Future Foods 9 (2024) 100331

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo

Biorefining of legume and grass biomasses: Technological properties and bioactivities of the green juice

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A R T I C L E I N F O

Keywords:
Green biorefinery
Phenolic acids
Flavonoids
Antioxidant activity
Antibacterial activity
Anti-inflammatory activity
Technological properties
Galega orientalis
Pisum sativum
Vicia faba
Trifolium pratense
Trifolium repens
Phleum pratense

A B S T R A C T

Five legume species (fodder galega, green pea, faba bean, red clover, and white clover) and one grass (timothy) grown under field conditions were investigated as potential future foods. Technological characteristics, such as emulsifying and foaming properties and nitrogen solubility indices, were determined from the green juices pressed from the whole plants. Secondly, the juices were evaluated for their antioxidative, antibacterial and anti-inflammatory properties. Faba bean green juice showed the highest emulsion stability and capacity, 66.0 ± 3.3 % and 18.0 ± 2.7 g/mg respectively, and second highest emulsion activity (58.4 ± 0.92 %). Considering the foaming properties, the highest activity was shown by white clover (433 ± 0.0 %) and green pea juices (411 ± 19.2 %). At the same time, the foam stability was remarkable for green pea juice (1078 ± 181.9 min) when compared to the other juices studied. The juices showed nitrogen solubility indices of about 50 % at pH 8, which were rather similar to those shown for the seeds of the same species in the literature. The antioxidant activities correlated with the total phenolic content. The highest antioxidant activity was shown by faba bean green juice both in the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) method. In addition, a weak positive correlation was observed between the total phenolic content and the antibacterial properties using *Escherichia coli* and *Staphylococcus aureus* strains, although mainly in the case of the latter strain, high inhibition readings were observed for all other plants than white clover. All juices showed some anti-inflammatory activity on human monocyte activation: the faba bean and red clover were the most efficient materials (IC$_{50}$ concentrations 49 ± 41 and 206 ± 68 mg/L, respectively).- These results suggest that the green juices tested may provide novel health-promoting food and feed ingredients in the future.

1. Introduction

Novel approaches are needed to transform the food system to be more resilient and sustainable in societal, economic, and environmental terms. Green biorefineries are of growing interest nowadays to produce various products, including new innovative food ingredients from green biomass that are abundantly available as are also the green crop residues or different grasses in a fresh or silage forms (Gaffey et al., 2023). The same authors also described residues from production as secondary green biorefinery feedstocks. Kromus et al. (2004) described the green biorefinery concept as an integrated technology that produces different compounds and energy via biogas production from the green biomass or the green parts of the plants. These compounds might be proteins, lactic acids, and fibers. During a green biorefinery process, the biomass is typically first separated into liquid and solid fractions, and soluble nutrients such as proteins are enriched in the green juice (Carlsson, 1997; Ayanfe et al., 2023). Ayanfe et al. (2023) investigated how the preservation method and press used affect the green juice yield and protein content. Their results suggested that twin screw press was the most efficient in the recovery of the green juices in terms of yield and also

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Abbreviations: BHA, butylated hydroxyanisol; CP, crude protein; DM, dry matter; EA, emulsion activity; EC, emulsion capacity; ES, emulsion stability; FA, foaming activity; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; LA, lysogeny agar; ORAC, oxygen radical absorbance capacity; RLU, relative light units; TPTZ, 2,4,6-Tris (2-pyridyl)-s-triazine.

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https://doi.org/10.1016/j.fufo.2024.100331
Received 2 January 2024; Received in revised form 23 February 2024; Accepted 15 March 2024

preservation has a positive effect when compared to the fresh biomass.

New sources of proteins for human foods and animal and pet feeds have been under study recently. It is a logical result of the transition of food/feed production systems towards more sustainable and economical production chains (Augustin and Cole, 2022). Also, unconventional plant proteins, typically not yet used in foods, could be utilized more effectively as food and feed ingredients in the future. Although novel food legislation in Europe (EFSA, 2023) may restrict the commercial use of proteins extracted from green biomass as food or feed ingredients at the moment, there is a great interest in screening the opportunities of such fractions for future applications (Gaffey et al., 2023).

Cultivation of legumes provides many ecosystem services such as nitrogen fixation, carbon sequestration and increased biodiversity, making them interesting raw materials for green biorefining (Jørgensen et al., 2022). In the current study, both perennial forage legumes, fodder galega (*Galega orientalis,* var. Gale), red clover, (*Trifolium pratense*, var. Selma), white clover (*Trifolium repens*, var. Lena), as well as annual species, green pea (*Pisum sativum*, var. Hulda), and faba bean (*Vicia faba*, var. Kontu) were evaluated as biomasses for green juice production. As a comparison, a grass species, timothy (*Phleum pratense*, var. Nuutti), was included to increase the variation in raw materials evaluated. The different species used in the current study represent different agronomic characteristics, but they are beyond the scope of the current discussion.

Although green biorefineries are continuously evolving, some gaps in knowledge still remain. Gaffey et al. (2023) described the need for future research in different topics such as paying more attention to waste materials as feedstock and increasing the co-products' role in the production. Besides, the authors mention the need for more research on sustainability issues and business models. Although studies on the processing technology are growing fast, studies on the food use of green juices remain scarce. Additionally, novel information is collected in this research, including the technological and functional properties of these green juices, which may, in turn, give new insight into how to utilize these in the most efficient way for product development.

The objectives of the current study were to i) produce green juices from the different plant biomasses by twin screw press, ii) screen the various technological properties to gain knowledge for which model food application they are the most suitable, and iii) determine the functional properties of green juices extracted from a range of green biomasses to evaluate their potential as functional future food ingredients.

2. Materials and methods

2.1. Green biomass production and fractionation

Green biomass of leguminous plant species fodder galega, green pea, faba bean, red clover, and white clover was harvested during the summer of 2021 in Jokioinen, Finland (60°48′N 23°29′E). In addition, gramineous forage grass, timothy, was harvested as a reference from Siikajoki, Finland (64°66′N, 25°09′E). The standing crops were cut at the field manually (except for timothy, which was harvested with a forage chopper), chopped, frozen at −20 °C and melted before processing. The green juice was separated using a laboratory scale twin screw press (Angel Juicer Ltd., Busan, South Korea) using a 300 g sample, and the measurement of liquid yield was taken when the screw press had reached a steady state. The raw materials and green juices were analysed for chemical composition as described by Ayanfe et al. (2023).

