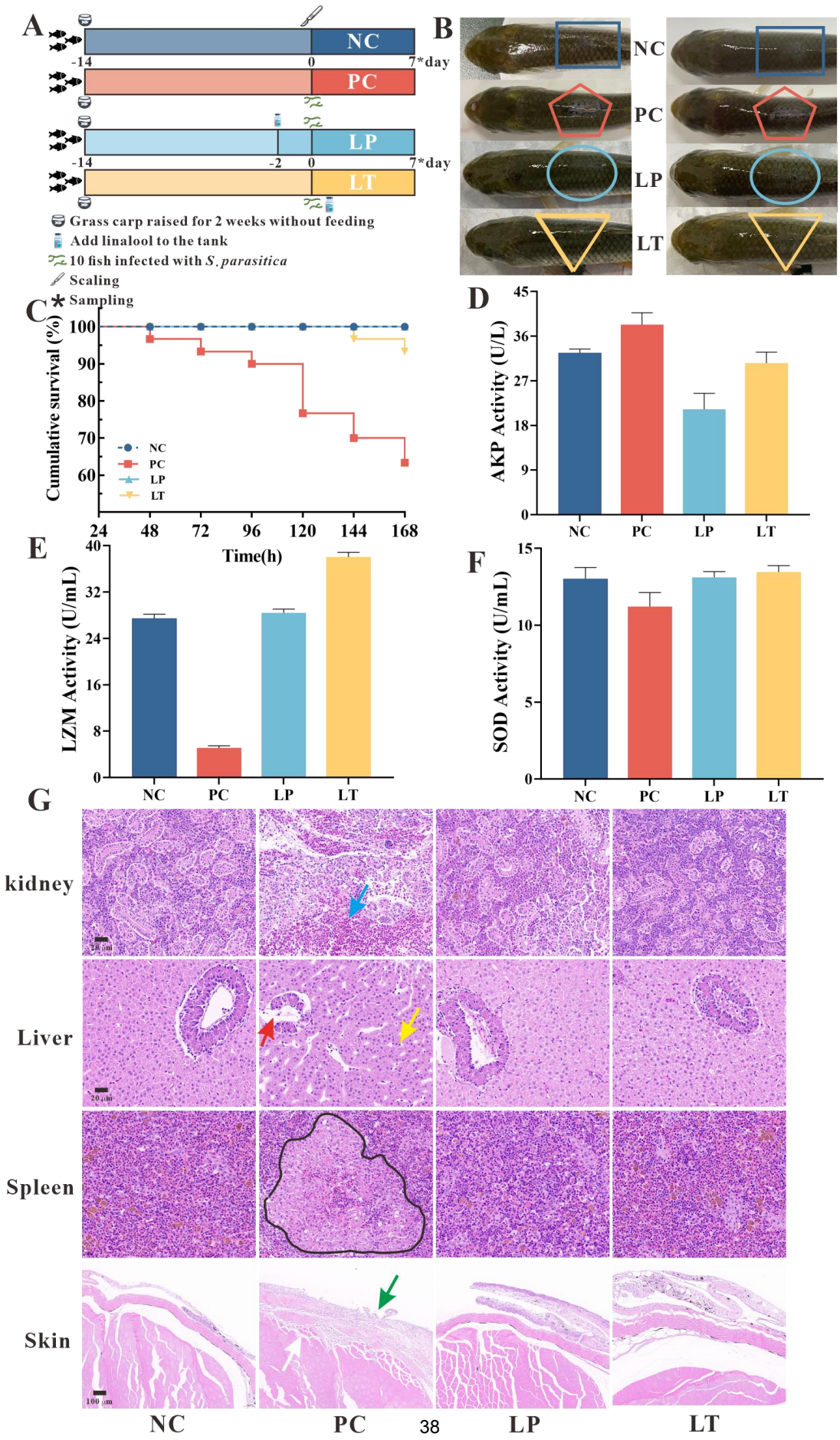
