

Research article

Bioactive metabolite profile and antioxidant properties of brown juice, a processed alfalfa (*Medicago sativa*) by-product



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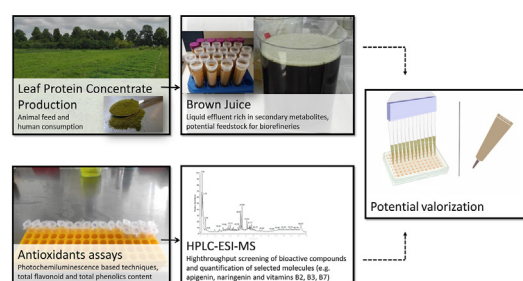
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HIGHLIGHTS

- Alfalfa brown juice possesses remarkable antioxidant properties.
- Broad range of flavonoids e.g., apigenin, luteolin, naringenin, tricetin found.
- Lacto-fermentation induced shift into aglycone molecules, thus improving bioactivity.
- Vitamin contents up to: B2 2042.7 ng mL⁻¹, B3 972.3 ng mL⁻¹ and B7 32.0 ng mL⁻¹.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, leaf protein concentrate (LPC) has gained increased attention in response to the constantly growing protein demand. Green biorefineries can become more economical by valorizing their by-products and reducing environmental risks. The current study describes the variations in the antioxidant capacity and phytochemical composition of a liquid by-product (referred to as brown juice (BJ)) obtained during the extraction of leaf protein concentrate (LPC) from the fresh biomass of alfalfa (*Medicago sativa* L.). Four varieties of alfalfa were investigated during three harvest times, i.e., August 2017 (first harvest), September 2017 (second harvest), and June 2018 (third harvest). Also, the fresh BJ was lacto-fermented to extend its preservation period but also modifying its composition. The results of different general phytochemical composition analyses and antioxidant assays revealed similar tendencies across different alfalfa varieties and harvest times. Most of the phytochemicals in the BJ identified by HPLC-MS/MS can be classified as flavonoids/flavonoid derivatives, e.g., apigenin, naringenin, luteolin, formononetin. Substantially, the lacto-fermentation process induced a switch into aglycones, e.g., apigenin content increased by an order of magnitude, while apigenin-7-O-glucuronide content was halved after lacto-fermentation. Additionally, several B vitamins were detected, including B2, B3, and B7. These results could provide a basis for various ways of industrial valorization but need to be strengthened by data generated from large-scale production.

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1. Introduction

The ever-increasing demand for sufficient protein supply brought increased attention to alternative and renewable protein sources (Pojić et al., 2018), including green biorefineries of leaf protein concentrate (LPC). Most of the protein in LPC comes from the chloroplast enzyme RuBisCo, the most abundant protein in the world (Bar-On and Milo, 2019), which is a technically undepletable resource with a relatively low environmental cost. The production of LPC was first described internationally almost a century ago (Ereky, 1933), initially to provide protein-rich animal feeds. After a decade, Pirie proposed to investigate the feasible use of LPC in human nutrition (Pirie, 1942). Lately, as the circular economy and bioeconomy concepts have been getting more attention (Ubando et al., 2020), and sustainability is at the forefront of scientific, governmental, and public interest (von Braun, 2018), the plant-based protein sources became trending and the green protein-biorefinery research intensifies (Bals and Dale, 2011; Kaszás et al., 2020; Kiel et al., 2015; Santamaría-Fernández et al., 2018; Santamaría-Fernández and Lübeck, 2020). In 2009, the European Food Safety Authority (EFSA) concluded that alfalfa LPC proposes no safety concern for human consumption at a maximum intake of 10 g/person/day (2009/826/EC), which established the expansion of LPC production. There have been examples of large-scale implementation of green biorefineries including LPC production such as the French 'France Luzerne' company (Grela and Pietrzak, 2014) or VEPEX in Hungary which was the first leaf protein plant in the world established in 1972 (Holló and Kralovszky, 2000). However, it is not yet widespread.

The European Commission has encouraged the development of the bioeconomy, including LPC production and other products based on green biomass with a strategic programme (European Commission, 2019). The strategy was updated in 2022 to assess the progress in the implementation of 2018 EU Bioeconomy Strategy and its Action Plan (European Commission, 2022). In a survey the gaps and challenges of the bioeconomy policy, considering the recent policy developments under the European Green Deal were assessed.

As a result of policy support and research efforts, the number of green biorefineries at pilot or commercial scale has been increasing in Europe in recent years (Santamaría-Fernández and Lübeck, 2020).

Alfalfa (*Medicago sativa*) is one of the most prevalent forage crops, an ideal subject for LPC production, though the technology is easily adaptable to other relevant species. Alfalfa fodder is commonly used in livestock production. Drying means a major environmental impact of the process (Gallego et al., 2011) and also significantly reduces protein content due to natural protease activity. Protein loss can be significantly reduced implementing leaf protein concentrate producing methods. Simple fractionation combined with an advanced biorefinery approach could provide a sustainable solution for the growing protein demand and meet the concepts of green industries and fit well in a circular economy as well. One of the main challenges with these processes is the handling of by-products. Besides the LPC, there is a substantial amount of fibre fraction, sometimes called press cake. The other by-product is referred to as deproteinized plant juice (DPJ), plant whey or brown juice (from hereon: BJ). It represents the largest fraction of the processed green biomass, amount to almost 50% of the green biomass (Bákonnyi et al., 2020) and making up to 13–15 % of the original dry matter content (Zanin, 1998). The BJ contains sugars, oligopeptides (Reddy et al., 1987), minerals, and valuable secondary metabolites (Bákonnyi et al., 2020). Its composition depends mainly on the applied technology and the input material (Santamaría-Fernández et al., 2018). It has a dry matter content varying between 4% and 15% (Zanin, 1998). BJ can be used in various ways, including L-lysine production (Thomsen et al., 2004), methane production through anaerobic digestion (Martinez et al., 2018; Santamaría-Fernández et al., 2018), or as a medium for microbes (Thomsen, 2005; Thomsen and Kiel, 2008; Weimer and Digman, 2013). Studies have shown the potential of alfalfa BJ in plant nutrition as fertilizer (Reddy et al., 1987) or biostimulant (Barna et al., 2021), which implies the

presence of phytohormones and other bioactive compounds in addition to the already identified components.

