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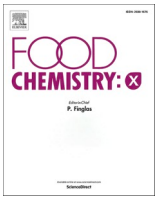

Metabolomics analysis of okara probiotic beverages fermented with *Lactobacillus gasseri* and *Limosilactobacillus fermentum* by LC-QTOF-MS/MS

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ABSTRACT

In this study, okara was fermented with probiotic strains *Lactobacillus gasseri* LAC 343 and *Limosilactobacillus fermentum* PCC, respectively. Significant increases in cell count (by 2.22 log CFU/mL for LAC and 0.82 log CFU/mL for PCC) and significant decreases in pH (by 1.31 for LAC and 1.03 for PCC) were found in fermented okara slurry. In addition, strain LAC tended to produce amino acids, while strain PCC depleted most amino acids. An untargeted metabolomic-based approach using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry was used to further understand the compositional changes and potential health benefits by identifying bioactive metabolites in fermented okara slurry. We successfully identified various beneficial bioactive compounds including γ-aminobutyric acid, indolelactic acid, D-phenyllactic acid, and *p*-hydroxyphenyllactic acid which had differences in fold-changes in okara slurry fermented with different strains. Our study indicated the feasibility of using probiotics to ferment okara for novel functional food development.

Introduction

Okara, or soybean residue, is a food side-stream from soy milk or tofu production. The major composition of okara is water which makes up 70–80 % (w/w) with a sawdust-like texture (Vong & Liu, 2016). While okara retains many nutrients including dietary fiber, protein, fat and isoflavones, these nutrients are largely untapped for human consumption due to poor okara palatability. On a dry basis, the bulk of it is made up of insoluble dietary fiber which is 40–50 % (w/w) of the content. Protein makes up 15–33 % (w/w) and 8–11 % (w/w) of okara is fat. A considerable amount of isoflavones is also present in okara, with glycosides (28.9 % w/w of total isoflavones dry weight) and aglycones (15.4 % w/w of total isoflavones dry weight) being the major ones (Vong et al., 2018; Vong & Liu, 2016; 2019).

The annual production of okara is around 10 million tons globally (Vong et al., 2018). However, okara is not well utilised due to its perishable nature and unpleasant organoleptic features. Several efforts have been made to address these problems. There are some existing drying methods to preserve okara (Ostermann-Porcel et al., 2017), and dried okara flour can be incorporated into food formulations as an ingredient. Physical processing methods like milling or chemical processing methods like treatment by acid or alkaline have also been applied to improve the physicochemical property of okara (Feng et al., 2021).

With consumers' developing awareness of environmental protection and physical well-being, the concept of functional foods is gaining more attention. Therefore, bio-valorisation of okara by probiotic fermentation to produce a functional food is another notable potential solution. It can be a highly desirable method since it not only gives access to the untapped nutrients in okara, but also helps alleviate the socio-eco-environmental problems caused by okara disposal.

Probiotics are live microorganisms that provide health benefits to the host when administered in an appropriate amount (Hill et al., 2014). The most commonly used strains of probiotics come from *Lactobacillus* and its recently derived novel genera (Zheng et al., 2020). Previously, Vong and Liu (2019) used *Lactobacillus* (now *Lacticaseibacillus*) *paracasei* to ferment okara and bio-transformed it into a functional probiotic drink. The final product contained significantly lower amounts of dietary fiber, higher amounts of free amino acids and isoflavone aglycones with a natural fruity flavor.

Lactobacillus gasseri LAC 343 and *Limosilactobacillus fermentum* PCC are two probiotic strains of interest to be used to ferment okara in the

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present study. *L. gasseri* strains have been reported to produce fructosyltransferases, which can convert sucrose into prebiotic fructans (Xu et al., 2020), while the *L. fermentum* strains have attracted attention due to their excellent ability to produce amylase, feruloyl esterase and phytase, which help these strains to utilize the abundant nutrients in the substrates (Jo et al., 2021). Previous studies have shown the feasibility of fermenting plant-based products using these two strains and found an enhancement in nutritional profile of the final product and potential health benefits towards human body (Jo et al., 2021; Xu et al., 2020). In addition, both *L. gasseri* and *L. fermentum* are reported to be able to regulate the immune system (Chamberlain, Hatch, & Garrett, 2019; Naghmouchi et al., 2020). Besides probiotic strains themselves, the functionality of the fermented product can also come from postbiotics. Postbiotics are the inanimate microorganisms including bioactive metabolites secreted by live probiotics and/or non-probiotics, or released after bacterial lysis, such as enzymes, peptides, polysaccharides, and organic acids (Salminen et al., 2021). These molecules may have anti-inflammatory, antioxidant, anti-obesogenic, antihypertensive, immunomodulatory activities which promote host well-being (Aguilar-Toalá et al., 2018; Chan et al., 2021). For example, a cell lysate suspension derived from *L. fermentum* BGHV110 showed hepatoprotective effects (Dinić et al., 2017). Lipoteichoic acid produced by *L. plantarum* exhibited immunomodulation effect (Kim et al., 2011). Cell free supernatants produced by *L. rhamnosus* GG showed potential anti-inflammatory effects (Cicenia et al., 2014).

Liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS/MS) has been applied in metabolomic studies of fermented okara to profile metabolites and analyse postbiotics. Gupta and Chen (2021) found that isoflavones abundancy changed from glucoside forms to their respective aglycone forms after fermenting with *Rhizopus oligosporus*. Chan et al. (2019) found a large reduction of enzymatic activity of α-glucosidase in okara fermented with *Eurotium cristatum* and concluded its potential anti-diabetic characteristic. Unlike targeted analysis that can only detect a limited number of features, untargeted metabolomics can provide a more comprehensive overview of most of the metabolites within a matrix without prior hypothesis.

Therefore, an untargeted metabolomics method (LC-QTOF-MS/MS) was utilised in this study to identify potential bioactive compounds, elaborate metabolites production after fermentation, explore potential postbiotics and gain more understanding about the potential therapeutical functions of probiotic-fermented okara.

Materials and Methods

Materials and probiotic strains

Fresh okara was obtained from Super Bean International Pte Ltd (Singapore) and kept at −20 °C before use. Lactic acid bacteria (LAB) *L. gasseri* LAC 343 (Morinaga, Tokyo, Japan) and *L. fermentum* PCC (Christian Hansen A/S, Hørsholm, Denmark) were obtained as lyophilised powder and stored at −80 °C before use (Vong & Liu, 2019).

Okara slurry preparation and enzymatic hydrolysis

The okara slurries were prepared according to the method of Vong et al. (2017) with slight modifications. After okara was thawed, its moisture content was measured with a moisture analyzer (MOC-120H Shimadzu, Kyoto, Japan). A total of 5 L of okara slurry (5 % w/v dry weight) was prepared with mineral water (Ice Mountain, Malaysia). The pH of the okara slurry was adjusted by adding lactic acid (90 %, w/w Merck, Singapore) to reach a final pH of 5.50. The okara slurry was boiled using a pressure cooker for 1 h and cooled down to 50 °C. The okara slurry was then treated with cellulase (1 % w/w, based on okara dry weight, Salus Nutra Inc., China) and pectinase (0.5 % w/w, based on okara dry weight, Salus Nutra Inc., China) for 2 h with constant stirring in a water bath at 50 °C. The enzyme-treated okara slurry was homogenised using a high-shear in-line mixer (Silverson Machines Ltd, Waterside, Chesham, Bucks., England) at 9,990 rpm for 30 min. Finally, the okara slurry was pasteurised at 95 °C for 25 min before storing it in a 4 °C fridge. The okara slurry was preheated in a 37 °C water bath before LAB inoculation.

