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Expression of terpene synthase-related genes in parents and offspring of *Flammulina filiformis* based on differences in volatile aroma components

Huan Lu [a], Wei Song [b], Xiao-Dong Shang [a], Jian-Yu Liu [a], Dan Zhang [a], Liang Li [b], Rui-Juan Wang [a,*], Xiao-Ting Zhai [c], Tao Feng [b]

[a] National Research Center for Edible Fungi Biotechnology and Engineering, Key Laboratory of Applied Mycological Resources and Utilization, Ministry of Agriculture, Shanghai Key Laboratory of Agricultural Genetics and Breeding, Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, No. 1000, Jinqi Road, Shanghai, China

[b] School of Perfume and Aroma Technology, Shanghai Institute of Technology, Shanghai 201418, China

[c] State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei 230036, China

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ABSTRACT

Flammulina filiformis (F. filiformis) is one of the four major edible types of fungus in the world and has been cultivated in China since 800 CE (Anno Domini). Some of the most essential criteria for evaluating the quality of *F. filiformis* are the types and contents of volatile components present. A focused study on screened the terpene synthase genes involved in the aroma of offspring and compared key terpenoids between parents and offspring, which is helpful for the development and application of *F. filiformis*. Firstly, the volatile aroma components of parent and offspring *F. filiformis* were extracted using two pretreatment procedures, and then were semi-quantified by an internal standard. Forty-eight, fifty-eight, and forty-eight volatile compounds were identified in parents and offspring of three different strains, and 15, 22, and 12 aroma compounds (OAVs $\geq$ 1) were further screened out via calculating their odor activity values (OAVs). Terpenoids, in particular linalool and eucalyptol, which contribute more to the aroma, result in the unique green and grassy aroma of the offspring. At last, the *F. filiformis* genome was resequenced and the coordinates of genes related to terpenoid synthase were determined. The results showed that Scaffolds, including scaffold3.t874 and scaffold9.t157 were connected to terpenoid synthesis of offspring (*No. 61523*). The variant genes g269 and g61 were related to terpenoid synthase sequences. This study provides a theoretical foundation for the cultivation of more diverse and unique varieties of *F. filiformis.*

1. Introduction

The most culinary and commercial value of edible mushrooms is derived from their organoleptic properties, such as aroma. C_8 aldehydes, alcohols, and ketones, which are the major contributors to mushroom aroma, generally produce mushroom-like or sweet odors (Sun et al., 2020; Xia et al., 2021). In addition, ketones and alcohols dominate in fresh mushrooms, and 43.3 % and 31.5 % of them are 3-octanone and 3-octanol, respectively (Fang, Yang, Kimatu, Zhao, An, & Hu, 2017; Yang et al., 2016). The volatile components of mushrooms vary with genetic background and age. Tietel et al. applied headspace gas chromatography-mass spectrometry to contrast the aroma components of three *Morchella* species and identified key age-related biomarkers to distinguish the age of mushrooms based on differences in aroma characteristics, including octanal, heptanal, 3-octen2-one, and benzeneacetaldehyde(Tietel & Masaphy, 2022).

Flammulina filiformis (F. filiformis), one of the most popular edible mushrooms, has been cultivated and consumed on a large scale in East Asia (Wang, Liu, Dai, Horak, Steffen, & Yang, 2018). It is estimated that *F. filiformis* is the most commonly cultivated species in modern farming systems (more than 4500 tons per day) (Li & Xu, 2022). Among them, Chinese production of *F. filiformis* in 2020 is 2,279,100 tons, and its exports reach 38,000 tons (China Edible Fungi Association, 2022). Due to their high protein content and low-fat content, consumption of *F. filiformis* is highly recommended. *F. filiformis* is renowned for its health benefits including antioxidant, cholesterol-lowering functions, memory improvement, and immune-modulatory properties (Chuang et al., 2020; Guo et al., 2019; Mahfuz et al., 2020; Wang et al., 2018; Yang et al.,

* Corresponding author.
E-mail address: wangruijuan@saas.sh.cn (R.-J. Wang).

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2015; Yeh, Ko, & Lin, 2014).

Highly sought-after *F. filiformis* has been studied for its unique aroma. *F. filiformis* commonly contain following C_8 compounds: benzeneacetaldehyde, (*E*)-2-octenal, 3-octanone, 3-octanol, and 1-octen-3-ol (Sun et al., 2020). Aroma compounds from other *Flammulina* species have previously been investigated. *No.61490* (*Flammulina* Strain number) has been recently identified to produce ester odorants including ethyl 2-methyl butyrate and ethyl isovalerate, with a fruity aroma (Wang et al., 2022). While previous studies only focused on other *Flammulina* species, it remains unclear which compounds are responsible for the special aroma of *No.61524* and *No.61532*. Furthermore, many publications have focused on the use of headspace solid-phase microextraction (HS-SPME) to isolate aroma compounds, resulting in insufficient research on compounds with low volatility.

Terpenoids are one of the main compounds that produce the aroma of basidiomycetes and are composed of the 5-carbon isoprenoid precursor isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP). It is necessary to study the difference in terpenoids between parents and offspring and the genes related to terpenoid synthase sequences, which is critical for further clarifying the mechanism of *F. filiformis* aroma generation. To date, no studies have been conducted on terpene synthase genes in *F. filiformis*.

In this study, the main objectives were to (i) screen for volatile aroma compounds in parents and offspring of *F. filiformis*, (ii) evaluate their relevance based on OAVs and compare the aroma profiles of parents and offspring of *F. filiformis*, and (iii) resequence *F. filiformis* and analyze coordinate information of terpenoid synthase-related genes on the genome to provide insight into changes in terpenoids aroma induced by the genetic process.