The main factor that induces the synthesis of plant secondary metabolites is the defence against stressors (Neugart et al., 2018) and adaptation to the environment. However, some compounds are known to play not only a secondary role, but also have a regulatory effect or even are primary metabolites (Craney et al., 2012; Erb and Kliebenstein, 2020). Many secondary metabolites have been used in traditional medicine and are still common in modern western medicine. However, many of them are yet to be discovered, as suggested by genome-mining approaches (Zhao et al., 2013), making plants an interesting target for drug discovery.

Fabaceae family (soybean, alfalfa, etc.) related flavonoids such as quercetin, biochanin A, genistein, erbstatin are considered potential treatment tools against tumorigenesis and inflammation, especially during menopause caused oestrogen decrease-related statuses (Bernatoniene et al., 2021; Manna, 2012). Alfalfa is also a rich source of biologically active phytochemicals (Goławska et al., 2014; Jasiński et al., 2009; Pecetti et al., 2006). Qualitative and quantitative studies have revealed a number of phenolic compounds, flavonoids and saponins (Rafinska et al., 2017). Phenolic compounds and flavonoids have remarkable antioxidant properties (Gulcin, 2020). The antioxidant activity is determined by the arrangement of functional groups around the nuclear structure (Heim et al., 2002).

Lactic Acid Bacteria (BAL) have an important role of producing of bioactive compounds. One of the methods used for the recovery of bioactive components is the long-established method of lacto-fermentation. This process is environmentally friendly and economically feasible. It can be used to effectively induce chemical changes in by-products from certain agricultural and food industry activities, whereby the chemical changes induced by lactic acid-producing bacteria (BAL) allow the formation of various valuable bioactive substances, either by hydrolysis or by transformation of substrates. The use of lactic acid bacteria in bio-processes is widespread, their importance is highlighted in oxido-reduction, demethylation, bioremediation (George et al., 2018), degradation of macromolecular substances such as indigestible polysaccharides, and conversion of undesirable flavours, which is very important in the food industry (Wang et al., 2021). Acidification of plant juices with lactic acid producing bacteria is a well-known alternative in green biorefining due to its many advantages (Lübeck and Lübeck, 2019; Thomsen et al., 2015; Novik et al., 2017).

The present study aimed to analyse the antioxidant properties and secondary metabolites of BJ. Different assays were applied to quantify the antioxidant capacity of the BJ and make a profile of its key bioactive molecules. Also, the current research aimed to unravel new valorization possibilities of BJ. Considering preservation is a key issue in the practical utilization of a product candidate, such as the perishable brown juice, lacto-fermentation was used for this purpose and followed the fermentation induced quantitative and qualitative changes of phyto-metabolites.

2. Materials and methods

2.1. Origin of BJ

The BJ was produced as previously described in detail by Bákonnyi et al. (2020). Briefly, the alfalfa fresh biomass was obtained from an irrigated field experiment, in which alfalfa varieties were sown in 4.3 m × 1.4 m plots arranged in a Randomized Block design with three replicates.

The main goal of the present project is the production of leaf protein concentrate (LPC), the fresh yield and crude protein content are of importance. Varietal choice is important in LPC production, as varieties with good leaf stem ratio, reduced lignin content, good remounting (regrowth) ability can result higher protein content and higher quantities of LPC. Four alfalfa varieties were tested, i.e., Legend, Jozsó, Dimitra and

Hunor-40. The native Hungarian variety Hunor-40 has high protein content, the Legend (American) is lignin-reduced variety, and the Dimitra (Italian) has outstanding leaf stem ratio with high protein content (Table 1).

Alfalfa fresh biomass was harvested once a month (May–October) in the green bud phenophase stage. However, in the present study, three harvests were performed for BJ production in order to have a comprehensive view of the seasonal changes throughout the vegetation period with following sampling dates: the first of August 2017 (first harvest), the first of September 2017 (second harvest), and the first of June 2018 (third harvest). During the fractionation process, the harvested above-ground alfalfa fresh biomass was cleaned manually by excluding weeds and removing any adhering particles. Cleaned alfalfa fresh biomass was fractionated into fibre and green juice using a twin-screw press Angel Juicer (5500, Angel Ltd., Praha, Czech Republic). The green juice was heated to 80 °C to coagulate mainly the chloroplastic and also the cytoplasmic proteins (Fári and Domokos-Szabolcsy, 2019). After thermal coagulation, the coagulant (LPC) was separated from BJ using moistened cotton cloth filter. The obtained BJ samples were stored at -20 °C until further analyses. BJ as it can spoil easily in few days, therefore, a lacto-fermentation method for the preservation of alfalfa at room temperature has been developed and applied to a variety Hunor-40 of alfalfa selected for its favourable properties. Hunor-40 is a reliable and common variety with good remounting features, high biomass yield in large-scale production. Additionally, it showed homogenous plots and favourable antioxidant parameters across the preliminary experiments. These traits altogether make it a good representative of commercially available alfalfa varieties and a good starting point for further studies on other species. The lacto-fermentation method, developed by Bákonfi et al. (2020) for BJ, was applied to prevent spoiling (due to high sugar and protein content) at room temperature. Briefly, after cooling, the BJ of Hunor-40 was inoculated with lactic acid bacterial cultures (*Pediococcus acidilactici*, *Lactobacillus paracasei*, *Lactobacillus plantarum*) at the rate of $1 \cdot 10^{11}$ CFU g⁻¹, and incubated at 35 °C for 48 h. The phytochemical analysis and quantification of selected bioactive molecules was performed from non-fermented and fermented BJ from these samples.