Okara slurry fermentation

The two probiotic strains in dry form were hydrated in sterile 0.65 % (w/v) saline and spread on an MRS (Merck, Darmstadt, Germany) agar plate, and then incubated at 37 °C for 48 h. A homogeneous colony of cells was then isolated, before propagating in MRS broth (7.8 g of Oxoid MRS broth powder in 150 mL of mineral water). Strains LAC 343 and PCC were further sub-cultured to obtain cells for preservation with the addition of glycerol (final concentration of 15 % v/v) as a cryoprotectant in a −80 °C freezer (Vong & Liu, 2019).

Before inoculation, the LAB cells were thawed and sub-cultured once in 9 mL of MRS broth (10 % v/v) and incubated at 37 °C for 24 h. The sub-culture was centrifuged at 4 °C, 8,800 x *g* for 10 min to discard the MRS broth, and the LAB cells were washed twice with 0.85 % (w/v) saline before reconstitution in 10 mL of saline. Two mL of the saline containing cells was then inoculated (1 % v/v) into 200 mL of sterilized okara slurry (autoclaved at 121 °C for 15 min). The inoculated okara slurries were incubated at 37 °C for 24 h, together with a control slurry without LAB inoculation.

For each probiotic strain, triplicate fermentations were performed. Sampling was done at 0 h and 24 h for pH measurement and viable cell count. Final samples (24 h) were collected for subsequent chemical analysis for both inoculated and control okara slurries, namely Control (Ctrl), LAC, and PCC.

Viable cell count and pH measurement

To determine the cell count in the okara samples at 0 h and 24 h, serial dilution was performed using 0.1 % peptone water (Oxoid Ltd., Basingstoke, Hants, UK) and suitable dilutions were plated on MRS agar using the pour plate method. The plates were incubated at 37 °C for 48 h before enumeration. The pH value of the okara samples was measured using a SevenCompact pH meter (Mettler Toledo), which was calibrated with pH buffers at 4.0, 7.0 and 9.0 before use.

Amino acid analysis

The analysis of amino acids was done by following a method modified from Liu et al (2021). Ctrl, LAC, and PCC samples (24 h) were centrifuged at 4 °C, 9,956 x *g* for 10 min, and the supernatant was collected and stored in a −20 °C freezer before use. Upon thawing, samples were centrifuged at 4 °C, 12,000 x *g* for 5 min, and the supernatant was filtered through a 0.2-μm Minisart RC 15 syringe filter. The filtered supernatant sample (800 μL) was added with 200 μL of sulfosalicylic acid (10 % w/v), and the solution was vortexed and kept in a 4 °C fridge overnight to precipitate proteins. The samples were then centrifuged at 4 °C, 12,000 x *g* for 5 min and the supernatant was filtered through a 0.2-μm Minisart RC 15 syringe filter before injecting into HPLC for analysis on a ARACUS Amino Acid Analyzer (MembraPure, Berlin, Germany). An amino acid standard mixture (Sigma-Aldrich, Singapore) was used to identify and quantify the amino acids in the samples, and Chromatography Data Handling System software was used to process the data.

Antioxidant capacity assays

Ctrl, LAC, and PCC samples (24 h) were centrifuged at 4 °C, 9,956 x *g* for 10 min, and the supernatant was collected and stored in a −20 °C freezer before use.

Total phenolic content (TPC)

The TPC of all samples was determined using a method of Isabelle et al. (2008) with modifications. Upon thawing, samples were centrifuged at 4 °C, 12,000 x *g* for 5 min, and the supernatant was filtered through a 0.2-μm Minisart RC 15 syringe filter. A gallic acid (Sigma-Aldrich, Singapore) standard solution within a range of 0.001–1 mg/mL was prepared, and 20 μL of each sample was mixed with 100 μL of 10 % (v/v) Folin-Ciocalteu reagent (Sigma-Aldrich, Singapore) followed by 80 μL of 7.5 % (w/v) Na_2CO_3 solution in a 96-well plate. Absorbance measurement at 765 nm with a microplate reader (Biotek PowerWave XS2 Microplate Spectrophotometer, Hampton, US) was performed with measurements done at every 1 min interval for 30 min and the highest reading towards the end was taken for data processing. Technical duplicates were prepared for each sample and the results were expressed as mg gallic acid equivalent (GAE) per liter (mg GAE/L) of okara slurry sample.

Oxygen radical absorbance capacity (ORAC)

The ORAC was measured for all samples following a method of Huang et al. (2002) with modifications. Upon thawing, samples were diluted 2x with HPLC-grade water, centrifuged at 4 °C, 12,000 x *g* for 5 min and the supernatant was filtered through a 0.2-μm Minisart RC 15 syringe filter. A Trolox standard solution (Sigma-Aldrich, Singapore) was prepared within a range of 0.001–0.25 mg/mL, and samples were diluted to four concentrations for analysis (dilution factor 6x, 18x, 54x, 162x). 2,2′-Azobis(2-methyl-propionamidine) dihydrochloride (AAPH) was dissolved in 75 mM phosphate buffer (pH 7.4) to a concentration of 153 mM, and a 1.39 μg/mL sodium fluorescein solution was prepared.

25 μL of each sample was mixed with 150 μL of the sodium fluorescein solution in a 96-well plate. After incubation at 37 °C for 10 min, 25 μL of AAPH solution was added. Fluorescence measurement with a microplate reader (Biotek Synergy HTX Multi-Mode Reader with Auto Pipette, Santa Clara, US) was performed with excitation filter set at 485 nm with 20 nm bandpass and emission filter set at 528 nm with 20 nm bandpass. A reading was taken at every 2 min interval for 5 h. The area under curve was calculated for data processing. Technical duplicates were prepared for each sample and the results were expressed as mg Trolox equivalent (TE) per liter (mg TE/L) of okara slurry sample.

LC-QTOF sample preparation

Sample preparation for LC-QTOF analysis was performed according to the method of Chan et al. (2021) with slight modifications. Ctrl, LAC, and PCC samples (24 h) were centrifuged at 4 °C, 9,956 x *g* for 10 min, and the supernatant was collected and frozen at −80 °C before freeze-drying (Buchi Standalone Freeze Dryer, Essen, Germany). The freeze-dried sample (0.1 g) was dissolved in 1 mL of LC-MS grade water to obtain a concentration of 100 mg/mL. An aliquot of 350 μL of dissolved sample was mixed with 1.4 mL of cooled acetonitrile to obtain a concentration of 20 mg/mL. Samples were kept at −20 °C for 1 h to precipitate the proteins, before centrifuging at 4 °C, 10,000 x *g* for 5 min. The supernatant was filtered through a 0.22-μm hydrophilic PTFE syringe filter and 1.0 mL was added into a HPLC vial for analysis on a LC-QTOF MS apparatus (Waters Xevo G2-XS QTOF MS). Technical duplicates were prepared for each sample. A standard was prepared using paracetamol (a small molecule that is not found in okara and would not be generated during fermentation) and added to each vial to obtain a concentration of 10.0 μg/mL. Quality control (QC) samples were prepared by mixing 200 μL of solution from each vial.

