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Sprouting alters metabolite and peptide contents in the gastrointestinal digest of soybean and enhances in-vitro anti-inflammatory activity

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ABSTRACT

Sprouting of soybeans can enhance the release of health-beneficial bioactive compounds, especially peptides, and metabolites, while gastrointestinal (GI) digestion alters their biotransformation and bioaccessibility. The present study aimed to evaluate the effect of soybean sprouting and GI digestion in modulating its anti-inflammatory activity. Soybeans were soaked in water overnight (Day 0) and sprouted for two and four days, subjected to simulated GI digestion, and human intestinal epithelial cells (Caco-2) were pretreated (2 h) with soybean sprout digest (SSD: 1000 μg/mL) before inflammation induction with IL-1β. Pre-treatment with Day 4 SSD specifically reduced the secretion of cytokine IL-8 by 19.5%. Sprouting for four days and GI digestion significantly increased the abundance of metabolites, including valine, isoleucine, citrulline, and trigonelline. Furthermore, the abundance of peptides with polar-hydrophilic and charged amino acids was explicitly accumulated in the Day 4 SSD up to 6-fold. These metabolites and peptides are potentially responsible for the observed anti-inflammatory effects.

1. Introduction

Soybean is one of the most used sources of plant proteins, which also contributes to a vast array of health benefits upon consumption (Eum et al., 2020). Soybean-derived phytochemicals and peptides can exhibit health-beneficial biological activities above and beyond their known nutritional value upon oral administration, followed by gastrointestinal (GI) digestion and absorption (Beermann, Euler, Herzberg, & Stahl, 2009; Minh, 2015). Earlier studies suggest that health-beneficial bioactive peptides and metabolites can be produced during GI digestion (Lo & Li-Chan, 2005; Nkhata, Ayua, Kamau, & Shingiro, 2018; Nolasco et al., 2021). Soybeans are a significant source of bioactive peptides and isoflavones, a class of flavonoids known for their health-beneficial biological activities (Xu & Chang, 2008). Therefore, soybean-derived peptides and metabolites potentially stimulate numerous health-beneficial physiological responses such as hypocholesterolemic, anti-inflammatory, anti-atherosclerotic, and immunomodulatory effects (Lammi, Zanoni, & Arnoldi, 2015; Lee et al., 2018; Mace et al., 2019).

Soybean is primarily consumed in two forms, raw or extracted soy products (e.g., fried, dried, or roasted whole soybeans, soy powder, soy protein isolate, soymilk, soybean oil, and soy butter), and fermented soy products (e.g., soy sauce, tofu, soy yogurt, and miso soup) (P. Singh, Kumar, Sabapathy, & Bawa, 2008). Along with these, in many cultures around the world, sprouting legume seeds is one of the methods to enhance the nutritive value and increase the health-beneficial biological compounds which are often associated with fermented soy products (Geng et al., 2022). During sprouting, the seed uses its reserve materials to synthesize cell constituents, causing a significant change in the biochemical characteristics (Ohanenye, Tsopmo, Ejike, & Udenigwe, 2020). Sprouting of soybean for 18 h or longer activates the endogenous proteases involved in the cleavage of storage proteins, which can facilitate the release of peptides (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018).

Additionally, the sprouting of soybean raises the levels of free amino acids (AAs), carbohydrates, dietary fibers, and other metabolites, subsequently increasing the density of the health-beneficial bioactive compounds (Ohanenye et al., 2020; Singh, Singh, Shevkani, Singh, &

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Kaur, 2017). The sprouting process could enhance the liberation of novel bioactive compounds upon consumption, primarily through GI digestion (Ohanenye et al., 2020). The present study hypothesized that soybean sprouting would facilitate the release of compounds with health-beneficial biological activities in a synergistic action with GI digestion; these bioactive compounds could potentially reduce chronic inflammation, one of the primary factors of developing metabolic disorders (Ginsberg & MacCallum, 2009). Chronic low-grade inflammation in the GI tract contributes substantially to developing systemic inflammation (Uhlig, 2013). Epithelial cells constitute one of the first barriers to pathogens and detrimental metabolites present or generated in the gut. The barrier can be compromised when inflammation, triggered by cytokines, induces cell apoptosis and intestinal barrier breach (Su et al., 2013). Therefore, the investigation of the anti-inflammatory activities of sprouted soybeans in the GI epithelial cells, one of the first cell types that dietary compounds come into contact with, is critical to prevent metabolic disorders. Thus, this study compared the anti-inflammatory activities in GI epithelial cells and chemical profiles of GI-digested soybean and its sprout to test this hypothesis.

2. Materials and methods

2.1. Sample preparation and soybean sprouting

Soybeans (*Glycine* max) used in this study were purchased from Ture Leaf Market (Salt Lake City, UT, USA), the genotype and variety of the soybeans are not known and represent a limitation of the study. The seeds were washed twice and soaked in tap water for 16 h at 25 °C in the dark. The seeds were transferred onto a wet paper towel and incubated at 25 °C in the dark for up to 5 days. The soybean sprouts were aligned on graph paper, and the image was taken every day after imbibition at the same time. The length of the soybean sprouts was measured using ImageJ by measuring the length of the lines drawn along the sprout roots compared to the scales on the graph paper. The root became ~ 7 cm long at the end of the incubation period. According to the size for dietary use, we decided to harvest on day 2 and day 4 for further analysis (Figure S1).

During the sprouting process, six pools of five samples were harvested as dry seed, day 0 (right after the 16 h of soaking), day 2 (48 h after soaking), and day 4 (96 h after soaking) and were flash-frozen in liquid nitrogen for future analysis of the chemical composition. Another 10 g of sprouts from each day were harvested for GI digestion without freezing. The experiment was repeated twice.

2.2. Metabolite profiling of soybean sprout and soybean sprout digest (SSD)

Metabolite contents were analyzed by gas chromatography-mass spectrometry (GC–MS; 7200 GC-QTOF system, Agilent, Santa Clara, CA) using the metabolite profiling protocol described in our previous study (Wase, Abshire, & Obata, 2022). Briefly, the frozen soybean samples were ground to a fine powder under liquid nitrogen temperature. Metabolites were extracted from 50 mg of the materials with 1.4 mL of methanol at 70 °C and mixed with 1.5 mL of water and 750 μL chloroform. Following phase separation, 50 μL aliquots of the upper aqueous fraction were dried to be derivatized by methoxyamination and trimethylation. Ribitol was added to the extraction solution as an internal standard, and a mixture of fatty acid methyl esters was included in the derivatization solution for retention time calibration. The GC–MS run was conducted in the TOF mode without MS/MS analysis. The peaks in the mixed samples were annotated to metabolites depending on their retention indexes and mass spectrum using MassHunter Unknowns analysis (Agilent) with manual curation. The peak heights of the representing fragments of annotated metabolites were determined in each sample by MassHunter Quantitative Analysis (Agilent). Following blank subtraction, the peak heights were normalized by that of ribitol and precise sample fresh weight to calculate the relative metabolite contents. The relative metabolite contents were further normalized by the mean of Day 0 samples and $\log_2$ transformed for the following analyses.