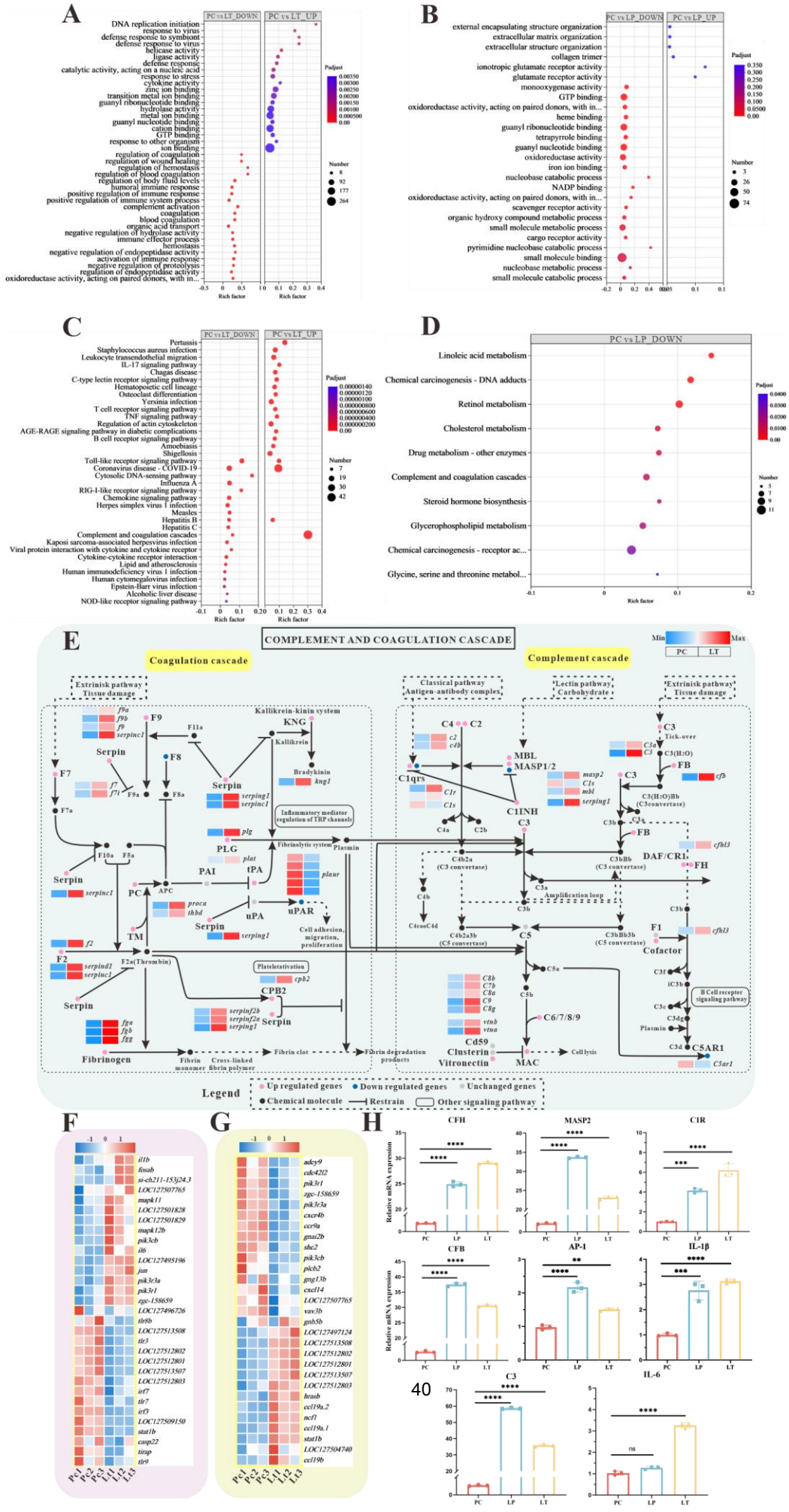


