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Modulating the aroma and taste profile of soybean using novel strains for fermentation

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ABSTRACT

A key factor influencing consumer acceptance of soybean products is the aroma and taste profile, which can be modulated through fermentation using unique microbial strains. This study aimed to identify and characterize novel microbial strains with the potential to enhance flavour profiles including umami, while reducing undesirable flavour notes such as beany aromas. The results showed an 800% (8-fold) increase in free amino acids in samples fermented with *Rhizopus oryzae*, which correlated with an increase in umami intensity as measured using an E-tongue. Samples fermented with *Neurospora crassa* also demonstrated an increase in methionine and cysteine, sulfur-containing amino acids that are deficient in raw soybean. Fermentation additionally resulted in a significant increase in fatty acids and alterations to the fatty acid profile. Notably, samples fermented with *Penicillium camemberti*, *Penicillium nalgiovense*, *Penicillium chrysogenum*, and *Leuconostoc mesenteroides* containing omega-3 fatty acids. Lastly, fermentation introduced desirable aroma compounds, including 'smoky', 'cheesy' and 'floral' notes, enhancing the sensory appeal of certain samples. This study demonstrates the innovative use of novel microbial strains in soybean fermentation as a promising strategy to modulate the aroma and taste profile of soybean products.

1. Introduction

In an era of escalating environmental concerns and a global shift towards more sustainable and ethical food choices, the demand for alternative protein sources has reached unprecedented levels. The agriculture and food sectors face the dual challenge of meeting the nutritional needs of a growing population while reducing the environmental footprint of food production. Since 1961, global meat consumption has quadrupled (Whitton et al., 2021), and the Food and Agriculture Organization (FAO) projects global meat production to increase by nearly 44 million metric tons by 2030 (Michele, 2021). Traditional livestock farming is linked to significant environmental issues, including deforestation, greenhouse gas emissions, and biodiversity loss. Additionally, the water and land resources required for animal farming exacerbate food insecurity and contribute to ethical concerns related to animal welfare, antibiotic use, and industrial farming practices. Excessive meat consumption has also been associated with increased risk of chronic diseases (Battaglia Richi et al., 2015).

While meat remains a key dietary source of protein, iron, zinc and vitamin B_{12} (McAfee et al., 2010), the need for more sustainable protein alternatives is driving a paradigm shift toward plant-based options. Soybean has emerged as a leading candidate due to its high protein content, which includes all essential amino acids, though it is relatively low in methionine and cysteine (T. Zhang et al., 2021). Soybeans are also an efficient protein source, with a lower environmental impact than animal-based proteins, offering the highest protein yield per unit of

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greenhouse gas emissions (González et al., 2011). Soy protein is widely used in the production of meat analogues, with high-moisture extrusion technology creating products that mimic the fibrous texture of real meat (T. Zhang et al., 2021).

However, one of the main barriers to wider adoption of soy-based meat alternatives is their undesirable flavour profiles, particularly the beany odour associated with soy, as well as a lack of desirable aromas. Soy protein's bulk composition may lack sufficient precursors for generating complex aroma compounds (L. Yang et al., 2023). Although synthetic flavouring agents are commonly added to mask off-flavours, consumers hesitancy around ultra-processed foods has limited the acceptance of soy-based products. Natural flavourings like hydrolysed vegetable protein and yeast extract (Zahari et al., 2022) have been explored but are costly, making these products less competitive.

Fermentation has emerged as a promising method to address these flavour challenges, offering a natural and effective way to enhance the taste, aroma and overall sensory quality of soybean-based products. Once primarily used as a preservation technique, fermentation now plays a key role in developing complex flavour profiles in a variety of foods. Soybean contains sugars, proteins, and lipids that act as precursors for flavour compounds during fermentation (Shahidi and Hossain, 2022). Microbial fermentation, in particular, has been shown to reduce the beany flavours of soy, enhancing its sensory attributes and increasing consumer acceptability (Elhalis et al., 2024). The type of microbial strains used in fermentation plays a crucial role in shaping the sensory profile of the final product. Different strains, such as bacteria, yeasts and mould species, have been widely studied for their ability to modulate flavour development in soy fermentation. Mould species are known for their proteolytic activity, which can break down soy proteins into peptides and free amino acids, generating umami and other savoury flavours (Elhalis et al., 2024), while yeasts can promote the production of volatile aroma compounds (Hu et al., 2019). By carefully selecting strains with specific proteolytic and lipolytic activities, it is possible to enhance the production of desirable flavour compounds, leading to a more palatable and appealing product. In this study, we explore the use of novel microbial strains to enhance the sensory qualities of fermented soybean products. We aim to demonstrate the potential of fermentation as a natural and effective method for transforming soybean into a highly palatable, nutritious protein source.

2. Materials and methods

2.1. Strains and chemicals

Table 1 provides an overview of the strains used in fermentation. AS and RO are fungal strains commonly used in soybean fermentation to create traditional fermented soybean products such as soy sauce and tempeh (Elhalis et al., 2024). PC, PN, PQ and PR are fungal strains commonly used in cheese and/or meat fermentation (Laranjo et al., 2019; Le Dréan et al., 2010). The yeast strains, PK and KM, are commonly isolated and are commonly employed in cocoa and coffee fermentation (Schwan et al., 2023), while DH is primarily utilized in meat and/or dairy fermentations (Laranjo et al., 2019).

Table 1
Strains used in fermentation.