2.3. Simulated gastrointestinal digestion of sprouted soybeans

Soybean sprouts at 0, 2, and 4 days of sprouting were subjected to *in-vitro* GI digestion as detailed in our previous study (Nolasco et al., 2021). The soybean sprouts were minced with a manual mincer (Kitchen Basics, ASIN: B00JX0ENHE) to mimic the human chewing process. Further samples hydrolysis included an oral phase in which the oral bolus consisted of the chewed soybean sprout, simulated salivary fluid, $CaCl_2$, and α-amylase from *Bacillus subtilis* (02100447-CF, MP Biomedicals, Santa Ana, CA) at a concentration of 75 U/mL of the oral phase volume. The oral bolus was stomached for 2 min and transferred to a jacketed beaker for further gastric hydrolysis. The oral bolus was mixed with simulated gastric fluid and $CaCl_2$. The solution pH was reduced and stabilized at 3 using a Titrando 902 pH-stat coupled with an 800 dosino device (Metrohm AG, Herisau, Switzerland). Later, pepsin from porcine gastric mucosa (P7012, Sigma Aldrich, St. Louis, MO) was added at 2000 U/mL to the gastric phase volume. The pepsin hydrolysis reaction was conducted for 2 h and titrated at pH 3. The total HCL consumed was recorded through Tiamo software (Metrohm AG, Herisau, Switzerland). The gastric digestion was terminated by increasing and stabilizing the pH to 7. Simulated intestinal fluid, $CaCl_2$, bile extracts porcine (B8631, Sigma Aldrich) at 10 mM in the intestinal phase volume was added. Following the pH stabilization, Pancreatin (P1750, Sigma Aldrich) was added, and intestinal digestion was conducted for 2 h. The pH was reduced to 6 to inactivate the intestinal digestion, and the SSD was centrifuged to obtain the supernatant containing the soluble peptides and metabolites. The supernatant was frozen and freeze-dried for future analysis.

The Degree of hydrolysis (DH) for both gastric and intestinal phases was calculated using the following equations:

Equation (1). Degree of hydrolysis in the gastric phase. $DH_{gastric}$

$$DH_{gastric} = 100 \times \frac{V(HCl) \times N(HCl)}{m(protein) \times h_{tot}} \times \frac{1}{(1 - \alpha(COOH))} \quad (1)$$

Source: (Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018).

Equation (2). Degree of hydrolysis in the intestinal phase. $DH_{intestinal}$

$$DH_{intestinal} = 100 \times \frac{V(NaOH) \times N(NaOH)}{m(protein) \times h_{tot}} \times \frac{1}{\alpha(NH_2))} \quad (2)$$

Note: where V is the volume of titrant (mL), N is normality (meq/mL), m is the protein mass (g), and h_{tot} is the number of peptide bonds per gram of proteins. α (NH2) = 0.44, α (COOH) = 0.09 (Mat et al., 2018). Equation Source: (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003).

2.4. Total peptide content analysis

The peptide content on the GI-digested soybean sprouts hydrolysate was quantified through Pierce™ Quantitative Fluorometric Peptide Assay kit (23290, Thermo Scientific, Waltham, MA). Quantitation of peptides via this method was achieved using an amine-reactive fluorescent detection reagent that specifically labels the N-terminus of peptides. The fluorescently labelled peptides were then detected at Ex 390 nm/Em 475 nm using a Biotek Synergy H1 microplate reader (Agilent). The samples were dissolved in double distilled water and diluted appropriately. The kit's peptide digest standard was used to generate a standard curve to quantify the peptide content through regression analysis.

2.5. Cell culture

Caco-2 (ATCC® HTB-37™) cells were grown in TPP T75 flasks

(TP90076, Midsci, St. Louis, MO, USA) using Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™, Manassas, VA, USA) supplemented with 1% Penicillin-Streptomycin (Gibco, 15140122, Waltham, MA, USA) cocktail (v/v) and 20% fetal bovine serum (v/v) (FBS: Gibco, 10437028, Waltham, MA, USA) at 37 °C and 5% CO_2 in a humidified condition. Media was replaced every 2–3 days. Caco-2 cells within passages 27–30 were used in this study.

2.6. In-vitro inflammation study of sprouted soybean digest

Caco-2 cells were seeded and grown in 48 well plates (50,000 cells/well) with EMEM media supplemented with 20% FBS and 1% Penicillin-Streptomycin (PS). The media was changed every 2 days until the cells became 80% confluent in 6–7 days. A low serum (EMEM + 1% FBS + 1% PS) media was used to dissolve the SSD and conduct the experiment. The growth media was removed at confluency, and the cells were quickly washed with Hanks' Balanced Salt Solution (HBSS). The cells were pretreated with 1000 μg/mL SSD from days 0, 2, and 4 or the control media for 2 h. Next, inflammation was induced by adding Interleukin-1β (IL-1β) to the media at a 25 ng/mL concentration and incubated for the next 24 h. After the experiment, cell-free supernatant media was collected for further analysis.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The cell-free supernatant media was used to evaluate the secretion of pro-inflammatory cytokine Interleukine-8 (IL-8) through ELISA (88–8086-88, ThermoFisher Scientific) by following the manufacturer's guidelines. The concentration of the IL-8 was quantified using a standard curve.

2.8. Peptide profile analysis of soybean sprout digests

An aliquot of the dried SSD samples was resuspended in water at 10 μg/μL. For the hydrophilic interaction liquid chromatography (HILIC) separation of the peptides with 2–3 amino acids, the samples were further diluted three times for a 16-μg injection. The separation of the peptides was done on a BEH-Amide 1.7 μm (2.1 × 100 mm, Waters) column using a Vanquish (ThermoFisher Scientific) HPLC at 40 °C and at a flow rate of 300 μL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 90% B to 40% B in 12 min, then back to 90% in 1 min. The data was acquired on a Q Exactive™ (QE)-HF mass spectrometer (MS) (ThermoFisher Scientific) using a positive ion mode. The mass range was 100 to 1000 *m/z* on single charge ions at 60,000 resolution, using an AGC target of 3e6 and a maximum ion time of 50 ms. The isolated ions were further fragmented by HCD using an isolation window of 1.6 *m/z* at a resolution of 15,000. For the separation of the larger peptides, samples were diluted 2 times and run by reverse phase chromatography (RPC) on an ACCQ-TAG ULTRA C18 1.7 μm (2.1 × 100 mm, Waters) column using the Vanquish HPLC at 40 °C and a flow rate of 300 μL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 2% B for 2 min, 2% to 35% B in 11 min, 35% to 90% B in 2 min, hold at 90% B for 1 min, then back to 2% in 0.5 min. The QE-HF was run in a data-dependent acquisition mode triggering on peptides with charge states 1 to 3 using a mass range of 100 to 1000 *m/z* at 60,000 resolution, with an AGC target of 3e6 and a maximum ion time of 50 ms. The isolated ions were further fragmented by HCD using an isolation window of 1.6 *m/z* and scanned at a resolution of 15,000.

