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Leveraging *Neurospora crassa* in Co-culture fermentations: Modulating the taste, aroma, and enhancing fatty aroma profiles in fermented soybean products

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ABSTRACT

Fermented soybean products offer nutritional benefits and complex flavour profiles shaped by microbial interactions. This study investigates the potential of *Neurospora crassa* (NC) in co-culture fermentations to enhance the sensory qualities of soybean-based products. After 72 h, significant growth was observed across all strains, with peak times varying by species. Moulds peaked at 72 h, yeasts at 48 h in monoculture but extended to 72 h in co-culture, while lactic acid bacteria (LAB) strains peaked at 48 h but showed enhanced growth when paired with NC. Sugar analysis revealed significant sucrose reductions in NC, *Rhizopus oryzae* (RO), and *Kluyveromyces marxianus* (KM) monocultures due to invertase activity, with further depletion in NC co-cultures. Free amino acid (FAA) levels were highest in mould-fermented samples, especially RONC, due to strong proteolysis. E-tongue analysis showed distinct taste profiles, with RO, RONC, and NC-*Lacticaseibacillus casei* (LC) samples exhibiting enhanced umami intensity. Fatty acid analysis revealed elevated levels in NC, RO, and RONC, linked to *de novo* synthesis. Volatile analysis indicated reduced beany notes and the emergence of 'meaty,' 'savoury,' and 'fatty' aromas. Overall, NC-based co-cultures improve flavour, umami, and nutritional quality, supporting their use in developing palatable, protein-rich, plant-based foods.

1. Introduction

Fermented foods play a crucial role in culinary traditions and have garnered considerable scientific interest due to their complex flavours and potential health benefits. Among these, fermented soybean products, such as soy sauce, miso, and tempeh, are particularly notable for their distinctive taste and aroma profiles. The flavour of these traditional fermented products arise from complex microbial interactions during fermentation (Elhalis et al., 2023). Despite their high protein content and nutritional advantages, fermented soybean products are primarily popular in Asia and less accepted in Western countries. This limited acceptance is largely attributed to undesirable 'beany' and 'green' aroma profiles (Nacchio et al., 2022), which pose challenges to their broader integration as alternative protein sources in global diets. Therefore, enhancing the sensory characteristics of these products through microbial fermentation strategies is critical to increasing consumer appeal and acceptance.

Microbial consortia in the realm of fermented foods plays pivotal roles in the development of sensory complexity. While traditional fermentations often rely on spontaneous microbial growth, the design of controlled co-culture systems presents an opportunity to fine-tune flavour and aroma outcomes. In this context, *Neurospora crassa* (NC), a filamentous ascomycete fungus, emerges as a promising fermentation agent. NC is well characterized genetically (Honda et al., 2020), easy to cultivate (Havlik et al., 2017), and capable of synthesizing flavour-relevant metabolites such as free amino acids and unsaturated fatty acids (Li et al., 2019; McKeon et al., 1997, p. 199). More importantly, NC possesses unique metabolic traits that enhance its fermentation potential. Its diverse protease system includes alkaline-active serine proteases and metalloproteases that effectively hydrolyze soybean

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proteins into free amino acids and peptides (Zheng et al., 2020). In addition, NC encodes multiple lipid desaturases, enabling conversion of saturated fatty acids into mono- and polyunsaturated fatty acids, which may serve as precursors for aroma-active aldehydes, alcohols and esters. These traits can not only improve digestibility and nutritional quality but also provide a biochemical foundation for flavour diversification (Gabrielides et al., 1982).

However, the use of NC alone may not be sufficient to achieve a broad spectrum of desired flavour modifications. Thus, the integration of NC into co-culture fermentation systems with functionally diverse microbial partners offers a rational approach to improve aroma complexity and taste modulation. The co-culture design in this study was based on the selection of complementary microorganisms, including *Rhizopus oryzae* (RO), known for robust proteolytic activity and traditional use in tempeh production; lactic acid bacteria (LAB) such as *Lacticaseibacillus casei* (LC) and *Lactiplantibacillus plantarum* (LP), which contribute acidification and production of buttery and fruity aroma compounds; and yeasts such as *Debaryomyces hansenii* (DH) and *Kluyveromyces marxianus* (KM), which are capable of synthesizing esters, alcohols, and other volatiles involved in flavour enhancement.

The rationale for this co-culturing strategy lies in the potential for synergistic enzymatic activity, cross-feeding of metabolic intermediates, and environmental modification (e.g., oxygen depletion by NC mycelia) that can collectively enable the formation of novel flavour compounds or enhance the levels of existing ones. For example, while NC can liberate fatty acids and amino acids through lipase and protease activity, LAB and yeast co-cultures can further transform these into aroma-active compounds such as esters, acetoin, and alcohols. In addition, LAB may benefit from glucose depletion by NC to activate citrate metabolism pathways that would otherwise remain dormant in monoculture fermentations. These microbial synergies are expected to not only mitigate off-flavours but also generate fatty, umami, and fruity aroma notes, leading to improved palatability.

Therefore, this study investigates the use of NC in co-culture fermentations with selected yeast and bacterial strains to enhance the sensory properties of soybean-based products. By elucidating the microbial interactions and resultant flavour transformations, we aim to provide a scientific basis for the application of co-culturing strategies in the development of flavour-optimized plant-based fermented foods.

2. Materials and methods

2.1. Microbial strain selection and culture conditions

Table 1 shows the strains used in fermentation. NC and RO are mould strains, in which NC has been used in soy fermented foods found in Indonesia and North Western China, while RO is commonly used to ferment tempeh (Bartholomai et al., 2022; Elhalis et al., 2023). The yeast strains, KM and DH, are commonly isolated and may be used in cocoa, coffee and dairy fermentation (Schwan et al., 2023), while DH is commonly used in meat and/or dairy fermentation (Laranjo et al., 2019). The LAB strains, LC and *Lactiplantibacillus plantarum* (LP) are commonly used in dairy fermentation (Yi et al., 2020).

Table 1
Strains used in fermentation.

Abbreviation	Type	Strain	Strain code/ origin
NC	Mould	*Neurospora crassa*	ATCC 46455
RO	Mould	*Rhizopus oryzae*	ATCC 20344
DH	Yeast	*Debaryomyces hansenii*	ATCC 10619
KM	Yeast	*Kluyveromyces marxianus*	MUCL 53775
LP	Lactic acid bacteria	*Lactiplantibacillus plantarum*	DSM 100813
LC	Lactic acid bacteria	*Lacticaseibacillus casei*	ATCC 334

Table 2
Umami-relevant free amino acids in fermented soybean samples after 72 h.