2.2. Technological properties

2.2.1. Emulsion properties

The emulsifying capacity, emulsion activity, and emulsion stability were determined according to the method by Satterlee et al. (1973) with minor modifications, described in Ben-Othman et al. (2023) and attached to the Supplementary Material 1 (S1).

2.2.2. Protein solubility

Protein solubility was determined using an in-house method. Protein dispersions (1 %, w/v) were prepared in water, and the pH was adjusted to 8 with 0.5 M or 0.1 M NaOH and stirred at room temperature for 2 h. The suspension was divided into two parts: one was stored at −20 °C until further analysis; the remainder was centrifuged at 3 000 × *g* for 10 min. The supernatants were filtered through a Whatman 42 filter, collected, and stored at −20 °C until analysis. The nitrogen content of the suspension and the filtered supernatant was determined using the Kjeldahl protocol. The nitrogen content of the samples was determined with an in-house Kjeldahl method according to the Association of Official Analytical Chemists (AOAC) method 2001.11, SFS EN ISO 20483:2013 and EN ISO5983-2. The correction factor of 6.25 was used in protein content calculations.

The nitrogen solubility index was expressed as

$$\mathrm{NSI}(\%) = (\text{concentration of nitrogen in supernatant} \,/\, \text{total nitrogen concentration}) \times 100$$

2.2.3. Foaming properties

The ability to form a foam depends on the ability of the protein to form an interfacial film, which can close air bubbles. Three methods are used to make foams: whipping, shaking, and letting air through the protein solution. In this study, a whipping method was used to make foams, and foam capacity and stability were measured as described by Ben-Othman et al. (2023). A detailed description of this methodology is attached to Supplementary material 1 (S1).

2.3. Determination methods for phenolic acids, flavonoids, total phenolic contents and antioxidant activities

2.3.1. Phenolic acids

Phenolic acids were determined in the raw materials after alkali and acid hydrolysis, according to Mattila and Hellström (2007). Briefly, freeze-dried samples (0.5 g) were homogenized with 10 mL of acidified 70 % methanol containing 2 g/L of butylated hydroxyanisol (BHA) and sonicated for 30 min. Next, 12 mL of water and 5 mL of 10 M NaOH were added, and the sample was stirred overnight at room temperature. Then, the solution was adjusted to pH 2, after which 2.5 mL of 13 M HCl was introduced. The sample was hydrolyzed for 30 min at 85 °C after which the liberated phenolic acids were extracted with diethyl ether-ethyl acetate (1:1 v/v) and analyzed by UHPLC-DAD according to conditions described by Hellström et al. (2020). Identification of the compounds was confirmed by UHPLC-QTOF/MS analysis applying a column and conditions described by Hellström et al. (2020).

2.3.2. Flavonoids by HPLC-DAD

Flavonoids were determined in raw materials after acid hydrolysis, according to Mattila et al. (2016). Briefly, the samples were hydrolyzed by refluxing the freeze-dried samples in 1.2 M HCl in 50 % aqueous methanol for 1 h and analyzed by HPLC-DAD. Flavonoid aglycons were identified from the chromatogram at 370 nm according to their typical

UV spectra similar to flavonols, flavones, or flavanones and were quantified as quercetin. In the red clover case, a sum of isoflavones (formonotein and biochanin A) were identified and quantitated at 280 nm.

Green juices were diluted with methanol (1:2 v/v), vortexed, centrifuged, filtered, and analyzed by HPLC-DAD similarly to the raw materials. Flavonoid sugar conjugates were identified from the chromatogram at 370 nm and expressed as sum quercetin. Isoflavones were identified and quantitated at 280 nm.

2.3.3. *Total phenolic content and antioxidant activity*

The samples were analysed in liquid form after centrifugation with 12,000 x g for 2 min in a centrifuge (model Eppendorf 5430) to remove the possible solid contents. The Folin–Ciocalteu method (Singleton et al., 1999) was used. The results are expressed as gallic acid equivalents per gram extract dry weight (mg GAE/g). The method has been published by Fidelis et al. (2023), and a detailed method description can be found in Supplementary Material 2 (S2).

The ferric-reducing antioxidant power (FRAP) method is based on the single electron transfer mechanism and measures the ability of antioxidants to reduce ferric Fe(III) to ferrous Fe(II) ions (Benzie and Strain, 1996). The results are expressed as μmol/g (extract dry weight) Fe(II) equivalents. The method has been published by Fidelis et al. (2023), and a detailed method descriptions can be found in the Supplementary material.

The oxygen radical absorbance capacity (ORAC) method measures the ability of potential antioxidants to prevent peroxyl radicals from harming a fluorescent substance fluorescein molecule. The assay was modified from that of Huang et al. (2002) and Prior et al. (2003). The results are expressed as Trolox equivalents (TE; μmol/g (extract dry weight)). The method has been published by Fidelis et al. (2023), and a detailed method description can be found in Supplementary Material 2 (S2).

2.4. *Bioactive properties*

2.4.1. *Antibacterial analyses*

A microplate method with bioluminescent indicator strains *Staphylococcus aureus* RN4220+pAT19 and *Escherichia coli* K12+pcGLS11 (Vesterlund et al., 2004) was used to study the antibacterial activity of the extracts. These strains have been constructed to produce a constant luminescent light signal, and antibacterial effects can be observed as a loss of emitted light signal intensity. The storage, cultivation, and test protocol has been previously described (Välimaa et al., 2020; Muilu-Mäkelä et al., 2022). The results are expressed as inhibition percentages (inhibition %) at the content of 4 mg/mL of each extract at 50 min of incubation. The method has been published by Fidelis et al. (2023) and detailed method description can be found in the Supplementary material 2 (S2).

2.4.2. *Anti-inflammatory activities*

For anti-inflammatory tests, the undissolved particles were removed from the juices by centrifugation and microfiltration. Contents of dry matter were measured, and the pH was adjusted to the physiological 7.4 as described in Pap et al. (2021). The anti-inflammatory effects of fractions on human THP-1 promonocyte respiratory burst (RB) responses were measured as described in Tompa et al. (2011). For a detailed description of reagents and methods, see Supplementary Material 3 (S3).

2.5. *Statistical analyses*

Experiments were run in triplicate, and the results were expressed as means ± standard deviation. To compare the treatments, the Brown-Forsythe test was used to check for equality of variances, and one-way ANOVA followed by the Duncan test was used to compare the mean values. Correlation analyses were calculated to measure the degree of association between pairwise variables. Probability values <0.05 were used to reject the null hypothesis, and the software TIBCO Statistica v. 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) was used.

