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Simultaneous improvement of aroma and bioactivity of *Spirulina platensis*: A novel approach by Basidiomycota fermentation

Can Xiang [a], Zihan Wang [b], Annu Ann Joseph [a], Sarah Greve [c], Ruotong Nie [a], Katrin Giller [c], Jianping Wu [b], Yanyan Zhang [a,*]

[a] Department of Flavor Chemistry (150h), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany
[b] Department of Agricultural, Food and Nutritional Science, University of Alberta, 4–10 Ag/For Building, Edmonton, Alberta T6G 2P5, Canada
[c] Department of Molecular Nutritional Science (140a), Institute of Nutritional Sciences, University of Hohenheim, Garbenstraße 30, 70599 Stuttgart, Germany

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ABSTRACT

Fermentation has emerged as a promising strategy to modify aroma profile of *Spirulina platensis*, yet the application of Basidiomycota for aroma improvement remains largely unexplored. In this study, a Basidiomycota–*S. platensis* fermentation system was established using edible mushroom strains to improve its sensory properties. Among twelve tested strains, *Stropharia rugosoannulata* showed the most pronounced aroma improvement after 56 h fermentation at 24 °C under shaking conditions. Sensory evaluation indicated that fermentation effectively reduced undesirable fishy and earthy odors and introduced floral, fruity, and sweetish notes. Further aroma profiling attributed these changes to a marked decrease in lipid-derived aldehydes and the formation of fruity ethyl esters. Ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, and ethyl 3-methylbutanoate were identified as key contributors to the fruity aroma. Precursor supplementation experiments suggested that involvement of branched-chain amino acid metabolism in the formation of the fruity ethyl esters via the Ehrlich pathway. In addition to aroma improvement, fermented *S. platensis* significantly reduced TNF-α-induced COX-2 expression and intracellular reactive oxygen species levels in EA.hy926 cells compared with the unfermented sample. Cytotoxicity assays using HepG2 cells showed no significant cytotoxic effects after fermentation. Overall, this study establishes a novel Basidiomycota–*S. platensis* fermentation system that improves aroma quality and enhances its anti-inflammatory and antioxidant potential under the present experimental conditions. The findings also provide supporting evidence for the proposed involvement of branched-chain amino acid metabolism in fruity ethyl ester formation and provide a basis for developing aroma-improved microalgal ingredients.

1. Introduction

Increasing pressure on global food systems has led to growing interest in alternative protein sources, including plant, microbial, insect, and algae based proteins, as complements or substitutes for conventional livestock-derived proteins (Carrillo-Huerta et al., 2025). Among these, microalgae represent a particularly promising option. Compared with other protein sources, such as soybean with a yield of approximately 0.6–1.2 t protein ha^{-1} $year^{-1}$, microalgae require no arable land and less water while achieving higher productivity of 4–15 t protein ha^{-1} $year^{-1}$ (Gupta et al., 2026). *Spirulina platensis* is a widely utilized microalga with a protein content reaching approximately 70% (Ragaza et al., 2020), providing all essential amino acids required by humans, with essential amino acids accounting for approximately 47% of total protein (Begum et al., 2024). Besides its high protein level, *S. platensis* also contains considerable amounts of polyunsaturated fatty acids (PUFAs, 1.7–3.8%), with ω-3 fatty acids accounting for up to 49% of the total fatty acids (Uzlaşır et al., 2024), as well as carotenoids and other bioactive compounds, including phycocyanin, chlorophyll *a*, flavonoids, and phenolic compounds (Lestingi, 2024).

* Corresponding author at: Department of Flavor Chemistry (150h), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany.
E-mail addresses: can.xiang@uni-hohenheim.de (C. Xiang), zihan18@ualberta.ca (Z. Wang), mx.annuann@gmail.com (A.A. Joseph), sarah.greve@uni-hohenheim.de (S. Greve), ruotong.nie@uni-hohenheim.de (R. Nie), katrin.giller@uni-hohenheim.de (K. Giller), jwu3@ualberta.ca (J. Wu), yanyan.zhang@uni-hohenheim.de (Y. Zhang).

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Despite its nutritional value and sustainable production, the wider application of *S. platensis* in food products is limited by its unfavorable sensory properties, which reduce consumer acceptance (Xiang et al., 2026). Its characteristic off-flavors are commonly described as fishy, marine-like, grassy, green, roasted, and seaweed-like, and are mainly attributed to chemical and enzymatic lipid oxidation during processing (Jia et al., 2024; Xiang et al., 2026).

Fermentation has long been used to improve the sensory properties of foods, primarily using fungi from Ascomycota (e.g., *Aspergillus*, *Penicillium*, and yeasts) and Mucoromycota (e.g., *Mucor* and *Rhizopus*) (Campbell-Platt, 1994). In contrast, Basidiomycota have received comparatively less attention, despite their diverse enzymatic systems involved in lignocellulose degradation and secondary metabolite production. These metabolic capabilities enable Basidiomycota to transform a wide range of precursors into aroma-active compounds. For example, *Pleurotus* spp. can synthesize and secrete commercially relevant flavor compounds, such as anisaldehyde and vanillin, under nitrogen-limited conditions (Gutiérrez et al., 1994). *Pleurotus ostreatus* can biotransform the terpene β-myrcene, naturally present in citrus peels, into aroma-active compounds with floral and citrus notes (Krings et al., 2008). Basidiomycota fermentation has been reported to reduce off-flavors while generating desirable aroma compounds. For instance, *Wolfiporia cocos* decreased hexanal and (*Z*)-6-nonenal associated with green off-notes in okara, while increasing floral compounds such as phenethyl alcohol and linalool (Wang et al., 2022). Similarly, *Cyclocybe aegerita* reduced aldehydes responsible for grassy off-flavors in soymilk, while enhancing cheese-like notes through the formation of short-chain fatty acids (Nedele et al., 2022). These studies demonstrate that Basidiomycota fermentation can substantially alter food aroma profiles.

For *S. platensis*, previous studies have explored the use of bacteria and yeasts to improve its sensory properties. Fermentation with lactic acid bacteria (LAB) and yeast strains has been shown to reduce pyrazines and carotenoid-derived compounds, while LAB, *Bacillus*, and yeasts decreased undesirable notes such as cardboard-like, marine, and earthy flavors. Among three yeast strains evaluated by Kurt et al. (2023), *Kluyveromyces marxianus* showed the most pronounced effects, with reduced marine and umami notes and increased fermented and rose-like aromas. However, the potential of Basidiomycota fermentation to modify the sensory properties of *S. platensis* remains largely unexplored.

In this study, edible Basidiomycota were explored as a fermentation strategy to improve the sensory quality of commercial *S. platensis*. Twelve strains were initially screened, and *S. rugosoannulata* was selected for further investigation based on its desirable aroma-modifying effects. Changes in aroma-active compounds were subsequently characterized to clarify the chemical basis of aroma remodeling during fermentation, while precursor supplementation experiments combined with molecular docking analysis were conducted to elucidate the formation mechanism of key fruity esters. In addition, the anti-inflammatory and antioxidant potential, as well as cytotoxicity, of fermented *S. platensis* were evaluated to further assess the biological effects of Basidiomycota fermentation.

2. Materials and methods

2.1. Materials and reagents

Commercial dried *S. platensis* powder was obtained from myvial GbR (Nattheim, Germany). Edible Basidiomycota strains were sourced from the German Collection of Microorganisms and Cell Cultures and the Institute of Food Chemistry and Biotechnology, Justus Liebig University Giessen (Giessen, Germany). Agar, malt extract, and peptone used for culture media preparation were purchased from Carl Roth (Karlsruhe, Germany).

Authentic standards (purity ≥95%) were used for the identification and semi-quantitative analysis of aroma-active compounds. Ethyl 2-methylpropanoate, 1-octen-3-ol, (*E*)-2-nonenal, and (*E,E*)-2,4-decadienal were obtained from Thermo Fisher Scientific (Sindelfingen, Germany). 2-Pentylfuran, 6-methyl-5-hepten-2-one, decanal, and α-ionone were purchased from Alfa Aesar (Karlsruhe, Germany). Hexanal, (*E*)-2-octenal, and saturated alkanes (C7–C40) were sourced from Merck (Darmstadt, Germany). Benzaldehyde, linalool, thymol, and β-ionone were obtained from Carl Roth (Karlsruhe, Germany). Ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and γ-nonalactone were obtained from J&K Scientific LLC (California, USA). 1-Octen-3-one was obtained from BLD Pharmatech GmbH (Reinbek, Germany). Analytical-grade solvents, including methanol, diethyl ether, and n-pentane, were obtained from VWR International (Darmstadt, Germany) and Fisher Scientific (Schwerte, Germany). Sodium chloride was purchased from VWR International (Darmstadt, Germany). L-Leucine, L-isoleucine, and L-valine (≥ 98% purity) were obtained from Merck, (Darmstadt, Germany).

