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Physiological evidence for the relationship between unsaturated fatty acid and cell permeability in extracellular polysaccharide synthesis and secretion *Agaricus bitorquis* (Quél.) Sacc. Chaidam

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In this study, the regulatory mechanism of exopolysaccharides from *Agaricus bitorguis* (Quél.) Sacc. Chaidam was investigated using *Lycium ruthenicum* Murr. Anthocyanins during cell growth and polysaccharide synthesis. The anthocyanin composition of *Lycium ruthenicum* Murr. was investigated by UHPLC-HRMS method, the anthocyanin derivatives of petunia ligand and proanthocyanidins, which was the main anthocyanin substance, and the results showed that the effect of LRA extract was better than that of the single component standard. Based on metabolomics, we have identified 2522 metabolites, including linoleic acid, α-linolenic acid and oleic acid were significantly up-regulated, and stearic acid, 16-hydroxypalmitic acid and 10-undecylenic acid were significantly down-regulated compared with the blank group. According to transcriptomics results and KEGG enrichment analysis, differential genes are mainly distributed in pathways such as unsaturated fatty acid metabolism and α-linolenic acid metabolism. By up-regulating stearyl CoA desaturase and acyl CoA oxidase, the content of α-linolenic acid, linoleic acid, and oleic acid increased the conversion of saturated fatty acids to unsaturated fatty acids, improved the fluidity of cell membranes, thereby enhancing the growth of ABSC mycelium.

Keywords: Anthocyanins, Cell membrane, Fluidity, Multi-omics joint analysis

Agaricus bitorguis (Quél.) Sacc. Chaidam (ABSC)is widely recognised as one of the most valuable and sought-after wild edible fungi due to its many beneficial properties. It has the characteristics of underground fruiting, huge fruiting bodies, strong stress resistance and rich nutrition[1]. It is rich in active substances such as polysaccharides, triterpenes and phenolic acids, and has been widely consumed by local herders as a natural food ingredient to resist hypoxia and improve physical strength[2]. At present, ABSC artificial cultivation technology is immature and large-scale cultivation has not been carried out. The traditional solid-state cultivation method has a long growth cycle and low yield. The results showed that the mycelium of ABSC obtained by liquid fermentation had the equivalent nutritional composition and medicinal value of the fruiting body, as well as obtained extracellular polysaccharides, phenolic acids and other functional active substances[3].

At present, liquid fermentation is widely used in the cultivation of edible and medicinal fungus due to its high quality of mushroom production, short production cycle and rapid mycelium germination[4]. Therefore, it is of great importance to conduct research on the regulation of extracellular polysaccharide synthesis and metabolism based on liquid culture and analysis of cell physiological characteristics of these rare edible mushrooms.

Lycium ruthenicum Murr. is a member of the *Solanaceae Lycium* L. *Lycium barbarum* L. contains a variety of bioactive compounds[5] such as the phenolic compounds, anthocyanins, proanthocyanidins, and polysaccharides[6]. Previous research showed that *Lycium ruthenicum* Murr. is a plant resource with high anthocyanidin content. Research has shown that anthocyanins have significant antioxidant, anti-inflammatory[7] and anti-aging activities, while improving lipid and glucose metabolism and regulating intestinal microbiota[8,9].

Therefore, *Lycium ruthenicum* Murr. Anthocyanins (LRA) may act as exogenous factors to improve the

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Abbreviations: ABSC, *Agaricus bitorquis* (Quél.) Sacc. Chaidam; EPS, Extracellular polysaccharides, LRA, *Lycium ruthenicum* Murr. (black wolfberry) anthocyanins; LRM, *Lycium ruthenicum* Murr. (black wolfberry)

fermentation environment to regulate fungal growth and metabolism, increase mycelial growth and polysaccharide synthesis. Fatty acid composition is essential for energy reserve and maintenance of membrane lipids[10]. Under adverse environmental conditions, cell membranes can sense stress and initiate responses to protect cells by adjusting stress perception and stiffness of the cell structure. Membrane integrity, function and fluidity are largely influenced by the lipid composition and unsaturated fatty acid content of plants[11,12]. Unsaturated fatty acids are synthesised by fatty acid desaturases, which introduce double bonds into the hydrocarbon chains of fatty acids. Fatty acid desaturases also play an important role in fatty acid metabolism and in maintaining the biological function of membranes[13]. Previous studies have shown that the environmental stresses such as drought, cold, salt and heat can induce changes in fatty acid composition, particularly linolenic acid. ABSC grows at high altitudes and is subject to environmental stresses that affect the levels of unsaturated fatty acids.

This project aims to analyse the relationship between differential genes and differential metabolites in the regulatory process of ABSC by combining transcriptomics and metabolomics analysis of LRA, and to explore the role of functional components of LRA on the fluidity of ABSC cell membranes. Based on the LRA regulation data, it provides a theoretical foundation and technical basis for liquid deep culture of mycelium and synthesis of polysaccharide functional factors, which can promote the basic research and industrial application of traditional edible fungal resources with characteristics in remote areas, and the research results have important theoretical significance and social benefits, and provide research examples for the exploitation and utilization of other special macrofungal resources.

Materials and Methods

Material

ABSC used in this study are preserved in the Food Engineering Laboratory of Qinghai University; *Lycium ruthenicum* Murr.; Petunia anthocyanin standard (99%), proanthocyanidin standard (99%).

Experimental methods

Liquid culture ABSC

In this study, the liquid culture of ABSC was used to conduct the fermentation for 4 days at 25°C and 100 rpm in 1000 mL flasks (300 mL liquid culture medium)[14].

Preparation of extracellular polysaccharides (EPS)

The filtered mycelium was weighed according to 1; 20 material to liquid ratio, ultrasonic treatment for 20 min. Then the filtrate was separated for purification and lyophilization[15].

Preparation of LRA

The LRM was mixed with 80% ethanol solution according to the material-liquid ratio of 20: 1, placed in an ultrasonic apparatus at 48°C for 25 min, the secondary extract was removed by low-temperature vacuum distillation, adsorbed and desorbed with AB-8, and the desorbed solution was concentrated and lyophilised to obtain the crude extract of LRA. At an earlier stage, the research team experimentally determined that the increase in EPS content and mycelial biomass was most effective when LRA was added up to (0.06 mg/mL), therefore, this experiment used the previous data and added anthocyanin at a concentration of 0.06 mg/mL[16].

Assay method

Structural characterization of mycelium

Each sample of mycelium was coated with gold powder at reduced pressure and examined using a scanning electron microscope[17].

Polysaccharide content determination

Phenol-sulfuric acid method[18].

Determination of biomass

Wet gravity method[19].

UPLC-HRMS analysis of the chemical composition of LRA

(1) Preparation of the sample solution

Weigh 20 mg of LRM, place it in a centrifuge tube (1.5 mL), add 0.3 mL of 40% methanol in water, vortex and mix, centrifuge at 16000 g 4℃ for 15 min, and remove the supernatant.

(2) Identification of compounds

The data obtained from XCMS extraction were then structural identified by standard spectrogram database matching. We conducted a comparison between the UPLC-HRMS results and the secondary map of the mass spectrometry database.

LC-MS/MS Analysis

LC-MS/MS analyses were performed using an UHPLC (1290 Infinity LC, Agilent Technologies)

coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) in Shanghai Applied Protein Technology Co., Ltd. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25-1000 Da, and the accumulation time for product ion scan was set at 0.05 s/spectra. The product ion scan is acquired using information dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with ± 15 eV; declustering potential (DP), 60 V(+) and −60 V(−); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

RNA Isolation and Illumina Sequencing

Total RNA was extracted using the Total RNA Miniprep Kit(Axygen, Hangzhou, China). RNA quality was assessed using the Agilent 2100 Bioanalyzer(Agilent Technologies, Santa Clara, CA, USA). ORF prediction was performed utilizing the TRINITY method. Gene expression and differential expression levels were determined using RSEM and edgeR software tools. To obtain sequences of high similarity, unigene sequences were first aligned to sequences lodged in the protein databases NR[20], KEGG[21], Swiss-Prot[22], and COG[23] (*e*-value <0.00001) using the blastx tool, and the nucleotide database NT (*e*-value <0.00001) using blastn[24].

