



# Bee pollen and bee bread nutritional potential: Chemical composition and macronutrient digestibility under in vitro gastrointestinal system

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## ABSTRACT

Bee pollen (BP) and bee bread (BB) have been often investigated as potential functional foods. Both bee products are generally characterized by their high nutritional content, with BB being referred as more digestible than BP, however, there is a lack of scientific studies proving this claim. Here, we present a comparative evaluation of the macronutrient digestibility of BP and BB after applying a simulated in vitro gastrointestinal digestive system, together with the evaluation of its nutritional value and chemical composition. The digestibility scores for protein content were calculated on average as 69% and 76% for BP and BB, respectively, whereas digestibility scores for soluble sugars varied depending on bee product and sugar type. The results demonstrated that the nutritional values of both bee products changed depending on their botanical origin but BB is more accessible in the intestinal lumen, especially regarding protein.

## 1. Introduction

Natural products such as bee pollen (BP) and bee bread (BB) are gaining more and more attention from the food industry because they are able to combine a rich macro and micronutrients content (Kieliszek et al., 2018) with therapeutic properties (Märgäoan et al., 2019), satisfying the consumers trend for natural and functional food (Bakour et al., 2019; Kieliszek et al., 2018). This interest lies on the composition of BP and BB, which includes: i) high-level of carbohydrate, protein, lipid, and fibre content (Dranca et al., 2020; Kieliszek et al., 2018), ii) a good source for vitamins C, K and E (Khalifa et al., 2020; Tomás et al., 2017) and minerals such as K, P, Mg or Ca (Mayda et al., 2020), iii) the presence of several bioactive compounds (e.g., phenolic acids, flavonoids and phenylamides) (Aylanc et al., 2021a), and iv) the potential health benefits associated with its consumption (Märgäoan et al., 2019).

BP is prepared by the bees, blending the pollen collected on the flowers with their own secretions, resulting in BP pellets containing thousands of pollen grains (Aylanc et al., 2021a). Honey bees transport those BP pellets to the hive and store them in the cells of the comb; the stored BP is additionally mixed with digestive enzymes secreted by the bees, honey, and organic substances. During this storage period, BP is converted to BB through lactic acid fermentation, under anaerobic

conditions, under the action of bacteria (*Pseudomonas* spp. and *Lactobacillus* spp.) and yeasts (*Saccharomyces* spp.) (Aylanc et al., 2021a). Both bee products serve as an important nutrient source during honey bees growth, from the larvae stage to the newly emerged young bees (Kieliszek et al., 2018). The functional properties and physicochemical composition of both bee products vary depending on the plant origin of the pollen, the nutrient status of the plant, the geographical conditions, the soil, and other factors such as the collection, storage, and degree of processing (Kieliszek et al., 2018). Besides, the transformation process of BP into BB, under fermentation conditions, induces some differences between these bee products. For example, amino acids, some vitamins (e.g. vitamin K), microbial activity, and free acidity levels are higher in BB (Kieliszek et al., 2018). Besides, the digestibility rate of BB is higher than BP due to the fact that the double-layered pollen wall was partially digested by bacterial enzymes (Aylanc et al., 2021a).

Numerous studies have been conducted to show the chemical composition and nutritional properties of both bee products and the positive effects on health, if consumed regularly. Those studies have generally focused on the physicochemical properties (Dranca et al., 2020; Tomás et al., 2017; Yang et al., 2013), antioxidant (Mayda et al., 2020), and antimicrobial activity (Bakour et al., 2019; Kaškonienė et al., 2020) of BP and BB. More recently, additional studies on antiobesity (Al-

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Hatamleh et al., 2020; Othman et al., 2019), anticancer (Khalifa et al., 2020; Wan-Omar et al., 2016), and anti-inflammatory activities (Khalifa et al., 2020; Li et al., 2017) were also explored. Nevertheless, the number of studies giving comparative nutritional composition and digestibility rates of BP and BB is still scarce and limited. Yesiltas et al. (2015) and Aylanc et al. (2021b) previously investigated the fates of phenolic compounds of BP and BB in the gastrointestinal tract. Additionally, Di-Cagno et al. (2019) evaluated the digestibility of proteins in BP fermented by a biotechnological process in the digestive system. These studies, but other reviews evaluating BP and BB, highlighted the unmet need to determine the digestibility degree of macro and micro-nutrient content of these bee products in the human gastrointestinal tract (Khalifa et al., 2020; Kieliszek et al., 2018). Furthermore, the number of studies highlighting the amino acid, fatty acid, and mineral content of these bee products is also very limited (Dranca et al., 2020; Mayda et al., 2020; Yang et al., 2013). The food potential of BP and BB is not limited to its direct consumption, and it is possible to find several studies where these bee products were incorporated as an ingredient: the addition of BP to the production of gluten-free bread (Conte et al., 2018) lead to the improvement of properties such as specific volume, texture, uniformity and an increase in the overall sensory acceptability while its use for biscuits (Krystyan et al., 2015) cause a significant increase on sugar, protein, ash, fibre, as well as the content of polyphenols and antioxidant potential.

Therefore, the aim of this study is to evaluate, for the first time, the changes in soluble sugars and protein content of different BP and BB, throughout an in vitro gastrointestinal digestion.

