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Characterization of the Key Aroma Compounds of Soybean Flavor in Fermented Soybeans with *Bacillus subtilis* BJ3-2 by Gene Knockout, Gas Chromatography–Olfactometry–Mass Spectrometry, and Aroma Addition Experiments

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Abstract: Soybean flavor is considered to be essential for the aroma quality of fermented soybeans (FS) with *Bacillus subtilis* BJ3-2 (BJ3-2) at 37 °C. However, the key aroma compounds of the soybean flavor must be further elucidated. In this study, two candidate genes (*sdaAA* and *katX*) of BJ3-2 involved in the control of soybean flavor production were screened using prior multi-omics data. FS samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* were analyzed by quantitative descriptive sensory analysis (QDA), gas chromatography–olfactometry–mass spectrometry (GC-O-MS), relative odor activity values (ROAV), and aroma addition experiments. The QDA revealed that the aroma profile of the soybean flavor in FS consisted of "sweaty", "smoky", "beany", "roasted", and "sweet" attributes. A total of 20 aroma-active compounds were detected, and 13 of them with ROAV > 1 were identified as key aroma compounds. Moreover, aroma addition experiments were conducted to further confirm the key aroma compounds of soybean flavor. Among them, 2-methylbutyric acid, 2,3,5-trimethylpyrazine, and guaiacol contributed higher aroma intensity values and ROAV, resulting in "sweaty", "roasted", and "smoky" attributes of soybean flavor in FS, respectively, while 1-octen-3-ol was associated with the "beany" attribute. These findings provide novel insights into the aroma attributes of soybean flavor in FS and a new strategy for revealing the key aroma compounds in fermented foods.

Keywords: *Bacillus subtilis*; fermented soybeans; soybean flavor; soybean flavor candidate gene; aroma compounds

Citation: Chen, Z.; Wu, Y.; Tong, S.; Jin, J.; Zhang, L.; Li, C.; Tan, Q.; Wen, F.; Tao, Y. Characterization of the Key Aroma Compounds of Soybean Flavor in Fermented Soybeans with *Bacillus subtilis* BJ3-2 by Gene Knockout, Gas Chromatography–Olfactometry–Mass Spectrometry, and Aroma Addition Experiments. *Fermentation* **2024**, *10*, 409. https://doi.org/10.3390/fermentation10080409

Academic Editors: Marìa Soledad Prats Moya, Ana Leahu and Cristina Ghinea

Received: 1 July 2024
Revised: 1 August 2024
Accepted: 6 August 2024
Published: 8 August 2024

1. Introduction

Fermented soybean foods are widely popular in East and Southeast Asian countries due to their unique flavor and nutritional value. *Bacillus subtilis* (*B. subtilis*) is a predominant functional bacterium in these foods [1]. During the fermentation of soybeans by *B. subtilis*, the exogenous enzymes secreted by *B. subtilis* (mainly proteases and lipases) can decompose the proteins and lipids into amino acids, fatty acids, and organic acids, which then form volatile compounds [2,3]. The soybean flavor is a special flavor formed by fermenting the raw material by microorganisms (namely Chi-flavor, a special flavor with an ammonia scent distinct from natto) [4]. Previous research has reported that the characteristics of *B. subtilis* BJ3-2 fermented soybean differ at different temperatures. For example, fermented soybeans at 37 °C enhance soybean flavor compared to those at 45 °C and 53 °C, while fermented soybeans at 45 °C and 53 °C enhance soy sauce-like flavor compared to those at 37 °C [5]. Although traditional soybean flavor products such as Douchi and

Chi-flavor-type Baijiu are popular and accepted worldwide due to their prominent soybean flavor [6,7], there are only a few reports on the study of soybean flavor. It is speculated that the synergistic effects of multiple metabolic pathways and multiple genes may produce soybean flavor [5]. Meanwhile, the key aroma compounds contributing to soybean flavor in fermented soybeans are poorly known.

Systematic characterization of the aroma-active compounds therein is a prerequisite for improving the flavor quality of fermented soybeans [8]. Food aroma compounds are usually studied using an integrated approach encompassing isolation, identification, and quantification of volatile compounds, a deep analysis of aroma compounds, and reconstitution the aroma using the key aroma compounds [9,10]. Gas chromatography–olfactometry–mass spectrometry (GC-O-MS), a combination of gas chromatography–olfactometry (GC-O) and gas chromatography–mass spectrometry (GC-MS), separates the volatile compounds in a complex sample through gas chromatography, enables the determination of which of these volatiles are aromatically active through olfactometric detection, and allows the identification of the compounds eluting from the column through mass spectrometry [11]. Thus far, the aroma compounds in several *B. subtilis* fermented soybean foods have been examined using GC-O-MS. Previous studies have demonstrated that pyrazines play a crucial role in the flavor of natto (a traditional Japanese food made from fermented soybeans), among which 2,3,5-trimethylpyrazine, 2,5-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine, and 2,3,5-methyl-6-ethylpyrazine substantially contribute to natto flavor [12]. Furthermore, Mathatheeranan et al. identified the dominant aroma compounds of thua nao (indigenous Thai fermented soybeans) fermented by commercial *B. subtilis* under controlled conditions including 2-methylbutyric acid, phenylethyl alcohol, benzaldehyde, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, and 2-methoxyphenol, which contribute to the fermented/sour, floral, almond-like, and nutty aromas of fermented soybeans [13]. More recently, GC-O-MS combined with relative odor activity values (ROAV) were applied to examine the aroma compounds of sufu [14], Yongchuan Douchi [6], typical fermented soy foods [15], and so on. In these studies, e-nose analysis and quantitative description sensory analysis (QDA) were used to analyze the aroma profiles of different fermented soy products. Differences in the aroma profiles of the various products have been identified and associated with key aroma compounds.

In general, microbial metabolism is crucial for producing flavor in fermented foods [16]. To elucidate the mechanism of soybean flavor and soy sauce-like flavor production by *B. subtilis* BJ3-2, Wu et al. measured and analyzed the transcriptome, proteome, and metabolome of *B. subtilis* BJ3-2 at three temperatures (37 °C, 45 °C, and 53 °C). The functions of differentially expressed genes (DEGs) have been analyzed by KEGG and GO enrichment analyses. These DEGs are enriched in the relevant metabolic pathways, including the glycine, serine, threonine, cysteine, and methionine metabolite pathways, among others [5]. Meanwhile, amino acids are substrates for the formation of aroma compounds [17]. The production of soybean flavor may be closely linked to these metabolic pathways. Nevertheless, the candidate genes that may regulate the production of soybean flavor need to be further investigated.

Therefore, this study aimed to (a) screen soybean flavor candidate genes from the analysis of multi-omics data; (b) obtain mutants with significantly altered soybean flavor by knocking out the soybean flavor candidate gene to better analyze the relationship between sensory attributes and aroma compounds of soybean flavor in fermented soybeans (FS); (c) characterize key aroma compounds of soybean flavor in FS using GC-O and ROAV; and (d) verify the correlation between key aroma compounds and sensory attributes by partial least squares regression (PLSR) analysis. In the present study, by employing multi-omics data, gene knockout, and the headspace solid-phase microextraction (HS-SPME) method, combined with GC-O-MS, ROAV, and aroma addition experiments, we have become the first to characterize the aroma of soybean flavor in FS samples with *B. subtilis* BJ3-2 and its different mutants. These data can help gain a deeper understanding of the key aroma compounds in the production of soybean flavor in fermented soybeans with *B. subtilis*.

They also provide a new strategy for the screening of soybean flavor candidate genes and the modification of fermentation flavor by genetic engineering.

2. Materials and Methods

2.1. Strains and Culture Conditions

B. subtilis BJ3-2 (GI: CP025941) strains were cultured on liquid Luria–Bertani (LB) medium or solid LB at 37 °C. Chloramphenicol (5 μg/mL) or erythromycin (1 μg/mL) were used for screening when necessary. *E. coli* DH5*α* was cultured on liquid or solid LB medium with ampicillin (100 μg/mL).