Abbreviation	Type	Strain	Strain code/ origin
AS	Mould	*Aspergillus sojae*	ATCC 42251
NC		*Neurospora crassa*	ATCC 44320
PC		*Penicillium camemberti*	ATCC 6985
PN		*Penicillium nalgiovense*	ATCC 46455
PQ		*Penicillium chrysogenum*	ATCC 10106
PR		*Penicillium roqueforti*	ATCC 10110
RO		*Rhizopus oryzae*	ATCC 20344
DH	Yeast	*Debaryomyces hansenii*	ATCC 10619
KM		*Kluyveromyces marxianus*	MUCL 53775
PK		*Pichia kluyveri*	Chr Hansen Holdings A/S
LC	Lactic acid bacteria	*Lacticaseibacillus casei*	ATCC 334
LM		*Leuconostoc mesenteroides* subsp. *mesenteroides*	ATCC 8293
LP		*Lactiplantibacillus plantarum*	DSM 100813
PA		*Pediococcus acidilactici*	ATCC 25742

2.2. Preparation of inoculum for fermentation

Selected fungal strains were cultured on Potato Dextrose Agar (PDA) and incubated at 25 °C for 7 days. The selected yeast strains were grown on Yeast-Peptone Dextrose (YPD) Agar (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 30 °C for 3 days. The selected lactic acid bacteria (LAB) strains were grown on De Man-Rogosa-Sharpe (MRS) Agar (Sigma-Aldrich) and incubated in an anaerobic jar with an AnaeroGen pack (ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated at 37 °C for 3 days.

After incubation, the fungal spores were collected using 5 mL of a 10% PBS solution then passed through a cell strainer (Corning Inc., Corning, New York, USA) to separate mycelia and the spores. The spores in the resulting supernatant were collected and counted using a hemacytometer. For yeast and bacteria, the cells were collected using 5 mL of 10% PBS solution and their optical density (OD) was measured using a Varioskan® spectrophotometer (ThermoFisher Scientific) at 600 nm.

2.3. Preparation of soybean for fermentation

Canadian non-GMO soybeans (*Glycine max)* were purchased from Hong Guan Huat Kee (Singapore). Soybeans were soaked at a 1:4 soybean-to-water ratio, with 0.05% w/w acetic acid added and incubated at 25 °C for 24 h. After incubation, the soybeans were thoroughly washed with tap water to remove residual acid and dehulled. Soybeans (500 g) were weighed into a 1 L glass bottle (Nalgene, Rochester, New York, USA) and autoclaved at 121 °C for 30min. After autoclaving, the soybeans were left to cool to 25 °C.

2.4. Inoculation and fermentation

The starting inoculum for each fungal strain was standardised to 6.0 log spores/g of rehydrated soybeans, while for yeast and LAB, the starting inoculum was 6.0 log CFU/g of rehydrated soybeans. The inoculum was added to the cooled rehydrated soybeans on covered petri dishes. Fermentation of the soybean samples was carried out in triplicates of 50 g at 25 °C for 72 h in a static incubator. Daily 5 g samples were aseptically collected for microbial and chemical analyses.

2.5. Microbial enumeration

Each sample (5 g) was weighed in a 50 mL Falcon tube, followed by the addition of 10 mL of a 10% PBS solution, and vortexed for 1min. The samples were diluted with a 10% PBS solution and plated at appropriate dilutions. Samples fermented with filamentous fungi were plated on Rose Bengal Agar (RBA) (Sigma-Aldrich) containing chloramphenicol (Sigma-Aldrich). Samples fermented with yeasts were plated on YPD agar containing chloramphenicol. Samples fermented with LAB were plated on MRS agar containing cycloheximide (Sigma-Aldrich). After plating, the RBA plates were incubated at 25 °C for 7 days, the YPD agar plates were incubated at 30 °C for 3 days and the MRS agar plates were incubated at 37 °C in an anaerobic jar with an AnaeroGen pack (ThermoFisher Scientific) for 3 days. Microbial counts were determined post-incubation, with results recorded in triplicate.

2.6. Preparation of samples for chemical analysis

Each sample (45 g) was frozen in liquid nitrogen for 20s and ground

in a coffee grinder (Rommelsbacher EGK 200, Rommelsbacher, Germany) for 1min. The resulting ground soybeans were stored in a 50 mL Falcon tube at −20 °C before further analysis.

2.7. pH

Ground soybeans (0.5 g) were weighed in a 5 mL centrifuge tube, followed by the addition of 1 mL of MilliQ H_2O. The samples were vortexed, and the pH of the beans was measured using a pH meter (LaquaAct, Horiba, Japan) and recorded in triplicate.

2.8. HPLC analysis for metabolite profiling

Ground soybeans (0.5 g) were weighed in a 5 mL centrifuge tube, followed by the addition of 2 mL of MilliQ H_2O. The samples were vortexed for 1min and centrifuged at 3405×*g* for 10min. The resulting supernatant was passed through a 0.22 μm filter (Claristep Syringeless Filters, Sartorius, Göttingen, Germany). Chromatographic analysis was performed on an Agilent Infinity 1200 liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA), coupled with a diode-array detector (DAD) and a refractive index detector (RID). Chromatographic separation was performed on an Aminex HPX-87H Column. The length of the column is 300 mm and the internal diameter is 7.8 mm. The fermentation metabolites were eluted isocratically with a mobile phase of 5 mol/m^3 H_2SO_4 at a flow rate of 0.6 mL/min. The RID temperature and column temperature was set at 35 °C, and the injection volume was 20 μL. Chromatic integration was analysed with Agilent Open Lab ChemStation (Agilent Technologies, Santa Clara, CA, USA) and exported to Microsoft Office Professional Plus 2016, Microsoft Excel (Microsoft Corporation, Albuquerque, NM, United States) for data analysis. Analyte concentrations of sugars, organic acids and ethanol were quantified using external standards with $R^2 \geq 0.98$.