LC-QTOF analysis

The samples were analysed using a Waters Xevo G2-XS QTOF MS system. The column used was CORTECS C18 column (2.1 mm × 100 mm, 1.6 μm) connected to a CORTECS T3 VanGuard column (2.1 mm × 5 mm, 1.6 μm) (Waters Pacific Pte. Ltd., Ireland). Both positive and negative modes of the electrospray ionisation (ESI) source were applied, with 2.5 kV and 2.3 kV ion spray capillary voltage respectively. The injection volume was 3 μL for each sample and the run time was 20.1 min. Gradient elution was applied using mobile phase A (0.1 % formic acid in water) and mobile phase B (acetonitrile) at a flow rate of 0.4 mL/min at 30 °C. The gradient condition was as follows: 0.0–2.0 min 5 % B, 2.0–10.0 min 5–70 % B, 10.0–15.0 min 70–80 % B, 15.0–15.5 min 80–95 % B, 15.5–16.5 min 95 % B, 16.5–16.6 min 95–5 % B, 16.6–20.1 min 5 % B.

The injection sequence was first acetonitrile as the blank, followed by background controls (1:4 v/v water: methanol) and then 12 injections of QC samples. After that, samples were injected into the system in a random sequence to ensure results validity and avoid biasness. One QC sample was run after every six samples.

Statistical analysis

The LC-QTOF data was uploaded to Progenesis QI coupled with METLIN database, and untargeted small molecules were identified. The identified compounds were then exported to EZ info. Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were then performed. OPLS-DA filtered out significant compounds using the following criteria: (1) a one-way analysis of variance (ANOVA) p-value < 0.05; (2) maximum fold-change ≥ 2; (3) minimum CV ≤ 30 %. After that, S-plots were generated and features with correlation $|p(\text{corr})[1]| > 0.75$ and covariation $|p[1]| > 0.05$ were picked as discriminating metabolites. The selected features were then transferred to Progenesis QI for further identification.

For cell count, pH, amino acids, TPC and ORAC, ANOVA with p-value at 95 % confidence interval was performed using SPSS® 20.0 software (Chicago, Illinois, USA), and data was organised in Microsoft Excel and presented as bar charts and tables.

Results and discussion

Viable cell count and pH changes

Changes in cell count and pH after fermentation by LAC and PCC are shown in Fig. 1A and Fig. 1B, respectively. There were significant increases in cell count, where the LAC and PCC population increased by 2.22 log CFU/mL and 0.82 log CFU/mL respectively. The final log CFU/mL of LAC was 8.69 and of PCC was 7.95 (Fig. 1A), which allowed both samples to fulfill 9 log CFU/100 mL serving to be regarded as a probiotic drink.

The pH of the fermented samples showed significant decreases ($p < 0.05$), while the pH of the control sample of uninoculated okara remained stable. LAC-fermented samples showed a larger pH decrease of 1.31, compared to the PCC-fermented samples with a pH decrease of 1.03 (Fig. 1B).

Both the cell count and pH changes showed that the LAB strains had indeed fermented the okara slurries. The larger pH decreases by LAC corresponded well with the larger increase in cell count and its homofermentative nature with the production of two moles of lactic acid per mole of glucose consumed. Conversely, the smaller decreases in pH by PCC concurred with the lower cell biomass increase and its heterofermentative fermentation of glucose with the formation of one mole of lactic acid per mole of glucose consumed. The findings also suggest that okara was a better growth matrix for LAC than PCC.

Changes in amino acids

Changes in amino acids in unfermented and fermented okara samples are shown in Fig. 1C. In both fermented samples, there was a

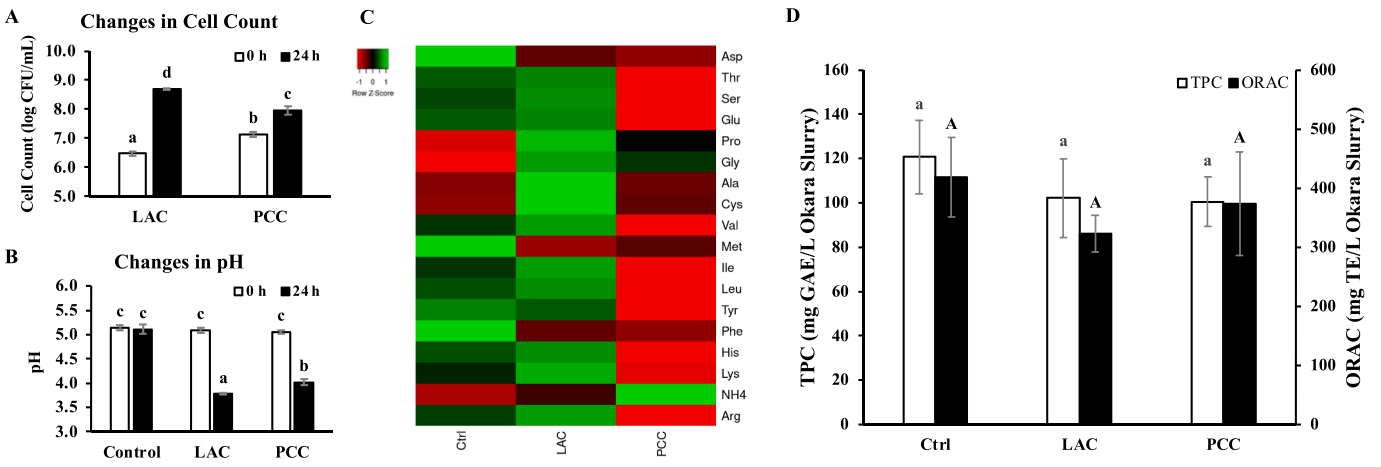

Fig. 1. Cell counts of fermented okara slurry (A), pH of unfermented and fermented okara slurry (B), heat map of amino acid compositions of unfermented and fermented okara slurry with green and red representing high and low concentrations respectively (C), and total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC) of unfermented and fermented okara slurry (D). $^{a, b, c, d, A}$ statistical analysis using ANOVA ($n \geq 3$) at 95 % confidence interval; lower-case letters correspond to TPC data and upper-case letters correspond to ORAC data in D. Ctrl = uninoculated okara incubated for 24 h, LAC = okara fermented by *L. gasseri* LAC 343, PCC = okara fermented by *L. fermentum* PCC.

decrease in aspartic acid, methionine and phenylalanine compared to the control, indicating that both strains had the ability to metabolise these amino acids (Fig. 1C, Table S1). In the LAC-fermented samples, more alanine, cystine, valine, isoleucine and arginine were produced while the rest of amino acids remained comparable to the control ($p < 0.05$). This is in contrast to what was found in the PCC-fermented samples, where most of the amino acids had been consumed except for proline, glycine, alanine, cystine, histidine, lysine and ammonium, which did not change ($p < 0.05$). This suggests that LAC was more proteolytic and peptidolytic than PCC, thus producing more amino acids than what was consumed. Zhan et al. (2023) reported that *Bacillus subtilis* DC-15 produced more histidine, lysine and phenylalanine after fermentation, indicating this strain might be more proteolytic than LAC and PCC. Therefore, okara fermented with different probiotic strains would have resulted in variation of the final amino acid profile.