2.2. Chemicals

Commercial standard n-alkanes (C7-C30), 2-octaol (≥99.0%), 2,3-butanedione (≥99.0%), 3-octanone (≥99.5%), and 3-ethyl-2,5-dimethylprazine (98%) were obtained from Macklin Biochemical Technology Co. (Shanghai, China). 2,3,5-Trimethylprazine (>98.0%), 2-ethylfuran (>98.0%), 2-pentylfuran (>98.0%), methyl 2-methylbutyrate (>98.0%), 2,4,5-trimethyloxazole (>98.0%), 2-heptaone (>98.0%), 2,5-dimethylprazine (>98.0%), 1-octen-3-ol (>98.0%), 2-methylbutyric acid (>98.0%), and guaiacol (>98.0%) were obtained from TCI Co., Ltd. (Shanghai, China). Propylene glycol (99%) and anhydrous ethanal (≥99.7%) were purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China).

2.3. Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

To validate the expression of *sdaAA* and *katX* in the multi-omics data, the total RNA of BJ3-2 was extracted, and the first strand of cDNA was synthesized using the FastKing method as a template (TIANGEN, Beijing, China). Then, the cDNA was detected by the CFX96 Touch PCR instrument (Bio-Rad, Hercules, CA, USA). The reaction system and the settings applied followed a previously described approach [18]. 16S rRNA was used as the internal reference gene. The primers used are listed in Table S1.

2.4. Construction and Transformation of Homologous Recombinant Knockout Vectors

HLarm is the homologous left arm of the target gene; HRarm is the homologous right arm; *cm* is the chloramphenicol gene; and *erm* is the erythromycin gene. DEDP is a double-exchange detection primer (Table S1) used to assess the success of the knockout. The genome of BJ3-2 was used as a template for PCR amplification of the HLarm and HRarm of *sdaAA* and *katX*, respectively. The *cm* was amplified by PCR using pHT01cas9-p43 as the template. The fragment and pUC18 were digested by the corresponding restriction endonuclease *Eco*R I, *Kpn* I, *Bam*H I, *Sal* I, and *Pst* I and then linked with T4 DNA ligase. The connected vector was then transformed into *E. coli* DH5*α*-competent cells. Finally, knockout vectors pUC18-(HLarm-*cm*-HRarm)/*sdaAA* and pUC18-(HLarm-*cm*-HRarm)/*katX* were constructed. Then, vectors were transformed into BJ3-2 and screened with chloramphenicol; the method used for the transformation was chemical transformation [19,20]. PCR validation of positively transformed colonies was performed using DEDP (Figures S1 and S2). Sequencing was then performed to validate the knockout strains (Figures S4 and S5). BJ3-2Δ*sdaAA* and BJ3-2Δ*katX* were obtained. In addition, the genome of BJ3-2 was used as a template to amplify the *sdaAA*, and the pMarA was used as a template for PCR amplification of the *erm*.

Another knockout vector, pUC18-*sdaAA*::*erm*, was constructed using the same method. pUC18-*sdaAA*::erm was transformed into BJ3-2 and screened with erythromycin. BJ3-2Δ*sdaAA* was obtained after validation by PCR using DEDP, and then pUC18-(HLarm-*cm*-HRarm)/*katX* was transferred into BJ3-2Δ*sdaAA* to obtain BJ3-2Δ*sdaAA*Δ*katX*. The double-knockout strain was validated by PCR using DEDP and sequencing (Figures S3 and S6). All primer synthesis and sequencing were performed by Tsingke Biotechnology Co., Ltd. (Beijing, China).

2.5. Preparation and Aroma Profile Evaluation of Fermented Soybeans (FS)

The preparation of FS samples was performed following the method previously described by Liu et al. [4], with slight modifications as follows: single colonies of BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* were inoculated into 5 mL of LB medium and shaken at 180 rpm for 14 h at 37 °C. Soybeans were then autoclaved at 121 °C for 20 min after soaking in water for 12-13 h. Appealed bacterial suspensions of BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* were inoculated onto cooled autoclaved soybeans (1%). Next, the soybeans and the bacterial suspensions were shaken and placed in the incubator at 37 °C for 72 h.

The aroma profile evaluation of FSs was assessed by quantitative descriptive analysis (QDA), and the sensory evaluation team consisted of 10 panelists from the School of Life Sciences, Guizhou University (4 males and 6 females, 20–27 years old). Each team member underwent a one-week sensory training course on fermented soybeans. Firstly, a descriptive test was conducted to elicit the sensory descriptor. Each FSs (5 g each) was put into 50 mL Teflon vessels, and each member smelled them to freely record the sensory descriptors. The panel then discussed and reached a unanimous agreement based on the frequency and intensity of the sensory attributes of soybean flavor. After that, five sensory attributes were selected to describe the soybean flavor, including "sweaty", "smoky", "beany", "roasted", and "sweet". Finally, for FS samples coded with 3-digit labels, the intensity of the aroma contribution was evaluated using a 6-point scale (0 = no smell, 1 = very weak, 2 = weak, 3 = moderate, 4 = strong, and 5 = very strong with significant irritation) [21], and each sample was analyzed three times. The flavor radar chart was plotted based on the obtained five-dimensional aroma wheel.

2.6. Extraction of Volatile Compounds in FS Samples by Using HS-SPME

HS-SPME extraction of FSs was performed according to Peng et al. [22] by putting 2.5 mL of distilled water, 0.625 g of NaCl, and 1.5 g of the fermented soybean sample into a 15 mL headspace vial. Samples were then pre-equilibrated in a 50 °C water bath (Dk-s24, Shanghai, China) for 20 min, after which the aged SPME (50/30 μm DVB/CAR/PDMS (2 cm); Bellefonte, PA, USA) was placed 1 cm above the liquid sample and adsorbed for 40 min. Next, SPME fiber was immediately inserted into the GC injection port (250 °C) of the GC-system and desorbed for 5 min. Each sample was analyzed three times.

2.7. Identification of Aroma-Active Compounds in FS Samples by GC-O-MS

GC-O-MS analysis was performed using an Agilent 8890B GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a polar capillary column DB-WAX (60 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies) and coupled to an olfactory detector (ODP4; Gerstel, Mülheim an der Ruhr, Germany) and an Agilent 5977B MS (Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer and the sniffer detection port received the volatile compounds separated by the capillary column in a 1:1 ratio. Humidified air was pumped into the sniffer arm at 40 mL/min. The following temperature program was applied: the column temperature was initially set at 40 °C and held for 3 min, ramped up to 130 °C at a rate of 3 °C/min and held for 5 min, and finally ramped up to 200 °C at a rate of 5 °C/min and held for 5 min. The GC inlet temperature was 250 °C, and high-purity helium was used as the carrier gas (He > 99.999%) and transported through the capillary column at a constant flow rate of 1.0 L/min. GC-O: The aroma intensity (AI) was evaluated using the approach of Peng et al. with minor adjustments (from a 5-point scale to a 6-point scale) [23]. Four panelists (2 males and 2 females, an average of 25 years old) were asked to record the AI values, as well as odor characteristics and retention time. A 6-point scale ranging from 0 (none) to 5 (extreme) was used to evaluate the aroma intensity. A higher number means that the compound has a stronger odor. Analyses was repeated three times by each panelist. Finally, the AI values were the average of four panelists.

For qualitative and quantitative analysis, volatile compounds were identified using mass spectra from the database (NIST 20.0) with reference to retention index (RI) and odor

characteristics from the literature. Some important compounds were further identified using standard compounds. Retention indices were calculated for individual compounds based on n-alkane standards from C7 to C30.