Sample	Concentration (mg/g wwb)				
	Glutamic acid (Glu)	Aspartic acid (Asp)	Methionine (Met)	Cysteine (Cys)	Total (Glu + Asp + Met + Cys)
Control	0.17 ± 0.04	0.07 ± 0.20	0.50 ± 0.50	0.00 ± 0.00	0.74
NC	4.41 ± 0.34	0.95 ± 0.10	0.72 ± 0.10	0.04 ± 0.00	6.12
RO	2.00 ± 0.14	0.48 ± 0.01	1.27 ± 0.06	0.09 ± 0.02	3.84
KM	0.00 ± 0.00	0.00 ± 0.00	0.89 ± 0.22	0.09 ± 0.00	0.98
DH	0.09 ± 0.11	0.03 ± 0.01	1.08 ± 0.01	0.10 ± 0.03	1.30
LP	0.09 ± 0.20	0.04 ± 0.01	0.30 ± 0.50	0.00 ± 0.00	0.43
LC	0.25 ± 0.02	0.11 ± 0.01	0.92 ± 0.67	0.50 ± 0.01	1.78
RONC	2.53 ± 0.12	0.86 ± 0.04	1.47 ± 0.03	0.13 ± 0.01	4.99
NCKM	1.97 ± 0.45	0.46 ± 0.08	0.58 ± 0.19	0.04 ± 0.01	3.05
NCDH	1.63 ± 0.47	0.49 ± 0.09	0.58 ± 0.12	0.04 ± 0.01	2.74
NCLP	3.47 ± 0.21	1.00 ± 0.07	0.67 ± 0.08	0.05 ± 0.01	5.19
NCLC	2.02 ± 0.18	0.28 ± 0.05	1.31 ± 0.03	0.06 ± 0.01	3.67

2.2. Soybean substrate preparation

Canadian non-GMO soybeans (*Glycine max,* variety S12J7*)* were purchased from Hong Guan Huat Kee (Singapore). The soybeans were soaked in water at 1:4 soybean-to-water ratio with the addition of 0.05 g/100 g w/w acetic acid (Sigma-Aldrich) and incubated at 25 °C for 24 h. After soaking, the soybeans were drained and washed thoroughly with tap water to remove residual acid. The post-soaking moisture content was determined gravimetrically to be 58.3 ± 0.4 g H_2O/100 g wet weight, which is within the optimal range for solid-state fermentation (55–60 g/100 g). Moisture was verified gravimetrically in triplicate after soaking and sterilisation. The beans were manually dehulled, achieving >95 % dehulling efficiency; hull removal was performed to minimize the influence of anti-nutritional factors and improve microbial accessibility to the cotyledon. Soybeans (500 g) were weighed into 1-L glass bottles (Nalgene, Rochester, New York, USA) and autoclaved at 121 °C for 30 min. After autoclaving, the soybeans were left to cool to 25 °C.

For the uninoculated control, soybeans underwent the same soaking, dehulling, autoclaving, and incubation procedures but were not inoculated with any microbial strains. The control was monitored to ensure that no spontaneous fermentation occurred. Residual sugars, organic acids, and volatiles in the control were measured at 0 h and 72 h to serve as reference points for comparison and are presented alongside the inoculated samples in all figures to enable direct comparison of microbial and non-microbial changes during fermentation. Detailed control data, including moisture content, sugar, and volatile profiles, are provided n Supplementary Tables S1 and S2.

2.3. Inoculation and fermentation procedure

NC was cultivated on Potato Dextrose Agar (PDA) and incubated aerobically at 25 °C for 7 days. DH and KM were grown on yeast extract peptone dextrose (YPD) Agar (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated aerobically at 30 °C for 3 days. LP was 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, NC spores were collected using 5 mL of a 10 g/100 g phosphate buffered salt (PBS) solution and passed through a cell strainer (Corning Inc., Corning, New York, USA) to separate the 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 g/100 g PBS solution (to keep inoculum preparation consistent with the mould strains) and quantified via measuring OD at 600 nm using a Varioskan® spectrophotometer

(ThermoFisher Scientific, Waltham, Massachusetts, USA). The CFU/mL of the yeast and bacteria strains were quantified using a standard curve for each individual strains.

The starting inoculum size for NC was 1.0×10^6 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 petri dishes with lids. Solid-state fermentation of the soybean samples was carried out in triplicates of 50 g at 25 °C for 72 h in a static incubator. Uninoculated soybeans were placed through the same incubation process and used as the 'control'. Daily samples of approximately 5 g were taken aseptically for microbial and chemical analyses.

2.4. Microbial enumeration

A sample (5 g) was weighed in a 50-mL Falcon tube, followed by the addition of 10 mL of a 10 g/100 g PBS solution, and vortexed for 1 min. The resuspended samples were serially diluted with 10 g/100 g PBS solution and plated. Samples fermented with NC, KM and DH were plated on Rose Bengal Agar (RBA) (Sigma-Aldrich) containing chloramphenicol (Sigma-Aldrich). Samples fermented with LP 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. The microbial counts were obtained after incubation in triplicate.

2.5. Sample preparation for chemical analysis

A sample of 45 g was frozen in liquid nitrogen for 20 s and ground in a coffee grinder (Rommelsbacher EGK 200, Rommelsbacher, Germany) for 1 min. The resulting ground soybeans were stored in a 50-mL Falcon tube at −20 °C before further analysis.

2.6. pH measurement

A sample of 0.5 g of ground soybeans was 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 samples was measured using a pH meter (LaquaAct, Horiba, Japan) in triplicate.

2.7. High-performance liquid chromatography (HPLC) for sugar and organic acid analysis

A sample of 0.5 g of ground soybeans was weighed in a 5-mL centrifuge tube, followed by the addition of 2 mL of MilliQ H_2O. The samples were vortexed for 1 min and centrifuged at 3405×*g* for 10 min. 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, 300 × 7.8 mm. The fermentation metabolites were eluted isocratically with a mobile phase of 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min. The RID and column temperatures were set at 35 °C, and the injection volume was 20 μL. Chromatic integration was analysed with Agilent Open Lab ChemStation (Agilent Technologies) and exported to Microsoft Office Professional Plus 2016, Microsoft Excel for data analysis. Analyte concentrations of sugars, organic acids and ethanol were quantified using external standards with $R^2 \geq 0.98$.

2.8. Amino acid analysis

Free amino acids were analysed with the ARACUS Amino Acid Analyser (MembraPure, Berlin, Germany). A 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 HulaMixer™ (ThermoFisher Scientific) for 30 min and centrifuged at 0 °C, 3405×*g* for 10 min. An aliquot of 800 μL of supernatant was added to 200 μL of 40 g/100 g trichloroacetic acid (TCA) in a 1-mL microcentrifuge tube and vortexed thoroughly for 10 min. The samples were then centrifuged at 4 °C at 25,830×*g* for 5 min. An aliquot of 100 μL of supernatant with 600 μL of 0.1 N HCl in the HPLC vial and the samples was used for analysis. 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.