The antibacterial effects of the green juices were analysed using one-sided ANOVA followed by post-hoc test and Bonferroni correction.

Comparisons of IC_{50} values of THP-1 cell activities between different green juices were made with Microsoft Excel, using the paired Student's *t*-test using the Bonferroni correction. The post hoc test was done

3. Results and discussion

3.1. *Composition of the original biomasses, green juices, and extraction rates*

The composition of the original biomasses and the green juices, as well as the extraction rates, are presented in Table 1. There was considerable variation in the composition of the raw materials as expected based on characteristics of different plant species, and also due to maturity of plants (the green peas were most mature at harvest) and environmental conditions (rainy weather before harvesting of timothy). The average dry matter (DM) concentration of the green juices was 97 g/kg, which aligns with previous data sets (Franco et al., 2019; Ayanfe et al., 2023), but it was highly variable between plant species. The DM concentration of the original biomass was positively correlated with the DM concentration of the extracted green juice (R^2= 0.94). The average crude protein (CP) concentration of the legume juices was 289 g/kg DM, which was relatively high, representing the high CP concentration of the original biomasses.

The extraction rates of green juice were high (on average, 73 % of fresh weight was captured in the green juice fraction) due to the highly efficient twin-screw press used for sample preparation (Franco et al., 2019). From the chemical components, the extraction rate (proportion of compound in the raw material captured into the liquid) was highest for ash and lowest for DM, while CP was intermediate. The average extraction rates were 39, 58, and 54 % for DM, ash, and CP, respectively.

Table 1

Composition of the biomasses and the green juices, and extraction rates.

	Fodder galega	Green pea	Faba bean	Red clover	White clover	Timothy
Date of harvest (2021)	9 June	6 August	13 August	16 June	16 June	18 August
Raw materials						
Dry matter (DM; g/kg)	128	278	188	171	171	118
Ash (g/kg DM)	83	49	62	110	113	78
Crude protein (g/kg DM)	270	131	234	193	224	186
Green juices						
DM (g/kg)	62	161	98	110	100	94
Ash (g/kg DM)	62	121	96	150	150	150
Crude protein (g/kg DM)	442	195	274	272	262	130
Extraction rates (%) of fresh biomass captured in the green juice						
Fresh weight	74	71	72	69	70	83
DM	35	41	37	44	41	36
Ash	27	102	57	61	41	57
Crude protein	58	61	44	63	55	45

Table 2

Technological properties of different green juices.

Sample	EA (%)	ES (%)	EC (g oil/ mg protein)	FA (%)	FS (min)	PSI (%)
Goat's rue	57.5 ± 2.0^{b}	61.5 ± 1.2^{b}	18.5 ± 1.3^{a}	367 ± 0.0^{b}	114 ± 7^{b}	69.2 ± 0.0
Green pea	59.5 ± 0.7^{a}	64.5 ± 2.0^{a}	14.4 ± 2.0^{a}	411 ± 19.2^{a}	1078 ± 182^{a}	66.7 ± 0.0
Faba bean	58.4 ± 0.92^{ab}	66.0 ± 3.3^{a}	18.0 ± 2.7^{a}	289 ± 19.2^{c}	193 ± 32^{b}	52.4 ± 0.0
Red clover	43.5 ± 1.0^{c}	56.3 ± 0.9^{c}	16.8 ± 5.9^{a}	172 ± 9^{d}	114 ± 6^{b}	50.0 ± 0.0
White clover	57.5 ± 0.6^{b}	57.7 ± 1.1^{c}	15.3 ± 1.6^{a}	433 ± 0.0^{a}	150 ± 39^{b}	50.0 ± 0.0
Timothy	6.85 ± 0.4^{d}	11.9 ± 0.7^{d}	16.6 ± 0.5^{a}	167 ± 0.0^{d}	114 ± 6.4^{b}	50.0 ± 0.0

Note: EA, emulsion activity; ES, emulsion stability; EC, emulsion capacity; FA, foam activity; FS, foam stability; PSI, protein solubility index. Mean values of triplicates ± standard deviation expressed on a wet basis. Means followed by different letters in the column differ statistically ($p < 0.05$).

3.2. Technological properties

Emulsion activity (EA, %), emulsion stability (ES, %) and emulsion capacity (EC, g/mg), foaming activity (FA, %) and foaming stability (FS, min), as well as the nitrogen solubility index (PSI, %) were measured in triplicate from the six different green juices. The results are compiled in Table 2.

3.2.1. Emulsion properties

Emulsion activity (EA) and stability (ES) were determined for all legume and grass juices. In emulsion activity, the highest value was measured for the green pea, but faba bean, fodder galega, and white clover were also close to it. Timothy juice, on the other hand, showed the lowest emulsion activity between all samples. Emulsion stability describes the ability of the sample to maintain the emulsion over time after the heat treatment. Faba bean and green pea showed the highest emulsion stability values, while the rest of the samples were significantly lower, and timothy showed the lowest value for emulsion stability.

Emulsion capacity (EC) is expressed through the amount of oil emulsified by protein in defined conditions. All the samples represented the same level of emulsifying capacity, fodder galega, and faba bean being the highest, while green peas showed the lowest value (Table 2).

3.2.2. Protein solubility

Solubility is an important functional parameter from the applicability point of view of the protein source, especially in beverages (Chang et al., 2022), but it is also necessary for other functionalities such as foaming and emulsification properties (Liang and Tang, 2013). The highest protein solubility index (PSI) was measured for the fodder galega, followed by the green pea and the faba bean. Hall and Moraru (2021) measured similar values for green pea (57 ± 2.0) and faba bean (58 ± 4.0) when the protein concentrate samples were prepared by air classification. Protein solubility of the samples is essential in the design of model foods, and low solubility of the plant proteins is a challenge for food industry applications (Hall and Moraru, 2021).

The protein solubility of the juices was evaluated, and the results are presented in Table 2. Faba bean had the lowest solubility at a pH 4–5 (isoelectric point), while at pH 7 it reached 43 % in the work of Alavi et al. (2021), while our results of the green juice showed 51 % solubility at the pH of 8. Saldanha do Carmo et al. (2020) also investigated the protein solubility of whole and dehulled peas, as well as faba beans in the pH range of 2–10. They observed that both fractions had even higher solubility when the pH was 9 or 10 in the case of the faba bean, and peas had lower solubility at pH 2 then the faba beans. In our study at pH 8, the results were the opposite; green peas juice had a solubility of 66.7 % and faba beans 52.4 %.

