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Composition, antioxidant and development-promoting activity of fermentation modified crude polysaccharides from stem and leaves of *Chenopodium album* L.

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Abstract

Chenopodium album L. (CAL) has many bioactive compounds and pharmacological activities. Fermentation is the preferred modification method of releasing target bioactive. We prepared two crude polysaccharides: SLC (the polysaccharide from the unfermented stem and leaves of CAL) and FSLC (the polysaccharide from the fermented stem and leaves of CAL). In vitro antioxidant and composition of SLC and FSLC were compared, and the effects of FSLC on antioxidant activity in the IPEC-J2 cells model and development-promoting activity in the zebrafish model were evaluated. The results revealed that FSLC possesses stronger DPPH, hydroxyl radical scavenging, and reducing power than SLC. The levels of total polysaccharide, polyphenol, and flavonoid, as well as the molar ratio of glucuronic acid increased in FSLC. Compared with SLC, the relative contents of protocatechuic acid, protocatechualdehyde, gentisic acid, vanillic acid, p-coumaric acid, quercetin, ferulic acid methyl ester, hispidulin, diosmetin, cinnamic acid, isorhamnetin, syringic acid and kaempferol in FSLC increased. In IPEC-J2 cells, antioxidant enzyme activities and GSH levels were significantly increased, while the MDA level was reduced by treatment with 0.25 mg/mL FSLC. In the zebrafish model, treatment with 25 ~ 300 µg/mL of FSLC had no harmful impact on the morphology and viability of embryos at 12 ~ 72 hpf. At 48 and 60 hpf, treatment with 100 ~ 300 µg/mL FSLC increased the hatching rate of embryos. At 72 hpf, treatment with 100 ~ 300 µg/mL FSLC could relieve morphological abnormalities caused by LPS in zebrafish and improve the hatching rate of embryos. Together, these results provide useful information on the potential for applying polysaccharides from the stems and leaves of CAL as natural feed additives to exert its antioxidant and development-promoting functions.

Keywords *Chenopodium album* L., Fermentation, Polysaccharides, Composition, Antioxidant activity, Development-promoting activity

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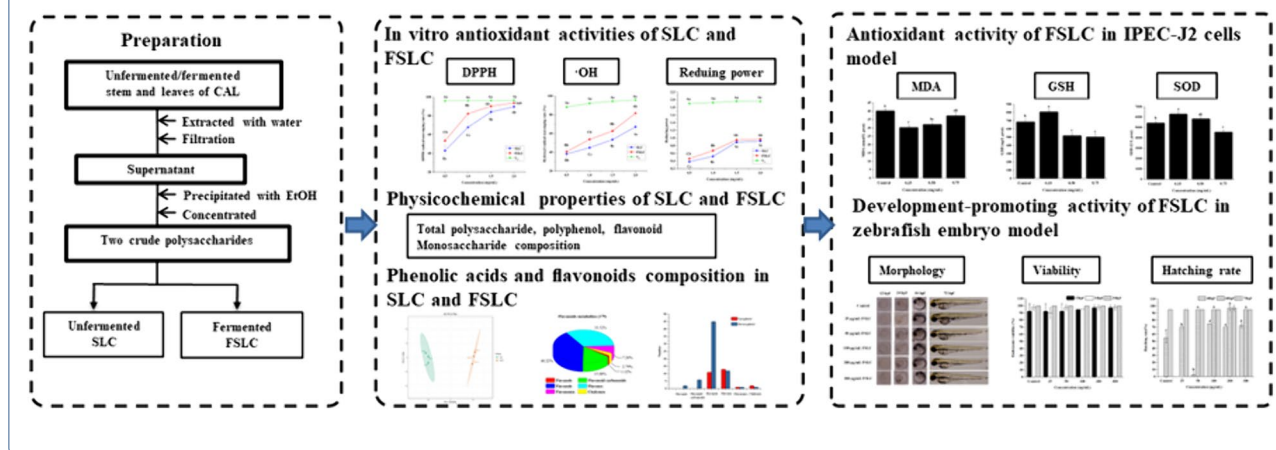
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Graphical Abstract



Introduction

Antibiotics have been used as feed additives in animal husbandry to promote growth performance and prevent diseases. In recent years, antibiotics have been banned worldwide. Oxidative stress is defined as an imbalance between the production of free radicals and reactive metabolites, leading to damage of important biomolecules and cells involved in chronic inflammation and a wide spectrum of diseases, including cancer, diabetes, cardiovascular, and neurological diseases [1]. As natural polysaccharides, plant polysaccharides, with no side effects, contribute to the potential value of treating or preventing disease caused by oxidative stress [2]. Ji et al. isolated a polysaccharide from *Ziziphus Jujuba* cv. *Muzao* exhibits in vitro antioxidant activity, especially in scavenging DPPH and hydroxyl radicals [3]. Long et al. found that dietary supplementation with *Lycium barbarum* polysaccharides improved growth performance, digestive enzyme activities, antioxidant capacity, and immune function of broilers [4]. Furthermore, Zhang et al. reported that glycyrrhiza polysaccharide improved the LPS-induced decline in serum antioxidant capacity and liver immune response [5]. Therefore, evidence supports the using plant polysaccharides as a promising natural feed additive instead of antibiotics.

Fermentation is a traditional food quality modification that affects the product's properties, such as antioxidant activity [6]. It has been considered a more effective and economical way to produce and extract active compounds in the food and pharmaceutical industries [7]. In Tao et al., the fermentation of *Artemisia* resulted in a significant increase in the yield of polysaccharides after process optimization. *Artemisia* polysaccharides showed a scavenging effect on DPPH,

ABTS, and hydroxyl radicals, and there was a dose-dependent relationship [8]. Chen et al. indicated that fermentation increased the content of wheat bran polysaccharides and enhanced its in vitro and in vivo antioxidant activity [9].

Chenopodium album L. (CAL) is an annual herb belonging to the *Chenopodium* genus of the *Chenopodiaceae* family [10], widely distributed in Asia, Europe, and North America, also called pigweed, lamb's quarter, goosefoot [11]. *Chenopodium album* has a high amount of dietary fiber [12], proteins [13], essential amino acids, carbohydrates, vitamins A and C [14]. It is not only used as a vegetable, salad, and animal feed but also for medicinal uses [15]. Several studies have reported the medicinal value of *Chenopodium album*. For instance, Arora et al. found that the extract of *Chenopodium album* could induce a significant reduction in rat paw edema (80.13%) after 21 days of treatment at 200 mg/kg per oral [16]. *Chenopodium album* can also act as an antiphlogistic and analgesic [17, 18] as well as antidiabetic and antihyperlipidemic [19] and against constipation and intestinal worms [20].

CAL is rich in bioactive compounds, such as polysaccharides, polyphenols, flavonoids [21], terpenoids, alkaloids, and saponins [17]. Hussain et al. revealed that the *Chenopodium album* has the potential for reducing oxidative stress and confirmed the potential for protecting the liver of the extract in rat models [15]. Xie et al. showed that fermented CAL positively affected the growth, nutrient digestibility, immunity, carcass characteristics, and meat quality of broilers [22]. He et al. isolated a polysaccharide from CAL using ultrasonic-assisted extraction. They evaluated its antioxidant activity in vitro, and the polysaccharide exhibited

strong scavenging activity against DPPH, ABTS, and hydroxyl radicals [23]. However, little research has been carried out on the characteristics and antioxidant and anti-inflammatory activity of CAL polysaccharides before and after fermentation.

The IPEC-J2 cell lines, isolated from pig small intestines, represent an established in vitro model to study the bioactivity of natural substances [24]. Zebrafish, as an animal model, has been widely used in toxicology and drug discovery studies investigating oxidative stress and inflammation due to its numerous advantages, such as its fecundity, rapid embryonic development, small size, transparent embryos, easy observability, and low cost [25]. In this study, we used the stems and leaves of CAL as the raw material and applied compound probiotics to ferment and modify the polysaccharides. Two crude polysaccharides, SLC (the polysaccharide from unfermented stem and leaves of CAL) and FSLC (the polysaccharide from fermented stem and leaves of CAL) were prepared with the aim of investigating their in vitro antioxidant activity and composition. The influence of FSLC on antioxidant activity in the IPEC-J2 cells model and development-promoting activity in the zebrafish model was further evaluated. Our results provide new evidence for the antioxidant enhancement effects of CAL polysaccharides and will promote the development and utilization of FSLC as green feed additives.

Materials and methods

Materials

Stem and leaves of CAL were harvested in Hohhot, Inner Mongolia Autonomous Region, China, from June to August 2022, stored and crushed under natural conditions. *Bacillus subtilis* (CGMCC 1.0892), *Lactobacillus plantarum* (CGMCC No. 1.12934), and *Saccharomyces cerevisiae* (CGMCC No. 2.1190) were purchased from the China General Microbiological Culture Collection Centre (Beijing, China). Pectinase was obtained commercially (Beijing Solarbio Technology Co., Ltd, Beijing, China). Corn meal and wheat bran were obtained from a local market. 1-Phenyl-3-methyl-5-pyrazolone (PMP) and ascorbic acid (V_C) were purchased from Sinopharm Chemical Reagent Co. Dulbecco's Modified Eagle's Medium (DMEM/F12), fetal bovine serum (FBS), insulin transferrin selenium (ITS), penicillin, and streptomycin were purchased from Gibco-BRL. All other chemicals and reagents were analytical grade.