Targeted metabolism of medium and long chain fatty acids

Metabolite extraction: Thaw the sample on ice and take 30 mg of the sample into a 2 mL glass centrifuge tube. Add 1 mL of chloroform methanol solution, sonicate for 30 min, take the supernatant, add 2 mL of 1% sulfuric acid methanol solution, place on an 80℃ water bath, methylate for half an hour, then extract with 1 mL of n-hexane, wash with 5 mL of pure water, and pipette 500 mL of the supernatant μL. Join 25 μL of methyl ortho Nonadecylic acid is used as the internal standard, mixed evenly and added into the injection bottle, and then tested by GC-MS. The injection volume is 1 μL. Split ratio 10:1, split injection.

Chromatographic Conditions: The sample was separated using a capillary column (Agilent 19091S—433UI:HP-5 ms, 30 m x 250 μM x 0.25 μM) in a gas chromatography system. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min. In the sample queue, a quality control (QC) sample was inserted at regular intervals to monitor and assess the stability and repeatability of the system.

Results

UPLC-HRMS analysis of the chemical composition of LRA

In this experiment, UHPLC-HRMS was used to collect data from LRA, and peak confirmation, secondary spectrum check, and then marking the peak number and numerical order in the positive and negative ion maps (Fig.1). The data were imported into the local TCM-related standard atlas database for secondary mass spectrum retrieval and comparison, and 1060 TCM chemical components were analysed and identified, and a total of 92 categories were identified. Among them, the top 6 compounds and their subcategories mainly include flavonoids, carboxylic acids, benzene, organic oxides, cassia bark acids and their derivatives, cytochalasins, coumarins and their derivatives, phenols, pyridines and their derivatives, fatty acyl groups and other compounds.

UHPLC-HRMS was used to study the composition of LRA and 11 anthocyanins were identified (Table 1). According to the sum of their peak areas in the total peak area, petunia anthocyanin derivatives accounted for about 90% of the total anthocyanins in LRM, and proanthocyanidins accounted for 4%, which were the major anthocyanins. The above study shows that LRM not only contains a large amount of anthocyanins, but also has more proanthocyanidins, among which anthocyanins are less stable and easily denatured. They were easily decomposed into anthocyanins and glycosides in aqueous solution. The antioxidant activity of the newly formed anthocyanins is much higher than that of similar anthocyanins, which can effectively reduce oxidative damage to cells and maintain cell viability[25].Glucoglycoside can also be used as a precursor for the synthesis of monosaccharides such as mannose and arabinose to enhance the synthesis of nucleotide monosaccharides by ABSC, which may eventually accelerate the synthesis of polysaccharides by fungal cells.

Effect of the addition of different anthocyanins on the fermentation of ABSC and its polysaccharide synthesis

According to the above identified LRA, the main materials are petunia anthocyanin and proanthocyanidins, so to investigate LRA and its main components on the influence of mycelial cell fermentation system. As well as it explored different anthocyanins substances on the influence of ABSC fermentation and polysaccharide synthesis. Early Group through single factor test proved that LRA extracts of 0.06 mg/mL, ABSC growth and polysaccharide synthesis promote the best effect, so the

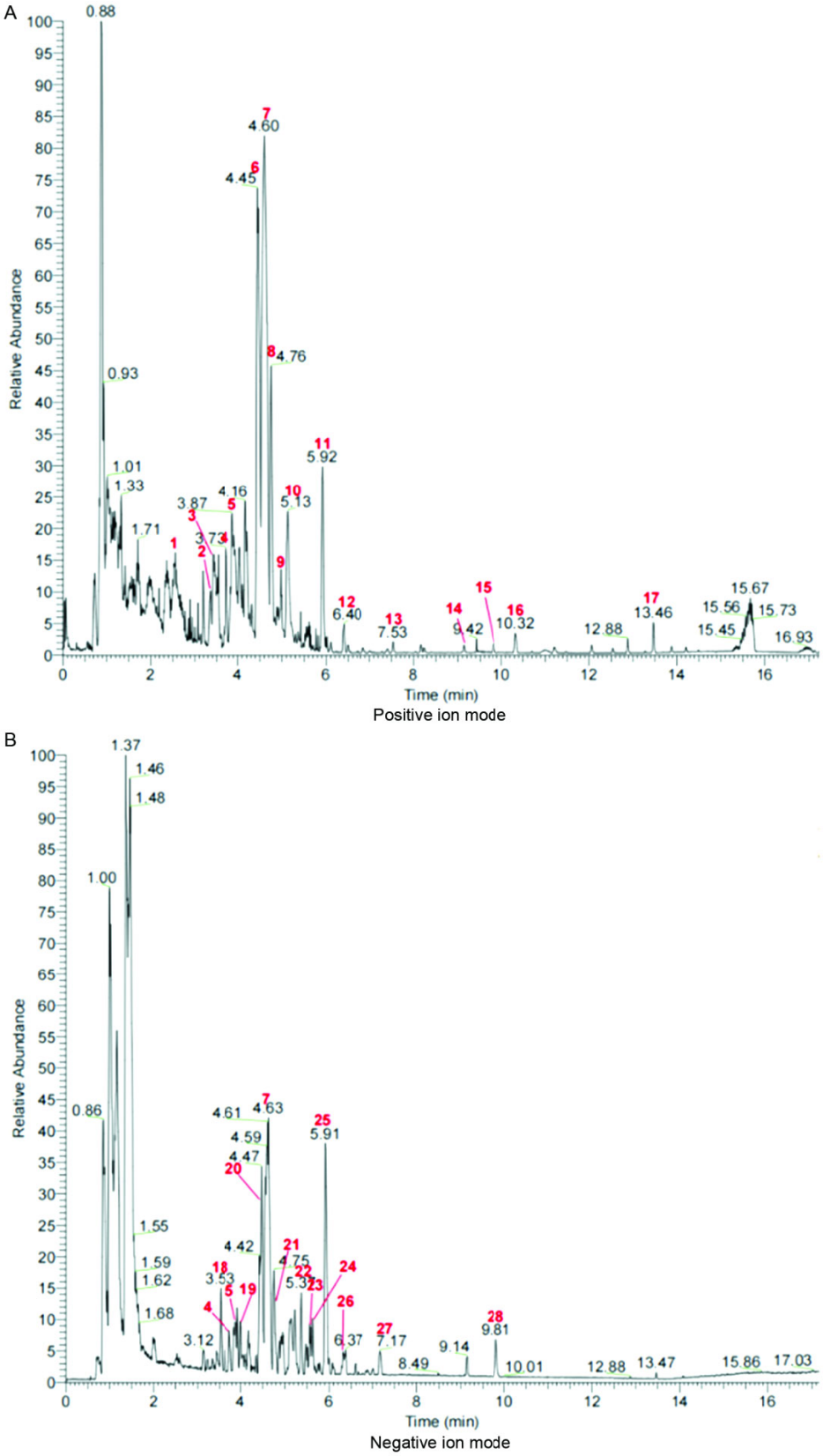

Fig. 1 — BPC diagram-mark peak

Table 1 — Structural identification of crude extracts of LRA

Sl. No.	Mass Noun	parent ion (*m/z*)	retention time (min)
1	Petunia-3-coumaranyl rucoside-5-glucoside	933	15.25
2	Petunia pigment-3-O-rint (coumaryl) -5-O-glucoside	317	14.25
3	Petunia pigment-3-O-kidney sugar (glucose-coumaroyl) -5-O-glucoside	316	13.16
4	Fphinidin-3-0-glucoside	465	4.26
5	Fphinidin-3-0- (6-0 acetyl) glucoside	919	4.90
6	Petunia-5-0-glucose	787	7.00
7	Mallow pigment-3-5 diglucoside	654	7.27
8	Anthocyanin-3-O- (6-O-acetylated) -glucoside	491	11.62
9	Seiya-3-rcoside	611	15.25
10	Peony element-3-caffeoyl racoside-5-glucoside	949	13.8
11	Petunia-3-0- (6-0-parmaranyl) rcoside-5-0-glucoside	933	4.88

optimal amount of anthocyanins extracts is 0.06 mg/mL, according to the above other glycosides material ratio conversion, petunia anthocyanins standard of 0.054 mg/mL, protoanthocyanins standard of 0.0024 mg/mL, dynamic determination of the ABSC liquid fermentation process.