3.2.3. Foaming properties

Foaming capacity (FC) is only one of the important indices in the protein functionality investigation. Still, the stability of the foam at different pH values also gives essential information (Kinsella, 1981).

Alavi et al. (2021) described the faba bean foaming properties as generally higher at pH 5 than at pH 7. The untreated faba bean sample reached 271 ± 3.6 %, while the best results were achieved with the previously heat-treated sample at pH 11 (559 ± 4.3 %). Our results showed a pH 5.5 higher foam capacity for the untreated faba bean sample, which accounted for 289 ± 9.6 %. The foam stability (FS) was also analyzed beside the foam capacity, and it showed similar results that Alavi et al. (2021) achieved with the non-treated and control faba bean sample (3.1–2.2 min), while the drainage of the half the amount in the present study was 3.25 min.

3.3. Phenolic acid content, flavonoid content, total phenolics, and antioxidant activity

3.3.1. Phenolic acids content

The contents of determined phenolic acids in different raw materials are presented in Table 3. The highest total content of phenolic acids was determined in timothy (849 mg/100 g DM) followed by fodder galega (795 mg/100 g DM). Fodder galega was remarkably rich in caffeic acid (621 mg/100 g DM). Pea had generally low and white clover moderately high contents of phenolic acids. Red clover was rich in pharmacologically active salicylic acid. Diferulic acids (as ferulic acid equivalents) could be determined only in timothy.

Table 3

Content of phenolic acids in the raw materials (mg/100 g DM, mean ± sd, $n = 3$).

	Fodder galega	Pea	Faba bean	White clover	Red clover	Timothy
Gallic acid	ND	ND	11.1 ± 0.8	7.28 ± 0.1	10.8 ± 0.7	8.92 ± 0.5
3,5-Dihydroxy benzoic acid (α-resorcyclic acid)	ND	ND	<LOQ	ND	<LOQ	<LOQ
3,4-Dihydroxy benzoic acid (protocatechuic acid)	14.4 ± 0.3	3.98 ± 0.4	10.6 ± 0.1	ND	6.51 ± 0.3	47.4 ± 1.6
2,5-Dihydroxy benzoic acid (gentisic acid)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
4-Hydroxy benzoic acid (*p*-hydroxy benzoic acid)	3.82 ± 0.9	3.39 ± 0.1	6.26 ± 0.2	7.94 ± 0.7	8.43 ± 0.7	10.7 ± 0.7
2,3-Dyhydroxy benzoic acid (pyrocatechuic acid)	ND	ND	ND	<LOQ	<LOQ	<LOQ
Vanillic acid	ND	ND	ND	ND	5.78 ± 0.2	7.70 ± 0.2
p-Coumaric acid	65.6 ± 3.3	21.0 ± 1.4	23.2 ± 2.0	59.9 ± 3.4	26.5 ± 0.7	278 ± 17
Caffeic acid	621 ± 38	6.35 ± 0.3	24.9 ± 2.0	209 ± 10	56.5 ± 0.3	102 ± 1.0
Ferulic acid	91.0 ± 5.9	10.3 ± 0.6	31.4 ± 2.6	114 ± 5.0	22.8 ± 0.7	305 ± 13
Sinapinic acid	ND	23.4 ± 1.2	17.8 ± 0.8	26.1 ± 1.0	5.75 ± 0.3	6.14 ± 0.4
Salicylic acid (2-hydroxy benzoic acid)	ND	ND	ND	11.0 ± 0.4	121 ± 2.0	60.7 ± 0.8
Diferulic acids	ND	ND	ND	ND	ND	26.6 ± 1.0
Total	795 ± 45	68.5 ± 3.4	114 ± 6	435 ± 18	264 ± 5.0	849 ± 29

Note: ND, not detected; <LOQ, < as the limit of quantification.

Previously published information on phenolic acids in vegetative parts of studied plant materials is very scarce. Caffeic acid, coumaric acid, and ferulic acid have been identified in timothy (Hartley and Jones, 1977; Mino and Harada, 1974) but the contents were not reported. Our study indicated that caffeic and vanillic acids were timothy's most abundant phenolic acids, followed by *p*-coumaric acid (Table 3).

Studies on galega have focused on *T. officianalis* while *T. orientalis* has been explored much less. Similarly, Samoilova et al. (2022) also found coumaric and caffeic acid in *T. orientalis*, aligning with our results. Besides, they reported the presence of gallic acid, and its polymer tannic acid, which could not be found in the current study. Furthermore, in the study of Samoilova et al. (2022), only soluble acids were measured, i.e., no hydrolysis was included, which was probably why they did not detect any other acids in their samples. According to our study, ferulic acid was the second most abundant phenolic acid in fodder galega after caffeic acid.

Caffeic, *p*-coumaric, and vanillic acids were the primary phenolic acids in faba bean. Generally, our results agree with the study of Mohamed et al. (2016) on shoots of faba bean, although they reported much higher contents of *p*-hydroxy benzoic acid. Some natural differences in the contents between different cultivars are expected. Also, environmental elements, including stress factors, have been found to influence the content of phenolic acids in faba beans (Mohamed et al., 2016).

Phenolic acids in pea seeds are well characterized, agreeing quite nicely with our results, but generally, the contents determined from the whole plant in the current study were higher than those determined in the seeds (Chahbani et al., 2018; Dueñas et al., 2004; Mattila and Hellström, 2007). Borges-Martinez et al. (2022) reported that the sprouts of germinated pea seeds had higher phenolic acid contents than the seeds, indicating the selective accumulation of phenolics in the vegetative parts in accordance with our study.

White clover had a higher total content of phenolic acids than red clover (Table 3). The major phenolic acid in white clover was caffeic acid, followed by ferulic acid, agreeing with Ahmad et al. (2020). Red clover was especially rich in salicylic acid. Kicel and Wolbis (2006) already reported the presence of salicylic acid in both white and red clovers. The total phenolic acid contents agree well with the study of Tava et al. (2019). Much lower phenolic acid contents in red clover were found by Chiriac et al. (2020), most probably because no hydrolysis step was included, and only soluble acids were determined.

