



Research article

Improving anti-hypertensive properties of plant-based alternatives to yogurt fortified with rice protein hydrolysate

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ABSTRACT

In recent years, an increasing demand of plant-based yogurt-like products for partially replacing milk products was related to both health and ethical needs. The objective of this study was to improve the functionality of yogurt-like products relevant for hypertension, using fortification with protein hydrolysate and lactic acid bacteria (LAB) fermentation of soy and Job's tears beverages. Broken rice was hydrolysed by protease enzymes. Plant-based beverages and yogurt-like products with and without rice protein hydrolysate were prepared by LAB fermentation and evaluated by *in-vitro* assays for inhibition of free radical scavenging and angiotensin-converting enzyme (ACE) activity. Biochemical changes caused by lactic acid bacteria metabolism were characterized using chemical analysis and ¹H NMR. The rice protein hydrolysate obtained using Alcalase[®] at pH 7.0 for 2 h at 60 °C showed the highest antioxidant activity and ACE inhibition. The ACE inhibition increased by 19.52% and 34.13% in Job's tears and soy yogurt-like products with hydrolysate, respectively. A strongly significant correlation ($R^2 = 0.84$) between *o*-phthalic dicarboxaldehyde (OPA) peptides and ACE inhibition was observed. These results indicated the promise of hydrolysate and LAB fermentation as a functional ingredient and process, respectively, for the food industry.

1. Introduction

Nowadays, plant-based alternatives to yogurt fermented by lactic acid bacteria (LAB) are increasing in the production volume and market share. Yogurt-like products produced from cereals, pseudocereals and legumes have been developed and studied (Montemurro et al., 2021). In addition, soybean has been widely used as a main raw material due to its high protein content (Donkor et al., 2005). Job's tears, or Chinese pearl barley (*Coix lacryma-jobi* L), belongs to the grass family Poaceae. Job's tears seeds are cultivated in East and South-East Asia, including China, Japan, Malaysia, Myanmar and Thailand. They have long been used in traditional Chinese medicine and as a nourishing cereal. Job's tears beverage is available on the market as an alternative health food in Japan and Thailand. The white-husked Job's tears contain protein and lipid content ranged 13.54%–13.18% DW and 4.86%–0.91% DW, respectively (Chai-siricharoenkul et al., 2011). Moreover, total phenolic content and DPPH radical scavenging ability of whole grain and degermed Job's tears flours ranged between 7.33 mg GAE/g to 8.18 mg GAE/g, and 5.40%–7.53%, respectively. However, the utilization of Job's tears as raw material for the production of plant-based beverages and yogurt-like products is limited. Furthermore, the utilization of industrial by-products, reducing

waste for added-value food products have been observed (Ferri et al., 2017). Broken rice contains about 80% starch and 8% protein and used as a raw material in starch products and as animal feed. The higher carbohydrate content and appropriate nutrient content in broken rice could serve as sources of carbon and nitrogen supplement in media for improving the growth of LAB (Amagliani et al., 2017; Cai et al., 2021; Pinciroli et al., 2019). Ferri et al. (2017) reported that the hydrolysis of rice protein produced antioxidant peptides. The presence of antioxidant peptides in fermented and plant-based products showed the promising inhibition of angiotensin-converting enzyme (ACE) activity, which is related to inhibitory action against hypertension (Donkor et al., 2005). Cavalheiro et al. (2020) reported that bioactive peptides enhanced the ACE inhibitory activity in a high-protein yogurt.

Metabolomics has been carried out by using mass spectrometry (MS) and nuclear magnetic resonance (NMR) for studying a wide range of metabolites in liquid milk (Zhang et al., 2018) and fermented dairy products (Lu et al., 2016; Settachaimongkon et al., 2014; Trimigno et al., 2020). However, the number of publications focusing on metabolomics as an analytical technique to study on biochemical changes in the production plant-based functional foods is rather limited (Kumar et al., 2017).

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It was hypothesized that lacto-fermentation of plant-based beverages affected by the addition of protein hydrolysates would result in improving of potential health benefits of plant-based yogurt-like. Therefore, the objective of this study was to improve the functionality of Job's tears and soy yogurt-like products via fortification with rice protein hydrolysate and LAB fermentation. In addition, the chemical, microbiological qualities, biochemical changes and inhibitory activity against ACE enzymes of yogurt-like products were determined.

2. Materials and methods

2.1. Plant materials

Soybean (*Glycine max*), Job's tears (*Coix lacryma-jobi* L.) and broken non-waxy rice (*Oryza sativa* L.) cultivar Thai Hom Mali (RD105) were purchased from the local market in Chiang Rai province, Thailand.

2.2. Lactic acid cultures

Mixed yogurt culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YF-L812 Yo-Flex[®] - Direct Vat Set cultures, DVS; size 10 pouch × 50 U) was obtained from Chr. Hansen, Hoersholm, Denmark.

2.3. Chemicals

Alcalase[®] (3.4.21.14 from *Bacillus licheniformis*), Flavourzyme[®] (232-752.2, from *Aspergillus oryzae*) and pepsin (232-629-3, from porcine gastric mucosa) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hipuryl-histidyl-leucine (HHL) (≥98%), ACE from rabbit lung (≥2.0 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium citrate, citric acid, disodium tetraborate decahydrate, sodium dodecyl sulphate (SDS), *o*-phthalic dicarboxaldehyde (OPA), dithiothreitol (DTT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used in the present study were of AR grade. Ultrapure water was generated and purified by using a Milli-Q water purification system (Merck Millipore, Bedford, MA, USA).

2.4. Proximate analysis

Proximate analysis including moisture, protein, fat, ash, crude fiber, and carbohydrates were determined of soybean, Job's tears and broken non-waxy rice by using the AOAC international method (2000).

Moisture content was determined by heating 2.00 g of each sample to a constant weight in a moisture can placed in a hot-air oven maintained at 103 ± 2 °C. The dry matter was used in the determination of the other parameters. In addition, total nitrogen content in 1.0 g samples was determined by the Kjeldahl method including digestion, distillation and titration in the Foss[™] protein 2006 tecater (FOSS Analytical AB, Sweden). Protein content was calculated by using % total nitrogen × 5.27 and 6.25 for soy and Job's tears proteins, respectively. Crude fat was obtained by extracting 1.0 g of each sample in a Soxhlet apparatus (Foss[™] soxtec avani 2055 tecater, FOSS Analytical AB, Sweden) using petroleum ether (boiling point range 40–60 °C) as the extraction solvent. Ash was determined by the incineration of 1.0 g samples placed in a muffle furnace maintained at 550 °C for 5 h. Furthermore, crude fibre was obtained by digesting 1.0 g of sample with H₂SO₄ and NaOH and digesting the residue in Foss[™] fibertec[™] 800 fiber analyzer (FOSS Analytical AB, Sweden). Each analysis was carried out in triplicate.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of rice protein was performed using the method of Ferri et al. (2017) with some modifications. Broken rice flour was mixed with deionized water to obtain 10.0% (w/v) total solids (w/w).