Reagents used for cell-based assays were obtained as follows. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Gibco Invitrogen (Burlington, ON, Canada). Secondary antibodies (goat anti-rabbit IRDye 680RD and donkey anti-rabbit IRDye 800CW) were obtained from LI-COR Biosciences (Lincoln, NE, USA). Dihydroethidium (DHE), digestive enzymes (α-amylase, pepsin, lipase, pancreatin), bile extract, Triton X-100, trypsin–EDTA, MTT, and dimethyl sulfoxide (DMSO) were sourced from Merck (Darmstadt, Germany). RPMI 1640 medium was obtained from Thermo Fisher Scientific (Sindelfingen, Germany). Hydrochloric acid was purchased from Carl Roth (Karlsruhe, Germany). Sodium hydroxide, phosphate salts, and other inorganic salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) were obtained from VWR International (Darmstadt, Germany) and AppliChem (Darmstadt, Germany).

2.2. Sample preparation

2.2.1. Fermentation

Fermentation experiments were performed following a previously established protocol with modifications adapted to the current system (Rigling et al., 2024). Edible Basidiomycota strains were cultivated on agar plates at 24 °C in darkness until sufficient mycelial growth (approximately 80% surface coverage) was achieved and subsequently stored at 4 °C prior to use. For preculture preparation, agar plugs (~1 cm^2) containing actively growing mycelia were transferred into 100 mL sterilized liquid medium and dispersed by homogenization. The precultures were incubated at 24 °C under shaking conditions (150 rpm) for 7 days in darkness.

To minimize the influence of residual nutrients, mycelial biomass was collected by centrifugation (2150 ×*g*, 10 min, 20 °C) and washed repeatedly with sterile ultrapure water. The washed biomass was then resuspended and used as inoculum (10%, *v*/v) for fermentation. The fermentation substrate consisted of a sterilized suspension of *S. platensis* (10 g/L in ultrapure water). Fermentation was carried out at 24 °C with shaking (150 rpm) in darkness for up to 100 h. Samples were collected at 4–6 h intervals to monitor the evolution of aroma characteristics.

2.2.2. Separation of mycelia and freeze-drying

At the end of fermentation, fungal biomass was removed from the culture by filtration. The resulting liquid fraction was frozen and subsequently subjected to lyophilization using a freeze dryer (LIO-3 S, FreezeDry GmbH, Germany) under reduced pressure (0.5 mbar) with a condenser temperature of −55 °C for 72 h. The obtained freeze-dried samples were collected and stored at −25 °C until further sensory and analytical evaluation.

2.3. Aroma analysis

2.3.1. Sensory evaluation

The aroma characteristics of *S. platensis* suspensions (20 g/L) before and after fermentation were evaluated by a trained descriptive sensory

panel. The panelists were trained and selected according to DIN EN ISO 8586:2014–05 (Liang et al., 2022). During the training sessions, aroma reference standards in the form of sniffing sticks were used to familiarize the panelists with the selected sensory descriptors (Table S1). The panel consisted of ten members (six females and four males, aged 25–32 years), all non-smokers with prior experience in sensory analysis. Ten descriptors, including grassy, fruity, fishy, mushroom-like, green, sweetish, floral, citrus-like, earthy, and musty notes, were used to describe the samples. Samples were presented under blind conditions in a randomized order using three-digit codes. The sensory evaluation was conducted in a single session, and each sample was evaluated in duplicate. The intensity of each attribute was rated on a scale from 0 to 5, where 0 indicated that the odor was not perceived and 5 indicated a strong intensity. The evaluation procedure followed our previously reported method (Xiang et al., 2026).

2.3.2. Qualitative and semi-quantitative analysis of aroma-active compounds

Aroma-active compounds were extracted by headspace solid-phase microextraction (HS-SPME) and analyzed using Gas Chromatography–Mass Spectrometry–Olfactometry (GC–MS–O) and GC–MS. GC–MS–O was applied to screen aroma-active compounds, while GC–MS was used for semi-quantitative analysis. The HS-SPME extraction procedure and GC–MS–O conditions were performed according to our previous study (Xiang et al., 2026) with slight modifications. GC–MS–O analysis was carried out on an Agilent 7890B gas chromatograph coupled with a 5977B mass spectrometer (Agilent Technologies, Waldbronn, Germany) and equipped with an olfactory detection port (ODP, Gerstel, Germany). GC–MS analysis for semi-quantification was performed on an Agilent 8890 gas chromatograph coupled with a 5977B mass spectrometer (Agilent Technologies, Waldbronn, Germany). To improve compound identification, two capillary columns with different polarities, a DB-WAX column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and an HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Agilent Technologies, Waldbronn, Germany), were employed.

For semi-quantitative analysis, a matrix-matched calibration approach was used to minimize interference from endogenous aroma compounds. Briefly, the sample matrix was treated with a mixture of methanol, diethyl ether, and *n*-pentane (2,1:1, *v*/v/v) and allowed to stand for 8 h. The solvent was then removed, and the extraction procedure was repeated three times. Subsequently, a series of standard solutions at different concentrations were spiked into the treated matrix to construct calibration curves, and semi-quantification was performed using the external standard method. Each calibration level was analyzed in triplicate. The calibration ranges, regression equations, coefficients of determination (R^2), repeatability expressed as relative standard deviation (RSD), limits of detection (LOD), and limits of quantification (LOQ) are summarized in Table S3. The odor activity value (OAV) of each compound was calculated as the ratio of its concentration to its odor threshold (OT) in water, with OT values obtained from literature.

2.4. Amino acid feeding experiment

To investigate the contribution of branched-chain amino acids to ester formation during fermentation, precursor feeding experiments were performed using valine, leucine, and isoleucine. Individual amino acids were dissolved in sterile water, filtered through 0.22 μm membranes, and added to the sterilized *S. platensis* suspension at a final concentration of 1 g/L prior to inoculation. A control without amino acid supplementation was prepared in parallel. Fermentation with *S. rugosoannulata* was conducted for 56 h under the same conditions described in Section 2.2. Samples were collected and analyzed for aroma compounds. Aroma compounds were determined using GC–MS equipped with a DB-WAX capillary column. Changes in aroma-related compounds were evaluated based on peak area responses. All experiments were performed in triplicate, with two parallel measurements for each replicate.

2.5. Molecular docking of alcohol acyltransferase (AAT)

Molecular docking was performed using AutoDock Vina (version 1.2.7) to investigate the interaction between the target protein AAT and branched-chain acyl-CoA intermediates involved in ester biosynthesis. As no experimentally validated AAT sequence from *S. rugosoannulata* is currently available, the amino acid sequence of the functionally related yeast AAT (UniProt ID: P40353, ATF1_YEAST) was retrieved from the UniProt database. The three branched-chain acyl-CoA intermediates, namely isobutyryl-CoA, isovaleryl-CoA, and *(S)*-2-methylbutanoyl-CoA, were obtained from the PubChem database in SDF format and converted into MOL2 format. The binding site was defined based on the predicted active pocket obtained from the Prank Web server (https://prankweb.cz/), with the grid center set at coordinates (−7.22, 4.86, 11.01). The grid box size was set to 36 × 36 × 36 Å to ensure sufficient coverage of the binding region. Prior to docking, the protein structure was prepared using AutoDock Tools (version 1.5.7). Protein–ligand complexes were visualized using PyMOL (version 3.2), and intermolecular interactions, including hydrogen bonds and hydrophobic interactions, were analyzed using Discovery Studio Visualizer 2025.

2.6. Bioactivity assays

2.6.1. EA.hy926 cell culture

EA.hy926 cells were maintained in DMEM containing 10% FBS, 1% penicillin–streptomycin, and 25 mM HEPES at 37 °C in a humidified incubator with 5% CO_2. Cells reaching 80–90% confluence were detached using trypsin–EDTA and seeded into 12-well plates. To minimize basal activity, cells were incubated for 12 h in medium supplemented with 1% FBS prior to treatment. Fermented and unfermented *S. platensis* samples were dispersed in culture medium at the indicated concentrations based on the dry weight of the original powder before being applied to the cells as described below.