Fermentation of stem and leaves of CAL and polysaccharides preparation

CAL's stem and leaves were fermented according to the fermentation conditions pre-screened in the laboratory. The details are as follows: The stem and leaves of CAL

were air-dried, crushed to 3~5 cm, and used as the main substrate for fermentation. The substrate medium (4 kg in total) consists of 70% CAL stem and leaves (w/w), 20% corn meal (w/w), 6% wheat bran (w/w), and 4% pectinase (enzyme activity 10,000 U/g) (w/w). The mixture was inoculated with 0.1% compound probiotics (*Bacillus subtilis*, *Lactobacillus plantarum*, and *Saccharomyces cerevisiae* mixed in a ratio of 1:1:1); while stirring evenly, distilled water was added to achieve a total moisture content of 50% in the system, with the temperature set at 28 °C to ferment for 24 h. The substrate before and after fermentation was dried (45 °C, 24 h) and extracted by hot water extraction (extraction temperature 80 °C, extraction time 60 min, material-water ratio 1:20). The extract was filtered, concentrated, and centrifuged (3500×g, 15 min). The supernatant was precipitated with three volumes of 80% ethanol to obtain the two crude polysaccharides: SLC (the polysaccharide from unfermented stem and leaves of CAL) and FSLC (the polysaccharide from fermented stem and leaves of CAL).

In vitro antioxidant activities of SLC and FSLC

The in vitro antioxidant activities of SLC and FSLC were estimated using three common methods, including DPPH, hydroxyl radical scavenging activities and reducing power. The DPPH, hydroxyl radical scavenging activities, and reducing power were analyzed according to our previous method [26, 27].

Chemical composition analysis of SLC and FSLC

The total polysaccharide contents of SLC and FSLC were determined by the phenol sulfuric acid method [28], for which glucose was used as a standard. The total polyphenol content of SLC and FSLC was adopted according to previous methods [29], and gallic acid was used as the standard. The total flavonoid content of SLC and FSLC was estimated using the colorimetric method [26].

Monosaccharide composition of SLC and FSLC

HPLC determined the monosaccharide composition of SLC and FSLC with PMP precolumn derivatization [9]. The sample (2.0 mg) was hydrolyzed by 0.2 mol/L trifluoroacetic acid at 120 °C for 2 h. After hydrolysis, the excess acid was removed by evaporation under nitrogen. Sodium hydroxide (0.1 mol/L) was added to dissolve the dried hydrolysates. The mixture was treated with 0.5 mol/L PMP in methanol and incubated at 70 °C for 30 min. After cooling to room temperature, the mixture was neutralized by adding 0.3 mol/L hydrochloric acid and then extracted with chloroform. The chloroform was discarded. The extraction was repeated three times. The aqueous phase was filtered through a 0.22 µm membrane. The resulting solution was analyzed using an Agilent

1100 HPLC system (Agilent, USA) with a C18 column (4.6×250 mm, 5 µm) and DAD detector. Elution was performed with a mixture of phosphate buffer solution (pH 7.0) and acetonitrile in a ratio of 82:18 (v/v) at a 1.0 mL/min flow rate. The detection wavelength was 250 nm.

Phenolic acids and flavonoids composition in SLC and FSLC

The composition of the phenolic acids and flavonoids compounds of the SLC and FSLC were performed using ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). The SLC and FSLC samples are freeze-dried by vacuum freeze-dryer (Scientz-100F) and crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Three biological replicates were performed for each group. Dissolve 100 mg of lyophilized powder with 1.2 mL 70% methanol solution, vortex 30 s every 30 min for 6 times in total, and place the sample in a refrigerator at 4 °C overnight. Following centrifugation at 12,000 rpm for 10 min, the extracts were filtrated (SCAA-104, 0.22 µm pore size; ANPEL, Shanghai, China) before UPLC-MS/MS analysis. HPLC and mass spectrum conditions were conducted using a reported method [29]. Based on the self-built database MWDB (Metware Biotechnology Co., Ltd. Wuhan, China) and public database of metabolite information, the qualitative analysis of substance was performed using secondary mass spectrometry data. The quantification of metabolites was performed using multiple reaction monitoring (MRM) mode analysis [29].

Antioxidant activity of FSLC in IPEC-J2 cells model

IPEC-J2 cells, donated by Dr Guoyao Wu of China Agricultural University, were used to evaluate the antioxidant enzyme activity of FSLC. The IPEC-J2 cells were grown in DMEM supplemented with FBS (10%), ITS (1%), and penicillin/streptomycin (1%) in a 75-cm² cell culture flask at 37 °C in a 5% CO₂ humidified incubator. FSLC was diluted in DMEM, and the concentrations used for the assay were 0.25–0.75 mg/mL, which are not cytotoxic to the cells. IPEC-J2 cells were seeded into 96-well plates at a density of 1×10⁵ cells/well and incubated for 24 h. Then, the cells were washed twice with phosphate-buffered saline (PBS) and treated with different concentrations of FSLC (0, control; 0.25, 0.50, and 0.75 mg/mL) for 24 h of exposure in a humidified incubator (5% CO₂, 37 °C). The activities of total antioxidative capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), levels of glutathione (GSH) and methane dicarboxylic aldehyde (MDA) were tested using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, China) according with the manufacturer's instructions.

The development-promoting activity of FSLC in zebrafish embryo model

Collection of zebrafish embryos

Dult wild-type zebrafish (strain AB; approximately 4–5 months old) were maintained in a temperature-controlled room at 28 °C fed thrice daily. On the previous day, 9 fishes (males and females in a ratio of 1:2) were kept in a hatchery with the following conditions: 28±0.5 °C, pH 7.0–7.5, conductivity 400–600 µS/cm and a 10/14 h dark/light cycle. In the morning (at a set light), the nine breeding fishes interbred for 1 h; the embryos were obtained from natural spawning and collected in Petri dishes.

Morphology, viability, and hatching rate after FSLC exposure

At 8 h post-fertilization (hpf), healthy embryos were placed in 24-well culture plates (20 embryos/wells, 4 wells/groups). Zebrafish embryos were exposed to FSLC for 12–72 hpf to measure the toxic effects over a continuing observation period. To examine the viability, hatching rate, and morphology of embryos/larvae, FSLC was dissolved in embryo medium and prepared into solutions of different concentrations (25, 50, 100, 200, 300 µg/mL) to assess the concentration-dependent toxicity. The embryo medium was renewed every 12 h, embryonic viability was evaluated at 12, 24, and 36 hpf, and larval hatching rate was evaluated at 48, 60, and 72 hpf. Photographs of the zebrafish development were taken under a stereomicroscope (SZX7, Olympus, Tokyo, Japan) during exposure. The stereomicroscope settings included a magnification of 25 and a scale bar of 5 mm.

Effects of FSLC on embryos response to LPS

After evaluating non-toxic concentrations of FSLC, these were used to counteract the influence of LPS in embryos. The application and the concentration of LPS to zebrafish embryos were according to the previous study [30]. LPS (4 µg/mL) was applied in embryos by immersion at 8 hpf for 2 h. After LPS exposure, the embryos were washed immediately with fresh embryo medium and transferred into a 24-well plate with 2 mL FSLC (100, 200, and 300 µg/mL). The embryos cultured without any treatment served as a control. Zebrafish embryos were reared to 72 hpf at 28.5 °C, and the embryo culture medium was renewed every 12 h. The larval hatching rate was evaluated at 72 hpf in each group, and images of the zebrafish were made under a camera lens (HIKROBOT, Hangzhou, China) and a stereomicroscope (SZX7, Olympus, Tokyo, Japan).

Statistical analysis

All experiments were performed in triplicate. The data are expressed as the mean±standard deviation (S.D.),

and one-way ANOVA followed by Duncan's multiple range tests in SAS were used to evaluate significant differences. The differences were accepted as significant at $P < 0.05$.

Metabolite data were $\log_2$ -transformed for statistical analysis to improve normality and were normalized. Principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) were used by R software to analyze the multivariate differences of metabolites. The metabolites with a fold change (FC) ≥ 2 or ≤ 0.5 , those with a variable importance in projection (VIP) ≥ 1 were considered significantly differential metabolites between SLC and FSLC groups.

Results and discussion

In vitro antioxidant activities

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and hydrogen peroxide, can attack lipids, proteins, and DNA [31], with potential impact on the whole organism and result in the occurrence of many diseases [1]. It is necessary to develop natural antioxidants from plants so that they can protect our bodies from harmful free radicals and prevent the occurrence of many diseases. We compared the DPPH, hydroxyl radical-scavenging activity, and reducing power between the SLC and FSLC. As shown in Fig. 1A, the DPPH radical scavenging capacity of SLC and FSLC was enhanced with increasing concentrations. When the concentration was between 0.5 and 0.15 mg/mL, the DPPH scavenging capacity of FSLC was significantly higher than that of SLC ($P < 0.05$). At the 2.0 mg/mL concentration, there was no significant difference between FSLC and SLC groups of DPPH scavenging rate. However, the FSLC exhibited comparable DPPH free-radical scavenging activity to that of V_C . The capacity of SLC and FSLC to scavenge hydroxyl radicals increased in a dose-dependent manner (Fig. 1B). When the concentration between 1.0 and 2.0 mg/mL, FSLC exhibited significantly higher hydroxyl radical-scavenging activity than those of SLC ($P < 0.05$). Figure 1C shows that the reducing power of FSLC and SLC were correlated with the concentration. The reducing power of FSLC was significantly higher than that of SLC at the concentration of 0.5–2.0 mg/mL. A recent report suggested that fermented *Polygonatum kingianum* polysaccharides fraction showed improved scavenging ability of DPPH radicals and total reduction power compared with the original polysaccharide [32], which is similar to those of our result. Song et al. revealed that fermented polysaccharides from bulbs of Lanzhou lily had higher DPPH radical ability, superoxide anion radical scavenging ability, and reducing efficiency than those of unfermented polysaccharides [33]. In the present study, we found that fermented polysaccharides

from the stem and leaves of CAL show superior potency than unfermented polysaccharides on antioxidants via enhancing the DPPH, hydroxyl radical-scavenging activity, and reducing power. This could be attributed to the change in the physicochemical properties and composition of polysaccharides after fermentation.