2.9. Fatty acid methyl ester (FAME) analysis via GC-FID

The fatty acid content in the fermented samples were extracted using the method adapted from GB 5009.168–2016 (J. 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.

2.10. Electronic tongue (E-tongue) taste profiling

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 30 min. After mixing, the samples were centrifuged at 3405×*g* for 30 min and the supernatant was collected for analysis. All samples were characterized at room temperature. The sensors and reference electrode were rinsed for 10 s with water between different samples. Each sample was measured for 120 s and the measurements were repeated 9 times due to system stability requirements. The signal value at the 120th s was extracted from the last 6 measurements and averaged to obtain the sensor signal raw data.

2.11. Analysis of volatile compounds for profiling using HS-SPME and GC-MS

Headsapce solid-phase microextraction (HS-SPME) is an analytical technique used for the extraction of volatile compounds from the headspace without the use of solvents. A 23 gauge 50/30 μm fibre coated with DVB/CAR/PDMS (Supelco, USA) was used for the extraction. Each sample was weighed 500 mg into a 20 ml headspace vial. 100 μl of 10 ppm 2,3-dimethoxytoluene was added as an internal standard and capped with a magnetic screw-top cap.

Gas chromatography mass spectrometry was used with Agilent 7890 B Gas Chromatography (GC) coupled with an Agilent 5977 B Mass Spectrometry Detector (MSD) (Agilent Technologies Inc., Santa Clara, CA, USA), together with a Gerstel Multipurpose Sampler (MPS) (Gerstel GmbH & Co.KG, Germany). With this system, the autosampler incubated the vial at 80 °C for 10 min followed by exposure of the fibre in the headspace for 30 min at 250 rpm. Subsequently, the fibre was desorbed at the GC inlet for 600 s at 250 °C. The column equipped was a polar DB-WAX UI (ultra inert) capillary column (30 m × 0.25 mm internal

diameter x 0.25 μm film thickness). Split ratio was set at 1:100 and the analysis were carried out with triplicates. Oven temperature was initially set at 35 °C, followed by 60 °C at 100 °C/min, next is 190 °C at 8 °C/min, and finally at 250 °C at 20 °C/min and held for 6.5 min. Helium was the carrier gas used and was set at a constant flow of 1.0 ml/min. The MS was operated in electron ionisation mode at 70eV, with a scan range of 35–450 m/z in full scan mode and an acquisition rate of 1.8scans/s. The source and transfer line temperatures were set at 230 °C and 150 °C respectively.

Alkanes C7 to C30 standards (Supelco) was also injected to calculate retention indices (RI) for sample profiling and compound identification. The compounds that were identified were compared based on mass spectra matches, RI with those available in NIST17 database and an internal library. Peaks were integrated using Agilent's Mass Hunter Unknowns analysis 10.0 with signal-to-noise threshold of 3. Compounds with areas of less than 0.05 % were filtered.

2.12. Calculation of odour activity values (OAVs)

Odour activity values (OAVs) were calculated to estimate the relative contribution of individual volatile compounds to the overall aroma profile. Compound concentrations μg/g wet weight) were obtained from GC-MS analysis as described above. Reported odour thresholds for each compound were obtained from the literature and are expressed in the same units (μg/g) (Van Gemert, 2011). The OAV of each volatile was determined using the following equation:

$$\mathrm{OAV} = \frac{T_i}{C_i}$$

where C_i is the measured concentration of compound I (μg/g) and T_i is the published odour threshold of the same compound (μg/g). Compounds with OAV >1 were considered aroma-active and likely to influence the sensory characteristics of the fermented samples, while compounds with OAV <1 were considered to have negligible individual impact. All calculations were performed in Microsoft Excel 365 (Microsoft Corporation, 2018), and the results were presented for further interpretation.

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).

3. Results and discussion

3.1. Changes in microbial population during fermentation

Fig. 1 illustrates the growth dynamics of microbial strains, with statistically significant differences observed between strains at each timepoint ($p < 0.05$). Fig. 1A shows monoculture fermentations, where all strains exhibited substantial proliferation by the end of the fermentation period. Fungal strains NC and RO reached peak counts at 72 h in both monoculture and co-culture conditions, consistent with prior observations that filamentous fungi exhibited slower, sustained biomass accumulation due to hyphal development. Yeast strains, in contrast, achieved maximal growth at 48 h in monoculture, but extended to 72 h in the co-culture with NC (Fig. 1B). This suggests early-stage metabolite competition, followed by metabolic adaptation and cross-feeding interactions, a phenomenon also reported in mixed yeast-fungal systems. LAB strains LP and LC consistently peaked at 48 h in monocultures (Fig. 1A) but demonstrated moderately enhanced growth in the co-cultures with NC (Fig. 1B), suggesting a potentially synergistic relationship (Todorov et al., 2024).

3.2. Substrate utilization and metabolite production

Fig. 2 presents sugar utilization profiles after 72 h. Fig. 2A depicts sugar utilization: NC, RO, and KM significantly depleted sucrose, likely due to active invertase secretion, which cleaves glucose and fructose (Sainz-Polo et al., 2013). Other monocultures retained higher residual sucrose, indicating limited or absent invertase activity. Interestingly, co-cultures with NC consistently showed more complete sucrose depletion, underscoring synergistic carbohydrate metabolism (possibly due to the consumption of glucose and fructose that would have reduced invertase repression and boosted sucrose hydrolysis). These findings align with prior work on mixed fungal-yeast fermentations, where enzymes from co-culture fermentation accelerates sugar breakdown and metabolite flux.

Fig. 2B shows acids, ethanol, and pH profiles. Citrate depletion was pronounced in all co-cultures and in RO monoculture, but minimal in NC or LAB monocultures. This is consistent with RO's known citrate lyase activity, which cleaves citrate into oxaloacetate and acetate thereby supplying essential precursors for fatty acid biosynthesis (e.g., acetyl-CoA from acetate feeds into malonyl-CoA formation and subsequent elongation steps (Chypre et al., 2012). Such citrate catabolism not only sustains central carbon metabolism but also provides metabolic flexibility under carbohydrate-limited conditions. In parallel, NC shows a metabolic preference for isocitrate lyase pathways, channelling carbon