For quantification, 8 µL of 2-octanol (0.18 mg/mL) was added to fermented soybeans as an internal standard, and all compounds were analyzed semi-quantitatively, and the relative concentration of each compound was calculated following the method of Zhao et al. [24]. The concentration of the volatile compounds is calculated from the peak areas of the volatiles and the concentration of the internal standard.

2.8. Calculation of Relative Odor Activity Values (ROAVs)

ROAVs were calculated based on the ratio of the relative content of volatile compounds to their absolute thresholds in water using the following formulae [25]:

$$\mathrm{ROAV} = \mathrm{Cx}/\mathrm{OTx}$$

where Cx is the average concentration of the compounds and OTx is the odor threshold of the compound. The thresholds used in this study were those in water obtained from published literature [26,27].

2.9. Aroma Addition Experiment

Aroma addition experiments were conducted to compare the aroma profile changes after the key aroma compounds screened from GC-O and ROAV were added to 1.5 g of FSs. Two FSs with BJ3-2 and BJ3-2Δ*sdaAA*Δ*katX*, which have greater differences in soybean flavor, were selected. The differences in concentrations of key aroma compounds in the two FSs were calculated from the semi-quantitative results (Table S5). All chemical solutions were prepared in water or propylene glycol solution and diluted to the corresponding concentrations [28] (Table S5). Then, 1.5 g of each of the two FSs matrices were placed into 15 mL Teflon vessels; 11 compounds were added to FSs with BJ3-2, and 2 compounds were added to FSs with BJ3-2Δ*sdaAA*Δ*katX* to achieve the same concentration in both FSs (Table S5). The two FSs were then equilibrated at 25 °C for 30 min. The aroma profiles of the two FSs were then evaluated by the ten panelists using QDA, and the flavor radar chart was drawn based on average.

2.10. Statistical Analysis

At least three replications of each experiment and sample analysis were conducted, and the results were reported as mean ± standard deviation. Data were statistically analyzed by SPSS software (version 26.0, Inc., Chicago, IL, USA) using a *t*-test to ascertain a significant difference between BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* at $p \leq 0.05$. Origin 2022 software (version 9.8.0.200) was used for plotting. The correlation between sensory attributes and key aroma compounds was analyzed by PLSR using the Unscrambler 9.7 version software (CAMO ASA, Oslo, Norway). TBtools v2.042 was used to make the heat map.

3. Results and Discussion

3.1. Screening of Soybean Flavor Candidate Genes of BJ3-2

To screen genes that may significantly impact the formation of soybean flavor, we conducted further analysis and screening of the prior multi-omics data from BJ3-2 incubated at different temperatures, as described in the literature [29]. Briefly, differential genes, proteins, and metabolites with $|\mathrm{Log_2FC\ (FOLDCHANGE)}| \geq 2$, $|\mathrm{Log_2FC}| \geq 1.5$, and $|\mathrm{Log_2FC}| \geq 1$ were screened according to p-value < 0.05 and from the transcriptome, proteome, and metabolome for KEGG pathway mapping. Co-analyzed genes and metabolites are mainly involved in the glycine, serine, and threonine metabolism; ABC transporter, arginine and proline metabolism; cysteine and methionine metabolism; and tryptophan metabolism metabolic pathways. Meanwhile, differential genes and proteins with $|\mathrm{Log_2FC}| \geq 2.5$ were screened from the joint analysis results, and a total of *sdaAA*

and *katX* were studied as candidate genes. In the transcriptome data, *sdaAA* was down-regulated by 2.50~fold (log_2FC) after incubating at 53 °C, and *katX* was down-regulated by 4.40~fold (log_2FC) after incubating at 53 °C (Table S2). RT-qPCR was further performed to confirm the gene expression of BJ3-2 (Table S3). As shown in Figure 1, the results show that the expression of *sdaAA* and *katX* was low at 53 °C and high at 37 °C, indicating the transcriptome data and the RT-qPCR results were consistent.

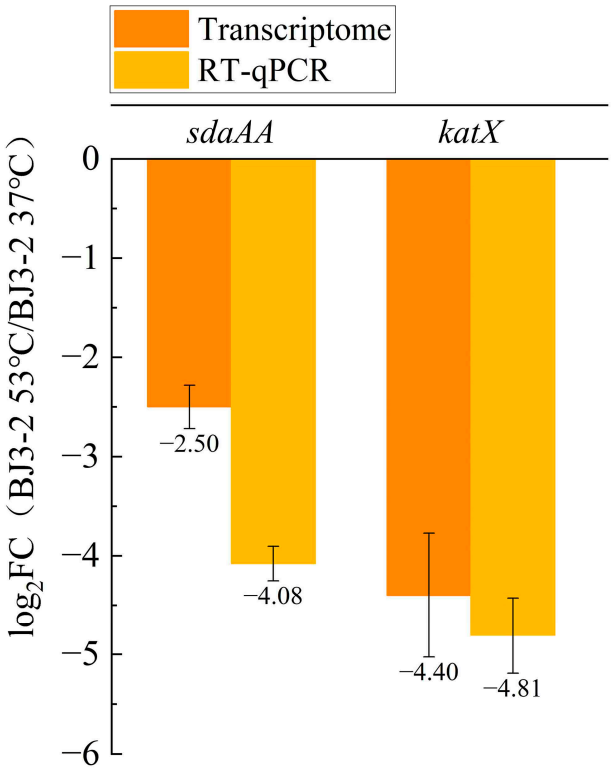

Figure 1. RT-qPCR verification of the expressions *sdaAA* and *katX*.

3.2. Knockout of sdaAA and katX Had No Significant Effect on the Morphology and Growth of BJ3-2

In this study, we constructed two single-knockout strains and one double-knockout strain by homologous recombination. After obtaining the knockout strain, it was determined whether the knockout affected the colonies' phenotypic characteristics and growth ability. The morphology of single colonies of BJ3-2 on solid plates was first identified. Colonies appeared white, dry, irregular in morphology, and internally wrinkled. The morphology of BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* colonies was comparable to that of BJ3-2 (Figure 2A,C,E,G).

Furthermore, the four strains (BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*) were stained with a blue-purple tint and a short stick form by Gram staining, as shown in Figure 2B,D,F,H. The growth curves were then used to determine the growth ability of the strains. The results are shown in Figure 2I. The OD values of BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* were similar to those of BJ3-2 at different time points. These findings showed that the morphology and growth of BJ3-2 were not significantly affected by the knockout of *sdaAA* and *katX*.