Physicochemical properties of SLC and FSLC

The physicochemical properties of SLC and FSLC are presented in Table 1. SLC and FSLC mainly contain total polysaccharide, polyphenol, and flavonoid. In comparison to SLC, the content of total polysaccharide, polyphenol, and flavonoid changed markedly in FSLC, which increased by 19.15%, 100.50%, and 69.39%, respectively. It illustrated that fermentation enhances the total polysaccharide, polyphenol, and flavonoid contents of crude polysaccharides from the stem and leaves of CAL. Wang et al. revealed that crude polysaccharides from fermented okra juice with various lactic acid bacteria had a 7.42–12.53% increase in total polysaccharides content compared to their un-fermented counterpart [34]. Polysaccharides are biomacromolecules with complex structures and are mainly composed of different ratios of monosaccharides and glycosidic bonds [35, 36]. The monosaccharide composition also changed noticeably after fermentation (Table 1). The molar proportion of xylose and arabinose in SLC was relatively high, while that in FSLC was relatively low. The molar ratio of glucose, glucuronic acid, and mannose in FSLC was increased by 46.97%, 376.12%, and 285.56%, respectively, compared with SLC. It is indicated that FSLC is a kind of weakly acidic heteropolysaccharide. Zhang et al. found that fermentation significantly increased glucuronic acid content in fermented *Eucheuma spinosum* polysaccharides [37]. The change in monosaccharide composition may be attributed to the effects of raw materials and extraction methods. Several previous studies have confirmed the presence of phenolics in crude polysaccharides [31, 38, 39]. Polyphenols are natural compounds found abundantly in plant-based products, which serve a vital function in the protection of the organism from external stimuli and in eliminating ROS [40]. Amodeo et al. showed the extracts of CAL had a higher content of phenolic compounds and flavonoids, which are effective antioxidants against lipid peroxidation [41]. In line with the current study, Ai et al. found that the yield of ginseng polysaccharides was significantly increased after fermentation, and fermented ginseng polysaccharides possess a higher capacity for scavenging hydroxyl and superoxide anion free radicals than unfermented ginseng polysaccharides [42]. Liu et al. proved that fermentation promoted the release of polyphenols from CAL [29]. Zhou et al. showed that fermentation with tea

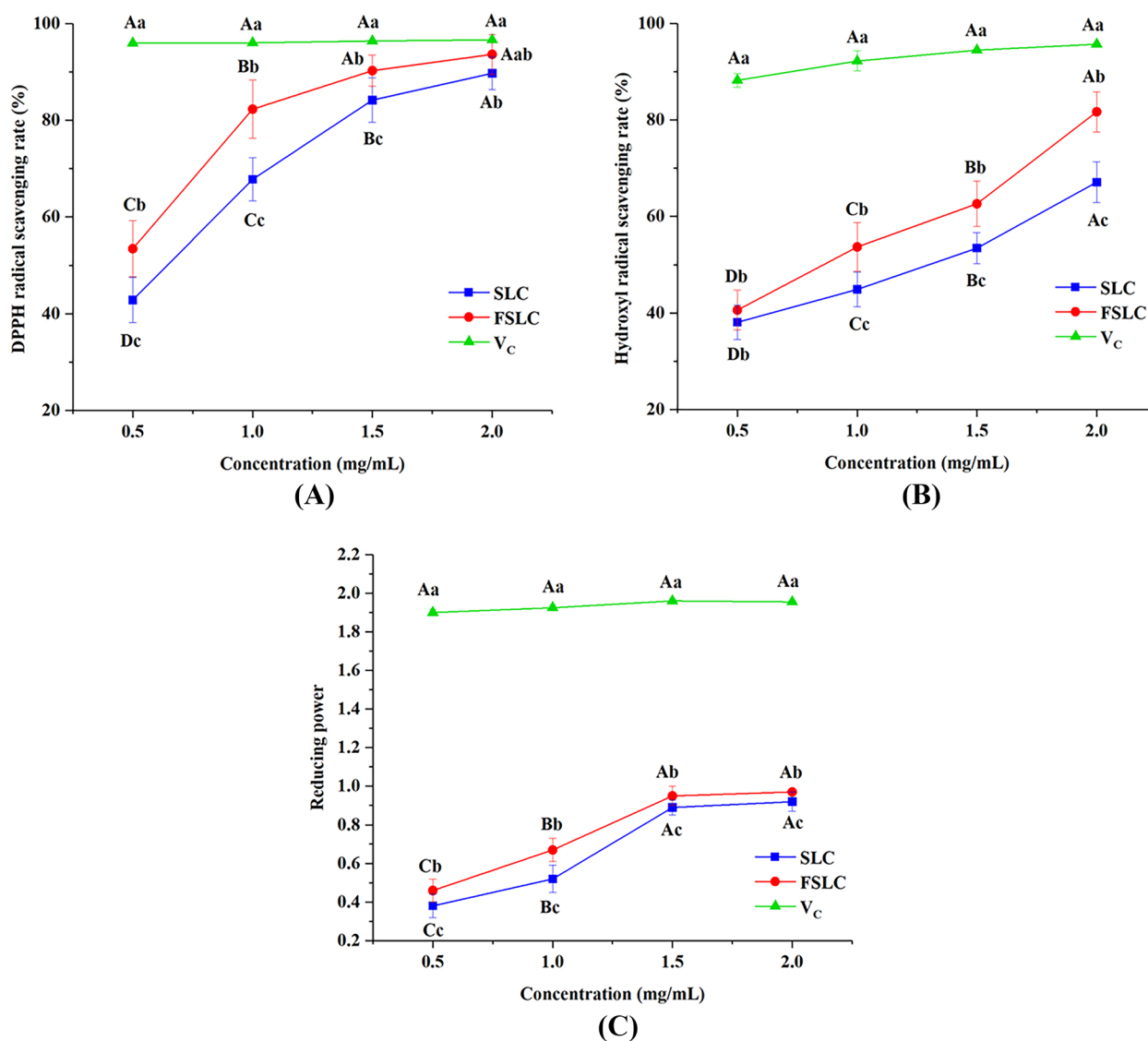


Fig. 1 Antioxidant activities of SLC and FSLC. **(A)** DPPH radical scavenging activity. **(B)** Hydroxyl radical scavenging activity. **(C)** Reducing power. Different lowercase letters denote statistically significant differences among SLC, FSLC, and V_c ($P < 0.05$), and different uppercase letters denote statistically significant differences among different concentrations within groups ($P < 0.05$)

residue could markedly increase polyphenolic concentrations and antioxidant activities of kombucha beverages [43]. During fermentation, microorganisms can secrete rich decomposing enzymes, such as protease, amylase, and cellulase [44], and these enzymes could be responsible for the changes in total polysaccharide content and monosaccharide composition. The destruction of cellular structures by enzymes and the hydrolysis of large polymeric phenolics by microorganisms may increase polyphenol and flavonoid contents in FSLC [45–47]. According to the physicochemical properties of SLC and FSLC, as mentioned (Table 1), it could be speculated that

the higher antioxidant capacity observed in FSLC could be attributed to its higher content of bound polyphenol and flavonoid when compared to SLC [48]. Polyphenols can be categorized into four categories according to their carbon skeleton: flavonoids, phenolic acids, lignans, and stilbenes [49]. So, we further analyzed the phenolic acids and flavonoids composition of SLC and FSLC.

Composition of phenolic acids in SLC and FSLC

Phenolic acids are hydroxyl derivatives of aromatic carboxylic acids, exhibiting antibacterial and anti-inflammatory activities due to their antioxidant properties [50].

Table 1 The physicochemical properties of SLC and FSLC

Index	SLC	FSLC	P-value
Total polysaccharide content (mg/g)	566.09 ^b ± 55.93	674.47 ^a ± 50.73	0.01
Total polyphenol content (mg/g)	51.78 ^b ± 7.95	103.82 ^a ± 17.64	0.01
Total flavonoid content (mg/g)	41.26 ^b ± 11.02	69.89 ^a ± 11.73	0.01
Monosaccharide composition (%)			
Mannose	2.84	10.95	
Ribose	0.63	2.42	
Rhamnose	6.01	8.26	
Glucuronic acid	2.01	9.59	
Galacturonic acid	14.22	6.54	
Glucose	25.59	37.61	
Galactose	7.75	7.28	
Xylose	26.43	7.15	
Arabinose	13.62	5.04	
Fucose	0.88	5.16	

There were significant differences between the data of groups of the same category with different superscript lowercase letters ($P < 0.05$)

A total number of 163 phenolic acids were target identified in SLC and FSLC. In the PCA score plot, two principal components (PC1 and PC2) were extracted to be 78.83% and 7.13%, respectively. The result of principal component analysis (PCA) showed that SLC and FSLC were clearly separated, and three biological replicates of each group were compactly gathered together (Fig. S1A), reflecting major changes in phenolic acids metabolite levels between SLC and FSLC. In addition, we used the supervised method, orthogonal partial least-squares discriminant analysis (OPLS-DA), to screen the variables responsible for differences among these two groups. Pair-wise comparisons were achieved by the OPLS-DA model, and the score plots are shown in Fig. S1B ($R^2X=0.796$, $R^2Y=0.995$, $Q^2=0.987$). In this model, R^2X and R^2Y were used to represent the interpretation rate to the X and Y matrices, respectively, and Q^2 represented the prediction ability. The Q^2 values of exceeded 0.9, demonstrating that the model was stable and reliable. The PCA and OPLS-DA score plots showed that the two groups were well-separated, suggesting that fermentation has a significant effect on the phenolic acid metabolite composition.

A volcano plot (Fig. S1C) visualized the difference in the expression level of phenolic acids metabolites between two groups. There were 100 significantly different phenolic acids metabolites between SLC and FSLC (45 up-regulated, 55 down-regulated). The differential phenolic acids metabolites between SLC and FSLC were further classified and compared (Table 2).