Some dihydroxy benzoic acids that could not be quantified by UHPLC-DAD were tentatively identified by UHPLC-MS according to molecular ions and MS^{E} fragments equal to 3,4-dihydroxy benzoic acid (Table S1). The retention order of dihydroxy benzoic acids in reversed-phase HPLC has been reported as follows: (1) 3,5-, (2) 3.4-, (3) 2,5-, (4) 2,6-, (5) 2,3-dihydroxy benzoic acid (Collins, 2022). Consequently, 2, 5-dihydroxy benzoic acid was tentatively detected in all samples, 3, 5-benzoic acid was detected in faba bean, red clover and timothy, 2, 6-dihydroxy benzoic acid in fodder galega, and 2,3-dihydroxy benzoic acid in clovers and timothy. 3,4-dihydroxy benzoic acid (protocatechuic acid) was detected and determined in all samples except white clover.

3.3.2. Flavonoid content

The flavonoid content as quercetin was determined as follows: fodder galega 54 ± 3, timothy 462 ± 39, green pea 1134 ± 49, faba bean 4913 ± 182 and white clover 4522 ± 343 µg/g DM. Red clover raw material contained flavonoids 121 ± 4 µg/g (DM) as quercetin and isoflavones as sum 14,919 ± 125 µg/g (DM).

The flavonoid content in fodder galega and timothy in the current study were lower than what was reported earlier by Vergun et al. (2020) for fodder galega (ca. 7–40 mg/g DM) and by Fariaszewska et al. (2020) for timothy (18–39 mg/g DM). The different sampling techniques and treatments of the raw materials might explain these differences. Neugart et al. (2015) reported a flavonoid content in the leaves of peas of ca. 5–8 mg/g DM and in faba bean leaves of ca. 9–13 mg/g DM, which also are somewhat higher than in our study. Carlsen et al. (2008) reported flavonoid contents in white clover leaves and stems as ca. 3–9 mg/g DM and Hofmann and Jahufer (2011) ca. 1.8–3.8 mg/g DM. Isoflavone content in red clover was in line with Saviranta et al. (2008) and Sivesind & Seguin (2005).

Flavonoid contents such as quercetin in green juices were found in timothy 740 µg/g (DM) (38 µg/g FM), fodder galega 13 µg/g (DM) (208 µg/g FM), green pea 293 µg/g (DM) (1822 µg/g FM), and faba bean 1130 µg/g (DM) (11,528 ug/g FM) (white clover juice sample was not available). In the red clover juice the content of isoflavones as a sum of formonotein and biochanin A was 962 µg/g (DM) (8742 µg/g FM).

3.3.3. Total phenolic content and antioxidant activity

The results of the total phenolic content and the ORAC and FRAP tests are summarized in Table 4. The lowest total phenolics were measured from the green pea (16.0 ± 0.5 mg GAE/g), timothy (22.4 ± 1.4 mg GAE/g), and fodder galega (25.3 ± 2.4 mg GAE/g) juices. Red clover shows antioxidant activity that may be attributed to the presence of different phenolics, mostly flavonoids, phenolic acids, and saponins (Esmaeili et al., 2015). The authors measured the total phenolic content of the red clover plant extracts in different solvents, and they found out that the extracts contained between 16.9 ± 1.2 and 46.9 ± 1.1 mg GAE/g phenolics depending on the solvent used and the type of the samples (in vivo, in vitro and callus obtained from in vitro culture). This is in accordance with our study where the juice from the red clover contained 39.7 ± 1.9 mg GAE/g total phenols. Also, Kazlauskaite et al. (2022) measured similar values from red clover extracts when comparing the effect of the solvent and the extraction method. The highest value was achieved using ethanol (50 % v/v) as the solvent, 54.1 ± 0.5, and 44.8 ± 0.9 mg GAE/g when water was employed as the solvent. Horvat et al. (2020) found 38.7 ± 1.9 to 60.0 ± 1.6 mg GAE/g material depending on the red clover variety, which is also in the range of our study. The white clover phenolics were significantly different from the composition of the red clover. The white clover contained 22.8 ± 1.8 mg GAE/g total phenolics. Faba bean is an excellent source of bioactive phenolic compounds, as the green juice contained 46.7 ± 5.1 mg GAE/g and was the highest among the species in this study. The compounds are mostly phenolics and tannins with bioactive compounds of pro-anthocyanidins, representing antioxidant function (Kumar et al., 2015). Chaieb et al. (2011) investigated the phenolic compound content of different faba bean cultivar seeds and measured significantly higher values than observed in the juice (118 to 158 mg GAE/g DW). Slightly lower values were measured by Baginsky et al. (2013) from immature faba bean seed varieties (817 to 1338 mg GAE/kg DW). However, the results showed still higher values of phenolics from the seeds than from the green juice of this study, likely because of the differences in the moisture content.

Legumes and grasses high in phenolic content may show antioxidant activity, and daily diet consumption may show beneficial health properties (Singh et al., 2017). The in vitro antioxidant capacities of the different green juices of this study were evaluated by the ORAC and FRAP methods, and the results are summarized in Table 2. The faba bean

Table 4
Total phenolic content and antioxidant activities of the green juice samples.

Sample	TPC (mg GAE/g)	ORAC (µM TE/g)	FRAP (µM Fe(II) eq/g)
Fodder galega	25.3 ± 2.4^{c}	644 ± 40^{d}	94.2 ± 8.8^{d}
Green pea	16.0 ± 0.5^{b}	244 ± 18^{e}	74.0 ± 1.6^{e}
Faba bean	46.8 ± 5.1^{a}	1745 ± 43^{a}	443 ± 7^{a}
Red clover	39.7 ± 1.9^{b}	977 ± 82^{c}	186 ± 13^{b}
White clover	22.8 ± 1.9^{c}	1048 ± 39^{b}	150 ± 14^{c}
Timothy	22.4 ± 1.4^{c}	923 ± 30^{c}	75.5 ± 7.2^{e}

Note: Mean values of triplicates ± standard deviation expressed on a dry basis. Means followed by the same letter in the column do not differ statistically ($p < 0.05$).

juice showed the highest antioxidant activity by the ORAC method, 1745 ± 43.1 μmol TE/g. Except for timothy (923 ± 30.5 μmol TE/g) and red clover juices (977 ± 82.3 μmol TE/g), all other samples showed statistical differences in the ORAC values ($p < 0.05$). The lowest activity was found in green pea green juice.

