Food Chemistry: X 31 (2025) 103153

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x

Leaf protein concentrate as a nutrient-dense protein source from vetch and triticale mixture: A comparative analysis of fermentation techniques☆

S. Reyhan Yavuz *, Tarek Alshaal, Wildan Suhartini, Nóra Bákonyi, Zoltán Kovács, László Kaszás, Szilvia Veres, Miklós G. Fári, Nevien Elhawat [1], Éva Domokos-Szabolcsy [1]

Institute of Applied Plant Biology, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, 4032 Debrecen, Hungary

ARTICLE INFO

Keywords:
Leaf protein concentrate
Green biomass
Common vetch
Triticale
Lactic acid fermentation
Lactobacillus fermentation
Yeast-bacteria fermentation

ABSTRACT

Green leaves from legumes and grasses have long been recognized as potential sources of protein for feed and food. Selecting an appropriate processing method is crucial for protein concentration and can also affect the composition of phytonutrients important for human and animal health. This study examines the extraction of leaf protein concentrate (LPC) from a mixture of fresh vetch-triticale biomass, using microwave coagulation (MWC) as the control method compared to fermentation processes, including lactic acid fermentation (LAF), induced *Lactobacillus* fermentation (LBF), and yeast-bacteria fermentation (YLBF). MWC yielded a higher percentage (11.2 %) of macroaggregate-containing LPCs with a crude protein content of 44.78 m/m%. The crude protein content of post-fermentation LPCs ranged from 35.14 to 38.50 m/m%, but SDS-PAGE analysis revealed that these LPCs were more readily solubilizable and contained higher levels of essential amino acids, such as methionine and isoleucine. Fermentation processes were also more effective at converting flavonoids into health-promoting aglycones, including chrysoeriol (248.5–283.9 μg g^{-1}), luteolin (242.6–304.1 μg g^{-1}), and isovitexin (72.6–91.6 μg g^{-1}). Among the fermentation methods, LBF and YLBF were preferable for accumulating these health-promoting flavonoids.

1. Introduction

The global demand for protein has been steadily rising due to population growth, urbanization, and a shift toward protein-rich diets (Tilman & Clark, 2014). At the same time, meeting this demand sustainably is increasingly challenging due to environmental and resource limitations associated with traditional protein sources, such as animal products and certain crops (Food and Agriculture Organization of the United Nations (Ed.), 2017). This has spurred interest in alternative protein sources that offer high protein content, along with desirable quality and digestibility profiles (Goksen et al., 2025; Malila et al., 2024). Among the various options, leafy biomass plants, especially those grown in low-input agricultural systems, represent a promising solution to address the protein supply gap for both human and animal consumption (Domokos-Szabolcsy, Alshaal, et al., 2023).

Protein is a crucial macronutrient essential for cellular structure, enzymatic activity, and immune function. However, establishing a sustainable protein supply chain remains a pressing global challenge (Food and Agriculture Organization of the United Nations (Ed.), 2017). The environmental impacts of intensive livestock farming, including greenhouse gas emissions, deforestation, and water pollution, highlight the urgent need for alternative, sustainable protein sources (Sá et al., 2020; Tilman & Clark, 2014). Animal feed production accounts for a significant share of global agricultural output, intensifying competition for resources. The protein conversion efficiency of livestock is notably low; for example, only 4 % of the protein in animal feed is converted into edible meat protein (Sabaté et al., 2015). Therefore, identifying protein sources that can be directly consumed by humans or efficiently incorporated into animal feed is critical for reducing resource use and enhancing food security (Espinosa-Marrón et al., 2022; Kaszás et al.,

---

☆ This article is part of a Special issue entitled: 'Non-Thermal Methodology for Fruit and Leaf Protein' published in Food Chemistry: X.

* Corresponding author.

E-mail addresses: reyhanyavuz@mailbox.unideb.hu (S.R. Yavuz), alshaal.tarek@agr.unideb.hu (T. Alshaal), wildan.suhartini@agr.unideb.hu (W. Suhartini), nbakonyi@agr.unideb.hu (N. Bákonyi), kovacs.zoltan@agr.unideb.hu (Z. Kovács), kaszas.laszlo@agr.unideb.hu (L. Kaszás), szveres@agr.unideb.hu (S. Veres), fari@agr.unideb.hu (M.G. Fári), elhawat.nevien@agr.unideb.hu (N. Elhawat), szabolcsy@agr.unideb.hu (É. Domokos-Szabolcsy).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fochx.2025.103153
Received 7 April 2025; Received in revised form 16 September 2025; Accepted 9 October 2025

2018).

Leafy plants and biomass crops have long been recognized as viable protein sources for sustainable food and feed production (Pirie, 1966; Pirie, 1987). These plants typically offer high protein content, a favorable amino acid profile, and the ability to thrive in diverse agroecological conditions, including marginal lands unsuitable for conventional agriculture (Domokos-Szabolcsy, Alshaal, et al., 2023; Malila et al., 2024). Additionally, many biomass crops, such as triticale (a hybrid cereal) and vetch (a legume), can be grown in low-input systems without irrigation or synthetic fertilizers, making them environmentally sustainable. Triticale and vetch are particularly well-suited for green biomass production. Triticale combines the robustness and high yield potential of wheat with the stress tolerance of rye, while vetch enhances soil fertility through nitrogen fixation (Pál & Zsombik, 2024; Pisulewska et al., 1991). When cultivated together as a mixed crop, these species form a symbiotic system that optimizes resource use, reduces weed pressure, and promotes ecological sustainability. Harvested in their vegetative state, these crops yield substantial green biomass that can be processed to extract leaf protein for various applications (Jensen et al., 2020).

Green leaf protein concentrate (LPC) is obtained from the soluble proteins in plant leaves, serving as a rich source of high-quality protein. LPC production involves mechanically pressing fresh biomass to extract green juice, which contains soluble proteins, chlorophyll, and other bioactive compounds (Kaszás et al., 2024). Precipitation methods are then used to isolate the protein, producing a concentrate suitable for feed or food applications (Hanczakowski et al., 1991).

LPC production offers multiple benefits. Firstly, it utilizes non-edible plant fractions, reducing competition with human food crops. Secondly, it supports circular economy principles by valorizing agricultural residues and minimizing waste (Kaszás et al., 2022). Lastly, LPC boasts a favorable nutritional profile, containing high levels of essential amino acids, bioactive peptides, and antioxidant compounds (Kaszás et al., 2020; Santamaría-Fernández & Lübeck, 2020). While the quantity of protein is vital for meeting nutritional demands, protein quality and digestibility are equally critical for its effectiveness in human and animal diets. Protein quality is evaluated based on its amino acid composition and the bioavailability of these amino acids post-digestion. High-quality proteins, like those in LPC, are rich in essential amino acids that the human body cannot synthesize and must obtain through diet (Millward & Jackson, 2004). Digestibility, defined as the proportion of ingested protein broken down into absorbable amino acids, affects the nutritional efficiency of protein sources. Plant-derived proteins often have lower digestibility than animal proteins due to anti-nutritional factors such as phytic acid and tannins (Samtiya et al., 2020; Tang et al., 2024). However, processing techniques like fermentation and thermal coagulation can significantly improve protein digestibility by reducing these anti-nutritional factors and breaking proteins into smaller, more bioavailable peptides.

Protein isolation from green juice is a pivotal step in LPC production, and the choice of precipitation method significantly affects the yield, quality, and functional properties of the final product. Traditional thermal coagulation, which involves heating green juice to denature proteins and promote aggregation, is a well-established method. However, alternative approaches, such as fermentation, are gaining interest for their ability to preserve bioactive compounds and enhance protein digestibility (Domokos-Szabolcsy et al., 2022).

Fermentation, driven by microorganisms or organic acids, has been investigated using *Lactobacillus*, lactic acid, and kefir cultures, each offering unique benefits (D'Almeida & de Albuquerque, 2025). *Lactobacillus* fermentation employs lactic acid bacteria to ferment green juice, lowering its pH and facilitating protein precipitation. This method not only isolates protein but also enriches the LPC with probiotics and bioactive metabolites (Bákonyi et al., 2020; Domokos-Szabolcsy, Alshaal, et al., 2023). Conversely, adding lactic acid directly to green juice encourages the growth of co-existing microorganisms, initiating natural fermentation (lactic acid fermentation). This approach is simple, cost-effective, and scalable for industrial use (Bákonyi et al., 2020). Kefir, a symbiotic culture of bacteria and yeast, provides a more complex fermentation system (Saleem et al., 2023).

This study aims to evaluate the protein concentration of a green forage mixture of vetch and triticale, commonly used in silage production, by comparing different fermentation methods to determine their suitability for feed and/or food applications. The three fermentation techniques assessed were natural lactic acid fermentation (LAF), *Lactobacillus* inoculum-induced fermentation (LBF), and yeast+*Lactobacillus* inoculum-induced fermentation (YLBF). A detailed analysis of the resulting LPCs was conducted to examine both qualitative and quantitative changes in proteins, as well as quantitative changes in phytochemical compositions relevant to human and animal health.

2. Materials and methods

2.1. Experimental design of growth conditions

Winter triticale (×*Triticosecale* Wittm. var. 'Dimenzio') and hairy vetch (*Vicia villosa* Roth. var. 'Paula') were cultivated in the demonstration garden of the University of Debrecen (47° 32′ 0" N, 21° 38′ 0" E) from autumn 2022 to spring 2023. The seeds of triticale and vetch were sown in a 4:1 ratio, consistent with conventional green forage technology. The field experiment was arranged in a Randomized Complete Block Design (RCBD) with three replicates. Each experimental plot measured 2.0 m × 2.5 m, with a 0.5 m row spacing between plots. Sowing occurred in October 2022, and fresh biomass was harvested in May 2023, when the vegetative shoots of both triticale and hairy vetch were fully developed, prior to flowering. The cultivation relied on rainfed conditions, with nutrient supplementation applied during the growing period. Meteorological data recorded during the experiment are provided in the supplementary material (Fig. S1).

2.2. Processing of fresh green biomass

The fresh green biomass of triticale and vetch mixture was harvested in the early morning when the air temperature was cooler to minimize the degradation of leaf proteins by proteases. The fresh biomass was then transported to the laboratory in an icebox. In the laboratory, 1 kg of fresh biomass was mechanically pressed using a twin-screw juicer (Angel Juicer 5500, Angel Ltd., South Korea) to extract the protein-rich green juice (GJ). The pressing process also yielded a fiber-rich pressed cake. The procedure was performed in triplicate. The following properties of the GJ were measured: pH (using a Mettler Toledo S20 Seven Easy pH meter, Switzerland), degrees Brix (using a RBR32-ATC manual refractometer, Polling, Germany), and electrical conductivity (EC) (using a Thermo Scientific Orion 209 A+ portable conductivity meter, Germany).