Phenolic acids can be further subdivided into two main types: C6-C1 phenolic acids and C6-C3 phenolic acids [51, 52]. C6-C1 phenolic acids have a hydroxybenzoic acid skeleton (e.g., protocatechuic, gallic, vanillic), while

C6-C3 phenolic acids have a hydroxycinnamic acid skeleton (e.g., caffeic, *p*-coumaric, ferulic) [51]. Ferulic acid, *p*-coumaric acid, caffeic, and cinnamic acid are major representative substances of C6-C3 phenolic acids. The relative contents of caffeic aldehyde (20.38 fold), *p*-coumaric acid (20.98 fold), ferulic acid methyl ester (21.02 fold), trans-4-hydroxycinnamic acid methyl ester (9.26 fold), cinnamic acid (7.02 fold), 4-methoxycinnamic acid (9.07 fold), ferulic acid ethyl ester (10.9 fold), *p*-coumaric acid methyl ester (4.08 fold) and dihydroferulic acid (9.03 fold) in FSLC were significantly up-regulated compared to SLC. In contrast, the relative contents of feruloylmalic acid, ferulic acid, isoferulic acid, 5-O-*p*-coumaroylquinic acid, 3-O-*p*-coumaroylquinic acid, *p*-coumaroylmalic acid and 1-O-feruloyl-D-glucose were significantly down-regulated. Ferulic acid (FA) is widely distributed in the plant kingdom and has a variety of biological activities, especially in oxidative stress and inflammation [52]. Bian et al. proved that in HEK293 cells treated with H₂O₂, pretreatment with FA (1 mM) can significantly improve cell survival rate, CAT and SOD levels [53]. Ferulic acid occurs mainly in various esters; its free state is rare [54]. Qiu et al. investigated the antioxidant effects of FA and lipophilized ferulate esters in fish oil-enriched milk [55]; the result revealed that methyl ferulate and ethyl ferulate more efficiently prevented lipid oxidation than ferulic acid. Earlier studies have demonstrated the antioxidant effects of dihydroferulic acid [56]. A study by Lee et al. revealed that dihydroferulic acid treatment improved the reduced viability of PC12 cells induced by H₂O₂ and enhanced the transcription levels of antioxidant genes [57]. It is reported that FA can be metabolized into other phenolic compounds with lower molecular weights,

Table 2 Relative content of part of phenolic acid differential metabolites in SLC and FSLC

Compounds	Relative content		VIP	FC	Type
	SLC	FSLC			
C6-C3 phenolic acids					
Caffeic aldehyde	4.58E+05	9.33E+06	1.15	20.38	Up
<i>p</i> -Coumaric acid	1.46E+05	3.05E+06	1.15	20.98	Up
Ferulic acid methyl ester	9.86E+04	2.07E+06	1.14	21.02	Up
Trans-4-hydroxycinnamic acid methyl ester	1.22E+05	1.13E+06	1.14	9.26	Up
Cinnamic acid	1.27E+05	8.92E+05	1.15	7.02	Up
4-Methoxycinnamic acid	7.17E+04	6.51E+05	1.14	9.07	Up
Ethyl ferulate	1.67E+04	1.82E+05	1.12	10.9	Up
<i>p</i> -Coumaric acid methyl ester	4.20E+04	1.71E+05	1.14	4.08	Up
1-O- <i>p</i> -Coumaroylglycerol	1.69E+04	1.15E+05	1.13	6.82	Up
Dihydroferulic Acid	1.61E+04	1.46E+05	1.10	9.03	Up
<i>p</i> -Coumaric acid ethyl ester	7.89E+03	7.14E+04	1.14	9.05	Up
Hydrocinnamic acid	9.87E+03	6.77E+04	1.14	6.86	Up
Feruloylmalic acid	3.17E+07	3.94E+06	1.14	0.12	Down
Ferulic acid	1.35E+07	1.54E+06	1.14	0.11	Down
Isoferulic acid	8.90E+06	9.32E+05	1.14	0.10	Down
Sinapinaldehyde	2.97E+06	3.47E+05	1.14	0.12	Down
5-O- <i>p</i> -Coumaroylquinic acid	1.75E+06	1.52E+05	1.12	0.09	Down
3-O- <i>p</i> -Coumaroylquinic acid	1.38E+06	1.06E+05	1.12	0.08	Down
<i>p</i> -Coumaroylmalic acid	1.62E+06	2.30E+05	1.11	0.14	Down
1-O- <i>p</i> -Coumaroyl-β-D-glucose	6.48E+06	2.55E+06	1.14	0.39	Down
<i>p</i> -Coumaric acid-4-O-glucoside	4.62E+06	2.21E+06	1.14	0.48	Down
1-O-Feruloyl-D-Glucose	2.77E+06	8.30E+05	1.14	0.30	Down
Feruloyltartaric acid	6.60E+05	1.53E+05	1.14	0.23	Down
Chlorogenic acid	6.88E+05	3.40E+04	1.13	0.05	Down
Cryptochlorogenic acid	4.19E+05	2.84E+04	1.14	0.07	Down
Sinapoylgucuronic acid	2.39E+05	7.96E+04	1.14	0.33	Down
1,3-O-Diferuloylglycerol	2.17E+05	4.32E+04	1.03	0.20	Down
3-O-Cinnamoyl-4,6-(S)-HHDP-β-D-glucose	1.72E+05	1.39E+04	1.13	0.08	Down
Ferulic acid-4-O-glucoside	1.67E+05	4.22E+04	1.10	0.25	Down
3-O-Feruloylquinic acid	1.64E+05	3.19E+04	1.02	0.19	Down
1-O-Galloyl-6-O- <i>p</i> -Coumaroyl-β-D-glucose	1.14E+05	1.38E+04	1.11	0.12	Down
C6-C1 phenolic acids					
4-Hydroxybenzoic acid	9.83E+06	2.36E+07	1.14	2.40	Up
Protocatechuic acid	8.79E+06	2.15E+07	1.12	2.45	Up
Protocatechualdehyde	5.95E+06	1.51E+07	1.15	2.54	Up
2-(Formylamino) benzoic acid	1.32E+06	1.13E+07	1.14	8.52	Up
Gentisic acid	3.14E+06	7.85E+06	1.12	2.50	Up
Vanillic acid	9.73E+05	3.38E+06	1.14	3.47	Up
3-Hydroxy-4-methoxybenzoic acid	3.52E+05	1.17E+06	1.09	3.33	Up
Syringic acid	2.39E+05	5.23E+05	1.09	2.18	Up
3-[(1-Carboxyvinyl)oxy]benzoic acid	4.53E+04	1.35E+05	1.02	2.99	Up
Vanillic acid methyl ester	3.32E+04	7.96E+04	1.13	2.39	Up
3-Hydroxy-4-isopropylbenzylalcohol-3-O-glucoside	2.20E+04	6.91E+04	1.12	3.15	Up
3-Aminosalicylic acid	1.17E+04	4.56E+04	1.13	3.90	Up
4-Hydroxy-3,5-diisopropylbenzaldehyde	1.71E+04	4.45E+04	1.12	2.60	Up
4-O-Methylgallic Acid	1.59E+04	3.99E+04	1.05	2.51	Up
1-O-Salicyl-D-glucose	1.33E+07	4.39E+06	1.14	0.33	Down

Table 2 (continued)

Compounds	Relative content		VIP	FC	Type
	SLC	FSLC			
Salicylic acid	2.82E+06	6.44E+05	1.14	0.23	Down
Vanillin	2.70E+06	3.19E+05	1.14	0.12	Down
4-Hydroxybenzaldehyde	2.62E+06	4.89E+05	1.14	0.19	Down
Benzoylmalic acid	2.61E+06	2.83E+05	1.14	0.11	Down
Benzamide	2.36E+05	1.07E+05	1.13	0.45	Down
Isovanillin	3.95E+05	3.18E+04	1.13	0.08	Down
Glucosyringic acid	4.42E+05	2.32E+04	1.15	0.05	Down
Methyl 4-hydroxybenzoate	3.36E+05	4.26E+04	1.13	0.13	Down
Benzoyltartaric acid	2.73E+05	3.28E+04	1.13	0.12	Down
Vnilloyltartaric acid	1.72E+05	4.57E+04	1.11	0.27	Down
4-Aminobenzoic acid	1.01E+05	3.27E+04	1.12	0.32	Down

such as dihydroferulic acid [58]. The relative contents of ferulic acid methyl ester, ferulic acid ethyl ester, and dihydroferulic acid in FSLC significantly up-regulated compared to SLC, while the feruloylmalic acid, ferulic acid, isoferulic acid, and 1-O-feruloyl-D-glucose were significantly down-regulated, this change may be driven by the action of enzymes produced during microbial fermentation. Additionally, cinnamic acid and its derivatives are a class of phenolic acids that have various pharmacological activities, including antioxidant [59] and anti-inflammatory [60]. Chao et al. revealed that the intake of cinnamic acid suppressed renal inflammatory cytokines release that consequently attenuated inflammatory stress in diabetic mice [61]. Wang et al. revealed that 4-methoxycinnamic acid could inhibit fungal cell wall synthesis, destroys the permeability of fungal cell membranes, and mediates the anti-inflammatory, immune response of the host [62]. *P*-Coumaric acid exists in free or conjugated forms with antioxidant and anti-inflammatory activities [63, 64]. Atul et al. found a protective effect of *p*-coumaric acid in neuroinflammation, cognitive impairment, and neuronal apoptosis induced by LPS [65]. Previous studies have shown that *p*-coumaric acid esters possess anti-inflammatory, antioxidation activities [66] and antifungal properties [67]. The relative contents of 5-O-*p*-coumaroylquinic acid, 3-O-*p*-coumaroylquinic acid, and *p*-coumaroylmalic acid in FSLC significantly down-regulated compared to SLC, while the *p*-coumaric acid was significantly up-regulated, the reason may be attributed to fermentation degrades these substances into small molecules of *p*-coumaric acid.