Figure 3. The *in vitro* anti-oomycete mechanisms revealed by transcriptome analysis. A-B) The GO enrichment of up and down regulated DEGs. C) The KEGG enrichment of DEGs. Significant enrichment was labeled as "*". $P < 0.05$, $P < 0.01$, and $P < 0.001$

981 were labeled as “*”, “**”, and “***”, respectively. D) Comparison of ribosome
982 biogenesis in eukaryotes pathway between the linalool treated mycelium and the
983 control group. E) Comparison of RNA polymerase pathway between the linalool
984 treated mycelium and the control group. F) The tertiary structure of NOP1. G)
985 Molecular docking of linalool with NOP1 involved enlarging the regions binding to
986 the NOP1 activation pocket to showcase the detailed amino acid structures. H)
987 Comparison of ABC transporters pathway between the linalool treated mycelium and
988 the control group. The red squares represented up-regulated genes, and the blue
989 squares represented down-regulated genes. I) Visual analysis of metabolic pathways
990 with iPath3.0. The figure represented gene set annotated pathways, red and green
991 represented pathways annotated by genes in different gene sets, respectively, and blue
992 represented pathways co-annotated by genes in two gene sets.


A
NC
PC
LP
LT
-14
0
7*day
-2
Grass carp raised for 2 weeks without feeding
Add linalool to the tank
10 fish infected with S. parasitica
Scaling
Sampling
B
NC
PC
LP
LT
C
Cumulative survival (%)
Time(h)
D
AKP Activity (U/L)
E
LZM Activity (U/mL)
F
SOD Activity (U/mL)
G
kidney
Liver
Spleen
Skin
NC
PC
LP
LT

994 **Figure 4.** Protective effect of linalool on grass carp infected with *S. parasitica*. A)
995 The experimental design. Grass carp were raised for 2 weeks without feeding, fish
996 without infection and linalool (Group NC), fish infected with *S. parasitica* (Group
997 PC), and 10 fish uninfected soaked water containing linalool for 2 days and then
998 1×10^6 spores /mL secondary zoospores were added (Group LP), and fish infected with
999 *S. parasitica* soaked for 7 days in water containing linalool (Group LT). B) The
1000 symptoms of *S. parasitica* infection in grass carps of different groups. C) The survival
1001 rates of grass carp infected with *S. parasitica* of different groups. D-F) Alkaline
1002 phosphatase (AKP), lysozyme (LYZ), and superoxide dismutase (SOD) activities in
1003 serum of grass carp of different groups. G) Histopathological analysis grass carp
1004 tissues in different groups. The arrows of different colors indicated: inflammatory cell
1005 infiltration in the kidney (blue arrow), cytoplasmic pyknosis (red arrow), nuclei
1006 displaced toward one side (yellow arrow), the red and white pulp was poorly
1007 demarcated, and a larger volume of melano-macrophage centers (black area), critical
1008 damage to the epithelium (green arrow) and myofiber (white arrow).


A
PC vs LT_DOWN
PC vs LT_UP
DNA replication initiation
response to virus
defense response to symbiont
defense response to virus
helicase activity
ligase activity
defense response
catalytic activity, acting on a nucleic acid
response to stress
cytokine activity
zinc ion binding
transition metal ion binding
guanyl ribonucleotide binding
hydrolase activity
metal ion binding
guanyl nucleotide binding
cation binding
GTP binding
response to other organism
ion binding
regulation of coagulation
regulation of wound healing
regulation of hemostasis
regulation of blood coagulation
regulation of body fluid levels
humoral immune response
positive regulation of immune response
positive regulation of immune system process