2. Materials and methods

2.1. Samples

Three strains of mature *F. filiformis* were acquired from the Shanghai Academy of Agricultural Sciences, as shown in Table 1. *F. filiformis* were wrapped in non-woven fabric, frozen in ice packs, and transported to the lab within 24 h. *F. filiformis* samples were cleaned in deionized water, mashed into mushroom puree with a JYL-C051 blender (Joyoung Company Limited, Shandong Province, China), and stored in a −18 °C refrigerator for further analysis.

2.2. Extraction of volatile compounds from F. filiformis

2.2.1. Headspace-solid phase microextraction (HS–SPME)

Mushroom homogenate (5.0 g) and 1,2-dichlorobenzene (10 μL, 100 mg/kg) were added to a 20 ml headspace bottle with a crimp aluminium cap, and the headspace bottle was placed in a water bath at 40 °C for 10 min for equilibration. A 1 cm 50/30 μm DVB-CAR-PDMS extraction fiber head (Supelco, St. Louis, USA) was used, with an extraction time of 45 min. Before extraction, the extraction fiber was conditioned at 250 °C for 10 min on Agilent 7890 gas chromatograph (Agilent, California, USA) to remove residual odor and impurities. The conditions were the same for the three *F. filiformis* strains (*No.61524, No.61490,* and *No.61532*).

2.2.2. Solvent-assisted flavor evaporation (SAFE)

Mushroom (30.0 g), 1,2-dichlorobenzene (300 μL, 100 mg/kg), and dichloromethane (200 ml) were added to a 500 ml conical tube. The extraction was performed three times at room temperature with dichloromethane supplementation each time. The sample was placed on a rotary evaporator (Shanghai Ya Rong Biochemical Instrument Factory, Shanghai, China) at 40 °C and concentrated to 150 ml.

Then, the 150 ml sample was subjected to solvent-assisted flavor

Table 1
F. filiformis strains and their origin.

	Strain	Origin	Color	Size of cap	Size of stipe	Fruiting body
parent	No.61524	China	Light yellow	Small	Thick	
	No.61490	China	White	Larger	Tiny	
offspring	No.61532	China	Yellow	Small	Thicker	

evaporation (SAFE) at 40 °C. The vacuum degree of the SAFE apparatus was on the order of magnitude of approximately 10^{-3} to 10^{-4} Pa and was achieved using a vacuum pump set (Glasbläserei Bahr, Manching, Germany). The distillate was concentrated using nitrogen purging to reach a final volume of 1 ml and was stored at −20 °C until analysis (Engel, Bahr, & Schieberle, 1999). The conditions were the same for the three *F. filiformis* strains (*No. 61524, No. 61490,* and *No. 61532*).

2.3. GC–MS analysis

An Agilent 6890A gas chromatograph and 5975C mass spectrometer were used (Agilent Technologies, Santa Clara, CA, USA). The fibers were desorbed for 5 min at 250 °C in the splitless inlet.

The conditions for GC were as follows: HP-Innowax column: 60 m × 0.25 mm × 0.25 μm (Agilent, California, USA); carrier gas flow rate: 1.8 ml/min; carrier gas: He; column temperature: initial, 40 °C; ramp, 3 °C/min to 90 °C; ramp, 2 °C/min to 150 °C; ramp, 8 °C/min to 230 °C; hold, 5 min; inlet temperature: 250 °C; injection volume: 1 μL.

The conditions for MS were as follows: ionization mode: EI; electron energy: 70 eV; transmission line temperature: 250 °C; ion source temperature: 230 °C; full scanning range: 30 ~ 450 *m/z*; scanning interval: 1 s.

2.4. Identification and semi-quantification of the aroma compounds

In order to identify the volatile compounds in *F. filiformis*, mass spectra were compared with the NIST Mass Spectral Library (2020 version), retention indices (RIs) with reference values (https://webbook.nist.gov/chemistry/). Standard compounds (*n*-alkanes C7–C30) were also analyzed on HP-INNOWAX under the same conditions described above. The *n*-alkane standards (C7 - C30) were purchased from Sigma-Aldrich (Shanghai, China) at least of analytical grade.

This study used the retention index method for qualitative analysis, which is the most widely used and highly recognized method internationally. Retention indices were calculated using the following equation:

$$RI_y = 100^*z + 100^*\frac{RT_y - RT_z}{RT_{z+1} - RT_z}$$

RI_y indicates the fraction retention index of compound y (where y is the fraction between Z and Z + 1); RT_z represents the retention time of *n*-alkanes with carbon atom number Z; and RT_{z+1} represents the retention time of *n*-alkanes with carbon atom number Z + 1.

For the GC–MS results, the internal standard method was selected for quantitative analysis. The internal standard method is a quantitative method in which a certain amount of internal standard substance added to the sample, and the mixed samples are subjected to MS analysis. Relative quantification was carried out using the internal standard 1,2-dichlorobenzene (100 mg/kg). The internal standard, 1,2-dichlorobenzene were purchased from Sigma-Aldrich (Shanghai, China). For peak area normalization, the area of each compound was divided by the inner standard area. Each aroma compound's concentration was calculated using internal standards, and a three-replication average was used.

2.5. Construction of sequencing libraries and illumina sequencing

2.5.1. Sample collection

A total of three *F. filiformis* strains DNA were extracted from individual hyphal with a Genomic DNA Kit (Quanshijin Biotechnology Co., Ltd., Beijing, China). DNA purity and concentrations were measured with a Nanodrop 1000 spectrophotometer (Thermofisher Scientific, Massachusetts, USA). The integrity of samples with an OD260/280 of 1.6–1.8 and a concentration of 50–1000 ng/μL were separated via 0.8 % agarose gel electrophoresis (AGE) to ensure sample quality. Samples exhibited a single distinct AGE band were diluted to 50 ng/μL and stored at −80 °C. Illumina NovaSeq platform (Illumina Inc., Hayward, California, USA) was chosen to perform the resequencing project. For Illumina paired-end sequencing, at least 1 μg genomic DNA was used for sequencing library construction for each sample. Paired-end libraries with insert sizes of ~ 450 bp were prepared following Illumina's standard genomic DNA library preparation procedure. Purified genomic DNA was sheared into smaller fragments of the desired size by Covaris, and T4 DNA polymerase was applied to generate blunt ends. After adding an 'A' base to the 3′ end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. The desired fragments were purified through gel electrophoresis and then selectively enriched and amplified by PCR. The index tag was introduced into the adapter at the PCR stage as appropriate followed by a library-quality test. Library sequencing was conducted by Shanghai Personalbio Technology Co., Ltd, and data analyses was conducted by Shanghai Biozeron Biotechnology Co., Ltd. Resequencing results of three *F. filiformis* were uploaded to NCBI (PRJNA903781, PRJNA903942, PRJNA903953).