2.2.1. Precipitation of soluble proteins from GJ

The soluble proteins in freshly extracted GJ were processed to produce LPC using four methods (Fig. 1). As a control, soluble leaf proteins in GJ were precipitated at 80 °C via microwave coagulation (MWC) technology, a standard thermal coagulation method. In addition to MWC, three fermentation techniques were compared for extracting soluble proteins from GJ. Lactic acid fermentation (LAF) involved adding 1 mL of 1 M lactic acid to 200 g of GJ, followed by anaerobic incubation at 35 °C for 48 h. *Lactobacillus* fermentation (LBF) utilized a commercial *Lactobacillus* inoculum (10^{11} CFU/g, containing *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Pediococcus acidilactici*), applied at a rate of 2 mg of dry weight inoculum per 200 g of GJ, with anaerobic incubation at 35 °C for 48 h, based on dosages from Bákonyi et al. (2020). Yeast+*Lactobacillus* fermentation (YLBF) employed a microbial inoculum (BiaRia Ltd., Biatorbágy, Hungary) containing *Lactococcus cremoris*, *Debaryomyces hansenii*, *Lactococcus lactis* subsp. *lactis*

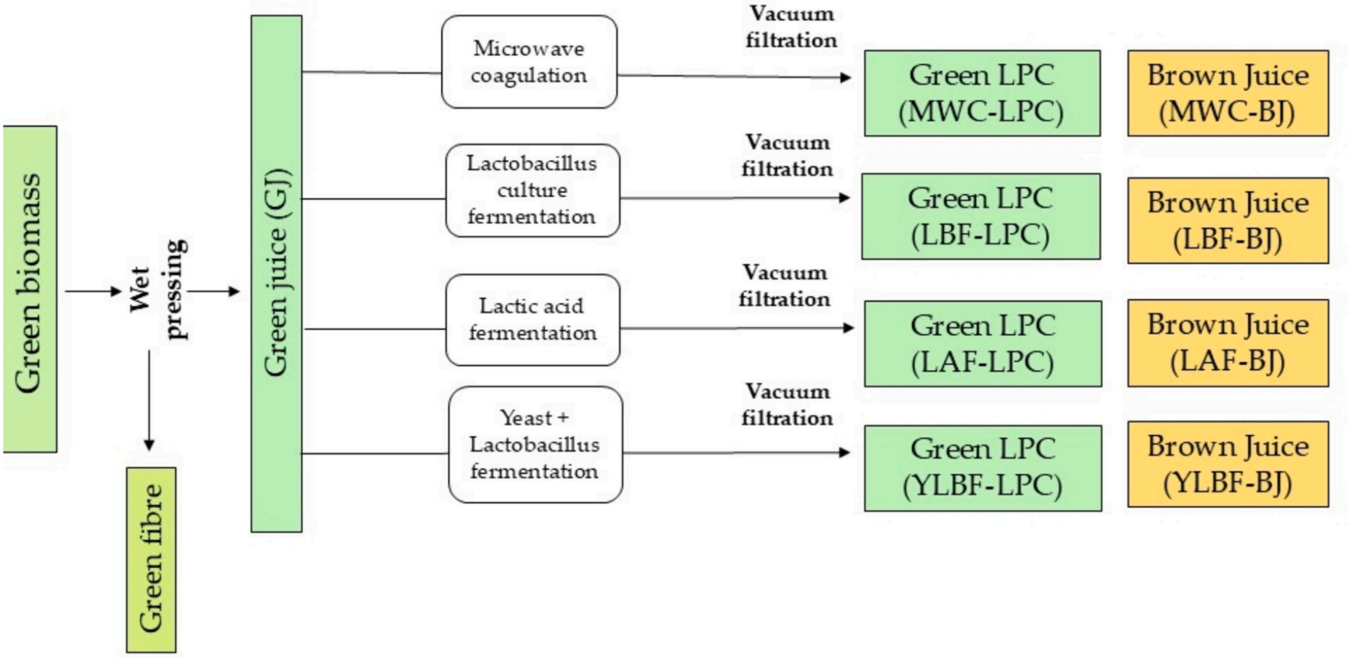

Fig. 1. Schematic flow chart of applied processes before freeze-drying each fraction.

biovar *diacetylactis*, *Candida colliculosa*, *Streptococcus thermophilus*, and *Leuconostoc mesenteroides*, supplemented with inulin and lactose. This inoculum was added at 10 mg of dry weight per 200 g of GJ, as per the manufacturer's recommendation, and incubated anaerobically at 24 °C for 36 h. Each fermentation method was conducted in quadruplicate, with pH measurements taken before and after fermentation. For the control, soluble proteins in GJ were isolated using MWC at 80 °C in a 400-watt microwave oven (Domokos-Szabolcsy et al., 2022). Post-fermentation, the resulting fluffy precipitate was filtered through a 35 μm mesh sieve using vacuum filtration, yielding LPC and a brown aqueous phase, termed brown juice (BJ) (Fig. 1). The pH, degrees Brix, and electrical conductivity (EC) of the brown juice were measured, along with the fresh weight of the LPC. The dry weight of the LPC was determined after freeze-drying (using an Alpha 1–4 LSC basic freeze dryer, Martin Christ Ltd., Germany) to calculate the dry matter content. The lyophilized samples were ground into powder, stored in sterile bags at −20 °C, and reserved for subsequent analyses.

2.3. Determination of crude protein

The crude protein content in freeze-dried GJ and LPC was determined using the Dumas method with an Elementar Vario Max Cube analyzer (Elementar Analysensysteme GmbH, Germany). Briefly, a 250 mg sample was placed in a combustion tube and burned in an oxygen-rich environment, which decomposed the nitrogen into nitrogen (N_2) and the carbon into carbon dioxide gas (CO_2). Nitrogen oxides were subsequently converted to nitrogen (N_2) in a reduction tube, while the carbon dioxide was captured in columns for later measurement. The instrument quantified the nitrogen produced, and after heating the CO_2 columns, it measured the carbon content. The nitrogen content was then used to calculate the crude protein content by applying a conversion factor of 6.25 (Sheen, 1991) as follows:

$$\text{Crude protein amount (\%)} = \text{Nitrogen content (\%)} \times 6.25$$

2.4. Determination of amino acid composition

The amino acid composition of the samples was analyzed using ultrahigh-pressure liquid chromatography (UHPLC) with a Waters Acquity H-Class Plus UPLC system (Waters, Milford, MA, USA). For sample preparation, 20 mg of powdered sample was placed in a 50 mL digestion tube and hydrolyzed with 6 M HCl in a microwave digestion unit for 1 h (CEM MARS One, Matthews, NC, USA). After hydrolysis, the pH of the sample was adjusted using 6 M NaOH, and the sample was filtered through a 3 kDa PES membrane filter (VWR International, Radnor, PA, USA). Amino acid separation was achieved using AccQTag pre-column derivatization chemistry. The hydrolyzed and neutralized samples were derivatized with an AccQ-Tag Ultra derivatization reagent kit (Waters, Milford, MA, USA) following the manufacturer's protocol. Derivatized amino acids were separated on an AccQ-Tag Ultra C18 column (1.7 μm; 2.1 × 100 mm, Waters, Milford, MA, USA) fitted with an Acquity in-line filter (0.2 μm; 2.1 mm, Waters, Milford, MA, USA). Chromatographic separation was achieved using a gradient elution with eluent A (100 % AccQ-Tag Ultra eluent A), eluent B (10 % AccQ-Tag Ultra eluent B in LC-MS grade water), eluent C (LC-MS grade water), and eluent D (100 % AccQ-Tag Ultra eluent B) at a flow rate of 0.6500 mL/min over an 11-min gradient. The elution profile for the UPLC separation is presented in Table 1, based on the method described by Guba et al. (2022). The column temperature was maintained at 54 °C. Data analysis was performed using Waters Empower 3 software (Waters, Milford, MA, USA) (Domokos-Szabolcsy et al., 2022).

2.5. Determination of protein expression pattern by SDS-PAGE

Protein expression changes in the fractions were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1D SDS-PAGE) according to the method outlined by Domokos-Szabolcsy et al. (2024). For sample preparation, 10 mg of freeze-dried material was combined with 250 μL of Laemmli 4× buffer (prepared for 100 mL as follows: 25 mL of 1 M Tris-HCl, 8 g SDS, 40 mL glycerol, 0.3 g DTT, and bromophenol blue). The samples were vortexed, incubated at 95 °C for 5 min, and then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected for further analysis. SDS-PAGE was conducted using a discontinuous polyacrylamide gel in a vertical Mini-Protean Tetra Cell gel system (Bio-Rad Inc., Hercules, CA, USA). The resolving gel was prepared at a concentration of 12.5 %, and the stacking gel at 9

Table 1
Elution profile of amino acid separation:Solvent A: 100 % AccQ-tag Ultra eluent A, solvent B: 10 % AccQ-tag Ultra eluent B in LC–MS grade water, solvent C: LC–MS grade water, solvent D: 100 % AccQ-tag Ultra eluent B.

Time (min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.00	0.65	10.00	0.00	90.00	0.00
0.29	0.65	9.90	0.00	90.10	0.00
3.50	0.65	9.90	0.00	90.10	0.00
4.60	0.65	9.90	25.00	65.10	0.00
5.49	0.65	9.00	80.00	11.00	0.00
7.10	0.65	8.00	25.00	57.90	9.10
7.30	0.65	8.00	15.60	57.90	18.50
7.50	0.65	8.00	12.00	57.90	22.10
8.20	0.65	7.80	0.00	77.20	15.00
8.30	0.65	4.00	0.00	36.30	59.70
8.55	0.65	4.00	0.00	36.30	59.70
8.60	0.65	4.00	65.00	26.00	5.00
9.20	0.65	4.00	60.00	36.00	0.00
9.70	0.65	10.00	0.00	90.00	0.00
10.90	0.65	10.00	0.00	90.00	0.00

%. Electrophoresis was performed with an initial constant voltage of 90 V for 15 min, followed by an increase to 160 V for 50–60 min to ensure optimal separation of protein bands. After electrophoresis, the gels were stained with Coomassie G250 solution, and protein bands were visualized and analyzed using the Bio-Rad ChemiDoc MP Imaging System.

2.6. Determination of phytochemical analysis

From the freeze-dried sample, 250 mg was extracted using a methanol:water (70:30) solution. The mixture was shaken and then placed in an ultrasonic bath for 2 h at room temperature in the dark. Subsequently, it was centrifuged at 6000 rpm for 5 min. The supernatant was filtered through a PTFE filter (0.22 μm pore size) to prepare the sample for analysis. Phytochemical analyses were performed using a Dionex Ultimate 3000RS UHPLC system (Thermo Fisher, USA) coupled to a Thermo Q Exactive Orbitrap hybrid mass spectrometer, equipped with a Thermo Accucore C18 analytical column (2.1 mm × 100 mm, 2.6 μm particle size), following the method of Kaszás et al. (2020). The flow rate was set to 0.2 mL/min, and the column temperature was maintained at 25 °C ± 1 °C. The mobile phase consisted of methanol (A) and water (B), both acidified with 0.1 % formic acid. The injection volume was 2 μL. The gradient profile was as follows: 0–3 min, 95 % B; 3–43 min, 0 % B; 43–61 min, 0 % B; 61–62 min, 95 % B; 62–70 min, 95 % B. The Thermo Q Exactive Orbitrap hybrid mass spectrometer (Thermo Fisher, USA) was fitted with an ESI source. Samples were analyzed in positive and negative ionization modes separately, with a capillary temperature of 320 °C and spray voltages of 4.0 kV (positive mode) and 3.8 kV (negative mode). The resolution was set to 35,000 for MS1 scans and 17,500 for MS2 scans, with a scanned mass range of 100–1500 *m/z*. For tandem MS (MS/MS) scans, the collision energy was set to 30 nominal collision energy units. Quantification was performed using an external calibration curve.