The acquired data from the HILIC and RPC separations were analyzed separately using Progenesis QI (Waters, v 2.4). The chromatograms were aligned, and the peaks were detected using a comprehensive set of algorithms, including isotope and adducts deconvolution, for a more accurate quantitative analysis. The library search used was NIST MS/MS v1.0. The compounds were filtered using a score of at least 30, a mass accuracy of < 3 ppm and isotopic similarity of at least 90. The MS/MS spectrum matching was further manually curated. Log2 ratios of peptide abundance in Day 4 and Day 2 over Day 0 SSD were calculated and used for further analysis. In addition, the RPC data was also analyzed using Proteome Discoverer 2.4 (ThermoFisher Scientific). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; v 2.6.2). Mascot was set up to search the cRAP_20150130 database, UniProt-soybean_UP000008827 (2019–11), and UniProt-soybean_UP000008827 database (20180326), assuming no specific digestion enzyme. Mascot was searched with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 ppm. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Scaffold (Proteome Software Inc., Portland, OR; v 4.8.9) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability, and protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides, with a false discovery < 1%. No significant peptide matches were reported here. Duplicates per treatment were analyzed through the described methodology.

2.9. Statistical analysis

Statistical analyses were conducted with the R platform (Team, 2018). Principal component analysis was performed by the prcomp function. The effects of the germination length on the contents of the metabolites and peptides were evaluated by one-way ANOVA using the aov function. In the analysis of peptides in the digested materials, only the peptides which showed significant effects by germination length ($P < 0.05$) were analyzed. Tukey's posthoc test evaluated the differences in the metabolite and peptide levels between the time points using glht and cld functions in the multicomp package ($P < 0.05$). The metabolites and peptides were clustered based on the time-course changes of their levels by the Gaussian mixed-effects model with embedded smoothing splines using the TMixClust R package (Golumbeanu, 2023). The number of clusters showing the largest average silhouette width was chosen for each analysis. The heatmaps were drawn by the pheatmap function with the metabolites and peptides arranged according to the clustering analysis results. A one-way ANOVA was performed to evaluate the effect of germination days in the DH analysis, peptide content, and the anti-inflammatory effect of germinated soybean at a 0.05 level of significance using Tukey's posthoc test with GraphPad Prism Software version 8.0.1 (San Diego, CA, USA). The data were expressed as mean ± standard deviation (SD).

3. Results

3.1. Chemical profiles of soybean sprout through time

The soybeans started germinating from Day 1 after imbibition. The sprout rapidly elongated between Days 3 and 4 and reached 165 mm on Day 5 (Figure S1). The following experiments were conducted until Day 4 since the sprouts on Day 5 were too long for general dietary use. To evaluate the chemical changes during soybean sprouting, the primary metabolite contents of the sprouted soybeans were analyzed by GC–MS before soaking (dry soy beans), after soaking (Day 0), and after 2 and 4 days of sprouting (Day 2 and 4, respectively). In the 95 detected metabolites (Supplementary Data 1), accumulations of 84 metabolites were significantly modulated by the sprouting period ($P < 0.05$). The global metabolite profile altered over the sprouting period was illustrated via the Principal Component Analysis (PCA) score plot along with the Principal Component (PC) 1 (Figure S2). Relative levels of metabolites showing significant effects of the sprouting period are shown as a heatmap in Fig. 1. The boxplots for individual metabolites are in Figure S3 and Supplementary Data 2. Most of the proteogenic amino

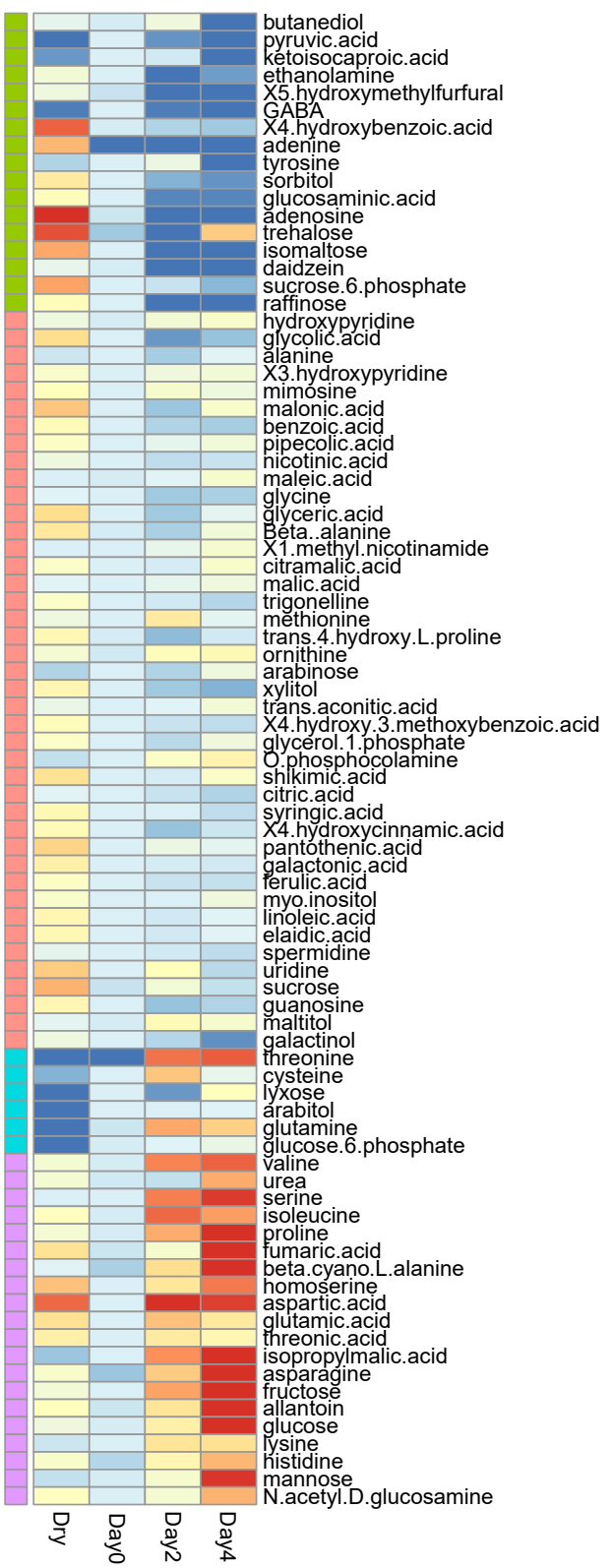

Fig. 1. Metabolite profiles of raw soybean sprouts. A heatmap showing the contents of individual metabolites in the dry soybean (Dry) and the sprouts at Day 0 (after imbibition), Day 2, and Day 4. Relative contents of 84 metabolites which showed significant effects by the sprouting period in ANOVA ($P < 0.05$), were analyzed. The levels of individual metabolites were normalized to those at Day 0, $\log_2$ transformed, and color-coded as indicated in the legend. The mixed effect model clustered the metabolites into four groups based on the patterns of time course changes. The colors of the boxes on the left indicate the clusters to which the metabolite belongs.

acids in cluster 4 include valine, isoleucine, proline, serine, methionine, aspartate, asparagine, glutamine, lysine, and histidine, accumulated during sprouting (Fig. 1 and Figure S3). The contents of the metabolites in cluster 3 (threonine, cysteine, lyxose, arabitol, glutamine, and glucose-6-phosphate) were very low in the dry soybeans and increased during the imbibition and sprouting processes. Levels of major monosaccharides, namely glucose and fructose, continuously increased up to eight times in cluster 4 (Fig. 1 and Figure S3) while oligosaccharides (sucrose and raffinose in cluster 1) and polyols (galactinol and sorbitol in cluster 1) (Fig. 1 and Figure S3) decreased two to eight times within the four days of sprouting.