Effect of different anthocyanins on mycelium biomass in fermentation of ABSC

During the liquid fermentation of ABSC, the mycelium biomass of each test group gradually decreased with the fermentation time(Fig. 2A). Compared with the blank group, at the highest peak at 72 h, the Anthocyanins extracts group had the highest mycelium biomass, followed by Procanthocyanins, Petunia group increased by 80.2%, 17.8% and 10.1%, respectively, (P<0.05). The growth rate of mycelium biomass in the Anthocyanins extracts was greater than the sum of the generations of the Proanthocyanidins, and Petunia groups,(Fig. 2B) with significant differences (P<0.05). There is a linear relationship between Anthocyanins extracts, and Proanthocyanidins Petunia anthocyanins group in bacterial biomass, R^2=0.9761(Fig. 2C). Therefore, in the liquid fermentation process of ABSC, Anthocyanins extracts have the best synergistic effect on mycelium biomass, and its regulation mechanism may be related to the

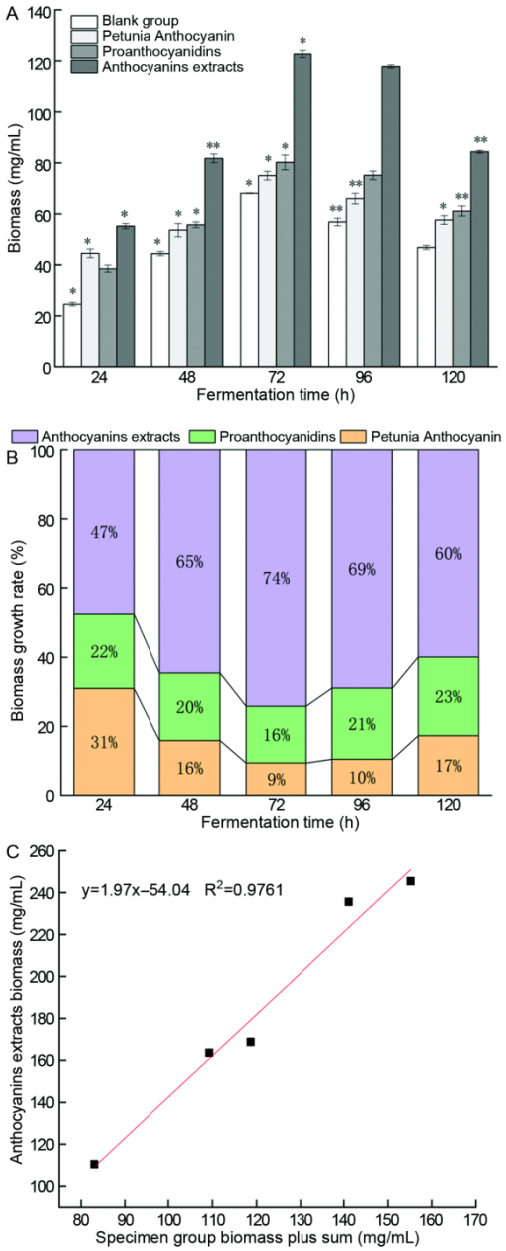

Fig. 2 — Effects of different anthocyanins on mycelium biomass. (A) Effect of anthocyanins on mycelial biomass; (B) Effect of different anthocyanins on the growth rate of mycelial biomass; and (C) Petunia Anthocyanin, Proanthocyanidins biomass addition, and the biomass of Anthocyanin extract S group. Note: P< 0.05 is * indicates significant difference, and P< 0.01 is **

synergistic effect of anthocyanins in crude extract such as Proanthocyanidin and Petunia anthocyanins.

Effect of different anthocyanins on EPS in fermentation of ABSC

EPS of each test group first increased and then decreased with the fermentation time (Fig. 3A). Compared with the blank group, Anthocyanins extracts

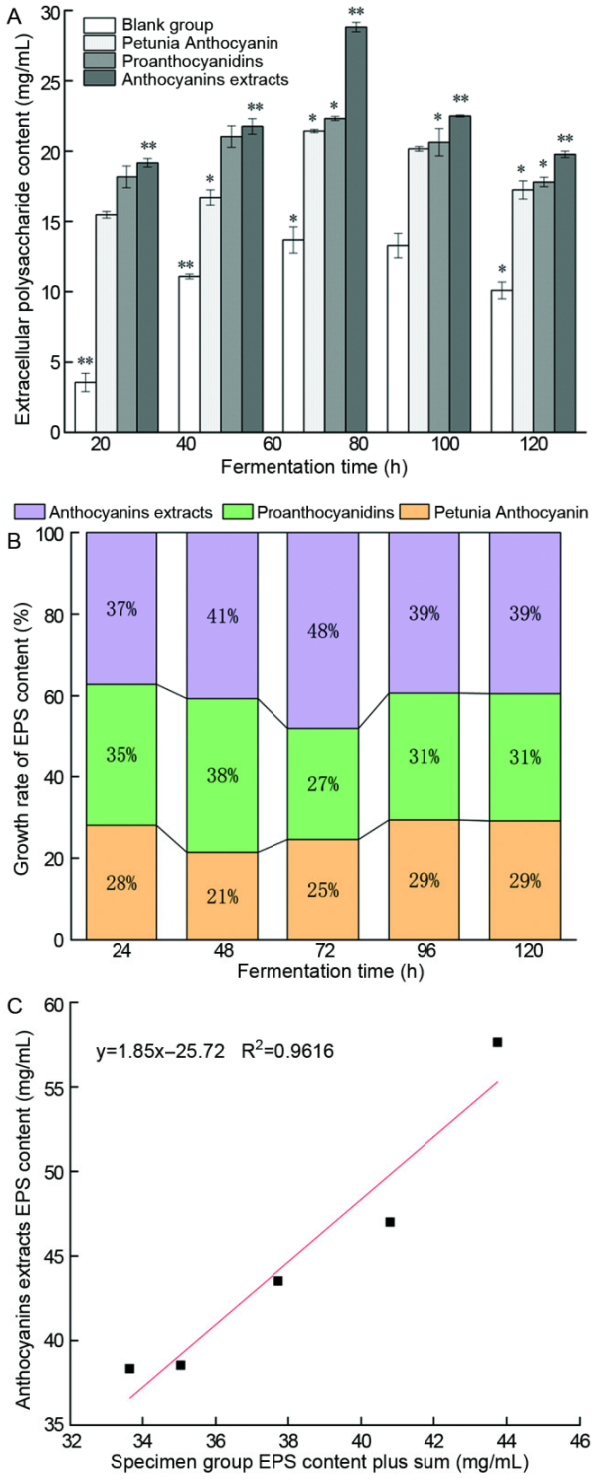

Fig.3 — Effect of different anthocyanins on the exopolysaccharides content. (A) Effect of different anthocyanins on extracellular polysaccharides;(B) Effect of different anthocyanins on the growth rate of fermentation broth polysaccharides; and (C) Petunia Anthocyanin, proanthocyanidin fermentation broth polysaccharide addition and the linear relationship between fermentation broth polysaccharides with crude extract of anthocyanins group

had the highest EPS, followed by Proanthocyanidins, and Petunia anthocyanin group ($P< 0.05$). At this time, the polysaccharide content of anthocyanins extracts, Proanthocyanidins, and Petunia increased by 110.6%, 63.2% and 56.6% (Fig. 3B), at 72 h, the growth rate of the anthocyanins extracts of 110.6% was similar to the number and 119.8% of the Proanthocyanidins, and Petunia standard group (Fig. 3C), there is a linear relationship between Anthocyanins extracts and Proanthocyanidins Petunia anthocyanins group, R^2=0.9616. Therefore, it is speculated that anthocyanins have a good effect on promoting the synthesis of exellular accharides and the regulation mechanism may be related to the synergistic effect of Anthocyanins extracts.