2.5.2. Sequence quality control and filtering

Raw data from high-throughput sequencing needs to be filtered to remove low-quality readings, base repetitions and other forms of artificial bias to produce high-quality sequences. The main steps of filtering are as follows: (1) the removal of spliced sequences; (2) the removal of reads with an average base mass value < 20 bp over a sliding window of 5 bases; (3) the removal of any read whose length is less than 50 bp from both ends.

2.5.3. Genome alignment

Reference genome alignment of the filtered reads was performed using BWA 0.7.12-r1039, using the BWA mem alignment. algorithm with the default parameters. The results were formatted and sorted using Picard 1.107, after which duplicate reads were identified using the MarkDuplicates function Picard 1.107.

2.5.4. Identification, annotation and difference analysis of SNP

Based on the comparison between the sample sequence and the reference genome, the Haplotypecaller module in GATK-4.1.2.0 was used to detect SNPs. Data screening and filtering criteria are as follows: (1) The filtering parameters of Genome Analysis Toolkit (GATK) for locus exclusion: mass depth (QD) < 10.0 rms mapping quality (MQ); (2) Allele types: Since SNPs are usually double alleles, more than two loci with different genotypes were screened out, SNP quality ≥ 20, SNP depth ≥ 4. According to SNP sequences, different SNP sites of three *F. filiformis* strains were found. Based on the annotation information of the reference genome, ANNOVAR is used to annotate the different SNP sites. If the SNP sites fall in the exon region of the gene and are non-synonymous mutations, they are considered as different genes. Finally, all genes in the genome were compared using BLASTP and NCBI NR library for functional annotation, and the target genes related to terpene synthetase were found according to the gene functional description. SNP of the offspring were filtered to remove the same terpene synthetase loci in the two parents, and only the genes related to the terpene synthetase of the offspring were obtained.

Illumina paired-end sequencing (PE150) was chosen to perform the resequencing project. For Illumina paired-end sequencing, at least 1 μg genomic DNA was used for sequencing library construction for each sample. Paired-end libraries with insert sizes of ~450 bp were prepared following Illumina's standard genomic DNA library preparation procedure. Purified genomic DNA was sheared into smaller fragments of the desired size by Covaris, and T4 DNA polymerase was applied to generate blunt ends. After adding an 'A' base to the 3′ end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. The desired fragments were purified through gel electrophoresis and then selectively enriched and amplified by PCR. The index tag was introduced into the adapter at the PCR stage as appropriate

followed by a library-quality test. After being quantified by TBS380, paired-end libraries were sequenced by Shanghai Biozeron Biotechnology Co., Ltd (Shanghai, China) with the Illumina HiSeq PE 2X151 bp read length.

2.6. Statistical analysis

In this study, to identify significant differences between samples, the aroma compounds identified by GC–MS were analyzed by one-way ANOVA using STATISTICS 8.1 (SAS Institute Inc., Cary, USA). Aroma profiles analysis were performed by using Radar Plot tools in Hiplot (https://hiplot.org), a comprehensive web platform for scientific data visualization (J. Li et al., 2022). A heatmap was drawn by TBtools (TBtools version:1.098691). The software GOatools (https://github.com/tanghaibao/GOatools) was used for enrichment analysis, and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was carried out by KOBAS (https://kobas.cbi.pku.edu.cn/home.do), the accuracy of which was tested by Fisher.