3.2. Degree of hydrolysis and peptide content of the soybean sprout GI digests

Changes in digestibility or GI digestibility (amount of peptide bond breakage due to GI enzymatic hydrolysis against the total amount of peptide bonds in proteins) modulate the release of free amino acids and peptides in the digestive system. The *in-vitro* simulated GI digestion of the sprouted soybean showed that the degree of hydrolysis (DH) in the gastric and intestinal phases significantly decreased over the sprouting period (Fig. 2A & B). The consequence of the decrease in DH was further evaluated by the measurement of the total peptide content of the SSD. Interestingly, the peptide content significantly increased in the Day 4 SSD compared to Day 0 and Day 2 (Fig. 2C), suggesting the increase in proteolysis with the progression of sprouting and loss of availability of the cleavage sites for GI proteolytic enzymes. Resulting in a decrease in DH but an increase in peptide content.

3.3. Anti-inflammatory effects of sprouted soybeans after gastrointestinal digestion

Modulation of the inflammatory responses induced by IL-1β (25 ng/mL) was examined on intestinal Caco-2 cells pretreated with the SSDs (1000 μg/mL). As shown in Fig. 2D, the Day 4 SSD could significantly ($P < 0.05$) reduce the inflammatory response by 19.5% with regards to the secretion of the pro-inflammatory cytokine, IL-8, when compared to the positive control (PC) and the Day 0 and 2 SSDs. The IL-8 production was reduced from 2147.88 ± 205.06 pg/mL for the PC to 1729.97 ± 118.04 pg/mL (~19.5% reduction) after Day 4 SSD pre-treatment. The pre-treatment with Day 0 and 2 SSDs did not exhibit a statistically significant reduction of IL-8 secretion when compared to the PC.

3.4. Chemical profiles of digested soybean sprout through time

Elicitation of the anti-inflammatory activity in the Day 4 sprouts reflects the alteration of the contents of bioavailable compounds in the

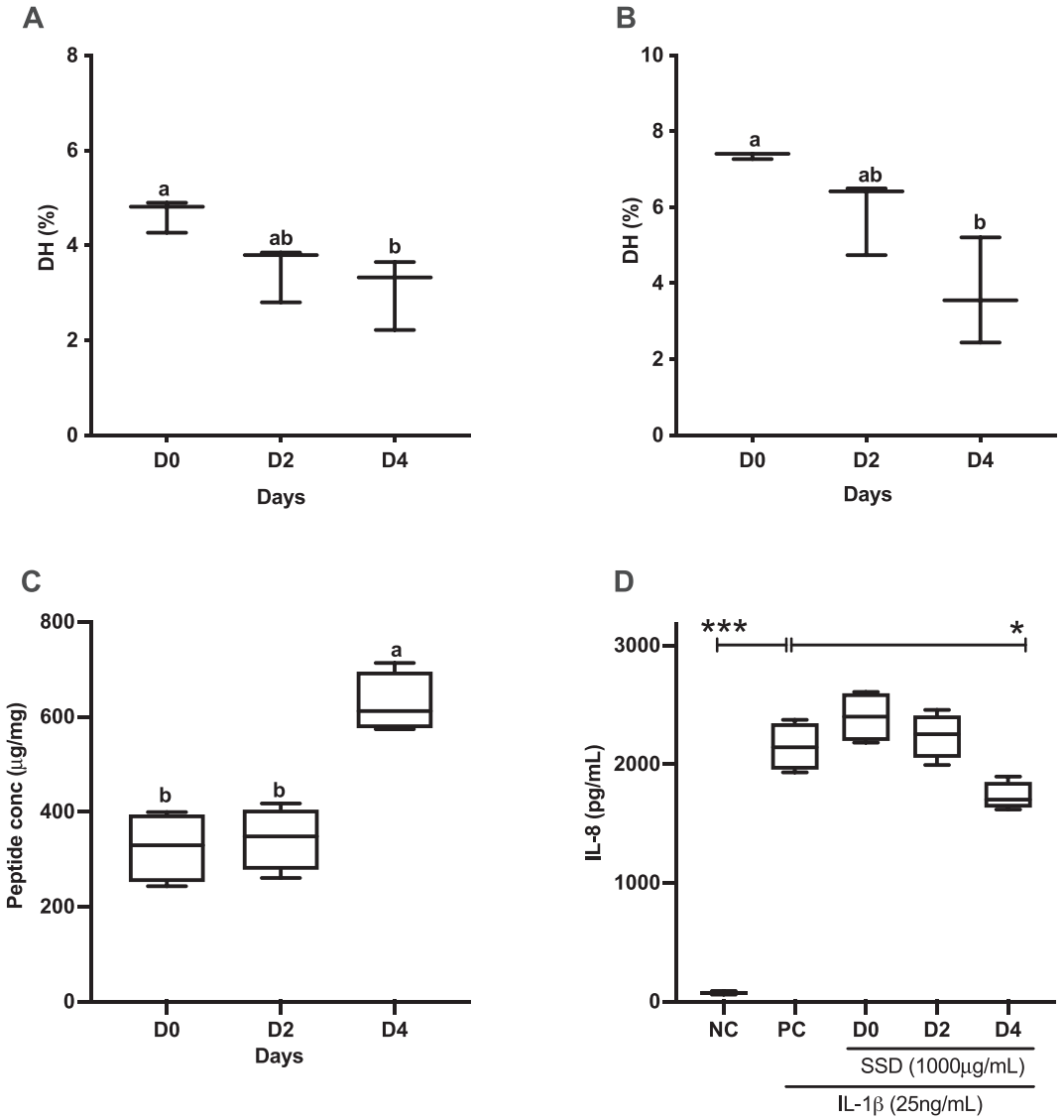

Fig. 2. Soybean sprout digest (SSD) degree of hydrolysis (DH), peptide content, and bioactivity (A) the DH in the gastric phase significantly decreased after 4 days of sprouting. (B) the DH in the intestinal phase also showed a decreasing trend in the digestibility significantly decreased after 4 days of sprouting. (C) the peptide content significantly increased after 4 days of sprouting; results were obtained from n = 3 independent experiments. (D) IL-8 cytokine secretion of Caco-2 after soybean sprout digest (SSD: 1000 μg/mL) pre-treatment and IL-1β (25 ng/mL) induction. Day 4 SSD significantly reduced the secretion of the pro-inflammatory cytokine IL-8 after the treatment ($P < 0.05$). *** indicates $P < 0.001$ and * indicates $P < 0.05$, respectively, results were obtained from n = 4 independent experiments.