complement activation
coagulation
blood coagulation
organic acid transport
negative regulation of hydrolase activity
immune effector process
hemostasis
negative regulation of endopeptidase activity
activation of immune response
negative regulation of proteolysis
regulation of endopeptidase activity
oxidoreductase activity, acting on paired donors, with in...
Rich factor
Padjust
Number
B
PC vs LP_DOWN
PC vs LP_UP
external encapsulating structure organization
extracellular matrix organization
extracellular structure organization
collagen trimer
ionotropic glutamate receptor activity
glutamate receptor activity
monooxygenase activity
GTP binding
oxidoreductase activity, acting on paired donors, with in...
heme binding
guanyl ribonucleotide binding
tetrapyrrole binding
guanyl nucleotide binding
oxidoreductase activity
iron ion binding
nucleobase catabolic process
NADP binding
oxidoreductase activity, acting on paired donors, with in...
scavenger receptor activity
organic hydroxy compound metabolic process
small molecule metabolic process
cargo receptor activity
pyrimidine nucleobase catabolic process
small molecule binding
nucleobase metabolic process
small molecule catabolic process
C
PC vs LT_DOWN
PC vs LT_UP
Pertussis
Staphylococcus aureus infection
Leukocyte transendothelial migration
IL-17 signaling pathway
Chagas disease
C-type lectin receptor signaling pathway
Hematopoietic cell lineage
Osteoclast differentiation
Yersinia infection
T cell receptor signaling pathway
TNF signaling pathway
Regulation of actin cytoskeleton
AGE-RAGE signaling pathway in diabetic complications
B cell receptor signaling pathway
Amoebiasis
Shigellosis
Toll-like receptor signaling pathway
Coronavirus disease - COVID-19
Cytosolic DNA-sensing pathway
Influenza A
RIG-I-like receptor signaling pathway
Chemokine signaling pathway
Herpes simplex virus 1 infection
Measles
Hepatitis B
Hepatitis C
Complement and coagulation cascades
Kaposi sarcoma-associated herpesvirus infection
Viral protein interaction with cytokine and cytokine receptor
Cytokine-cytokine receptor interaction
Lipid and atherosclerosis
Human immunodeficiency virus 1 infection
Human cytomegalovirus infection
Epstein-Barr virus infection
Alcoholic liver disease
NOD-like receptor signaling pathway
D
PC vs LP_DOWN
Linoleic acid metabolism
Chemical carcinogenesis - DNA adducts
Retinol metabolism
Cholesterol metabolism
Drug metabolism - other enzymes
Complement and coagulation cascades
Steroid hormone biosynthesis
Glycerophospholipid metabolism
Chemical carcinogenesis - receptor ac...
Glycine, serine and threonine metabol...
E
COMPLEMENT AND COAGULATION CASCADE
Coagulation cascade
Complement cascade
Min
Max
PC
LT
Extrinisk pathway
Tissue damage
Classical pathway
Antigen-antibody complex
Lectin pathway
Carbohydrate
Kallikrein-kinin system
KNG
Kallikrein
Bradykinin
Serpin
Inflammatory mediator regulation of TRP channels
Fibrinolytic system
Plasmin
PLG
PAI
tPA
uPA
uPAR
PC
APC
TM
Cell adhesion, migration, proliferation
Plateletativation
CPB2
Fibrinogen
Fibrin monomer
Cross-linked fibrin polymer
Fibrin clot
Fibrin degradation products
C1qrs
C1INH
MBL
MASP1/2
C3
C4
C2
C4b2a (C3 convertase)
C3bBb (C3 convertase)
DAF/CR1
FH
Amplification loop
C4b2a3b (C5 convertase)
C3bBb3b (C5 convertase)
C5
C6/7/8/9
Cd59
Clusterin
Vitronectin
MAC
Cell lysis
Cofactor
F1
C5AR1
B Cell recepter signaling pathway
Legend
Up regulated genes
Down regulated genes
Unchanged genes
Chemical molecule
Restrain
Other signaling pathway
F
G
H
CFH
MASP2
C1R
CFB
AP-1
IL-1β
C3
IL-6
Relative mRNA expression
PC
LP
LT
Pc1
Pc2
Pc3
Lt1
Lt2
Lt3