## 2. Material and methods

### 2.1. Chemicals and reagents

Methanol, sulphuric acid, petroleum ether, toluene, pentanoic acid, diethyl ether, acetonitrile, potassium phosphate, sodium bicarbonate, sodium hydroxide, hydrochloric acid, and calcium chloride dihydrated were purchased from Fisher Scientific (Pittsburgh, PA, USA). Kjeldahl catalyst tablets, potassium chloride, sodium chloride, and ammonium carbonate were purchased from Panreac Applichem (Barcelona, Spain). Magnesium chloride hexahydrated was purchased from Acros Organics (Pittsburgh, PA, USA).

Human salivary  $\alpha$ -amylase (A1031-1KU), porcine pepsin (P6887), porcine pancreatin 4 $\times$  USP specifications (P1750), bile bovine (B3883), fructose, glucose, saccharose, maltulose, maltose, and LAA21-L-amino acids kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI pure system, Houston, TX, USA).

### 2.2. Bee pollen and bee bread samples

Three BP and BB samples were collected in hives located at different apiaries, in the northeast of Portugal by local beekeepers and following the hygienic procedures defined within the HACCP protocols. BP samples were received as fresh pollen pellets, while BB was received within the frames (see (Aylanc et al., 2021b) for more details). BP samples were named BP-A1, BP-A2 and BP-A3, while BB samples were named BB-A1, BB-A2 and BB-A3. Within the laboratory facilities, BP samples were cleaned from debris of wood and dead bee parts, and frozen honeycombs were crushed manually to extract BB. Upon extraction, the samples were freeze-dried (FreeZone 4.5 model 7750031, 117 Labconco, Kansas City, MO, USA) and stored in a desiccator at room temperature, with silica gel. For analytical purposes, the samples were previously subjected to ground, 20 mesh, with a blender (Moulinex A320, Groupe SEB, Mayenne, France).

Based on the palynological analysis (Aylanc et al., 2021b), *Plantago* sp., *Crepis capillaris* (Asteraceae family), *Cytisus striatus* (Fabaceae family) and *Castanea sativa* were found as the dominant pollen types

(relative frequency >45 %) in BP-A1, BP-A2, BP-A3, and BB-A2 samples, respectively. *Castanea sativa* and *Rubus* sp. were classified as accessory pollen level (15–45 %) in the BB-A1, while *Jasione montana*, *Cytisus striatus* and *Castanea sativa* were found at the accessory pollen level in BB-A3.

### 2.3. Free acidity and pH

To measure the pH, 5 mL of distilled water was added to 2 g of sample and evaluated using a pH meter (Hanna Instruments, HI5521-02 Model, Smithfield, RI, USA). The free acidity of the samples was achieved by titration to pH 8.30 with 0.05 mol.mL<sup>-1</sup> NaOH using a potentiometric titrator (Hanna Instruments, HI 902 Model, Smithfield, RI, USA).

### 2.4. Nutritional, chemical composition and energy value

Water, ash, lipid, protein, and dietary fibre were performed according to the procedure proposed by the Association of Official Analytical Chemists (AOAC, 2016). The water content was determined using a moisture analyzer (PMB 53 Moisture Analyzer, Adam Equipment, Milton Keynes, UK) following the AOAC 925.45 Method. Ash content was determined by incineration (Ivymen Furnace N-22L, JP Selecta, Barcelona, Spain) at 550 $\pm$ 5 °C according to the AOAC 923.05 Method. The AOAC 989.05 Method was adopted to determine the total lipid content (with a petroleum ether solvent using a Soxhlet apparatus) of the samples. Total dietary fibre content was performed using the fibre assay kit (Megazyme K-TDFR-200A, Wicklow, Ireland) following the AOAC 985.29 Method.

The protein content (N  $\times$  6.25, for both bee products or blank) for raw and digested samples was estimated according to the macro-Kjeldahl technique using the automatic Kjeldahl steam distillation unit (Pro-Nitro A, JP Selecta, Barcelona, Spain), following the AOAC 920.87 Method.

The estimated (by difference) available carbohydrate (FAO/WHO, 1998) and total energy value (Carocho et al., 2020) of BP and BB were calculated using the following Eqs. (1) and (2), respectively:

$$\text{Carbohydrate}(g/100gdw) = 100 - (g \text{ ash} + g \text{ lipids} + g \text{ proteins} + g \text{ dietary fibers}) \quad (1)$$

$$\text{Energy} \left( \frac{\text{kcal}}{100gdw} \right) = 4 \times (g \text{ proteins} + g \text{ carbohydrates}) + 2 \times (g \text{ dietary fibers}) + 9 \times (g \text{ lipids}) \quad (2)$$

#### 2.4.1. Amino acids profile

Sample preparation for amino acids analysis was carried out according to the method outlined by Lima et al (2019). Briefly, 2.5 g of each sample was added into flasks containing 5 mL of water: acetonitrile (50:50) (v/v) and 1.5 mM *N*-Acetyl-L-Tyrosine (as internal standard) solution. The mixture was vortexed for 5 min and then sonicated for 10 min at room temperature. The samples were immediately centrifuged at 4 °C at 10,000 g for 10 min (Heraeus Multifuge X1R – Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was filtered through a 0.22  $\mu$ m membrane filter and stored at -4 °C.

The chromatographic analysis was performed in a UPLC-DAD-MS/MS chromatographic system (Dionex Ultimate 3000 UPLC instrument from Thermo Scientific, Waltham, MA, USA) equipped with a diode-array detector (DAD) and coupled to Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) with an ESI source. The operating conditions were carried out as previously determined by the authors (Reis Lima et al., 2019). The results were given as mg per 100 g dw.