2.2. General phytochemical composition analysis

2.2.1. Determination of total phenolics contents (TPC)

The TPC in the BJ was determined according to the method of Boór and Bélafiné Bakó (2010). The reagent forms a blue complex with the hydroxide group of phenols and phenol-like compounds, and its absorption can be inferred from the light absorption of the solution. All phenolics content results were calculated using the formula ' $y = 0.0991x$ ' ($R^2 = 0.9854$) obtained from the gallic acid calibration curve. Calibration points: 0, 1.02; 2.04; 3.06; 4.08; 5.1 µg/ml The reaction mixture consisted of 1250 µl Folin-Ciocalteu reagent:DW (1:10), methanol:DW (80:20), 50 µl sample and after 1 min incubation at room temperature 1000 µl Na₂CO₃ resulting in a total volume of 2500 µl. Absorbance was measured at 760 nm (Ultrospec 2100 pro, Amersham BioSciences spectrophotometer). One representative sample from each plot was carried over for TPC and TFC measurements. The obtained results were expressed as µg mL⁻¹ gallic acid equivalent (GAE) of liquid brown juice samples.

Table 1. Alfalfa varieties and their characteristics.

Variety	Color of flowers	Year of state recognition	Origin of the variety
Legend	Blue	2008	American
Jozsó	Mixed	1996	Hungarian
Dimitra	Purple	no data	Italian
Hunor-40	light purple, dark blue	1989	Hungarian

Source: NÉBIH, National Variety List (2018).

2.2.2. Determination of total flavonoid contents (TFC)

The determination of TFC in the BJ was carried out according to Kim et al. (2003). The basis of the determination is that flavonol and flavone-type compounds form a complex with aluminium chloride (AlCl₃) in an acid medium in a stoichiometric reaction. The colour intensity of the resulting complex is suitable for the quantitative determination of the flavonoids in the solution. During the measurement, the aluminium-containing reagent solution was made of 5 mL of 10 g mL⁻¹ AlCl₃, 5 mL of 1 M L⁻¹ KOAc (potassium acetate), 75 mL of methanol, and 140 mL of water. The standard curve was generated using rutin ($y = 0.0231x$, $R^2 = 0.998$). Absorbance of the BJ samples (0.5 mL) was measured at 415 nm using UV/VIS-Spectrophotometry (Ultrospec 2100 pro, Amersham BioSciences spectrophotometer). The obtained results were expressed as µg mL⁻¹ rutin equivalent (RE) of liquid BJ samples.

2.3. Antioxidant assays for the determination the antioxidant capacity of BJ

The determination of antioxidant capacity of BJ was performed by ACL, ACW photochemiluminescence (PCL) method described in Nemes et al. (2015) and distributed under the name Photochem® by Analytik Jena AG (Jena, Germany). The samples were diluted with either distilled water (water-soluble antioxidant capacity; ACW) or methanol (lipid-soluble antioxidant capacity; ACL) to fit the calibration range.

In this assay, the photochemical generation of superoxide anion radical (O₂•-) is combined with the sensitive detection by using chemiluminescence. The assay is initiated by optical excitation of photosensitizer (S), resulting in the generation of O₂•-. Two different protocols were used: ACW and ACL, so both hydrophilic and lipophilic antioxidants can be measured separately (Popov and Lewin, 1994, 1996). Values are expressed in µg mL⁻¹ ascorbic acid equivalent (AAE) for ACW and Trolox equivalent (TE) for ACL results. Representative samples from two plots were used for ACW and ACL measurements. The obtained results were expressed as µg mL⁻¹ of liquid brown juice samples.

2.4. Screening and quantification of phytochemicals in BJ by HPLC-MS/MS

In this study, the non-fermented and fermented BJs of Hunor-40 were used for detailed metabolite profiling. For identification and quantification of phytochemicals an UHPLC-ESI-ORBITRAP-MS/MS hyphenated system was used (Dionex Ultimate 3000RS UHPLC system/Thermo Fisher, Waltham, MA, USA/coupled to a Thermo Q Exactive Orbitrap hybrid mass spectrometer). Column: Thermo Accucore C18 analytical column, 100/2.1 mm, 2.6 µm particle size.

2.4.1. Sample preparation

0.5 mL BJ was extracted with 25 mL methanol:water (70:30) solution. The mixture was stirred at 150 rpm for 2 h at room temperature. The hydro-alcoholic extracts were filtered using a 0.22 µm PTFE syringe filter.

2.4.2. Screening for bioactive phytochemicals

Qualitative analysis was carried out to determine the phytochemical compounds in Hunor-40 BJ. Separation of bioactive molecules was achieved under the following conditions. Flow rate: 0.2 mL min⁻¹; column oven temperature: 25 ± 1 °C; mobile phase: methanol (A) and water (B), both acidified with 0.1% formic acid. Gradient program: 0–3 min, 95% B; 3–43 min, 0% B; 43–61 min, 0% B; 61–62 min, 95% B; and 62–70 min, 95% B. The injection volume was set to 2 µL. For screening a mix sample of Hunor-40 containing all three harvests was used to get the profile of possibly abundant secondary metabolites.

2.4.3. Quantification of bioactive phytochemicals

Chromatographic separation for quantitative analysis was carried out by the same HPLC-MS/MS system mentioned above. Briefly, 1 µL

solution was injected into the column. The flow rate was set to 0.2 mL min⁻¹ and the column oven temperature to 25 °C ± 1 °C. The mobile phase consisted of water (A) and methanol (B) with the following gradient profile: 0–2 min, 95% A; 2–20 min, 100% B; 20–22 min, 100% B; 22–23 min, 95% A and 23–30 min, 95% A.