Hydroxybenzoic, vanillic, protocatechuic, gentisic acid, and syringic acid are the most popular C6-C1 phenolic acids. Compared with SLC, the relative contents of 4-hydroxybenzoic acid (2.40 fold), protocatechuic acid

(2.45 fold), protocatechualdehyde (2.54 fold), 2-(formylamino) benzoic acid (8.52 fold), vanillic acid (3.47 fold), gentisic acid (2.50 fold), 3-hydroxy-4-methoxybenzoic acid (3.33 fold) 2,6-dimethoxybenzaldehyde (6.55 fold), and syringic acid (2.18 fold) in FSLC were significantly up-regulated, while the relative contents of 1-O-salicyl-D-glucose, salicylic acid, vanillin, 4-hydroxybenzaldehyde, benzoylmalic acid, benzamide, and isovanillin were significantly down-regulated. Benzoic acids serve as precursors for a wide variety of essential compounds, including 4-hydroxybenzoic acid and 2-aminobenzoic acid [68]. 4-Hydroxybenzoic acid can be converted into more useful compounds that have potential biotechnological applications in food, pharmacy, fungicides, etc. [69]. During fermentation, one reason for the decrease in 4-hydroxybenzaldehyde content may be metabolized into 4-hydroxybenzoic acid [70]. Protocatechualdehyde is a naturally occurring compound resulting from phenolic acids' degradation [71] that has antioxidant activity [72]. Chang et al. found that protocatechualdehyde showed a strong DPPH free radical scavenging activity and inhibitory effects on LPS-induced nitric oxide (NO) production and COX-2 mRNA expression in RAW264.7 macrophages [73]. Protocatechuic acid is a water-soluble benzoic acid derivative, reported to have antioxidant and anti-inflammatory properties [74], like improving the macrophage endogenous antioxidant potential [75] and inhibiting monocyte adhesion molecules that play a role in anti-inflammatory effect [76]. Vanillic acid is an oxidized form of vanillin, which helps to manage/reduce oxidative stress due to its antioxidant potential [77]. Naganna et al. demonstrated that vanillic acid could enhance insulin secretion and protect pancreatic β cells from H₂O₂-induced oxidative stress [78]. Oxidation reaction during fermentation may partly explain the relative

decline of vanillin and isovanillin. It is reported that gentisic acid can effectively scavenge hydroxyl radical and organohaloperoxyl radical, significantly reducing the levels of gamma radiation-induced damage to lipids and proteins in rat liver mitochondria [79]. Similarly, syringic acid has also been proven to have antioxidant activity and is beneficial to human health [80, 81].

As discussed above, fermentation altered the composition of phenolic acids in the polysaccharide from the stem and leaves of CAL and increased the relative contents of *p*-coumaric acid, cinnamic acid, ferulic acid methyl ester, protocatechuic acid, protocatechualdehyde, vanillic acid,

gentisic acid, and syringic acid, which are likely causes of the enhancement of the antioxidant activity of FSLC.

Composition of flavonoids in SLC and FSLC

Flavonoids are the most common group of polyphenolic compounds, which are classified into several subgroups such as flavones, flavonols, and chalcones, possessing various activities such as anti-inflammatory, antioxidant, etc. [21]. A total number of 179 flavonoids were target identified in SLC and FSLC. Flavonols, flavones, and flavonoid carbonoside are the main flavonoids, which account for more than 88% of the flavonoids in SLC and FSLC (Fig. 2). The PCA was conducted on the flavonoids between two groups (Fig. S2A). The PCA components clearly separated SLC and FSLC groups, indicating that fermentation substantially affects flavonoids metabolite composition. The score plots generated from the intergroup comparison in OPLS-DA are shown in Fig. S2B. According to the results ($R^2X=0.7676$, $R^2Y=0.98$, $Q_2=0.966$), Q_2 values is greater than 0.9, demonstrating that the model could be applied to further screen for differential flavonoids metabolites.

There were 94 significantly different flavonoids metabolites between SLC and FSLC (27 up-regulated, 67 down-regulated) (Fig. S2C). The differential flavonoids metabolites between SLC and FSLC were classified and compared (Fig. 3A). As shown in Fig. 3A, the differentially accumulated metabolites are mainly concentrated in three subgroups: flavonols, flavones, and flavonoid carbonoside. These differential metabolites were further classified and analyzed (Fig. 3B). As shown in Fig. 3B, the

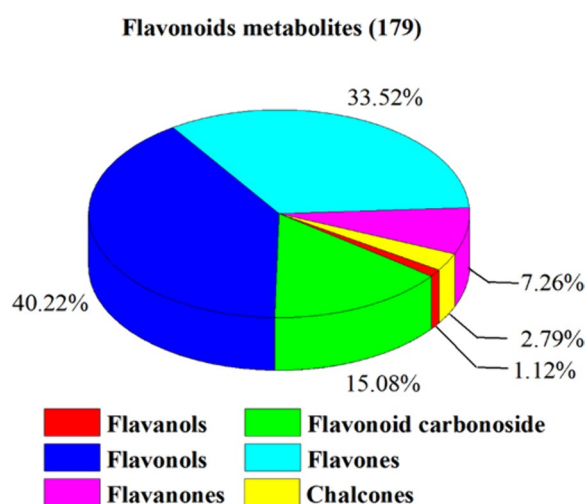


Fig. 2 Flavonoids classification chart according to subgroups

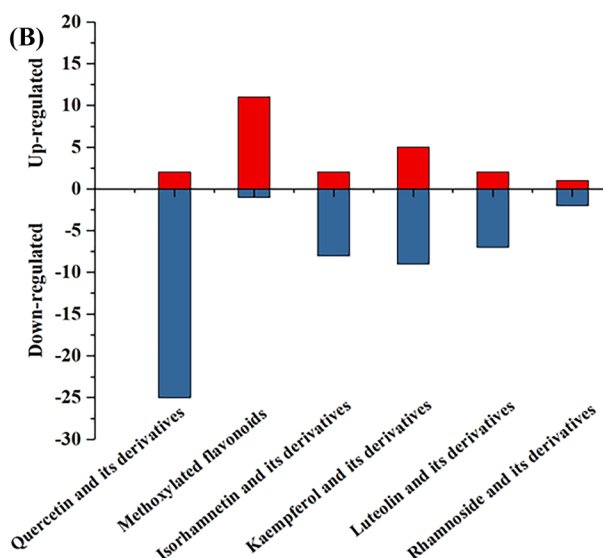
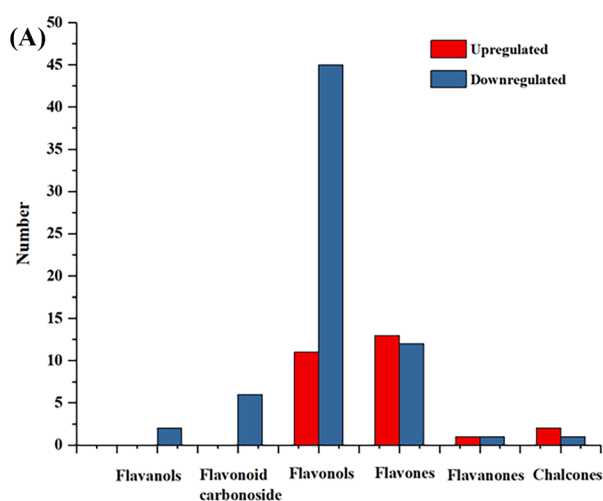


Fig. 3 Differentially accumulated flavonoids metabolites between SLC and FSLC. (A) Classification of differentially expressed metabolites. (B) The number of main differential metabolites

main up-regulated metabolites in FSLC are methoxylated flavonoids and kaempferol and its derivatives, and mainly down-regulated metabolites are quercetin and its derivatives, isorhamnetin and its derivatives, kaempferol and its derivatives and luteolin and its derivatives.

The relative content of some flavonoids' differential metabolites in SLC and FSLC is shown in Table 3. Fermentation resulted in the higher accumulation of quercetin (6.45 fold), 5,7,2'-trihydroxy-8-methoxyflavone (3.20 fold), 6,7,8-tetrahydroxy-5-methoxyflavone (3.44 fold), hispidulin (3.48 fold), diosmetin (3.4), kaempferol-7-O-rhamnoside (4.83 fold), isorhamnetin (24.11 fold), kaempferol (28.49 fold), luteolin (2.06 fold), nobiletin (43.40 fold), tangeretin (17.45 fold), eupatilin (2.65 fold) and rhamnetin (4.61 fold) (Table 3). Whereas, isorhamnetin-3-O-rutinoside (narcissin), rhamnetin-3-O-glucoside, quercetin-3-O-galactoside (hyperin), kaempferol-3-O-(6"-malonyl) glucoside, quercetin-3-O-rutinoside-7-O-rhamnoside, quercetin-3-O-glucoside (isoquercitrin), kaempferol-3-O-rutinoside (nicotiflorin), luteolin-7-O-neohesperidoside (lonicerin) and quercetin-3-O-sophoroside-7-O-rhamnoside relevant content decreased in FSLC compared to SLC (Table 3).