Sodium citrate buffer (pH 7.0) was prepared using 50 mM sodium citrate and then the pH was adjusted with 50 mM citric acid. Hydrolysis of rice solution was carried out by using the proteases Alcalase[®], Flavourzyme[®] and pepsin (2% v/v) at pH 7 for 2 h at 50, 60 and 70 °C. The activity of enzyme was inactivated at 90 °C for 5 min, followed by rapid cooling to room temperature using an ice bath. The solution was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant obtained was termed 'crude hydrolysate' and stored at -20 °C before analysis.

2.6. Plant-based beverages and yogurt-like samples

Soybean and Job's tears grain was boiled at 100 °C for 10 min in order to peel. The peeled soy bean and Job's tears grain was dried at 65 °C for 6 h in a cabinet drier. After drying, soy and Job's tears flour was prepared by using a grain miller and then packed in a high-density polyethylene bag for storage. The plant-based beverages including JB and SB were prepared at 10% w/v soy and Job's tears flour with distilled water, fortified with 5% (w/v) rice protein hydrolysate before being pasteurized at 85 °C for 15 min and cooled to 40 °C. Consequently, the commercial freeze-dried starter culture YF-L812 (250 L: 50 U of DVS culture) was added and thoroughly mixed for 10–15 min. The fermentation was recommended at 45 °C and terminated at 8 h when final pH ranged between 4.5 (Grasso, Alonso-Miravalles & O'Mahony 2020). During fermentation, the samples including JY, SY, RJ, RJY, RSB and RSY were sampled at 0, 1, 2, 4 and 8 h in plastic cups and kept at -20 °C in a freezer before analysis. The fermentation and analysis were performed in triplicate.

2.7. Preparation of yogurt-like product water soluble extracts

A 10 g sample of plant-based beverages and yogurt-like products with and without rice protein hydrolysate was extracted using the method of Shori and Baba (2014) with some modifications. The sample was homogenized with 2.5 ml of sterile distilled water. The pH of samples was determined. Subsequently, the pH of mixture was acidified to pH 4.0 with HCl (0.1 M). The acidified mixture was then heated in a water bath (45 °C) for 10 min followed by centrifugation (5000 g, 10 min, 4 °C). Sodium hydroxide solution (0.1 M) was added to adjust the pH of the supernatant to pH 7.0. The neutralized supernatants were re-centrifuged (5000 g, 10 min, 4 °C) and the supernatant was collected and stored in a -20 °C freezer before analysis.

2.8. Enumeration of LAB

Total LAB enumeration was performed by using 3M Petrifilm[®] Lactic Acid Bacteria Count Plate alternative methodology (3M MN, USA; <https://www.3m.com>). The number of colonies was expressed as log cfu/g.

2.9. Determination of degree of hydrolysis (DH)

The DH was measured using OPA according to the method of Nielsen et al. (2001). The OPA reagent was prepared by dissolving disodium tetraborate decahydrate (7.620 g) and SDS (0.200 g) in deionized water (150 mL), followed by OPA (40 mg/mL in ethanol; 4 mL) and DTT (44 mg/mL in water; 1 mL). The mixture was rinsed and made up to 200 mL with deionized water. The crude hydrolysate sample was prepared in series dilution using deionized water. An aliquot of OPA reagent (3 mL) was mixed with the test sample (400 µL). After the incubation for 2 min at room temperature, the absorbance of the mixture was measured at 340 nm using a spectrophotometer (Biochrom-Libra S22, UK). A deionized water was used as a blank. A standard calibration curve was developed by a sample of 0.97 mM serine (400 µL) and treated as described above. The DH percentage was calculated as the ratio of the number of broken peptide bonds (h) to the total number of bonds per unit weight (h (tot))

(Eq. (1)). Measurement of the sample, blank, and standard was carried out in triplicate.

$$DH (\%) = [h/h(\text{tot})] \times 100 \quad (1)$$

where

h (tot) = total number of bonds per unit (for most proteins this is 8 g equivalents/kg protein)

h = number of peptide bonds broken (calculated by Eq. (2)).

$$\text{Serine-NH}_2 = \frac{(\text{OD sample} - \text{OD blank})}{(\text{OD standard} - \text{OD blank})} \times 0.9516 \text{ meqv/L} \times 0.1 \times \frac{100}{X} \times P \quad (2)$$

where

Serine-NH₂ = serine NH₂/g protein (meqv)

X = sample (g)

P = protein content in sample (%)

0.1 = sample volume (L)

h = (serine-NH₂ - β)/α meqv/g protein (α and β for rice protein are estimated to be 1.00 and 0.40, respectively).

2.10. OPA assay

The peptide content in water-extracted plant-based samples was determined using the OPA method as described by Shori and Baba (2014) with some modifications.

The OPA solution was prepared using 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% (w/w) SDS, OPA (40 mg dissolved in 1 mL of methanol) and 100 μL of β-mercaptoethanol. The final volume was adjusted to 50 mL using distilled water. The reagent was prepared fresh and used within 2 h of preparation. An aliquot of water-extracted plant-based samples (10 μL) was mixed with 1.0 mL of OPA reagent in a 1.5 mL cuvette. The solution was mixed briefly by inversion and incubated for 2 min at room temperature. The absorbance reading was measured at 340 nm (Biochrom-Libra S22, UK). A standard curve of peptide concentration was developed by preparing a different concentration of tryptone standards (0.25, 0.5, 0.75, 1.00, 1.25 and 1.50 mg/mL). The absorbance of the tryptone standard solution was recorded at 340 nm. The concentration of OPA peptides in samples was expressed as mg/g.

2.11. Determination of pH

Samples were homogenized in distilled water (1:9 ratio w/v) prior to determine pH by using a digital pH meter (model DM22, Mettler-Toledo Switzerland).

2.12. Determination of total titratable acidity (TTA)

TTA was determined following the AOAC Method 947.05 (AOAC International, 2006). A 1 mL of water-extracted plant-based sample was mixed with 9 mL of dH₂O. A 2–3 drops of 0.1% phenolphthalein were added as pH indicator. The mixture was then titrated with 0.1 N NaOH under continuous stirring until the development of a consistent pink colour. The amount of acid produced during fermentation was calculated and expressed as the percentage lactic acid equivalent (Eq. (3)).

$$TTA\% = 10 \times V_{\text{NaOH}} \times 0.1 \times 0.009 \times 100 \quad (3)$$

where

10 = dilution factor

V_{NaOH} = volume of NaOH required to neutralize the acid

0.1 = normality of NaOH.