2.9. Amino acid analysis

Free amino acids were analysed with the ARACUS Amino Acid Analyser (MembraPure, Berlin, Germany). Standard norvaline solution was used as an internal standard. Samples (0.4 g) were weighed in a 5 mL centrifuge tube followed by the addition of 350 μL of 10 mM norvaline and 3650 μL of 0.1 N HCl. The samples were placed in a Hula-Mixer™ (ThermoFisher Scientific) for 30min and centrifuged at 0 °C, 3405×*g* for 10min. An aliquot of 800 μL of supernatant was added to 200 μL of 40% trichloroacetic acid (TCA) in a 1 mL microcentrifuge tube and vortexed thoroughly for 10min. The samples were then centrifuged at 4 °C at 25,830×*g* for 5min. An aliquot of 100 μL of supernatant with 600 μL of 0.1 N HCl in the HPLC vial and the samples were analysed. The separation was carried out in a lithium system column kit (MembraPure) consisting of a pre-column and a separation column (150 mm × 4 mm). Eluent solutions (A-F), wash and derivatization solutions containing ninhydrin were purchased from MembraPure. The eluent flow rate and reactor temperature were set at 200 μL/min and 130 °C, respectively. All amino acids were detected at 570 nm except for proline, which was detected at 440 nm. A calibration factor programmed by MembraPure was used to quantify the amino acids.

2.10. Fatty acid compositional analysis

The fatty acid content in the fermented samples were extracted using the method adapted from GB 5009.168-2016 (J. Zhang, 2019). The fatty acid profiles were analysed with a GC-FID (Agilent 7908 A). The fatty acid peaks were identified by comparison of retention times with those of standards from a Supelco 37 component fatty acid methyl ester mix. Results were expressed as a percentage of total fatty acids or fatty acid group in each sample.

2.11. Electronic tongue (e-tongue) analysis

The samples were analysed using the ASTREE e-tongue (Alpha MOS, Toulouse, France) to acquire taste-related data. The signals were measured using 7 potentiometric sensors (AHS, ANS, CPS, CTS, NMS, PKS, SCS) and an AgCl reference electrode was used. Samples (0.3 g) were weighed into a 50 mL Falcon tube and 30 mL of MilliQ water was added. The samples were vortexed to resuspend in water and mixed in a rotary shaker for 30min. After mixing, the samples were centrifuged at 3405×*g* for 30min and the supernatant was collected for analysis. All samples were characterised at room temperature. The sensors and reference electrode were rinsed for 10s with water between different samples. Each sample was measured for 120s and the measurements were repeated 9 times due to system stability requirements. The signal value at the 120th second was extracted from the last 6 measurements and averaged to obtain the sensor signal raw data.

2.12. GC-MS

For volatile profiling, 0.5 g of sample was weighed into a 20 mL headspace vial followed by the addition of 100 μL of internal standard (1 ppm). The samples were run through GC-MS (GC: Agilent 7890 B GC; MS: Agilent 5977 B MSD). Headspace-solid phase microextraction was used to extract volatile compounds in the samples. The incubation time was 10min, and the incubation temperature was set at 40 °C. The extraction time was set at 30min. Volatile profiling was conducted using a DB-WAX UI (30 m × 0.25 mm) column. The inlet temperature was set at 250 °C and the split ratio, at 100:1. The flow rate was set at 1 mL/min. Target analytes were quantified by comparing their peak areas against an external calibration curve of target standards. Untargeted analysis was carried out by deconvolution and annotation of the mass spectra by matching the retention index and mass spectral profile against an in-house library (RI window = 20, MS match threshold = 80%). The amount of aroma compounds in the samples was normalized to the internal standard (2,3-dimethoxytoulene) aliquoted into each of the samples prior to GC analysis.

2.13. Statistical analysis

All data reported were obtained from three independent experiments. All data were expressed as mean values and standard deviations (SD). Principal component analysis (PCA) of selected volatile compounds was performed using R Studio (R Core Team, 2021) Analysis of variance (ANOVA) was conducted using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) with the Data Analysis ToolPak to evaluate significant differences between groups. Tukey's Honestly Significant Difference (HSD) post-hoc test was applied to identify significant pairwise differences, using the standardized range statistic q for multiple comparisons. Results were considered statistically significant at $p < 0.05$. Significant differences between means were indicated using letter groupings, with means sharing the same letter not differing significantly at the specified confidence level.