Quercetin is a popular flavonoid present as glycosides in various plants [82], and it has a wide range of biological actions, including anti-inflammatory and antioxidant [83]. An earlier report confirmed that quercetin inhibits cytokine and inducible nitric oxide synthase expression by inhibiting the NF- κ B pathway [84]. Oh et al. evaluated the antioxidant potential of quercetin and its derivatives; quercetin showed the highest radical scavenging activity among all tested samples [85]. Kaempferol and its glycoside derivatives have many beneficial functions, including antioxidant [86] and anti-inflammatory effects [87]. Shafek et al. reported that kaempferol has higher DPPH free radical scavenging potential than two kaempferol glycosides (kaempferol 3-O- β -D-glucopyranosyl (1 $\rightarrow$ 2) β -D-xylopyranoside and kaempferol 3-O- α -L-arabinopyranosyl (1 $\rightarrow$ 2) β -D-galactopyranoside) [88]. Similarly, compared with its glycosides' derivatives, kaempferol efficiently inhibited T-cell proliferation and NO release [87]. Isorhamnetin belongs to the flavonol class and exhibits significant antioxidant [89] and anti-inflammatory [90] effects. Wang et al. demonstrated that isorhamnetin has a potential protective effect that protects against oxidative stress in human RPE cells [91]. Yang et al. showed that isorhamnetin could alleviate LPS-induced acute lung injury by inhibiting Cox-2 expression in male BALB/c mice [92]. Luteolin could protect against H₂O₂-induced oxidative stress and ameliorate ROS and superoxide generation [93]. In nature, flavonoids almost all exist as their O-glycoside or C-glycoside forms in plants [94]. Previous studies have reported that

flavone glycosides are difficult to absorb in the intestinal tract and need to be bio-transformed into free aglycones before entering the systemic circulation to exert their effects [95]. Fermentation has been shown to hydrolyze the glycosides in safflower to aglycones that are more easily absorbed by the body, and fermented safflower exhibited a significant increase in hydroxyl radical scavenging ability and inhibition of hepatic oxide production [96]. Noticeably, our result demonstrated that fermentation promotes the degradation of flavonoid glycosides to flavonoid aglycone, significantly increasing the relative contents of quercetin, kaempferol, isorhamnetin, and luteolin.

What's more, fermentation significantly increased the methoxylated flavonoids, including 5,7,2'-trihydroxy-8-methoxyflavone, 6,7,8-tetrahydroxy-5-methoxyflavone, hispidulin, diosmetin, nobiletin, tangeretin, eupatilin. As an active ingredient in a number of traditional Chinese medicinal herbs, hispidulin had strong antioxidant potential [97] and induced apoptosis in human hepatoblastoma cancer cells [98]. Diosmetin has been reported to possess a strong antioxidant activity by scavenging intracellular reactive oxygen species [99], relieving inflammatory cell infiltration and suppressing the NF- κ B signaling pathway [100]. Nobiletin and tangeretin are the most studied polymethoxylated flavone, exhibiting strong antioxidant properties [101]. Pretreatment with nobiletin protected PC12 cells against H₂O₂-induced cytotoxicity by restoring GSH and SOD contents, diminishing MDA levels, and scavenging ROS formation [102]. Kou et al. found that tangeretin can enhance the activity of antioxidant enzymes, reduce the level of oxidative stress and injury, and protect myocardial morphology and ultrastructure [103].

As mentioned above, fermentation changed flavonoids composition of the polysaccharides from stem and leaves of CAL, promoted the accumulation of quercetin, hispidulin, diosmetin, isorhamnetin, kaempferol, luteolin, nobiletin and tangeretin. The enhancement of antioxidant activity of FSLC could be attributed to the increase of relative contents of quercetin, hispidulin, diosmetin, isorhamnetin, kaempferol, luteolin, nobiletin and tangeretin.

Effect of FSLC on antioxidant activity in IPEC-J2 cells model

FSLC demonstrated stronger in vitro antioxidant activities than SLC, so we further studied the effect of FSLC on the enzyme activity in IPEC-J2 cells. Overproduction of ROS can cause oxidative stress, which may lead to lipid peroxidation in cells [1]. MDA is a product of the lipid peroxidation process, which reflects the degree of lipid peroxidation in vivo [104]. In Fig. 4A, it is apparent that the MDA levels of the cells treated with FSLC at the concentration of 0.25–0.50 mg/mL were significantly

Table 3 Relative content of part of flavonoids differential metabolites in SLC and FSLC

Compounds	Relative content		VIP	FC	Type
	SLC	FSLC			
Quercetin and its derivatives					
Quercetin	3.24E + 05	2.09E + 06	1.14	6.45	Up
Quercetin-3-O-(2"-O-galactosyl) glucoside	1.19E + 05	3.37E + 05	1.14	2.84	Up
Quercetin-3-O-rutinoside-7-O-glucoside	1.66E + 06	5.00E + 04	1.12	0.03	Down
Quercetin-3-O-sophoroside-7-O-rhamnoside	1.65E + 06	6.06E + 04	1.12	0.04	Down
Quercetin-3-O-rutinoside (rutin)	4.53E + 06	4.04E + 05	1.13	0.09	Down
Quercetin-3-O-glucoside (Isoquercitrin)	1.48E + 07	2.00E + 06	1.13	0.14	Down
Quercetin-3-O-rutinoside-7-O-rhamnoside	2.25E + 07	3.23E + 06	1.10	0.14	Down
Quercetin-3-O-galactoside (Hyperin)	1.33E + 07	1.79E + 06	1.14	0.13	Down
Quercetin-3-O-xyloside (Reynoutrin)	1.17E + 07	3.68E + 06	1.12	0.32	Down
Quercetin-3-O-(2"-O-Rhamnosyl) rutinoside	1.01E + 07	2.43E + 06	1.11	0.24	Down
Quercetin-4'-O-glucoside (Spiraeoside)	1.97E + 06	3.47E + 05	1.11	0.18	Down
Methoxylated flavonoids					
5,7,2'-Trhiyrox-8-methoxyflavone	3.71E + 05	1.19E + 06	1.12	3.20	Up
6,7,8-Tetrahydroxy-5-methoxyflavone	2.82E + 05	9.70E + 05	1.13	3.44	Up
Hispidulin	2.75E + 05	9.56E + 05	1.12	3.48	Up
Diosmetin	2.80E + 05	9.51E + 05	1.13	3.40	Up
Nobiletin	1.25E + 03	5.43E + 04	1.15	43.40	Up
Tangeretin	2.40E + 03	4.18E + 04	1.14	17.45	Up
Eupatilin	8.29E + 03	2.20E + 04	1.08	2.65	Up
5,6,7-Tetrahydroxy-8-methoxyflavone	2.48E + 05	4.53E + 04	1.12	0.18	Down
Isorhamnetin and its derivatives					
Isorhamnetin	2.18E + 04	5.25E + 05	1.15	24.11	Up
Isorhamnetin-3-O-rutinoside (Narcissin)	2.59E + 07	2.57E + 06	1.12	0.10	Down
Isorhamnetin-3-O-neohesperidoside	2.46E + 07	2.44E + 06	1.13	0.10	Down
Isorhamnetin-7-O-glucoside (Brassicin)	2.24E + 06	7.25E + 05	1.00	0.32	Down
Isorhamnetin-3-O-rutinoside-4'-O-glucoside	1.86E + 05	1.94E + 04	1.13	0.10	Down
Kaempferol and its derivatives					
Kaempferol	8.17E + 03	2.33E + 05	1.14	28.49	Up
Kaempferol-3-O-rhamnoside (Afzelin)(Kaempferin)	1.45E + 05	1.45E + 05	1.12	5.01	Up
Kaempferol-7-O-rhamnoside	1.42E + 05	6.86E + 05	1.12	4.83	Up
Kaempferol-3-O-(6"-p-Coumaroyl)glucoside (Tiliroside)	2.50E + 04	1.78E + 05	1.15	7.13	Up
Kaempferol-3-O-rutinoside (Nicotiflorin)	2.44E + 07	5.70E + 06	1.12	0.23	Down
Kaempferol-3-O-neohesperidoside	2.62E + 07	5.95E + 06	1.12	0.23	Down
Kaempferol-3-O-(6"-malonyl) glucoside	1.10E + 05	9.23E + 03	1.15	0.08	Down
Kaempferol-3-O-sophoroside-7-O-rhamnoside	1.70E + 05	2.16E + 04	1.12	0.13	Down
Kaempferol-3-O-neohesperidoside-7-O-glucoside	1.74E + 05	2.51E + 04	1.11	0.14	Down
Luteolin and its derivatives					
Luteolin	2.65E + 04	5.46E + 04	1.10	2.06	Up
Luteolin-7-O-(6"-caffeoyl) rhamnoside	2.17E + 04	1.96E + 05	1.15	9.01	Up
Luteolin-7-O-neohesperidoside (Lonicerin)	2.59E + 07	6.16E + 06	1.12	0.24	Down
Luteolin-8-C-glucoside (Orientin)	3.74E + 05	1.06E + 05	1.12	0.28	Down
Luteolin-6-C-glucoside-7-O-rhamnoside	3.29E + 05	7.34E + 04	1.11	0.22	Down
Luteolin-7-O-glucoside (Cynaroside)	5.34E + 05	1.13E + 05	1.10	0.21	Down
Luteolin-6-C-glucoside (Isoorientin)	4.39E + 05	1.88E + 05	1.13	0.43	Down
Rhamnetin and its derivatives					
Rhamnetin	2.74E + 03	1.26E + 04	1.06	4.61	Up
Rhamnetin-3-O-Glucoside	9.82E + 05	7.68E + 04	1.14	0.08	Down

Table 3 (continued)

Compounds	Relative content		VIP	FC	Type
	SLC	FSLC			
Rhamnetin-3-O-Glucoside	2.25E+07	2.32E+06	1.13	0.10	Down

decreased compared with the control group. GSH, as a non-enzymatic antioxidant, has physiological functions, including free radical scavenging, anti-oxidation, and electrophile elimination [105]. Compared with the control, treatment with FSLC at a concentration of 0.25 mg/mL significantly increased GSH content (Fig. 4B). Antioxidant defense systems of living organisms include enzymes and non-enzymatic defenses, enzyme antioxidant defense systems including SOD, CAT, GSH-Px, and T-AOC [106]. SOD is the first line of cellular defense against oxidative damage [107]. As shown in Fig. 4C, the SOD activity of cells treated with 0.25 mg/mL FSLC was significantly increased. Treatment with FSLC at 0.25 and 0.75 mg/mL significantly improved the CAT activity of cells (Fig. 4D). After being stimulated with 0.25 mg/mL of FSLC, GSH-Px activity of cells was significantly increased (Fig. 4E). It is observed that the T-AOC of cells treated with 0.25 mg/mL FSLC was significantly increased (Fig. 4F). Fang et al. suggested that enzymatic degradation of polysaccharides from *Gracilariopsis lemaneiformis* pretreatment can significantly reduce reactive oxygen species and MDA levels, improve antioxidant enzyme activity and alleviate oxidative damage in HFL1 cells [108]. Similarly, Qian et al. revealed that the polysaccharide from *Auricularia auricula* effectively ameliorated oxidative stress in human hepatocellular carcinoma (HepG2) cells by inhibiting reactive oxygen species generation, decreasing the content of MDA and increasing the activities of SOD, GSH-Px, and CAT [109]. These results indicated that 0.25 mg/mL FSLC treatment could alleviate lipid peroxidation, enhance the antioxidant enzyme secretion, and reduce oxidative stress in IPEC-J2 cells. Thus, FSLC might be a suitable source of natural antioxidants.