2.6.2. Measurement of reactive oxygen species (ROS)

Intracellular ROS production was evaluated using DHE staining with slight modifications (Wang et al., 2026). Following sample pretreatment (2.5 mg/mL, 1 h), cells were exposed to tumor necrosis factor-α (TNF-α, 10 ng/mL) for 6 h. Thereafter, DHE (20 μM) was applied and incubation continued for 30 min at 37 °C. After washing with phenol red-free DMEM, fluorescence images were obtained using an Olympus IX81 microscope, and signal intensity was quantified with ImageJ.

2.6.3. Measurement of cyclooxygenase-2 (COX-2) expression

To evaluate inflammation-related protein expression, EA.hy926 cells seeded in 12-well plates were treated with samples (100 μg/mL) for 18 h, followed by stimulation with TNF-α (10 ng/mL) for 6 h. Protein expression was analyzed by Western blot with slight modifications based on a previously reported method (Wang et al., 2023). Briefly, cells were lysed in Laemmli buffer containing 2% DTT and denatured by heating. The protein samples were separated by 9% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin in TPBS and subsequently incubated overnight at 4 °C with primary antibodies against COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at a dilution of 1:1000. After incubation with the corresponding secondary antibodies for 1 h at room temperature, protein signals were visualized using a LI-COR Odyssey imaging system and quantified with Image Studio Lite 5.2. The expression level of COX-2 was normalized to GAPDH.

2.6.4. Assessment of EA.hy926 cell viability

Cell viability was evaluated using the MTT assay under the same treatment conditions as those used for the COX-2 assays. EA.hy926 cells

were seeded into 96-well plates and cultured as described in Section 2.6.1. Untreated cells served as the negative control, whereas cells treated with TNF-α were included as the inflammatory control. Subsequently, 5 μL of MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated for 2 h at 37 °C. The culture medium was then removed, and 100 μL of solubilization solution (10 g SDS in 99.4 mL DMSO containing 0.6 mL acetic acid) was added to dissolve the formazan crystals. After incubation for 5 min at room temperature, absorbance was measured at 570 nm using an Agilent Technologies BioTek Epoch microplate spectrophotometer. Cell viability was expressed as a percentage relative to untreated control. All experiments were performed in triplicate with three parallel wells for each treatment.

2.7. Cytotoxicity assay

2.7.1. Simulated gastrointestinal digestion

The gastrointestinal digestion of fermented and non-fermented *S. platensis* was simulated using a standardized static model adapted from the INFOGEST protocol without major modifications (Brodkorb et al., 2019). For the oral phase, samples (0.5 g) were mixed with simulated salivary fluid (2 mL), and the pH was adjusted to 7.0 prior to the addition of α-amylase (150 U/mL). The system was incubated briefly at 37 °C under continuous agitation. Gastric digestion was initiated by adding simulated gastric fluid (2 mL) and adjusting the pH to 3.0, followed by the addition of pepsin (2000 U/mL) and lipase (60 U/mL). The mixture was maintained at 37 °C for 2 h. For the intestinal phase, simulated intestinal fluid (4 mL) was introduced, and the pH was readjusted to 7.0. Bile extract (40 mg/mL) and pancreatin (100 U/mL) were added, and digestion proceeded for an additional 2 h. Following digestion, samples were centrifuged (2150 ×*g*, 10 min), and the supernatants were collected, filtered (0.22 μm), and stored at −25 °C for subsequent experiments.

2.7.2. HepG2 cell culture

HepG2 cells were maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO_2. Cells were propagated in 25 cm^2 culture flasks and passaged at 80–90% confluence using 0.25% trypsin–EDTA at a ratio of 1:6, typically twice weekly. Fresh culture medium was supplied every two days.

2.7.3. Assessment of HepG2 cell viability

Cell viability was evaluated using the MTT assay as described in Section 2.6.4 with slight modifications. Briefly, HepG2 cells were seeded in 96-well plates at a density of 1.8×10^4 cells per well and allowed to attach for 24 h. The medium was then replaced with 200 μL of fresh culture medium containing in vitro digested samples at different concentrations (0.2, 0.5, 1, 2, 5, 8, and 10%, *v*/v), followed by incubation for another 24 h. Cells cultured in standard medium served as the negative control, while 0.1% Triton X-100 was used as the positive control. Absorbance was measured at 590 nm with a reference wavelength of 660 nm using an Agilent Technologies BioTek Epoch microplate spectrophotometer. Cell viability was expressed as a percentage relative to the untreated control.

2.8. Statistical analysis

All experiments were carried out with a minimum of three independent replicates, and the results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using SPSS Statistics 29.0 (IBM Corp., Armonk, NY, USA). Differences among groups for bioactivity and cytotoxicity were evaluated by one-way analysis of variance (ANOVA). Differences in sensory descriptor intensities between unfermented and fermented *S. platensis* were analyzed using paired-samples *t*-tests. For the cytotoxicity assays, technical replicate values that deviated by more than 20% from the corresponding sample mean were treated as outliers and excluded prior to analysis. Based on this criterion, one data point was removed from the unfermented sample, while three data points were excluded from the fermented sample. Data visualization, including radar plots and bar graphs, was conducted using Origin 2025 (OriginLab, MA, USA) and GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $p < 0.05$, and highly significant differences were considered at $p < 0.01$.

3. Results and discussion

3.1. Establishment of a Basidiomycota–S. platensis fermentation system for aroma improvement

Previous studies have shown that co-fermentation of *S. platensis* with *Lactobacillus plantarum* and *Bacillus subtilis* can reduce undesirable algal off-flavors and generate aroma compounds such as acetoin, thereby imparting creamy notes (Bao et al., 2018). In contrast, the potential of Basidiomycota fermentation, despite its diverse enzymatic capabilities, has not yet been explored for improving the aroma of *S. platensis*.

In this study, twelve edible Basidiomycota strains were used to ferment *S. platensis* suspension to evaluate their effects on sensory properties and to establish a Basidiomycota–*S. platensis* fermentation system capable of improving its aroma. The initial odor of *S. platensis* was characterized by fishy and earthy notes, consistent with previous reports (Sahin et al., 2022; Xiang et al., 2026). Fermentation was carried out at 24 °C under shaking conditions (150 rpm) in the dark for up to 98 h. Samples were collected every 4–6 h for sensory evaluation to determine the fermentation time point showing the most pronounced aroma changes. The odor attributes and their intensities at the selected fermentation time points for each strain were summarized in Table S2. Strain selection was based on the sensory characteristics observed at these time points, with particular consideration given to the reduction of the original fishy and earthy off-odor attributes together with the development of desirable aroma notes. Distinct aroma profiles were generated depending on the strain. For example, *Pleurotus sapidus* produced lemony and mushroom-like notes after 80 h of fermentation, whereas *Lentinula edodes* generated a slight sweet aroma after 61 h. Fermentation with *Polyporus umbellatus* resulted in paper-like and lemony notes. Among all strains, fermentation with *Stropharia rugosoannulata* resulted in a favorable sensory profile. After 56 h of fermentation, the original fishy and earthy attributes of *S. platensis* were no longer perceived during sensory evaluation, while sweetish and lemony notes were observed. Repeated fermentation experiments showed that fermentation of sterilized *S. platensis* suspension (10 g/L, 121 °C for 15 min) with *S. rugosoannulata* at 24 °C and 150 rpm under dark conditions reproducibly improved the aroma profile of *S. platensis* after 56 h of fermentation. Based on these results, *S. rugosoannulata* was selected for subsequent experiments, and a stable Basidiomycota–*S. platensis* fermentation system (Fig. 1A) was established.