#### 2.4.2. Fatty acids profile

The samples were extracted with petroleum ether in a Soxhlet apparatus for 4 h. Fatty acids were determined by gas-liquid chromatography with mass spectrometry detection (GC-MS) based on the following *trans*-esterification procedure: fatty acids were methylated with 4.45 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v) and 0.55 mL of internal standard (pentanoic acid; 0.5 mg.mL<sup>-1</sup>), for 12 h in a water bath at 50 °C and 160 g; then, 3 mL of deionized water was added, to obtain phase separation; the fatty acid methyl esters were recovered with 3 mL of diethyl ether by shaking in a vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous to eliminate the water; the sample was recovered in a vial, and filtered before injection with 0.22 µm nylon filter.

The fatty acid profile was analysed with a Perkin Elmer system (GC Clarus® 580 GC module and Clarus® SQ 8 S MS module, Shelton, CT, USA) gas chromatograph, equipped with DB-WAX fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 6.1, Perkin Elmer, Shelton, CT, USA). The oven temperature was programmed as 50 °C for 1 min, 50–200 °C, at 25 °C.min<sup>-1</sup>, and subsequently at 3 °C.min<sup>-1</sup> up to 230 °C, and then held isothermal for additional 23 min. The transfer line temperature was set as 250 °C; ion source temperature, 230 °C; carrier gas, helium, adjusted to a linear velocity of 1 mL.min<sup>-1</sup>; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. Split injection (1:50) was carried out at 250 °C. For each analysis, 1 µL of the sample was injected in the GC. The peaks identification was based either on: (i) the comparison of the obtained spectra with those of the NIST mass spectral library; (ii) confirmed using the linear retention indices calculated from the retention times of an *n*-alkane mixture (C7–C40) (Supelco, Bellefonte, PA, USA) analysed under identical conditions; (iii) with the comparison with published data, and when possible; (iv) with commercial standards. The quantitation was carried out using the relative values directly obtained from peak total ion current (TIC) peak area relative to the internal standard. The results were expressed as g per 100 g of sample dw.

#### 2.4.3. Mineral content analysis

The mineral elements were analysed by atomic absorption spectroscopy (AAS) using a Perkin Elmer PinAAcle 900 T Spectrometer (Waltham, MA, USA). Potassium, sodium, calcium, magnesium, zinc, and iron were analysed by flame ionization AAS, while atomic absorption spectrophotometry in a graphite chamber was applied for manganese, copper, cadmium, and lead.

The sample preparation was carried out through microwave-assisted extraction, using a MARS 5 Digestion Microwave System (CEM Corporation, Matthews, NC, USA). Approximately, 1 g of the sample was weighed into a PTFE digestion tube followed by the addition of 10 mL of concentrated nitric acid. The digestion was performed by setting the ramp temperature program: 15 min until 200 °C with a power of 1200 W, followed by additional 15 min at the same temperature and power conditions. After cooling down, the resulting solutions were diluted up to 50 mL with deionized water and analysed by AAS, with prior treatment for specific elements. For the determination of potassium and sodium, the sample was diluted in a cesium chloride solution (1 g.L<sup>-1</sup>); for calcium and magnesium, the sample was diluted in a lanthanum chloride solution (1 g.L<sup>-1</sup>); for manganese and copper, a magnesium nitrate solution (1 g.L<sup>-1</sup>) was used as a matrix modifier; and iron and zinc were directly analysed. The quantification of elements was achieved by comparing the absorbance responses with a calibration prepared from

commercial standard solutions (Panreac Applichem, Barcelona, Spain) within the following ranges: Ca, Fe and K (0.25 to 5 ppm), Na (0.125 to 2.5 ppm) Mg and Zn (0.0625 to 1.25 ppm), Cu and Pb (25 to 100 ppb), Mn (10 to 40 ppb) and Cd (1.25 to 4 ppb).

#### 2.4.4. Soluble sugars profile

The sugar extraction procedure was done accordingly to the previously described method (Tomás et al., 2017). Approximately, 2 g of sample was mixed with 30 mL of water and stirred under mechanical agitation for 30 min. The mixture was then filtered through Whatman filter paper (grade 40) into a 50 mL volumetric flask. After adding 5 mL of methanol, the final volume was adjusted with water. An aliquot of this solution was filtered through a 0.22 µm membrane into a 2 mL vial, and kept in the freezer until analysis. Mono- and disaccharides, which are known as the most abundant carbohydrate types in BP and BB, were individually analysed by high-performance liquid chromatography coupled with a refractive index detector (HPLC-RI) in an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany) a degasser (Knauer Smartline 5000, Berlin, Germany), an autosampler (Jasco AS-2057, Tokyo, Japan) and a RI detector (Knauer Smartline 2300, Berlin, Germany). Data acquisition and remote control of the HPLC system were done by ClarityChrom software (Knauer, Berlin, Germany). The chromatographic separation was obtained with a column Eurospher 100–5 NH<sub>2</sub> (4.6 × 250 mm, 5 mm, Knauer) kept at 30 °C. The mobile phase was composed by acetonitrile/water, 80:20 (v/v) at a flow rate of 1.5 mL.min<sup>-1</sup>. The results were expressed as g per 100 g of sample dw.