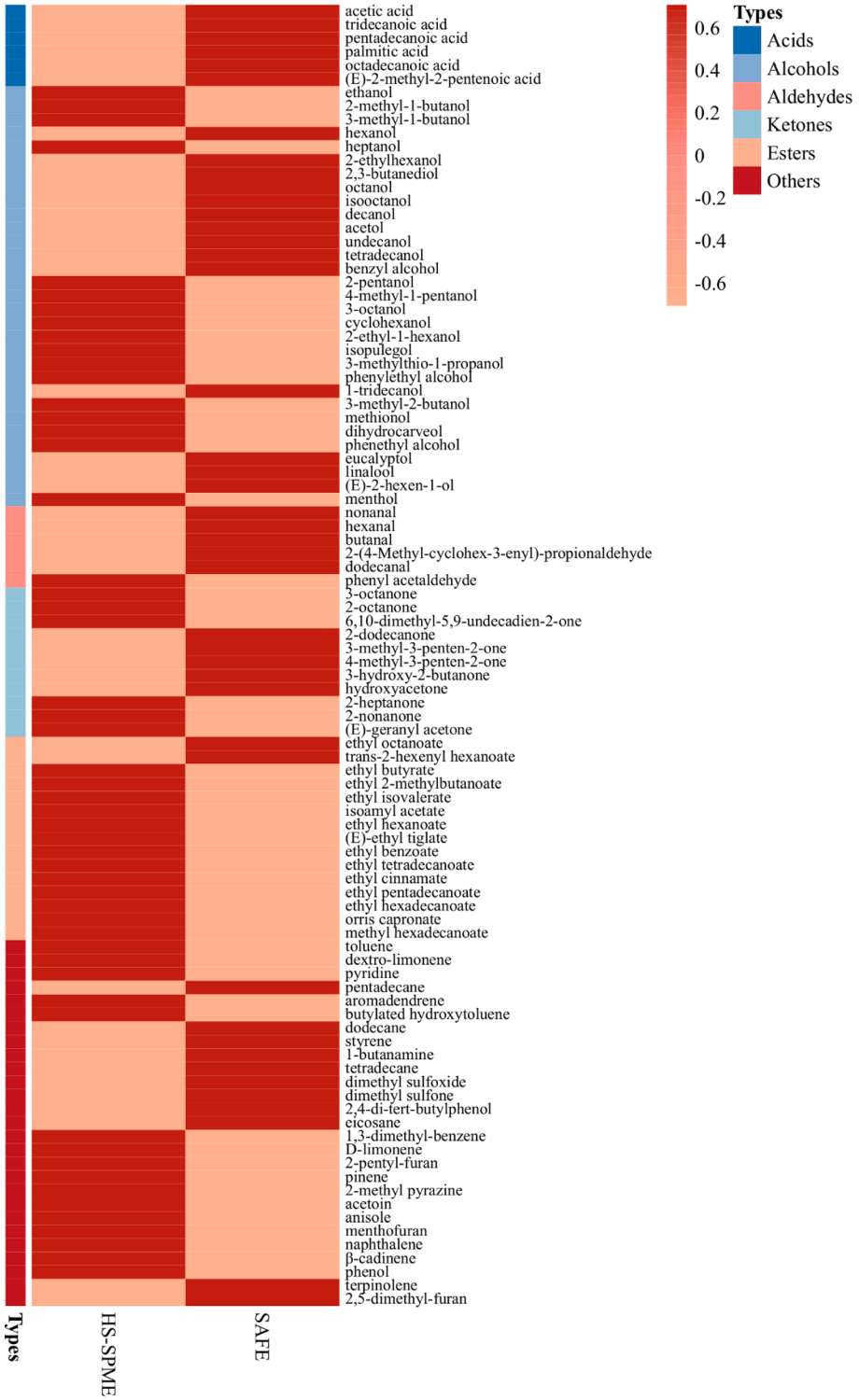

Fig. 1. Heatmap of volatile compounds in the *F. filiformis*. The concentration and types of each kind of compounds under two pretreatment methods.

3. Results and discussions

3.1. Identification of aroma compounds by GC–MS

The aroma compounds of three *F. filiformis* strains were extracted by HS-SPME and SAFE, and analyzed by GC–MS. As shown in Fig. S1, 48, 58, and 48 aroma compounds were identified from *No. 61524, No. 61490,* and *No. 61532*, respectively. Of these, the aroma compounds that were shared by parent and offspring *F. filiformis* included dodecane, palmitic acid (fatty), dodecanoic acid (fatty), nonanal (waxy), eicosane, 2,3-butanediol (fruity), 3-hydroxy-2-butanone (creamy), ethanol (alcoholic), 1-butanamine (ammonia-like fishy), linoleic acid (fatty), hexanal (sweaty), pyridine (putrid), dodecanal (waxy), 2-octanone (earthy), styrene (sweet), acetic acid, heneicosane (waxy), and acetone (fruity).

Further comparing the aroma extraction methods, in *No. 61524* samples, HS-SPME and SAFE detected 20 and 33 aroma compounds respectively; in *No. 61490* samples, 42 and 19 aroma compounds were detected by HS-SPME and SAFE, respectively; and in *No. 61532* samples, 31 and 18 aroma compounds were detected by HS-SPME and SAFE, respectively. In general, HS-SPME is an efficient method for the extraction of aroma substances from *F. filiformis*. Different varieties of mushrooms with the same pretreatment method will yield different aroma compounds. For example, with the SAFE extraction method, more compounds were extracted from *No. 61524* than from *No. 61490*. Choosing the appropriate aroma extraction method plays an important role in the accurate study of the aroma compounds of *F. filiformis*.

The data in Table S1, Table S2 and Table S3 showed that the aroma compounds of *F. filiformis* identified by the different extraction methods are quite different. HS-SPME was recognized as an effective technique to extract aromatic compounds from mushroom (Jung et al., 2021). According to the heatmap of volatile compounds based on two pretreatment methods (Fig. 1), HS-SPME technique was more effectual for the extraction of ketones, esters, and alcohols from *F. filiformis* samples. By HS-SPME method, high concentration of ethanol, 3-methyl-1-butanol, 3-octanone, 2-octanone, and ethyl hexadecanoate were isolated. It has been reported that 3-octanone was the major volatile component in *F. filiformis*, imparting a mushroom-like scent (Yang et al., 2016). As a result, a single extraction technique is not adequate to analyze the

Table 2
OAVs of key aroma-active compounds in three kinds of *F. filiformis*.