Effect of FSLC on development-promoting activity in zebrafish embryo model

Effects of FSLC on morphology, viability, and hatching rate of zebrafish embryos

Since, to date, FSLC has never been used on zebrafish; different concentrations were assessed to allow us to find the safe concentration. In order to identify the suitable concentration and time points used in the following experiments, FSLC ranging from 25, 50, 100, 200, to 300 µg/mL concentration was applied to evaluate the embryonic viability and hatching rate and observe

the morphology of zebrafish. As presented in Fig. 5A, FSLC concentration from 25 to 300 µg/mL did not alter the zebrafish morphology from 12 to 72 hpf. Then, we confirmed the viability and hatching rate of zebrafish embryos in response to various concentrations of FSLC exposure. As shown in Fig. 5B, compared with the control, treatment with 25–300 µg/mL of FSLC had no dramatic impact on embryonic viability ($P>0.05$). It is interesting that at 48 hpf, the zebrafish embryos in the control group did not hatch, while the incubation of zebrafish embryos was observed in the 50–300 µg/mL FSLC treatment group (Fig. 5C). At 48 and 60 hpf, treatment with 100–300 µg/mL FSLC significantly improved the hatching rate of embryos ($P<0.05$, Fig. 5C). There was no differences were observed for hatching rate of embryos exposed to different concentrations of FSLC compared to control group at 72 hpf. These data illustrate that there are no harmful effects of FSLC (25–300 µg/mL) on zebrafish embryonic development and treatment with 100–300 µg/mL FSLC increased the hatching rate of embryos. To investigate the effects of FSLC on the development-promoting action of zebrafish, FSLC at concentrations 100–300 µg/mL was selected in the follow-on study.

Effects of FSLC on the morphology and hatching rate of zebrafish embryos response to LPS

LPS is an endotoxin molecule, and its administration to cells or animals induces strong immune responses [110]. Previous reports have demonstrated that LPS treatment caused toxic impacts on phenotypic changes in zebrafish embryos, such as yolk sac edema size, tail bending, and heart rate. As presented in Fig. 6A, LPS 4 µg/mL induced abnormalities, like large yolk sac and body axis curvature in zebrafish compared with the control group, which is consistent with previous reports [111]. While the treatment with FSLC (100–300 µg/mL) after LPS-induced had no morphological abnormalities, illustrating FSLC showed protective effects against the toxicity of LPS. As shown in Fig. 6B, when the zebrafish embryos were treated with 4 µg/mL LPS, the hatching rate dropped significantly compared with the control group. The hatching rate of FSLC treatment from 100 to 300 µg/mL after LPS-induced significantly improved compared to the LPS and control group ($P<0.05$). As a nutritional cache, the yolk sac serves nutrients for the zebrafish from embryo to

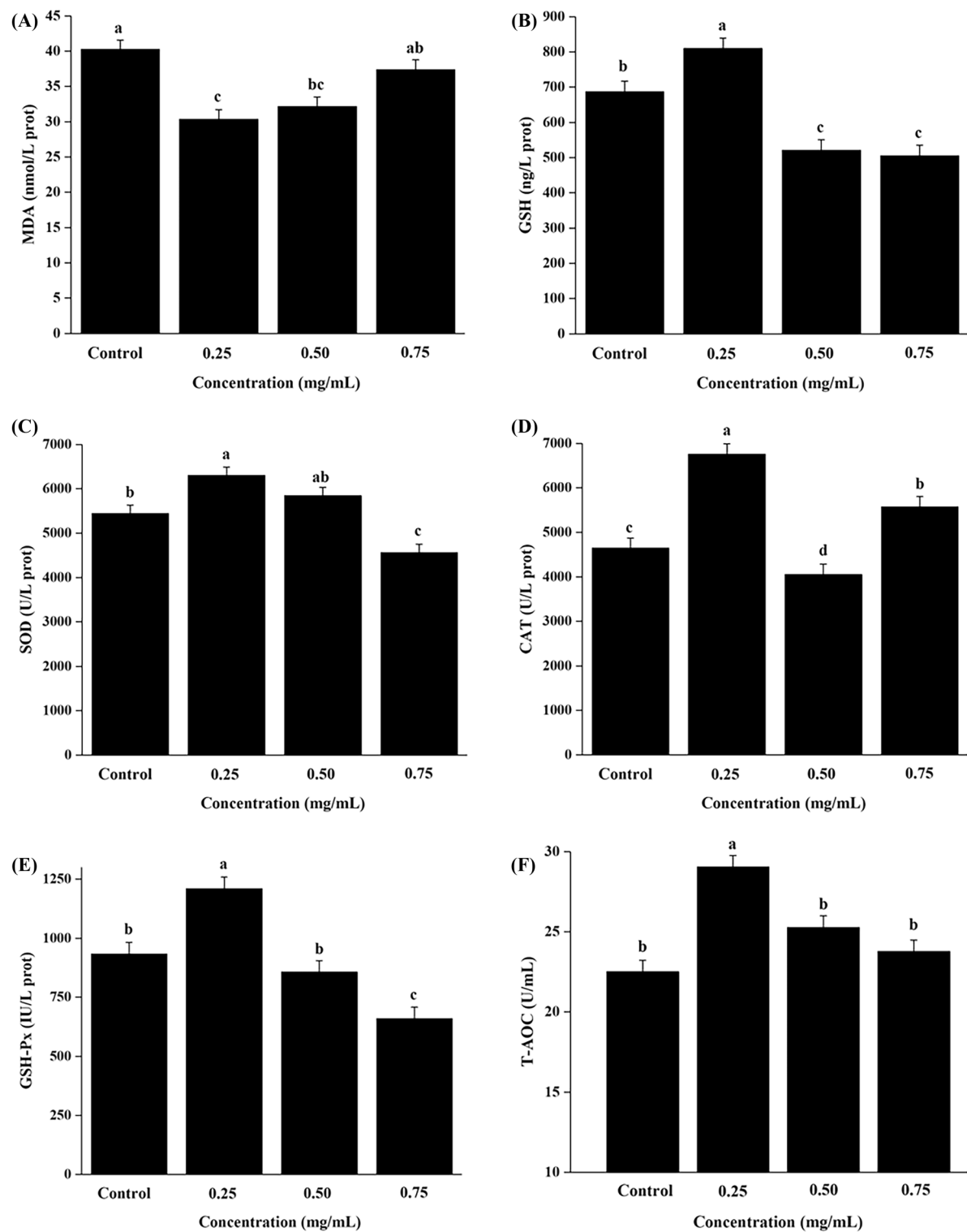


Fig. 4 Effect of FSLC on the contents of MDA and GSH and antioxidant enzymes in IPEC-J2 cells. MDA content (A), GSH content (B), SOD activity (C), CAT activity (D), GSH-Px activity (E), T-AOC activity (F). Bars of the group labeled with different lowercase letters denote statistically significant differences ($P < 0.05$)