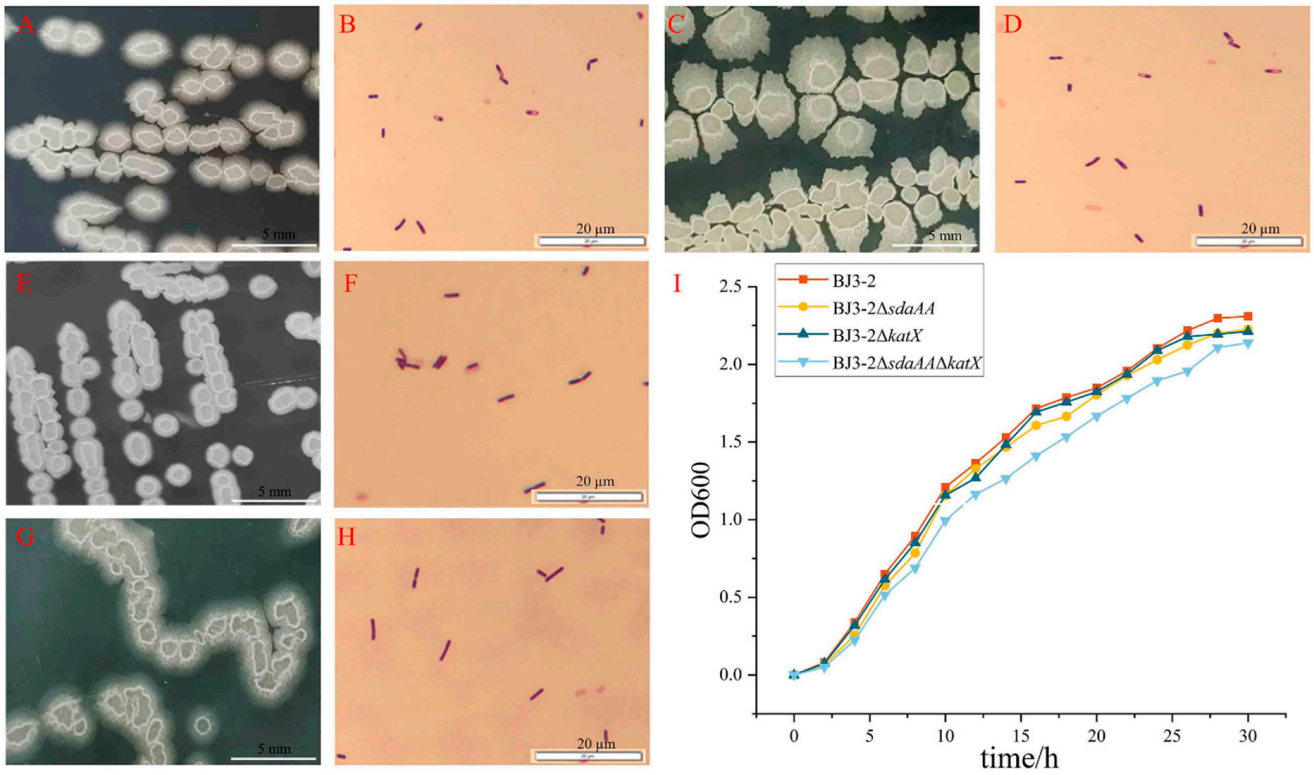

Figure 2. Colony morphology and growth curves of BJ3-2, BJ3-2Δs*daAA*, BJ3-2Δ*katX*, and BJ3-2Δs*daAA*Δ*katX*. (**A**) Colony morphology of BJ3-2. (**B**) Gram staining of BJ3-2. (**C**) Colony morphology of BJ3-2Δs*daAA*Δ*katX*. (**D**) Gram staining of BJ3-2Δs*daAA*Δ*katX*. (**E**) Colony morphology of BJ3-2Δ*sdaAA*. (**F**) Gram staining of BJ3-2Δs*daAA*. (**G**) Colony morphology of BJ3-2Δ*katX*. (**H**) Gram staining of BJ3-2Δ*katX*. (**I**) Growth curves of BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*.

3.3. Sensory Analysis of Soybean Flavor in FSs with BJ3-2, BJ3-2ΔsdaAA, BJ3-2ΔkatX, and BJ3-2ΔsdaAAΔkatX at 37 °C

Quantitative descriptive analyses (QDAs) were applied to assess the differences in aroma profiles of four FSs after fermentation of soybeans with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* at 37 °C (Figure 3A). As shown in Figure 3B, the aroma profiles of soybean flavor from FSs were described by five sensory attributes: "sweaty", "smoky", "beany", "roasted", and "sweet". BJ3-2 was more frequently associated with the "sweaty (4.5 ± 0.45)" attribute, whereas the other attributes were at low levels, probably because acids obscure other attributes. BJ3-2Δ*sdaAA* and BJ3-2Δ*katX* had diminished "sweaty" attributes and enhanced "smoky", "beany", and "sweet" attributes compared to BJ3-2. BJ3-2Δ*sdaAA*Δ*katX* presented the lowest intensity of the "sweaty (2.3 ± 0.32)" attribute and the highest intensity of the "beany (2.6 ± 0.12)" and "roasted (3.1 ± 0.33)" attributes, with higher differences from BJ3-2. In addition, the "sweet" attribute had the lowest intensity of soybean flavor in FSs, and knockout of *sdaAA* and *katX* resulted in an increase in the intensity of the "sweet" attribute. The QDA results suggest that knockout of *sdaAA* and *katX* has a substantial effect on the flavor formation of soybean flavor and also validate the analysis of the mechanism of soybean flavor formation using multi-omics data.

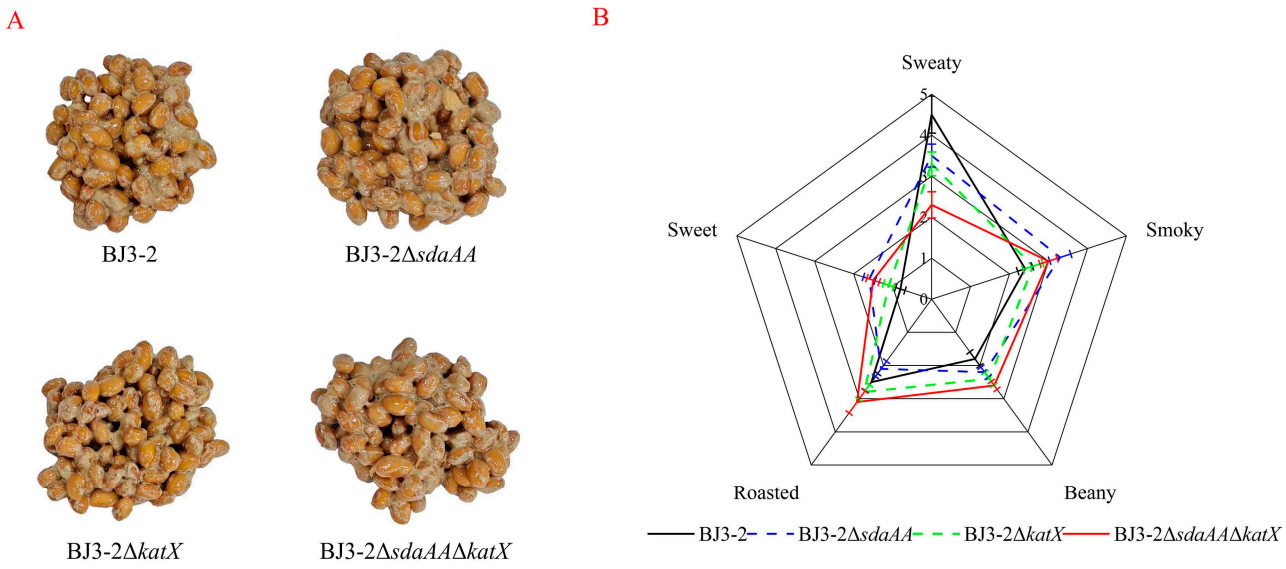

Figure 3. Aroma profile analysis of soybean flavor in fermented soybean samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*. (**A**) Fermented soybean samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* at 37 °C. (**B**) Aroma profile analysis of soybean flavor in fermented soybean samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* via QDA (panel number = 10).

3.4. Identification of Aroma-Active Compounds of Soybean Flavor in FSs with BJ3-2, BJ3-2ΔsdaAA, BJ3-2ΔkatX, and BJ3-2ΔsdaAAΔkatX by GC-MS-OSME

GC-MS-OSME was conducted to investigate the aroma-active compounds in four FSs. OSME (odor-specific magnitude estimation) is often used to determine the contribution of aroma-active compounds to the flavor based on aroma intensity (AI); higher AI values indicate a greater contribution to the flavor [25,30]. A total of 20 aroma-active compounds were detected, including 6 ketones, 5 pyrazines, 3 alcohols, 2 furans, 1 ester, 1 phenol, 1 acid, and 1 other (Table S4).