GI-digested sprouts. As the soybean sprout harvested on Day 4 specifically showed anti-inflammatory activity, the compounds increased in the SSD specifically at this time point are probably associated with the bioactivity. The metabolite levels in the SSDs were analyzed by GC–MS analysis. The global metabolite profiles of the SSDs from Day 0, Day 2, and Day 4 sprouts were distinctly separated in the PCA analysis (Figure S4). Among the 99 detected metabolites (Supplementary Data 3), ANOVA revealed 51 of them were significantly affected by the sprouting period ($P < 0.05$). These metabolites were clustered into five groups depending on the time course changes, and their relative abundance was shown as a heatmap in Fig. 3. The metabolites in Cluster 2 to 5 accumulated in the digests of day 4 sprouts compared to day 0. Eighteen metabolites accumulated explicitly in the digests of the day 4 sprout (Fig. 4, plots for all metabolites are in Supplementary Data 4), including nine amino acids (Ala, Val, Asp, Gln, Asn, His, Tyr, methionine sulfoxide, and citrulline), five sugars and related metabolites (lyxose, xylitol, fructose, galactose, and *N*-acetylmannosamine), and four other metabolites (malate, isopropyl malate, trigonelline, and inosine).

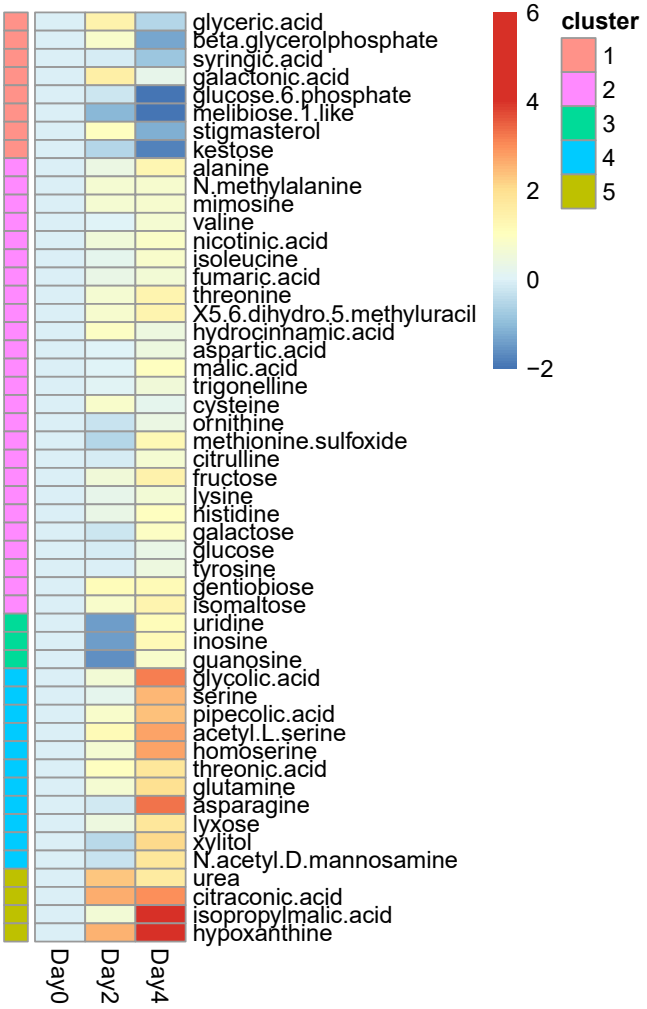

Fig. 3. Metabolite profiles of soybean sprout digest. A heatmap showing the contents of individual metabolites in the sprouts at Day 0 (after imbibition), Day 2, and Day 4 digest by simulated gastrointestinal digestion. Relative contents of 51 metabolites that showed significant effects by the sprouting period in ANOVA ($P < 0.05$) were analyzed. The levels of individual metabolites were normalized to those at Day 0, log2 transformed, and color-coded as indicated in the legend. The mixed effect model clustered the metabolites into five groups based on the patterns of time course changes. The colors of the boxes on the left indicate the clusters to which the metabolite belongs.

3.5. Peptide profiles in the digested soybean sprouts

A total of 150 di- and tripeptides by the RPC and 110 by the HILIC were detected as single chromatographic peaks in the SSDs (Supplementary Data 5), and the ANOVA analysis showed that 81 of them for RPC and 63 of them for HILIC were significantly modulated by the sprouting period ($P < 0.05$) (Supplementary Data 6). The peptides were clustered into 4 groups for RPC analysis, and for HILIC, they were clustered into 5 groups (Fig. 5). The abundance of 60 peptides out of 81 from RPC (Supplementary Data 5 and 6) and 47 out of 63 from HILIC (Supplementary Data 5 and 7) significantly increased in the Day 4 sprouts, indicating an effect of sprouting. Interestingly, 25 peptides were detected by both RPC and HILIC separation, and their abundance profile was found to be similar; however, except for 4 peptides, the changes were found to be significant most of the time only in one set, which indicates that both separations provide complementary information. Many peptides with increased abundance up to 6-fold (75% of the peptides with significant changes) also correlates with the earlier findings of higher peptide content observed in the Day 4 GI digested sample. The peptides in higher abundance in the Day 4 GI digested sprouts are potentially responsible for the observed anti-inflammatory effect.

4. Discussion

The changes in the metabolite profiles in soybeans by sprouting and GI digestion can produce multiple bioactive compounds. The main findings of the study are: i) sprouting generated simple sugars and amino acids, ii) proteolysis during sprouting increased the peptide content in SSD, iii) inflammation was reduced (by 19.5%) by metabolites and peptides present in Day 4 SSD samples through a potential synergistic effect, and iv) some of the metabolites and peptides identified in Day 4 SSD samples indicates their potential anti-inflammatory activity. The current study shows that the GI digest of sprouted soybean exerts anti-inflammatory activity in intestinal epithelial cell culture, unlike the non-germinated soybean, indicating that the release of bioactive compounds depends on sprouting. The changes in the metabolic profile during sprouting are most likely due to the degradation of storage carbohydrates, lipids, and proteins to produce the energy and cellular building blocks required for germination and seedlings growth (Ohanenye et al., 2020; Palmiano & Juliano, 1972; Wanasundara, Wanasundara, & Shahidi, 1999). The activity of endogenous enzymes such as α-amylase, proteases, and lipases has been attributed as one of the factors of macronutrient breakdown (Nkhata et al., 2018). The degradative tendency can also be seen in the decrease of oligosaccharides (e.g., sucrose, trehalose, isomaltose, and raffinose) and increase of monosaccharides (e.g., glucose, fructose, and mannose) over the sprouting period before GI digestion (Fig. 1). Most of the proteogenic amino acids also accumulated over the sprouting, most likely derived from storage protein degradation (Fig. 1). The increase of major sugars and free-amino acids can modulate the bioavailability of nutrients and thus enhance the nutritional values of soybean since they are more absorbable during dietary intake than macro storage molecules (Bueno et al., 2020).