3. Results and discussion

3.1. Changes in microbial population, pH, sugars, and metabolites during fermentation

Soybean is a valuable nutrient source ideal for various fermentation processes, containing approximately 40% protein, 20% oil and 33% carbohydrate (Hou et al., 2009). Throughout fermentation, all microbial strains demonstrated substantial growth, with fungal growth peaking at 72 h and yeasts and bacteria at 48 h (Fig. 1). An increase in pH, from 5.56 ± 0.02 in control samples to 6.96 ± 0.17 in DH-fermented samples, was observed for samples fermented with filamentous fungi and yeast. In contrast, a decrease in pH, to 4.46 ± 0.10 was observed in samples

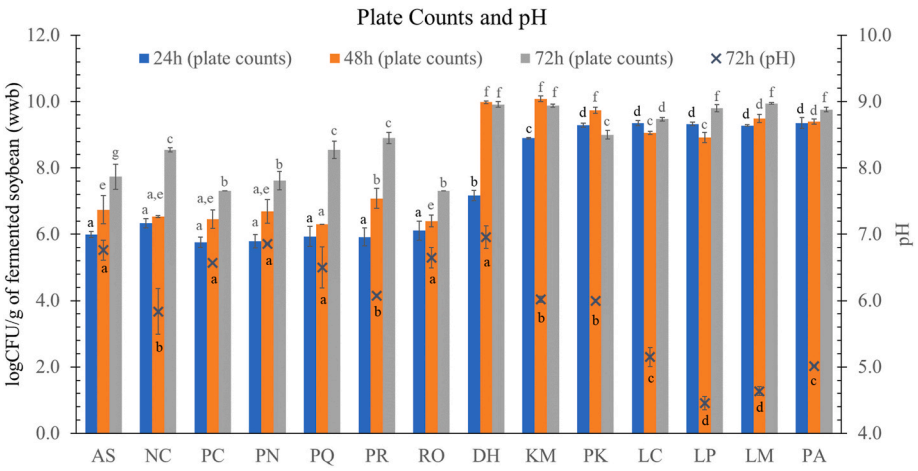

Fig. 1. Microbial populations of fermented soybean across fermentation and pH values of fermented samples at the end of 72 h of fermentation. The control was uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

fermented with LP, corresponding to LAB fermentation (Fig. 1).

Sucrose, the major sugar in soybeans, along with glucose, fructose, raffinose and stachyose, serves as a potential primary carbon and energy source for these microbes (Yazdi-Samadi et al., 1977) (Fig. 2). The fungal strains preferentially consumed sucrose, reducing its concentration significantly, from 15.98 ± 0.45 mg/g wwb in control samples to 0 mg/g wwb in RO-fermented samples. Notably, *Penicillium* strains exhibited a stronger preference for fructose over glucose (Sharma et al., 2014). Among the yeast strains, DH, known for its semi- and non-fermentative metabolism (Wrent et al., 2014), left high residual sugar levels (sucrose: 11.52 ± 5.13 mg/g wwb, glucose: 6.08 ± 2.16 mg/g wwb, fructose: 6.90 ± 4.01 mg/g wwb) post-fermentation.

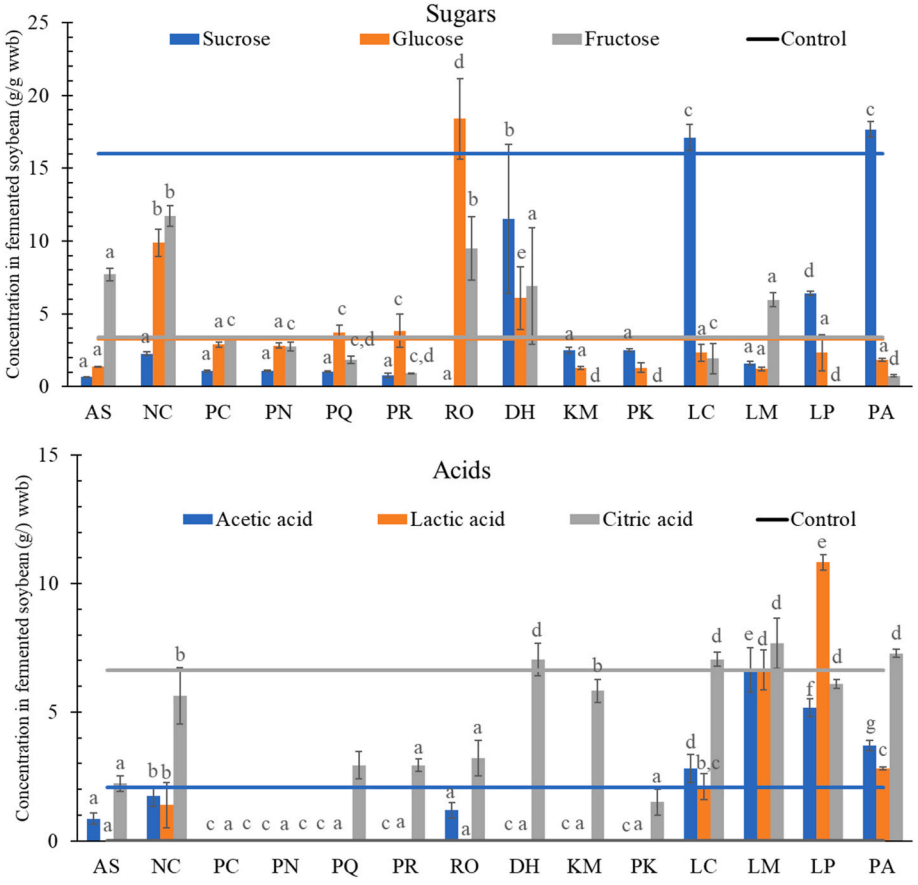

Fig. 2. Sugar and metabolite profiles of fermented soybean after 72 h of fermentation. The control was uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

Despite this, DH achieved substantial growth, suggesting its ability to utilize other carbon and energy sources present in soybean for proliferation (Fig. 2). Viana et al. (2006) reported that DH can produce α-galactosidase, which degrades stachyose and raffinose into simple sugars for consumption (Viana et al., 2006).