2.13. Determination of polar metabolites by ¹H NMR

Water-extracted plant-based samples were analysed by 1D ¹H NMR using a 500 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operated under full automation.

The test sample was prepared by mixing a 150 μL aliquot of water-extracted plant-based samples with 650 μL of deuterium oxide (99 atom % D) in a 0.5 mm diameter cryogenic NMR probe. The ¹H NMR spectra were baseline-corrected, phase-corrected, aligned and calibrated based on the peak of the internal standard (sodium trimethylsilylpropylsulfonate, DSS). For each spectrum, chemical shift (δ) across the ranges of 0.00–10.00 ppm was segmented (binning) with an interval of 0.04 ppm. Metabolites were identified by means of the Chenomx NMR suite 8.6 library (Chenomx Inc., Alberta, Canada), and from the literature (Lu et al., 2016; Settachaimongkon et al., 2014; Trimigno et al., 2020).

2.14. Determination of total phenolic content (TPC)

The TPC was determined by Shori and Baba (2014) method with some modifications. Briefly, water-extracted plant-based samples (1.0 mL) were mixed with 1.0 mL of 95% ethanol and 5 mL of distilled water. After the mixtures were mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 v/v with distilled water) and incubated for 5 min at room temperature. Na₂CO₃ (1.0 mL, 5% w/v) was then added to the mixture before re-incubation for 60 min at room temperature. The absorbance at 725 nm of the mixture was measured using a UV-visible spectrophotometer (Biochrom-Libra S22, Cambridge, UK). A standard curve of gallic acid (10–50 μg/mL) in methanol was prepared and used for calculating TPC. The result was expressed as μg of gallic acid equivalent (GAE)/mL.

2.15. Determination of antioxidant activity (AOX)

The determination of antioxidant activity was carried out by using the method of Thaipong et al. (2006) with some modifications. A water-extracted plant-based sample (1 mL) was diluted with deionized water (9 mL). The diluted sample (50 μL) was mixed with 50 mM Tris buffer solution (100 μL, pH 7.4) and freshly prepared 0.1 mM DPPH in methanol (850 μL). The test solution was shaken vigorously and incubated in the dark at room temperature for 30 min. The absorbance of test solution at 515 nm was then measured by using a UV-visible spectrophotometer (Biochrom-Libra S22, Cambridge, UK). A standard curve was prepared by using Trolox (5–100 mM TE/mL in methanol). AOX was calculated and expressed as mM TE/mL.

2.16. Determination of ACE inhibitory activity

The determination of ACE inhibitory activity was performed using the method described by Cavalheiro et al. (2020) with some modifications. The procedure is based on the release of hippuric acid from HHL catalysed by the action of ACE. HHL (3.8 mM) was prepared in 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. A water extract (35 μL) was mixed with HHL (3.8 mM, 200 μL). The test solution then incubated at 37 °C for 5 min. The reaction started with the addition of 20 μL of ACE solution (0.1 U/mL in borate buffer). The mixture was re-incubated at 37 °C for 30 min before adding 250 μL of 1 M HCl to terminate the reaction. The hippuric acid formed in the reaction was extracted by the addition of 1.5 mL of ethyl acetate followed by vortexing for 15 s and centrifugation for 10 min at 700 g at 20 °C. After centrifugation, 1 mL of the organic phase (ethyl acetate) was transferred to a new tube and evaporated in a water bath for 30 min at 100 °C. The remaining residue was dissolved in 1 mL of ultrapure water and the absorbance of the solution was measured at 228 nm in a UV-visible spectrophotometer (Biochrom-Libra S22, Cambridge, UK) using ultrapure water as a blank. The percentage of ACE inhibition was calculated according to Eq. (4):

$$\text{ACE inhibition (\%)} = [1 - (A - C)/(B - D)] \times 100 \quad (4)$$

where

A is absorbance with ACE, HHL and sample; B is absorbance with ACE and HHL without sample; C is absorbance with HHL and sample; and D is absorbance with HHL without ACE and sample.

2.17. Statistical analysis

All experiments were performed and assays were determined in triplicates. The results were expressed as the mean \pm standard deviation. One-way analysis of variance (ANOVA) for means comparison was used for determining significant difference at $p < 0.05$ by Duncan test. In addition, Pearson's correlation (R^2) formulas are calculated to find how strong a relationship is between data including OPA peptides and ACE inhibition. Both statistical procedures were performed using the IBM SPSS statistics package version 19 (SPSS Inc., Chicago, IL, USA). Metabolomics data from 1D ^1H NMR were normalized by a Pareto normalization procedure before multivariate statistical analysis. Principal component analysis (PCA) and heat-map visualization were performed using MetaboAnalyst 5.0 <https://www.metaboanalyst.ca>

3. Results and discussion

3.1. Proximate analysis of raw materials

The chemical composition of soy, Job's tears and broken rice was significant difference ($p < 0.05$) (Table 1). Soy flour and Job's tears flour contained a protein content of 36.74 % DW and 15.24 % DW, respectively while broken rice flour had 7.22 % DW of protein. The fat content of soybean ranged from 13.2 % DW to 24.6 % DW and mean fat concentration was 19.5 % DW. In addition, Job's tears flour and broken rice flour had 6.82 % DW and 0.18 % DW fat content, respectively.

The content of fat and protein in soy flour was consistent with that reported by Assefa et al. (2019). The protein concentration ranged from 27.3 % DW to 45.4 % DW. Similarly, Luithui and Meera (2019) reported a protein content of 12.66 % DW to 13.64 % DW and fat content of 4.28 % DW to 5.36 % DW in Job's tears flour. In addition, soy flour contained 34.49 % DW carbohydrate while higher carbohydrate content, 71.0 % DW and 89.38 % DW, was observed in Job's tears and rice flours, respectively.

Broken rice flour had a protein content of 7.22%, two-fold and five-fold lower than that in soy and Job's tears flours, respectively. A lower protein content in rice flour means it might not be a good protein source to develop plant-based yogurt-like products. Unlike soy flour, rice flour does not contain phytoestrogens, protein allergens and is derived from non-genetically modified rice (Bocquet et al., 2019). Therefore, the potential of rice protein as a raw material for developing a functional ingredient is a good alternative choice.

3.2. Rice protein hydrolysate

Rice protein was hydrolysed using the proteolytic enzymes including Alcalase[®], Flavourzyme[®] and pepsin at pH 7, for 2 h and at 50, 60 and

Table 1. Proximate analytical results of raw materials.