1009

1010 **Figure 5.** Global transcriptomic analysis after *S. parasitica* infection and linalool
1011 treatment in grass carp and in-depth analysis of crucial KEGG pathway and DEGs. A-
1012 B) The GO enrichment of up and down regulated DEGs in LT and LP groups. C-D)
1013 The KEGG enrichment of up and down regulated DEGs in LT and LP groups. E)
1014 Complement and coagulation cascades pathway between *S. parasitica* infection and
1015 linalool treatment in the spleens of grass carp. F) 31 DEGs related to Toll-like
1016 receptor signaling pathway. G) 29 DEGs related to chemokine signaling pathway. The
1017 red squares represented up-regulated genes, and the blue squares represented down-
1018 regulated genes. H) Expression levels of *cfh, masp2*, *c1r*, *c3*, *cfb*, *ap-1*, *il-1β*, and *il-6*
1019 in the spleen revealed variations among the PC, LP, and LT groups. For RT-qPCR, the
1020 results were presented as the means ± SD and were analyzed using independent t-tests
1021 (**$p < 0.05$, ** $p < 0.001$, ****$p < 0.0001$).

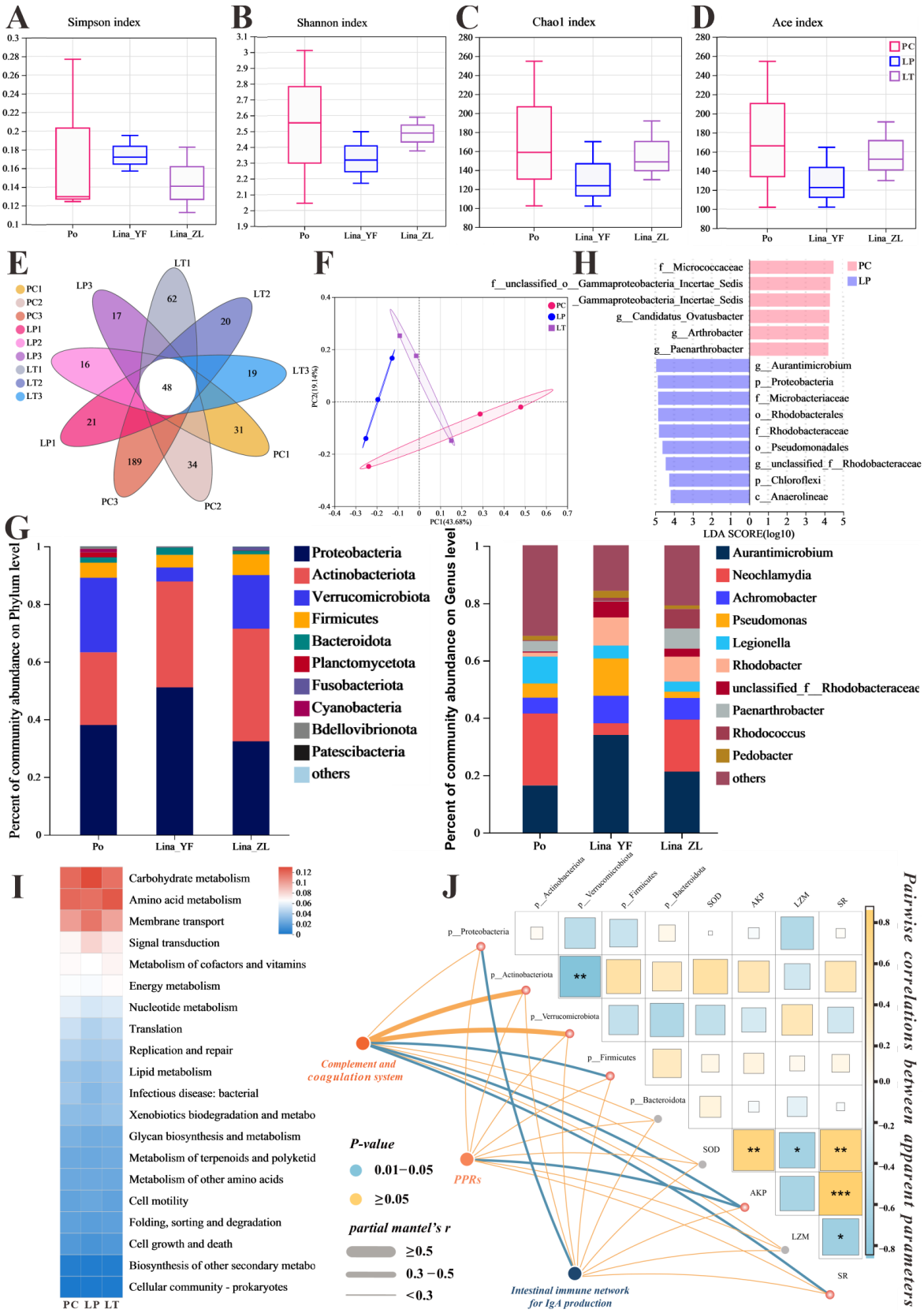


Figure 6. The effect of linalool on regulating gut microbiota of grass carp infected with *S. parasitica* and correlation analysis. A-D) The α diversity index comparison

among the different groups. E) The OTUs petal map of PC, LP, and LT groups. F) PCoA using Bray–Curtis distance revealed variations among the PC, LP, and LT groups (ANOSIM R = 0.3086, P = 0.061). G) Relative abundance of the top 10 species in the gut from the different groups (phylum and genus levels). H) Column chart of LDA value distribution. Discriminative biomarkers identified by linear discriminant analysis effect size (LEfSe) with logarithmic LDA score greater than 3.0. I) Heat map of the differences in predicted functional metabolisms within gut bacterial KEGG pathways. J) The correlation layout of potential related immune genes, specific microbial species, and physical characteristics. Pairwise comparisons of characterizations are presented, with a color gradient and block size denoting Pearson's correlation coefficients.