Using the FRAP method, faba bean juice showed again the highest antioxidant value, 443 ± 7.3 μM Fe(II) eq/g, followed by the red clover and the white clover juices, the values being 186 ± 12.7 μM Fe(II) eq/g and 150 ± 14.3 μM Fe(II) eq/g, respectively. The results for the red clover are in good agreement with the results of Kazlauskaite et al. (2022). In their work, the FRAP values for red clover were 102 ± 1.6 and 193 ± 1.9 mg Fe(II) eq./g, respectively. The FRAP values of timothy and green pea juices did not show significant differences. The antioxidant activity measured by ORAC and FRAP correlated with each other ($r = 0.97$) and the total phenolic content ($r_{ORAC} = 0.83$ and for $r_{FRAP} = 0.89$). A correlation between total phenolic content and ORAC was also found in the work of Chen et al. (2023) when different pea cultivars were compared. The authors also described the need for more than one antioxidant assay for a comprehensive study due to the other reactive oxygen species and differences in the mechanisms.

3.4. Bioactivity properties

3.4.1. Antibacterial activity

A study was undertaken to find out the antibacterial potential of the six green juices on the gram-negative *E. coli* and gram-positive *Staphylococcus aureus* strains. The inhibition values differed significantly between the plants (Fig. 1A and B) in the case of *E. coli*, where the red clover juice showed the highest (92.7 %) and green pea juice had lowest (17.6 %) inhibition. In the case of the gram-positive *Staphylococcus aureus*, faba bean showed the highest inhibition (94.3 %) followed by red clover (91.9 %). The lowest inhibition was observed for white clover (30.1 %). These values indicate excellent results for the plant materials used in this study, as in the work of Pundarikakshudu et al. (2001), a simple water extract of galega did not exhibit any effect on the various gram-positive and gram-negative bacteria. However, in this study, we used juice pressed from fresh plant material, while Pundarikakshudu et al. (2001) utilized cold-water extracts of the dried plant material, which likely influenced the results.

The correlation analysis showed some correlation of inhibition with the total phenolic content, with R^2 values being 0.61 in the case of *E. coli* and 0.42 in the case of *S. aureus*. The results indicate that besides the phenolics, other compound groups such as proteins may also play a role in the bacterial inhibition. These compounds might contain some antibacterial proteins in the samples (Tienaho et al., 2019; Tienaho et al., 2020). Sitohy and Osman (2010) also found that esterified legume proteins resulted in lower turbidity of the samples against gram-positive and gram-negative bacteria compared to the control. They suggested that legume proteins could be safe preservatives for different food preparations.

3.4.2. Anti-inflammatory properties

The effects on phagocyte functions were studied using human THP-1 promonocytes, one of the best and least un-ubiquitous phagocyte models (Chanput et al., 2014). The results are from 8 separate measurements and reflect the cell activation as outer cell membrane superoxide radical producing oxidase complex. The results are expressed as the chemiluminescence (CL) (relative light units, rlu) peak activities representing the highest level of radical-producing reaction (Fig. 2A and B) and as areas of the kinetic CL curves regarded as measures of total radical production during the RB (Fig. 3A and B).

All the plant juice samples had inhibitory effects on phagocytosis receptor mediated activation of normal state and inflammatory phagocytes and on their oxidase enzyme activities (Figs. 2A and B, 3A and B) (Table 5). There were significant differences in inhibitory activities between samples the faba bean sample being the most active and red clover being the second potent (Table 5). Timothy green juice showed nearly as high activity as red clover in primed cells. Still, it was less active with non-primed ones. Green pea and timothy samples had significantly stronger inhibitory effects on inflammatory primed leukocytes than on non-primed ones (Table 5). One explanation could be that the compounds in these green juices prevent the LPS priming reaction. After LPS priming, THP-1 promonocytes exert 30–80 % higher CL response to yeast particles than non-primed (Tompa et al., 2011). Thus, inhibition of the priming reaction would result in stronger inhibition, which is seen as a lower IC_{50} concentration. This kind of mechanism could be beneficial in preventing widespread diseases like type 2 diabetes, vascular disease, and neural meta-inflammation. The anti-inflammatory properties of faba bean have earlier been reported in a few in vivo studies. In an experimental high sucrose diet rat model, dietary supplementation with dried ground faba bean reduced oxidative

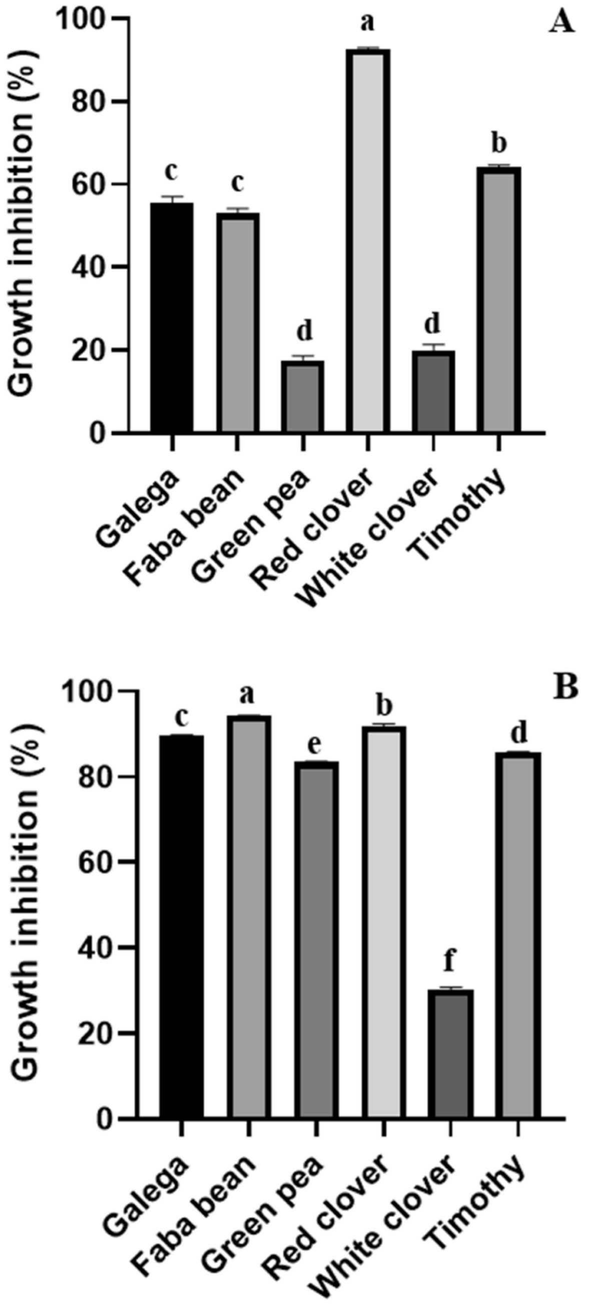

Fig. 1. The effect of the green juices from different green plant biomasses on the *E. coli* (A) and *S. aureus* (B) growth inhibition.