Chemical composition (% DW)	Raw materials		
	Soy flour	Job's tears flour	Broken rice flour
Carbohydrates	34.49 \pm 0.04 ^c	71.0 \pm 0.25 ^b	89.38 \pm 0.20 ^a
Proteins	36.74 \pm 0.31 ^a	15.24 \pm 0.22 ^b	7.22 \pm 0.12 ^c
Fats	18.84 \pm 0.25 ^a	6.82 \pm 0.02 ^b	0.18 \pm 0.01 ^c
Moisture	9.31 \pm 0.08 ^b	2.18 \pm 0.09 ^c	11.89 \pm 0.11 ^a
Crude fibre	4.87 \pm 0.15 ^a	3.30 \pm 0.02 ^b	1.65 \pm 0.01 ^c
Ash	5.05 \pm 0.02 ^a	3.55 \pm 0.13 ^b	1.56 \pm 0.01 ^c

All data are expressed as the mean \pm SD (n = 3).

^{a-c} Different letters within the same row indicate a significant difference ($p < 0.05$).

70 °C, respectively. The DH ranged from 12.69% to 33.96% (Table 2). The DH indicates the percentage of bonds available for proteolytic hydrolysis that were actually cleaved; it is a standard parameter for monitoring the level of proteolysis (Samaei et al., 2021). At 60 °C the DH was higher for Alcalase[®] than for Flavourzyme[®] and pepsin. In order to extend the range of applications of rice protein ingredients in food products, hydrolysed rice protein ingredients with a DH ranging from 17.7% to 24.2% have been developed using enzymatic hydrolysis (Amagliani et al., 2017).

The TPC, including phenolic compounds and phenolic amino acids of peptides, in the hydrolysate is shown in Table 2. Rice protein hydrolysate contained a TPC ranging between 0.94 μg GAE/mL in pepsin rice protein hydrolysate at pH 7 and 70 °C to 7.33 μg GAE/ml in Alcalase[®] rice protein hydrolysate at pH 7 and 60 °C. In addition, the antioxidant properties of hydrolysates were evaluated by DPPH scavenging assays. Antioxidant activity of rice protein hydrolysate varied from 20.44 mM TE/mL to 27.99 mM TE/mL. The highest AOX was observed in pepsin rice protein hydrolysate at pH 7 and 60 °C. In addition, enzymatic hydrolysis increased the antioxidant properties of proteins extracted from rice bran (Thamnarathip et al., 2016a; Thamnarathip et al., 2016b). Samaei et al. (2021) reported that the hydrolysis of protein allowed the release of specific and inactive protein fragments within the parent proteins, becoming active when free in the solution.

In addition, the management of human blood pressure related to ACE inhibitory activity. An *in vitro* ACE-inhibitory activity assay was used to screen the anti-hypertensive potential of hydrolysates produced using different protease enzymes and hydrolysis temperatures. Hydrolysates, produced from Alcalase[®], showed ACE inhibitory activity (69.61%) followed by those from Flavourzyme[®] (56.86%) and pepsin (22.55%). The ACE activity of Alcalase[®] protein hydrolysate was the highest (69.61%) when the hydrolysis was carried out at pH 7 and 60 °C.

A higher significant relation coefficient (R^2) between DH and TPC (0.73, $p < 0.05$) and DH and ACE (0.74, $p < 0.05$) was observed. The hydrolysis of broken rice by using protease had a strong positive effect on the ACE inhibitory activity. Several reports in the literature indicated that ACE inhibitory activity increased as the MW of the peptide fractions decreased and was dependent on the presence of hydrophobic residues (aromatic or branched-chain) and on their position in the amino acid sequence (Aluko, 2015; Cavalheiro et al., 2020).

However, a non-significant relation coefficient between DH and AOX (0.05, $p \geq 0.05$) was found. An inverse correlation between DH and AOX for the protein extracted from hemp bran and hydrolysed with different enzymes was also observed (Das, 2015). During hydrolysis, a mixture of small peptides and free amino acids is formed. Their antioxidative properties depend on the size of the peptides generated and the nature and sequence of their amino acid residues. In these mixtures, low-MW peptides are generally the most active for specific factors (easier steric interaction with the substrate, higher hydrophilicity and solubility, exposure of hydrophobic side chains) and their combination (Samaei et al., 2021).

In addition, hydrolysis of proteins using enzymes including Alcalase[®], Flavourzyme[®] and Neutrase[®] significantly ($p < 0.05$) affected the properties of Riceberry bran protein hydrolysate including protein content, protein yield, TPC and antioxidant activity (ABTS and FRAP), whereas the hydrolysis duration had no significant effect ($p \geq 0.05$) on those properties (Thamnarathip et al., 2016a).

Interestingly, the significant relation coefficient (R^2) between TPC and ACE was 0.81 ($p < 0.05$). Therefore, the optimal hydrolysis conditions, providing the highest ACE inhibition, using Alcalase[®] at pH 7 and 60 °C, were used to obtain rice protein hydrolysate for fortifying plant-based beverages before LAB fermentation. Alcalase[®] is an alkaline endopeptidase. It hydrolyses glutenin, the main protein accounting for 60%–80% of the total protein in rice (Pantao et al., 2020).

3.3. LAB cell count

Changes in the viability of LAB cells in plant-based yogurt-like products with and without hydrolysate are shown in Figure 1 (A). The

Table 2. Rice protein hydrolysis conditions, total phenolic content, antioxidant activity and ACE inhibition.

Enzyme	pH	Temperature (°C)	Degree of hydrolysis DH (%)	Total phenolic content, TPC (µg GAE/mL)	Antioxidant activity, AOX (mM TE/mL)	ACE inhibition (%)
Alcalase®	7	50	26.47 ± 1.44 ^c	6.62 ± 0.02 ^b	23.39 ± 1.04 ^{bc}	29.41 ± 5.09 ^{de}
	7	60	33.96 ± 2.96 ^a	7.33 ± 0.17 ^a	25.38 ± 2.46 ^{ab}	69.61 ± 4.49 ^a
	7	70	29.58 ± 1.54 ^{bc}	3.38 ± 0.13 ^d	26.15 ± 0.36 ^{ab}	17.65 ± 2.94 ^f
Flavourzyme®	7	50	33.94 ± 1.31 ^a	3.32 ± 0.07 ^d	27.33 ± 0.51 ^a	37.25 ± 4.49 ^d
	7	60	31.01 ± 2.93 ^{ab}	6.47 ± 0.13 ^b	25.30 ± 2.06 ^{ab}	56.86 ± 8.11 ^b
	7	70	30.89 ± 2.68 ^{ab}	5.34 ± 0.34 ^c	20.44 ± 1.49 ^c	48.04 ± 3.40 ^c
Pepsin	7	50	17.44 ± 0.78 ^d	1.76 ± 0.07 ^f	22.98 ± 0.18 ^{ab}	19.61 ± 7.40 ^f
	7	60	16.50 ± 3.00 ^d	2.98 ± 0.07 ^e	27.99 ± 3.26 ^a	22.55 ± 1.70 ^{ef}
	7	70	12.69 ± 1.43 ^e	0.94 ± 0.03 ^g	25.27 ± 0.72 ^{ab}	13.73 ± 3.40 ^f

All data are expressed as the mean ± SD (n = 3).