Based on the literature describing typical alfalfa secondary metabolite profile and screening of mixed Hunor-40 BJ 16 molecules were selected. For quantitative analysis nicotinamide (≥99.5%); nicotinic acid (≥98%); riboflavin (≥98%); isoquercitrin (primary reference standard); naringenin (≥95%); biochanin A; coumestrol (≥95%); formononetin (≥98%); luteolin (≥98%); apigenin (≥98%); liquiritigenin (phyproof Reference Substance); tricin (phyproof Reference Substance); Apigenin 7-O-glucuronide (primary reference standard); biotin (≥99%); quercetin (≥95%); genkwanin (≥98%); ferulic acid (≥99%) as external standards were applied (Merck-Sigma, Darmstadt, Germany). Working standard solutions were prepared daily by dilution of the stock solutions with methanol.

2.4.4. Mass spectrometry conditions

The capillary temperature was 320 °C. Samples were ionized separately, using an ESI source with 4.0 kV in positive ionization mode and 3.8 kV in negative ionization mode, respectively. Mass spectrometry parameters: resolution of 35,000 for MS1 scans and 17,500 for MS2 scans, scanning between 100–1500 m/z. The tandem MS/MS scans were run on 30 normalized collision energy units. The difference between measured and theoretical molecular ion masses was <5 ppm in each case. Data acquisition and processing was done in Thermo Trace Finder 2.1 software based on own and public internet databases (Metlin, Mass Bank of North America, m/z Cloud). After processing, manual checking of the results was carried out in the Thermo Xcalibur 4.0 software (Thermo-Fisher, Waltham, MA, USA).

2.5. Statistical analysis

Levene's Test was used to test for equality of variances and Shapiro-Wilk test for checking normal distribution. The results of the

experiments showed significant interaction between the two independent variables (Harvest time and Variety), afterwards they were subjected to Kruskal-Wallis test in R (version 4.0.3) and the means were compared by Dunn's Test at $p < 0.05$.

3. Results and discussion

3.1. TPC, TFC, ACW and ACL of alfalfa BJ

Two phytochemical composition analysis (TPC and TFC) and two different antioxidant assays (ACW and ACL) were used to get a comprehensive view on the antioxidant potential of BJ. Table 2 showed the results of different phytochemical composition analysis and antioxidant assays in the BJ of the four alfalfa varieties within three different harvests.

In the upper section of Table 2 the data for varieties are shown as Means ± SD of the three harvest times. In the second section, data for the harvest times are presented as Means ± SD of all varieties. The results of the experiments showed significant interaction between the two independent variables, therefore they were subjected to one-way analysis afterwards. Means of the interactions in the same column followed by the different lowercase letters are significantly different according to Dunn's test ($P \leq 0.05$). Data are Means ± SD. Concentrations are expressed in Gallic Acid Equivalent (TPC), Rutin Equivalent (TFC), Ascorbic Acid Equivalent (ACW) and Trolox Equivalent (ACL) in $\mu\text{g mL}^{-1}$.

The TPC of BJ significantly varied across the examined alfalfa varieties and harvest times (Table 2). Considering the interaction between alfalfa varieties and harvest time, the second harvest of Hunor-40 had the highest values from all samples ($2613.5 \pm 575.2 \mu\text{g mL}^{-1}$). On the other side of the scale, the first cut of Jozsó had significantly lower values ($995.6 \pm 126.7 \mu\text{g mL}^{-1}$), the lowest among all alfalfa varieties and harvest times.

Both alfalfa varieties and harvest times significantly influenced the TFC in the BJ (Table 2). Similarly, to the total phenolics content, it was found that in terms of TFC the second harvest of Hunor-40 proved to be superior ($241.3 \pm 50.1 \mu\text{g mL}^{-1}$), while the first cut of Jozsó contained

Table 2. Results of general phytochemical composition analysis (TPC, TFC) and antioxidant assays (ACW, ACL) from alfalfa brown juice samples from different varieties and harvest times.

		TPC $\mu\text{g mL}^{-1}$	TFC $\mu\text{g mL}^{-1}$	ACW $\mu\text{g mL}^{-1}$	ACL $\mu\text{g mL}^{-1}$
Variety					
Legend		1547.7 ± 513.0	168.7 ± 27.5	308.2 ± 326.5	426.6 ± 201.6
Jozsó		1510.3 ± 468.6	107.5 ± 54.2	281.6 ± 167.0	600.4 ± 169.5
Dimitra		1611.2 ± 474.8	159.1 ± 39.3	470.8 ± 155.9	661.4 ± 159.9
Hunor-40		1986.8 ± 568.7	211.8 ± 46.2	404.2 ± 398.4	648.6 ± 297.1
Harvest					
1 st harvest		1611.6 ± 607.1	133.6 ± 51.3	335.1 ± 249.8	661.5 ± 101.7
2 nd harvest		1628.8 ± 732.8	153.8 ± 70.9	519.3 ± 327.1	619.3 ± 282.8
3 rd harvest		1839.5 ± 288.2	196.7 ± 30.1	202.0 ± 190.0	509.4 ± 231.4
Harvest X Variety					
1 st harvest	Legend	1895.4 ± 329.9 ab	139.1 ± 18.1 bcd	691.4 ± 135.1 ab	701.3 ± 127.2 ab
	Jozsó	995.6 ± 126.7 b	64.6 ± 13.3 d	115.0 ± 11.6 c	528.9 ± 20.5 bcd
	Dimitra	2051.8 ± 600.8 ab	172.2 ± 59.4 abc	335.6 ± 70.3 bc	666.7 ± 44.9 abc
	Hunor-40	1503.5 ± 736.9 ab	158.6 ± 10.5 abc	198.6 ± 13.3 bc	749.3 ± 0.3 ab
2 nd harvest	Legend	1170.5 ± 611.1 ab	177.5 ± 3.1 abc	102.9 ± 7.1 c	314.6 ± 73.9 d
	Jozsó	1622.9 ± 238.4 ab	89.3 ± 29.0 cd	478.6 ± 19.0 abc	509.9 ± 49.5 bcd
	Dimitra	1108.3 ± 128.3 ab	114.9 ± 34.6 bcd	658.3 ± 56.8 ab	662.3 ± 251.1 abcd
	Hunor-40	2613.5 ± 575.2 a	241.3 ± 50.1 a	870.1 ± 117.8 a	990.3 ± 15.2 a
3 rd harvest	Legend	1591.8 ± 160.5 ab	192.5 ± 12.9 ab	41.4 ± 13.8 c	318.2 ± 74.3 d
	Jozsó	1912.2 ± 180.4 ab	168.4 ± 15.9 abc	334.4 ± 15.8 bc	708.4 ± 232.3 ab
	Dimitra	1673.4 ± 73.5 ab	190.2 ± 27.3 ab	418.6 ± 32.3 abc	657.2 ± 209.9 abcd
	Hunor-40	1843.3 ± 60.2 ab	235.6 ± 16.3 a	13.8 ± 2.6 c	353.7 ± 86.0 cd