The standard compounds used for quantification included: Apigenin (≥95.0 %), Luteolin (≥98 %), Kaempferol (analytical standard), Quercetin (analytical standard), Chrysieriol (analytical standard), Tricin (analytical standard), Naringenin (analytical standard), Isoquercitrin (analytical standard), Rutin (analytical standard), Astragalin (analytical standard), Isovitexin (analytical standard), Nicotinic acid (≥95.0 %), Nicotinamide (≥99.5 %), Riboflavin (analytical standard), p-Coumaric acid (analytical standard), Caffeic acid (≥98 %), Ferulic acid (USP reference standard), Chlorogenic acid (analytical standard), Neochlorogenic acid (analytical standard), and Cryptochlorogenic acid (analytical standard).

2.7. Determination of total polyphenol (TPC) and flavonoid (TFC) content

The same hydroalcoholic extract used for quantitative phytochemical analysis was also utilized for measuring total polyphenols and flavonoids. The Folin–Ciocalteu reagent was applied to the supernatants (Singleton et al., 1999). Absorbance was measured at 760 nm for total polyphenol content (TPC) and at 415 nm for total flavonoid content (TFC) using a UV-160 A spectrophotometer (Shimadzu, Japan). A standard curve was prepared using varying concentrations of gallic acid. The TPC and TFC concentrations in the samples were expressed as milligrams of gallic acid equivalent (for TPC) and rutin equivalent (for TFC) per gram of dry weight (DW).

2.8. Statistical evaluation

Data analysis was conducted using Microsoft Excel 2016 and the SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was performed, with one-way ANOVA used to compare differences among treatments and two-way ANOVA applied to assess interactions between treatments. Mean separation was carried out using Tukey's post hoc test, and significant differences were considered at $p \leq 0.05$. The results are presented as mean values ± standard deviation.

3. Results and discussion

3.1. Yields and characterization of processed green biomass fractions

In continental climates, annual grasses like triticale are commonly used in green forage practices for silage production. Legumes are frequently combined with cereals in mixtures to enhance the nutritional value of swards for ruminant feed, primarily due to the high nitrogen content of legumes. Additionally, vetch, a leguminous green manure crop, shows promise in crop rotation systems for improving soil health (Pál & Zsombik, 2024). The fibrous components of grasses can also serve as a structural support, improving the efficiency of LPC extraction (Pisulewska et al., 1991). This study evaluated the suitability of a vetch and triticale green fodder mixture for protein concentration by comparing fermentation methods with MWC. The fresh green matter yield of the vetch + triticale mixture was 34.2 t ha^{-1} (data not shown), significantly higher than the yield reported by Pisulewska et al. (1989) for a different variety and growing season. However, their vetch-barley forage mixture yielded a comparable 32–34 t ha^{-1}. After fractionation of the vetch + triticale green biomass, the fiber-to-GJ ratio was 18 % and 82 %, respectively (Fig. 2/A), which is lower than that of crops like broccoli or Jerusalem artichoke (Domokos-Szabolcsy et al., 2022; Kaszás et al., 2020). Further processing of green juice for protein concentration revealed significant differences based on the method used. MWC and LAF achieved the highest LPC yields (11–12 %), whereas YLBF produced a significantly lower yield (~6 %) from the triticale + vetch green juice. LPC yield was inversely correlated with the brown aqueous fraction, known as brown juice (BJ). Specifically, MWC resulted in the lowest BJ yield (88 %), while YLBF produced the highest (~94 %). Overall, thermal coagulation proved more efficient than fermentation for LPC production, with yeast+*Lactobacillus* fermentation being the least effective. In green biorefinery experiments, BJ is typically the most abundant by-product, regardless of plant species (Kaszás et al., 2024; Kovács et al., 2023). Consequently, efforts are underway to valorize its nutrients for applications such as single-cell protein production or biofertilizer use (Barna et al., 2022).

The initial pH of the green juice was 5.80. After fermentation, the pH of the filtered BJ dropped below 4.0, regardless of the method used (Fig. 2/D). In contrast, MWC had no significant effect on pH. This pH reduction during fermentation is advantageous, as GJ or BJ at pH 5.8 is susceptible, whereas fermented BJ (pH ~3.8) can be stored at room

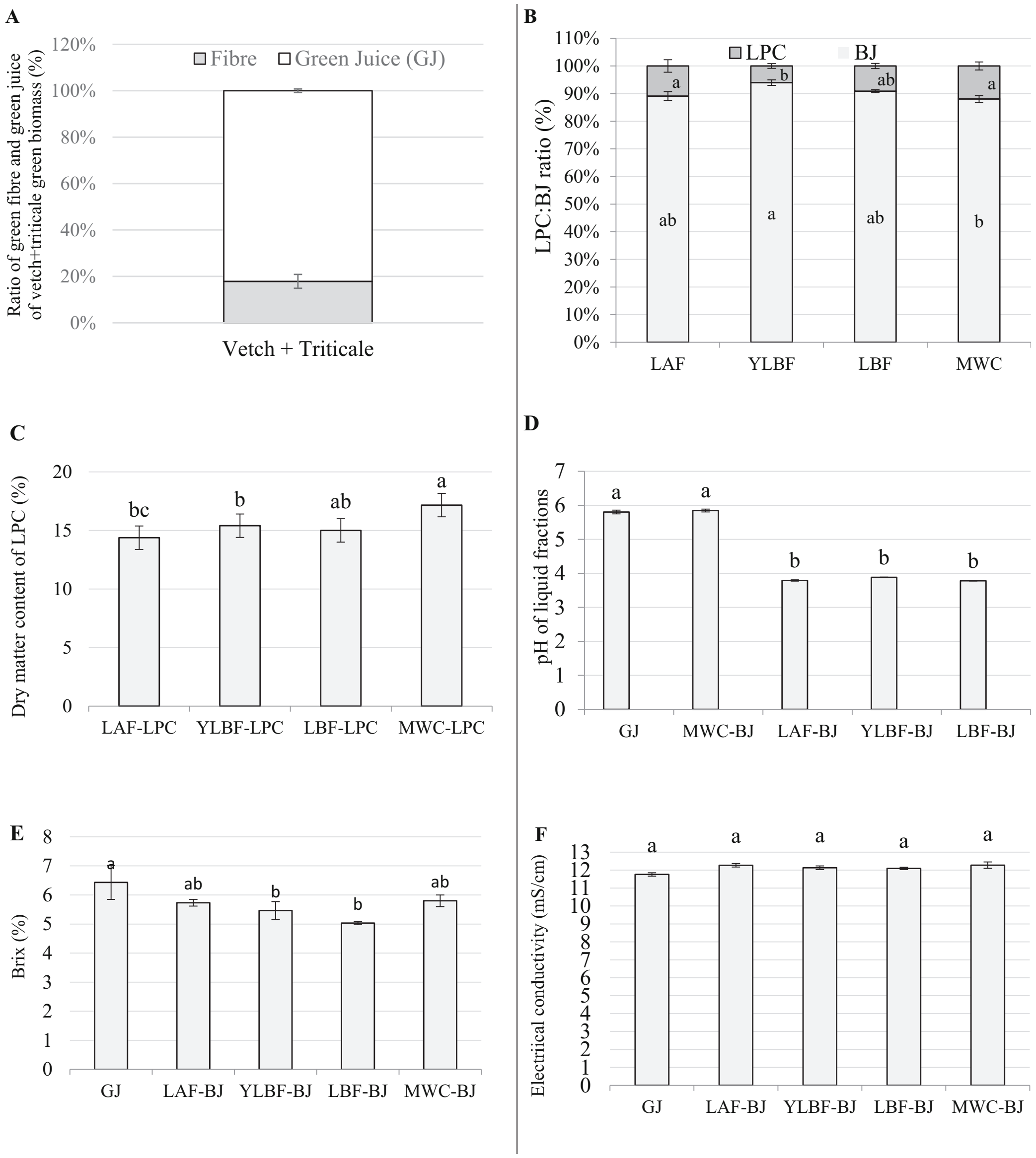

Fig. 2. (A) Ratio of green fiber and green juice, (B) yield percentage of brown juice and LPC fresh mass obtained after lactic acid (LAF), yeast+*Lactobacillus* (YLBF), *Lactobacillus* (LBF) fermentations, and microwave coagulation (MWC), (C) Dry matter content of LPC, (D) pH of green juice and brown juices obtained after microwave coagulation (MWC) and lactic acid (LAF), yeast+*Lactobacillus* (YLBF), and *Lactobacillus* (LBF) fermentations, (E) brix of green juice and brown juices obtained after microwave coagulation (MWC) and lactic acid (LAF), yeast+*Lactobacillus* (YLBF), and *Lactobacillus* (LBF) fermentations, (F) electrical conductivty of green juice and brown juices obtained after microwave coagulation (MWC) and lactic acid (LAF), yeast+*Lactobacillus* (YLBF), and *Lactobacillus* (LBF) fermentations. Different letters on bars are significant according to the Tukey's test at $p \leq 0.05$. Data are means±SD ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

temperature for extended periods without spoilage risk. This decrease reflects organic acid production, which is essential for protein coagulation and isolation. Pérez-Vila et al. (2022) noted that pH reduction during fermentation improves protein solubility and extraction efficiency. MWC, as a thermal process, does not alter pH, consistent with Segatto et al. (2022), who reported that thermal methods induce protein denaturation and coagulation without pH changes. The brix value of MWC-treated BJ was comparable to that of untreated GJ (~6.5 %), whereas the brix value of fermented BJ decreased alongside pH, indicating reduced water-soluble sugar content during fermentation (Fig. 2/E). This reduction occurs because microorganisms utilize sugars in the green juice as a substrate to produce organic acids, such as lactic acid. EC is commonly measured during milk fermentation, where its increase is attributed to lactose conversion to lactic acid and the solubilization of calcium and phosphorus salts bound to casein (Mucchetti et al., 1994). However, no literature was found on EC changes during plant GJ fermentation. During pressing, soluble mineral salts released from leaf tissue cell organelles are primarily present in the high-water-content GJ. Contrary to findings in milk fermentation, EC did not change significantly across the processing methods used for green juice. As shown in Fig. 2/F, EC values ranged from 11.76 to 12.28 mS cm^{-1} in both GJ and fermented BJ.