#### 2.5. In vitro gastrointestinal digestion

The static in vitro digestion model was performed according to a previously described method (Minekus et al., 2014). It consists of three sequential phases: oral, gastric, and intestinal digestion. Briefly, 5 g of sample was crushed in a mortar, to simulate the mastication process, and mixed with 3.5 mL of simulated saliva fluid stock solution. This step is followed by the addition of 0.5 mL α-amylase solution of 1500 U.mL<sup>-1</sup>. Then, the pH was adjusted to 7 with 1 mol.L<sup>-1</sup> NaOH, and incubated in a water bath in darkness, at 37 °C, for 2 min, with constant shaking. In the gastric phase, the oral bolus was mixed with 7.5 mL of the simulated gastric fluid stock solution, followed by 1.6 mL pepsin solution of 25,000 U.mL<sup>-1</sup>. Then, the pH was adjusted to 3 with 1 mol.L<sup>-1</sup> HCl and incubated for 2 h under the same conditions as described for the oral phase. In the final stage, the gastric chyme was mixed with 11 mL of simulated intestinal fluid stock solution, 5 mL of pancreatin solution of 800 U.mL<sup>-1</sup>, 2.5 mL of bile (160 mmol.L<sup>-1</sup> in fresh bile), and NaOH (to adjust the pH 7). NaOH and HCl solutions (both 1 mol.L<sup>-1</sup>) were used to set the pH back to 7 and incubated under the same conditions as the previous phases. Finally, the resulting samples, from three digestion phases, were centrifuged for 15 min at 10,000 g at 4 °C, with the soluble and pellet fractions stored at –32 °C, until further analysis. Each BP and BB sample was digested in triplicate and the replicates were mixed for analysis. A blank experiment was made using 5 mL of pure water instead of the 5 g of sample, with no significant results (digestibility below 0.05 % for proteins and sugars). The digestibility (%) was described as the total protein content, or individual soluble sugars released (soluble fractions) in the in vitro digestion system compared, to the amount of total protein content, or individual soluble sugars extracted in the raw samples, and calculated according to Eq. (3):

$$\text{Digestibility}(\%) = \left( \frac{\text{total protein or individual soluble sugars released in the in vitro digestion system}}{\text{total protein content or individual soluble sugars in the raw sample}} \right) \times 100 \quad (3)$$

## 2.6. Data analysis

All analyses were performed in triplicate and the data were denoted as mean  $\pm$  standard deviation (SD). The data statistical analysis was made using GraphPad Prism version 8 (San Diego, CA, USA). One-way analysis of variance followed by Tukey's multiple comparison test was conducted to see whether there is a statistical significance.  $p < 0.05$  was considered as significant.

## 3. Results and discussion

### 3.1. Nutritional composition

Table 1 summarizes the mean and standard deviations of the data obtained from the chemical analysis of BP and BB samples. The average pH value was measured as 4.7 and 4.0 for BP and BB, respectively, which is in agreement with the values found in the literature (Aylanc et al., 2021a). The acidity of BP and BB is mainly due to the presence of organic acids such as oxalic, succinic, malic, citric, tartaric, gluconic, and lactic acids found in these natural bee products (Bakour et al., 2019; Dranca et al., 2020). Its value may vary significantly according to the bee products, for example, it is well known that BB has higher free acidity than BP as a fermentation product (by lactic acid bacteria) (Kieliszek et al., 2018). Our results confirm that the free acidity of BB is higher than BP; mean  $273 \pm 2$  meq.kg<sup>-1</sup> and  $176 \pm 4$  meq.kg<sup>-1</sup>, respectively.

The ash content is a quality parameter that can vary depending on the botanical origin of the pollen; BP samples had an average ash content of  $3.3 \pm 0.3$  g.100 g<sup>-1</sup> while BB samples had a mathematically higher average of  $3.5 \pm 0.2$  g.100 g<sup>-1</sup>, although with no statistical significance. Indeed, the difference is more significant between pollen samples rather than between products, with the highest and lowest value found for *Plantago* sp. (BP-A1) and *Crepis capillaris*. (BP-A2), respectively. Overall, the results for the ash content of BP and BB samples in the current study are in accordance with the data from previously reported works (Dranca et al., 2020; Kostić et al., 2015). Recommendations for BP refer that it should not exceed 6 % (Campos et al., 2015).

The water content of BP and BB is an important factor which may affect the activity of microorganisms, deterioration, and the shelf life of these products and so, for consumption, it is normally subject to previous dehydration. The water content of BP samples varied between  $4.8 \pm 0.3$  g.100 g<sup>-1</sup> (BP-A2) and  $5.7 \pm 0.3$  g.100 g<sup>-1</sup> (BP-A3), while for BB samples were between  $7.4 \pm 0.9$  g.100 g<sup>-1</sup> (BB-A3) and  $7.8 \pm 0.4$  g.100 g<sup>-1</sup> (BB-A2). The water content for BP samples was below the commercially proposed maximum limit (6 g.100 g<sup>-1</sup>) with an average value of  $5.3 \pm 0.5$  g.100 g<sup>-1</sup> (Campos et al., 2015). For BB there is no

specified water content limit established, but compared to some previously reported studies, all BB samples had an acceptable level with an average value of  $7.6 \pm 0.2$  g.100 g<sup>-1</sup> (Ismail et al., 2018; Tomás et al., 2017). While the maximum water content is important to guarantee the microbial safety of the product, water content  $< 3$  % should also be avoided, since it may cause unwanted odour and taste due to discoloration and chemical reactions that may occur (Kieliszek et al., 2018).