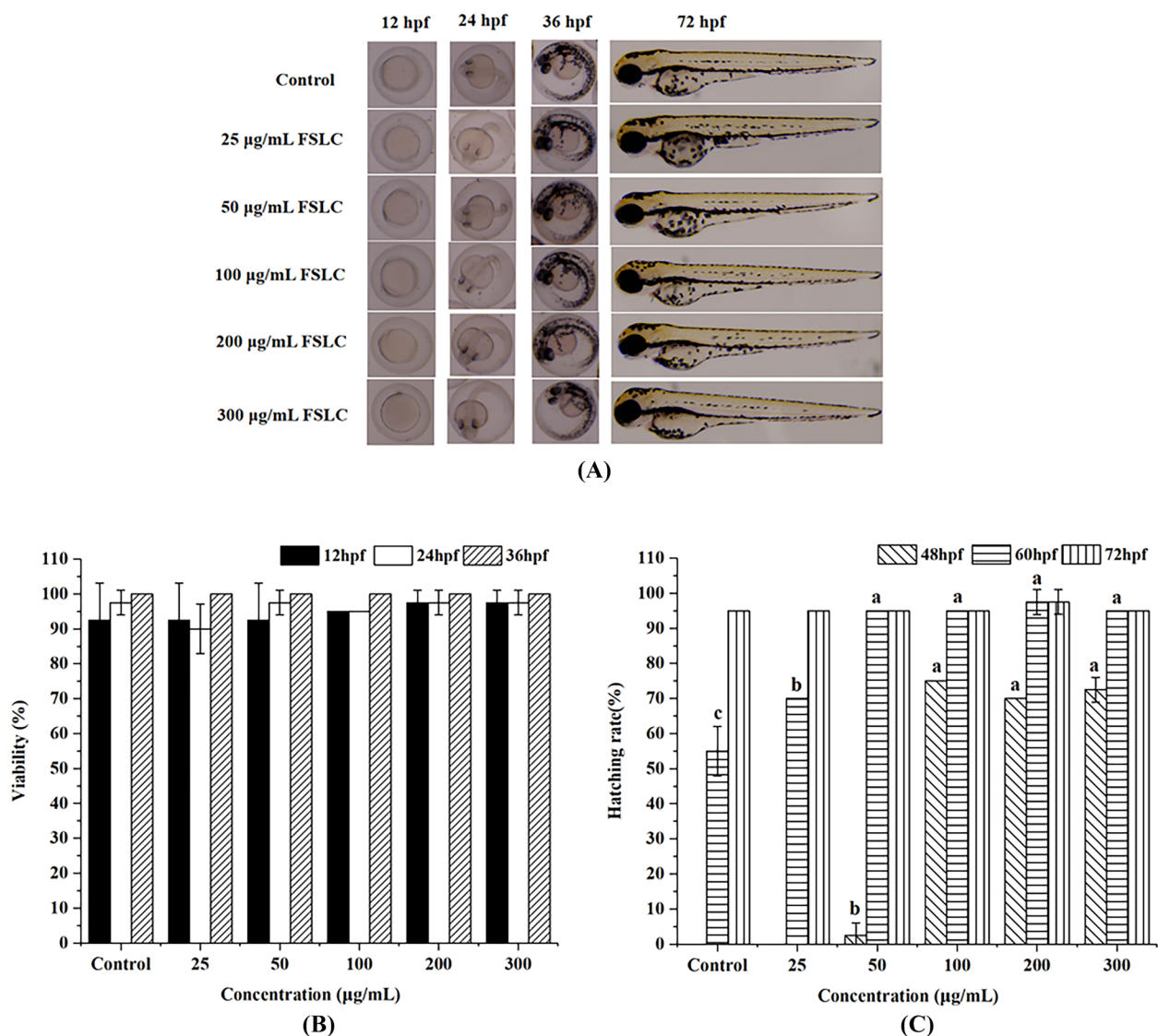


Fig. 5 The morphology, viability, and hatching rate in zebrafish are caused by different concentrations of FSLC exposure. **(A)** Morphological images at 12–72 hpf. **(B)** Results for embryonic viability. **(C)** Results for hatching rate in zebrafish. Bars of the groups labeled with different lowercase letters denote statistically significant differences ($P < 0.05$)

juvenile fish [112]. So, the size of the yolk sac is crucial to embryo development. At 72hpf, the zebrafish in the LPS-induced group had developmental retardation, and less yolk consumption. Fortunately, with the intervention of FSLC, the LPS-induced morphology abnormalities were not present, and the hatching rate of zebrafish was significantly increased. Wang et al. indicated that sulfated polysaccharides isolated from *Sargassum fulvellum* improved the survival rate, reduced cell death, reactive oxygen species, and NO levels of LPS-stimulated zebrafish [113]. Furthermore, Wang et al. reported that a sulfated polysaccharide from *Saccharina japonica* played a protective role against LPS-induced inflammatory responses in

zebrafish, increasing the survival rate, reducing the yolk sac edema size, and inhibiting cell death and the production of intracellular reactive oxygen species (ROS) and NO [114]. Hereby, the results demonstrated that treatment with 100–300 $\mu\text{g/mL}$ FSLC could relieve morphological abnormalities of zebrafish caused by LPS and improve the hatching rate of embryos, which indicates that FSLC possesses a development-promoting effect.

Conclusion

SLC and FSLC were obtained from unfermented stems and leaves of CAL and fermented stems and leaves of CAL via hot water extraction and ethanol precipitation.

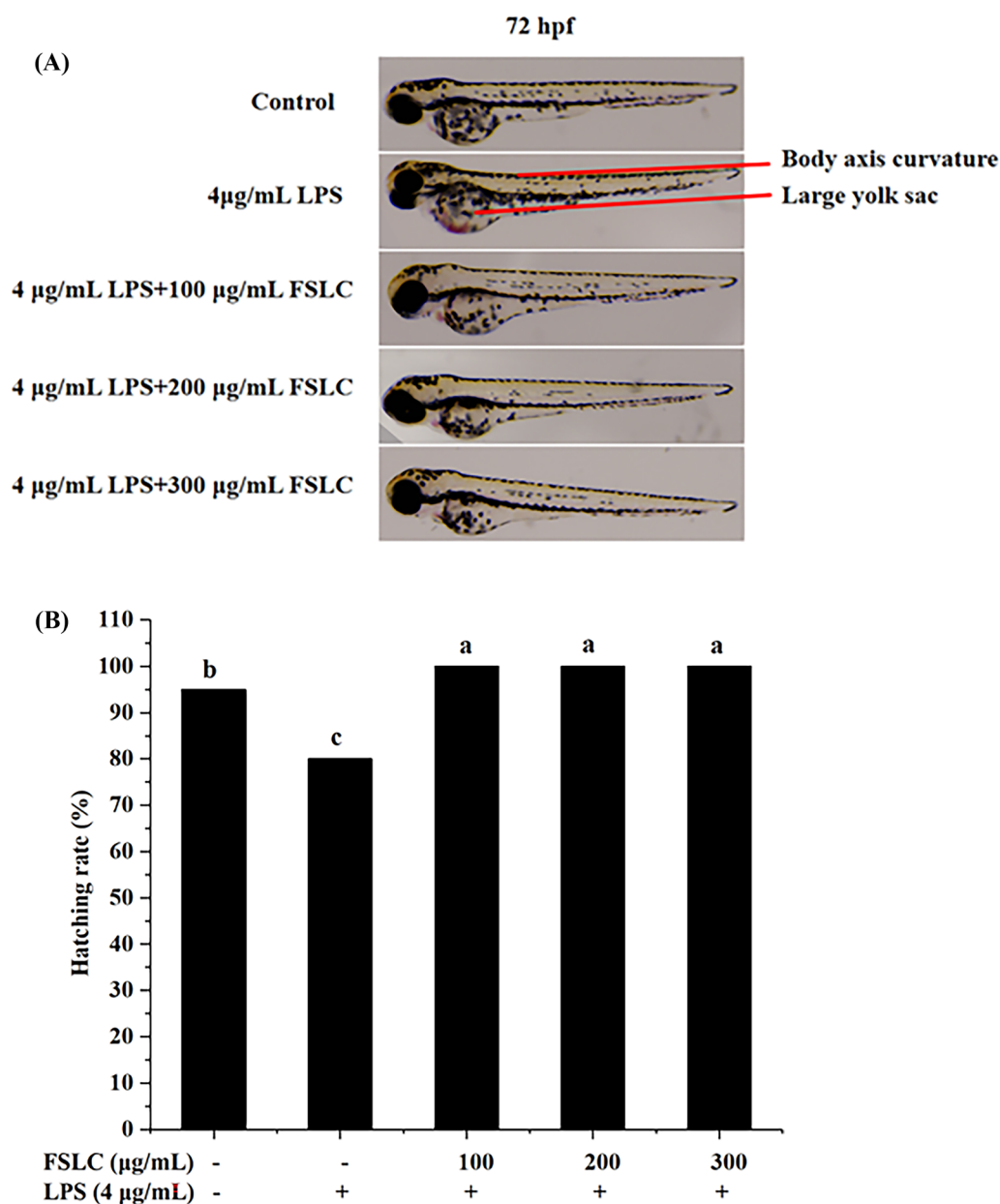


Fig. 6 Effects of FSLC on LPS exposure on morphology and hatching rate of zebrafish. **(A)** Morphological images at 72 hpf. **(B)** Results for hatching rate in zebrafish. Bars of the groups labeled with different lowercase letters denote statistically significant differences ($P < 0.05$)

In vitro antioxidant activity, FSLC exhibits stronger DPPH, hydroxyl radical-scavenging activity, and reducing power than SLC. The levels of total polysaccharide, polyphenol, and flavonoid, and the molar ratio of glucuronic acid increased in FSLC. Moreover, in comparison with SLC, the relative content of protocatechuic acid, protocatechualdehyde, gentisic acid, vanillic acid, p-coumaric acid, quercetin, ferulic acid methyl ester, hispidulin, diosmetin, cinnamic acid, isorhamnetin, syringic acid, and kaempferol increased in FSLC. Our results also indicated that

treatment with 0.25 mg/mL FSLC could reduce MDA levels and increase antioxidant enzyme activities and GSH levels in IPEC-J2 cells. Notably, treatment with 100–300 μ g/mL FSLC increased the hatching rate of zebrafish embryos at 48 and 60 hpf, could relieve morphological abnormalities caused by LPS, and improve the hatching rate of embryos at 72hpf. These results together suggested that FSLC could be a potential natural feed additives to protect against oxidative damage and development-promoting effects. However, the structural features of FSLC and its molecular

mechanism on antioxidant is still unclear. Therefore, the antioxidant mechanism and structure–activity relationship of FSLC will be further studied in future work.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-024-00610-z>.

Additional file 1: **Figure S1.** Phenolic acids metabolites identified in SLC and FSLC. **A** The two-dimensional scatter plot of the PCA model. **B** The score plot of the OPLS-DA model. **C** Volcano plot of the differential phenolic acids metabolites between SLC and FSLC. **Figure S2.** Flavonoids metabolites identified in SLC and FSLC. **A** The two-dimensional scatter plot of the PCA model; **B** The score plot of the OPLS-DA model. **(C)** Volcano plot of the differential flavonoids metabolites between SLC and FSLC.

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Author contributions

N. L. methodology, investigation, formal analysis, writing-original draft, and writing-review and editing. Y. W. methodology, validation and formal analysis. XP. A. conceptualization, methodology, data curation and project funding acquisition. JW. Q. supervision, project administration and funding acquisition. BY. W. methodology and formal analysis. J. D. methodology. WW. W. methodology. All authors contributed to the article and agreed to the published version of the manuscript.

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Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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