the least amount of flavonoids ($64.6 \pm 13.3 \mu\text{g mL}^{-1}$), less than the third of the former one. Overall, the TFC and TPC showed similar tendencies. The TFC of the first and second harvest of Hunor-40 was significantly higher than the TFC of the first cut of Legend, first and second cut of Jozsó, second cut of Dimitra. Except for Dimitra, higher values were found for the 2nd and 3rd harvests compared to the 1st harvest. The TFC content increased from the first harvest to the third harvest recording the highest value ($196.7 \pm 30.1 \mu\text{g mL}^{-1}$) in the third harvest, regardless of the alfalfa variety. The plants were under stress due to harvesting, which can explain the observed tendencies.

The ACW results presented in Table 2 significantly varied due to the alfalfa varieties and harvest times and are in line with the results described above. Regarding the interaction between alfalfa varieties and their harvest times, the Legend, Hunor-40, and Dimitra varieties possessed the highest ACW content in the first ($691.4 \pm 135.1 \mu\text{g mL}^{-1}$), second ($870.1 \pm 117.8 \mu\text{g mL}^{-1}$), and third ($418.6 \pm 32.3 \mu\text{g mL}^{-1}$) harvest, respectively.

The BJ extracted from the first harvest displayed the highest ACL values ($661.5 \pm 101.7 \mu\text{g mL}^{-1}$), regardless of the alfalfa variety. The Hunor-40 variety revealed the highest significant ACL values in the first ($749.3 \pm 0.3 \mu\text{g mL}^{-1}$) and second ($990.3 \pm 15.2 \mu\text{g mL}^{-1}$) harvests among all tested alfalfa varieties; however, it showed the lowest ACL content ($353.7 \pm 86.0 \mu\text{g mL}^{-1}$) in the third harvest. The results were relatively low compared to the previously published studies, although they examined solid plant material from intact plants. Wu et al. (2022) investigated the extraction and antioxidant capacity of *Medicago sativa* and found the total antioxidant capacity under the optimized conditions 15.76 mg/g and $28.79 \mu\text{mol Trolox/g}$. Starowicz et al. (2021) measured the antioxidant capacity in different plant species of which clover, related to lucerne have 873.00 ± 35.98 and $20.62 \pm 0.72 \mu\text{mol Trolox/g DM}$ for ACW and ACL respectively. Purkiewicz et al. (2020) found that in the orange carrot juice (pressed by high-speed juicer) the antioxidant capacity was 4.02 ± 0.03 and $85.43 \pm 0.46 \mu\text{mol Trolox/mL DM}$ for ACW and ACL respectively.

ACW and ACL results changed seemingly similarly across the season. The few lower results (Legend 3rd harvest: $41.4 \pm 13.8 \mu\text{g mL}^{-1}$, Hunor-40 3rd harvest: $13.8 \pm 2.6 \mu\text{g mL}^{-1}$ ascorbic acid equivalent) in ACW could be attributed to the quick degradation of water-soluble antioxidants. However, all measurements resulted in higher ACL values compared to ACW, despite the high water content of alfalfa BJ.

In spite of the fact that the different methods (TPC, TFC, ACW, and ACL) showed similar tendencies, still they should be used in conjunction with each other to provide reliable data. The above-mentioned results revealed that the quality of the BJ depends on the alfalfa variety and its harvest time. The Hunor-40 variety is considered the best among all tested alfalfa varieties, where it exhibited the highest TPC, TFC contents, and the second highest ACW and ACL contents.

3.2. Screening of phytochemicals in alfalfa BJ

Ultra-high performance liquid chromatography-electrospray ionization-Orbitrap/mass spectrometry qualitative analysis (UHPLC-ESI-ORBITRAP-MS/MS) was carried out to identify the possible bioactive phytochemicals in non-fermented and fermented BJ obtained from Hunor-40.

47 phytochemicals were successfully identified in non-fermented BJ, whereas 31 phytochemicals were found in BJ after fermentation (Table 3). Several bioactive compounds, such as vitamins, flavons, iso-flavons, flavonoids, phenolic acids, organic acids, fatty acids and terpenes were identified.

Molecules belonging to the class of B vitamins and their derivatives were detected in non-fermented and fermented BJ, i.e., riboflavin (B2), nicotinic acid and nicotinamide (B3) and biotin (B7) each of them important precursors of or actually cofactors themselves for metabolic processes e.g., synthesis of fatty acids and amino acids (isoleucine, valine) and gluconeogenesis. Moreover, qualitative analysis revealed the

presence of Trigonelline (N-methylnicotinamide), a metabolite of nicotinamide, which is an alkaloid with a wide range of regulatory functions in plants (Minorsky, 2002) and various beneficial effects on animal models and human health (Garg, 2016).