A total of 19, 20, 20, and 18 aroma-active compounds were found in BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* FS samples, respectively (Table 1). Eight compounds with relatively high AI values coexisted in all FSs, including 2,3-butanedione (1.74 ± 0.20–2.58 ± 0.16), methyl 2-methylbutyrate (2.32 ± 0.14–2.98 ± 0.20), 2,4,5-trimethyloxazole (1.54 ± 0.46–2.48 ± 0.11), 2,5-dimethylpyrazine (2.89 ± 0.27–3.04 ± 0.12), 2,3,5-trimethylpyrazine (3.02 ± 0.17–3.46 ± 0.09), 1-octen-3-ol (2.65 ± 0.18–3.04 ± 0.16), 2-methylbutyric acid (3.34 ± 0.27–3.85 ± 0.24), and guaiacol (2.91 ± 0.37–3.12 ± 0.28). Therefore, these compounds are regarded as important contributors to soybean flavor. The compound 2-methylbutyric acid, known as an off-flavor compound in *Bacillus*-fermented foods [31,32], showed the highest AI (3.85 ± 0.24) in BJ3-2, followed by BJ3-2Δ*sdaAA* (3.58 ± 0.16), BJ3-2Δ*katX* (3.46 ± 0.14), and BJ3-2Δ*sdaAA*Δ*katX* (3.34 ± 0.27). In this study, through the aroma characterization of 2-methylbutyric acid in GC-O and its standard compounds, we concluded that 2-methylbutyric acid is responsible for the "sweaty" attribute.

The aroma compounds 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine have previously been reported in natto [12]. These pyrazines typically provide a "roasted" or "nutty" attribute. In this study, these three pyrazines showed different AI values among the four FSs. Guaiacol also showed a relatively higher AI value in four FSs, and it has a smoky flavor, which should be related to the "smoky" attributes of soybean flavor. 1-Octen-3-ol imparts a mushroom flavor, which is here related to the "beany" attribute. In addition, 2,4,5-trimethyloxazole contributed to the "roasted" attribute and 2-pentylfuran to the "beany" attribute.

Table 1. GC-O/AI and ROAV analysis of aroma-active compounds of soybean flavor in fermented soybean samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*.

	GC-O (AI)				ROAV			
Compounds	**BJ3-2**	**BJ3-2Δ*sdaAA***	**BJ3-Δ*katX***	**BJ3-2Δ*sdaAA*Δ*katX***	**BJ3-2**	**BJ3-2Δ*sdaAA***	**BJ3-2Δ*katX***	**BJ3-2Δ*sdaAA*Δ*katX***
3-Methyl-2-butanone	0.16 ± 0.03	0.1 ± 0.02	0.13 ± 0.03	0.17 ± 0.05	0.1 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
2-Ethylfuran	1.25 ± 0.09	1.37 ± 0.12	1.85 ± 0.06	1.45 ± 0.26	3.30 ± 0.04	5.38 ± 1.09	6.70 ± 2.72	4.68 ± 0.27
2,3-Butanedione	1.74 ± 0.20	2.1 ± 0.18	2.35 ± 0.23	2.58 ± 0.16	13.72 ± 2.96	10.06 ± 0.38	29.19 ± 7.18	54.08 ± 5.94
Methyl 2-methylbutyrate	2.72 ± 0.17	2.98 ± 0.20	2.32 ± 0.14	2.49 ± 0.09	56.80 ± 8.96	109.30 ± 18.00	55.16 ± 7.72	48.45 ± 8.24
2-Heptanone	1.14 ± 0.08	1.05 ± 0.04	1.26 ± 0.09	1.31 ± 0.08	1.12 ± 0.59	1.69 ± 0.10	2.64 ± 0.53	2.06 ± 0.16
2,4,5-Trimethyloxazole	2.17 ± 0.12	1.54 ± 0.46	2.32 ± 0.22	2.48 ± 0.11	17.33 ± 0.94	4.20 ± 0.32	17.98 ± 1.78	18.27 ± 1.08
2-Pentylfuran	1.06 ± 0.04	1.12 ± 0.13	1.53 ± 0.08	1.35 ± 0.11	1.63 ± 0.12	2.39 ± 0.49	3.41 ± 1.24	2.90 ± 0.88
3-Octanone	1.5 ± 0.09	1.54 ± 0.10	1.38 ± 0.16	1.62 ± 0.12	2.93 ± 0.83	1.87 ± 1.60	4.29 ± 0.27	2.96 ± 0.52
2-Octanone	0.95 ± 0.07	0.37 ± 0.09	0.54 ± 0.02	0.00 ± 0.00	1.35 ± 0.39	0.72 ± 0.14	0.64 ± 0.25	0.00 ± 0.00
2,5-Dimethylpyrazine	2.89 ± 0.27	2.95 ± 0.35	2.93 ± 0.14	3.04 ± 0.12	67.54 ± 2.72	88.58 ± 0.46	76.63 ± 1.35	79.50 ± 0.55
2-Nonanone	1.05 ± 0.15	0.19 ± 0.07	0.84 ± 0.17	0.32 ± 0.28	1.01 ± 0.09	0.64 ± 0.05	1.15 ± 0.10	0.79 ± 0.08
1-Hexanol	0.43 ± 0.04	0.45 ± 0.05	0.54 ± 0.12	0.57 ± 0.16	0.13 ± 0.01	0.15 ± 0.02	0.17 ± 0.01	0.20 ± 0.01
2-Ethyl-5-methylpyrazine	0.00 ± 0.00	0.24 ± 0.06	0.19 ± 0.12	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
2,3,5-Trimethylpyrazine	3.02 ± 0.17	3.27 ± 0.14	3.35 ± 0.19	3.46 ± 0.09	185.94 ± 9.72	147.72 ± 1.56	208.56 ± 6.54	212.19 ± 3.40
1-Octen-3-ol	2.65 ± 0.18	2.74 ± 0.24	2.95 ± 0.14	3.04 ± 0.16	45.85 ± 1.43	52.28 ± 3.59	53.02 ± 1.49	55.10 ± 2.82
3-Ethyl-2,5-dimethylpyrazine	1.76 ± 0.23	1.83 ± 0.19	1.94 ± 0.35	1.68 ± 0.17	2.19 ± 0.30	2.16 ± 0.01	3.06 ± 0.23	3.93 ± 0.11
Tetramethylpyrazine	0.42 ± 0.04	0.58 ± 0.06	0.45 ± 0.02	0.38 ± 0.09	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
3-Octanol	0.56 ± 0.06	0.73 ± 0.10	0.94 ± 0.05	1.23 ± 0.07	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
2-Methylbutyric acid	3.85 ± 0.24	3.58 ± 0.16	3.46 ± 0.14	3.34 ± 0.27	591.46 ± 9.51	341.41 ± 3.40	327.74 ± 11.86	241.16 ± 10.44
Guaiacol	2.91 ± 0.37	3.08 ± 0.18	2.88 ± 0.24	3.12 ± 0.28	164.85 ± 19.76	248.76 ± 31.59	179.23 ± 22.06	236.32 ± 5.65

Note: Orange color in the table indicates compounds that are common to both GC-O and ROAV > 1. Data are presented as mean ± SD.

Several other compounds, including 2-ethylfuran, 2,3-butanedione, methyl 2-methylbutyrate, 2-heptanone, and 3-octanone, were associated with the "sweet" attribute, as these compounds were perceived as having a pleasant flavor, neutralizing the overall flavor of soybean. Concentration differences mainly caused differences in AI values in fermented soybean samples from different strains. Therefore, these compounds need to be quantified and relative odor activity values calculated.

3.5. Key Aroma Compounds of Soybean Flavor in FSs with BJ3-2, BJ3-2ΔsdaAA, BJ3-2ΔkatX, and BJ3-2ΔsdaAAΔkatX Identified via ROAV

The contribution of aroma-active compounds to overall flavor is related to concentration and odor threshold values. After semi-quantification using the internal standard method, ROAV was further calculated to determine the contribution of aroma-active compounds to soybean flavor. Aroma-active compounds with ROAV > 1 were considered to be the contributors to the soybean flavor of FSs. As shown in Table 1, there were 15, 13, 14, and 13 compounds with ROAV > 1 in FSs with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*, respectively.