Among the LAB strains, only LM and LP effectively utilized sucrose (Fig. 2). LM has been reported to produce sucrose phosphorylase, which converts sucrose into fructose and glucose-1-phosphate in the presence of phosphate (Schacht et al., 2005). Meanwhile, LP possesses a two-component enzyme system that enhances its carbohydrate utilization capabilities (Cui et al., 2021). LP-fermented samples exhibited the highest lactic acid concentration (10.83 ± 0.31 mg/g wwb), correlating with its homofermentative nature and the lowest observed pH (Figs. 1 and 2). A significant increase in acetic acid (6.63 ± 0.86 mg/g wwb) was observed for LM-fermented samples, aligning with LM's heterofermentative nature, which involces converting acetyl phosphate to acetic acid (Punia Bangar et al., 2022). Organic acid production, such as lactic acid and acetic acid, is crucial not only for preserving fermented soybean but also for influencing flavour perception. For instance, lactic acid has been reported to enhance saltiness intensity in soy sauce (Singracha et al., 2017).

Significant reductions in citric acid were observed for fungi-fermented samples, except for those fermented with NC (Fig. 2). Fungal strains can produce citrate lyase, which metabolizes citrate for the endogenous biosynthesis of fatty acids (Chypre et al., 2012). However, NC only produces isocitrate lyase (Rogers and McFadden, 1977), explaining the lack of citrate consumption during fermentation. Interestingly, citrate consumption was not observed in the LAB fermentations (Fig. 2), even though some LAB are known to degrade citrate to produce aromatic compounds such as diacetyl, which imparts buttery and dairy aromas. Citrate degradation varies among LAB species and strains, as it is depends on the presence of genes encoding citrate lyase and citrate permease, which facilitate citrate internalization and catabolism into oxaloacetate, respectively (Eicher et al., 2024).

3.2. Free amino acid and e-tongue profiles

Soybean protein primarily consists of glycinin and β-conglycinin, which function as the main storage protein for germinating seeds (Yazdi-Samadi et al., 1977). During fermentation, microbes produce alkaline and acidic proteases that hydrolyse these proteins into free amino acids (FAAs) (Fig. 3), altering the taste profile of the soybeans. E-tongue analysis revealed distinct taste differences between unfermented and fermented samples, with fermented samples forming three distinct clusters based on their taste profiles (Fig. 4). LAB-fermented grouped into a single cluster, likely due to the sour taste resulting from increased lactic acid levels. PN, AS and NC also formed a separate cluster, while the remaining samples were loosely grouped (Fig. 4), highlighting the influence of fermentation on taste, potentially due to variations in FAA profiles.

FAAs contribute to flavour as precursors to volatile flavour compounds formed during fermentation, the Maillard reaction and Strecker degradation (Zhao et al., 2016). Among fungi-fermented samples, RO exhibited the highest increase in FAAs (Fig. 3) and umami intensity (Fig. 5). FAAs increased by over 800%, from 0.93 ± 0.05 mg/g wwb in unfermented samples to 89.93 ± 4.64 mg/g wwb in RO-fermented samples, likely due to high protease activity by these fungi, which hydrolyse soybean storage proteins.

Umami, described as a savoury or meaty taste, is influenced not only by FAAs, but also by peptides, such as γ-glutamyl peptides (Wang et al., 2022). In samples like those fermented with PC, high umami intensity was observed despite lower FAA levels (23.06 ± 1.31 mg/g wwb) (Fig. 5). This suggests that peptides, generated through protease activity, play a significant role in enhancing umami intensity. For example, PC produces proteases that hydrolyse proteins into peptides, which have been shown to increase the umami intensity of Camembert cheese during ripening (Mane and McSweeney, 2020).

Yeast- and LAB-fermented samples exhibited only modest increases in FAA levels (Fig. 3, Table S1). However, certain strains such as DH (2.54 ± 0.40 mg/g wwb) and PK (2.63 ± 0.03 mg/g wwb) led to relatively higher FAA levels. DH is commonly used in fermented sausage and cheese to hydrolyse proteins into FAAs, as it produces proteases A and D, which share homology to cathepsin D, a protease found naturally in fermented meat products such as dry sausages (Bolumar et al., 2008).

Despite lower FAA levels, LAB fermentation resulted in higher umami intensity compared to the control. LAB strains are known to produce extracellular proteases and peptidases, which hydrolyse soy protein into peptides. These peptides likely contributed to the umami intensity of the fermented soybeans (Ren and Li, 2022).