Lipids are one of the main macronutrients with a significant contribution to the nutritional value of BP and BB, and vary depending on the botanical origin (Khalifa et al., 2020; Kieliszek et al., 2018). According to the data given in Table 1, the highest lipid content in BP samples was found for BP-A2 with a value of  $9.3 \pm 0.3$  g.100 g<sup>-1</sup>, which can be associated with a rich composition in *Crepis capillaris* pollen, while the lowest lipid content was determined in BP-A3 with a value of  $2.0 \pm 0.1$  g.100 g<sup>-1</sup>. For the BB samples, the lipid content ranged between  $3.0 \pm 0.3$  to  $4.7 \pm 0.4$  g.100 g<sup>-1</sup>, Table 1. There were significant differences between the minimum and maximum values for BP samples, in contrast with the similar values found among BB samples. Nevertheless, all values are within the proposed limits (1–13 g.100 g<sup>-1</sup>) for BP quality standards (Campos et al., 2015) and consistent with the results reported for BP and BB in different countries (Dranca et al., 2020; Kostić et al., 2015; Tomás et al., 2017).

Regarding the total protein content in both bee products, among BP samples, BP-A1 and BP-A2 ( $\approx 22$  g.100 g<sup>-1</sup>) had the highest protein content with a remarkable difference compared to the lower value of BP-A2 ( $15.1 \pm 0.4$  g.100 g<sup>-1</sup>), Table 1. The difference among samples was statistically significant ( $p < 0.05$ ), and could be attributed to the pollen's botanical origin (Bakour et al., 2019; Mayda et al., 2020). On the contrary, the BB-A1 ( $23.3 \pm 0.7$  g.100 g<sup>-1</sup>), BB-A2 ( $22.7 \pm 0.5$  g.100 g<sup>-1</sup>) and BB-A3 ( $21.7 \pm 0.4$  g.100 g<sup>-1</sup>) samples had quite close values to each other, with no statistically significant difference ( $p > 0.05$ ) among samples. The activity of lactic acid bacteria may have contributed to the levelling of protein content in BB (Herbert & Shimanuki, 1978), despite their botanical origin differences. The obtained results are in accordance with those reported by Yang et al. (2013) (14–29 g.100 g<sup>-1</sup>), Tomás et al. (2017) (14–22 g.100 g<sup>-1</sup>), and Kostić et al. (2015) (15–27 g.100 g<sup>-1</sup>).

Dietary fibre, also known as analogous carbohydrates, represents a considerable component of both bee products, averaged  $5.4 \pm 0.6$  g.100 g<sup>-1</sup> for BP samples, while BB averaged  $4.4 \pm 0.5$  g.100 g<sup>-1</sup>. These substances consist mainly of non-starch polysaccharides, such as arabinoxylans,  $\beta$ -glucan, or cellulose and correspond to the edible part of plants hardly digested by human enzymes. Its incorporation into the human diet brings positive health effects such as promoting the growth of beneficial bacteria in the intestine and preventing diseases such as

**Table 1**  
Chemical and nutritional composition of BP and BB.

Sample	pH	Free acidity (mEq.kg <sup>-1</sup> )	Ash (g.100 g <sup>-1</sup> )	Water (g.100 g <sup>-1</sup> )	Lipid (g.100 g <sup>-1</sup> )	Protein (g.100 g <sup>-1</sup> )	Fiber (g.100 g <sup>-1</sup> )	Carbohydrate (g.100 g <sup>-1</sup> )	Energy (kcal.100 g <sup>-1</sup> )
BP-A1	4.7 $\pm 0.0^a$	186.9 $\pm 6.5^b$	3.7 $\pm 0.5^a$	5.4 $\pm 0.3^b$	4.2 $\pm 0.1^b$	21.2 $\pm 0.7^b$	5.4 $\pm 0.3^a$	65.8 $\pm 0.7^a$	394.9 $\pm 1.9^c$
BP-A2	4.7 $\pm 0.0^a$	163.0 $\pm 12.5^b$	2.7 $\pm 0.1^b$	4.8 $\pm 0.3^b$	9.3 $\pm 0.3^a$	15.1 $\pm 0.4^c$	4.9 $\pm 0.7^{ac}$	68.4 $\pm 0.9^a$	425.9 $\pm 2.8^a$
BP-A3	4.7 $\pm 0.0^a$	178.6 $\pm 5.9^b$	3.4 $\pm 0.2^a$	5.7 $\pm 0.3^b$	2.0 $\pm 0.1^d$	21.5 $\pm 1.3^{ab}$	5.8 $\pm 0.8^a$	67.2 $\pm 2.1^a$	384.7 $\pm 1.8^d$
Mean	4.7 $\pm 0.0$	176.2 $\pm 12.1$	3.3 $\pm 0.5$	5.3 $\pm 0.5$	5.2 $\pm 3.7$	19.3 $\pm 3.6$	5.4 $\pm 0.5$	67.1 $\pm 1.3$	401.8 $\pm 21.5$
BB-A1	4.0 $\pm 0.1^b$	291.9 $\pm 18.4^a$	3.5 $\pm 0.1^a$	7.7 $\pm 0.8^a$	3.0 $\pm 0.3^c$	23.3 $\pm 0.7^a$	4.7 $\pm 0.4^{ab}$	64.5 $\pm 0.8^a$	391.5 $\pm 1.7^c$
BB-A2	4.0 $\pm 0.0^b$	257.5 $\pm 14.1^a$	3.6 $\pm 0.3^a$	7.8 $\pm 0.4^a$	3.1 $\pm 0.3^c$	22.7 $\pm 0.5^{ab}$	4.8 $\pm 0.6^{ab}$	64.9 $\pm 0.6^a$	391.4 $\pm 3.7^c$
BB-A3	4.0 $\pm 0.0^b$	270.5 $\pm 14.7^a$	3.3 $\pm 0.1^{ab}$	7.4 $\pm 0.9^a$	4.7 $\pm 0.4^b$	21.7 $\pm 0.4^{ab}$	3.8 $\pm 0.4^{bc}$	67.3 $\pm 1.4^a$	402.8 $\pm 1.1^b$
Mean	4.0 $\pm 0.0$	273.3 $\pm 17.4$	3.5 $\pm 0.2$	7.6 $\pm 0.2$	3.6 $\pm 1.0$	22.6 $\pm 0.8$	4.4 $\pm 0.6$	65.6 $\pm 1.5$	395.2 $\pm 6.5$