Among these compounds, the highest values of ROAV were for 2-methylbutyric acid (ROAV 241.16 ± 10.44–591.46 ± 9.51), 2,3,5-trimethylpyrazine (ROAV 147.72 ± 1.56–212.19 ± 3.40), and guaiacol (ROAV 164.85 ± 19.76–248.76 ± 31.59), implying they contribute significantly to the aroma of soybean flavor in FSs. Relatively lower ROAV for 2,3-butanedione (ROAV 10.06 ± 0.38–54.08 ± 5.94), methyl 2-methylbutyrate (ROAV 48.45 ± 8.24–109.3 ± 18.00), 2,4,5-trimethyloxazole (ROAV 4.2 ± 0.32–18.27 ± 1.08), 2,5-dimethylpyrazine (ROAV 67.54 ± 2.72–88.58 ± 0.46), and 1-octen-3-ol (ROAV 45.85 ± 1.43–55.1 ± 2.82). The lowest ROAV were found for 2-ethylfuran (ROAV 3.3 ± 0.04–6.7 ± 2.72), 2-heptanone (ROAV 1.12 ± 0.59–2.64 ± 0.53), 2-pentylfuran (ROAV 1.63 ± 0.12–3.41 ± 1.24), 3-octanone (ROAV 1.87 ± 1.60–4.29 ± 0.27), and 3-ethyl-2,5-dimethylpyrazine (ROAV 2.16 ± 0.01–3.93 ± 0.11). These compounds are also closely related to the aroma formation of soybean flavor in FSs. Taken together, the ROAV calculations for most of the compounds were in agreement with the AI values of the GC-O, suggesting a high degree of agreement between the two methods. Of these 20 aroma-active compounds from GC-O, 13 were detected with ROAV > 1 in all FSs, indicating their crucial influence on the formation of soybean flavor in FSs. The 13 key aroma compounds are listed in Figure 4. The chemical structures of these compounds were further identified by comparing the retention index (RI) and aroma profile with those of the standard compounds and used for further analysis.

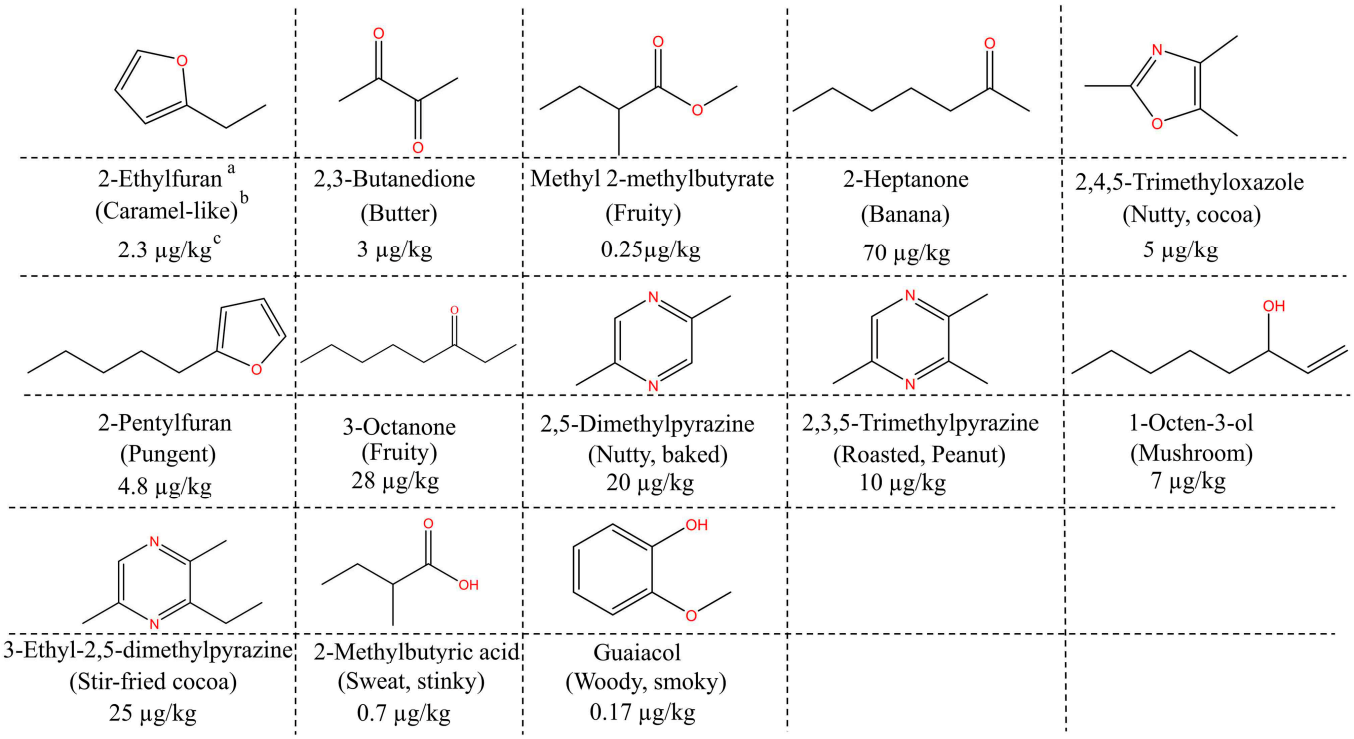

Figure 4. Information on thirteen key aroma compounds from GC-O (AI) combined with ROAV. [a] Thirteen key aroma compounds from GC-O combined with ROAV; [b] aroma characteristics of key aroma compounds; [c] odor thresholds of compounds in water reported in the literature.

3.6. Relationship between Key Aroma Compounds and Sensory Attributes of Soybean Flavor in FSs

Partial least squares regression (PLSR) was performed to investigate the relationship between sensory attributes and key aroma compounds in the four FS samples. The X-matrix was set as the thirteen key aroma compounds, and the Y-matrix was set as five sensory attributes. The PLSR consisted of two significant PCs explaining 75% of the X-variance and 92% of the Y-variance (Figure 5), while the inner ellipse and outer ellipse explained 50% and 100% of the variance, respectively. All variables are located outside the small ellipse except 2-ethylfuran (1) and 3-octanone (7), which shows that these variables were well explained by the PLSR model. The low values of RMSEC (0.11) and RMSRP (0.19) also indicate that the regression model performs well. As shown in Figure 5, the closer to the variable Y, the higher the correlation between X and Y [33]. The compound 2-Methylbutyric acid (12) was highly associated with the "sweaty" attribute. The "smoky" attribute was strongly influenced by guaiacol (13). In addition, 2,3,5-trimethylpyrazine (9) and 1-octen-3-ol (10) showed robust correlation with "roasted" and "beany" attributes, respectively. In addition, the "sweet" attribute was weakly associated with certain aroma compounds. This might be because more compounds are implicated in the formation of this sensory attribute. These results are consistent with the GC-O and ROAV analyses (Table 1).