No.	Compounds	OAV						Aroma description
		No. 61524		*No. 61490*		*No. 61532*		
		SPME	SAFE	SPME	SAFE	SPME	SAFE	
Acids								
1	pentadecanoic acid	–	1	–	–	–	–	fatty
2	octadecanoic acid	–	7	–	–	–	–	fatty
Alcohols								
3	2-ethylhexanol	<1	1	–	–	–	–	citrus
4	2,3-butanediol	<1	3	<1	2	–	2	creamy
5	decanol	–	3	–	–	–	–	fatty
6	undecanol	–	1	–	–	–	–	waxy
7	3-methyl-1-butanol	–	–	30	–	4	–	fermented
8	octanol	–	–	–	36	–	–	mushroom
9	(E)-2-hexen-1-ol	–	–	–	–	–	29	fruity
10	hexanol	–	–	–	–	<1	2251	herbal
11	eucalyptol	–	–	–	–	–	6326	herbal
12	linalool	–	–	–	–	–	13,936	green
Aldehydes								
13	hexanal	–	359	–	1367	–	654	green
14	butanal	–	1271	–	–	–	–	chocolate
15	dodecanal	–	19,406	–	31,537	–	12,774	aldehydic
16	nonanal	–	–	–	4327	–	1093	aldehydic
Ketones								
17	2-dodecanone	–	17	–	–	–	–	citrus
18	3-hydroxy-2-butanone	–	11,951	–	16,322	<1	7824	buttery
19	3-octanone	–	–	133	–	–	–	mushroom
20	4-methyl-3-penten-2-one	–	–	–	75,366	–	–	vegetable
Esters								
21	ethyl octanoate	<1	1285	<1	1382	–	–	waxy
22	*trans*-2-hexenyl hexanoate	–	13	–	38	–	–	green
23	ethyl butyrate	–	–	8	–	–	–	fruity
24	ethyl 2-methylbutanoate	–	–	178	–	–	–	fruity
25	ethyl isovalerate	–	–	872	–	90	–	fruity
26	isoamyl acetate	–	–	13	–	–	–	fruity
27	ethyl hexanoate	–	–	3017	–	–	–	fruity
28	ethyl hexadecanoate	–	–	3	–	–	–	waxy
Others								
29	styrene	–	78	<1	83	<1	57	balsamic
30	tetradecane	–	6			–	–	waxy
31	D-limonene	–	–	1	–	–	–	citrus
32	2-pentyl-furan	–	–	1	–	–	–	fruity
33	naphthalene	–	–	385	–	–	–	pungent
34	terpinolene	–	–	–	54	–	–	herbal

F. filiformis aroma profile. It is necessary to combine several pretreatment methods with GC–MS (Yao et al., 2021). The Solvent Assisted Flavor Evaporation (SAFE) method is a gentle, efficient way to extract volatiles from complex matrices. Especially for volatiles with relatively high boiling points, SAFE could maintain high recovery rates (Xu et al., 2019). Linalool (13936) and eucalyptol (6326) with high OAV and boiling point, could be detected only by SAFE method in *F. filiformis* extracts, not by HS-SPME method. Consequently, multiple pretreatment techniques may provide comprehensive information on *F. filiformis* scent properties.

3.2. Aroma contribution of F. filiformis

The corresponding OAVs were calculated using the concentration and odor threshold of aroma compounds, and the compounds with an OAV $\geq$ 1 were selected, as shown in Table 2 below. The substances with higher OAVs in *No. 61524* were dodecanal (19406), 3-hydroxy-2-butanone (11951), ethyl octanoate (1285), and butanal (1271). Dodecanal provided mushrooms with a citrus peel-like aroma, butanal had a musty and green aroma, 3-hydroxy-2-butanone had a cream aroma, and ethyl octanoate provided a fruity aroma. There were abundant acids and ketones, which made a large contribution to the overall aroma of *No. 61524*. Acids can react with alcohol to produce esters. Therefore, esters and ketones were the most prevalent and gave the mushroom a fruity aroma. The substances with higher OAVs values in *No. 61490* were 4-methyl-3-penten-2-one (75366), dodecanal (31537), 3-hydroxy-2-butanone (16322), hexanal (1367), nonanal (4327), ethyl hexanoate (3017), and ethyl octanoate (1382). It is worth noting that aldehydes and ketones contributed substantially to the overall aroma of *No. 61490*, and alcohols were also the characteristic aroma compounds of *F. filiformis*. Octanol detected in *No. 61490* is a typical eight-carbon compound, which is the main aroma component of most edible fungi and has a characteristic mushroom aroma. In addition, 3-octanone is also an eight-carbon compound. In the three *F. filiformis* strains, there were 6 terpenoids with OAVs $\geq$ 1 (7, 11, 12, 26, 31, 34). The terpenoids with higher OAVs in offspring (*No. 61532*) were eucalyptol (6326), and linalool (13936). Eucalyptol had a unique herb aroma, hexanal provided a green fragrance, and linalool detected in *F. filiformis* provided the woody rose aroma.

Compounds with an OAV $\geq$ 1 from the three *F. filiformis* strains were selected for heatmap analysis (the darker the color is, the higher the relative content of the substance) and cluster analysis. Fig. S2 showed that the aroma components of parents and offspring had a certain genetic correlation according to the clustering conditions, and more unique aroma components were produced in offspring. Specifically, some of volatile compounds were identified between offspring and two different parents. First, the common aroma compounds between the parent (*No. 61524*) and offspring *F. filiformis* included 2-methyl-1-butanol (leathery, cocoa), 3-methyl-1-butanol (alcoholic, banana), pentadecanoic acid, hexanol (fruity, sweet), 2-ethyl hexanol (citrus, floral), and heptanol (leafy, violet). The common aroma compounds between another parent (*No. 61490*) and offspring *F. filiformis* (*No. 61532*) included (E)-ethyl tiglate, ethyl benzoate, ethyl hexadecanoate, ethyl isovalerate, ethyl cinnamate, phenol, naphthalene, D-limonene, menthofuran, cyclohexanol, 2-methyl pyrazine, ethyl tetradecanoate, and ethyl pentadecanoate. Finally, 13 compounds, such as phenylacetaldehyde, linalool, and eucalyptol, were unique to offspring. Among terpenoids, linalool (13936) and eucalyptol (6326), which contribute more to the aroma, give offspring the unique green aroma. Linalool was proved to be a key aroma metabolite in blueberry fruit, and eucalyptol was found to be sharply reduced in ripe fruit. Genes encoding terpene synthases were also found in the genomic region associated with these two compounds (Ferrao, Johnson, Benevenuto, Edger, Colquhoun, & Munoz, 2020). This phenomenon is also common in plants such as flowers (Campos, Vieira, Santos, Jorge, Marques, & Boaro, 2019; Zhou et al., 2023). The aroma compounds of *L. fauriei* (♀), *L. 'Tuscarora'* (♂), and their fragrant hybrids differed markedly. Additionally, the hybrids were detected special compounds that were unavailable from the parents, such as (Z)-3-hexen-1-ol (Zhou et al., 2023). The production of terpenoids in mushrooms may be influenced by hybridization (Campos et al., 2019).