The increased abundance of amino acids like valine and isoleucine (Fig. 1) in sprouted soybean indicates the onset of proteolysis of the storage proteins (González-Montoya et al., 2018). Proteolysis can modify the soybean protein structure and affect further digestion. An earlier study reported the changes in the electrophoretic protein profile of the soybean proteins after an 80 h germination period (Bueno et al., 2020). Moreover, the free amino acid groups released during the germination period and before digestion increase significantly between 0 and 56 h from 80 to 104 h (Bueno et al., 2020). In the present study, the DH decreased by sprouting (Fig. 2). It should be noted that DH was measured by the pH-stat method, which monitors the hydrogen ion released by the cleavage of peptide bonds. Therefore, the DH appears lower in already hydrolyzed foods (Spellman et al., 2003). The low DH

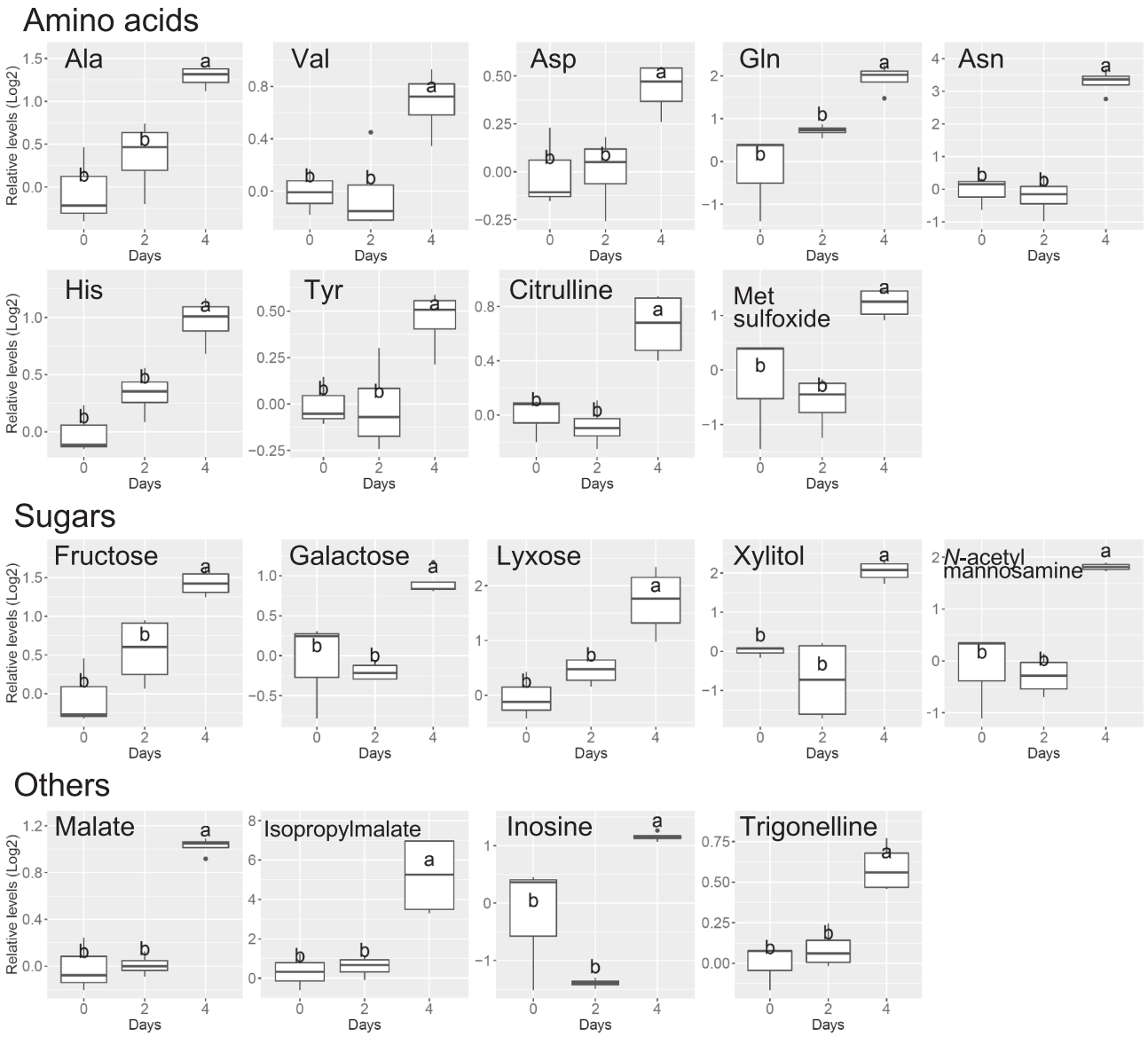

Fig. 4. Metabolite contents in soybean sprout digests. The boxplots show the relative metabolite contents in the soybean sprouts at Day 0 (after imbibition), Day 2, and Day 4 digest by simulated gastrointestinal digestion. The metabolites (i.e., Amino acids, Sugars, and other specific types) increased specifically in the Day 4 sprout digests were shown. The boxplot for all metabolites are in Supplementary Data 4. The boxplots show the minimum, first quartile, median, third quartile, and maximum contents of metabolites (n = 4). The points indicate the metabolite contents in the outliers. One factor ANOVA was conducted to examine the variation in metabolite contents among sprouting periods, and the letters indicate the results of following the Tukey HSD test ($P < 0.05$).

of the Day 4 soybean sprout suggests protein hydrolysis during sprouting, leading to higher peptide and amino acid contents observed in the metabolite profiling (Fig. 1). Thus, sprouting initiates a proteolysis process, and hydrolysis of the storage proteins might contribute to the low DH, as observed in Day 4 SSD (Fig. 2). These results also indicate the possibility of producing novel and unique peptide sequences during GI digestion after four days of sprouting, which might not be released in the gastrointestinal tract from imbibed soybean (Day 0).

In the present study, the GI digest of Day 4 sprouted soybean specifically exhibited anti-inflammatory properties in GI epithelial cells by reducing IL-8 secretion (Fig. 2D). IL-8 is a post-injury chemoattractant cytokine responsible for neutrophil trafficking in the inflammatory regions (Griffith, Sokol, & Luster, 2014). Additionally, intestinal epithelial cell-derived IL-8 actively promotes gene expression related to cellular differentiation through Toll-Like Receptor-2 (TLR2) and TLR5 ligation (Rossi et al., 2013). Thus, the reduction in IL-8 secretion by the Day 4 SSD indicates its potential role in controlling chronic inflammation by regulating neutrophil recruitment and inhibiting cell differentiation. However, these subsequent effects and the effects on other inflammatory parameters need to be verified in future in-vivo studies. The anti-inflammatory result also suggests that GI digestion and four days of sprouting are acting together to facilitate the release of bioactive compounds. The metabolite analysis of the GI digested sample revealed the accumulation of amino acids, sugars, and other metabolites (malate, isopropyl malate, trigonelline, and inosine) in Day 4 SSD. Although many amino acids and sugars accumulated on Day 4, it is highly unlikely they have anti-inflammatory activities considering their general availability in various food sources. Thus, other metabolites accumulated in Day 4 SSD likely contribute to the observed anti-inflammatory bioactivity. Out of these metabolites, trigonelline is reported to be accumulated in a crop plant, *Moringa oleifera* (commonly called drumstick) (Mathur & Kamal, 2012), which exhibits anti-inflammatory health benefits (Khalili, Alavi, Esmaeil-Jamaat, Baluchnejadmojarad, & Roghani, 2018). However, no downregulation of IL-8 secretion was