3.2. Quantitative and qualitative analysis of proteins in LPC

The freeze-dried GJ from pressed fresh biomass initially contained 26.02 m/m% crude protein (Table 1). The fermentation of GJ significantly increased the crude protein content in LPC fractions due to microbial activity, consistent with findings by Bákonyi et al. (2020). Specifically, the crude protein content rose to 38.5 m/m% in YLBF-LPC, 36.89 m/m% in LBF-LPC, and 35.14 m/m% in LAF-LPC. Santamaría-Fernández et al. (2017) reported comparable crude protein contents of 35–37 m/m% in LPC derived from clover-grass mixtures and alfalfa using LBF. Additionally, Çabuk et al. (2018) noted that fermentation enhances protein yield by degrading cell walls and releasing proteins. In comparison, MWC resulted in the highest crude protein content relative to GJ (Table 2). This aligns with Santamaría-Fernández and Lübeck (2020), who highlighted that thermal coagulation methods like MWC are highly effective for protein extraction due to their ability to denature proteins and promote coagulation. Table 2 also presents the proteinogenic amino acid composition of GJ and LPCs. The true protein content, calculated as the sum of amino acids, correlates directly with crude protein content but is consistently lower. The concentration of individual amino acids generally varies with the true protein content depending on the fermentation or microwave treatment applied; however, glutamine (Gln) was not detected in any treatment.

The GJ had a total amino acid content of 12.382 g 100 g^{-1}, with prominent levels of aspartic acid (Asp) at 1.549 g 100 g^{-1}, glutamic acid (Glu) at 1.600 g 100 g^{-1}, and arginine (Arg) at 0.775 g 100 g^{-1}. These amino acids are typically the most abundant in plant proteins (Domokos-Szabolcsy, Yavuz, et al., 2023), and consistent with this, they were also found in the highest concentrations in LPCs, regardless of the treatment applied. The amino acid composition of LPCs was significantly enhanced by the protein coagulation method. MWC yielded the highest total amino acid content (31.462 g 100 g^{-1}), with elevated levels of leucine (3.244 g 100 g^{-1}) and arginine (2.355 g 100 g^{-1}), aligning with Chandran et al. (2024), who noted that thermal methods improve the extraction of essential amino acids. Fermentation techniques also resulted in substantial amino acid contents, particularly for glutamic acid and aspartic acid, which are vital for protein synthesis and neurological functions, as reported by Msheliza et al. (2024). LAF-LPC sample had a total amino acid content of 24.811 g 100 g^{-1}, while LBF-LPC sample contained 23.261 g 100 g^{-1}. YLBF-LPC sample exhibited a total amino acid content of 24.181 g 100 g^{-1}, with especially high concentrations of glutamic acid (3.119 g 100 g^{-1}), aspartic acid (2.969 g 100 g^{-1}), and isoleucine (1.676 g 100 g^{-1}). Notably, isoleucine was more abundant in YLBF-LPC than in MWC-LPC (Table 2).

Table 2

Crude protein content (m/m%) and Amino acid contents (g/100 g sample) in green juice (GJ) and leaf protein concentrate (LPC) obtained from Triticale+Vetch fresh biomass mixture after microwave coagulation (MWC), lactic acid (LAF), *Lactobacillus* (LBF), and yeast+*Lactobacillus* (YLBF) fermentations. Data are presented as mean ± SD. Different letters above the columns show significant differences according to Tukey's test at the level of $p \leq 0.05$.

	Green juice	MWC-LPC	LAF-LPC	LBF-LPC	YLBF-LPC
Crude protein (m/m%)	26.02e	44.78a	35.14d	36.89c	38.50b
AA (g/100 g)					
NH3	0.109 ± 0.003	0.198 ± 0.036	0.236 ± 0.005	0.219 ± 0.016	0.180 ± 0.000
His	0.280 ± 0.002	0.810 ± 0.053	0.580 ± 0.074	0.569 ± 0.020	0.286 ± 0.000
Asn	0.190 ± 0.063	0.394 ± 0.047	0.285 ± 0.026	0.364 ± 0.031	0.503 ± 0.000
Ser	0.634 ± 0.021	1.581 ± 0.202	1.238 ± 0.204	1.068 ± 0.023	1.208 ± 0.000
Gln	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Arg	0.775 ± 0.052	2.355 ± 0.242	1.506 ± 0.299	1.256 ± 0.004	1.753 ± 0.000
Gly	0.687 ± 0.030	1.808 ± 0.181	1.441 ± 0.190	1.324 ± 0.037	1.434 ± 0.000
Asp	1.549 ± 0.078	3.631 ± 0.320	2.943 ± 0.422	2.631 ± 0.156	2.969 ± 0.000
Glu	1.600 ± 0.149	1.859 ± 0.469	2.842 ± 0.464	2.530 ± 0.090	3.119 ± 0.000
Thr	0.571 ± 0.014	1.549 ± 0.194	1.158 ± 0.190	1.033 ± 0.015	1.111 ± 0.000
Ala	0.831 ± 0.054	2.060 ± 0.191	1.803 ± 0.250	1.641 ± 0.080	1.852 ± 0.000
Pro	0.645 ± 0.021	1.696 ± 0.137	1.238 ± 0.152	1.171 ± 0.068	0.101 ± 0.000
Cys	0.094 ± 0.010	0.201 ± 0.039	0.066 ± 0.066	0.125 ± 0.002	1.102 ± 0.000
Lys	0.688 ± 0.082	1.818 ± 0.233	1.368 ± 0.254	1.182 ± 0.015	1.015 ± 0.000
Tyr	0.546 ± 0.020	1.668 ± 0.092	1.105 ± 0.124	1.055 ± 0.041	0.485 ± 0.000
Met	0.140 ± 0.012	0.599 ± 0.066	0.402 ± 0.064	0.449 ± 0.001	0.913 ± 0.000
Val	0.636 ± 0.004	1.913 ± 0.108	1.340 ± 0.187	1.331 ± 0.048	1.092 ± 0.000
Ile	0.454 ± 0.019	1.552 ± 0.059	1.072 ± 0.144	1.120 ± 0.041	1.676 ± 0.000
Leu	1.058 ± 0.003	3.244 ± 0.273	2.379 ± 0.332	2.272 ± 0.094	1.827 ± 0.000
Phe	0.752 ± 0.037	2.244 ± 0.133	1.694 ± 0.182	1.699 ± 0.063	1.556 ± 0.000
Sum	12.38 ± 0.228	31.46 ± 1.083	24.81 ± 3.320	23.26 ± 0.840	24.18 ± 0.000

Overall, these results indicate that MWC is highly effective for extracting elevated amino acid concentrations, while fermentation methods also demonstrate significant extraction efficiency, particularly for glutamic acid and aspartic acid. These findings offer valuable insights into the efficacy of various protein isolation techniques for extracting amino acids from biomass, which is critical for optimizing green protein production from plant sources.

The protein concentration process significantly affects the fate of soluble proteins in GJ. SDS-PAGE revealed distinct differences in the protein expression patterns of LPC compared to GJ (Fig. 3). Due to its high soluble protein content, GJ exhibited the greatest number of protein bands (Fig. 3). In contrast, MWC-LPC displayed the fewest visible bands, as microwave treatment above 80 °C coagulates most proteins, although imperfect coagulation allows a small fraction of proteins to be

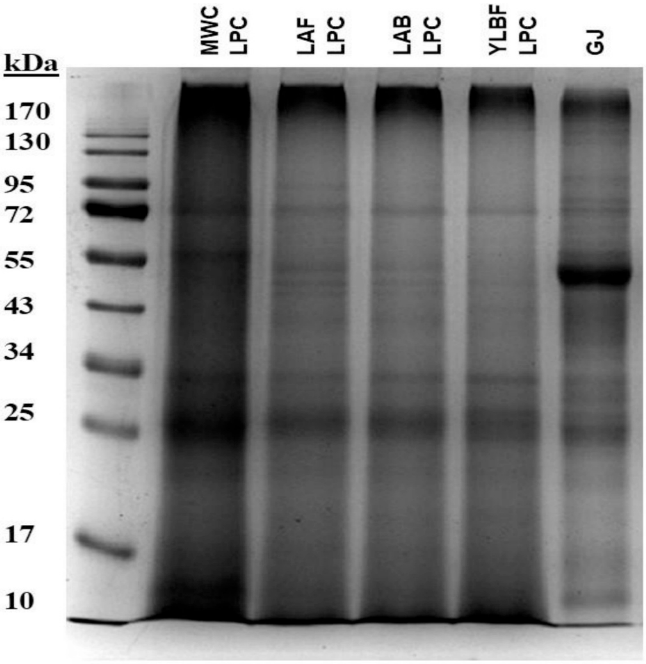

Fig. 3. Protein pattern as SDS-PAGE of fractions that obtained by various processes of Triticale-Vetch leaves: freeze-dried green juice (GJ); lactic acid fermented leaf protein concentrate (LAF-LPC); yeast+*Lactobacillus* fermented leaf protein concentrate (YLBF-LPC); *Lactobacillus* fermented leaf protein concentrate (LAB-LPC); microwave coagulated leaf protein concentrate (MWC-LPC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resolubilized. High-abundance proteins in C3 plants, such as the Rubisco large subunit (55 kDa) and small subunit (14 kDa), are more likely to be solubilizable. Nynäs et al. (2021) reported that the thermal stability of Rubisco subunits varies across plant species, attributed to the protein environment, and is typically below 66 °C, with a faint presence of the large subunit observed above 80 °C in sugar beet. Fermentation methods resulted in more protein bands in LPCs compared to MWC-LPC. LAF alters protein content and composition due to enzymatic activity and the presence of acids, with proteolysis by lactic acid bacteria (*Lactobacillus* spp.) contributing to the increased number of bands by hydrolyzing proteins to release amino acids for microbial use (Emkani et al., 2022). Among the fermented fractions, YLBF-LPC showed the fewest visible bands between ~43 kDa and ~ 55 kDa. Fermentation precipitates a fraction of proteins near their isoelectric points (around pH ~4), where they become insoluble. Additionally, the higher number of protein bands in fermented LPCs is partly due to proteins from the microbial background.

3.3. Quantification of phytochemicals in LPC

The GJ initially contained 259 μg g^{-1} of TPC and 63 μg g^{-1} of TFC. Fermentation significantly increased both TPC and TFC compared to GJ (Fig. 4/A, B), whereas MWC only significantly elevated TFC. Among fermentation methods, LAF increased TPC to 294 μg g^{-1} and TFC to 139 μg g^{-1}, while YLBF yielded the highest values, with TPC at 305 μg g^{-1} and TFC at 149 μg g^{-1}. These findings align with Domokos-Szabolcsy, Yavuz, et al. (2023), who noted that fermentation enhances the release of phenolic compounds, which contribute to the antioxidant properties and nutritional value of LPC. Similarly, Emkani et al. (2022) reported that LAF increases TPC in legumes, with outcomes varying based on the legume type, fermenting microorganisms, and fermentation process. The increase in TPC likely results from the release of these compounds from plant cell walls due to structural degradation or enzymatic transformation during fermentation. Ng et al. (2011) also observed that fermentation increases the TPC in plant tissues, attributing enhanced antioxidant activity to phenolic compounds acting as reducing agents, hydrogen donors, and singlet oxygen quenchers.