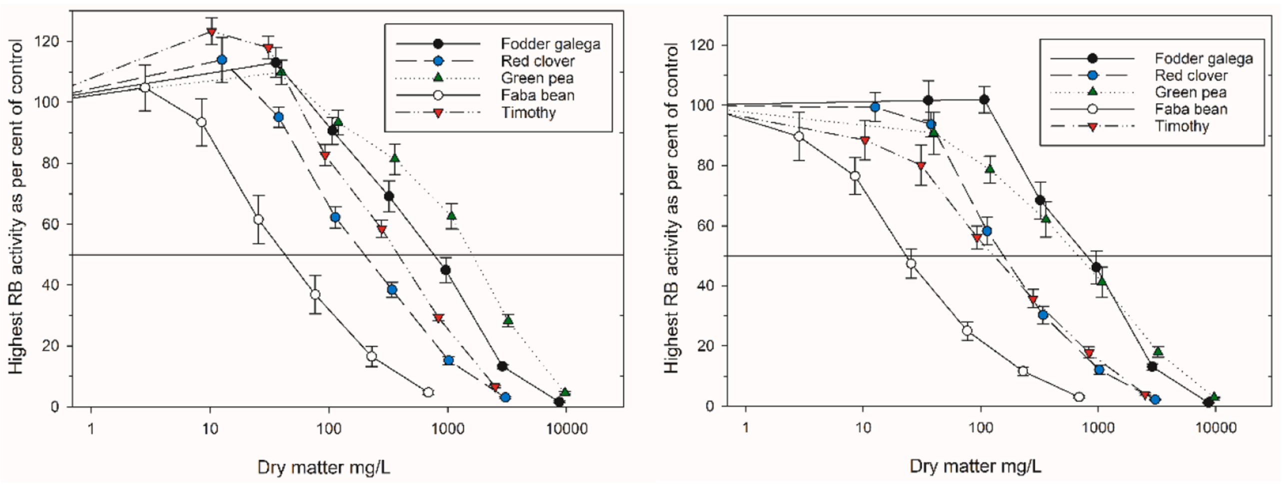

Fig. 2. A (left) and 2B (right). Effects of green juices from different green plant biomasses on peak respiratory burst (RB) activity of human THP-1 promonocytes. Fig. 2A represent the effect on ``normal state'' promonocytes (no priming) and 2B on promonocytes primed to inflammatory cells with *E. coli* LPS (10 μg/mL). The RB response to opsonized zymosan was measured as luminol enhanced chemiluminescence for 90 min. The points represent the mean of eight independent experiments (n = 8) and the error bars represent the standard error of the mean (S.E.M).

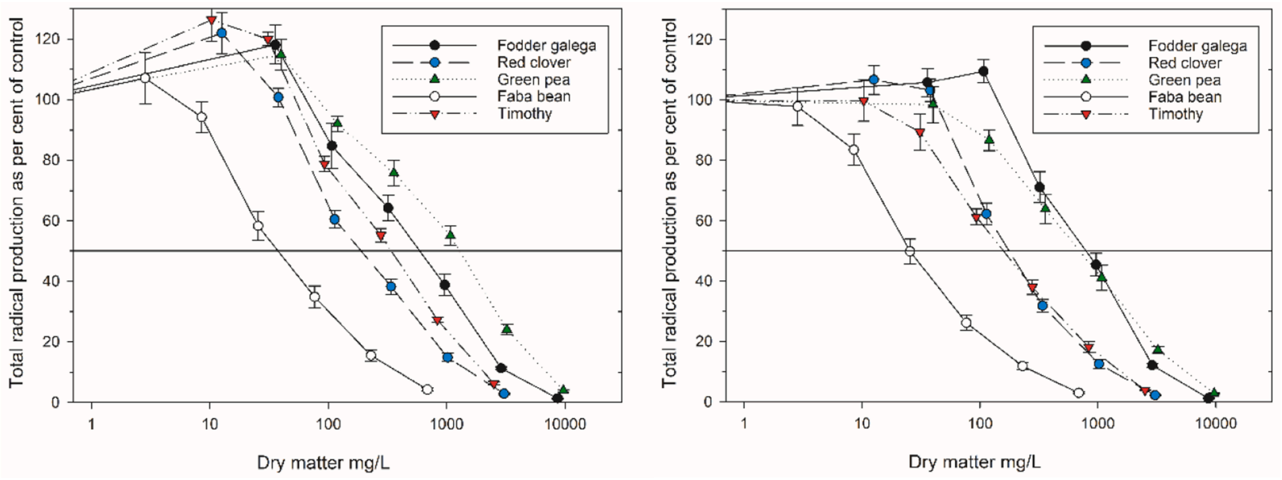

Fig. 3. A (left) and 3B (right). The effects of extracts from different green plant biomasses on the total radical production (area sum) of human THP-1 promonocytes during the RB. In Fig. 3A are the dose response curves of ``normal state'' leukocytes (no priming) and in 3B) those of inflammatory cells (primed with 10 μg/mL of *E. coli* LPS). The total oxygen radical production in response to serum opsonized zymosan was measured for 90 min as area of luminol enhanced chemiluminescence curves (the peak activities are show in Fig. 2A and B. The n = 8 and the error bars represent S.E.M.

Table 5
The IC_{50} concentrations (sample concentration that inhibits 50 % of the free radical production) of the samples for maximum reaction rate (Max CL) during respiratory burst (RB) activity of human THP-1 promonocytes.