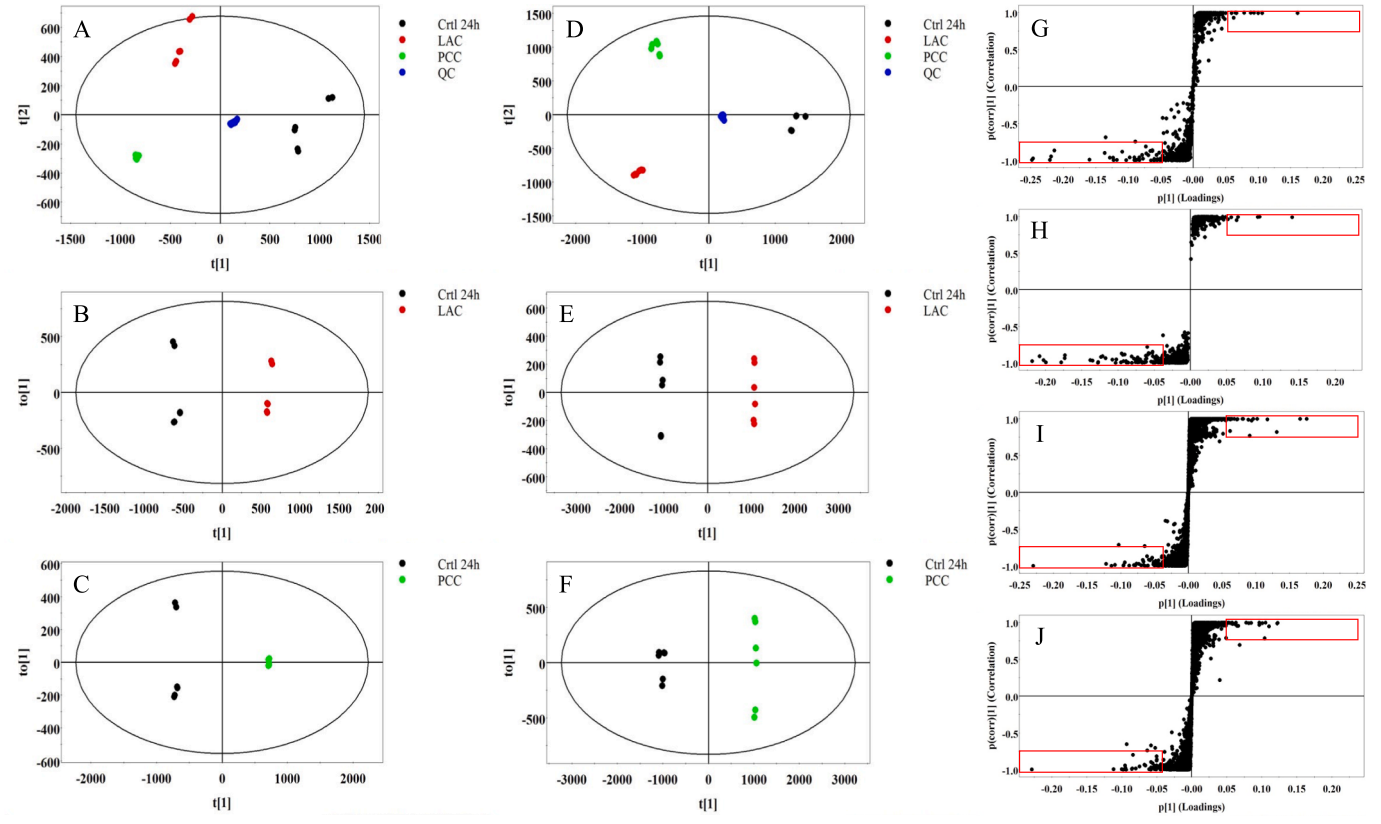

Fig. 2. PCA analysis of unfermented and fermented okara slurry (n = 6) in positive (A) and negative (D) mode; OPLS-DA plot (positive mode) of Ctrl 24 h and LAC (B), Ctrl 24 h and PCC (C); OPLS-DA plot (negative mode) of Ctrl 24 h and LAC (E), Ctrl 24 h and PCC (F); S-plots from OPLS-DA analysis (positive mode) of Ctrl 24 h and LAC (G), Ctrl 24 h and PCC (H); S-plots from OPLS-DA analysis (negative mode) of Ctrl 24 h and LAC (I), Ctrl 24 h and PCC (J). Ctrl 24 h = uninoculated okara slurry incubated for 24 h; LAC = okara slurry fermented by *L. gasseri* LAC 343; PCC = okara slurry fermented by *L. fermentum* PCC; QC = quality control samples.

Antioxidant capacity

The changes of TPC and ORAC are illustrated in Fig. 1D. In both analyses, there were slight decreases in the absolute values of the fermented samples, but the decreases were statistically insignificant and hence comparable to the control ($p < 0.05$). A possible reason for the insignificant changes in TPC could be due to the enzyme pre-treatment of the okara slurry before fermentation. The enzymes could have broken down the cell walls of the okara matrix, releasing some phenolic compounds bound to the cell walls, thus the okara slurry would already contain relatively high amounts of phenolic compounds before fermentation. The probiotic strains thus would not produce significantly more phenolic compounds during fermentation, or may even metabolise the existing phenolic compounds, resulting in insignificant changes to the total phenolic content. Interestingly, the ORAC results were similar to that of TPC where no significant changes were seen after fermentation. It has been reported that some amino acids like tyrosine, phenylalanine and methionine have the ability to react with radicals (Pownall et al., 2010; Stadtman et al., 2002). This may be part of the reason why there was a dip in ORAC since these amino acids decreased after fermentation (Fig. 1C), but this change was not enough to significantly affect the ORAC value.

LC-QTOF analysis

PCA analysis of unfermented and fermented samples (24 h) in positive and negative modes are shown in Fig. 2A and Fig. 2D respectively. QC samples are situated in the middle while the other samples formed their own cluster away from each other, indicating variations between the sample compositions. OPLS-DA showed that both LAC-fermented (Fig. 2B and Fig. 2E) and PCC-fermented (Fig. 2C and Fig. 2F) samples differed from the unfermented control. The goodness of fit of the OPLS-

Table 1
Putative identification of metabolites distinguished in unfermented and probiotic fermented okara slurry (24-h incubation).