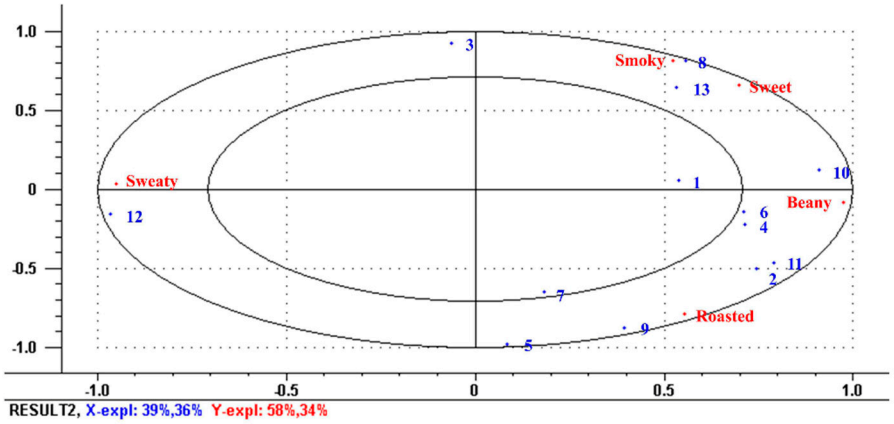

Figure 5. The correlation loading plots between the concentration of the thirteen key aroma compounds (X matrix) and scores of sensory attributes (Y matrix) in FSs with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*; the validation method is full cross-validation; ellipses indicate r^2 = 0.5 and 1.0, respectively. Numbers indicate the following compounds: 1, 2-ethylfuran; 2, 2,3-butanedione; 3, methyl 2-methylbutyrate; 4, 2-heptanone; 5, 2,4,5-trimethyloxazole; 6, 2-pentylfuran; 7, 3-octanone; 8, 2,5-dimethylpyrazine; 9, 2,3,5-trimethylpyrazine; 10, 1-octen-3-ol; 11, 3-ethyl-2,5-dimethylpyrazine; 12, 2-methylbutyric acid; 13, guaiacol.

*3.7. Changes in Key Aroma Compounds in FSs between BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX***

Based on semi-quantitative results, significant difference analyses ($p \leq 0.05$) were performed on the content of 13 key aroma compounds between the four FSs with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*.

As shown in Figure 6, in comparison to FSs with BJ3-2, the content of 2-methylbutyric acid in FSs with BJ3-2Δ*katX*, BJ3-2Δ*sdaAA*, and BJ3-2Δ*sdaAA*Δ*katX* was significantly lower, with BJ3-2Δ*sdaAA*Δ*katX* having the lowest content. The lowest content of 2-methylbutyric acid in BJ3-2Δ*sdaAA*Δ*katX* also explains well why it possesses the lowest intensity of "sweaty" attributes (Figure 3B). This compound may have a positive correlation with the intensity of "sweaty" attributes. 2,3,5-Trimethylpyrazine was decreased in BJ3-2Δ*sdaAA* and significantly increased in both BJ3-2Δ*katX* and BJ3-2Δ*sdaAA*Δ*katX*. Meanwhile, in contrast to BJ3-2, the "roasted" attribute of BJ3-2*sdaAA* was weakened, whereas the "roasted" attribute of BJ3-2Δ*katX* and BJ3-2Δ*sdaAA*Δ*katX* was enhanced (Figure 3B), indicating that 2,3,5-trimethylpyrazine was associated with the "roasted" attribute. Guaiacol and 1-octen-3-ol were increased in BJ3-2Δ*katX*, BJ3-2Δ*sdaAA*, and BJ3-2Δ*sdaAA*Δ*katX*, which is consistent with the QDA results (Figure 3B) of enhanced "smoky" and "beany" attributes of soybean flavor in FSs. Several other compounds also showed some variations in the content of the four FSs. In summary, knockout of *sdaAA* and *katX* resulted in a significant increase or decrease in the content of key aroma compounds, which mainly resulted in a significant decrease in 2-methylbutyric acid and a significant increase in 2,5-dimethylpyrazine, 2-ethylfuran, 2-pentylfuran, 2-heptanone, 1-octen-3-ol, and guaiacol, thus affecting the soybean flavor.

Additionally, KEGG pathway analysis shows that *sdaAA* (which encodes serine dehydratase) is involved in metabolic pathways, mainly including glycine, serine, and threonine metabolism and cysteine and methionine metabolism. *katX* (encodes catalase) is involved in metabolic pathways, mainly tryptophan metabolism, glyoxylate metabolism, and dicarboxylate metabolism. This implies that the knockout of *sdaAA* and *katX* may impact the production of aroma compounds by affecting these metabolic pathways, leading to altered sensory attributes in FS with its mutants. However, the roles and specific regulatory mechanisms of *sdaAA* and *katX* in the production of soybean flavor still require further investigation.

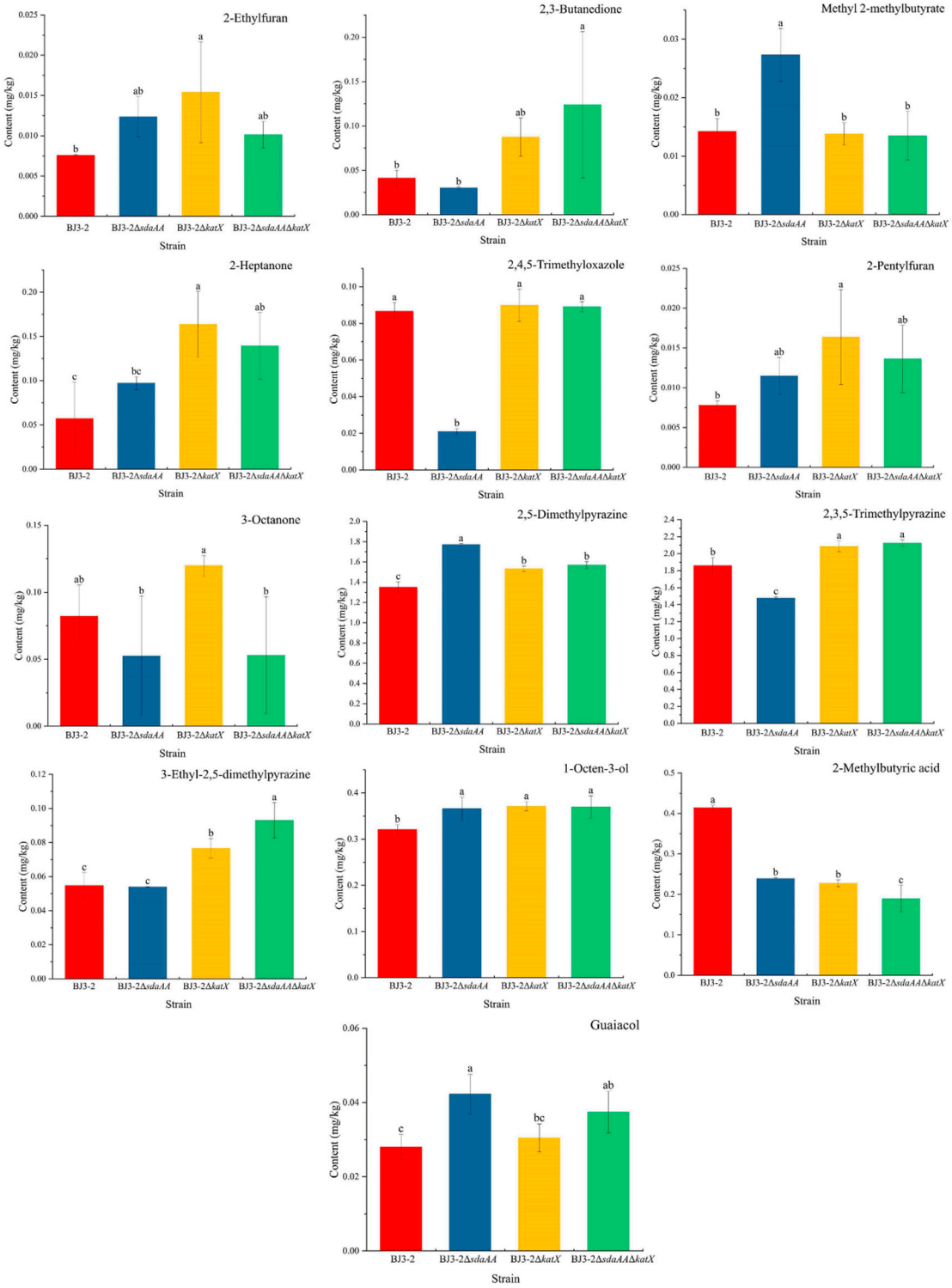

Figure 6. Changes in content of key aroma compounds among the four fermented soybean samples with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*. Significant differences between groups are indicated by letters a, b, c.