In conclusion, compared with Petunia anthocyanin and Proanthocyanidins, Anthocyanins extracts could effectively promote the biomass and polysaccharide production of ABSC. Therefore, Anthocyanins extracts was determined as the best regulator, and the mechanism of regulating the growth and polysaccharide metabolism of ABSC was further explored.

Structural characterization analysis of mycelium

In order to study the effect of the addition of crude extract of LRA and its main components (Petunia and Proanthocyanidins) on the morphology of ABSC, they were cultured and fermented in the media of different anthocyanins[26]. After observing the hyphae morphology with a magnification of 1000 times by SEM, it was found that the mycelium structure of ABSC changed differently after adding anthocyanin extracts (Fig. 4). The blank group had finer hyphae and a rough, dry, and unsmooth surface. Petunia anthocyanin group and Proanthocyanidins group had slight folds and creases. The anthocyanins extracts group was the thickest and longest, with smooth and complete surface. Therefore, we speculate that the addition of LRA leads to increased membrane fluidity and altered permeability, which facilitates more nutrient exchange and secretion of metabolites, thus accelerating the growth and synthesis of polysaccharide in ABSC.

Untargeted metabolomics analysis of ABSC

To fully elucidate cell membrane permeability during ABSC fermentation, we add LRA during fermentation for all subsequent transcriptomic and non-targeted metabolomic analyses; use hyphae without LRA as a control. The non-targeted metabolomic analysis was performed to further

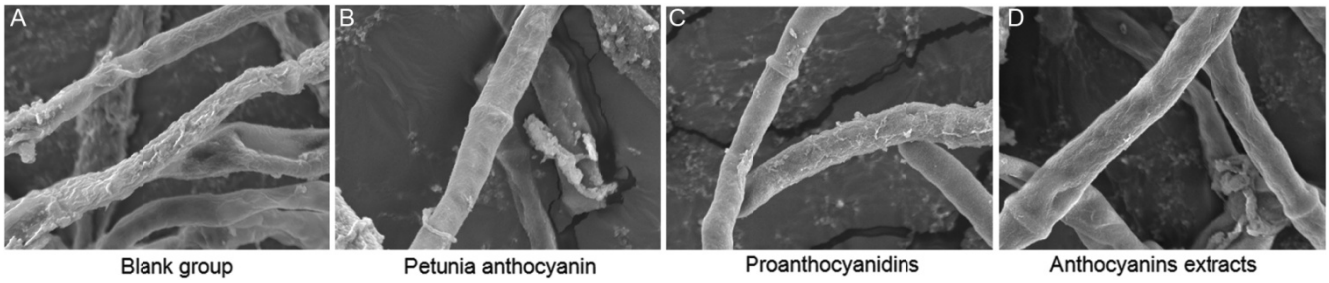

Fig.4 — Effect of different anthocyanins on the mycelial structure (×1000)

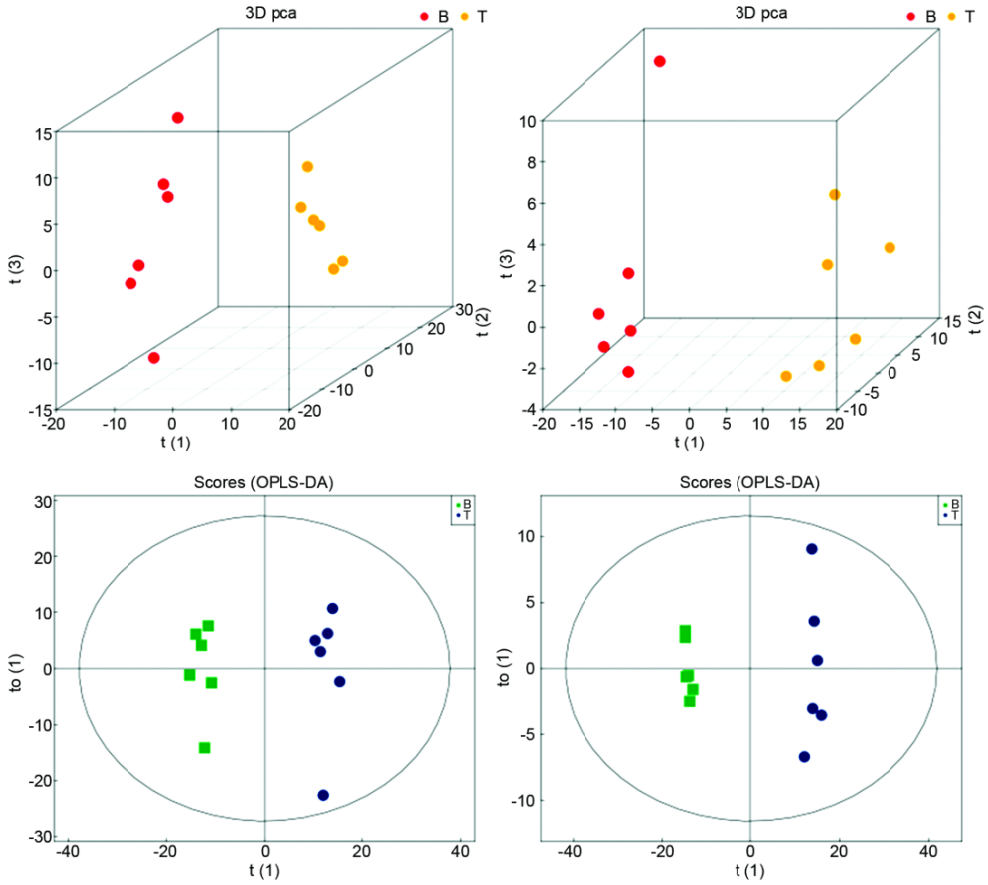

Fig.5 — Stability metabolomics analysis of ABSC: (A) Positive and negative, ion mode 3D PCA score map; and (B) positive and negative ion mode OPLS-DA score map

investigate the possible mechanism of unsaturated fatty acid biosynthesis induced by LRA. To compare the different metabolite compositions of LRA in different media, mycelia from different media were subjected to LC-MS analysis. The sample repetition correlation plot showed that the sample reproducibility was good (Fig. 5A). The OPLS-DA model can discriminate between two sets of samples, indicating the good quality and high reliability of the metabolomic data. The metabolomic data can be used for subsequent analyses. To screen the metabolites, the metabolites were subjected to the OPLS-DA method. The R^2 in both positive and negative ion modes was higher than 0.5 and the sample points were within a 95% confidence interval, indicating that the model is reliable with a good fit (Fig. 5B). LRA-induced metabolite changes in ABSC, indicating that the original model was stable and reliable and there was no overfitting.