Metabolites	RT (min)	*m/z*	Mass Error (ppm)	Adducts	Formula	MS/MS fragments	Fold-change (compared to control)	
							PCC	**LAC**
Sugars								
L-(+)-Glucose*	0.76	203.0528	1.03	$[M + Na]^+$	$C_6H_{12}O_6$	163.0597	↓ 12.0	↓ 2.8
Ribulose	0.66	149.0450	−3.56	$[M-H]^-$	$C_5H_{10}O_5$	131.0345	↓ 1.1	↓ 1.0
Organic acids and derivatives								
Isocitric acid*	0.72	191.0193	−2.34	$[M-H]^-$	$C_6H_8O_7$	175.0244, 173.0087, 117.0190	↓ 8.9	↑ 2.5
Succinic acid*	0.98	117.0188	−4.69	$[M-H]^-$	$C_4H_6O_4$	99.0083, 73.0293	↑ 30.4	↑ 7.9
Citric acid	0.64	191.0194	−1.88	$[M-H]^-$	$C_6H_8O_7$	101.0241, 89.0238, 85.0293, 59.0134	↓ 1.2	↓ 1.1
Malic acid	0.69	133.0140	−2.06	$[M-H]^-$	$C_4H_6O_5$	115.0034, 87.0084	↓ 5.7	↑ 1.4
Fumaric acid	0.69	115.0033	−3.28	$[M-H]^-$	$C_4H_4O_4$	71.0135	↑ 3.2	↑ 1.4
Gluconic acid*	0.62	195.0506	−2.40	$[M-H]^-$	$C_6H_{12}O_7$	179.0557, 161.0454	↓ 7.7	↓ 1.4
Methylmalonic acid*	0.72	117.0189	−3.99	$[M-H]^-$, $[M + FA-H]^-$	$C_4H_6O_4$	103.0033, 73.0290	↑ 18.1	↑ 4.7
Nucleoside and pyrimidine								
Cytidine*	0.65	244.0936	3.29	$[M + H]^+$, $[M + K]^+$	$C_9H_{13}N_3O_5$	112.0513	↓ 3.3	↓ 1.1
Cytosine*	0.66	112.0512	5.66	$[M + H]^+$	$C_4H_5N_3O$	95.0248, 94.0406	↓ 5.6	↓ 1.2
Amino acid derivatives								
Pyroglutamic acid	0.64	128.0348	−4.18	$[M-H]^-$	$C_5H_7NO_3$	94.0296	↑ 1.3	↑ 1.5
γ-Aminobutyric acid	0.62	104.0707	1.10	$[M + H]^+$	$C_4H_9NO_2$	87.0448, 86.0605	↑ 1.9	↑ 2.3
Indolelactic acid*	5.15	204.0661	−2.30	$[M-H]^-$	$C_{11}H_{11}NO_3$	186.0556, 158.0605, 142.0657, 130.0658	↑ 790.5	↑953.6
	5.15	206.0813	0.46	$[M + H]^+$, $[M + Na]^+$	$C_{11}H_{11}NO_3$	146.0610, 130.0653, 118.0657, 115.0545	↑ 7.6	↑ 9.1
Indole-3-acetaldehyde	1.44	160.0755	−1.29	$[M + H]^+$	$C_{10}H_9NO$	130.0656, 115.0545	↑ 10.0	↑ 14.2
Indole-3-ethanol	5.66	162.0918	3.08	$[M + H]^+$	$C_{10}H_{11}NO$	144.0797, 130.0662	↑ 5.6	↓ 1.2
5-Hydroxyindole-3-acetic acid	5.15	190.0504	−2.83	$[M-H]^-$	$C_{10}H_9NO_3$	146.0603, 144.0449	↑ 606.0	↑ 696.0
D-Phenyllactic acid	4.12	165.0552	−2.98	$[M-H]^-$	$C_9H_{10}O_3$	96.9598	↑ ∞	↑ ∞
2-Phenylacetaldehyde	8.24	121.0649	0.95	$[M + H]^+$	C_8H_8O	103.0547, 93.0701, 91.0547	↓ 1.1	↑ 1.1
4-Hydroxyphenyllactic acid*	3.28	181.0502	−2.54	$[M-H]^-$	$C_9H_{10}O_4$	163.0396, 135.0446, 134.0368, 119.0497	↑ 86.3	↑ 94.5
3,4-Dihydroxyphenylacetic acid	5.88	167.0343	−3.90	$[M-H]^-$	$C_8H_8O_4$	133.0284, 123.0446	↓ 1.1	↓ 1.6
Fatty acids and derivatives								
Linoleic acid	13.87	279.2326	−1.43	$[M-H]^-$, $[M+FA-H]^-$	$C_{18}H_{32}O_2$	261.2222	↑ 1.7	↑ 2.1
(±)12,13-DiHOME*	7.43	315.2528	−0.54	$[M+H]^+$	$C_{18}H_{34}O_4$	279.2304, 113.0967, 85.1023	↑ 15924.1	↓ ∞
(±)9-HpODE*	9.10	311.2225	−0.98	$[M-H]^-$, $[M+FA-H]^-$	$C_{18}H_{32}O_4$	293.2114, 275.1994, 197.1179	↓ 1.5	↓ 1.6
9-oxoODE	10.60	293.2113	−3.20	$[M-H]^-$	$C_{18}H_{30}O_3$	249.2220, 185.1179	↓ 2.1	↓ 2.9
13-oxoODE	10.45	293.2114	−2.81	$[M-H]^-$	$C_{18}H_{30}O_3$	275.2013, 205.1226	1.0	↓ 1.1
9(*S*)-HpOTrE*	8.48	309.2067	−1.53	$[M-H]^-$	$C_{18}H_{30}O_4$	291.1964, 223.1697, 185.1178, 171.1021	↓ 1.7	↓ 1.9
12-OPDA*	8.46	293.2115	1.40	$[M+H]^+$	$C_{18}H_{28}O_3$	275.2005	↓ 2.6	↓ 2.0

Metabolites are listed according to their sequence to be discussed; those with * are significant features.
↓Indicates a decrease in amount of metabolites found in probiotic fermented okara slurry;↑Indicates an increase in amount of metabolites found in probiotic fermented okara slurry.
Abbreviations: mass to charge ratio – m/z; retention time – RT; LAC = okara fermented by L. gasseri LAC 343; PCC = okara fermented by L. fermentum PCC; 12,13-DiHOME = 12,13-dihydroxy-9-octadecenoic acid; 9-HpODE = 9-hydroperoxy-10,12-octadecadienoic acid, 9-oxoODE = 9-oxo-10,12-octadecadienoic acid, 13-oxoODE = 13-oxo-9,11-octadecadienoic acid, 12-OPDA = 12-oxophytodienoic acid, 9(S)-HpOTrE = 9-hydroperoxyoctadeca-10,12,15-trienoic acid.

DA model (Fig. S1) was verified to have parameters of $R^2Y \geq 0.997$ and $Q^2 \geq 0.996$ before proceeding to pick out the significant features that contributed to the differences.

Fig. 2G–J show the S-plots of detected features of unfermented and fermented samples. From the S-plots, the significant features were identified in the red boxes and were used in the investigation of potential metabolic pathways.