3.8. Aroma Addition Experiment

Based on our analyses and further validation of key aroma compounds, BJ3-2 and BJ3-2Δ*sdaAA*Δ*katX*, which have greater differences in aroma profiles, were selected for an aroma addition experiment. Aroma addition experimental models were prepared by adjusting the contents of key aroma compounds in the two samples to be consistent based

on the difference in concentrations of the 13 key aroma compounds in the two FSs. After that, it was determined whether there was a change in the aroma profiles of soybean flavor in the two FSs or whether the aroma profiles converged to verify the effect of these key aroma compounds. As shown in Figure 7, QDA was applied to access the aroma profiles of the two FSs after adding the aroma compounds. The aroma profiles of both samples changed after the addition of the differential aroma compounds, and both samples converged in "sweaty", "smoky", "roasted", and "beany" attributes but still differed in "sweet". This may be due to the fact that the "sweet" attribute of BJ3-2 is masked by the strong intensity of the "sweaty" attribute. The result of the aroma addition experiment suggests that these compounds are closely related to the aroma profiles of soybean flavor and also validates these substances as key aroma compounds of soybean flavor in FS.

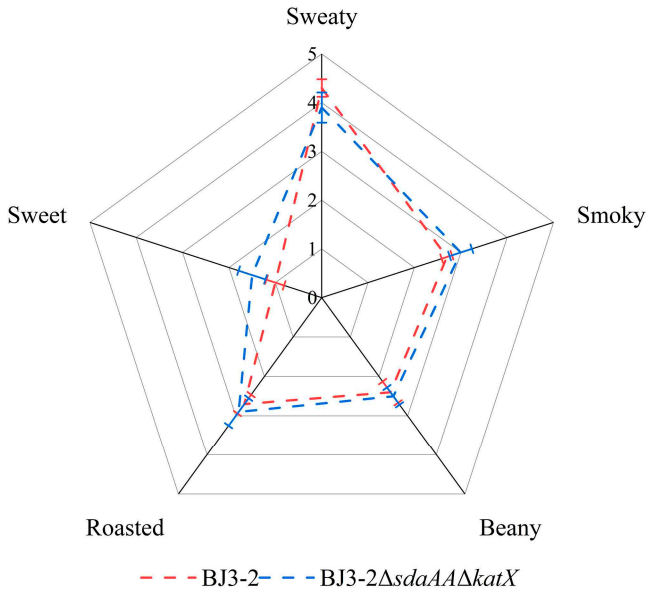

Figure 7. Aroma profile of soybean flavor in fermented soybean samples with BJ3-2 and BJ3-2Δ*sdaAA*Δ*katX* after addition of aroma compounds (panel number = 10).

3.9. Source of Key Aroma Compounds of Soybean Flavor in Fermented Soybeans

The results of the key aroma compounds of soybean flavor in FS showed that 2-methylbutyric acid produced the main compounds contributing to "sweaty" attributes. Moreover, 2-methylbutyric acid was identified as a short-chain fatty acid. The source of 2-methylbutyric acid may be due to the ability of *B. subtilis* to metabolically convert isoleucine, leucine, and valine to 2-methylbutyric acid during fermentation [34]. Meanwhile, 2-methylbutyric acid can be converted to methyl 2-methylbutyrate [35].

Furthermore, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine have been detected in a number of fermented foods, such as white wine, curd, and natto [12,36,37]. Pyrazines can be produced by non-enzymatic Maillard reactions and enzymatic reactions caused by *B. subtilis* [13]. During fermentation, exogenous enzymes secreted by *B. subtilis* hydrolyze proteins and carbohydrates into monosaccharides and free amino acids, respectively. These pyrazines can be produced from glucose and L-threonine [3].

Guaiacol is also found to be an important aroma compound and can be produced by the degradation of vanillin by the enzyme vanillin dehydrogenase in *B. subtilis* [38]. Moreover, 2,3-butanedione usually has a pleasant, creamy, or buttery taste and can be metabolized by *B. subtilis* to produce α-acetolactate (which can be converted to acetoin) and 2,3-butanedione (which can also be converted to each other under the catalysis of acetoin/butanediol dehydrogenase) [39]. Cysteine/alanine has been reported to react with 2,3-butanedione to form 2,4,5-trimethyloxazole [40], and 2-heptanone can also be produced by the Maillard reaction or biosynthesized from acid [41]. The metabolic pathways of

3-octanone and 1-octen-3-ol in fungi are well defined, whereas the biosynthetic pathway in *B. subtilis* has not been elucidated. The fungal pathway is mainly based on the oxidative degradation of unsaturated fatty acids [42,43].

4. Conclusions

In this study, the aroma profiles and key aroma compounds of soybean flavor in FS with *B. subtilis* BJ3-2 and its knockout mutants at 37 °C were analyzed using QDA, GC-O-MS, ROAV calculation, and aroma addition experiments. QDA results revealed that the overall aroma of soybean flavor in FSs consists of "sweaty", "smoky", "roasted", "beany", and "sweet" attributes. In addition, the knockout of soybean flavor candidate genes (*sdaAA* and *katX*) did not significantly affect the morphology and growth of BJ3-2 but had a greater effect on the aroma profiles of soybean flavor in FSs with BJ3-2. A total of 20 aroma-active compounds were detected by GC-O-MS from four FSs with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX*, and 13 key aroma compounds were identified as key aroma compounds according to their high ROAVs. Furthermore, PLSR revealed a good correlation between the key aroma compounds and sensory attributes. Knockout of *sdaAA* and *katX* resulted in a significant increase or decrease in the content of key aroma compounds. Finally, combined with the aroma addition experiments, 2-methylbutyric acid, 2,3,5-trimethylpyrazine, guaiacol, and 1-octen-3-ol were identified as the aroma compounds responsible for the main aroma profiles of soybean flavor. The findings of this study provide new insights into the analysis of the soybean flavor of fermented soybeans produced by *B. subtilis* BJ3-2, which is important for quality control and improvement of the aroma of fermented soybeans.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10080409/s1, Table S1: Primers used in this study; Table S2: Transcriptome data at 37 °C and 53 °C; Table S3: Reverse transcription–quantitative real-time PCR at 37 °C and 53 °C; Table S4: Relative content (ug/kg) of volatile compounds in FSs by HS-SPME-GC-O-MS (μg/kg); Table S5: Data used in the aroma addition experiment; Figure S1: Construction and transformation of *sdaAA* homologous recombination knockout vector; Figure S2: Construction and transformation of *katX* homologous recombination knockout vector; Figure S3: Construction and transformation of *sdaAA* and *katX* homologous recombination knockout vector; Figure S4: Sequencing of BJ3-2Δ*sdaAA*; Figure S5: Sequencing of BJ3-2Δ*katX*; Figure S6: Sequencing of BJ3-2Δ*sdaAA*Δ*katX*; Figure S7: Fermented soybeans with BJ3-2, BJ3-2Δ*sdaAA*, BJ3-2Δ*katX*, and BJ3-2Δ*sdaAA*Δ*katX* at 37 °C.

Author Contributions: Data curation, Z.C., F.W. and Y.T.; Formal analysis, Z.C., Q.T. and F.W.; Funding acquisition, Y.W.; Methodology, Z.C.; Project administration, S.T. and C.L.; Validation, J.J.; Writing—original draft, Z.C.; Writing—review and editing, Y.W. and L.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (Grant No. 32060585).

Institutional Review Board Statement: As the experimental samples used in the study were consumed in daily life, the study was exempted from ethical review and approval.

Informed Consent Statement: Informed consent was obtained from all panelists involved in the study. Participants agreed to the use of their data/answers. The study strictly adhered to a protocol designed to protect the rights and privacy of all participants.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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