In Fig. 2, we could visually observe the difference in the aroma profiles of the parents and offspring. *No. 61490* indicates the strongest alcoholic and green notes, while *No. 61524* contains a weaker alcoholic note and a stronger fruity note. It may be attributed to the higher concentration of esters (fruity) in mushrooms (Fig. S2) (Hou et al., 2021). The overall aroma of the offspring (*No. 61532*) was dominated by green aroma, with the weakest fruit aroma. In *F. filiformis*, green scent is often associated with alcohols or aldehydes, which have been reported previously in several mushrooms (Yin, Fan, Fan, Shi, Yao, & Gao, 2019). It is consistent with *F. filiformis'* more significant green aroma that higher concentrations of alcohols and aldehydes in *No. 61532* and *No. 61490* (Fig. S2).

3.3. Terpene biosynthetic pathway analysis of F. filiformis

A major component of basidiomycetes aroma is terpenoids, which consist of the 5-carbon isoprenoid precursor isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP). Synthetically, these isomers are derived from the plastic methyl erythritol phosphate (MEP) pathway or mevalonic acid (MVA) pathway in the cytoplasm/endoplasm and peroxisome. Terpene synthases (TPSs) are key enzymes in terpenoid biosynthesis, and the reaction between IDP and DMADP leads to the formation of *cis*-pentenyl diphosphate or *trans*-pentenyl diphosphate, and pentenyl diphosphate is further transformed into terpenes with different structures by enzymes in the terpene synthase (TPS) family (Chen et al., 2022; Chen et al., 2016; Li, Li, Leng, Hu, Wu, & Dou, 2021). *F. filiformis* also contains various types of terpenoids. According to the resequencing results of *F. filiformis* and the coordinate information of terpenoid synthase-related genes on the genome, 12 genes related to terpene synthase were identified in three *F. filiformis* strains (Table S4). And according to the results of function annotation, only scaffold 3.t874 and scaffold 9.t157 were found to be related to terpenoid synthase in the offspring (*No. 61523*). The genes of offspring are selected and rearranged by parent spore mononuclear hybridization, the agronomic traits (such as the color of offspring fruiting body between two parents) and flavor substances of the offspring have changed greatly. This may lead to changes in regulatory genes and metabolic pathways between parents and offspring, it may also be one of the reasons why linalool and eucalyptol are not detected in offspring. In the next experiment, two parents and offspring will be selected for transcriptome analysis at different growth stages, to find out the differential genes regulating terpenoid synthesis during the growth of parents and offspring and make further verification. As shown in Fig. 3, there were 4 terpenoids with an OAV $\geq$

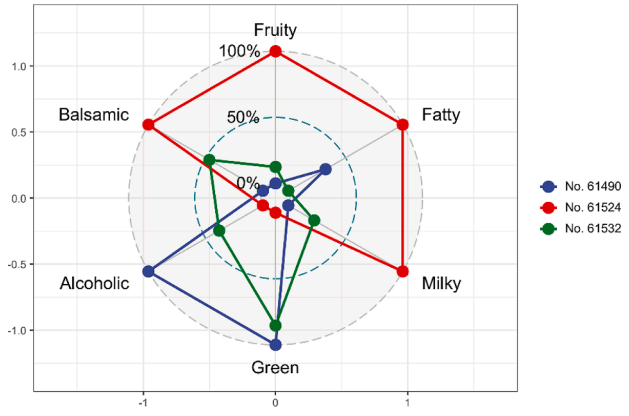

Fig. 2. Aroma profile radar plot of three kinds of *F. filiformis*.