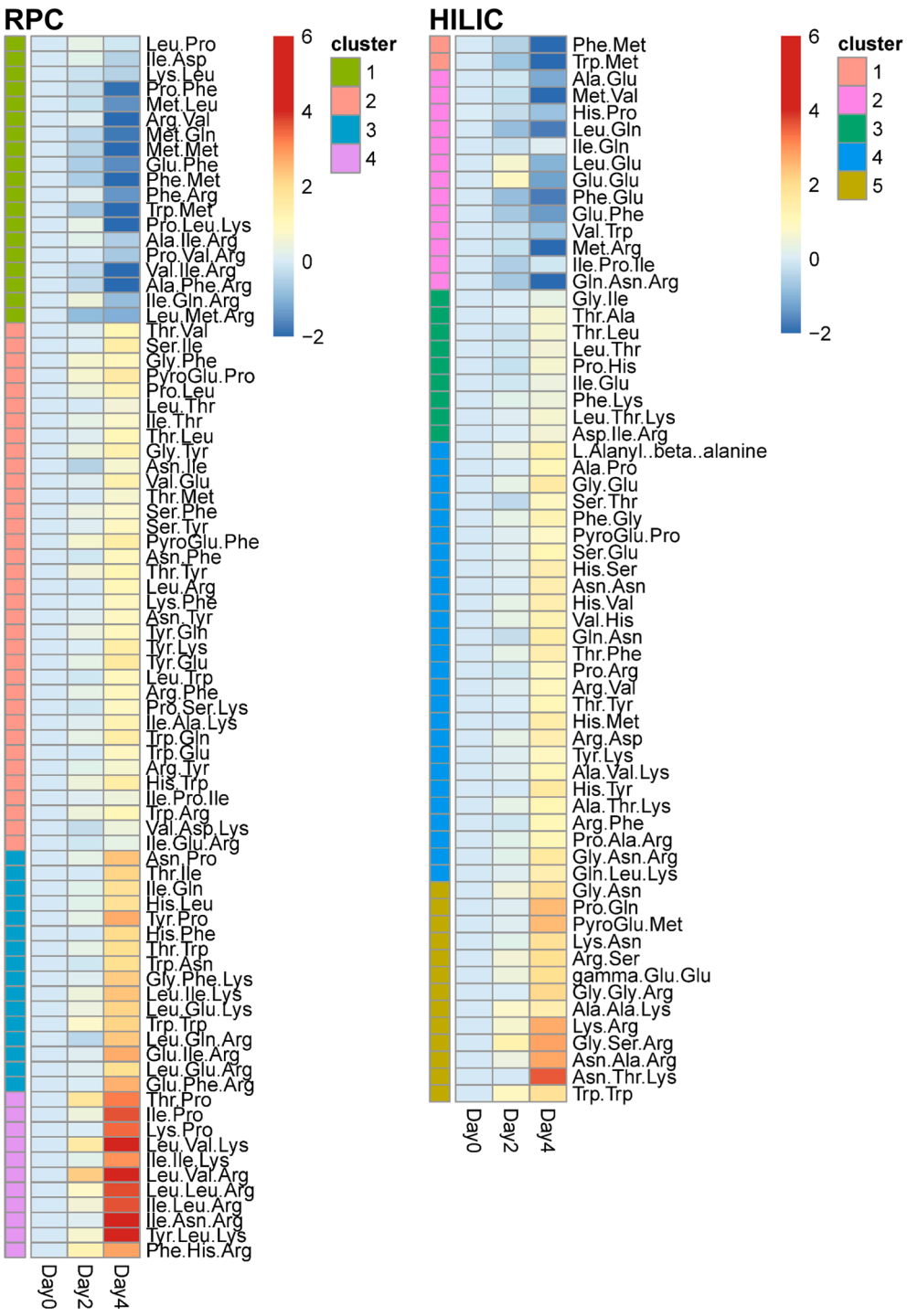

Fig. 5. Oligopeptide profiles of soybean sprout digests. A heatmap showing the contents of individual oligopeptides in the sprouts on Day 0 (after imbibition), Day 2, and Day 4 were digested by simulated gastrointestinal digestion. Oligopeptides were identified and quantified by LC-MS/MS analysis with two LC separations (RPC and HILIC, on the left and right panels, respectively). Relative contents of peptides which showed significant effects by the sprouting period in ANOVA ($P < 0.05$) were analyzed. The levels of individual peptides were normalized to those at Day 0, $\log_2$ transformed, and color-coded as indicated in the legend. The peptides were clustered into five groups based on the patterns of time course changes by mixed effect model. The clusters to which the peptide belongs are indicated by the colors of the boxes on the left.

observed with a trigonelline pre-treatment at 0.3, 0.6, 1.25, and 2.5 mmol/L (Figure S5), indicating trigonelline is not the sole compound exhibiting anti-inflammatory activity.

Metabolites in black soybean, such as isoflavones, increased on Day 4 of the sprouting (Ren, Wang, Liu, Wang, & Wang, 2017). Isoflavones have been attributed as antioxidant and anti-inflammatory molecules (Ren et al., 2017). Phenolics and flavonoids also present in soybean sprouts have shown antioxidant activity (Silva et al., 2013). However, the increase of isoflavones and phenolics during the soybean sprouting alone does not usually correlate to anti-inflammatory activity (Eum et al., 2020). Previous results suggest that metabolites such as organic acids and volatiles and interactions between several metabolites could reduce inflammation (Silva et al., 2013). Although phenolics were not quantified in the current study due to technical limitations, they may synergistically exert anti-inflammatory activity with the metabolites accumulated in Day 4 SSD.

Apart from bioactive metabolites, the release of peptides during GI digestion could modulate biological activities. Previous studies have identified anti-inflammatory peptides from germinated soybeans and black soybeans treated with high hydrostatic pressure. Protein extract after germination reduced the secretion of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as PGD2 in RAW 264.7 cells, stimulated with LPS (González-Montoya et al., 2018; Kim et al., 2018). Similar peptides with anti-inflammatory activities may be produced during

sprouting and GI digestion. The peptide analysis showed an increased abundance of tripeptides with polar-hydrophilic, positively charged amino acids, Arg and Lys in their C-terminal and non-polar-hydrophobic amino acids, Leu, Ile, Tyr, Glu, Phe, and Val in their N-terminal, in the Day 4 SSD (Fig. 5).