The notable metabolites are presented in Table 1 together with their respective retention times (RT), experimental mass to charge ratios (*m/z*), mass errors, adducts, molecular formulae, MS/MS fragments and fold-changes. These metabolites were then mapped into proposed fermentative pathways of LAC and PCC as displayed in Fig. 3. Overall, we observed a partial tricarboxylic acid (TCA) cycle, pentose phosphate metabolism, pyrimidine metabolism, amino acid metabolism and fatty acid metabolism, which are elaborated in subsequent sections.

Central metabolism (partial TCA cycle & pentose phosphate pathway) and pyrimidine metabolism

Glucose is the major sugar source utilised by the probiotic strains. It decreased by 12.0-fold in the PCC-fermented samples and 2.8-fold in the LAC-fermented samples (Table 1). Glucose might be catabolised into pyruvate via homo- or heterofermentative pathways, where the pyruvate subsequently entered the partial TCA cycle (Fig. 3). In the PCC-fermented samples, isocitrate decreased by 8.9-fold and succinic acid increased by 30.4-fold (Table 1). In the LAC-fermented samples, the amount of isocitrate increased by 2.5-fold and succinic acid increased by 7.9-fold (Table 1). Citric acid showed a decrease after fermentation in

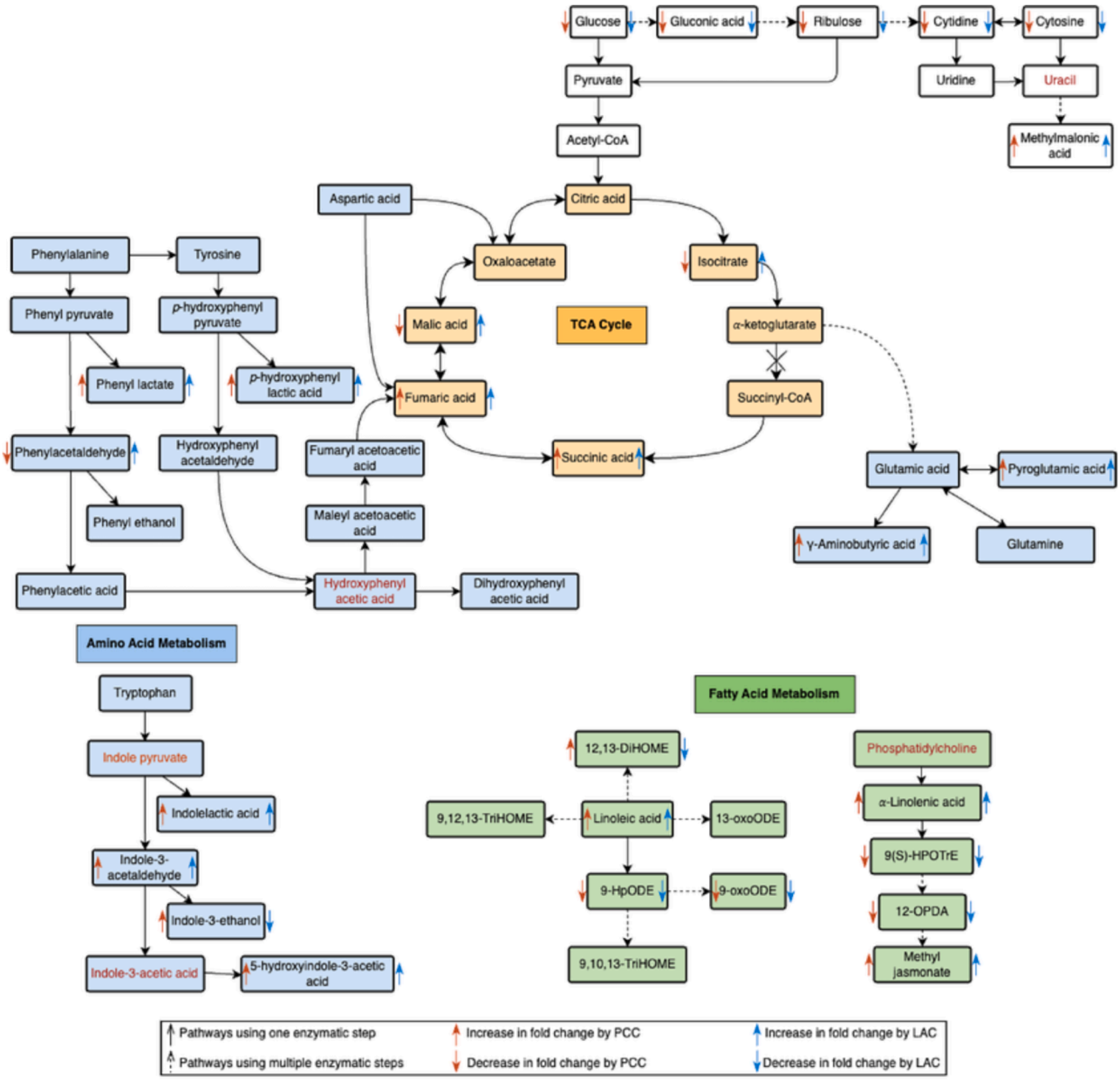

Fig. 3. Proposed metabolic pathways during okara fermentation by *L. gasseri* LAC 343 and *L. fermentum* PCC. Notes for compound names in red: uracil, conjugated forms were detected, suggesting that the singular uracil compound was formed before conjugation; hydroxyphenyl acetic acid, hydroxyphenyl acetic acid *O-b*-D-glucoside was detected, suggesting hydroxyphenyl acetic acid was formed before the substitution; indole pyruvate, although not detected in the samples the generation of this compound was deduced from the detection of subsequent metabolites; indole-3-acetic acid, hydroxyindole-3-acetic acid was detected, suggesting that indole-3-acetic acid was formed before that; phosphatidylcholine: substituted form of phosphatidylcholine was found, refer to Table S2.

both fermented samples (Table 1). This indicates that both strains had converted some citric acid into succinic acid during fermentation via the reductive sequence of the partial TCA cycle (citrate → oxaloacetate → malic acid → fumaric acid → succinic acid). This was further confirmed by the detection of malic acid (5.7-fold decrease and 1.4-fold increase in PCC and LAC samples respectively) and fumaric acid (3.2-fold and 1.4-fold increase in PCC and LAC samples respectively). Succinic acid increased in fermented okara slurry for both strains, but the increase in fold change differed significantly where the content in the PCC-fermented samples (30.4-fold increase) was much higher than that of the LAC-fermented samples (7.9-fold increase). The increased levels of succinic acid and decreased levels of citric acid followed a similar trend to that reported by Zhan et al. (2023), where *B. subtilis* DC-15 was used to ferment okara. Moreover, multiple studies have confirmed that the reductive sequence of the partial TCA cycle is a common metabolic pathway found in LAB fermentation (Chan et al., 2021; De Vuyst & Leroy, 2020; Loh et al., 2021; Zhao et al., 2021). However, we reason that the bulk of increased succinic acid observed in this study would likely have stemmed from aspartic acid metabolism via this route: aspartic acid → oxaloacetate → (malic, fumaric) → succinic acid, given the substantial consumption of aspartic acid in both PCC and LAC fermented okara slurry (Fig. 1C, Table S1). By the action of aminotransferase, aspartic acid could have been converted to oxaloacetate, or through another pathway, aspartic acid could have also been transformed into fumaric acid by the reaction of an aspartase (also known as aspartate-ammonia lyase) (Fernández & Zúñiga, 2006).