3.2. Effects of fermentation on aroma profile

3.2.1. Changes in sensory attributes after fermentation

To validate the preliminary strain screening results, ten trained panelists were recruited to evaluate sensory changes after fermentation. As shown in Fig. 1B, the dominant odor attributes of unfermented *S. platensis* were musty (2.2), fishy (2.0), and green (1.8), consistent with previous reports (Xiang et al., 2026). After fermentation, the aroma profile shifted markedly, with floral (2.2), fruity (1.9), and sweetish (1.6) notes becoming predominant. Concurrently, the intensities of undesirable attributes, including musty, fishy, grassy, green, and earthy, decreased to 1.1, 0.3, 0.6, 0.7, and 0.9, respectively. Similar improvements in algal off-flavors have been reported for fermentations involving lactic acid bacteria and other microorganisms (Kurt et al., 2023). In

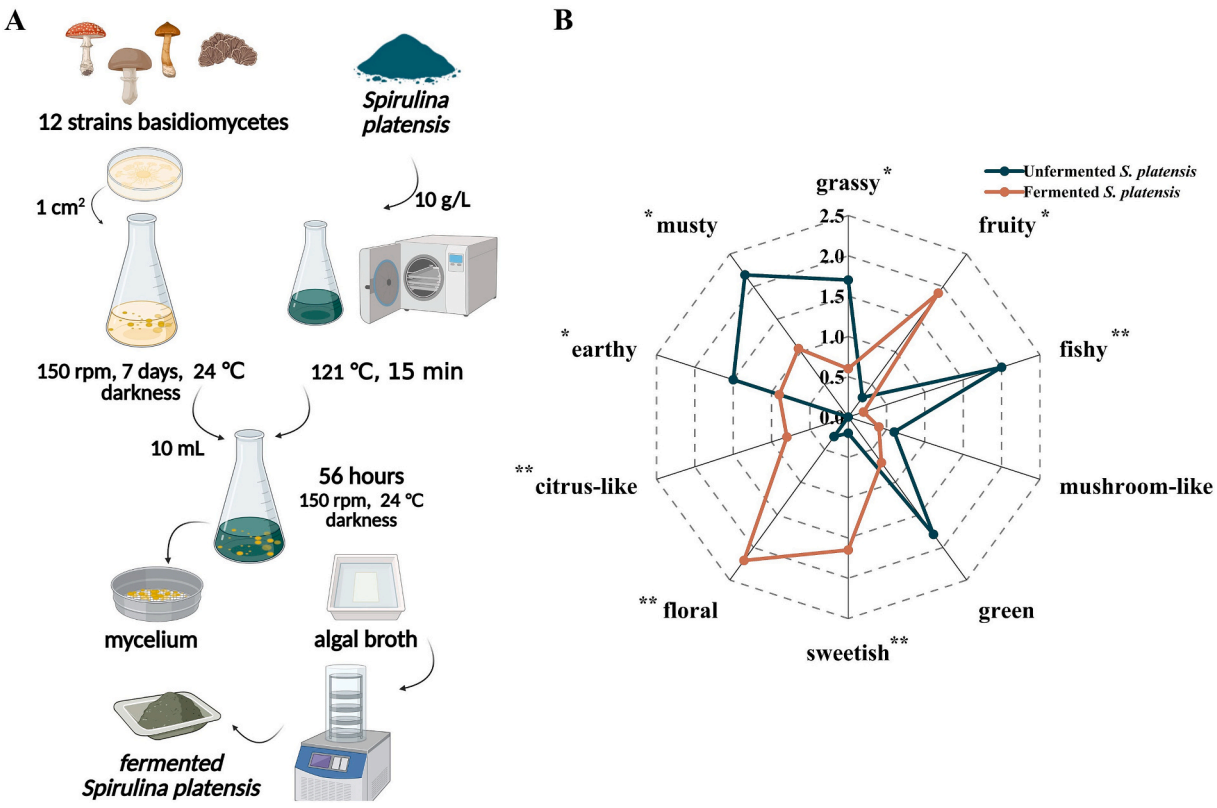

Fig. 1. Illustration of the established Basidiomycota–*Spirulina platensis* fermentation system (A) and radar plot of sensory attributes of unfermented and fermented *Spirulina platensis* (B). Asterisks indicate significant differences between the two samples for individual sensory descriptors, as determined by paired-samples *t*-tests (*$p < 0.05$, **$p < 0.01$).

addition, yeast fermentation of *S. platensis* has been shown to reduce "seaweed" and "earthy" notes while introducing floral and fruity attributes (Zhao et al., 2025). The reduction of undesirable odor attributes and the emergence of floral and fruity notes observed in the present study suggest that fermentation with *S. rugosoannulata* substantially altered the aroma profile of *S. platensis*. The underlying molecular basis of these sensory changes is further discussed below.

3.2.2. Aroma-active compounds and their contribution to aroma changes

To elucidate the chemical basis underlying the observed sensory changes, aroma-active compounds were qualitatively analyzed. As shown in Table 1, a total of 24 odor-active compounds were sniffed across both samples, including 20 in unfermented *S. platensis* and 16 in fermented *S. platensis*. Several compounds associated with fishy, floral, and waxy notes were no longer detected after fermentation, whereas fruity ethyl esters and benzaldehyde, which imparts an almond-like aroma, were detected exclusively in the fermented sample. These changes are consistent with the sensory evaluation results, showing a reduction in fishy and green odor and the emergence of fruity notes after fermentation.

To further characterize the odor differences, 17 aroma-active compounds that were detected on both columns and identified using four complementary approaches were selected for semi-quantitative analysis. Their OAVs were subsequently calculated. As shown in Table 2, nine compounds in unfermented *S. platensis* exhibited OAVs greater than 1. Among these, (*E*)-2-nonenal (fatty, green; OAV = 2085) showed the highest contribution, followed by β-ionone (floral, woody; OAV = 382), (*E,E*)-2,4-decadienal (fatty, deep-fried; OAV = 231), and α-ionone (floral, raspberry-like; OAV = 76). Additional contributors included hexanal (green, grassy; OAV = 61), 1-octen-3-ol (mushroom-like; OAV = 40), (*E*)-2-octenal (fatty, nutty; OAV = 35), decanal (OAV = 35), and γ-nonalactone (caramel-like; OAV = 1). Sixteen compounds with OAVs >1 were identified in fermented *S. platensis*. Fruity ethyl esters became dominant contributors, including ethyl 2-methylbutanoate (OAV = 2699), ethyl 2-methylpropanoate (OAV = 1011), and ethyl 3-methylbutanoate (OAV = 227). In addition, 1-octen-3-one (mushroom-like; OAV = 400), and linalool (citrus-like, floral; OAV = 236) showed a high contribution. Several compounds present in the unfermented sample were also detected after fermentation, including (*E,E*)-2,4-decadienal (OAV = 1128), (*E*)-2-nonenal (OAV = 960), hexanal (OAV = 58), (*E*)-2-octenal (OAV = 211), 1-octen-3-ol (OAV = 51), α-ionone (OAV = 8), β-ionone (OAV = 89), and decanal (OAV = 22). Benzaldehyde (almond-like; OAV = 1), 2-pentylfuran (herbal-like; OAV = 4), and γ-nonalactone (OAV = 1) were also detected.

In unfermented *S. platensis*, the highest OAVs were observed for the lipid-derived aldehydes (*E*)-2-nonenal, (*E,E*)-2,4-decadienal, and hexanal, which was consistent with the sensory evaluation showing pronounced green and fishy odor attributes (Du et al., 2021). After fermentation, the highest OAVs were observed for the newly formed ethyl esters, particularly ethyl 2-methylbutanoate and ethyl 2-methylpropanoate, which was in agreement with the increased fruity aroma detected during sensory evaluation. However, several lipid-derived aldehydes, including (*E*)-2-nonenal, (*E*)-2-octenal, and (*E,E*)-2,4-decadienal, still exhibited OAVs greater than 1 after fermentation, whereas the intensities of the green and fishy odor attributes were reduced. This observation suggests that OAVs estimate the potential contribution of individual odorants but do not fully reflect aroma perception in complex volatile mixtures. The high OAVs of the newly formed ethyl esters may have reduced the perceptual impact of the remaining aldehydes through odor interactions such as masking (An et al., 2024). In addition, the concentrations of several aldehydes associated with green and fatty odors decreased after fermentation, which may also have contributed to the reduced perception of these odor attributes.

Fermentation led to substantial changes in the aroma profile of

Table 1
Aroma-active compounds identified in unfermented and fermented *Spirulina platensis*.