Earlier studies have reported the importance of non-polar, hydrophobic amino acids in the N-terminal as one of the major structural requirements of peptides to exhibit anti-inflammatory activity (Guha & Majumder, 2018). These structural features of peptides with potential anti-inflammatory activity were absent in the digest of Day 0, but the abundance of these peptides increased with sprouting time (Day 2 and Day 4 digests), further suggesting the potential combinatorial role

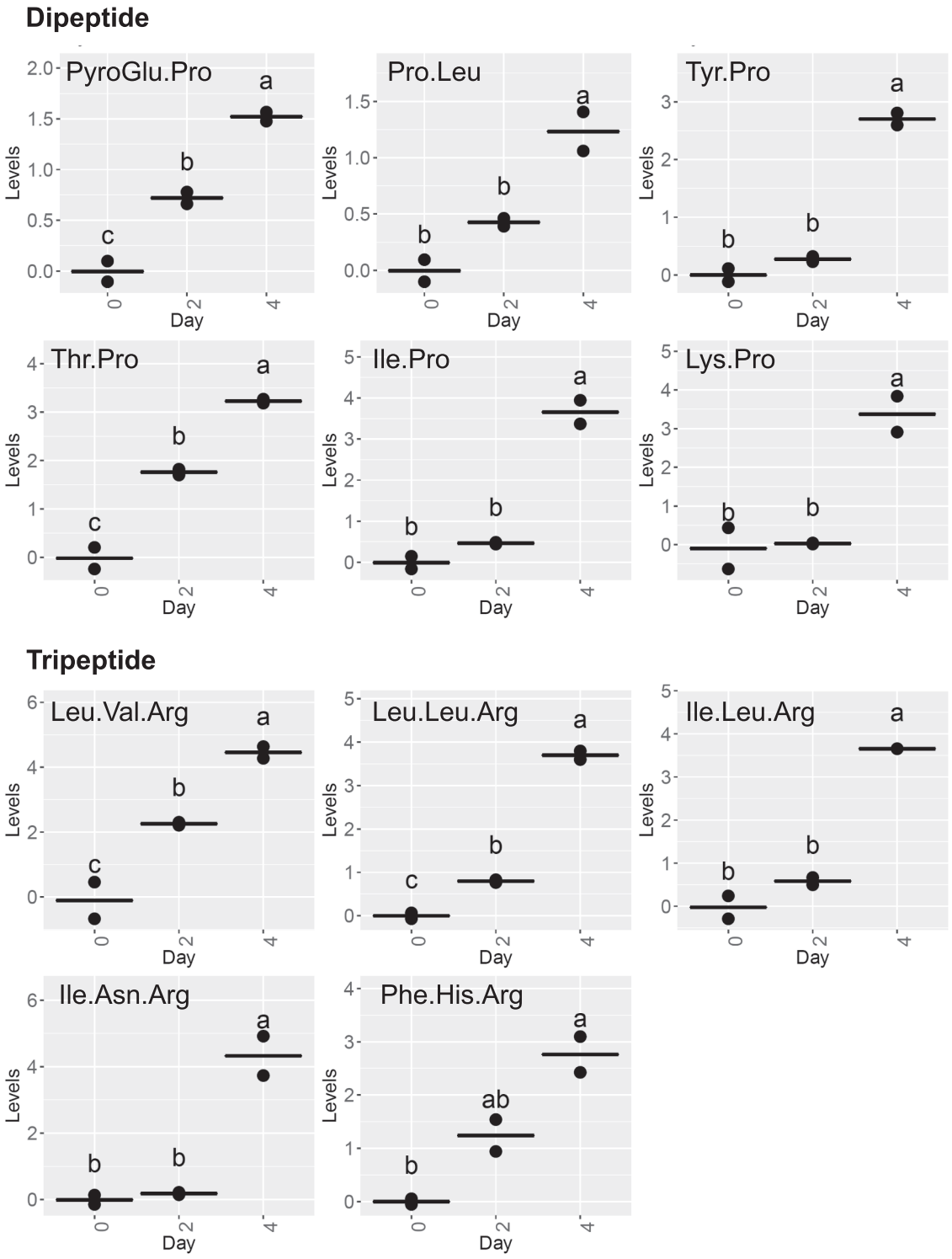

Fig. 6. Peptides contents in soybean sprout digests. The boxplots show the relative peptides contents in the soybean sprouts at Day 0 (after imbibition), Day 2, and Day 4 digest by simulated gastrointestinal digestion. The peptides (Di and tripeptides) increased specifically in the Day 4 sprout digests were shown. The boxplots for all peptides are in Supplementary Data 6 and 7. One factor ANOVA was conducted to examine the variation in peptide contents among sprouting periods, and the letters indicate the results of following the Tukey HSD test ($P < 0.05$).

of sprouting and digestion (Fig. 5) in release of peptides with specific structural features. It is also reported that proline-rich peptides, such as Val-Pro-Pro and Ile-Pro-Pro, have the potential to exhibit anti-inflammatory properties (Nakamura et al., 2013). Peptides rich in Pro are more resistant to intestinal digestion and can remain bioavailable when consumed orally (Ohara et al., 2010).

The present study identified increased abundances of proline-rich di-peptides (Pro-Leu, Tyr-Pro, Thr-Pro, Ile-Pro, and Lys-Pro) in the Day 4 SSD sample (Fig. 6), additionally, the abundances of C-terminal arginine-rich tri-peptides (Leu-Val-Arg, Leu-Leu-Arg, Ile-Leu-Arg, Ile-Asn-Arg, Phe-His-Arg) in the Day 4 SSD sample (Fig. 6) indicates the potential role of C-terminal Pro for di-peptides and Arg for tri-peptides in exhibiting the observed anti-inflammatory effects. Apart from charge, hydrophobicity, peptide length, molecular weight, and isoelectric point, the position of specific amino acid at specific location also plays a pivotal role in exhibiting anti-inflammatory activity (Gu et al., 2019; Hall, Reddivari, & Liceaga, 2020; Lin, Liao, Bai, Wu, & Wu, 2017). The increased abundance of di- and tripeptides complies with all these structural requirements, suggesting their potential role in exhibiting the observed anti-inflammatory activities. However, further studies are necessary to elucidate and for complete understanding of structure–function relationship of peptides and anti-inflammatory activities.

5. Conclusions

This study suggests that sprouted soybean after four days is preferable over raw soybeans and Day 2 sprouts to release the bioactive compounds which exhibit anti-inflammatory activity after GI digestion. We could show the substantial alteration in the chemical composition of sprouting soybeans and their GI digests. The peptides and metabolites accumulated explicitly in the Day 4 SSD likely contribute to the synergistic anti-inflammatory bioactivity, although identifying the exact bioactive molecules requires additional future studies. These results indicated the potential use of soybean sprouts as a value-added food ingredient to develop functional foods to prevent chronic inflammatory disorders. Further research with animal models is required to investigate the potential of soy sprouts to show anti-inflammatory activities *in-vivo*.

CRediT authorship contribution statement

Emerson Nolasco: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Inga Krassovskaya:** Formal analysis, Data curation. **Kelvin Hong:** Formal analysis, Data curation. **Kali Hansen:** Formal analysis, Data curation. **Sophie Alvarez:** Formal analysis, Data curation. **Toshihiro Obata:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kaustav Majumder:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.jff.2023.105780.

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