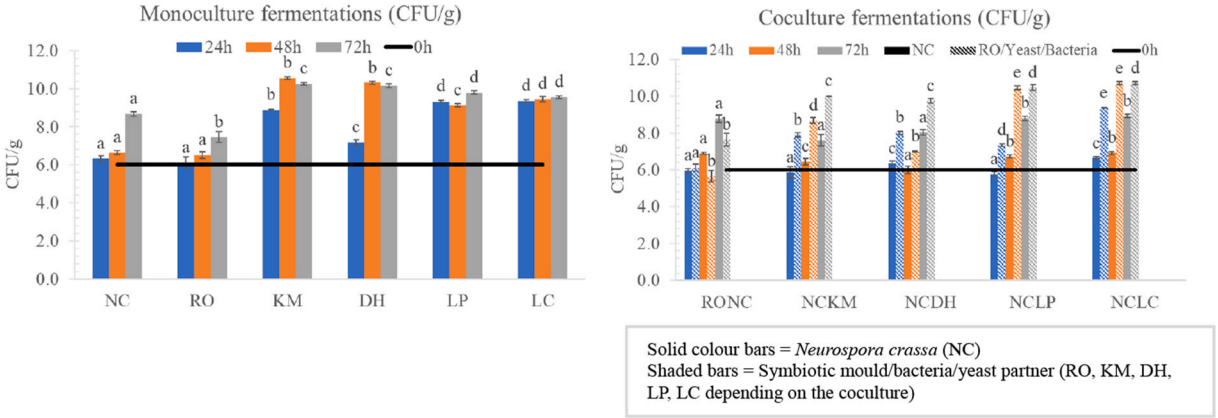

Fig. 1. (A) Microbial populations of monoculture fermentations across 72 h. The black line represents the initial inoculum concentration. (B) Microbial populations of co-culture fermentations across 72 h. The black line represents the initial inoculum concentration. The solid colour bars represent the population of NC while the shaded bars represent the population of the co-culture yeast or bacterial strain. Different letters above the bars indicate statistically significant differences among samples (ANOVA with Tukey HSD, $p < 0.05$).

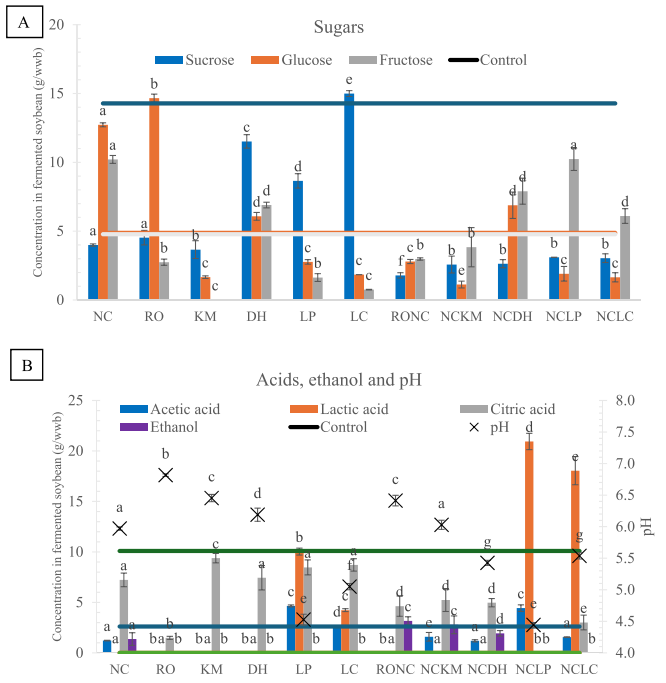

Fig. 2. (A) Substrate utilization and (B) end-product metabolite profiles for fermented soybean after 72 h of fermentation. The control was uninoculated soybean. The straight lines represent the levels in the uninoculated soybean, and the bars represent the levels in the fermented samples. Different letters above the bars indicate statistically significant differences among samples (ANOVA with Tukey HSD, $p < 0.05$).

through the glyoxylate cycle to support growth and metabolite production (Rogers & McFadden, 1977). Although some LAB strains are capable of citrate metabolism leading to diacetyl and acetoin production (Hugenholtz et al., 2000), this pathway was not active in monoculture fermentations in our study. In the co-cultures, however, complete citrate consumption (particularly in NCLP) may suggest activation of citrate lyase under conditions of glucose depletion and reduced oxygen availability, as reported by (Antranikian & Giffhorn, 1987; Laëtitia et al., 2014). While no direct measurements of dissolved oxygen or redox potential were performed in this study, we hypothesize that NC's glucose utilization and the formation of a mycelial blanket could have contributed to localized oxygen limitation, thereby favouring this metabolic pathway.

Lactic acid accumulation was observed in all LAB-fermented samples (Fig. 2B), with concentrations nearly doubling in NCLP and NCLC co-cultures. This enhancement is likely due to the increased LAB growth observed in co-culture (Fig. 1B), consistent with enhanced lactic acid production in fungal-bacterial systems reported by (Liang et al., 2025). Despite higher lactic acid concentrations, pH values did not differ significantly from monocultures, which may be due to concurrent buffering via ammonia release from proteolysis, a process observed in high-protein fermentations (Owusu-Kwarteng et al., 2022), or due to simultaneous reduction of citrate, which also contributes to acidity.

Ethanol was detected in several samples, particularly in RONC and NC-yeast co-cultures (Fig. 2B). NC's ability to produce ethanol from cellulose-derived sugars has been documented (Waters et al., 2017), suggesting that its enzymatic breakdown of plant polysaccharides supports downstream fermentation. Although KM is a known ethanol producer from glucose (Bilal et al., 2022), ethanol was absent in its monoculture, likely due to its redirection into secondary metabolites such as 2-phenylethyl alcohol, a known aroma compound in yeast fermentation (Kim et al., 2014). This underscores the dynamic shifts in metabolic priorities when microorganisms are grown alone versus in consortia.

3.3. Free amino acid and e-tongue profiles

Fig. 3 presents the free amino acid (FAA) profiles of the soybean samples after 72 h of fermentation. Among the monocultures, mould-fermented samples, particularly those inoculated with NC and RO, exhibited significantly higher FAA concentrations than the uninoculated control. This outcome aligns with previous studies reporting high extracellular protease activities in filamentous fungi, which degrade complex plant proteins into peptides and soluble amino acids (Kumar et al., 2005; Li et al., 2019). The RONC co-culture showed the highest FAA levels, significantly exceeding both NC and RO monocultures, suggesting synergistic proteolysis between NC and RO. This effect is likely driven by complementary enzyme profiles, with NC secreting alkaline-active serine proteases (Zheng et al., 2020) and RO producing a broad spectrum of proteases. Given that RONC samples maintained a mildly alkaline pH (Fig. 2), conditions were favourable for enhanced protein hydrolysis, an effect previously observed in other solid-state co-culture systems (Olagunju et al., 2025).