No.	Aroma-active compound	CAS number	Odor impression	Samples		RI (DB-Wax)		RI (HP-5MS)		Identification method
				S. platensis	fermented *S. platensis*	calculated	literature	calculated	literature	
1	ethyl 2-methylpropanoate	97–62-1	fruity, sweetish		+	981	975	758	755	O, MS, RI, S
2	ethyl 2-methylbutanoate	7452-79-1	fruity, sweetish		+	1051	1051	852	850	O, MS, RI, S
3	ethyl 3-methylbutanoate	108–64-5	fruity		+	1072	1072	856	859	O, MS, RI, S
4	hexanal	66–25-1	green, grassy	+	+	1077	1077	798	800	O, MS, RI, S
5	2-pentylfuran	3777-69-3	vegetable, herbal	+	+	1230	1231	991	990	O, MS, RI, S
6	6-methyl-2-heptanone	928–68-7	fruity	+		1238	1237	937	932	O, MS, RI
7	octanal	124–13-0	green, fruity	+	+	1289	1291	–	–	O, MS, RI
8	1-octen-3-one	4312-99-6	mushroom-like	+	+	1297	1297	980	979	O, MS, RI, S
9	6-methyl-5-hepten-2-one	110–93-0	fresh	+	+	1333	1333	987	987	O, MS, RI, S
10	(*E*)-2-octenal	2548-87-0	fatty, nutty	+	+	1421	1421	1056	1056	O, MS, RI, S
11	1-octen-3-ol	3391-86-4	mushroom-like	+	+	1448	1448	991	990	O, MS, RI, S
12	decanal	112–31-2	soapy, citrus-like	+	+	1496	1497	1195	1195	O, MS, RI, S
13	unknown	–	floral, sweetish	+		1509	–	–	–	–
14	benzaldehyde	100–52-7	almond-like		+	1522	1522	966	966	O, MS, RI, S
15	(*E*)-2-nonenal	18,829–56-6	fatty, green	+		1530	1530	1176	1172	O, MS, RI, S
16	linalool	78–70-6	citrus-like, floral	+	+	1546	1546	1095	1097	O, MS, RI, S
17	2-pentylpyridine	2294-76-0	sweat-like	+		1575	1572	–	–	RI
18	unknown	–	fishy	+		1717	–	–	–	–
19	2,5-dimethyl benzaldehyde	5779-94-2	fresh, waxy	+		1731	1705	–	–	MS, RI
20	unknown	–	citrus	+		1754	–	–	–	–
21	(*E,E*)-2,4-decadienal	25,152–84-5	cooked fishy	+	+	1807	1807	1318	1318	O, MS, RI, S
22	α-ionone	127–41-3	floral	+		1854	1854	1435	1430	O, MS, RI, S
23	β-ionone	14,901–07-6	wood-like, floral	+	+	1939	1937	1492	1488	O, MS, RI, S
24	γ-nonalactone	104–61-0	caramel, sweetish	+	+	2020	2020	1362	1361	O, MS, RI, S

Note: -: not available. +: detected by olfactometry. Compounds were identified based on odor description (O), mass spectrometry (MS), retention index (RI), and comparison with authentic standards (S).

Table 2
Concentrations and odor activity values (OAVs) of aroma-active compounds in unfermented and fermented *Spirulina platensis*.

No.	Aroma-active compounds	Concentration (μg/L)		Odor threshold (μg/L)	OAV	
		unfermented	fermented		*S. platensis*	Fermented *S. platensis*
1	ethyl 2-methylpropanoate	ND.	89.96 ± 9.56	0.089 [a]	ND.	1011
2	ethyl 2-methylbutanoate	ND.	21.59 ± 1.45	0.008 [a]	ND.	2699
3	ethyl 3-methylbutanoate	ND.	5.22 ± 0.53	0.023 [a]	ND.	227
4	hexanal	272.93 ± 9.07	259.61 ± 6.04	4.5 [b]	61	58
5	2-pentylfuran	2.88 ± 0.21	25.54 ± 4.48	6 [b]	<1	4
6	1-octen-3-one	ND.	20.00 ± 1.12	0.005 [b]	ND.	400
7	6-methyl-5-hepten-2-one	1.51 ± 0.05	2.72 ± 0.43	50 [d]	<1	<1
8	(*E*)-2-octenal	106.15 ± 1.37	634.45 ± 26.64	3 [b]	35	211
9	1-octen-3-ol	40.19 ± 1.09	51.23 ± 5.29	1 [b]	40	51
10	decanal	70.14 ± 1.77	44.55 ± 3.38	2 [b]	35	22
11	benzaldehyde	16.52 ± 0.52	466.81 ± 17.7	350 [b]	<1	1
12	(*E*)-2-nonenal	208.49 ± 7.34	96.04 ± 6.51	0.1 [b]	2085	960
13	linalool	ND.	51.98 ± 7.74	0.22 [b]	ND.	236
14	(*E,E*)-2,4-decadienal	16.17 ± 0.48	78.94 ± 3.78	0.07 [c]	231	1128
15	α-ionone	30.56 ± 2.99	3.34 ± 0.31	0.4 [b]	76	8
16	β-ionone	2671.45 ± 95.43	624.58 ± 15.9	7 [b]	382	89
17	γ-nonalactone	37.58 ± 1.13	29.66 ± 1.99	27 [c]	1	1

Note: ND: not detected. Odor threshold in water values were obtained from [a]: Munafo JR. et al. (2016), [b]: Xiang et al. (2026), [c]: Liang et al. (2023), [d]: Yang et al. (2022)

S. platensis, including both the accumulation and reduction of specific odor-active compounds. Specifically, the levels of (*E*)-2-nonenal and decanal decreased significantly after fermentation, indicating that these aldehydes were further transformed or degraded during the process. (*E*)-2-Nonenal is generally formed via oxidative cleavage of unsaturated fatty acids such as oleic acid or linoleic acid, whereas decanal is associated with fatty acid β-oxidation or oxidative degradation (Shahidi & Hossain, 2022). During fermentation, microbial metabolism can convert these aldehydes into the corresponding alcohols or acids through redox reactions (Hazelwood et al., 2008). In addition, reactions with amino compounds, such as Schiff base formation, may also contribute to their decline (Matsuo, 1954). A similar trend was observed for α-ionone and β-ionone, whose concentrations also decreased after fermentation. These compounds originate from the oxidative cleavage of carotenoids such as β-carotene (Paparella et al., 2021), but are chemically reactive and can undergo further oxidation, reduction, or structural rearrangements. Microbial activity, together with changes in fermentation conditions such as pH and redox state, may further affect

their stability (Luo et al., 2013).

In contrast to the decreasing compounds described above, several odor-active compounds accumulated during fermentation. Although (*E*)-2-octenal, (*E,E*)-2,4-decadienal, and benzaldehyde were already identified in unfermented *S. platensis*, their levels increased significantly after fermentation, suggesting further accumulation or enhanced formation during this process. This increase appears to be associated with intensified lipid oxidation and amino acid metabolism. (*E*)-2-octenal and (*E,E*)-2,4-decadienal are typically formed through the oxidative degradation of PUFAs such as linoleic acid. During fermentation, microbial activity may promote both lipoxygenase-mediated reactions and non-enzymatic oxidation, thereby accelerating the formation of these aldehydes (Shahidi & Hossain, 2022). Benzaldehyde, on the other hand, is likely derived from phenylalanine metabolism, potentially via the Ehrlich pathway or related oxidative reactions. In addition, the disruption of cellular structures during fermentation may facilitate the release of lipid and protein substrates, providing increased availability of precursors for the formation of these volatile compounds (Huang et al., 2024). The elevated levels of these aldehydes point to a concurrent enhancement of lipid oxidation and amino acid metabolism during fermentation.

Besides the compounds showing increased abundance, several aroma-active compounds were detected exclusively in fermented *S. platensis*, suggesting that they were newly formed during fermentation. Among them, 1-octen-3-one and linalool were detected exclusively in fermented *S. platensis*. 1-Octen-3-one is widely recognized as a key mushroom-like aroma compound reported in the fruiting bodies of Basidiomycota (Wu et al., 2005), as well as during Basidiomycota fermentation processes (Ibrahimi et al., 2020). Its formation is generally associated with lipid oxidation of linoleic acid (Su et al., 2022) and arachidonic acid. However, arachidonic acid was not detected in *S. platensis* (Senila et al., 2025), suggesting that 1-octen-3-one is more likely derived from linoleic acid oxidation in this system. Linalool, which imparts a citrus-like aroma, may be formed via multiple pathways, including enzymatic release from glycosidically bound precursors, de novo biosynthesis via the methylerythritol phosphate or mevalonate pathways, or microbial biotransformation of terpenoid precursors such as geraniol and nerol (Demyttenaere et al., 2000; Zhang et al., 2024). In the present study, geraniol and nerol were not detected in either unfermented or fermented samples, and no evidence was found for glycosidically bound precursors in *S. platensis*. Therefore, linalool formation is more likely associated with de novo biosynthesis from geranyl pyrophosphate via microbial terpenoid metabolism.