It is also surprising that the isocitrate amount in the LAC-fermented samples increased (Table 1), indicating other pathways that may lead to its production. Isocitrate has been shown to exhibit antioxidant capacity (Fig. 4) (Mok et al., 2019). Besides this metabolic pathway, LAB can also degrade malic acid into lactic acid through malolactic fermentation (Liu et al., 2023). There was a slight increase of lactic acid in both PCC- and LAC-fermented samples (1.3-fold and 1.2-fold increase respectively) (Table S2). It should be noted that lactic acid was added during okara slurry preparation to adjust the pH. Therefore, the fold-change may not accurately indicate the actual fold-change caused by fermentation.

Gluconic acid, one of the significant features, decreased by 7.7-fold and 1.4-fold in PCC- and LAC-fermented samples respectively (Table 1). Pentose phosphate pathway operated in LAB constituted the Entner-Doudoroff pathway, which has been found in some bacteria (Kornecki et al., 2020) and a specific genome study has revealed its presence in LAB (Salvetti et al., 2013). Glucose can be degraded into gluconic acid through the Entner-Doudoroff pathway and further be transformed into pentoses (Kornecki et al., 2020; Nieto-Peñalver et al., 2014; Salvetti et al., 2013). Pyruvate was then produced from pentose and entered TCA cycle (Salvetti et al., 2013). This gives a possible explanation for the slight decrease in ribulose after fermentation (1.1-fold and 1.0-fold for PCC and LAC respectively). As the significant features included cytosine (5.6-fold decrease in PCC and 1.2-fold decrease in LAC) and cytidine (3.3-fold decrease in PCC and 1.1-fold decrease in LAC), pyrimidine metabolism is possibly operated by both probiotic strains. Cytidine is formed when cytosine is attached to a ribose ring. Cytidine can be deaminated into uridine which is then cleaved to form uracil (Krasuski, Michnowska-Swincow, & Jarzembowski, 2007). Conjugated forms of uracil were detected in both PCC- and LAC-fermented samples, suggesting that pyrimidine metabolism had led to the production of uracil, though the conjugated form of uracil would likely be less bioavailable. Uracil and uracil derivatives have been shown to possess biological potential, especially anti-viral and anti-tumor activities. Additionally, they also possess herbicidal, insecticidal, and bactericidal activities (Pałasz & Cież, 2015). Methylmalonic acid, another product of pyrimidine metabolism, showed 18.1-fold and 4.7-fold increase in PCC- and LAC-samples respectively (Table 1).

Glutamic acid metabolism

Glutamic acid is an amino acid present in okara. It decreased significantly in the PCC-fermented samples compared to the unfermented control (Fig. 1C, Table S1). Glutamine and glutamic acid degradation led to the formation of pyroglutamic acid (1.3-fold increase in PCC- and 1.5-fold increase in LAC-fermented okara), which is the cyclic lactam of glutamic acid (Montevecchi et al., 2011). Pyroglutamate possesses anti-bacterial activity (Chan et al., 2021) and has a potential osmo-protection function (Kumar & Bachhawat, 2011) (Fig. 4). On the

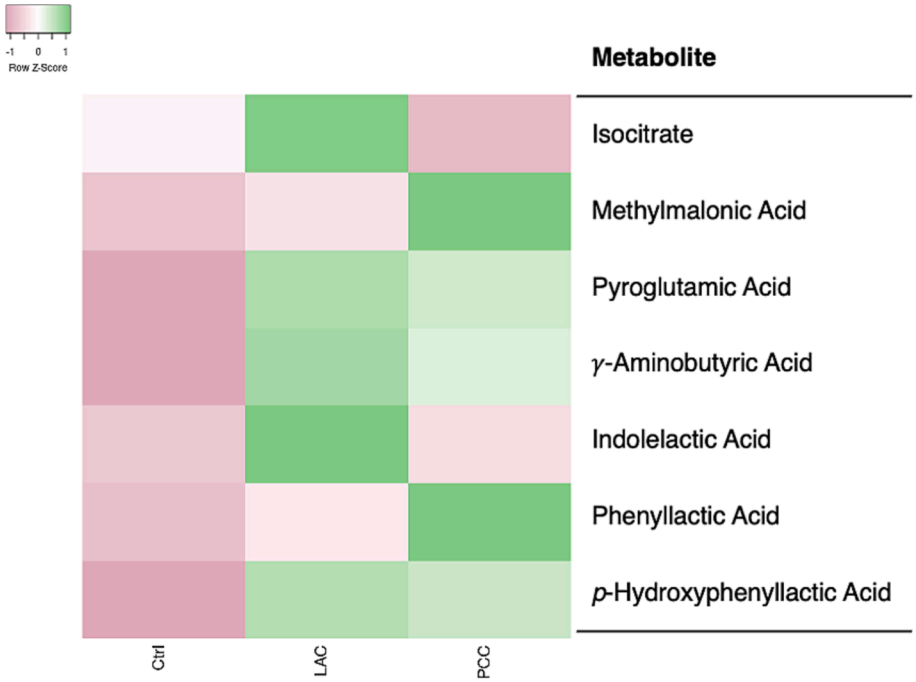

Fig. 4. Heatmap of identified metabolites, where pink and green represent low and high abundance respectively. Ctrl = uninoculated okara slurry incubated for 24 h, LAC = okara slurry fermented by *L. gasseri* LAC 343, PCC = okara slurry fermented by *L. fermentum* PCC.

other hand, pyroglutamic acid catabolism could also generate glutamate in the presence of the enzyme 5-oxoprolinase. This might be one reason that there was no significant change in the amount of glutamic acid in LAC-fermented samples since pyroglutamic acid can function as a reservoir of glutamate, and in this case, the equilibrium possibly favoured glutamate formation.

Another metabolic pathway utilising glutamic acid was through the production of γ-aminobutyric acid (GABA) with a 1.9-fold increase and a 2.3-fold increase in PCC- and LAC-fermented samples respectively. Some LAB have been shown to produce glutamate decarboxylase to catalyse the production of GABA. GABA can be an inhibitory neurotransmitter in the central nervous system and has a direct influence on personality and stress management (Fig. 4) (Dhakal et al., 2012).