NCLP also demonstrated a stronger umami intensity, comparable to RONC and significantly higher than NC and RO. FAA analysis (Table 2) showed that NCLP had the highest aspartic acid content, significantly higher than RO, LC, and all yeast samples, further supporting its role in driving umami perception. However, in PCA (Fig. 4), NCLP clustered further from RO and RONC, likely due to its higher lactic acid production and associated sourness, which distinguished its taste profile despite its strong umami contribution. The pronounced umami observed in NCLP is further supported by the known proteolytic capacity of LP, which has been shown to enhance umami and nutritional quality in a range of plant-based fermentations. For instance, solid-state fermentation of soybean protein meal with *L. plantarum* Lp6 increased protein content to 60.15 % and significantly improved FAA levels and protein solubility (Amadou et al., 2011). Similarly, LP enhanced umami-active compounds in shiitake mushrooms (Chen et al., 2021) and modulated aroma and taste in chestnut–soybean milk blends (Sun et al., 2025). These findings support the synergistic role of LP in co-culture with NC, where NC likely contributes peptides through its strong proteolytic activity, and LP further hydrolyses these peptides into free amino acids and converts citrate into organic acids. The release of glutamic and aspartic acids, combined with acidification-driven flavour modulation, intensifies umami perception in NCLP fermentations. NCLC < while showing a lower umami intensity (0.41 g MSG/100 mL) compared to RONC and NCLP, clustered alongside RO and RONC in the PCA analysis. This suggests that synergy between NC and LC may arise from complementary proteolytic activities, where NC generates peptides from soybean proteins and LC further degrades these peptides into free amino acids and organic acids that contribute to flavour development. Such cooperative mechanisms between filamentous fungi and LAB have also been reported in fruit and vegetable fermentations, where LC produced stronger umami notes than other LAB strains due to its enhanced proteolytic and peptidase activity (Cui et al., 2019).

(Fig. 6) provides a detailed visual representation of the e-tongue sensor responses across seven taste dimensions: astringency (AHS), pungency (PKS), saltiness (CTS), umami (NMS), sourness (CPS), bitterness (ANS), and aftertaste bitterness (SCS). The most prominent differentiators were observed in the umami (NMS) and aftertaste bitterness (SCS) dimensions. Co-cultures such as RONC and NCLC displayed broader and significantly higher responses across these axes compared to the control and yeast monocultures, indicating intensified umami and lingering savoury (=umami) taste. By contrast, monoculture treatments were clustered more closely with the control, reflecting significantly lower taste complexity. These results were consistent with the quantitative umami intensity estimations (Fig. 5), where NCLC, RONC, and RO showed the highest levels. Taken together, these findings demonstrate that increased amino acid release through proteolysis, coupled with the generation of lipid-derived volatiles such as 2,4-decadienal and hexanal, acted synergistically to produce a significantly more complex and

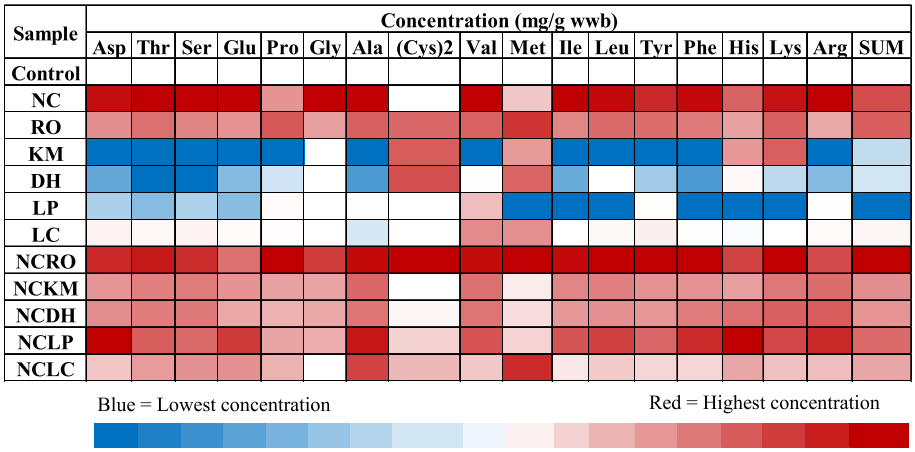

Fig. 3. Heatmap showing the relative abundance of various amino acids across different fermented soybean samples. The columns represent the samples, while the rows indicate individual amino acids. The colour intensity reflects concentration levels, with red hues indicating higher concentrations, blue hues indicating lower concentrations, and white hues representing the concentrations found in the uninoculated (control) sample. Red hues show an increase in amino acid concentration compared to the control, while blue hues indicate a decrease. The e-tongue profiling and PCA (Fig. 4) supported these findings, with RO, RONC, and NCLC clustering separately from the control and yeast monocultures. PC1 and PC2 explained 61.5 % and 23.85 % of the variance, respectively, accounting for 85.35 % overall, indicating that most variations in taste profiles were captured within the first two components. NC and NCKM formed a separate cluster, likely influenced by proteolysis and alcohol/ester production by KM (Besançon et al., 2024), whereas LAB monocultures (LP, LC) and the NCLP co-culture grouped together, consistent with their high lactic acid content and resulting sourness. The uninoculated control grouped closely with NCDH, suggesting minimal deviation from baseline. A breakdown of umami-relevant FAAs revealed that RONC contained significantly more glutamic acid than the uninoculated control, together with elevated methionine and cysteine, providing sulfur-containing precursors for aroma-active compounds. These results indicate that RONC not only increased the FAA pool but also enriched key umami contributors such as Glu and Asp, which directly stimulate the T1R1/T1R3 receptor complex and enhance umami perception (Amin et al., 2020).