Among the newly generated compounds, fruity ethyl esters were particularly prominent after fermentation, including ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, and ethyl 3-methylbutanoate, which are likely key contributors to the fruity aroma of fermented *S. platensis*. These esters are widely recognized as important aroma compounds in wine and various fermented foods (Chung, 2000; Pino & Queris, 2011). The formation of ethyl esters requires ethanol as an ethyl donor. Ethanol was detected in both unfermented and fermented *S. platensis*, and the ratio of the ethanol peak area to that of the internal standard (thymol) decreased significantly after fermentation, from 1.11 ± 0.20 to 0.67 ± 0.02. This observation is consistent with the proposed involvement of ethanol in ethyl ester formation during fermentation.

Besides ethanol availability, the acids required for ester formation were likely derived from branched-chain amino acid metabolism. In *S. cerevisiae*, branched-chain amino acids such as valine, leucine, and isoleucine can be metabolized through the Ehrlich pathway to generate higher alcohols and their corresponding acids (Hazelwood et al., 2008). Briefly, these amino acids undergo transamination and decarboxylation to form the corresponding aldehydes, namely 2-methylpropanal, 3-methylbutanal, and 2-methylbutanal. These aldehydes can subsequently be reduced to higher alcohols or oxidized into the corresponding branched-chain acids. The resulting acids are then converted into acyl-CoA intermediates by acyl-CoA synthetase, after which AAT catalyzes esterification with ethanol (Liu et al., 2023), leading to the formation of ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, and ethyl 2-methylbutanoate. In the present fermentation system, *S. platensis* provides these branched-chain amino acids (Da Fontoura Prates et al., 2020), while Basidiomycota have been reported to possess both acyl-CoA synthetase (Oghenekaro et al., 2016) and AAT (Sun et al., 2021), supporting the feasibility of this pathway during fermentation. These results suggest that fruity ester formation was linked to ethanol production and branched-chain amino acid catabolism during fermentation. Precursor supplementation experiments were subsequently performed to further assess the proposed pathway.

3.2.3. Role of branched-chain amino acids in ester formation

Supplementation with isoleucine, leucine, and valine increased the levels of ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, and ethyl 2-methylpropanoate to 23-, 79-, and 7-fold of the control group, respectively (Fig. 2A–C). These results are consistent with the proposed conversion of branched-chain amino acids into the corresponding fruity esters in the present fermentation system. In the isoleucine supplementation group, 2-methylbutanal and 2-methylbutanoic acid, which were not detected in the control, became detectable after supplementation, whereas 2-methylbutanol remained undetected. This product distribution suggests that the aldehyde intermediate may have been preferentially oxidized to the corresponding acid rather than reduced to the alcohol (Hazelwood et al., 2008). In contrast, supplementation with leucine or valine increased the levels of the corresponding branched-chain aldehydes, alcohols, and acids simultaneously, suggesting that both the oxidative and reductive conversions of the aldehyde intermediates may have occurred during fermentation. In addition, supplementation with one branched-chain amino acid partially suppressed the formation of esters derived from the other two amino acids, suggesting potential competition among branched-chain amino acid metabolic pathways. Ethanol levels also decreased significantly after amino acid supplementation. These findings suggest that the formation of fruity ethyl esters in the *S. platensis–S. rugosoannulata* fermentation system is likely associated with the branched-chain amino acid metabolism and subsequent esterification reactions involving ethanol.

Comparison of the relative peak areas showed that the three corresponding ethyl esters reached comparable final levels after amino acid supplementation, despite their markedly different fold increases relative to the respective controls. In contrast, the branched-chain acids exhibited substantially different accumulation levels, with 3-methylbutanoic acid showing the highest relative peak area, followed by 2-methylbutanoic acid and 2-methylpropanoic acid (Table S4). The accumulation of branched-chain acids was not fully consistent with the formation of the corresponding ethyl esters, suggesting that precursor availability alone is insufficient to explain the observed ester profiles and that downstream enzymatic steps involved in ester biosynthesis may also contribute. Among these steps, AAT catalyzes the final esterification reaction using acyl-CoA derivatives rather than free acids as acyl donors. Therefore, a preliminary molecular docking analysis was subsequently performed to explore whether differences in substrate recognition by AAT might contribute to the observed ester formation patterns.

3.2.4. Preliminary molecular docking using a reference yeast AAT model

As no functionally validated AAT from *S. rugosoannulata* has been reported to date, a functionally related yeast AAT model was employed as a reference model for the docking analysis. Isobutyryl-CoA, isovaleryl-CoA, and *(S)*-2-methylbutanoyl-CoA, representing the corresponding acyl-CoA intermediates derived from valine, leucine, and isoleucine metabolism, respectively, were selected as the putative acyl donors. The docking analysis was intended to provide a preliminary and indirect assessment of potential substrate recognition and should not be interpreted as direct evidence of the substrate specificity or catalytic properties of the native *S. rugosoannulata* enzyme.

All three branched-chain acyl-CoA intermediates were successfully

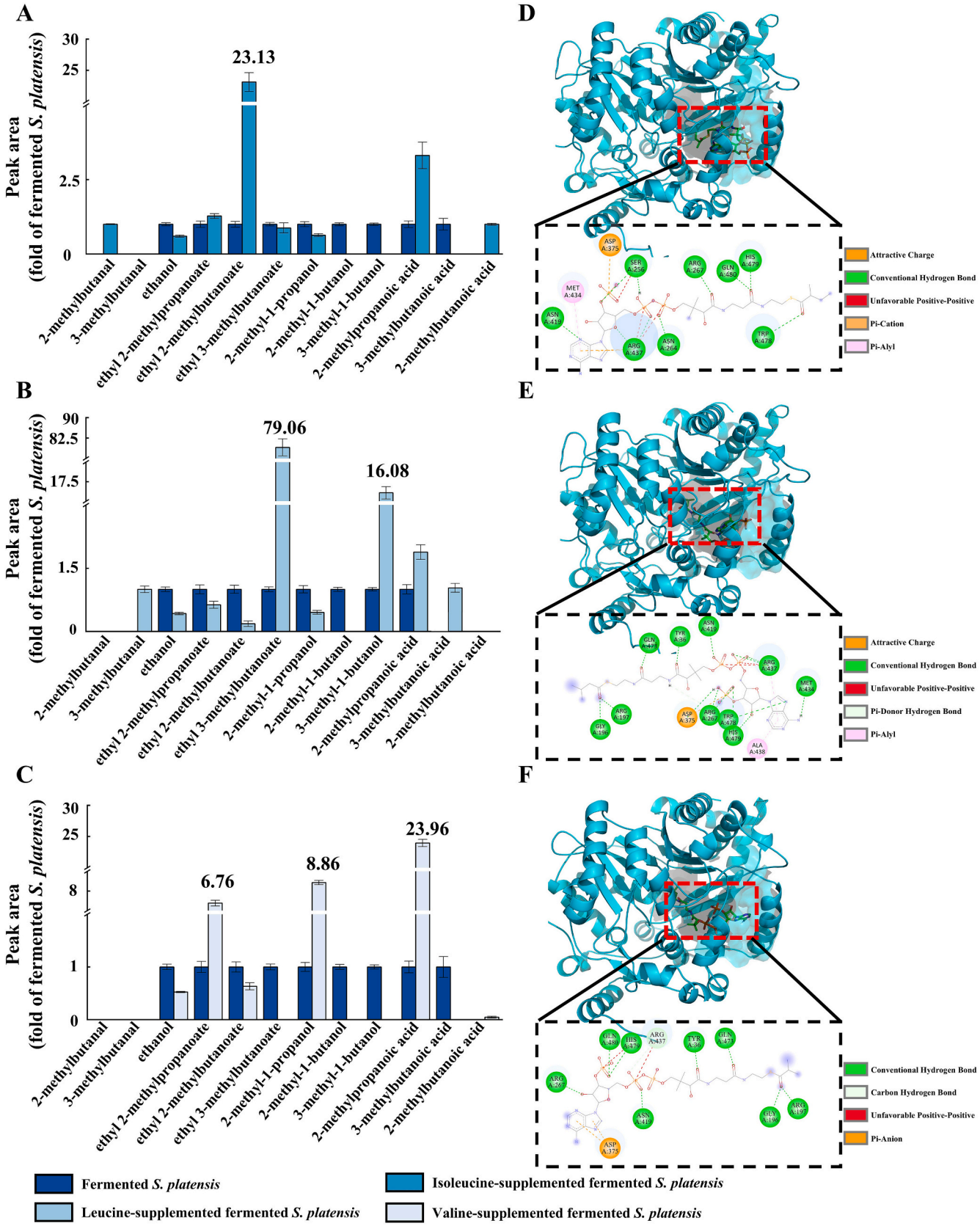

Fig. 2. Effects of branched-chain amino acid supplementation on aroma compound formation in fermented *Spirulina platensis* and molecular docking of branched-chain acyl-CoA with reference yeast AAT model. (A–C) Relative peak areas of aroma compounds, expressed as fold changes relative to fermented *S. platensis* without supplementation, in samples supplemented with isoleucine (A), leucine (B), and valine (C), respectively. (D—F) Predicted binding modes of (*S*)-2-methylbutanoyl-CoA (D), isovaleryl-CoA (E), and isobutyryl-CoA (F) with the AAT model. The upper panels show the predicted binding poses within the binding pocket, whereas the lower panels illustrate the predicted ligand–protein interactions identified by molecular docking.