To investigate the metabolite compositions, the non-targeted metabolite profiling of extracts was carried out. The metabolites extracted from muscles

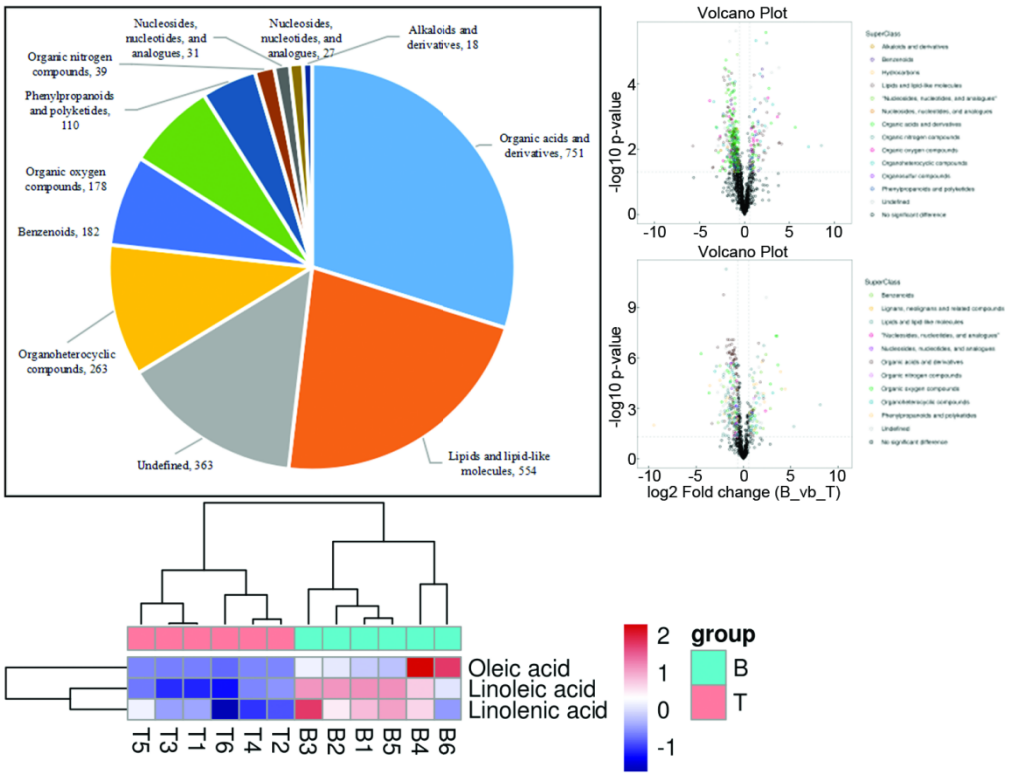

Fig. 6 — Stability analysis of metabolites: (A) metabolites; (B) volcano map; and (C) KEGG Pathway

were screened using UPLC-QTOF/MS[27]. To demonstrate the differences in spectra among different types of samples, a series of liquid chromatography are illustrated in (Fig. 6). Under optimized elution conditions of ultra-high performance liquid chromatography, the elution periods of the main period were around 0–0.5 min, 0.5–7.0 min, 7.0–8.0 min, 8.0–9.0 min, 9.0–9.1 min, and 9.1~12.0 min.

Based on m/z values and the standard libraries (METLIN, and HMDB–Human Metabolome Database) by comparison, a total of 2,522 metabolites were identified using metabolomic analysis of which 1,688 and 834 metabolites were identified in positive and negative ion modes, of which organic acids and their derivatives accounted for the largest proportion of 29.8%, followed by lipids and lipid molecules, accounting for 22.0% (Table 2).

In this experiment, a total of 261 differential metabolites were obtained by screening the significantly different metabolites in the samples of OPLS-DA VIP (Variable Importance in the Projection) >1, and significance was set to p-value<0.05. Using PCA and OPLS-DA diagram analysis[28], we observed that the metabolites of the samples before and after the treatment of LRA were

Table 2 — Metabolite count statistics

Detection mode	Metabolites quantity
Positive ion mod	1688
Negative ion mod	834

significantly different (Fig. 6). The number of significant differential metabolites was obtained after identification and screening (Table 3), whichwere related to the biosynthesis of unsaturated fatty acids. A total of eight differential metabolites were found to be related to biosynthesis of unsaturated fatty acids, of which linoleic acid, α-Linolenic acid, oleic acid were up-regulated, stearidonic acid, 16-Hydroxypalmitic acid, 10-undecynoic acid, citraconic acid, mevalonic acidwere down-regulated.

Among the fatty acid differential metabolites, the contents of unsaturated fatty acids such as oleic acid, linoleic acid and α-linolenic acid after LRA treatment in were increased by 3.51, 1.58 and 1.63 times, respectively, stearic acid. The content of saturated fatty acids such as 16-hydroxypalmitic acid was reduced by 4.68 and 5.43 times, respectively, with significant differences ($P< 0.05$), which showed after LRA treatment could promote the conversion of saturated fatty acids to unsaturated fatty acids.

Studies have shown that increased content of unsaturated fatty acids helps to improve cell membrane fluidity[29-31], which in turn promotes the growth of mycelial cells[32] and the secretion of exopolysaccharides. In addition, the content of 10-undecynoic acid was reduced by 4.25 times compared with the blank group, and the difference was significant ($P< 0.05$), indicating that LRA could inhibit the synthesis of 10-undecylenic acid. Studies have shown that 10-undecylenic acid is a specific inhibitor of cytochrome P450 4A1 catalyzing ω-hydroxylation reaction[33], So LRA can effectively promote ω-hydroxylation to produce α-linolenic acid.

RNA extraction and transcriptomic analysis

Transcriptomic profiling and DEGs

To further explore the mechanism underlying the LRA-mediated effect on EPS synthesis in ABSC, RNA-Seq analysis was conducted to evaluate the transcriptomic response of ABSC in a medium containing LRA. Transcriptomic data revealed a total of 68,061 transcripts, average GC content was found to be 52.94%, and the Q30 score was between 93.9% and 94.7% (Table 4).

After filtering, the GC content distribution and the sequencing error rate were calculated, and clean reads are retrieved for subsequent analysis. Then, clean reads were assembled through Trinity software. The length of transcripts and cluster sequences (unigenes) were counted, respectively (Table 5). The average length of transcripts was 1559 bp, while the N50 and the N90 were found to be 3379 bp and 601 bp, respectively. The average length of unigenes (non-redundant sequences) was 962 bp, and the N50 and the N90 were 2085 bp and 318 bp, respectively.

The transcriptome sequencing was performed on an Illumina Hiseq 2500 platform (Table 6). To improve the quality and reliability of data, raw reads containing sequencing adapter or N (N represents that the base sequence is uncertain), and low-quality reads (reads with a base number of Q*phred* ≤ 20 accounting for more than 50% of the whole read) were removed from the original data.

Table 3 — Differential metabolic pathway

Category	Compound Name	Affiliate access	VIP	P-value	Content
Fatty acids	α-Linolenic acid	Biosynthesis of unsaturated fatty acids	4.01	0.0044	↑
	Oleic acid		1.13	0.0145	↑
	10-undecynoic acid	Fatty acid biosynthesis	3.94	0.0005	↓
	Oleic acid		1.13	0.0145	↑
	Linoleic acid	Linoleic acid metabolism	22.70	0.0000	↑
	Stearidonic acid	α-Linolenic acid metabolism	10.36	0.0015	↓
	Citraconic acid	Valine, leucine and isoleucine biosynthesis, C5-Branched dibasic acid metabolism, 2-Oxocarboxylic acid metabolism	1.47	0.0000	↓
	Mevalonic acid	Biosynthesis of secondary metabolites	1.82	0.0000	↓
	Stearidonic acid		10.36	0.0015	↓
	16-Hydroxypalmitic acid	Cutin, suberine and wax biosynthesis	9.27	0.0028	↓
	Oleic acid		1.13	0.0145	↑

Table 4 — Summary of raw RNA-Seq data of ABSC grown in medium with and without LRA

Sample	Raw reads	Clean reads	Clean bases	Q20(%)	Q30(%)	GC(%)
B1	61150528	60372332	8.11G	97.88	93.96	52.83
B2	69803164	68982836	9.27G	97.89	94.01	52.82
B3	66530792	65801208	8.89G	97.95	94.1	52.83
T1	53489854	53222480	7.24G	98.22	94.7	53.34
T2	61274648	60629230	8.22G	97.84	93.9	53.09
T3	59410618	58642024	7.9G	97.98	94.2	52.73