Results are expressed on the dry weight of bee pollen (BP) and bee bread (BB), except water content. Each value is the mean $\pm$ SD, n = 3. In each column, different superscript letters means significantly different ( $p < 0.05$ ).

colonic diverticulosis and constipation (Kulathunga & Simsek, 2022). According to the results reported by Bárbara et al. (2015) on 21 different BP samples, the average dietary fibre content was 3.6 %, while several studies on BB showed that the total dietary fibre content may vary from 3 % to 8 % (Bakour et al., 2019), in agreement with the results reported in the current study.

The most abundant macronutrient in both BP and BB are the carbohydrates, with the content average of  $67.1 \pm 1.3 \text{ g} \cdot 100 \text{ g}^{-1}$  for BP and  $65.6 \pm 1.5 \text{ g} \cdot 100 \text{ g}^{-1}$  for BB, with no statistically significant difference ( $p > 0.05$ ) between them. The available carbohydrate content includes simple carbohydrates, such as those present in honey and added by the bees to BB, but also may include starch and polyols. The energetic values for the samples ranged from  $384.7 \pm 1.8$  (BP-A3) to  $425.9 \pm 2.8 \text{ kcal} \cdot 100 \text{ g}^{-1}$  (BP-A2), with no statistical differences between BP and BB. These results are in the same range as those reported by Yang et al. (2013) and Dranca et al. (2020). These findings regarding the chemical composition and nutritional value of BP and BB indicate that the plant origin of pollen could be the main factor determining the nutritional properties of these bee products rather than the difference between BP and BB.

### 3.2. Amino acid composition

Even though the nutritional value of BP and BB is often associated with the high protein content, the true value of the protein contained in

**Table 2**  
Free amino acid composition of the BP and BB samples ( $\text{mg} \cdot 100 \text{ g}^{-1}$ ).

Amino acid	BP-A1	BP-A2	BP-A3	BB-A1	BB-A2	BB-A3
<b>Essential amino acids</b>						
Histidine	1.42 $\pm 0.1^b$	3.96 $\pm 0.3^a$	0.41 $\pm 0.3^c$	0.44 $\pm 0.0^c$	0.31 $\pm 0.1^d$	0.27 $\pm 0.1^d$
Lysine	1.60 $\pm 0.8^b$	1.16 $\pm 0.2^c$	2.59 $\pm 0.2^a$	0.68 $\pm 0.6^d$	0.52 $\pm 0.1^e$	0.39 $\pm 0.0^f$
Threonine	0.12 $\pm 0.0^b$	0.08 $\pm 0.0^{cd}$	0.18 $\pm 0.0^a$	0.11 $\pm 0.02^b$	0.07 $\pm 0.0^c$	0.10 $\pm 0.0^{bd}$
Valine	22.33 $\pm 2.9^b$	76.07 $\pm 3.2^a$	2.11 $\pm 0.3^f$	17.36 $\pm 9.2^c$	5.51 $\pm 0.8^e$	6.70 $\pm 0.9^d$
Methionine	38.55 $\pm 3.1^a$	20.58 $\pm 0.9^c$	10.63 $\pm 1.1^d$	38.28 $\pm 4.6^a$	23.84 $\pm 1.5^b$	19.04 $\pm 1.2^c$
Leucine – Isoleucine	0.93 $\pm 0.1^c$	1.56 $\pm 0.0^b$	2.03 $\pm 0.2^a$	0.66 $\pm 0.2^d$	0.45 $\pm 0.0^e$	0.34 $\pm 0.0^f$
Phenylalanine	9.74 $\pm 0.7^b$	2.59 $\pm 0.2^f$	36.68 $\pm 2.6^a$	6.18 $\pm 1.4^c$	4.42 $\pm 0.4^e$	5.07 $\pm 1.4^d$
Tryptophan	0.99 $\pm 0.2^c$	1.14 $\pm 0.2^b$	1.88 $\pm 0.2^a$	ND	ND	ND
<b>Total</b>	<b>75.68</b>	<b>107.14</b>	<b>56.51</b>	<b>63.71</b>	<b>35.12</b>	<b>31.91</b>
<b>Non-essential amino acids</b>						
Arginine	0.12 $\pm 0.1^b$	0.12 $\pm 0.0^b$	0.47 $\pm 0.0^a$	0.07 $\pm 0.0^c$	0.05 $\pm 0.0^c$	0.05 $\pm 0.0^c$
Cysteine	ND	ND	ND	$\pm 0.0^a$	$\pm 0.0^b$	$\pm 0.0^c$
Cystine	1.47 $\pm 0.1^e$	1.25 $\pm 0.1^f$	5.94 $\pm 1.0^a$	2.53 $\pm 0.3^b$	2.11 $\pm 0.2^c$	2.02 $\pm 0.4^d$
Asparagine	3.62 $\pm 0.3^b$	3.66 $\pm 0.4^b$	11.21 $\pm 1.7^a$	1.82 $\pm 1.6^c$	0.82 $\pm 0.2^d$	0.65 $\pm 0.1^d$
Glutamic acid	0.14 $\pm 0.0^b$	0.09 $\pm 0.0^c$	0.44 $\pm 0.0^a$	0.09 $\pm 0.0^c$	0.08 $\pm 0.0^c$	0.03 $\pm 0.0^d$
Serine	7.46 $\pm 0.8^a$	4.14 $\pm 1.0^d$	6.29 $\pm 0.4^b$	5.35 $\pm 0.9^c$	2.94 $\pm 0.1^e$	2.53 $\pm 0.0^f$
Alanine	53.24 $\pm 1.9^b$	63.83 $\pm 3.2^a$	51.39 $\pm 2.0^b$	24.26 $\pm 1.8^c$	15.29 $\pm 1.6^d$	10.90 $\pm 1.2^e$
Aspartic acid	0.59 $\pm 0.1^b$	0.51 $\pm 0.1^c$	0.90 $\pm 0.2^a$	0.31 $\pm 0.0^d$	0.16 $\pm 0.0^e$	0.22 $\pm 0.1^e$
Proline	0.72 $\pm 0.1^c$	0.95 $\pm 0.1^b$	2.07 $\pm 0.1^a$	0.57 $\pm 0.4^d$	0.29 $\pm 0.0^e$	0.18 $\pm 0.0^f$
Tyrosine	1.98 $\pm 0.3^b$	1.34 $\pm 0.2^d$	1.63 $\pm 0.2^c$	3.25 $\pm 0.7^a$	1.68 $\pm 0.1^c$	2.00 $\pm 0.1^b$
<b>Total</b>	<b>69.34</b>	<b>75.89</b>	<b>80.34</b>	<b>41.70</b>	<b>24.34</b>	<b>19.10</b>