^{a-g} Different letters within the same column indicate a significant difference (p < 0.05).

number of LAB colonies in JB, SB, RJB and RSB were significantly (p < 0.05) lower than those in JY, SY, RJY and RSY. In general, the LAB cell count increased significantly (p < 0.05) during fermentation, reaching values that were, on average, 1 log cycle higher at the end of the fermentation. There were 5.40, 5.40, 5.45 and 5.46 log cfu/g of LAB in JY, SY, RJY and RSY at 8 h. Fermentable sugars and proteins are growth factors for LAB. Broken rice hydrolysate has also been used as additional carbon source for biomass and pigment production (Cai et al., 2021). In addition, Donkor et al. (2005) reported the on average, the fermentation

time ranged between 6 h and 6.5 h for probiotic soy yogurt and between 9 h and 9.5 h for the control soy yogurt, longer than the times observed for milk-based yogurt.

3.4. Changes in pH

Figure 1 (B) shows pH value of yogurt was measured during fermentation. No significant differences (p ≥ 0.05) in initial pH, ranging between 6.53 to 6.81, were recorded for any of the plant-based

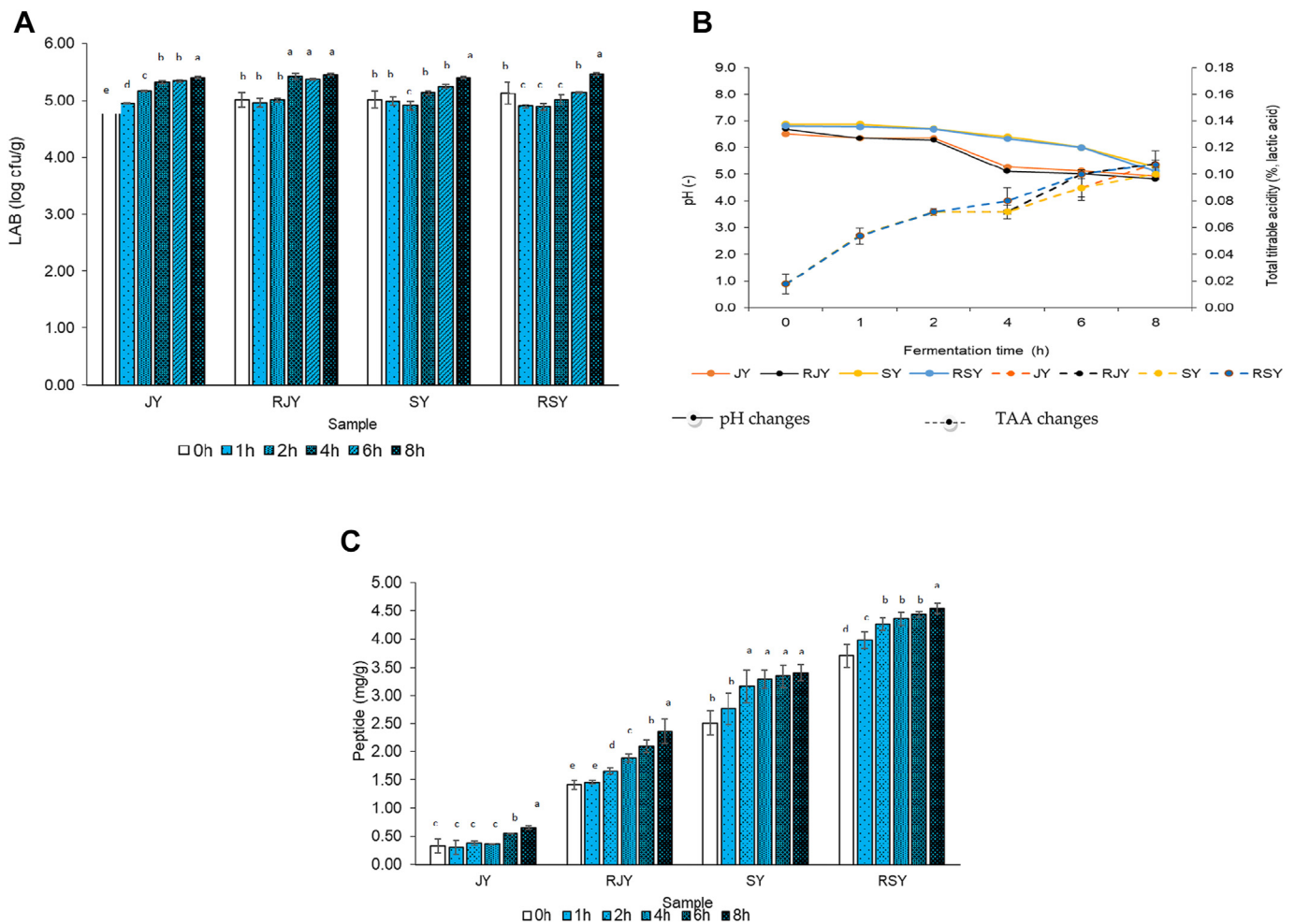


Figure 1. Shows Lactic acid bacteria cell viability of plant-based yogurt-like products during fermentation (A); Changes of pH and total titratable acidity during fermentation Job's tears and soy yogurt-like products with and without hydrolysate (B) and OPA peptides during fermentation Job's tears and soy yogurt-like products with and without hydrolysate (C). ^{a-e} Different letters above the bars indicate a significant difference (p < 0.05). JY = Job's tears yogurt-like product; RJY = Rice protein hydrolysate-fortified Job's tears yogurt-like product; SY = Soy yogurt-like product and RSY = Rice protein hydrolysate-fortified soy yogurt-like product.

beverages. The pH value of JB (6.53) and RJB (6.69) was lower than that of SB (6.87) and RSB (6.81). There was a lag phase of about 4 h at the beginning of the incubation before the pH of plant-based yogurt-like products started to reduce. In addition, the pH value of plant-based yogurt-like products began to reduce significantly ($p < 0.05$) from 4 h to 8 h. The fortification of hydrolysate into RJB and RSB was not shown to reduce pH value in RJY and RSY. The JB and RJB samples showed lower pH readings than those of SB and RSB. All yogurt-like products, including JY, RJY, SY and RSY, reached constant pH at 8 h. At the end of fermentation, the final pH values varied from 4.82 to 5.28. Grasso, Alonso-Miravalles and O'Mahony (2020) reported that the final pH values of commercial yogurt-like products including soy, coconut, cashew, almond and hemp ranged between 3.99 to 4.58.