Samples	Concentration (mg/g wwb)																	
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	$(Cys)_2$	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Sum
Control																		
AS																		
NC																		
PC																		
PN																		
PQ																		
PR																		
RO																		
DH																		
KM																		
PK																		
LC																		
LM																		
LP																		
PA																		

Fig. 3. Heat map of free amino acid profiles of the fermented soybean samples after 72 h of fermentation. The control was uninoculated soybean. Each row represents the FAA profile of an individual fermented samples, and each column corresponds to a specific amino acid. The colour gradient from green to red indicates increasing FAA concentrations, with green representing low concentrations and red representing high concentrations. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

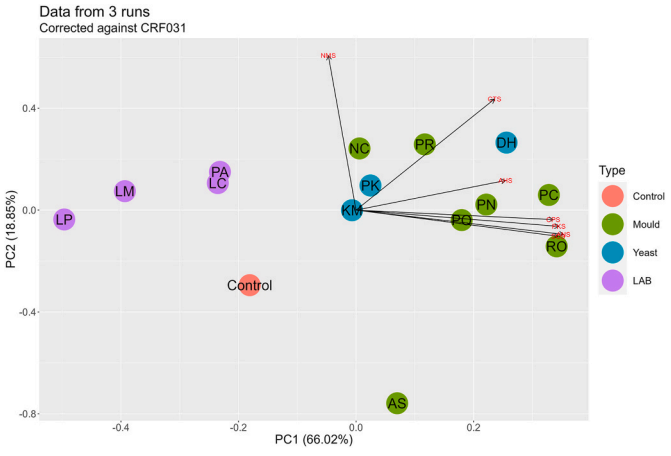

Fig. 4. Taste map of fermented soybean samples after 72 h of fermentation. The control was uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

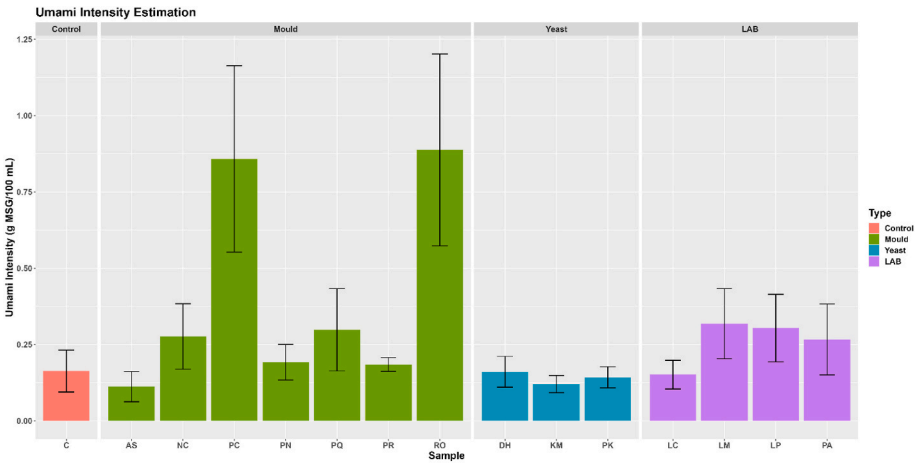

Fig. 5. Umami intensity estimation of soybean samples after 72 h of fermentation. The control was uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

3.3. *Fatty acid profile*

Soybean oil typically contains 10% palmitic acid (C16:O), 4% stearic acid (18:0), 23% oleic acid (18:1), 51% linoleic acid (18:2) and 7–10% linolenic acid (18:3) (Sharma et al., 2014). The fermented samples showed an increase in fatty acid content (Fig. 6), which can be attributed to fermenting microorganisms utilizing the carbohydrates and lipids present in soybean as substrates for carbon and energy sources, as well as for fatty acid synthesis. This increase in fatty acid content may enhance the aroma profiles of fermented soybeans, as mono- and polysaturated fatty acids, such as oleic and linoleic acids, are key aroma precursors associated with cooked meat aroma (Dinh et al., 2021).

Interestingly, several fermented samples did not have linoleic acid by the end of fermentation (Fig. 6). Similar observations have been reported in soy sauce fermentation, where linoleic acid decreased following *koji* fermentation (Zou et al., 2019). This decrease could be due to the rapid oxidation of linoleic acid, given its high degree of unsaturation, or its involvement in the generation of aroma compounds (Zou et al., 2019).

The presence of α-linolenic acid (ALA) was detected in samples fermented with DH and LM. Certain yeast strains are known to synthesize fatty acids, including α-linolenic acid (Cordova and Alper, 2018). ALA is involved in key aroma-generating reactions, such as its interaction with cysteine and ribose, which plays a crucial role in producing cooked beef aromas (Elmore and Mottram, 2006). Additionally, the breakdown of ALA can lead to the formation of seafood-like aromas (Peinado et al., 2016). Therefore, the increase in fatty acids during fermentation can result in the formation of novel aroma compounds, enhancing the sensory attributes of fermented soybeans.

3.4. *Volatiles profile*

The PCA of aroma compounds reveals distinct differences between unfermented and fermented soybean samples (Fig. 7). Unfermented samples exhibited higher levels of green and beany aroma compounds, such as hexanal, 2-hexenal, and 2-pentyl furan (Table S2). These compounds, particularly hexanal due to its potent odour at low concentrations, are considered off-flavours (L. Yang et al., 2023). They are generated during soybean processing through the oxidation of polyunsaturated fatty acids by lipoxygenases (LOX) and auto-oxidation. Heat treatment is commonly applied to raw soybean to reduce LOX activity and degrade formed during soaking (L. Yang et al., 2023). However,