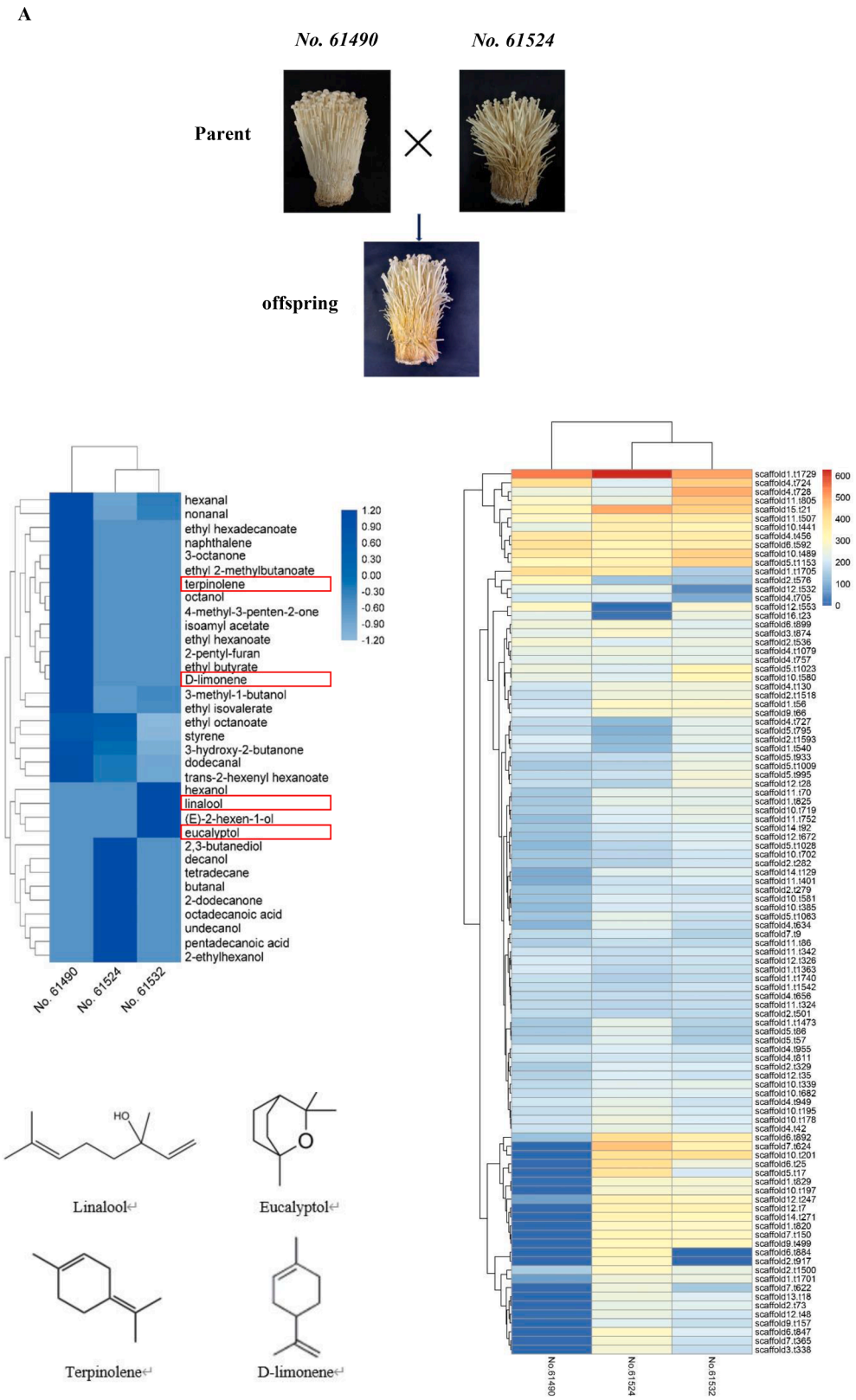

Fig. 3. Heatmap of volatile compounds and terpenoid synthase-related sequences in three kinds of *F. filiformis*. (A) Terpenoids and scaffolds characterized by the highest OAV (OAVs $\geq$ 1) in the three *F. filiformis* strains; (B) Overview of gene sequences related to offspring terpene synthases.

1 in the three *F. filiformis* strains, among which eucalyptol and linalool contribute the most to the aroma in the offspring (*No. 61523*), and the aroma contribution of the other 2 terpenoids was higher in the parent (*No. 61490*), which may be related to the differences in the expression of TPS enzymes of different terpenoids during the growth and development of *F. filiformis*(Table S4). According to the results of resequencing

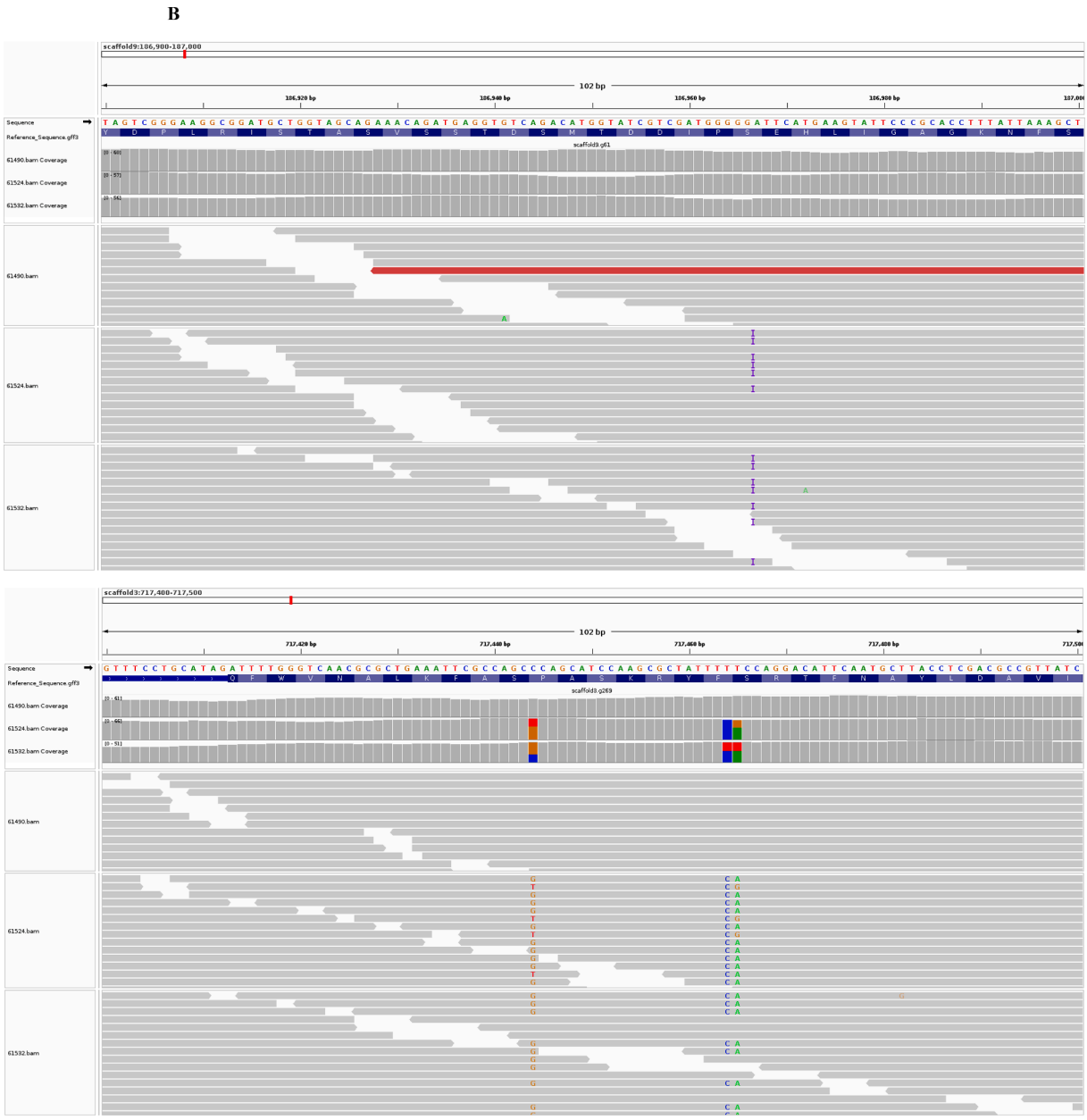

Fig. 3. (*continued*).

annotation, 45 and 90 genes related to terpene synthetase of parent (*No. 61490*) and parent (*No. 61524*), respectively, and 87 genes related to terpene synthetase of progeny *No. 61523* were found in *F. filiformis* (Table S5, Table S6, Table S7). Among them, the offspring terpene synthase-related genes were filtered to remove the same sites contained in the two parents, and the specific genes g269 and g61 related to terpene synthase in the offspring were found. These two related genes will be verified in the future to clarify the mechanism of *F. filiformis* flavor formation.