3.5. Changes in TTA

The TTA of a fermented dairy product measures the equivalent percentage (%) of lactic acid present in the yogurt during fermentation. An almost linear increase in TTA for JY, RJY, SY and RSY during fermentation was observed (Figure 1 (B)). In general, pH values decreased while the concentration of TTA (% lactic acid) increased, indicating the efficiency of LAB for lactic acid fermentation of plant-based beverages. The TTA for all plant-based yogurt-like products showed no significant differences ($p \geq 0.05$) during the 8 h of fermentation. LAB had the same rate of TTA production during fermentation. The level of lactic acid was increased significantly ($p < 0.05$) every hour, approaching its maximum values of 0.11% (lactic acid) for all samples except SY.

3.6. Concentration of OPA peptides

The concentration of OPA peptides in JY, RJY, SY and RSY was significantly increased ($p < 0.05$) during fermentation (Figure 1 (C)). JB and SB had 0.32 and 2.51 mg/g of OPA peptides, respectively. When the hydrolysate was fortified into JB and SB at hour 0, there was a 340.87% increase in OPA peptides in fresh JB compared to fresh RJB and a 84.84% increase in fresh SB compared to fresh RSB. The concentrations of OPA peptides in JY and RJY at hour 8 of fermentation were lower ($p < 0.05$) than those of SY and RSY. At the end of fermentation, the OPA peptide concentration was increased by 104.29%, 67.38%, 35.66% and 22.56%, with regard to JY, RJY, SY and RSY, respectively. The highest OPA peptide content was observed in RSY (4.54 mg/g), followed by SY (3.40 mg/g).

3.7. Polar metabolites determined by ^1H NMR

Polar metabolite profiles were determined by ^1H NMR. A total of 11 metabolites, including carbohydrates and organic acids, were identified based on the Chemomx database and previous studies including those of Settachaimongkon et al. (2014), Lu et al. (2016) and Trimigno et al. (2020).

The primary role of dairy starter cultures is the acidification of milk by converting lactose into lactic acid (Settachaimongkon et al., 2014). In our study, LAB including *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* exhibited acidifying capacity in plant-based beverages. The main carbohydrates including glucoses and maltose were converted into lactate in JY, RJY, SY and RSY. In addition, lactate, acetate and ethanol can be simultaneously generated from their carbohydrate metabolism (Trimigno et al., 2020). An increase in metabolites during fermentation could directly indicate the activity of LAB. This was confirmed by lower pH, more colonies of LAB and a higher concentration of lactic acid (TTA) as previously mentioned. Increases in other organic acids and free amino acids were also observed in JY, RJY, SY and RSY.

3.8. PCA and heat maps

For multivariate analysis, a total of 214 bins were introduced as variables for the analyses. The similarities and differences between Job's

tears products are displayed by the overall PCA score plot (Figure 2 (A)). The first principal component (PC1) accounted for 51.3% of the total variance while the second principal component (PC2) accounted for 18.7% of the variance. PC1 and PC2 explained 74.4% of the total variance. The clear distinction among JB, JY, RJB and RJY samples was rather larger and they could be separated. In addition, it was evident that the metabolite profiles of the soy samples could be distinguished along the separate PCA score plot (Figure 2 (B)). The soy samples fermented with and without hydrolysate were clearly separated from each other. The results indicate metabolic activity of LAB during the lacto-fermentation of plant-based yogurt-like products, which corresponds with a distinctive polar metabolite profile, as demonstrated by multivariate analysis.

Heat-map visualization combined with hierarchical cluster analysis is shown in Fig.2 (C) and (D) for Job's tears and soy products, respectively. The results show that JB and SB contained a high abundance of metabolites in the sugar group including sucrose, glucose and maltose. During lacto-fermentation, decreases in metabolites in the sugar group were observed in all beverage samples without hydrolysate (JB and SB) and corresponded with an increase in lactate concentration. Increases in other organic acids (e.g. acetate, pyruvate) and free amino acids including phenylalanine, alanine, tyrosine, leucine and proline were clearly observed in the samples with hydrolysate and fermented with LAB. Trimigno et al. (2020) indicated that high concentrations of pyruvate are found in the lacto-fermentation of dairy yogurt. Moreover, acetate is a product in the metabolism of pyruvate. Two important aroma compounds, diacetyl and acetoin, were produced from the metabolism of pyruvate. Alanine is produced via proteolysis by LAB.

Metabolites including the sugar group and amino acids were detected in high abundance in the plant-based samples with hydrolysate (RJB and RSB). Lacto-fermentation of RJB and RSB resulted in an increase in lactate concentration. However, sugars and amino acids were detected in high abundance in RJY and RSY. Similar to JB and SB, the samples with hydrolysate and fermented with LAB contained organic acids and free amino acids.

The dendrogram shows that Job's tears and soy samples with and without hydrolysate could be well grouped according to their metabolite profiles. Similar to what was observed for PCA, the plant-based samples with and without hydrolysate and fermented with LAB could be clearly assigned into different clusters.

3.9. TPC

Figure 3 (A) shows the TPC in plant-based beverages and yogurt-like products. The initial TPC of fresh JB, RJB, SB and RSB was 5.53, 9.84, 12.91 and 15.73 μg GAE/mL, respectively. In general, LAB fermentation led to higher values of TPC in plant-based yogurt-like products compared to the initial values. At the end of fermentation, the TPC concentration was increased by 10.53%, 5.26%, 24.31% and 20.58%, with regard to JY, RJY, SY and RSY, respectively. The highest TPC was observed in RSY (18.96 μg GAE/mL), followed by SY (16.05 μg GAE/mL). Fermentation of cooked soy meal by *Bacillus subtilis* and simulated *in vitro* gastrointestinal digestion causes the release of microbial enzymes, enhancing the content of phenolics and flavonoids including genistin, daidzin, glycitin and malonylgenistin (Dajanta et al., 2013; Freitas et al., 2019). Similarly, it seems that lactic acid fermentation enhances the conversion of phenolic compounds and the depolymerization of phenolic compounds with a high molecular weight (Rodríguez et al., 2009).