Table 5 — Statistical analysis of length distribution of transcripts and unigenes

	min	max	median	mean	N50	N90
Transcript	201	28676	669	1559	3379	601
Unigene	201	28676	402	962	2085	318

Note: ↑ indicates up-regulation of metabolites,↓ indicates down-regulation of metabolite

Table 6 — Statistical table of gene annotation success rate

Type	Number of Unigenes	Percentage (%)
Annotated in NR	37430	54.99
Annotated in SwissProt	7720	11.34
Annotated in PFAM	17750	26.08
Annotated in GO	14729	21.64
Annotated in KO	18448	27.11
Annotated in all Databases	3265	4.8
Annotated in at least one Database	37605	55.25
Total Unigenes	68061	100

Functional annotation of KEGG and GO genes

As compared with the control, 48 genes were up-regulated and 181 genes were down-regulated (Fig. 7). In the GO function annotation, 10259 genes are annotated, mainly divided into three categories: molecular function (MF), biological process (BP), and cellular component (CC). The number of genes annotated to biological processes was much more than molecular function and cell components. After KO annotation of genes, they can be classified according to the KEGG metabolic pathway they participate in, and the results are shown in the figure below. The KEGG metabolic pathway involves cellular processes and environmental information processing Information Processing, Genetic Information Processing, Metabolism, Organic Systems (Organismal Systems).

KEGG metabolic pathway analysis of DEGs

The Volcano Plot was constructed to visualize the distribution and expression of differential genes in Blank conditions and LRA group (Fig. 8A). The KEGG enrichment scatter plot of the differential gene was for visual display of KEGG enrichment analysis(Fig. 8B). In this experiment, a total of 20 pathway entries with the most significant enrichment were selected to display. KEGG enrichment was measured using the Rich factor, FDR, and the number of genes enriched in these pathways. Differential gene clustering was used to determine the altered gene expression between different groups. According to the similarity of gene expression in each sample, the genes were clustered and analyzed to visually display the expression of genes in different samples to obtain relevant information about biological problems. The outcome of the analysis is represented in (Fig. 8C).

To further investigate the effect of LRA on ABSC cell membranes during fermentation, transcriptomic analysis was performed. Of these DEGs, five are mapped to three pathways associated with Biosynthesis of unsaturated fatty acids (Table 7).

Integrated transcriptomic and metabolomic analysis

The metabolic pathway of unsaturated fatty acids is mainly concentrated in the ω-3 and ω-6 unsaturated fatty acid families. The upregulation of gene expression of acyl-CoA thioesterase enzyme, stearoyl-CoA desaturase and acyl-CoA oxidase led to upregulation of oleic acid, linoleic acid, docosahexaenoic acid (DHA), and eicosatrienoic acid, and the down-regulation of stearic acid content led to the transfer of lecithin to linolenic acid, resulting in upregulation of linolenic acid content. This shows that LRA regulates the mycelium growth and exopolysaccharide secretion of ABSC by promoting the biosynthesis of unsaturated fatty acids (Fig. 9).

Targeted metabolomics validation test for medium and long chain fatty acids

SCD1, stearyl-coenzyme A desaturase; ACOX1, acyl-CoA oxidase; PLA2G, secretory phospholipase A2; LOX2S, lipoxygenase; FADS2, acyl-CoA 6-desaturase; fadA, acetyl-CoA acyltransferase (Fig. 10).

According to the analysis of KEGG enrichment in the metabolic pathway, the significantly different metabolites and their pathways (Table 8). The main pathways include biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, lipoic acid metabolism, α-linolenic acid metabolism, the most important of which is unsaturated fatty acid metabolism. Consistent with the above conclusions of metabolome and transcriptome, the upregulation of the expression of stearoyl-CoA desaturase and acyl-CoA oxidase gene led to an upregulation of oleic acid and linoleic acid content, and the downregulation of stearic acid content led to the transfer of lecithin to linolenic acid, resulting in an upregulation of linolenic acid content. In linolenic acid metabolism, acyl-CoA oxidase gene and acyl-CoA desaturase gene are up-regulated to generate oleic acid, octadecenoic acid and stearic acid, which increase the content of intracellular unsaturated fatty acids. This shows that LRA regulates the mycelium growth and exopolysaccharide secretion of ABSC by promoting the biosynthesis of unsaturated fatty acids[34]. Therefore, based on medium and long chain fatty acid targeted metabolomics, the regulatory channel of anabolic unsaturated fatty acids under the influence of LRA can be verified.