GO and KEGG enrichment analyses were carried out on terpenoid synthase-related differential genes identified by *F. filiformis* resequencing (Fig. 4). Through enrichment analysis, the significantly differential genes were further mapped into related compound synthesis pathways. For example, in GO enrichment analysis, a relatively large number of differential genes were enriched in the functions of the vacuole, storage vacuole, fungal-type vacuole, and lytic vacuole, and the most enriched pathways were related to the functions of the synaptonemal complex and synaptonemal structure. In KEGG enrichment analysis, the greatest number of differential genes was enriched in the functions of variable types of *N*-glycan biosynthesis, arginine and proline biosynthesis, and ribosome biosynthesis in eukaryotes, and the greatest number of pathways was enriched in the functions of glycosphingolipid biosynthesis-ganglio series and glycosaminoglycan degradation. Among these functional enrichment pathways, the differential genes of *F. filiformis* mainly focus on fission, degradation, and biosynthesis, which indicates that terpenoids and the flavor formations of *F. filiformis* strains differ in their functional gene differences and enrichment pathways.

4. Conclusion

This study applied two pretreatment methods to extract the volatile aroma components of parents and offspring of *F. filiformis* and found a relationship between parental aroma profiles and their offspring. Three main odorants (hexanal, dodecanal, and 3-hydroxy-2-butanone) were present in parent and offspring mushrooms. Eucalyptol and linalool were present in offspring mushrooms resulting in a unique herb aroma.

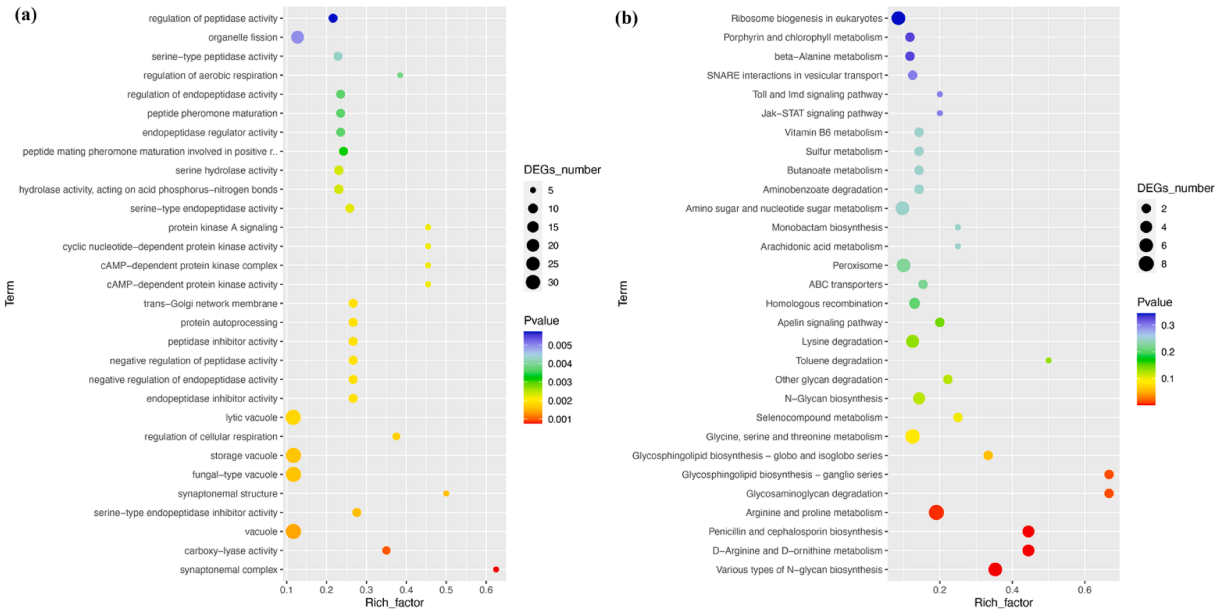

Fig. 4. Differential gene enrichment analysis of three kinds of *F. filiformis*: (a) GO enrichment analysis, (b) KEGG enrichment analysis.

The differences between parental and offspring aromas from related genes needed to be further studied.

According to the resequencing results of *F. filiformis* and the coordinate information of terpenoid synthase-related genes on the genome, scaffolds connected to terpenoid synthesis of offspring (*No. 61523*) were identified, including scaffold3.t874 and scaffold9.t157. The variant genes g269 and g61 were related to terpenoid synthase sequences. These two related genes will be validated in the future to clarify the mechanism of the aroma generation of *F. filiformis*. This research provided a theoretical basis for the future cultivation of *F. filiformis* with unique aroma profiles.

Data availability

The data presented in this study are available in this article.

CRediT authorship contribution statement

Huan Lu: Writing – original draft. **Wei Song:** Writing – original draft. **Xiao-Dong Shang:** Methodology. **Jian-Yu Liu:** Software. **Dan Zhang:** Visualization. **Liang Li:** Investigation. **Rui-Juan Wang:** Writing – review & editing. **Xiao-Ting Zhai:** Writing – review & editing. **Tao Feng:** Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2022.100156.

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