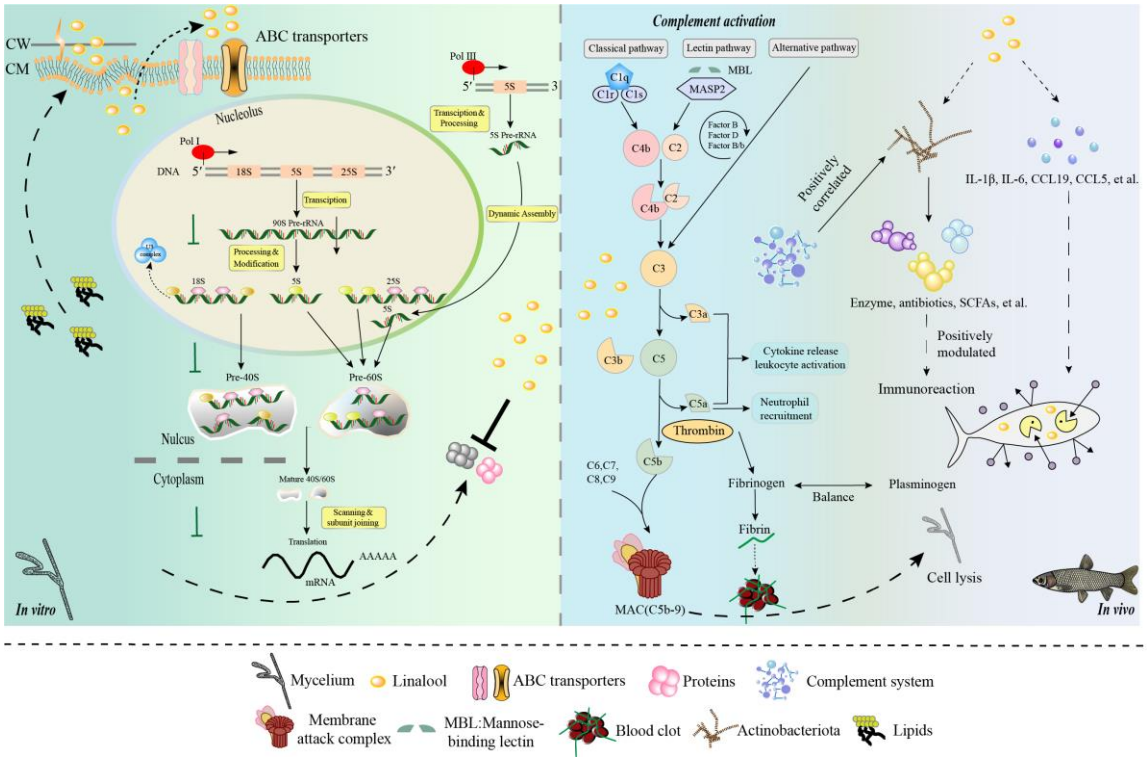


Figure 7. Model diagram of the mode of action of linalool on *S. parasitica* and grass carp. *In vitro*, (1) linalool influenced DNA transcription, tRNA transport, rRNA processing and maturation (5.8S, 18S, and 25S), and the biogenesis and assembly of ribosome subunits (40S and 60S) in the cell, which might lead to the reduction of S. parasitica growth; (2) Linalool disrupted the cell membrane, and the upregulation of

1042 glycerophospholipid metabolism likely represents the cell's response to cope with this
1043 damage; (3) ABC transporters contributed to metabolic resistance by pumping linalool
1044 out of the cell. *In vivo*, (1) Linalool enhanced the complement and coagulation system
1045 which in turn activated host immune defense and lysate *S. parasitica* cells; (2)
1046 Linalool promoted wound healing, tissue repair, and phagocytosis to cope with *S.*
1047 *parasitica* infection; (3) Linalool positively modulated the immune response by
1048 increasing the abundance of beneficial Actinobacteriota; (4) Linalool stimulated the
1049 production of inflammatory cytokines (*il-1β* and *il-6*) and chemokines (*ccl19* and
1050 *ccl5)* to lyse *S. parasitica* cells.

1051 Table 1. Primers for qPCR were used in this study.

Primer name	Primer sequence 5′-3′
c3-F	ACAAGCCCATCTACACGCCT
c3-R	GAAACCTCAGGAATGGCGTA
cfb-F	AGAATGGAGAGGTGACCCCA
cfb-R	CCGCTATCCTGACACACTCG
c1r-F	GGGTTTCGGAGGGTTTGA
c1r-R	CCTGTATTGCCACATCTTTCA
cfh-F	ATAGGAGGGAAATGTGGGC
cfh-R	TCCCGTCCACGCCATC
masp2-F	TGGGCACCAGGTTCATCG
masp2-R	GCTGTTTGACTACGCCCATCTT
il-1b-F	ATCTCCTCGTCTGCTGGGTG
il-1b-R	TTGAGAGTTTCAGTGACCTCCTTC
il-6-F	AAAATCTGACCTCAACCCTGG
il-6-R	TGTCCACCCTTCCTCTTGCT
ap-1-F	TCTCACTCAACAGATGCCAAAC
ap-1-R	CTTAGCGGCTGCGAGTTTAT
β-ActinF	GCTATGTGGCTCTTGACTTCG
β-ActinR	GGGCACCTGAACCTCTCATT

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