Sample	Fodder galega	Red clover	Green pea	Faba bean	Timothy
IC_{50} (mg/L) non-primed	684 ± 286^{b}	206 ± 68^{d}	1603 ± 417^{a}	49.4 ± 41.9^{e}	377 ± 85^{c}
IC_{50} (mg/L) LPS-primed	758 ± 404^{a}	161 ± 60^{b}	778 ± 435^{a***}	25.7 ± 11.6^{c}	141 ± 66^{b***}

Note: The values are the means ± S.D. The letters indicate statistical differences between the samples (inside horizontal lines) the same letter indicating no difference. Differences between non-primed and LPS-primed cells (vertical column) are expressed with * (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

damage and inflammation-derived colonic injury (Eskandrani, 2021). In a rat paw edema model, vicine (a heterocycle glucoside from faba bean) showed anti-inflammatory and oxidative stress-reducing effects (Husein 2012). Methanolic extracts from the Egyptian faba bean cultivar “Sakha 3’’ had beneficial anti-inflammatory and neuroprotective effects in a mouse model of Parkinson’s disease. The extracts were administered as subcutaneous injections (600 mg/kg) every second day 9 times (Abdel-Sattar et al., 2021). These published studies align with our results obtained by using leukocyte models. Faba beans are known to contain many phytochemicals e.g., polyphenols, which are expected to be beneficial for humans (for review, see Turco et al., 2016). This study is, however, the first when the anti-inflammatory effect of juice from green biomass of faba bean plant is demonstrated.

The anti-inflammatory and health-beneficial properties of red clover cover effects on serum lipids, hypertension, menopause, and oxidative stress and have been well-known for a long time in traditional medicine, reviewed by Akbaribazm et al. (2021). The anti-inflammatory effects have been reported in cell models (Ramos et al., 2012; Lee et al., 2020a) and animal models (Widyarini et al., 2001, 2006; Lee et al., 2020b; Akbaribazm et al., 2020). Our results are parallel with these studies.

Interestingly, our green juice extract from timothy showed nearly as high anti-inflammatory activity as red clover. To the best of our knowledge, there is no earlier published data on anti-inflammatory effects in timothy. Timothy pollen is an allergen, and most research is focused on the allergic reactions that the pollen proteins may evoke. According to our results, the green biomass of timothy also contains anti-inflammatory compounds.

The fodder galega and green pea were the least active in our cell model. In general, plants contain a multitude of phytochemicals such as phenolic compounds, terpenoids, and salicylates which often inhibit hyperactive leukocytes (Lojek et al., 2014) and, thus, as part of the diet, reduce the risk of diseases that have an aetiology of long-term silent inflammation with overactive leukocytes (Aune et al., 2017; Yan et al., 2022). These include, among others, cardiovascular diseases (Siti et al., 2015), metabolic syndrome and type II diabetes, chronic depression (Rahimian et al., 2022), as well as older age neurodegenerative diseases (Maleki and Rivest, 2019). Thus, the green juices tested may provide novel health promoting food and feed ingredients in the future.

3.5. Future prospects

As the human population is growing, the demand for sustainable protein sources increases as well. The overconsumption of animal proteins is not only of health concern, but also contributes to adverse environmental impacts which the animal protein production generate. New protein sources of plant origin inevitably have to be brought to the market to feed the population. These protein sources need to be sustainable and affordable with minimal processing principles.

Green biorefineries make it possible to process green biomass with simple processing schemes into valuable protein sources. First, the solid and liquid fractions are separated to liberate the proteins from the fiber matrix and concentrate them. This liquid stream was the object of this work, but it is also possible to include another processing step to harvest the protein fraction in pure form by acid precipitation. However, the intact green juices showed valuable technological properties essential for model food development. The plant materials are also abundant in different phenolic compounds and phenolic acids released to the juice fraction together with the protein.

The analysis of the technological properties of proteins, e.g., foaming and emulsifying properties, assists in evaluating the texture forming potential of the protein-protein texture forming potential from the pressed juices of the green biomass. In addition to functionality, the detailed composition of the fractions as such, including nutrient contents and antinutritional compounds, and their behaviour/reactions in prototype product applications need to be clarified before further steps towards novel food ingredients can be taken. The European Food Safety Authority strictly regulates the acceptability of new food sources, and yet, it needs to be proven that green juices from various green biomasses are fully safe for humans, i.e., they are non-toxic and do not cause allergic reactions before they can be accepted as novel food ingredients.

4. Conclusions

Although novel food legislation in Europe currently restricts the commercial use of proteins extracted from green biomass as food or feed ingredients, there is a great interest in screening the opportunities of such fractions for future applications. This study demonstrated the feasibility of legume and grass raw materials to recover green juices with potential for food-grade materials. The technological and functional properties of the juices were analysed with positive results, although apparent differences were identified between the plant species. Despite the differences, the findings of this study revealed that faba bean green juice is especially promising for future food development as it has good emulsifying properties (emulsifying activity of 58.4 ± 0.92 %, emulsion stability of 64.5 ± 2.0 % and emulsion capacity of 18.0 ± 2.7 g/mg), but also generated foam with a foam activity value of 289 ± 19.2 % and foam stability value of 193 ± 32.5 min. Besides, it was outstanding with the total phenolic content of 46.8 ± 5.1 mg GAEg/g, having the highest ORAC and FRAP antioxidative values of 1745 ± 43.1 μmol TE/g and 443 ± 7.3 μM Fe(II) eq/g, respectively. In the antibacterial tests, faba bean showed the highest inhibition (94.3 %) in the case of the gram-positive *Staphylococcus aureus*. Among the samples investigated in the study, faba bean juice showed the strongest anti-inflammatory properties, with the IC_{50} concentration being 49.4 ± 41.9 mg/L, and the lowest activity in green pea with IC_{50} being 1603 ± 417 mg/L. The results indicate that the green juices tested mayprovide novel sustainable and health promoting, in the future, novel sustainable and health-promoting food and feed ingredients.

Ethical statement

The authors confirm that the study did not involve experimentation on human or animal subjects.

CRediT authorship contribution statement

Nora Pap: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Data curation, Conceptualization. **Daniel Granato:** Writing – original draft, Methodology, Formal analysis. **Eila Järvenpää:** Writing – original draft, Methodology, Data curation, Conceptualization. **Jenni Tienaho:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Pertti Marnila:** Writing – original draft, Methodology, Investigation, Data curation. **Jarkko Hellström:** Writing – original draft, Formal analysis, Data curation. **Juha-Matti Pihlava:** Writing – original draft, Formal analysis, Data curation. **Marcia Franco:** Writing – original draft, Methodology. **Tomasz Stefański:** Writing – original draft, Methodology. **Marketta Rinne:** Writing – original draft, Supervision, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by the Natural Resources Institute Finland in the GreenFood project (project no. 41007-00214900). The authors thank Marja Kallioinen and Ulla Jauhiainen for the technical assistance in experimental research.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2024.100331.

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