19 different flavons were found in non-fermented BJ, mainly various types of apigenin, luteolin, tricrin and chrysoeriol. Lacto-fermentation not only stabilized alfalfa BJ, but also changed its chemical composition. Examining the micro- and macroelements in BJ before and after lacto-fermentation Bákonyi et al. (2020) reached a similar conclusion. Non-fermented BJ contained the glycosides of certain molecules (e.g., molecules with a suffix -glucuronide or -glucoside), though several flavon and isoflavon compounds cannot be found in fermented BJ. During the fermentation process in the BJ glycosidic bonds were presumably hydrolysed, thus the molecules became available and active. Bacteria utilize the sugar chain in their metabolism, and probably that catalysed the process, reducing the number of compounds to 8 in fermented BJ.

Most of the phenolic acids vanished after fermentation. Only the ubiquitous plant cell wall component, ferulic acid, remained and was mostly reduced into dihydroferulic acid.

During lacto-fermentation bacteria consume sugars and secrete several organic acids as a result of their metabolism, which lead to the accumulation of organic acids in fermented BJ. There was no change in terpenes and fatty acid components during fermentation, except traumatic acid, which could be detected in fermented BJ, though, at a very low level.

3.3. Quantification of phytochemicals in alfalfa BJ

As the qualitative analysis confirmed, the group of flavonoids is the richest secondary metabolite class in BJ. Based on this information, the quantity of selected flavonoids was measured. Additionally, due to their known physiological effects, some vitamins were included in the quantitative analyses. From the phenolic acids, ferulic acid was found in measuring range and included in the final table. The concentration of these bioactive components in the BJ of Hunor-40 variety during three harvest times was measured before and after the fermentation process (Table 4).

The quantified secondary metabolites were mostly identified previously in alfalfa (Rafińska et al., 2017) with their role in plant physiology more or less well described. The following discussion mainly focuses on the possible exploitation of the individual components or the complex matrix altogether.

The concentration of bioactive components in the BJ heavily depended on the fermentation process of BJ and the harvest time of alfalfa fresh biomass. The second harvest displayed the lowest concentration of most detected bioactive components. The fermentation process of the BJ resulted in a decrease in the concentration of riboflavin, nicotinic acid, nicotinamide, biotin, genkwanin, apigenin-7-O-glucuronide, isoquercitrin, liquiritigenin, and ferulic acid. On the contrary, the concentration of apigenin, luteolin, tricrin, formononetin, biochanin A, quercetin, and naringenin was higher in fermented BJ than in non-fermented BJ.

Regarding the non-fermented BJ, riboflavin, nicotinic acid, biotin, and genkwanin concentrations slightly dropped down from the first harvest to the second harvest, then increased again in the third harvest; however, the highest concentrations corresponded to the first harvest. For example, riboflavin concentration in the first, second, and third harvests was 2043 , 1214 , and 1800 ng mL^{-1} , respectively. Biotin concentration varied from 32.0 ng mL^{-1} (in the first harvest) to 16.7 ng mL^{-1} (in the second harvest) and 25.9 ng mL^{-1} (in the third harvest). Likewise, genkwanin concentration was 42.4 ng mL^{-1} (in the first harvest) and reduced to 15.8 ng mL^{-1} (in the second harvest), then increased to 26.4 ng mL^{-1} (in the third harvest). Otherwise, apigenin-7-O-glucuronide, luteolin, formononetin, biochanin A, and liquiritigenin concentrations gradually increased from the first harvest to the third harvest. For instance, apigenin-7-O-glucuronide concentration increased from 1851 ng mL^{-1} (in the first harvest) to 3234 ng mL^{-1} (in the third harvest). In

Table 3. Identified phytochemicals in non-fermented and fermented BJ obtained from fresh alfalfa biomass (*Medicago sativa* var. Hunor-40).