docked into the predicted binding pocket of the AAT model. Isovaleryl-CoA showed the lowest predicted binding energy (−9.345 kcal/mol), followed by isobutyryl-CoA (−9.183 kcal/mol) and (*S*)-2-methyl-butanoyl-CoA (−7.927 kcal/mol). Despite differences in binding affinity, the three ligands occupied similar regions within the predicted binding pocket (Fig. 2D–F). The three ligands adopted broadly similar binding orientations within the predicted binding pocket. Several conserved hydrogen-bonding interactions involving Arg267, Asn419, and His479, together with a conserved π-anion interaction with Asp375, were predicted for all three ligands. Additional hydrogen bonds with Tyr36, Gly196, Arg197, Gln473 or Gln480 were observed depending on the substrate.

The docking results indicate that the three branched-chain acyl-CoA intermediates could be accommodated within the predicted binding pocket of the surrogate yeast AAT model. However, the predicted binding scores did not correspond directly to the final abundances of the respective ethyl esters. This lack of correspondence suggests that the observed ester profiles cannot be explained by the docking scores alone and may also depend on factors such as precursor conversion, acyl-CoA availability, ethanol availability, and enzyme abundance. Therefore, evidence for the involvement of branched-chain amino acid metabolism in ester formation was derived primarily from the precursor-supplementation experiments, whereas the docking analysis provided only complementary and indirect structural information.

3.3. Effects of fermentation on anti-inflammatory and antioxidant potential in TNF-α stimulated EA.hy926 cells

Besides its desirable aroma characteristics, the bioactive properties of fermented *S. platensis* were studied in endothelial cells. As a key enzyme in vascular inflammation, COX-2 catalyzes the production of various bioactive lipids that act as mediators regulating vascular tone through vasoconstrictive and vasodilatory signaling, thereby contributing to the pathophysiology of hypertension (Chowdhury et al., 2010). Under hypertensive conditions, COX-2 expression is typically upregulated by pro-inflammatory cytokines such as TNF-α (Wang & DuBois, 2010). Therefore, COX-2 protein expression in TNF-α stimulated vascular EA.hy926 cells was used as a representative inflammatory biomarker to compare the effects of fermented and unfermented *S. platensis* (Wang et al., 2026).

As shown in Fig. 3A and B, TNF-α stimulation markedly increased COX-2 protein level compared with the untreated control, indicating an inflammatory response. The COX-2 level in cells treated with unfermented *S. platensis* was comparable to that in the TNF-α-treated group, indicating that the unfermented sample did not exhibit inhibitory effects. This observation differs from previous reports, which may be attributed to differences in extraction methods or sample preparation (Abu-Taweel et al., 2019). In contrast, fermented *S. platensis* significantly reduced ($p < 0.05$) COX-2 level compared with both the TNF-α-treated group and the group treated with TNF-α in combination with unfermented *S. platensis*. COX-2 levels in the fermented group were not significantly different from those in untreated cells, suggesting that fermentation reduced TNF-α-induced COX-2 expression under the

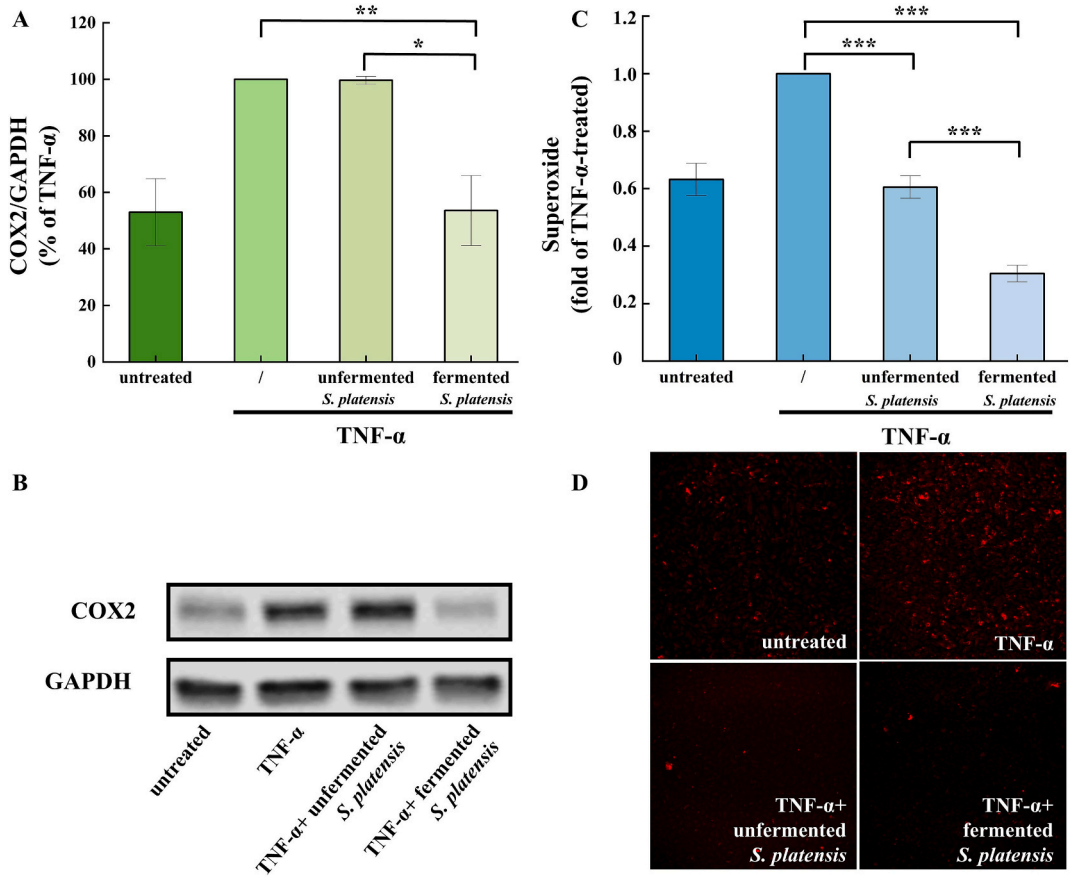

Fig. 3. Effects of unfermented and fermented *Spirulina platensis* on TNF-α-induced inflammation and oxidative stress in EA.hy926 cells. (A) Relative COX-2 expression normalized to GAPDH and expressed as a percentage of the TNF-α-treated group. (B) Representative Western blot analysis of COX-2 and GAPDH. (C) Intracellular superoxide levels expressed as fold change relative to the TNF-α-treated group. (D) Fluorescence images showing intracellular ROS levels under different treatments. Statistical significance is indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

present experimental conditions. This enhancement may be associated with fermentation-induced changes in bioactive compounds, such as phenolics, bioactive peptides, or pigments including phycocyanin (Elbandy, 2023). Consistent with this, previous studies have reported that lactic acid bacteria fermentation of *S. platensis* increases the levels of bioactive compounds and improves its anti-inflammatory properties (Liu et al., 2011).