Tryptophan, phenylalanine and tyrosine metabolism

Indolelactic acid is a bioactive metabolite that is able to help regulate the gut microbiome due to its antibacterial and antifungal properties (Fig. 4) (Roager & Licht, 2018). It showed a drastic increase by 790-fold in the PCC-fermented samples and 954-fold in the LAC-fermented samples (Table 1). There was also an increase of indole-3-acetaldehyde by 10.0-fold and 14.2-fold for PCC- and LAC-fermented samples respectively (Table 1). The presence of both these compounds suggested that tryptophan metabolism proceeded first via the action of aminotransferase to produce indole pyruvate (Fernández & Zúñiga, 2006). Indole pyruvate was not detected in the samples, which may indicate that it reacted with dehydrogenases and decarboxylases to produce indolelactic acid and indole-3-acetaldehyde respectively. Strain PCC might have further reduced indole-3-acetaldehyde to produce indole-3-ethanol with a 5.6-fold increase. On the other hand, there was no exceptional change for indole-3-ethanol in the LAC-fermented samples, which could be ascribed to the larger increase in indole-3-acetaldehyde by LAC. 5-Hydroxyindole-3-acetic acid was also detected with a large fold-change of 600–700 for both strains (Table 1), and this was possibly the hydroxylated form of indole-3-acetic acid produced from indole-3-acetaldehye after the action of dehydrogenase.

From phenylalanine metabolism, D-phenyllactic acid had infinite fold-changes in both PCC- and LAC-fermented samples (Table 1), which was possibly produced from the sequential action of aminotransferase to produce phenyl pyruvate, followed by the action of dehydrogenase. D-Phenyllactic acid has antifungal and antioxidant property as it has been shown to decrease the production of reactive oxygen species (Fig. 4) (Beloborodova et al., 2012). On the other hand, there were insignificant changes to 2-phenylacetaldehyde with 1.1-fold decrease and increase in the PCC- and LAC-fermented samples respectively (Table 1). Similarly, phenylpyruvate and 2-phenylethanol were also detected but without significant changes. This suggests that the metabolism of phenylalanine by PCC and LAC largely favoured the production of D-phenyllactic acid from phenyl pyruvate. Also, compared to tryptophan which was not detected in the samples, the metabolism of phenylalanine seemed more sluggish with a 2-fold decrease after fermentation and this result agreed well with the changes in amino acids shown in Fig. 1C and Table S1.

In the metabolism of tyrosine, *p*-hydroxyphenyllactic acid increased by 86.3- and 94.5-fold in the PCC- and LAC-fermented samples respectively (Table 1), which suggests that it has been produced via *p*-hydroxyphenylpyruvate by the action of dehydrogenase (Fernández & Zúñiga, 2006). *p*-Hydroxyphenyllactic acid has antifungal and antioxidant property similar to D-phenyllactic acid (Fig. 4), thus it might be able to protect the body from oxidative damage (Beloborodova et al., 2012). A glucose substituted *p*-hydroxyphenylacetic acid was also detected in the fermented samples with less than 2-fold increase (Table S2). The production of this compound suggests that the reaction continued from *p*-hydroxyphenylpyruvate, which was first reacted by a decarboxylase to *p*-hydroxyphenylacetaldehyde, followed by a dehydrogenase to produce *p*-hydroxyphenylacetic acid (Fernández & Zúñiga, 2006). The same precursor can also be deduced from the presence of dihydroxyphenylacetic acid detected in the samples (Table 1).

This metabolism continued as either maleylacetoacetic acid and fumarylacetoacetic acid were detected (Table S2). They could lead to the production of fumaric acid and continue into the reductive branch of the partial TCA cycle (Fig. 3).

Fatty acid metabolism

Fatty acids in okara consist of mainly polyunsaturated fatty acids. Linoleic acid and α-linolenic metabolites were detected in the PCC- and LAC-fermented samples.

Linoleic acid showed a 1.7-fold increase in the PCC- and 2.1-fold increase in LAC-fermented samples (Table 1). This indicates that more linoleic acid was produced than broken down. 12,13-DiHOME, one of the end metabolites of linoleic acid metabolism and a significant feature, increased 1924.1-fold after fermentation with PCC but showed an infinity-fold decrease in the LAC-fermented samples (Table 1). Another pathway of linoleic acid metabolism was through the intermediate 9-HpODE, which can be converted to 9-oxoODE (Fig. 3). Both 9-HpODE (1.5-fold and 1.6-fold decrease in the PCC- and LAC-fermented samples respectively) and 9-oxoODE (2.1-fold and 2.9-fold decrease in PCC- and LAC-fermented samples respectively) were found in both fermented samples. Other end products of linoleic acid metabolism such as 13-oxoODE (1.1-fold decrease in the LAC-fermented samples), 9,12,13-TriHOME (1.2-fold decrease in the PCC-fermented samples and 1.4-fold increase in the LAC-fermented samples respectively) and 9,10,13-TriHOME (1.2-fold decrease in both fermented samples) were all detected in both fermented samples (Table S2). Although there was a slight increase in 9,12,13-TriHOME in the LAC-fermented samples, this change was insignificant, making it difficult to conclude how linoleic acid was utilised by LAC.

Phosphatidylcholine is a class of glycerophospholipid naturally present in all mammalians and is the most abundant phospholipid in eukaryotic cellular membrane (Thomas et al., 2016). Phosphatidylcholine was likely the precursor for α-linolenic acid, where 1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenedioyl)-*sn-glycero*-3-phosphatidylcholine was detected in our samples with a 3.2-fold and 1.9-fold decrease in the PCC- and LAC-fermented samples respectively, while α-linolenic acid showed a 1.3-fold increase in both fermented samples (Table S2). α-Linolenic acid is an essential omega-3 fatty acid and has potential health benefits such as protection against cardiovascular diseases, modulation of the inflammatory response, and improvements to both central nervous system function and behavior (Stark et al., 2008). α-Linolenic acid might have been further transformed to methyl jasmonate with a 3.2-fold and 4.2-fold increase in the PCC- and LAC-fermented samples respectively (Table S2). The pathway was proposed as intermediate metabolites such as 9(*S*)-HpOTrE (1.7-fold and 1.9-fold decrease in PCC- and LAC-fermented samples respectively) and 12-OPDA (2.6-fold and 2.0-fold decrease in PCC- and LAC-fermented samples respectively) were identified as significant features (Table 1).

Conclusion

With the aid of LC-QTOF-MS/MS analysis, notable metabolites were identified in the okara slurry fermented with *L. gasseri* LAC 343 and *L. fermentum* PCC, and possible metabolic pathways were deduced. Similar metabolites were found in both the LAC- and PCC-fermented samples, though there were differences in the fold-changes. Some bioactive compounds such as indolelactic acid, D-phenyllactic acid, and *p*-hydroxyphenyllactic acid were identified, which have been reported to show antioxidant, antibacterial/antifungal, anticancer, and anti-inflammatory properties. Our study showed that metabolomics (untargeted) analysis can be coupled with targeted analysis to give a more comprehensive understanding of potential postbiotics in fermented foods. However, this study reported just the metabolic changes during the fermentation of okara slurry by *L. gasseri* LAC 343 and *L. fermentum*

PCC. More work can be done to quantify the notable bioactive metabolites that were produced. In addition, performing clinical trials would help to ascertain if the fermented okara slurry can indeed bring health benefits after consumption.

CRediT authorship contribution statement

Zihan Gao: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Melody Chang Zhou:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Jing Lin:** Methodology, Visualization. **Yuyun Lu:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing. **Shao Quan Liu:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101178.

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