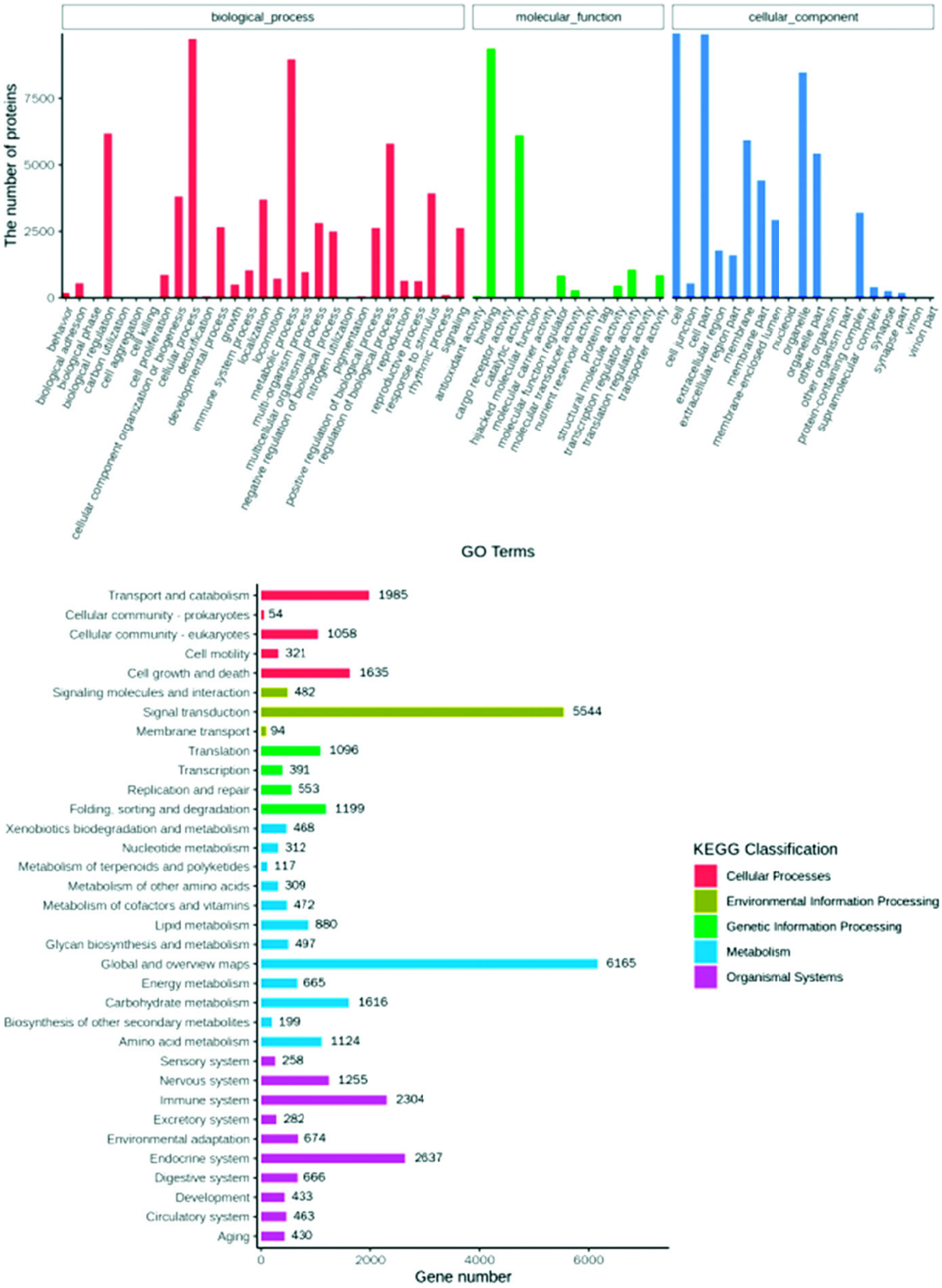

Fig. 7 — Functional annotation of KEGG and GO genes

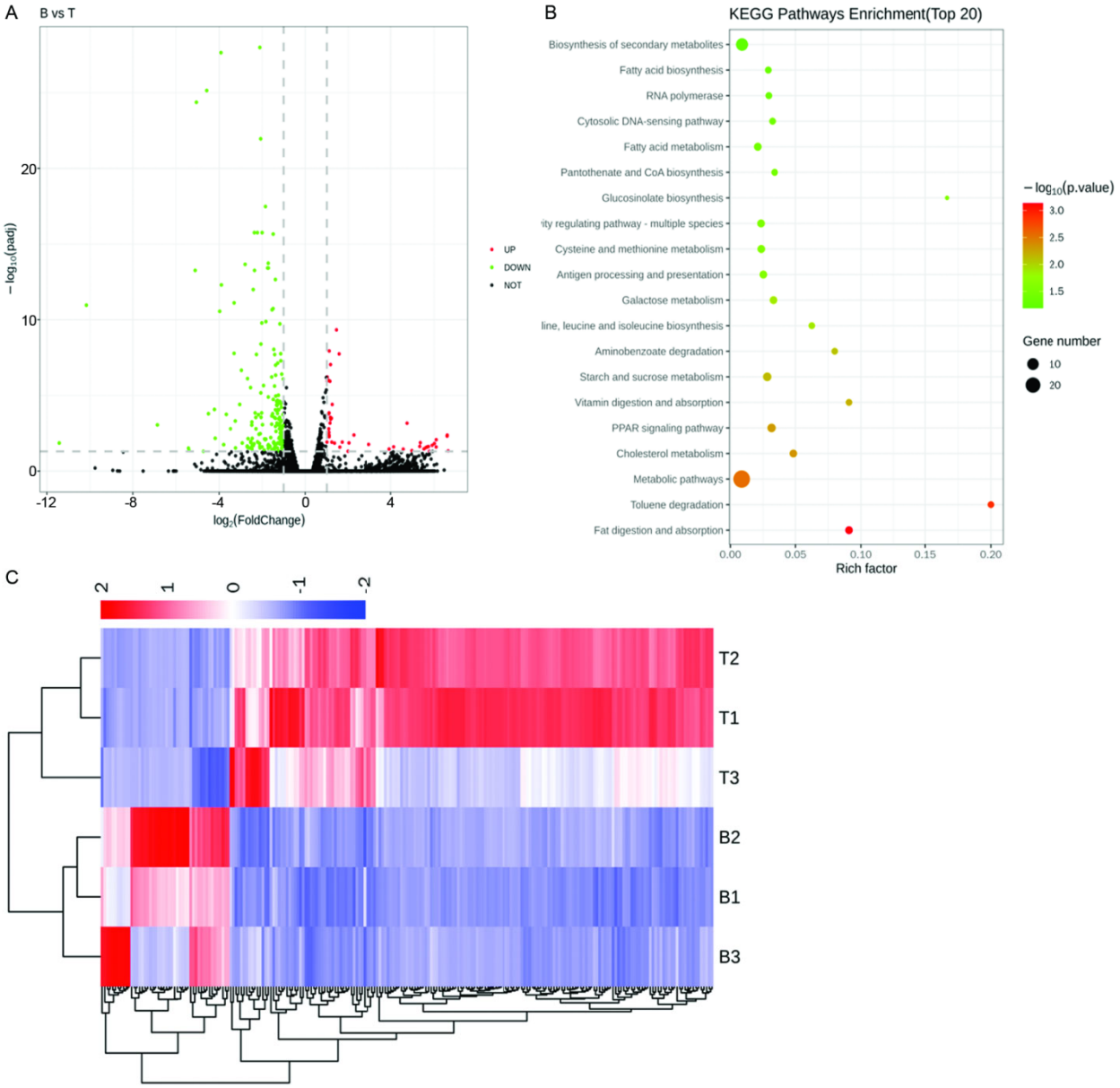

Fig. 8 — Analysis of the transcriptomic data. Transcriptome sequencing of ABSC under LRA. A(A) Volcano plot showing the DEGs between LRA and control groups. The X-axis indicates fold change of gene expression (threshold, | log2(Treat/Control)>1), while the Y-axis indicates the statistically significant level (threshold, q-value<0.005); (B) KEGG pathway enrichment of DEGs. The rich factor represents the ratio of the number of DEGs to the total number of annotated genes in this pathway; and (C) Clustered heatmap for DEGs detected from differential gene note

Table 7 — Differentially expressed genes in access

Enzyme name	Affiliate access	variety
α Fatty acid synthase subunit	Unsaturated fatty acid metabolism	↑
β Fatty acid synthase subunit		↑
Acyl-Coenzyme A oxidase		↑
stearyl-coenzyme A desaturase		↑
Acyl-Coenzyme A thioesterase		↑

Note: ↑ indicates up-regulation of metabolites,↓ indicates down-regulation of metabolite

Discussion

Unsaturated fatty acids have an important effect on the fluidity of cell membranes. Cell membranes are composed of lipid bilayers that contain saturated and unsaturated fatty acids[35]. The main feature of unsaturated fatty acids is the existence of a double-bond structure in their carbon chains, resulting in the carbon chains that cannot be completely arranged in a straight line, but take on a curved shape, making unsaturated

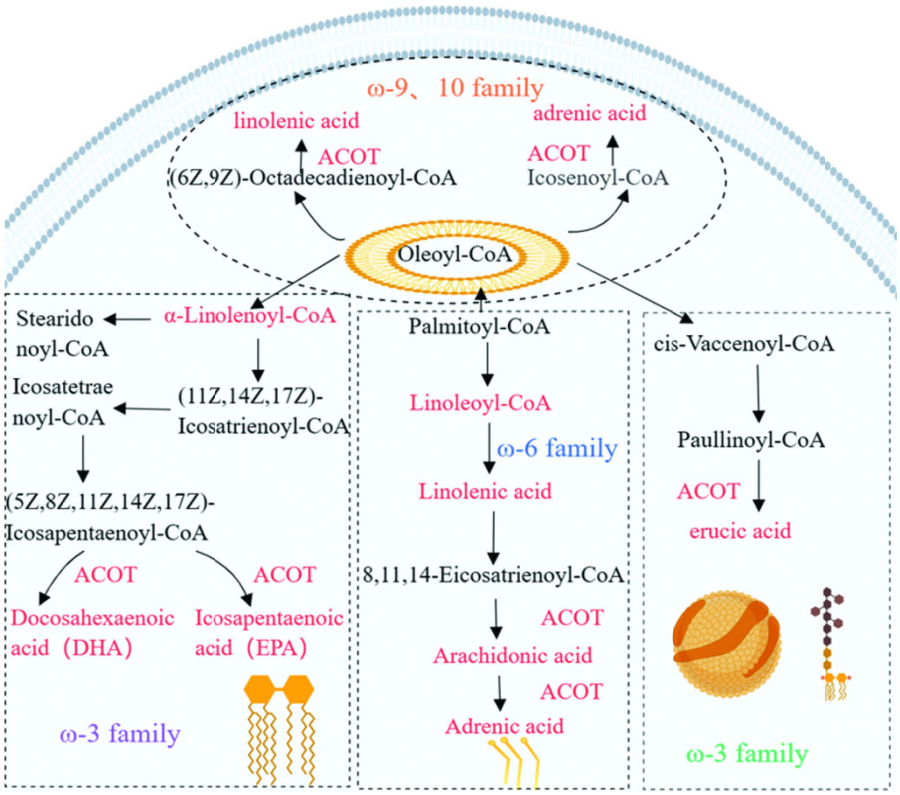

Fig. 9 — During fermentation, the regulatory mechanism of ABSC hyphal cell membrane is regulated by LRA. Green indicates downward adjustment of content/expression, and red indicates upward adjustment of content/expression. ACOT, Acyl-Coenzyme A thioesterase