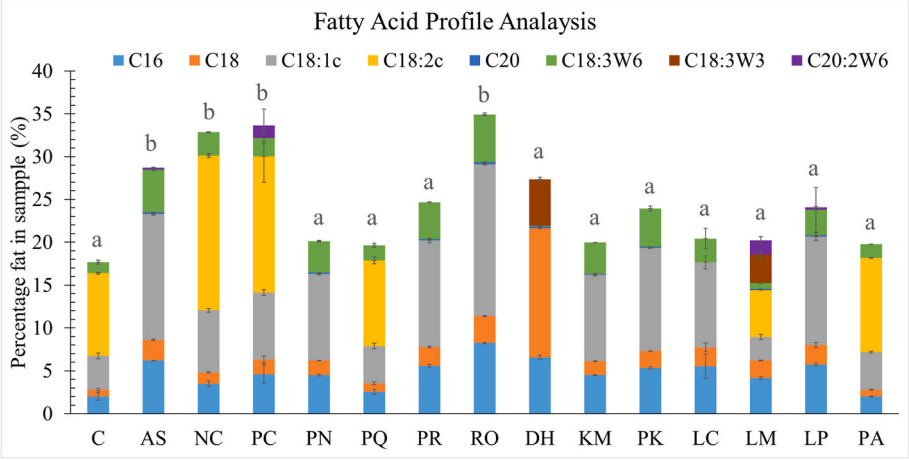

Fig. 6. Fatty acid profile analysis of fermented soybean samples after 72 h of fermentation. The control was uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

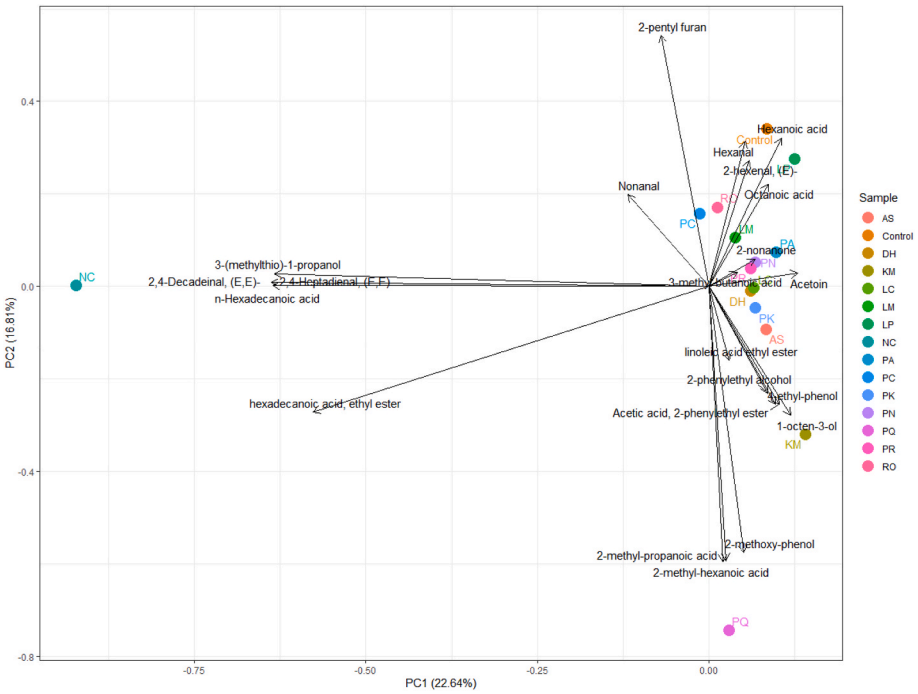

Fig. 7. PCA analysis and aroma compounds comparison of fermented samples after 72 h of fermentation. The control used is uninoculated soybean. (AS: Aspergillus sojae; NC: Neurospora crassa; PC: Penicillium camemberti; PQ: Penicillium chrysogenum; PR: Penicillium roqueforti; RO: Rhizopus oryzae; DH: Debaryomyces hansenii; KM: Kluyveromyces marxianus; PK: Pichia kluyveri; LC: Lacticaseibacillus casei; LP: Lactiplantibacillus plantarum; LM: Leuconostoc mesenteroides subsp. mesenteroides; PA: Pediococcus acidilactici).

complete removal of these compounds is challenging due to their binding to glycinin or interactions with amino acid residues, making short-term heat treatment insufficient (Zhou and Cadwallader, 2006).

Most fermented samples showed an absence of hexanal (Fig. 7 and Table S2). Fermentation generally reduces hexanal levels as microbial enzymes break down protein structures, release bound hexanal, and convert it into hexanol or further oxidize it into hexanoic acid, both of which contribute to unique aromas (Vong and Liu, 2017). However, samples fermented with PC showed an increase in green and beany compounds. PC is known to produce significant amounts of lipoxygenase (Karahadian et al., 1985), which exhibits high substrate specificity for linoleic acid at pH 6.5 and 8.0, aligning with the pH of the fermentation medium (Fig. 1). The robust lipoxygenase activity in PC likely accounts for the increased levels of aldehydes and beany aroma compounds in these samples.

Some fermented samples, particularly those with NC, exhibited higher concentrations of fatty and oily aroma compounds, including n-hexedecanoic acid, 2-octenal, 2,4-decadienal (E,E)-, 2,4-heptadienal (E, E)- and ethyl ester hexadecenoic acid (Fig. 7 and Table S2). NC has been reported to produce polyunsaturated fatty acids *de novo* and have the capability of incorporating exogenous fatty acids into its lipids (McKeon et al., 1997), which could explain the release of palmitic acid during fermentation (Fig. 6). Samples fermented with DH also exhibited high levels of linoleic acid ethyl ester (Table S2), a compound characteristic of fatty aromas. DH is known to inducing strong lipolysis, hydrolysing lipids to release fatty acids that undergo esterification or alcoholysis reactions with ethanol to form ethyl esters (Ramos-Moreno et al., 2021). NC-fermented samples also contained 3-(methylthio)-1-propanol derived from methionine, contributing meaty and onion-like aromas (Fig. 7 and Table S2). Although raw soybean has low methionine production, NC has been reported (S. Yang et al., 2014) to facilitate the production of methionine (Table S1), which can subsequently undergo reactions to form 3-(methylthio)-1-propanol.