Results are expressed on the dry weight of bee pollen (BP) and bee bread (BB). Each value is the mean  $\pm$  SD,  $n = 3$ . In each row, different superscript letters means significantly different ( $p < 0.05$ ). ND = not detected.

these bee products is determined by the digestibility degree, as well as the presence and quantity of essential amino acids. Here we evaluate the free amino acids found in BP and BB.

The UPLC-DAD-MS/MS technique allowed us to identify and quantify a total of 18 free amino acids in BP and BB, Table 2. While all essential amino acids were detected in BP samples: histidine, lysine, threonine, valine, methionine, leucine, isoleucine, phenylalanine, and tryptophan, the BB samples exhibit all but the latter. The absence or low concentration of tryptophan in BB was also mentioned in a previous study (Kieliszek et al., 2018) and may be linked with the energy conversion required by bacteria during the fermentation process of BB. In general, BP samples had higher concentrations of essential amino acids compared to BB, and on average, valine ( $33.5 \text{ mg} \cdot 100 \text{ g}^{-1}$ ) represented the highest value, followed by methionine ( $23.3 \text{ mg} \cdot 100 \text{ g}^{-1}$ ), phenylalanine ( $16.3 \text{ mg} \cdot 100 \text{ g}^{-1}$ ) and histidine ( $1.9 \text{ mg} \cdot 100 \text{ g}^{-1}$ ), respectively. For BB, methionine was found to be the highest level in all samples: BB-A1 ( $38.28 \pm 4.6 \text{ mg} \cdot 100 \text{ g}^{-1}$ ), BB-A2 ( $23.84 \pm 1.5 \text{ mg} \cdot 100 \text{ g}^{-1}$ ), and BB-A3 ( $19.04 \pm 1.2 \text{ mg} \cdot 100 \text{ g}^{-1}$ ).

BP and BB exhibited also a rich profile in terms of non-essential amino acids. All non-essential amino acids were detected in BP samples, except for glutamine, cysteine, and glycine. On the other hand, BB samples lacked only glutamine and glycine, which was also reported for BP and BB samples collected from Turkey (Bayram et al., 2021), and China (Yang et al., 2013). Like for the essential amino acids, the total amount of non-essential amino acids was higher for BP samples, particularly due to the higher amounts observed for asparagine, serine, and alanine, which contribute mostly to the overall amount. Even though for BB the amount of cysteine or tyrosine was higher than in BP, their contribution is significantly less to the total amount. Unlike other studies that reported higher levels of amino acids in BB as a result of the fermentation of BP (Bayram et al., 2021; Kieliszek et al., 2018), this was not observed for our samples. This behaviour may be explained by the differences in the botanical origin of the samples that affect their protein content (Khalifa et al., 2020), but also by the fact that some microorganisms can consume amino acids in the ambient as carbon and energy sources for their metabolic activities (Kieliszek et al., 2018).

### 3.3. Fatty acid composition

The similarities between the fatty acid profile of BB samples were more evident than between BP samples, which may reflect the closeness between the botanical origin of BB, where *Castanea sativa* and *Rubus* sp. pollen are common, accounting for at least 35 % of the total, while BP samples have a clear difference in the botanical origin (Aylanc et al., 2021b). A total of 12 different fatty acids were detected in the tested samples, Table 3, nine of which were saturated and three unsaturated. The main fatty acids, for both BP and BB, were linolenic acid, with a mean value of  $1.01 \text{ g} \cdot 100 \text{ g}^{-1}$ , palmitic acid,  $0.92 \text{ g} \cdot 100 \text{ g}^{-1}$ , and oleic acid,  $0.47 \text{ g} \cdot 100 \text{ g}^{-1}$ . Caprylic acid and 14-methylpalmitic acid were detected only in BP-A1 and BP-A2 samples, in which *Plantago* sp. and *Crepis capillaris* were dominant, respectively, while heptadecanoic acid and lignoceric acid were observed only in BB samples, with average values of  $0.13 \text{ g} \cdot 100 \text{ g}^{-1}$  and  $0.41 \text{ g} \cdot 100 \text{ g}^{-1}$ , respectively. Current studies on the fatty acid content of these bee products demonstrate that they are good sources of fatty acids. Yang et al. (2013) identified fatty acids such as linolenic acid and palmitic acid in different BP samples ( $n = 12$ ), with high concentrations. In another study, Mayda et al. (2020) reported that fatty acids such as lauric acid, palmitic acid, oleic acid and linolenic acid were the major fatty acids found in both BP and BB. In line with the results reported by the previous works, our results showed that the fatty acid composition of both bee products may differ depending on the botanical origin of the pollen or the processing and storage conditions. Nevertheless, it must be highlighted that the lipid extractable fraction of bee pollen cannot be evaluated only based on the fatty acid composition, since the non-polar fraction may include many other compounds such as phospholipids, di and triglycerides, sterols and