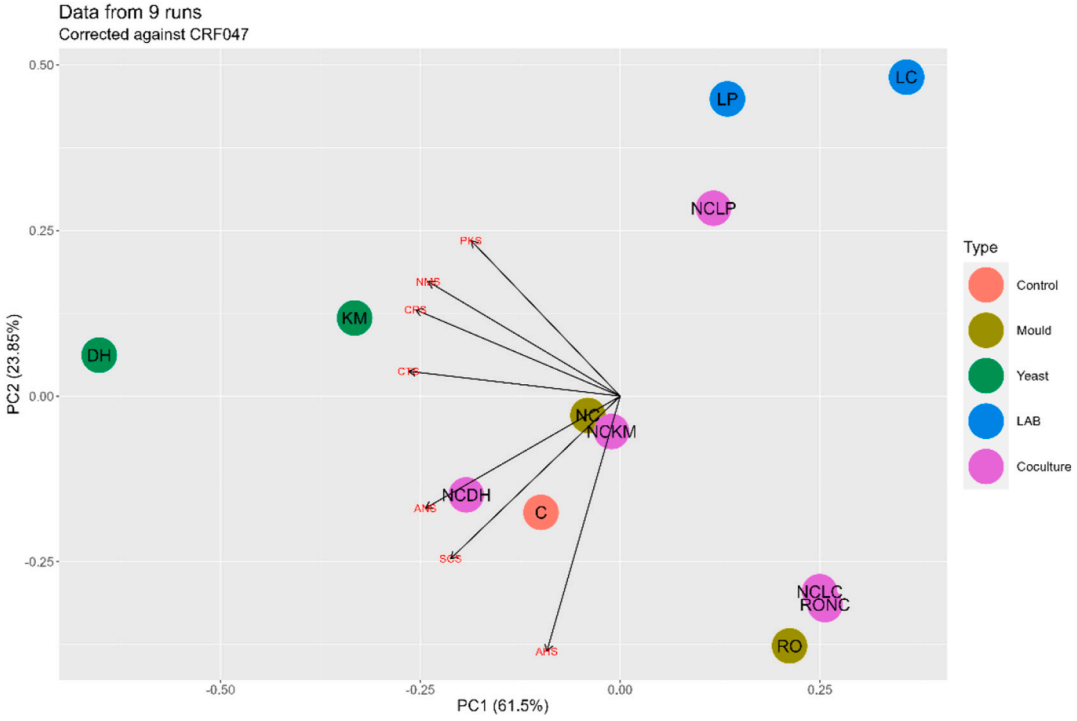

Fig. 4. PCA of e-tongue profile of fermented soybeans after 72 h of fermentation. The control was uninoculated soybean.

distinctly "meaty" flavour profile in RONC, NCLP, and NCLC. This integrated modulation of taste- and aroma-active metabolites underscores the importance of tailored microbial combinations and highlights the functional complementarity between fungal and bacterial strains in shaping the sensory quality of fermented soybeans.

3.4. Fatty acid profile

Fig. 7 illustrates the fatty acid profiles of soybean samples after 72 h of fermentation. Clear and significant differences were observed among treatments. NC and RO monocultures exhibited the highest total fatty acid contents, significantly greater than the uninoculated control ($p < 0.05$), with NC enriching particularly C18:2c and C18:3ω3. RONC also showed a pronounced increase in fatty acids, significantly higher than

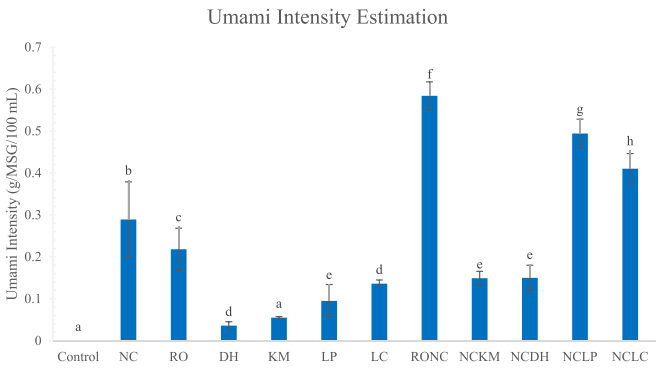

Fig. 5. Umami intensities of fermented soybeans after 72 h of fermentation. The control was uninoculated soybean. Different letters above the bars indicate statistically significant differences among samples (ANOVA with Tukey HSD, $p < 0.05$).

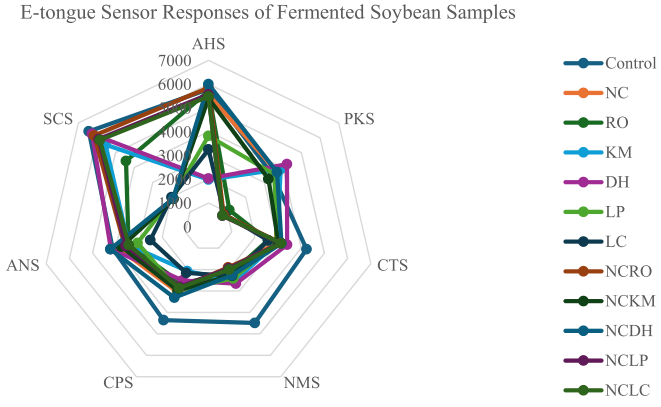

Fig. 6. Electronic tongue radar plot showing sensor response patterns across seven taste attributes for all fermented and control soybean samples. Taste dimensions include astringency (AHS), pungency (PKS), saltiness (CTS), umami (NMS), sourness (CPS), bitterness (ANS), and aftertaste bitterness (SCS).

most co-cultures but lower than NC and RO. This increase suggests that *de novo* fatty acid synthesis may contribute, in addition to the hydrolysis of intrinsic soybean lipids mediated by fungal lipases. Filamentous fungi such as NC and RO are known to possess a robust *de novo* fatty acid synthesis pathway (Cocetta et al., 2020), which involves the carboxylation of acetyl-CoA to malonyl-CoA via acetyl-CoA carboxylase, followed by iterative elongation steps catalyzed by fatty acid synthase, leading to the production of long-chain saturated fatty acids. By contrast, yeast and LAB monocultures contained significantly lower fatty acid levels, not significantly differing from the control. Co-cultures such as NCLP and NCLC showed intermediate levels, significantly higher than KM and DH but lower than NC and RO, indicating that co-culturing with LAB enhanced but did not fully replicate the strong fatty acid enrichment observed in mould monocultures.

Beyond the total fatty acid content, fungal fermentations promoted marked increases in unsaturated fatty acids, especially oleic acid (C18:1c) and linoleic acid (C18:2c), and α-linolenic acid (C18:3ω3). NC, RO, and RONC were significantly enriched in C18:2c relative to control, yeast, and LAB monocultures, while NCLP and NCLC also exhibited significantly higher levels of C18:3ω3 compared to DH and KM. This trend corroborates previous findings that oleaginous fungi produce key desaturase enzymes that introduce double bonds at specific portions along fatty acid chains (Zhang et al., 2022). For example, Δ9-desaturase (Ole1) mediates the conversion of stearic acid (C18:0) to oleic acid (C18:1), while Δ12-desaturase further modifies oleic acid (C18:1) to yield linoleic acid (C18:2). The higher levels of these unsaturated fatty acids observed in the NC and RO treatments may therefore reflect a combined effect of enhanced desaturase activity and selective lipase-mediated release under the fermentation conditions employed.

The enrichment of unsaturated fatty acids is also of sensory significance. These molecules are known precursors of volatile aroma compounds, particularly lipid-derived aldehydes and acids formed through oxidative degradation. Compounds such as (E,E)-2,4-decadienal and 2-methylbutanoic acid, which contribute fatty, meaty, and umami-associated notes, are commonly derived from linoleic and α-linolenic acid oxidation (Dinh et al., 2021). Similar patterns have been documented in fungal fermentation of other plant matrices, where increases in unsaturated fatty acids correlate with improved aroma complexity and savoury character. Thus, the observed lipid transformations in NC-, RO-, and RONC-fermented soybeans not only reflect enhanced fungal metabolic activity but also contribute to the improved aroma profiles discussed in Section 3.5.