Compound	Formula	Non-fermented BJ	Fermented BJ
Vitamins			
Riboflavin	C ₁₇ H ₂₀ N ₄ O ₆	+	+
Nicotinic acid	C ₆ H ₅ NO ₂	+	+
Nicotinamide	C ₆ H ₆ N ₂ O	+	+
Biotin	C ₁₀ H ₁₆ N ₂ O ₃ S	+	+
Flavonoids			
Flavanones			
Naringenin-6,8-di-C-glucoside (4',5,7-Trihydroxyflavanone 6,8-C-glucoside)	C ₂₇ H ₃₂ O ₁₅	+	+
Naringenin (4',5,7-Trihydroxyflavanone)	C ₁₅ H ₁₂ O ₅	+	+
Liquiritigenin (4',7-Dihydroxyflavanone)	C ₁₅ H ₁₂ O ₄	+	+
Flavonols			
Quercetin-3,4'-di-O-glucoside	C ₂₇ H ₃₀ O ₁₇	+	
Isoquercitrin	C ₂₁ H ₂₀ O ₁₂	+	+
Flavans			
Apigenin-4'-O-glucuronide-7-O-[glucuronyl-(1→2)-glucuronide]	C ₃₃ H ₃₄ O ₂₃	+	
Apigenin-O-glucoside-O-glucuronide	C ₂₇ H ₂₈ O ₁₆	+	
Apigenin-7-O-[feruloyl-(→2)-[glucuronyl-(1→3)]-glucuronyl-(1→2)]glucuronide	C ₄₃ H ₄₂ O ₂₆	+	
Apigenin-4'-O-glucuronide-7-O-[feruloyl-(→2)-glucuronyl-(1→2)-glucuronide]	C ₄₃ H ₄₂ O ₂₆	+	
Apigenin (4',5,7-Trihydroxyflavone)	C ₁₅ H ₁₀ O ₅	+	+
Chrysoeriol-4',7-di-O-glucuronide	C ₂₈ H ₂₈ O ₁₈	+	
Chrysoeriol-7-O-glucuronide	C ₂₂ H ₂₀ O ₁₂	+	
Chrysoeriol (3'-Methoxy-4',5,7-trihydroxyflavone)	C ₁₆ H ₁₂ O ₆	+	+
Genkwanin	C ₁₆ H ₁₂ O ₅	+	+
Luteolin-di-O-glucuronide	C ₂₇ H ₂₆ O ₁₈	+	
Luteolin-4'-O-glucuronide-7-O-[feruloyl-(→2)-glucuronyl-(1→2)]glucuronide]	C ₄₃ H ₄₂ O ₂₇	+	
Luteolin-7-O-glucuronide	C ₂₁ H ₁₈ O ₁₂	+	
Luteolin (3',4',5,7-Tetrahydroxyflavone)	C ₁₅ H ₁₀ O ₆	+	+
Tricin-7-O-glucuronide	C ₂₃ H ₂₂ O ₁₃	+	
Tricin-7-O-[feruloyl-(→2)-glucuronyl-(1→2)-glucuronide]	C ₃₉ H ₃₈ O ₂₂	+	+
Tricin (3',5'-Dimethoxy-4',5,7-trihydroxyflavone)	C ₁₇ H ₁₄ O ₇	+	+
4',7-Dihydroxyflavone	C ₁₅ H ₁₀ O ₄	+	+
Methoxy-tetrahydroxyflavone	C ₁₆ H ₁₂ O ₇	+	+
3'-Methoxy-4',5,5',7-tetrahydroxyflavone-7-O-glucuronide	C ₂₂ H ₂₀ O ₁₃	+	
Isoflavones			
Alfalone (4',7-Dimethoxy-6-hydroxyisoflavone)	C ₁₇ H ₁₄ O ₅	+	+
Formononetin (7-Hydroxy-4'-methoxyisoflavone)	C ₁₆ H ₁₂ O ₄	+	+
Ononin (Formononetin 7-O-glucoside)	C ₂₂ H ₂₂ O ₉	+	
Biochanin A (4'-Methylgenistein)	C ₁₆ H ₁₂ O ₅	+	+
Chalcones			
Isoliquiritigenin	C ₁₅ H ₁₂ O ₄	+	+
Coumestans			
Coumestrol	C ₁₅ H ₈ O ₅	+	+
9-O-Methylcoumestrol	C ₁₆ H ₁₀ O ₅	+	+
Medicagol	C ₁₆ H ₈ O ₆	+	+
Sativol	C ₁₆ H ₁₀ O ₆	+	
Pterocarpan			
Medicarpin (3-Hydroxy-9-methoxypterocarpan)	C ₁₆ H ₁₄ O ₄	+	
Methylnissolin	C ₁₇ H ₁₆ O ₅	+	
Phenolic acids			
Ferulic acid	C ₁₀ H ₁₀ O ₄	+	
Dihydroferulic acid	C ₁₀ H ₁₂ O ₄		+
Caffeic acid	C ₉ H ₈ O ₄	+	
Feuloylglucose	C ₁₆ H ₂₀ O ₉	+	
Sinapic acid (Cis isomer)	C ₁₁ H ₁₂ O ₅	+	
Sinapic acid (Trans isomer)	C ₁₁ H ₁₂ O ₅	+	
Unknown hydroxybenzoic acid derivative	C ₁₆ H ₁₀ O ₆		+
Alkaloids			

(continued on next page)

Table 3 (continued)

Compound	Formula	Non-fermented BJ	Fermented BJ
Trigonelline	C ₇ H ₇ NO ₂	+	+
Organic acids			
Benzoyltartaric acid	C ₁₁ H ₁₀ O ₇	+	+
Hydroxyphenyllactic acid	C ₉ H ₁₀ O ₄		+
Dihydroxy-phenylpropionic acid	C ₉ H ₁₀ O ₄		+
3-Phenyllactic acid	C ₉ H ₁₀ O ₃		+
Terpenes			
Dihydroactinidiolide	C ₁₁ H ₁₆ O ₂	+	+
Fatty acid/dicarboxylic acid			
12-Hydroxydodecanoic acid	C ₁₂ H ₂₄ O ₃	+	+
Azelaic acid (Nonanedioic acid)	C ₉ H ₁₆ O ₄	+	+
Dodecanedioic acid	C ₁₂ H ₂₂ O ₄	+	+
Dihydroxydodecanoic acid	C ₁₂ H ₂₄ O ₄	+	+
Traumatic acid or isomer	C ₁₂ H ₂₀ O ₄		+

the same line, the concentration of formononetin increased from 66.7 (first harvest) to 295.0 ng mL⁻¹ (third harvest). Also, liquiritigenin concentration varied from 1.5 ng mL⁻¹ in the first harvest to 41.6 ng mL⁻¹ in the third harvest. On the other hand, the following bioactive compounds showed higher concentration in the fermented BJ of the third harvest compared to the first and second harvests: riboflavin, nicotinamide, apigenin-7-O-glucuronide, luteolin, formononetin, biochanin A, liquiritigenin, and quercetin. Vitamin content was slightly decreased in all fermented BJ samples.

As mentioned above, most mapped molecules in BJ can be classified as flavonoids. Flavonoids in general provide versatile health improving effects by reducing oxidative stress, prevent chronic inflammatory diseases (Birt and Jeffery, 2013) and play a key role in improving cardiovascular conditions (Hertog et al., 1993).