A hyphenated analytical method was employed to analyze the phenolic compound composition in GJ derived from triticale and vetch green biomass (data not shown). Based on these findings, phenolic compounds were selected for quantitative analysis during fermentation and MWC (Figs. 5 and 6; Table 3). Flavonoids in the processed triticale+vetch green biomass fractions were categorized into two groups based on their concentrations (Fig. 5/A, B). Major flavone components, including tricin (3′,5′-dimethoxy-4′,5,7-trihydroxyflavone), isovitexin (apigenin-6-C-glucoside), apigenin (4′,5,7-trihydroxyflavone), chrysoeriol (3′-methoxy-4′,5,7-trihydroxyflavone), and luteolin (3′,4′,5,7-tetrahydroxyflavone), ranged from 6.1 to 304.1 μg g^{-1} (Fig. 5/A). In the GJ fraction, concentrations were tricin (6.1 μg g^{-1}), apigenin (20.0 μg g^{-1}), chrysoeriol (25.3 μg g^{-1}), luteolin (40.3 μg g^{-1}), and isovitexin (55.9 μg g^{-1}). Protein concentration methods significantly increased the levels of these flavone aglycones or monoglycosides. Consistent with TFC, fermentation led to a greater increase in these flavone components compared to MWC. Specifically, anaerobic fermentation methods, i.e., LAF-LPC, LBF-LPC, and YLBF-LPC, resulted in elevated concentrations of apigenin (68.1–112.3 μg g^{-1}), luteolin (242.6–304.1 μg g^{-1}), and chrysoeriol (248.5–285.5 μg g^{-1}), demonstrating the effectiveness of fermentation in extracting these compounds. However, no consistent differences were observed among the three fermentation methods. Minor flavonoid components, including naringenin (4′,5,7-trihydroxyflavanone), kaempferol (3,4′,5,7-tetrahydroxyflavone), astragalin (kaempferol-3-O-glucoside), quercetin (3,3′,4′,5,7-pentahydroxyflavone), isoquercitrin (quercetin-3-O-glucoside), and rutin (quercetin-3-rutinoside), were present at ≤38.9 μg g^{-1} (Fig. 5/B; Table 3). Quercetin concentrations, like those of major flavones, increased significantly with protein concentration methods, whether thermal or fermentation-based, which is beneficial due to its potent antioxidant properties. The chelating activity of iron in flavonoids is influenced by resonance structures, such as the 4-keto group and the 2–3 double bond, or modifications like methylation of 3-OH and 5-OH groups (Hur et al., 2014). In contrast, fermentation drastically reduced the concentrations of rutin and isoquercitrin, glycosylated quercetin derivatives, from 38.9 μg g^{-1} and 5.3 μg g^{-1}, respectively, to below the detection limit in post-fermentation LPC. Microorganisms producing β-glucosidase can convert rutin to quercetin (Shin et al., 2016). Gao et al. (2024) suggested that high-molecular-weight flavonoids degrade into smaller molecules during biochemical reactions. Thus, the increased levels of flavonoid aglycones, such as quercetin, likely result from the release of bound flavonoids during fermentation through microbial and enzymatic activity, rather than continuous biosynthesis. Previous experiments with broccoli confirmed that aglycones of kaempferol and quercetin derivatives were the most abundant post-fermentation compounds in LPCs (Domokos-Szabolcsy et al., 2022). Due to their high hydrophilicity and molecular weight, flavonoid

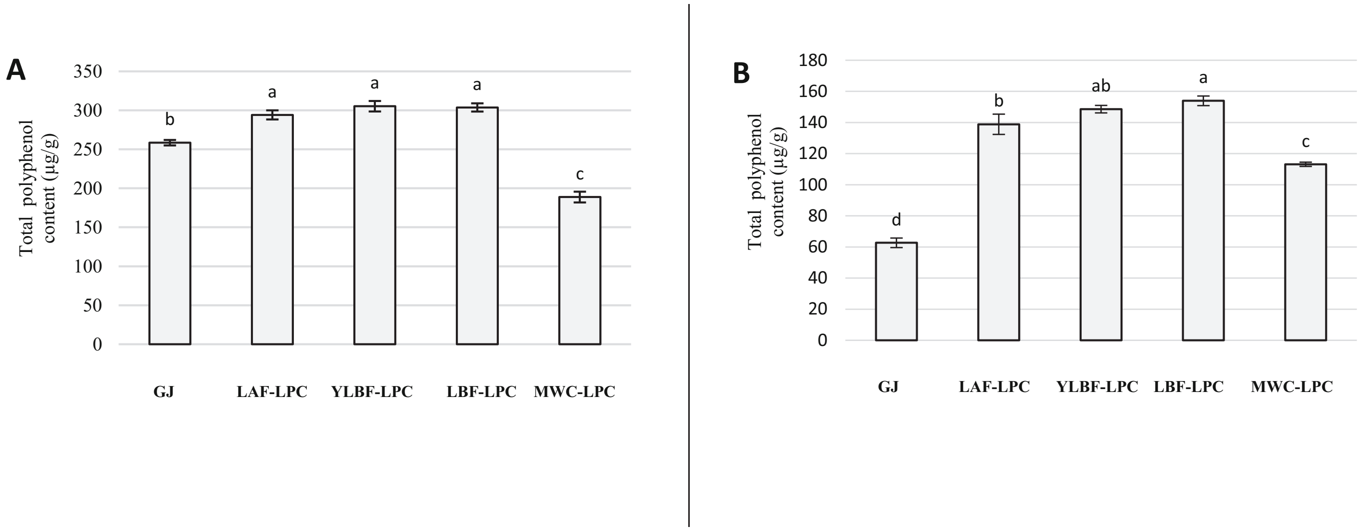

Fig. 4. (A) total polyphenol content (TPC), (B) total flavonoid content (TFC), of green juice and leaf protein content (LPC) obtained from Triticale+Vetch fresh biomass mixture after microwave coagulation (MWC), lactic acid (LAF), *Lactobacillus* (LBF), and yeast+*Lactobacillus* (YLBF) fermentations. Different letters on bars are significant according to the Tukey's test at $p \leq 0.05$. Data are means±SD ($n = 3$).

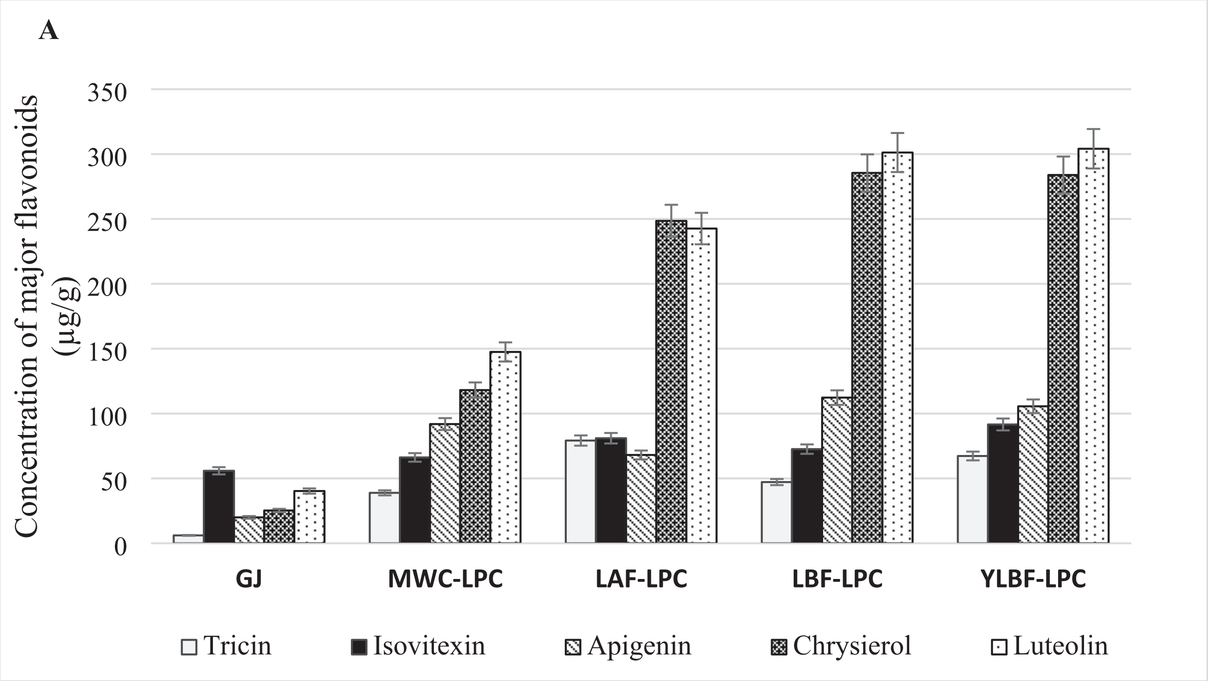

Fig. 5. Concentration of (A) major flavonoids, (B) chlorogenic acid isomers, (C) phenolic acids in green juice (GJ), leaf protein concentrate (LPC) fractions obtained from Triticale+Vetch fresh biomass mixture after microwave coagulation (MWC), lactic acid (LAF), *Lactobacillus* (LBF), and yeast+*Lactobacillus* (YLBF) fermentations.

glycosides exhibit impaired absorption in the intestinal cells of healthy adults, requiring conversion to aglycones by β-glucosidases for uptake into peripheral circulation. Aglycones and their metabolites are more bioavailable and bioactive than their precursor glycosides (Gaya et al., 2020).

With the exception of chlorogenic acid, phenolic compounds were detected at lower concentrations than the major flavonoids. Fig. 5/D details the concentrations of p-coumaric acid, caffeic acid, and ferulic acid in various samples. In the GJ sample, concentrations were 4.7 μg g^{-1} for p-coumaric acid, 1.4 μg g^{-1} for caffeic acid, and 13.4 μg g^{-1} for ferulic acid. The MWC-LPC sample showed significantly higher levels, with p-coumaric acid at 16.0 μg g^{-1}, caffeic acid at 5.3 μg g^{-1}, and ferulic acid at 36.0 μg g^{-1}, suggesting that thermal coagulation enhances the extraction of these compounds. In fermentation samples, including LAF-LPC, LBF-LPC, and YLBF-LPC, p-coumaric acid ranged from 1.5 to 10.8 μg g^{-1}, caffeic acid from 3.5 to 8.5 μg g^{-1}, and ferulic acid from 6.6 to 33.1 μg g^{-1}. Notably, LAF-LPC exhibited the highest ferulic acid concentration (33.1 μg g^{-1}) among the fermentation samples. Controlled fermentation with lactic acid bacteria (*Lactobacillus* spp.) facilitates the conversion of simple phenolics and the depolymerization of high-molecular-weight phenolic compounds, increasing their solubilizable forms (Dueñas et al., 2005). Phenolic acids, such as p-coumaric, caffeic, and ferulic acids, offer significant health benefits, with ferulic acid linked to anti-cancer, anti-inflammatory, and neuroprotective properties (Ancy Jenifer et al., 2021). In vitro studies indicate that p-coumaric acid has lower free radical scavenging and reducing activity against DPPH, superoxide, and ABTS radicals compared to caffeic and ferulic acids (Mathew et al., 2015). However, in cellular assays using human lung (A549) and colon adenocarcinoma (HT29-D4) cell lines, p-coumaric acid demonstrated comparable or greater capacity to eliminate reactive oxygen species than ferulic acid (Pei et al., 2015). Additionally, p-coumaric, caffeic, and ferulic acids can mitigate insulin resistance in diabetic rats by increasing adiponectin production (Pei et al., 2015). Ferulic acid also inhibits cancer cell growth, reduces inflammation, and protects against neurodegenerative diseases like Alzheimer's by reducing oxidative stress in the brain (Ancy Jenifer et al., 2021). The high ferulic acid levels extracted via LAF (33.1 μg g^{-1}) and MWC (36.0 μg g^{-1}) indicate that LPC is a valuable source of this compound for functional foods and nutraceuticals. Caffeic acid, another phenolic acid detected in LPC, exhibits antioxidant and anti-inflammatory properties, with studies by Fessard et al. (2017) showing its ability to reduce oxidative stress and inflammation, making it valuable for preventing chronic diseases like cardiovascular disorders and diabetes. The elevated caffeic acid levels in fermentation samples, particularly in LAF (8.5 μg g^{-1}), suggest that fermentation enhances the extraction of this solubilizable compound, positioning LPC as a promising source for functional foods.