3.5. *Volatiles profile*

The PCA of volatile compounds revealed clear differentiation among fermented and unfermented soybean samples (Fig. 8), suggesting substantial microbial modulation of the aroma profile. The unfermented control exhibited high levels of beany and grassy compounds such as hexanal and 2-pentylfuran, both associated with undesirable green notes (Tao et al., 2022). However, these compounds exhibited low odour activity values (OAV <1) across all samples, indicating limited sensory relevance despite their frequent citation as markers of raw soybean aroma.

In contrast, several microbially derived compounds exhibited OAV >1, confirming their potential contribution to the aroma of fermented samples. For example, 3-(methylthio)-1-propanol, detected exclusively in NC monoculture, displayed an OAV >2 and is known to impart meaty and onion-like notes (Deed et al., 2019). Its formation is consistent with methionine catabolism, and previous studies have shown that NC expresses methionine synthase, supporting its *de novo* synthesis (Yang et al., 2014). Interestingly, this compound was absent in co-cultures, possibly due to microbial competition or consumption.

Co-culture samples involving NC also exhibited significantly elevated levels of ethyl esters, notably ethyl oleate and linoleic acid ethyl ester, which impart sweet, fatty, and fruity notes (Cui et al., 2023; Martínez-Ruiz et al., 2018). While previous studies have reported that NC alcohol dehydrogenases (ADH1 and ADH3) show limited carboxylate ester-forming activity (Park et al., 2007), ester biosynthesis in fungi is more commonly attributed to alcohol acyltransferases. These findings suggest that NC may possess multiple enzymatic routes capable of contributing to ester formation. This interpretation is further supported by reports of ester production in mixed fungal-yeast systems, where alcohol dehydrogenase, esterase, or acyltransferase activity contributed to the observed metabolite profiles (Wang et al., 2022).

Among all detected volatiles, 2,4-decadienal exhibited the highest OAV and was most abundant in RONC samples. Known for its strong chicken-like, fatty, and fried aroma (Cui et al., 2023; Sun et al., 2020), this compound arises from linoleic acid oxidation, likely facilitated by fungal lipoxygenases.

NCKM samples showed elevated levels of 2-phenylethanol (OAV >1), a compound contributing sweet and floral notes. This is consistent with the Ehrlich pathway active in KM (Kim et al., 2014, p. 201), which metabolizes phenylalanine. Enhanced proteolysis by NC likely increased phenylalanine availability, promoting this aroma pathway. Additionally, aroma-active compounds included 2-methyl-propanoic acid and 2-methyl-hexanoic acid (OAV >1), detected in KM co-cultures and associated with cheesy and savoury notes (Arellano-Plaza et al., 2017). These branched-chain acids derived from valine and isoleucine metabolism, respectively, through aminotransferase and α-keto acid dehydrogenase activity, previously reported in yeast and LAB (Chen et al., 2024).

In LAB co-cultures (NCLP), acetoin (OAV >1), a buttery compound,

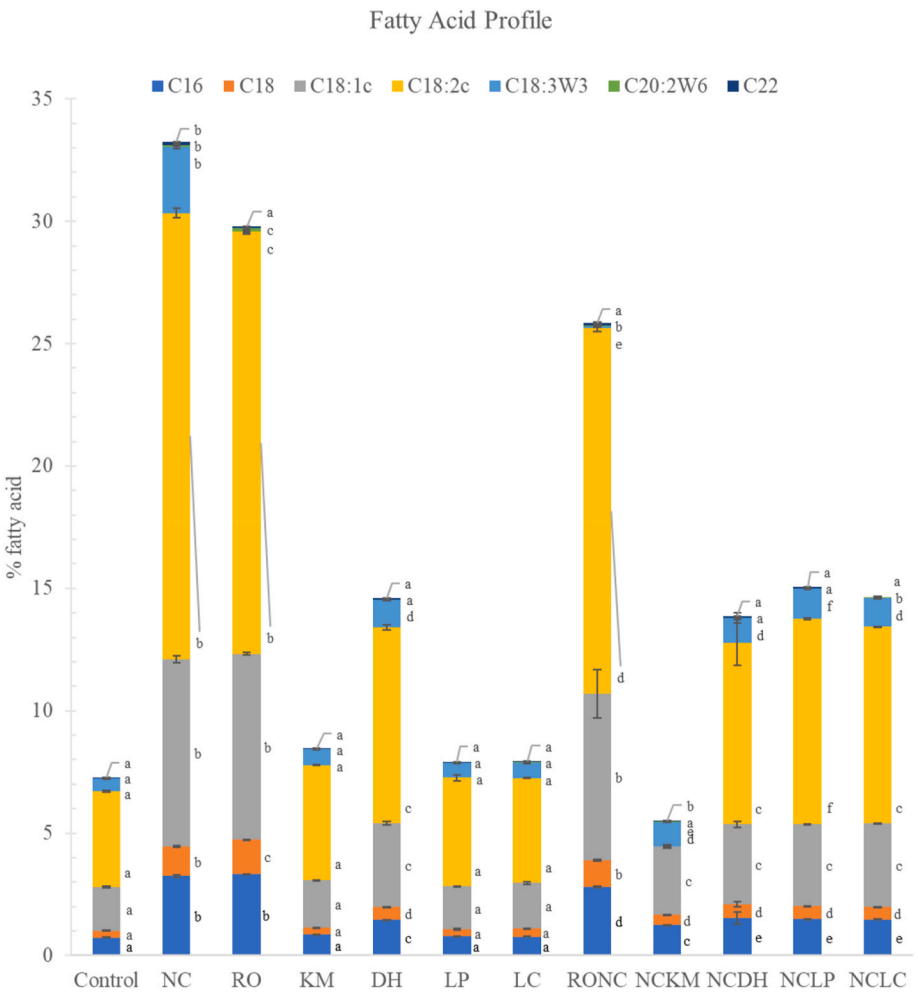

Fig. 7. Fatty acid profile analysis of fermented soybean samples after 72 h of fermentation. The control was uninoculated soybean. Different letters above the bars indicate statistically significant differences among samples (ANOVA with Tukey HSD, $p < 0.05$).

was observed. LP is known to produce acetolactate decarboxylase (Tsau et al., 1992), but the absence of acetoin in LP monoculture and its presence in co-culture suggests that NC may create microaerophilic conditions via its mycelium network, favouring acetoin accumulation (Cheynier et al., 2010). Additionally, citric acid depletion (Fig. 2) supports citrate-derived acetoin formation via pyruvate (Shimazu et al., 1985). Conversely, NCLC samples showed elevated ethyl acetate (OAV >1), a compound known to impart fruity notes. Its formation likely results from NC-derived esterase activity acting on ethanol and acetic acid, although further enzymatic studies are needed to confirm this reaction.