3.10. AOX

AOX, measured using the DPPH scavenging assay, is shown in Figure 3 (B). The results show that DPPH scavenging inhibition increased during the fermentation of JB, RJB, SB and RSB. At the end of fermentation, DPPH inhibition was increased by 76.69%, 9.19%,

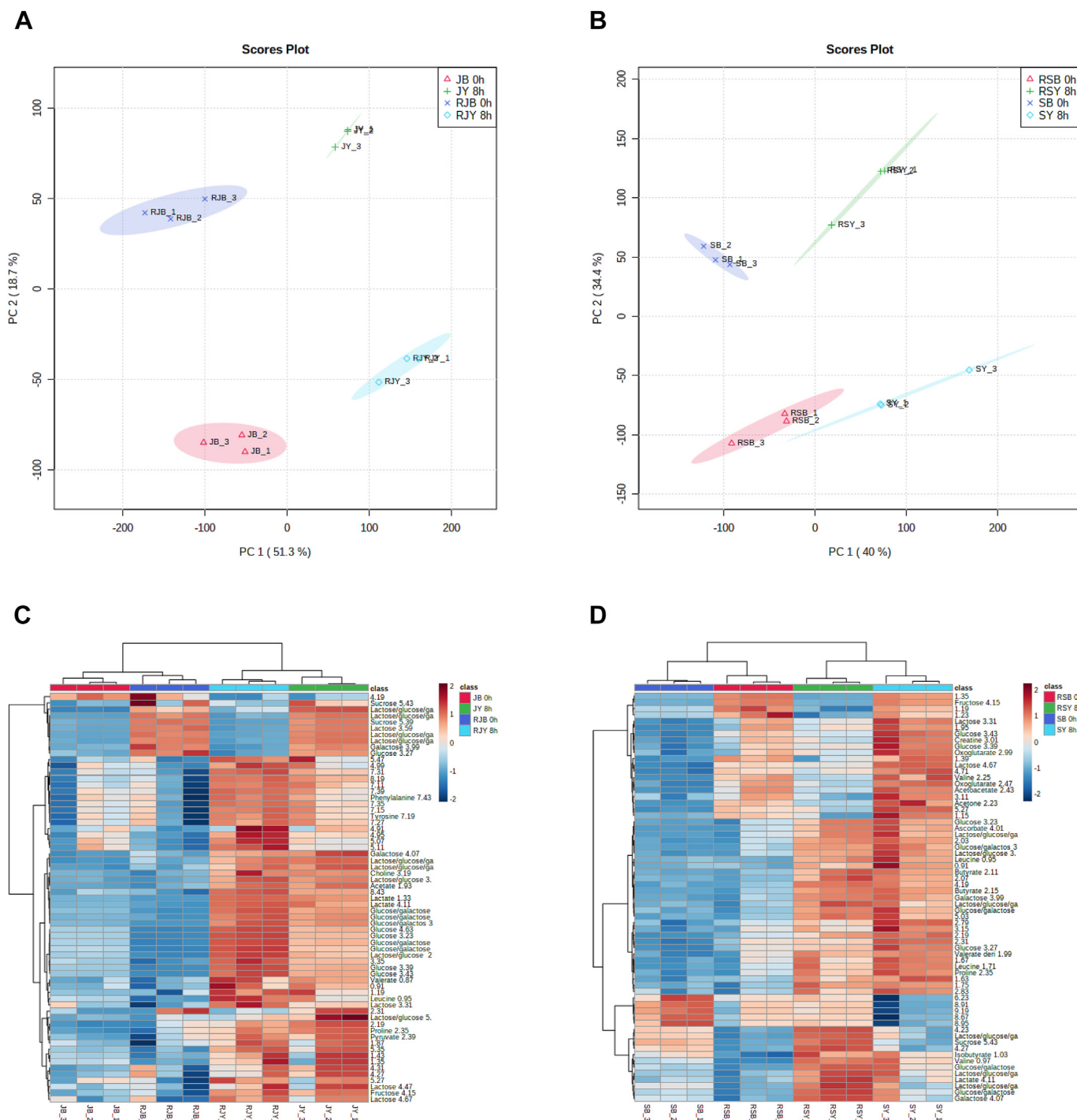


Figure 2. Displays PCA score plots for overall comparison of Job's tears products with and without hydrolysate (A); PCA score plots for overall comparison of soy products with and without hydrolysate (B); Heat map and hierarchical clustering of metabolite profiles from Job's tears products (C); Heat map and hierarchical clustering of metabolite profiles from soy products (D). JB = Job's tears beverage; JY = Job's tears yogurt-like product; RJB = Rice protein hydrolysate-fortified Job's tears beverage; RY = Rice protein hydrolysate-fortified Job's tears yogurt-like product; SB = Soy beverage; SY = Soy yogurt-like product; RSB = Rice protein hydrolysate-fortified soy beverage; RSY = Rice protein hydrolysate-fortified soy yogurt-like product. Red indicates a higher content of the corresponding compound while blue indicates a lower content of the corresponding compound.

32.81% and 18.130%, with regard to JY, RY, SY and RSY, respectively. The highest DPPH inhibition was observed in RSY (30.04 mM TE/mL), followed by RSB (25.34 mM TE/mL). These results indicated that the AOX activity of plant-based yogurt-like products was enhanced by the fermentation. In addition, Cáceres et al. (2019) reported fermentation significantly ($p < 0.05$) enhanced TPC and AOX in yogurt-like products from germinated brown rice compared to their non-fermented counterparts.

3.11. ACE inhibitory activity

All plant-based beverages and yogurt-like products with and without protein hydrolysate had *in vitro* ACE inhibitory activity as shown in Figure 3 (C). The percentage of ACE inhibition by JB and RJB was significantly lower than that by SB and RSB. LAB fermentation of JB and RJB resulted in an increase of ACE inhibition in JY and RY, by 15.87% and 19.52%, respectively. ACE inhibition of RJB and RY had no