Although present at lower concentrations than ferulic and caffeic acids, p-coumaric acid contributes to the antioxidant and anti-inflammatory properties of LPC. Pérez-Vila et al. (2022) noted that p-coumaric acid reduces oxidative stress and inflammation, making it a valuable compound for promoting overall health. Its presence in LPC, particularly in MWC samples (16.0 μg g^{-1}), underscores LPC's potential as a source of this solubilizable compound for functional foods and nutraceuticals.

Chlorogenic acids were the most abundant phenolic acids in GJ and LPCs. Three isomers—3-O-caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA), and 5-O-caffeoylquinic acid (5-CQA)—were identified in GJ with a characteristic $[M + H]^+$ ion at *m/z* 355.1029. Among these, 3-CQA (chlorogenic acid) was predominant (Fig. 5/C). This finding aligns with Alcázar Magaña et al. (2021), who reported that 3-CQA is the most common isomer in plant species. Chlorogenic acids (CQAs) act as antioxidants or Michael acceptors, targeting the Keap1-Nrf2 pathway, a key regulator of cellular resistance to oxidative stress. In vitro studies indicate that the ability to activate Nrf2 varies among CQA isomers. Generally, protein concentration methods led to a significant decrease in chlorogenic acid concentrations. In GJ, chlorogenic acid was present at 225.0 μg g^{-1}, neochlorogenic acid at 14.0 μg g^{-1}, and cryptochlorogenic acid at 3.1 μg g^{-1}. The MWC-LPC sample showed reduced levels of chlorogenic acid (159.9 μg g^{-1}) and neochlorogenic

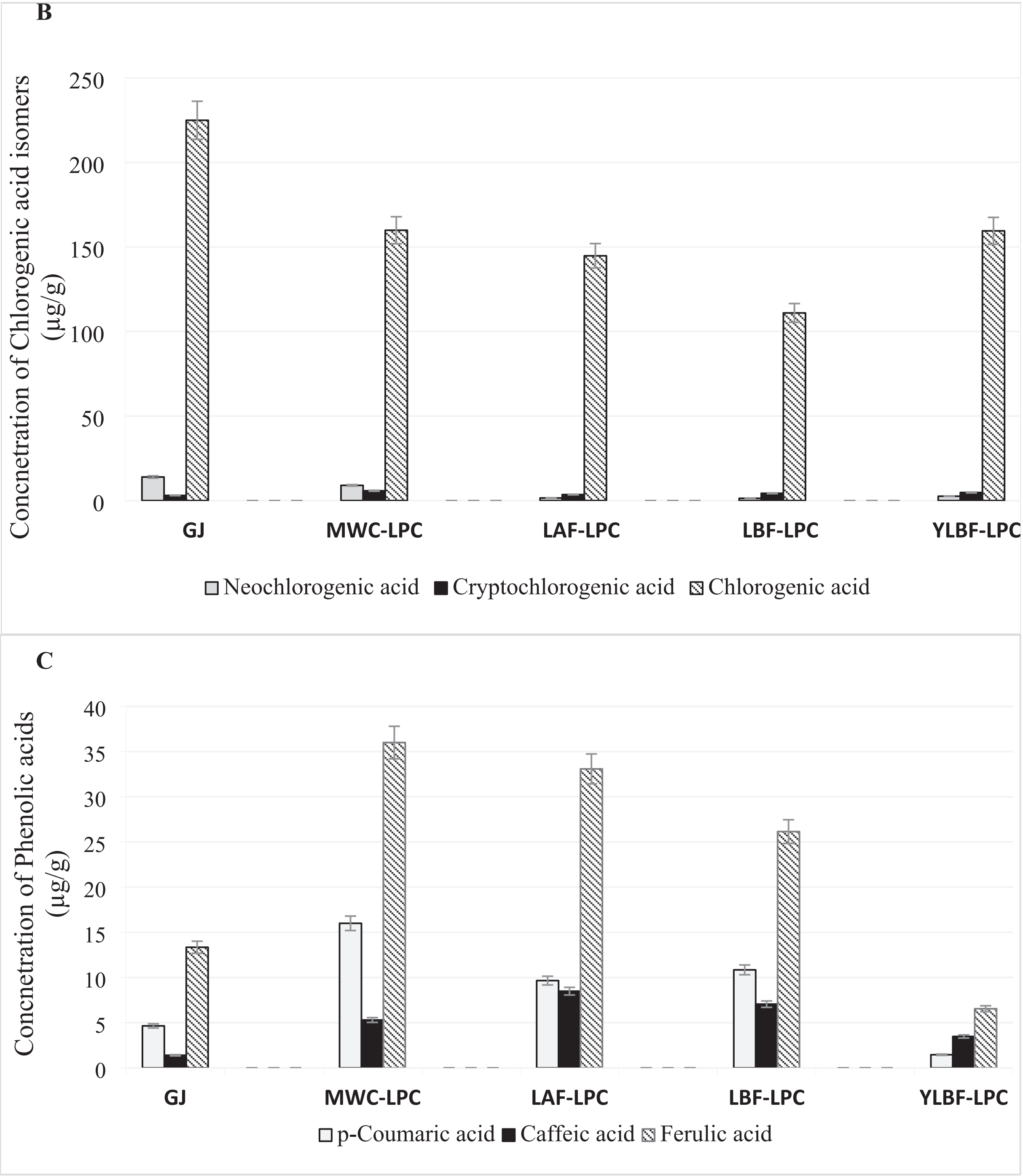

Fig. 5. (*continued*).

acid (9.0 µg g^{-1}) but a higher concentration of cryptochlorogenic acid (5.8 µg g^{-1}) compared to GJ. In fermentation samples, including LAF-LPC, LBF-LPC, and YLBF-LPC, chlorogenic acid ranged from 111.0 to 159.5 µg g^{-1}, neochlorogenic acid from 1.4 to 2.6 µg g^{-1}, and cryptochlorogenic acid from 3.6 to 4.8 µg g^{-1}. Notably, YLBF-LPC exhibited the highest cryptochlorogenic acid concentration (4.8 µg g^{-1}) among fermentation samples.

These findings are consistent with and build upon existing literature on extracting bioactive compounds from plant biomass. For instance, Zhao and Shah (2016) reported that fermentation enhances the bioavailability of flavonoids and phenolic acids, making them more solubilizable for human absorption. Similarly, Domokos-Szabolcsy, Yavuz, et al. (2023) highlighted that thermal methods, such as MWC, improve the extraction of heat-stable compounds like phenolic acids and vitamins. This study further demonstrates that fermentation techniques, particularly LAF and LBF, preserve heat-sensitive compounds like luteolin and chrysoeriol, enhancing the versatility and nutrient density of LPC.

Our findings demonstrate that fermentation techniques, particularly YLBF and LBF, significantly enhance the yield and quality of LPC while preserving bioactive compounds. However, MWC yielded the highest LPC output, with the greatest dry matter and protein content, establishing it as the most efficient method for protein extraction. Although MWC is the most effective for protein recovery, fermentation provides a valuable alternative, especially for applications requiring heat-sensitive bioactive compounds. The elevated levels of luteolin, chrysoeriol, and

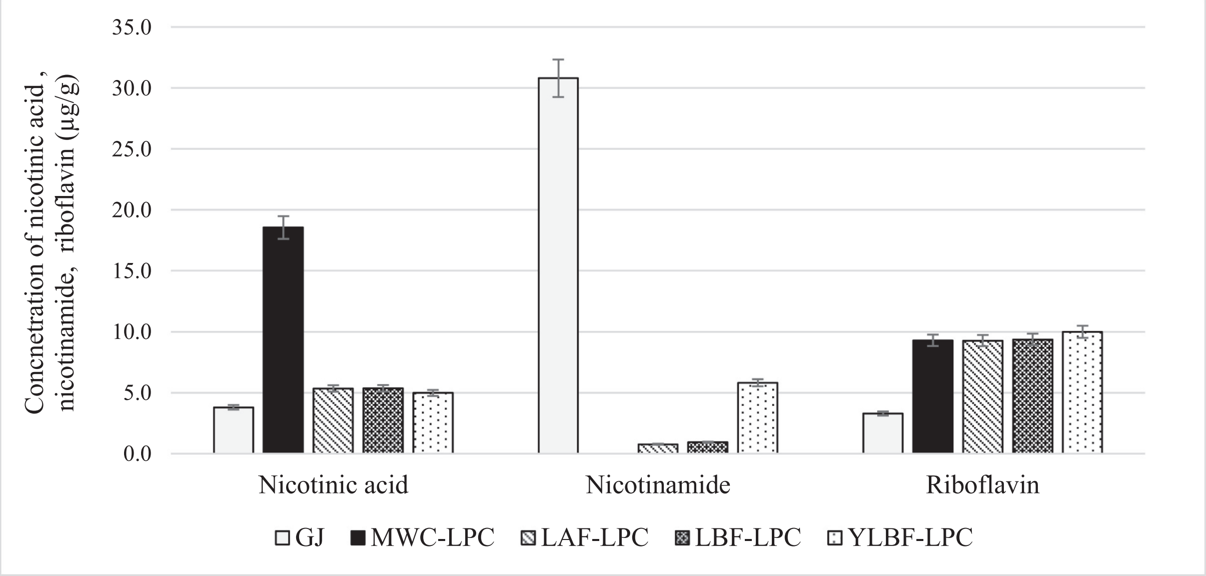

Fig. 6. Contents of nicotinic acid, nicotinamide, and riboflavin in green juice (GJ), leaf protein concentrate (LPC) fractions obtained from Triticale+Vetch fresh biomass mixture after microwave coagulation (MWC), lactic acid (LAF), *Lactobacillus* (LBF), and yeast+*Lactobacillus* (YLBF) fermentations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Concentration of minor flavonoids in green juice (GJ), leaf protein concentrate (LPC) fractions obtained from Triticale+Vetch fresh biomass mixture after microwave coagulation (MWC), lactic acid (LAF), *Lactobacillus* (LBF), and yeast+*Lactobacillus* (YLBF) fermentations. nd: no data.