TNF-α is also known to induce the production of reactive oxygen species (ROS) (Vlahopoulos et al., 1999). Therefore, assessing ROS levels in TNF-α-treated cells provides insight into potential antioxidative effects of *S. platensis* (Jahandideh et al., 2016). As shown in Fig. 3C and D, both unfermented and fermented *S. platensis* significantly reduced ($p < 0.001$) superoxide levels compared with the TNF-α-treated group, indicating that *S. platensis* possesses intrinsic antioxidant potential regardless of fermentation. Previous studies have also demonstrated that *S. platensis* extracts exhibit antioxidant activity, which has been attributed to bioactive compounds such as flavonoids, β-carotene, phycocyanobilin, vitamin A, and α-tocopherol (Hirata et al., 2000; Wang et al., 2007). Treatment with fermented *S. platensis* resulted in a significantly greater reduction in superoxide levels, suggesting that fermentation further reduced intracellular ROS accumulation compared with the unfermented sample. This enhancement may be related to metabolic activities during fermentation that increase the availability or transformation of antioxidant compounds. Consistent with this, previous studies have shown that fermentation of *S. platensis* with *Lactobacillus plantarum* increased DPPH radical scavenging activity by up to 60% after 24 h (Castro et al., 2019), and similar improvements have been reported using lactic acid bacteria and *Bacillus* strains (Aboobacker et al., 2025; Niccolai et al., 2019).

To assess whether the observed reductions in COX-2 expression and intracellular ROS accumulation might have been influenced by cytotoxic effects, the viability of EA.hy926 cells was evaluated under the same treatment conditions. As shown in Fig. S1, no significant differences in cell viability were observed among the treatment groups ($p > 0.05$), indicating that none of the treatments significantly affected EA.hy926 cell viability under the experimental conditions. These findings support the interpretation that the observed changes in COX-2 expression and intracellular ROS accumulation were unlikely to be attributable to differences in cell viability.

Overall, fermentation with *S. rugosoannulata* reduced TNF-α-induced COX-2 expression and intracellular ROS accumulation in EA.hy926 cells compared with unfermented *S. platensis*. These observations indicate an improvement in the anti-inflammatory and antioxidant potential of the fermented product under the present experimental conditions. Since only one inflammatory marker and one oxidative stress marker were evaluated, further studies incorporating additional biomarkers and positive controls are warranted to provide a more comprehensive assessment of its biological activities. Furthermore, the biological activities were compared on an equivalent dry-weight basis, which reflects the practical use of the final product. Future studies normalizing specific bioactive components, such as proteins, peptides, or phenolic compounds, would help clarify their individual contributions to the observed biological effects.

3.4. Effect of fermentation on cytotoxicity

In vitro cytotoxicity assays are commonly used to screen for compounds that may impair basic cellular functions (Tolosa et al., 2015). The HepG2 cell line is widely recognized as a useful model for early-stage cytotoxicity evaluation (Westerink & Schoonen, 2007), and the MTT assay is extensively applied for assessing cell viability (Ghasemi et al., 2023; Hanelt et al., 1994). In the present study, the cytotoxicity of digested *S. platensis* samples before and after fermentation was evaluated using HepG2 cells treated with different concentrations of the digested samples, followed by MTT-based cell viability measurement. As shown in Fig. 4, cell viability of untreated cells was set at 100%. Treatment with DMSO alone significantly reduced cell viability, which may be attributed to the inhibitory effect of high DMSO concentrations (10%) on HepG2 cell proliferation (Nguyen et al., 2020). Cells treated with *S. platensis* samples exhibited viability above 80% across all tested concentrations, indicating the absence of significant cytotoxicity. These results are consistent with previous reports showing that *S. platensis* extracts do not induce cytotoxic effects in Vero and BJ cell lines, and only slightly reduce viability in HepG2 cells while remaining above 50% even at higher concentrations (Santiago-Cruz et al., 2025). Moreover, no significant differences ($p > 0.05$) were observed between fermented and unfermented samples at the same concentrations, suggesting that fermentation did not adversely affect the cytocompatibility of *S. platensis* after in vitro digestion.

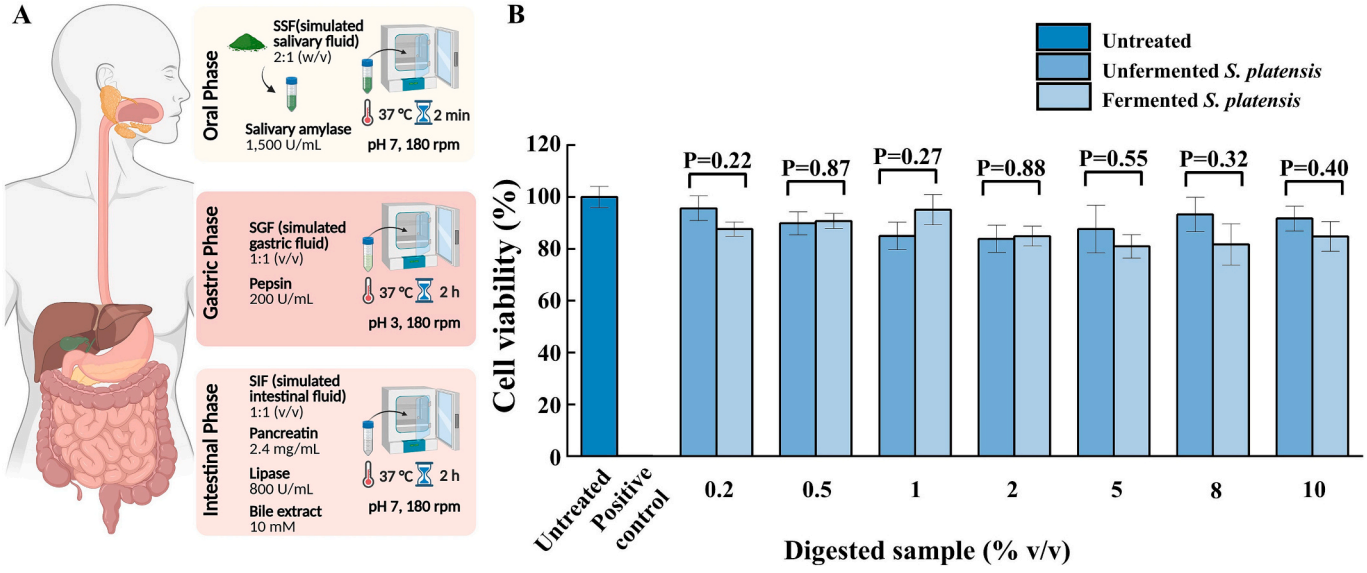

Fig. 4. Schematic diagram of the in vitro simulated digestion process (A) and cell viability of HepG2 cells treated with digested unfermented and fermented *Spirulina platensis* (B).

4. Conclusion

This study established a stable *S. rugosoannulata–S. platensis* fermentation system capable of improving the sensory quality of *S. platensis*. After 56 h of fermentation, the original musty and fishy odors were substantially reduced and replaced by fruity and floral notes. These sensory changes were associated with decreases in several off-flavor aldehydes and ionones, together with the formation of 1-octen-3-one, linalool, and branched-chain ethyl esters. Among them, ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, and ethyl 3-methylbutanoate were identified as the major contributors to the fruity aroma of the fermented product. Precursor supplementation experiments provided supporting evidence for the proposed involvement of branched-chain amino acid catabolism via the Ehrlich pathway in the formation of these esters The differences in ester accumulation among the precursor groups further suggest that factors beyond precursor availability may also have affected the final ester levels during fermentation. In addition, fermentation reduced TNF-α-induced COX-2 expression and intracellular ROS accumulation in EA.hy926 cells without introducing significant cytotoxicity. These findings suggest that Basidiomycota fermentation represents a promising strategy for improving the aroma of *S. platensis* while enhancing its anti-inflammatory and antioxidant potential under the present experimental conditions. Future work should focus on optimizing fermentation conditions and evaluating its applicability in other microalgal substrates and food systems.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used AI-assisted tools (including large language model-based services) to support language translation and linguistic refinement of the manuscript. After using these tools, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Studies in human

This study was performed in compliance with relevant laws, regulatory frameworks and guidelines where the research took place.

This study was approved by the University of Hohenheim.

(Approval No. 2025/22_Xiang)

CRediT authorship contribution statement

Can Xiang: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation. **Zihan Wang:** Writing – review & editing, Visualization, Investigation, Data curation. **Annu Ann Joseph:** Writing – review & editing, Investigation. **Sarah Greve:** Writing – review & editing, Supervision, Methodology. **Ruotong Nie:** Writing – review & editing, Resources, Methodology. **Katrin Giller:** Writing – review & editing, Supervision, Resources. **Jianping Wu:** Writing – review & editing, Supervision, Resources. **Yanyan Zhang:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Informed consent and patient details

Written informed consent to take part in the study and to publish the article has been obtained from all participants or their legal representatives. The privacy rights of participants have been observed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.120516.

Data availability

Data will be made available on request.

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