Although this study demonstrates the potential of non-traditional co-cultures to enhance soybean flavour, several limitations remain. Industrial feasibility was not evaluated, and scaling up co-culture fermentations presents significant challenges. Maintaining stable strain ratios is difficult under industrial conditions where oxygen transfer, pH, and temperature gradients can shift microbial dynamics. Co-cultures also carry a greater risk of contamination than monocultures, requiring stricter inoculation control and monitoring technologies to ensure reproducibility. Sensory assessment in this work relied solely on instrumental tools. While the e-tongue and OAV analysis provide quantitative insights into taste and aroma, they cannot capture the complexity of human perception, particularly synergistic/interactive effects between volatile and non-volatile compounds. Human sensory validation, following standardized protocols such as ISO 8586, will therefore be necessary to evaluate attributes such as umami intensity, reduction of beany notes, and overall acceptability. Nutritional fortification outcomes were also only partially addressed. Although increases in free amino acids were observed, essential amino acids such as lysine were not quantified, nor was the degradation of anti-nutritional factors (e.g., trypsin inhibitors, phytic acid) examined. Mechanistic interpretation of elevated fatty acid levels also remains limited, as (isotopic) tracer experiments (e.g., ^{13}C-acetyl-CoA labelling) that could distinguish between *de novo* synthesis and hydrolytic release were beyond the scope of this study. These factors are critical for evaluating the broader nutritional and biochemical impacts of co-culture fermentation. Future studies should therefore incorporate pilot-scale trials, sensory validation, isotopic tracer-based metabolite tracking, and comprehensive nutritional profiling to support industrial translation.

4. Conclusion

This study demonstrates that co-culture fermentations involving *Neurospora crassa* represent a promising strategy to improve the sensory quality of soybean-based products. Compared to monocultures, co-cultures consistently reduced undesirable “beany” off-flavours while generating aroma-active compounds that enriched savoury, fatty, and fruity sensory attributes. Among the combinations tested, *Rhizopus oryzae-Neurospora crassa* and *Neurospora crassa-Lactiplantibacillus plantarum* emerged as particularly promising, highlighting the importance of pairing functionally complementary strains. RONC exhibited an approximately 25–30 % increase in total umami-relevant FAAs compared to NC or RO monocultures, including more than a 20-fold

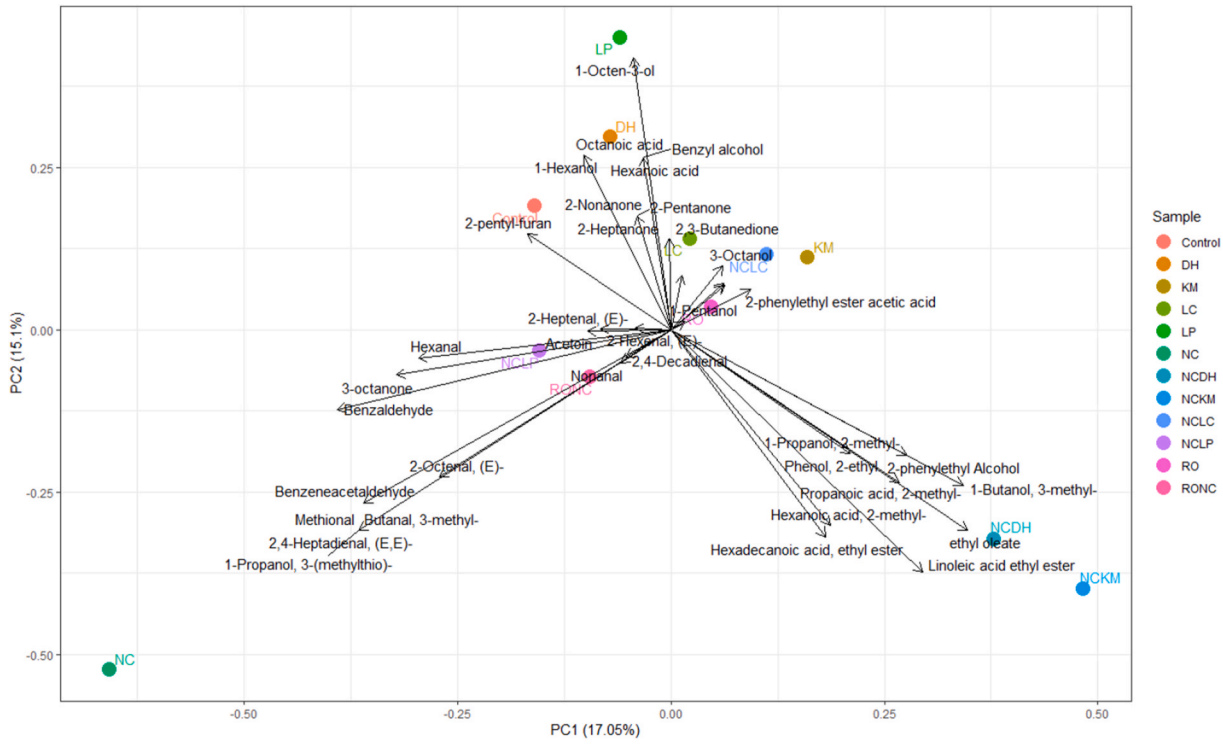

Fig. 8. PCA analysis and aroma compounds comparison of fermented samples after 72 h of fermentation. The control used is uninoculated soybean.

increase in glutamic acid relative to the control. This translated into the highest umami intensity, accompanied by elevated levels of 2,4-decadienal and ethyl esters that imparted savoury, fatty, and sweet notes. NCLP also showed strong synergistic potential, producing the highest aspartic acid levels and significantly higher umami intensity. Its volatile profile, marked by acetoin and ethyl acetate, contributed buttery and fruity notes, while lactic acid production enhanced balance by masking off-flavours. Although NCLP clustered separately from RONC due to its higher acidity, it nonetheless provided a distinct sensory outcome characterized by freshness and flavour complexity. Taken together, these findings highlight that *Neurospora crassa*-based co-cultures not only outperform single-strain fermentations in terms of flavour enhancement but also diversify the sensory profile through complementary metabolic activities. By combining proteolysis, amino acid enrichment, and volatile production, these co-cultures offer a robust platform for flavour optimization in plant-based fermentation. This work provides a scientific basis for developing more palatable and nutritionally enriched soybean-derived ingredients, supporting the broader acceptance of fermented plant proteins in global diets.

CRediT authorship contribution statement

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

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.118651.

Data availability

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

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