Concentration of minor flavonoids (%)	Naringenin	Kaempferol	Astragalin	Isoquercitrin	Rutin	Quercetin
GJ	0.2	0.5	4.5	5.3	38.9	0.4
MWC-LPC	1.1	8.2	0.7	1.3	18.5	20.9
LAF-LPC	1.0	nd	nd	nd	nd	17.3
LBF-LPC	1.3	nd	nd	nd	nd	23.7
YLBF-LPC	1.1	nd	nd	nd	nd	25.3

ferulic acid extracted through fermentation are particularly noteworthy due to their nutritional and medicinal significance. Furthermore, the amino acid profiles obtained from both thermal and fermentation methods underscore LPC's potential as a high-quality, solubilizable protein source for human and animal nutrition.

3.4. Quantitative analysis of vitamins of GJ

Fig. 6 presents the concentrations of nicotinic acid, nicotinamide, and riboflavin in various samples. In the GJ sample, nicotinic acid was detected at 3.8 μg g^{-1}, nicotinamide at 30.8 μg g^{-1}, and riboflavin at 3.3 μg g^{-1}. The MWC-LPC sample showed higher concentrations of nicotinic acid (18.6 μg g^{-1}) and riboflavin (9.3 μg g^{-1}), but nicotinamide was undetectable (nd). In fermented LPC samples, including LAF-LPC, LBF-LPC, and YLBF-LPC, nicotinic acid levels were relatively low (5.0–5.4 μg g^{-1}), while riboflavin levels remained consistent (9.3–10.0 μg g^{-1}). Nicotinamide was present in low amounts in LAF-LPC (0.8 μg g^{-1}) and LBF-LPC (0.9 μg g^{-1}), with a higher concentration in YLBF-LPC (5.8 μg g^{-1}). Vitamins such as nicotinic acid (niacin) and riboflavin (vitamin B2) are essential for metabolic processes and overall health. Niacin supports energy conversion from food and maintains healthy skin and nerves, while riboflavin is critical for energy production and the metabolism of fats, drugs, and steroids. The elevated riboflavin levels in fermented LPC samples (9.3–10.0 μg g^{-1}) indicate that LPC is a valuable source of this vitamin, particularly for addressing dietary deficiencies.

Fermentation techniques maintained consistent riboflavin levels, which is significant given riboflavin's role in metabolic processes (Fessard et al., 2017). Studies have shown that fermentation by lactic acid bacteria (*Lactobacillus* spp.) can enhance riboflavin concentrations (Yang et al., 2024). Compared to other plant-based sources like leafy greens or legumes, LPC provides a concentrated, sustainable source of these solubilizable vitamins. Domokos-Szabolcsy et al. (2023) noted that fermentation and thermal methods improve vitamin extraction from plant biomass, positioning LPC as a promising solution for addressing vitamin deficiencies in populations with limited dietary diversity. While fermentation resulted in lower nicotinic acid levels, it consistently preserved riboflavin, highlighting the distinct advantages of fermentation and thermal methods in extracting specific bioactive compounds from biomass.

4. Conclusion

This study investigated the potential for concentrating protein in green fodder mixtures of peas and triticale, commonly used in silage production, by comparing three fermentation methods with MWC as a control. The results demonstrated that MWC yielded a higher proportion of easily separable LPC with greater crude protein content compared to fermentation methods. However, fermentation led to higher accumulation of essential amino acids, such as methionine and isoleucine, which contribute to true protein content in fermented LPCs. Additionally, fermented LPCs contained more solubilizable proteins, whereas the denatured protein aggregates in MWC-LPC were less readily resolubilized, potentially impacting digestibility, particularly for food applications. Beyond changes in protein quality, fermentation methods offered significant advantages in enhancing the quantity of bioactive compounds in LPCs. Fermentation resulted in substantial increases in health-promoting aglycones, including tricin, chrysoeriol, and luteolin, due to the depolymerization of high-molecular-weight flavonoids. Among the fermentation methods, LBF and YLBF were more effective in accumulating these flavonoids. However, this study has limitations, such as the small-scale experimental design and the need for further optimization of extraction techniques to improve efficiency and scalability. Additionally, the economic feasibility and sensory properties of LPC-based products require further exploration to ensure consumer acceptance.

CRediT authorship contribution statement

S. Reyhan Yavuz: Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Tarek Alshaal:** Writing – review & editing, Validation, Investigation, Conceptualization. **Wildan Suhartini:** Investigation, Formal analysis. **Nóra Bákonyi:** Writing – review & editing, Validation, Investigation. **Zoltán Kovács:** Investigation. **László Kaszás:** Investigation. **Szilvia Veres:** Investigation. **Miklós G. Fári:** Resources, Investigation. **Nevien Elhawat:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Éva Domokos-Szabolcsy:** Writing – review & editing, Validation, Supervision, Investigation, 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.

Acknowledgement

This work has been implemented with the TKP2021-EGA-20 support provided by the National Research, Development, and Innovation Fund of Hungary. The research project was also funded by the Stipendium Hungaricum scholarship. The authors would like to thank Kruppa Mag Ltd. for the vetch and triticale seeds provided from their own breeding stock.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.103153.

Data availability

Data will be made available on request.

References

Alcázar Magaña, A., Kamimura, N., Soumyanath, A., Stevens, J. F., & Maier, C. S. (2021). Caffeoylquinic acids: Chemistry, biosynthesis, occurrence, analytical challenges, and bioactivity. *The Plant Journal: For Cell and Molecular Biology, 107*(5), 1299–1319. https://doi.org/10.1111/tpj.15390

Ancy Jenifer, A., Vasanthy, M., Thamarai Selvi, C., Ravindran, B., Chung, W. J., & Chang, S. W. (2021). Treatment of coffee cherry pulping wastewater by using lectin protein isolated from Ricinus communis L. seed. *Journal of Water Process Engineering, 39*, 101742. https://doi.org/10.1016/j.jwpe.2020.101742

Bákonyi, N., Kisvarga, S., Barna, D. O., Tóth, I., El-Ramady, H., Abdalla, N., … Fári, M. G. (2020). Chemical traits of fermented alfalfa Brown juice: Its implications on physiological, biochemical, anatomical, and growth parameters of Celosia. *Agronomy, 10*(2), 247. https://doi.org/10.3390/agronomy10020247

Barna, D., Alshaal, T. O., Tóth, I., Cziáky, Z., Fári, M. G., Domokos-Szabolcsy, É., & Bákonyi, N. (2022). Bioactive metabolite profile and antioxidant properties of brown juice, a processed alfalfa (*Medicago sativa*) by-product. *Heliyon, 8*(11), Article e11655. https://doi.org/10.1016/j.heliyon.2022.e11655

Çabuk, B., Nosworthy, M. G., Stone, A. K., Korber, D. R., Tanaka, T., House, J. D., & Nickerson, M. T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology, 56*(2), 257–264. https://doi.org/10.17113/ftb.56.02.18.5450

Chandran, A. S., Kashyap, P., & Thakur, M. (2024). Effect of extraction methods on functional properties of plant proteins: A review. *eFood, 5*(3), Article e151. https://doi.org/10.1002/efd2.151

D'Almeida, A. P., & de Albuquerque, T. L. (2025). Is it possible to produce meat without animals? The potential of microorganisms as protein sources. *Fermentation, 11*(1), 24. https://doi.org/10.3390/fermentation11010024

Domokos-Szabolcsy, É., Alshaal, T., Elhawat, N., Kovács, Z., Kaszás, L., Béni, Á., & Kiss, A. (2024). Enhanced oligopeptide and free tryptophan release from chickpea and lentil proteins: A comparative study of enzymatic modification with bromelain, Ficin, and papain. *Plants, 13*(21), 3100. https://doi.org/10.3390/plants13213100

Domokos-Szabolcsy, É., Alshaal, T., Koroknai, J., Kovács, S., Tóth, C., Csilléry, G., … Fári, M. G. (2023). Phytochemical evaluation of the fruits and green biomass of determinate-type sweet pepper (*Capsicum annuum L. fasciculatum*) grown in terrestrial bioregenerative life-support research facilities. *Journal of Plant Interactions, 18*(1), Article 2268118. https://doi.org/10.1080/17429145.2023.2268118

Domokos-Szabolcsy, É., Elhawat, N., Domingos, G. J., Kovács, Z., Koroknai, J., Bodó, E., … Bákonyi, N. (2022). Comparison of Wet Fractionation Methods for Processing Broccoli Agricultural Wastes and Evaluation of the Nutri-Chemical Values of Obtained Products. *Foods, 11(16), Article 16*. https://doi.org/10.3390/foods11162418

Domokos-Szabolcsy, É., Yavuz, S. R., Picoli, E., Fári, M. G., Kovács, Z., Tóth, C., … Elhawat, N. (2023). Green biomass-based protein for sustainable feed and food supply: An overview of current and future prospective. *Life, 13*(2), 307. https://doi.org/10.3390/life13020307

Dueñas, M., Fernández, D., Hernández, T., Estrella, I., & Muñoz, R. (2005). Bioactive phenolic compounds of cowpeas (*Vigna sinensis L.*) modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *Journal of the Science of Food and Agriculture, 85*(2), 297–304. https://doi.org/10.1002/jsfa.1924

Emkani, M., Oliete, B., & Saurel, R. (2022). Effect of lactic acid fermentation on legume protein properties, a review. *Fermentation, 8*(6), 244. https://doi.org/10.3390/fermentation8060244

Espinosa-Marrón, A., Adams, K., Sinno, L., Cantu-Aldana, A., Tamez, M., Marrero, A., … Mattei, J. (2022). Environmental impact of animal-based food production and the feasibility of a shift toward sustainable plant-based diets in the United States. *Frontiers in Sustainability, 3*. https://doi.org/10.3389/frsus.2022.841106

Fessard, A., Kapoor, A., Patche, J., Assemat, S., Hoarau, M., Bourdon, E., … Remize, F. (2017). Lactic Fermentation as an Efficient Tool to Enhance the Antioxidant Activity of Tropical Fruit Juices and Teas. *Microorganisms, 5*(2), 23. https://doi.org/10.3390/microorganisms5020023

Food and Agriculture Organization of the United Nations (Ed.). (2017). *The future of food and agriculture: Trends and challenges. (Food and Agriculture Organization of the United Nations)*.