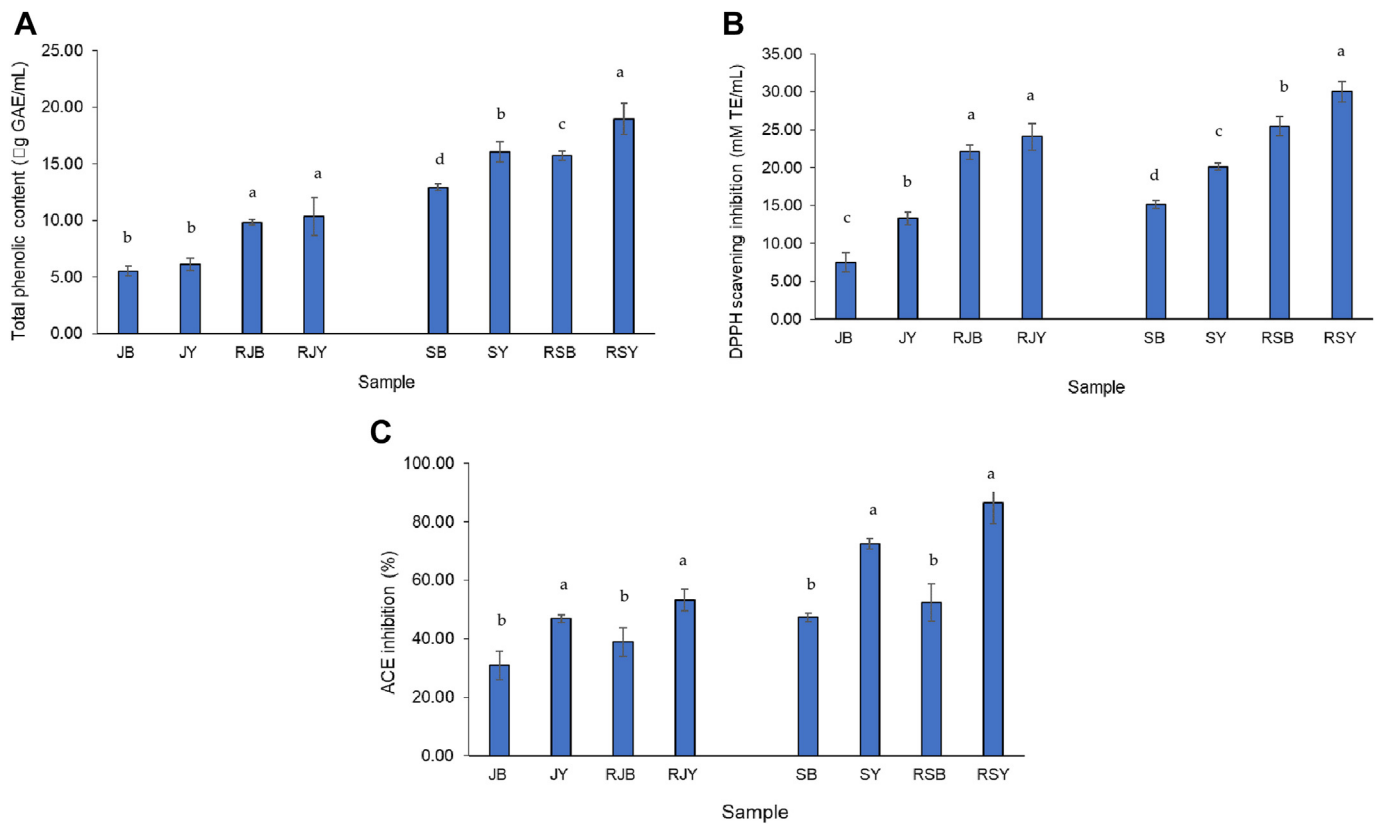


Figure 3. Shows Total phenolic compounds (A); Antioxidant activity (B); ACE inhibitory activity (C). ^{a-d} Different letters above the bars indicate a significant difference ($p < 0.05$). JB = Job’s tears beverage; JY = Job’s tears yogurt-like product; RJB = Rice protein hydrolysate-fortified Job’s tears beverage; RJY = Rice protein hydrolysate-fortified Job’s tears yogurt-like product; SB = Soy beverage; SY = Soy yogurt-like product; RSB = Rice protein hydrolysate-fortified soy beverage; RSY = Rice protein hydrolysate-fortified soy yogurt-like product.

significant effect ($p \geq 0.05$). In addition, the ACE inhibitory activity of soy beverage and soy yogurt-like products showed significant differences ($p > 0.05$). Interestingly, the percentage of ACE inhibition of SB and RSB increased by 25.10% and 34.13%, respectively after LAB fermentation. Higher ACE inhibitory activity of 86.51% and 72.43% was observed in soy yogurt-like product with and without hydrolysate, respectively. Donkor et al. (2005) reported that probiotic soymilk fermentation resulted in 69.7% ACE inhibition at the end of a 28-day storage period. A significant strongly positive correlation ($R^2 = 0.84$) between the OPA peptides and ACE inhibition was observed, indicating that the extent of ACE inhibition might have been partially dependent on the extent of the proteolytic activity.

3.12. Pearson correlation matrix

Table 3 shows Pearson correlation matrix for plant-based beverage and yogurt-like products with and without rice protein hydrolysates. A

Table 3. Pearson correlation matrix for plant-based beverage and yogurt-like products with and without rice protein hydrolysates.

	TPC	OPA	AOX	ACE
TPC ^a	-	0.96 ^b	0.77	0.81
OPA	-	-	0.83	0.83
AOX	-	-	-	0.70
ACE	-	-	-	-

TPC = Total phenolic content; OPA = OPA peptides; AOX = Antioxidant activity; ACE = ACE inhibition.

^a n = 24.

^b Significant level at $p < 0.05$.

correlation (R^2) between TPC and OPA, AOX and ACE was significant ($p < 0.05$). ACE inhibition correlated significantly ($R^2 = 0.83$, $p < 0.05$) with OPA peptide values. Pinciroli et al. (2019) reported that peptides in the majority rice proteins are capable to interact with the sites of both ACE enzyme and inhibits its function.

4. Conclusion

The LAB fermentation of plant-based beverages fortified with broken rice protein hydrolysate resulted in higher concentrations of released bioactive ACE inhibitors and metabolites, providing plant-based protein alternatives to yogurt of high quality and with health benefits. The percentage of ACE inhibition of plant based-beverages increased by 51.29%–53.04% after LAB fermentation while higher ACE inhibitory activity of 86.51% and 72.43% was observed in soy yogurt-like products with and without hydrolysate, respectively. This improvement of ACE inhibition in yogurt-like products is partly due to more free peptides. At the end of fermentation, the OPA peptide concentration was increased by range 22.56%–35.66% and 67.38%–104.29% for soy and Job’s tears yogurt-like products with and without rice hydrolysate, respectively. The significant correlation ($R^2 = 0.83$, $p < 0.05$) between ACE inhibition and OPA peptide values was observed. In addition, the high abundance of metabolites in the sugar group and those containing nitrogen provided by the protein hydrolysate affected the acidification capacity of LAB and the metabolite profile in yogurt-like products. The addition of protein hydrolysate could be considered as a technological strategy to enhance the bioactive potential of yogurt-like products. Further studies using tandem MS-based proteomics to confirm the identification of bioactive peptides obtained from the lacto-fermentation of plant-based products and *in vivo* studies using animal and/or human models are also recommended.

Declarations

Author contribution statement

Prinya Wongsu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kittikan Yuenyongrattanakorn: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Waranya Pongvachirint, Anusara Auntalarok: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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