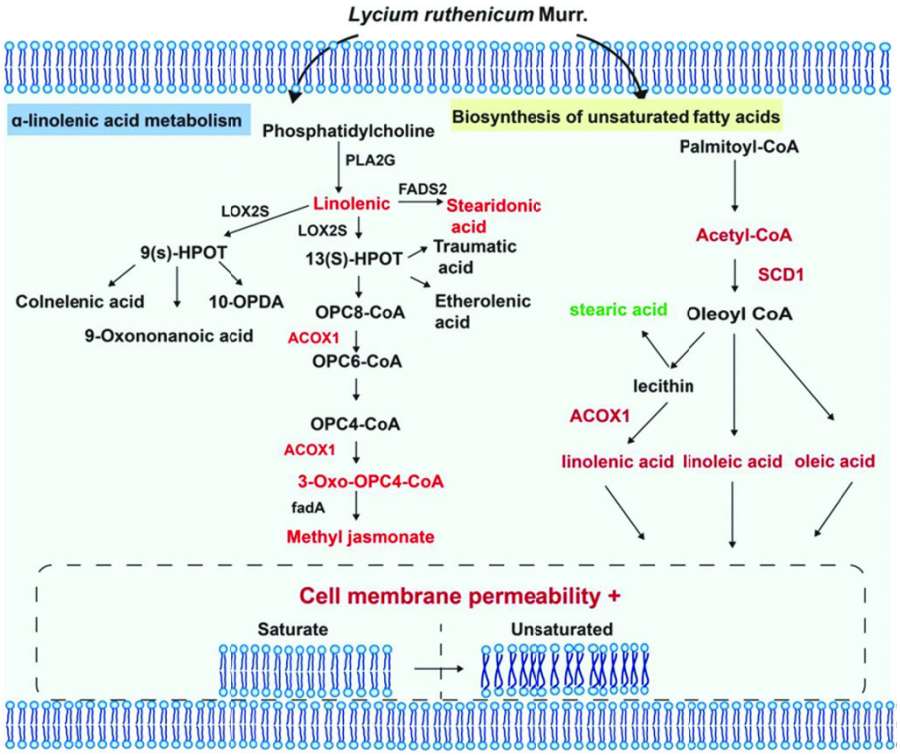

Fig. 10 — Biosynthesis and metabolism pathway of unsaturated fatty acids under treatment with LRA

Table 8 — Differential metabolic pathways

category	Metabolite name	Affiliate access	P-value	content
Fatty Acids	Methyl linolenate	Biosynthesis of unsaturated fatty acids	0.0000	↑
	Methyl tetracosanoate		0.0001	↓
	cis-11-Eicosenoic acid methyl ester		0.0108	↓
	Methyl palmitate		0.0389	↑
	Methyl palmitate	Fatty acid biosynthesis	0.0389	↑
	Methyl palmitoleate		0.0000	↑
	Methyl myristate		0.0015	↑
	Methyl dodecanoate		0.0000	↑
	Methyl linolenate	alpha-Linolenic acid metabolism	0.0000	↑
	Methyl octanoate	Lipoic acid metabolism	0.0217	↓
	cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester	NA	0.0001	↓
	Methyl heptadecanoate	NA	0.0065	↓
	cis-11,14-Eicosadienoic acid methyl ester	NA	0.0024	↓
	Methyl tricosanoate	NA	0.0160	↓
	Methyl heneicosanoate	NA	0.0175	↓
	Methyl docosatetraenoate	NA	0.0215	↑

Note: ↑ indicates up-regulation of metabolites,↓ indicates down-regulation of metabolite

fatty acids more fluid than saturated fatty acids. The fluidity of the cell membrane is critical to the function of the cell, and it determines the rate at which the cell membrane moves in and out of material, as well as the interaction of molecules inside and outside the cell. Proper unsaturated fatty acid content can improve the fluidity of cell membranes, thereby promoting the normal function of cells.

Pepe[36], McLennan PL[37] showed that increasing the content of phospholipid omega-3 polyunsaturated fatty acids in cardiomyocytes can increase the stability of cell membranes and produce anti-arrhythmic effects. Kang[38] and other studies found thatω-3 polyunsaturated fatty acids dissolve in the form of free fatty acids on the hydrophobic inner surface of the cell membrane phospholipids, and do not directly participate in the composition of the cell membrane in covalent bonds or other forms. It is important to note that excessive intake of unsaturated fatty acids may also negatively affect the fluidity of cell membranes. Moderate intake and balanced fatty acid composition are essential for the health of cell membranes.

In summary, we used transcriptomic and metabolomic data to identify differential genes and differential metabolites[39] involved in the regulation of key metabolic pathways of unsaturated fatty acids by LRA. The outcomes of this study showed that, mycelial growth and EPS synthesis of ABSC may not only be related to a single gene or metabolite, but also multipled metabolic pathways, involving multiple regulatory pathways and signaling systems. However, how these candidate genes or metabolites are involved in regulation demands further in-depth study and discussion.

Conclusion

In this study, the regulatory mechanism of exopolysaccharides from ABSC was investigated using LRA during cell growth and polysaccharide synthesis. The composition of anthocyanins in LRM was studied by UHPLC-HRMS method, and a total of 11 anthocyanins were identified, according to the sum of its peak areas accounting for the total peak area, the anthocyanin derivatives of petunia ligand accounted for about 90% of the total anthocyanin content in LRM, and proanthocyanidins accounted for 4%, which was the most important anthocyanin substance. In this paper, the effects of 0.06 mg/mL Anthocyanins extract on the growth and polysaccharide synthesis of ABSC were dynamically analyzed, and the results showed that the crude extract group and main components of LRM had a promoting effect on the growth and polysaccharide synthesis of ABSC and the effect of anthocyanins extract was better than that of single-component standard. Anthocyanins extract and main components of anthocyanins of LRM can affect the apparent structure of the mycelium of ABSC, so that the mycelium of ABSC appears folded, and the mobility and permeability of mycelium cells are increased.

Through metabolomics analysis of the regulation effect of LRA in ABSC, 40 differential metabolites

were screened, and it was found that LRA can upregulate linoleic acid α- Linoleic acid, linolenic acid, and oleic acid are down-regulated, including stearic acid, 16-hydroxypalmitic acid, and 10-undecylic acid. Screening out bio-anabolic pathways of metabolic unsaturated fatty acids. According to the sequencing and annotation results, a large number of genes in the mycelium of ABSC after LRA treatment were differentially expressed, and in the KEGG enrichment analysis, it was found that the genes of the unsaturated fatty acid metabolism pathway showed up-regulated expression during the regulatory process, and the content of α-linolenic acid, linoleic acid and oleic acid were increased by up-regulating stearoyl-CoA desaturase and acyl-CoA oxidase. Increased conversion of saturated fatty acids to unsaturated fatty acids improved the fluidity of cell membranes.

In summary, LRA promoted the synthesis of unsaturated fatty acids and improves cell membrane fluidity by up-regulating unsaturated fatty acids and up-regulating or down-regulating the expression of key enzymes, thereby improving the growth of mycelium of ABSC.

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Conflict of interest

All authors declare no conflict of interest.

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