Fermentation also introduced floral aromas, particularly in samples fermented with yeasts KM and PK. These yeasts produced high amounts of 2-phenylethyl alcohol, which is synthesised from glucose and L-phenylalanine to through the Ehrlich and shikimate pathway (Mitri et al., 2022).

Fungi-fermented samples, such as those with AS and PN, exhibited mushroom-like and earthy odours due to the formation of 1-octen-3-ol from linoleic acid oxygenation. While 1-octen-3-ol is often considered an off-flavour in soymilk (Feng et al., 2023), it has been reported that 1-octen-3-ol is the major odour compound in high quality chicken meat (Jin et al., 2021). Additionally, Xiao et al. (2024) (Xiao et al., 2024) noted that 1-octen-3-ol enhances saltiness when added to low-concentration NaCl solutions and can improve the overall umami intensity of traditional Chinese salty meat products.

Cheesy and dairy-like aromas were also observed in fermented samples, particularly those with PQ and PR. These samples contained branched-chain carboxylic acids, such as 2-methyl-1-propanoic acid, 2-methyl-hexanoic acid and 3-methyl-1-butanoic acid, which likely resulted from reactions between free fatty acids (FFAs) and FAAs in the fermenting medium (Altenbach et al., 2010). High levels of ketones, such as 2-nonanone – a methyl ketone with fruity and blue cheese aromas – were also detected. PR is known to produce methyl ketones during blue cheese ripening through a robust lipase system that converts FFAs to keto-acids via β-oxidation and then decarboxylated to form methyl ketones (Karahadian et al., 1985). The complete consumption of linoleic acid in PR-fermented samples (Fig. 6) further underscores the lipolytic activity of PR, contributing to the conversion of PUFAs to methyl ketones.

LAB-fermented samples exhibited cheesy and fatty aromas, likely due to the enzymatic oxidation of hexanal to hexanoic acid. LP-fermented samples also contained octanoic acid, possibly produced via LP-catalysed oxidation of octanal, imparting a fatty and cheesy aroma. Acetoin, which provides a buttery and creamy aroma, was also detected in LAB-fermented samples, except those fermented with LM. Acetoin is synthesised through conversion of pyruvate by acetolactate synthase to acetolactate, followed by the decarboxylation of acetolactate to acetoin by acetolactate decarboxylase (Cesselin et al., 2021).

4. Conclusion

Fermenting soybeans with unique microbial strains holds significant promise for enhancing the aroma and taste profiles of soybean-based products, offering an innovative approach to improving their sensory appeal. The sensory attributes of fermented soybean products reflect the intricate interplay between microbial metabolism, substrate utilization, and the resulting chemical changes that impact sensory attributes. Microbial growth, sugar consumption, and organic acid production are interconnected processes that shape the fermentation environment. Fungal and yeast strains exhibit distinct sugar utilization preferences, which not only influence the sugar profile but also impact pH levels and the production of metabolic byproducts such as organic acids. Fungal fermentation typically results in higher pH due to reduced acid production, while LAB strains lower the pH through the generation of lactic and acetic acids, contributing to flavour development and preservation. The production of FAAs and peptides, driven by microbial protease activity, directly affects the taste profile, like RO. Additionally, fermentation alters the fatty acid composition, contributing to aroma development. These fatty acids, along with volatile aroma compounds, play a pivotal role in creating the sensory complexity in fermented products. Interactions between lipids, amino acids, and microbial enzymes generate unique flavour and aroma profiles. Ultimately, the balance between microbial activity, nutrient utilization, and metabolic outputs drives the sensory evolution of fermented soybean products, linking these factors to sensory indicators.

Several limitations were identified in this study. The fermentation conditions, such as temperature, oxygen levels, and substrate composition, may not fully replicate industrial or traditional settings, potentially limiting the generalisability of the findings. Furthermore, focusing on the endpoint of fermentation (72 h) without analysing intermediate stages overlooks the dynamic changes in microbial populations, metabolite production and sensory attributes that occur over time. Although strain-specific metabolite activities were characterised, interactions between strains in co-culture systems were not evaluated, which could significantly influence fermentation outcomes. The e-tongue analysis, while valuable for detecting taste differences, cannot fully capture the complexity of flavour perception due to the absence of human sensory panel evaluations. Further exploration of microbial consortia and optimisation of fermentation processes offer significant potential not only for enhancing the organoleptic properties of soybean-based foods but also for developing novel, flavourful products that increase the acceptability of soy as a sustainable protein source. Continued research in this area is crucial to fully realise the potential of microbial fermentation in addressing the global demand for nutritious and sustainable food options.

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CRediT authorship contribution statement

Xin Hui Chin: Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing, Writing – original draft. **Ryan Soh:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Geraldine Chan:** Data curation, Formal analysis, Methodology. **Pnelope Ng:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Aaron Thong:** Methodology, Writing – review & editing. **Hosam Elhalis:** Writing – review & editing. **Kanagasundaram Yoganathan:** Supervision, Conceptualization. **Yvonne Chow:** Writing – review & editing, Supervision. **Shao Quan Liu:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100933.

Data availability

No data was used for the research described in the article.

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