Gao, Y., Zhou, H., Wang, Y., Nussio, L. G., Yang, F., & Ni, K. (2024). Insights into fermentation with lactic acid bacteria on the flavonoids biotransformation of alfalfa silage. *Chemical and Biological Technologies in Agriculture, 11*(1), 73. https://doi.org/10.1186/s40538-024-00594-w

Gaya, P., Peirotén, Á., & Landete, J. M. (2020). Expression of a β-glucosidase in bacteria with biotechnological interest confers them the ability to deglycosylate lignans and flavonoids in vegetal foods. *Applied Microbiology and Biotechnology, 104*(11), 4903–4913. https://doi.org/10.1007/s00253-020-10588-x

Goksen, G., Karabulut, G., Keklik, M., & Martínez-Sanz, M. (2025). Comprehensive review of the digestibility of novel alternative protein sources: Current status and challenges. *Food Chemistry, 489*, Article 145013. https://doi.org/10.1016/j.foodchem.2025.145013

Guba, A., Bába, O., Tőzsér, J., Csősz, É., & Kalló, G. (2022). Fast and sensitive quantification of AccQ-tag Derivatized amino acids and biogenic amines by UHPLC-UV analysis from complex biological samples. *Metabolites, 12*(3), 272. https://doi.org/10.3390/metabo12030272

Hanczakowski, P., Szymezyk, B., & Skraba, B. (1991). Composition and nutritive value of native and modified green fraction of leaf protein from lucerne (Medicago sativa). *Journal of the Science of Food and Agriculture, 56*(4), 495–501. https://doi.org/10.1002/jsfa.2740560409

Hur, S. J., Lee, S. Y., Kim, Y.-C., Choi, I., & Kim, G.-B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chemistry, 160*, 346–356. https://doi.org/10.1016/j.foodchem.2014.03.112

Jensen, E. S., Carlsson, G., & Hauggaard-Nielsen, H. (2020). Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: A global-scale analysis. *Agronomy for Sustainable Development, 40*(1), 5. https://doi.org/10.1007/s13593-020-0607-x

Kaszás, L., Alshaal, T., El-Ramady, H., Kovács, Z., Koroknai, J., Elhawat, N., Nagy, É., Cziáky, Z., Fári, M., & Domokos-Szabolcsy, É. (2020). Identification of bioactive phytochemicals in leaf protein concentrate of Jerusalem artichoke (*Helianthus tuberosus L.*). *Plants, 9*(7), 889. https://doi.org/10.3390/plants9070889

Kaszás, L., Alshaal, T., Kovács, Z., Koroknai, J., Elhawat, N., Nagy, É., El-Ramady, H., Fári, M., & Domokos-Szabolcsy, É. (2022). Refining high-quality leaf protein and valuable co-products from green biomass of Jerusalem artichoke (*Helianthus tuberosus L.*) for sustainable protein supply. *Biomass Conversion and Biorefinery, 12*(6), 2149–2164. https://doi.org/10.1007/s13399-020-00696-z

Kaszás, L., Kovács, Z., Koroknai, J., Elhawat, N., Fári, M., Cziáky, Z., Alshaal, T., & Domokos-Szabolcsy, É. (2024). Identification of key parameters and phytochemical composition of fiber and brown juice by-products from green biomass of Jerusalem artichoke (*Helianthus tuberosus L.*). *Biomass and Bioenergy, 188*, Article 107332. https://doi.org/10.1016/j.biombioe.2024.107332

Kaszás, L., Kovács, Z., Nagy, E., Elhawat, N., Abdalla, N., & Domokos-Szabolcsy, E. (2018). Jerusalem artichoke (*Helianthus tuberosus L.*) as a potential chlorophyll source for humans and animals nutrition. *Environment Biodiversity and Soil Security, 2* (1), 1–20. https://doi.org/10.21608/jenvbs.2018.2942.1022

Kovács, Z., Soós, Á., Kovács, B., Kaszás, L., Elhawat, N., Razem, M., … Domokos-Szabolcsy, É. (2023). Nutrichemical alterations in different fractions of multiple-harvest alfalfa (*Medicago sativa L.*) green biomass fortified with various selenium forms. *Plant and Soil, 487*(1), 173–195. https://doi.org/10.1007/s11104-023-05917-8

Malila, Y., Owolabi, I. O., Chotanaphuti, T., Sakdibhornssup, N., Elliott, C. T., Visessanguan, W., … Petchkongkaew, A. (2024). Current challenges of alternative proteins as future foods. *npj Science of Food, 8*(1), 53. https://doi.org/10.1038/s41538-024-00291-w

Mathew, S., Abraham, T. E., & Zakaria, Z. A. (2015). Reactivity of phenolic compounds towards free radicals under in vitro conditions. *Journal of Food Science and Technology, 52*(9), 5790–5798. https://doi.org/10.1007/s13197-014-1704-0

Millward, D. J., & Jackson, A. A. (2004). Protein/energy ratios of current diets in developed and developing countries compared with a safe protein/energy ratio: Implications for recommended protein and amino acid intakes. *Public Health Nutrition, 7*(3), 387–405. https://doi.org/10.1079/PHN2003545

Msheliza, E. A., Umar Abdulkadir, R., Zongoro, A. J., & Hussein, J. B. (2024). Influence of fermentation and roasting on the amino acid profile of infant food created from mixtures of Sorghum and soybean. *Contemporary Agriculture, 73*(1–2), 6–16. https://doi.org/10.2478/contagri-2024-0002

Mucchetti, G., Gatti, M., & Neviani, E. (1994). Electrical conductivity changes in Milk caused by acidification: Determining factors. *Journal of Dairy Science, 77*(4), 940–944. https://doi.org/10.3168/jds.S0022-0302(94)77029-6

Ng, C.-C., Wang, C.-Y., Wang, Y.-P., Tzeng, W.-S., & Shyu, Y.-T. (2011). Lactic acid bacterial fermentation on the production of functional antioxidant herbal *Anoectochilus formosanus Hayata*. *Journal of Bioscience and Bioengineering, 111*(3), 289–293. https://doi.org/10.1016/j.jbiosc.2010.11.011

Nynäs, A.-L., Newson, W. R., & Johansson, E. (2021). Protein fractionation of green leaves as an underutilized food source—Protein yield and the effect of process parameters. *Foods, 10*(11), 2533. https://doi.org/10.3390/foods10112533

Pál, V., & Zsombik, L. (2024). Effect of common vetch (*Vicia sativa L.*) green manure on the yield of corn in crop rotation system. *Agronomy, 14*(1), 19. https://doi.org/10.3390/agronomy14010019

Pei, K., Ou, J., Huang, J., & Ou, S. (2015). p-Coumaric acid and its conjugates: Dietary sources, pharmacokinetic properties and biological activities. *Journal of the Science of Food and Agriculture, 96*(9), 2952–2962. https://doi.org/10.1002/jsfa.7578

Pérez-Vila, S., Fenelon, M. A., O'Mahony, J. A., & Gómez-Mascaraque, L. G. (2022). Extraction of plant protein from green leaves: Biomass composition and processing considerations. *Food Hydrocolloids, 133*, Article 107902. https://doi.org/10.1016/j.foodhyd.2022.107902

Pirie, N. W. (1966). Leaf protein as a human food. *Science, 152*(3730), 1701–1705. https://doi.org/10.1126/science.152.3730.1701

Pirie, N. W. (1987). *Leaf protein: And its by-products in human and animal nutrition*. Cambridge University Press.

Pisulewska, E., Hanczakowski, P., & Pisulewski, P. (1989). Yield, composition and nutritive value of leaf protein concentrates from mixed forages of cereals and legumes. *Animal Feed Science and Technology, 27*(1–2), 117–125. https://doi.org/10.1016/0377-8401(89)90136-3

Pisulewska, E., Hanczakowski, P., Pisulewski, P., & Szymczyk, B. (1991). The changes of the yield, composition and nutritive value of leaf protein extracted from vetch and cereal mixtures during three years cultivation. *Journal of the Science of Food and Agriculture, 55*(2), 197–205. https://doi.org/10.1002/jsfa.2740550205

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2020). Plant proteins as high-quality nutritional source for human diet. *Trends in Food Science & Technology, 97*, 170–184. https://doi.org/10.1016/j.tifs.2020.01.011

Sabaté, J., Sranacharoenpong, K., Harwatt, H., Wien, M., & Soret, S. (2015). The environmental cost of protein food choices. *Public Health Nutrition, 18*(11), 2067–2073. https://doi.org/10.1017/S1368980014002377

Saleem, K., Ikram, A., Saeed, F., Afzaal, M., Ateeq, H., Hussain, M., Raza, A., Rasheed, A., Asghar, A., & Asif Shah, M. (2023). Nutritional and functional properties of kefir: Review. *International Journal of Food Properties, 26*(2), 3261–3274. https://doi.org/10.1080/10942912.2023.2280437

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: An overview. *Food Production, Processing and Nutrition, 2*(1), 6. https://doi.org/10.1186/s43014-020-0020-5

Santamaría-Fernández, M., & Lübeck, M. (2020). Production of leaf protein concentrates in green biorefineries as alternative feed for monogastric animals. *Animal Feed Science and Technology, 268*, Article 114605. https://doi.org/10.1016/j.anifeedsci.2020.114605

Santamaría-Fernández, M., Molinuevo-Salces, B., Kiel, P., Steenfeldt, S., Uellendahl, H., & Lübeck, M. (2017). Lactic acid fermentation for refining proteins from green crops and obtaining a high quality feed product for monogastric animals. *Journal of Cleaner Production, 162*, 875–881. https://doi.org/10.1016/j.jclepro.2017.06.115

Segatto, M. L., Stahl, A. M., Zanotti, K., & Zuin, V. G. (2022). Green and sustainable extraction of proteins from agro-industrial waste: An overview and a closer look to Latin America. *Current Opinion in Green and Sustainable Chemistry, 37*, Article 100661. https://doi.org/10.1016/j.cogsc.2022.100661

Sheen, S. J. (1991). Comparison of chemical and functional properties of soluble leaf proteins from four plant species. *Journal of Agricultural and Food Chemistry, 39*(4), 681–685. https://doi.org/10.1021/jf00004a011

Shin, N. R., Moon, J. S., Shin, S.-Y., Li, L., Lee, Y. B., Kim, T.-J., & Han, N. S. (2016). Isolation and characterization of human intestinal *enterococcus avium* EFEL009 converting rutin to quercetin. *Letters in Applied Microbiology, 62*(1), 68–74. https://doi.org/10.1111/lam.12512

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *, Vol. 299. Methods in enzymology* (pp. 152–178). Academic Press. https://doi.org/10.1016/S0076-6879(99)99017-1.

Tang, J., Yao, D., Xia, S., Cheong, L., & Tu, M. (2024). Recent progress in plant-based proteins: From extraction and modification methods to applications in the food industry. *Food Chemistry: X, 23*, Article 101540. https://doi.org/10.1016/j.fochx.2024.101540

Tilman, D., & Clark, M. (2014). Global diets link environmental sustainability and human health. *Nature, 515*(7528), 518–522. https://doi.org/10.1038/nature13959

Yang, X., Hong, J., Wang, L., Cai, C., Mo, H., Wang, J., Fang, X., & Liao, Z. (2024). Effect of lactic acid Bacteria fermentation on plant-based products. *Fermentation, 10*(1), 48. https://doi.org/10.3390/fermentation10010048

Zhao, D., & Shah, N. P. (2016). Lactic acid bacterial fermentation modified phenolic composition in tea extracts and enhanced their antioxidant activity and cellular uptake of phenolic compounds following in vitro digestion. *Journal of Functional Foods, 20*, 182–194. https://doi.org/10.1016/j.jff.2015.10.033