The amount of genkwanin decreased by 30 % on average in fermented BJ in comparison to the non-fermented BJ. Genkwanin is a methylated form of apigenin, therefore it might have been demethylated into apigenin which can explain the observed outstanding change related

to apigenin, also known as 4',5,7-trihydroxyflavone. It had values in non-fermented BJ a bit above 200 ng mL⁻¹ (219.2, 207.5, 204.1 ng mL⁻¹ for first, second and third harvest, respectively) and increased by more than a magnitude (2114.4–2846.8 ng mL⁻¹) as a result of the fermentation. As seen from the screening, this drastic increase could also be connected to the conversion of glycosidic forms of apigenin into its bioactive aglycone form, resulting in a less water-soluble substance. Apigenin is one of the most abundant flavonoids in general. It possesses anticancer properties (Madunić et al., 2018) and diverse preferable pharmacological properties (Hu et al., 2016), thus making the BJ a potential candidate for cancer prevention research. Its significance is emphasized more by the fact that edible sources of flavones are scarce (Manach et al., 2004). Likewise, luteolin, tricetin, formononetin, biochanin A and naringenin content also increased consequently. Tricetin was the second most abundant quantified flavonoid with little changes connected to fermentation. Tricetin is mainly found in monocots and reported also in alfalfa as the monomer of lignin (Lan et al., 2016; Ralph, 2020). Similarly to most other flavonoids, tricetin has promising antioxidant properties beneficial for human health and it

Table 4. Concentration (ng mL⁻¹) of bioactive compounds in non-fermented and fermented BJ of alfalfa (*Medicago sativa* var. Hunor-40) within three harvest times.

	1 st harvest		2 nd harvest		3 rd harvest	
	Non-fermented BJ	Fermented BJ	Non-fermented BJ	Fermented BJ	Non-fermented BJ	Fermented BJ
Vitamins						
Riboflavin	2042.7	1660.6	1214.0	1093.4	1800.5	1694.7
Nicotinic acid	972.3	365.6	578.3	302.8	708.5	338.8
Nicotinamide	nd [*]	nd.	nd.	nd.	64.9	2.9
Biotin	32.0	25.9	16.7	14.6	25.9	21.9
Flavonoids						
Genkwanin	42.4	30.2	15.8	11.0	26.4	19.2
Apigenin	219.2	2846.8	207.5	2629.9	204.1	2114.4
Apigenin-7-O-glucuronide	1850.8	1190.7	2987.8	1291.9	3233.5	1597.9
Luteolin	8.7	73.5	19.9	136.7	29.1	153.6
Tricetin	978.0	990.9	842.0	892.9	835.5	949.2
Formononetin	66.7	210.7	148.7	280.6	295.0	336.6
Biochanin A	5.1	10.7	6.1	11.3	8.1	20.9
Isoquercitrin	31.6	5.8	58.2	10.2	nd.	nd.
Liquiritigenin	1.5	0.3	15.0	3.6	41.6	4.6
Quercetin	nd.	2.9	nd.	3.6	nd.	3.9
Naringenin	27.5	48.7	34.2	65.7	19.8	40.4
Phenolic acids						
Ferulic acid	154.6	117.5	143.7	126.7	127.4	116.1

* not detected.

has a documented strong tumor suppressing effect (Zhou and Ibrahim, 2010). There was a 5.3–8.4x increase in luteolin content after fermentation, contributing to the increased abundance of bioactive compounds in fermented BJ. As Table 3 shows, several tricetin and luteolin glycosyl derivatives were found in BJ. Even though no quantitative measurements were made of them, it is plausible that, similarly to apigenin, the glycosyl side chains were used up during fermentation by microorganisms and this is the reason for the significant increase in aglycones.

Naringenin, commonly found in citrus fruits, was proposed as a candidate in therapeutics against a wide range of diseases and proven to be potent against the most significant inflammatory conditions (Alam et al., 2014). Content of isoquercitrin, and liquiritigenin dropped in BJ after fermentation. The ferulic acid content was 9–24% lower in fermented BJ than in non-fermented BJ. Ferulic acid has a wide range of positive effects on the cellular level (Kumar and Pruthi, 2014), still, its presence might be negligible due to its relatively low concentration in BJ and plenty of common foods being a good source of ferulic acid (Mattila and Hellström, 2007).

BJ, as a residual from agricultural processing has been proved to contain valuable antioxidants. Fermented and non-fermented alfalfa BJ presented a broad range of bioactive molecules, which implies that it could be further processed, extracted, purified into value-added products. Despite the versatility of BJ and the promising results obtained from this study, further extensive experiments are needed including monitoring samples from production at scale.

4. Conclusions

The present study aimed to investigate the variations in the TFC, TPC, antioxidant capacity (including ACW, and ACL) and phytochemical composition of a liquid fraction obtained during the isolation of leaf proteins from fresh biomass of alfalfa using the green biorefinery technique upon the harvest time and alfalfa variety. The results illustrated that the TPC and TFC were the highest in the BJ obtained from the Hunor variety during the third harvest. The highest ACW and ACL were noticed in the BJ of Dimitra variety during the second and first harvests, respectively. The HPLC MS/MS analyses showed that the BJ is rich in B vitamins, including riboflavin, nicotinic acid, nicotinamide, and biotin. Moreover, applying the lacto-fermentation process for the fresh BJ markedly influenced its phytochemical composition, where all the detected B vitamins showed lower contents in the fermented BJ than in the non-fermented one. Also, these vitamins changed considerably within the harvest time as the highest contents corresponded to the first harvest. The findings obtained from this study should be strengthened by data generated from large-scale production in future studies and could be extended to other relevant crop species, e.g., grasses and legumes.

E-supplementary data of this work can be found in online version of the paper.

Declaration

Author contribution statement

Döme Barna; Éva Domokos-Szabolcsy; Nóra Bákonny: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tarek Alshaal: Analyzed and interpreted the data; Wrote the paper. Ibolya O. Tóth; Zoltán Cziáky: Performed the experiments.

Miklós Gábor Fári: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/suppl. material/referenced in article.

Declaration of interest's statement

The authors declare the following conflict of interests: The University of Debrecen registered a patent (WO-2019150144-A1) related to the biomass processing method used in this article with É. D-Sz and MG. F. as inventors. The authors declare there are no other conflicts of interest.

Additional information

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