**Table 3**  
Fatty acid composition of the BP and BB samples (g.100 g<sup>-1</sup>).

Fatty acid		BP-A1	BP-A2	BP-A3	BB-A1	BB-A2	BB-A3
Caprylic acid	C8:0	0.05±0.0 <sup>b</sup>	0.09±0.0 <sup>a</sup>	ND	ND	ND	ND
Lauric acid	C12:0	0.08±0.0 <sup>b</sup>	0.10±0.0 <sup>a</sup>	ND	0.06±0.0 <sup>c</sup>	ND	ND
Pentadecanoic acid	C15:0	ND	ND	ND	0.06±0.0 <sup>a</sup>	ND	ND
Azelaic acid	–	ND	0.07±0.0 <sup>b</sup>	ND	ND	ND	0.09±0.1 <sup>a</sup>
Palmitic acid	C16:0	0.91±0.0 <sup>c</sup>	0.32±0.0 <sup>d</sup>	1.04±0.2 <sup>bc</sup>	1.14±0.0 <sup>a</sup>	1.01±0.2 <sup>b</sup>	1.10±0.3 <sup>ac</sup>
Heptadecanoic acid	C17:0	ND	ND	ND	0.13±0.0 <sup>b</sup>	0.11±0.0 <sup>b</sup>	0.16±0.0 <sup>a</sup>
14-methylpalmitic acid	–	0.13±0.0 <sup>a</sup>	0.07±0.0 <sup>b</sup>	ND	ND	ND	ND
Oleic acid	C18:1	0.52±0.1 <sup>c</sup>	0.12±0.0 <sup>e</sup>	0.74±0.2 <sup>a</sup>	0.43±0.0 <sup>d</sup>	0.55±0.1 <sup>b</sup>	ND
Linoleic acid	C18:2	0.60±0.0 <sup>b</sup>	ND	0.64±0.2 <sup>a</sup>	ND	ND	ND
Linolenic acid	C18:3	1.32±0.1 <sup>a</sup>	ND	1.09±0.2 <sup>b</sup>	0.68±0.3 <sup>d</sup>	0.96±0.0 <sup>c</sup>	ND
Behenic acid	C22:0	ND	0.07±0.0 <sup>b</sup>	ND	0.32±0.0 <sup>a</sup>	ND	ND
Lignoceric acid	C24:0	ND	ND	ND	0.43±0.0 <sup>b</sup>	0.35±0.1 <sup>c</sup>	0.47±0.0 <sup>a</sup>

Results are expressed on the dry weight of bee pollen (BP) and bee bread (BB). Each value is the mean ± SD, n = 3. In each row, different superscript letters means significantly different (p < 0.05). ND = not detected.

**Table 4**  
Minerals (mg.100 g<sup>-1</sup>) and soluble sugar (g.100 g<sup>-1</sup>) composition of BP and BB.

Element	BP-A1	BP-A2	BP-A3	BB-A1	BB-A2	BB-A3
K	514.8 ±3.6 <sup>d</sup>	356.4 ±9.5 <sup>f</sup>	531.7 ±4.4 <sup>c</sup>	497.2 ±2.5 <sup>e</sup>	564.2 ±0.6 <sup>b</sup>	691.0 ±1.1 <sup>a</sup>
Na	5.7 ±0.4 <sup>f</sup>	12.3 ±0.5 <sup>b</sup>	8.8 ±0.2 <sup>d</sup>	19.5 ±0.1 <sup>a</sup>	7.1 ±0.8 <sup>e</sup>	10.6 ±0.1 <sup>c</sup>
Ca	31.9 ±2.2 <sup>d</sup>	40.4 ±2.8 <sup>c</sup>	88.4 ±1.2 <sup>a</sup>	55.7 ±6.4 <sup>b</sup>	59.5 ±0.6 <sup>b</sup>	85.7 ±2.2 <sup>a</sup>
Mg	69.2 ±0.2 <sup>dc</sup>	30.1 ±0.9 <sup>e</sup>	67.8 ±2.7 <sup>d</sup>	72.2 ±0.4 <sup>c</sup>	76.8 ±2.3 <sup>b</sup>	80.5 ±1.4 <sup>a</sup>
Fe	66.6 ±6.0 <sup>a</sup>	37.9 ±2.8 <sup>b</sup>	20.2 ±1.2 <sup>c</sup>	40.4 ±1.3 <sup>b</sup>	16.7 ±2.1 <sup>d</sup>	6.4 ±0.2 <sup>e</sup>
Mn	8.0 ±0.6 <sup>a</sup>	5.5 ±1.3 <sup>b</sup>	9.1 ±0.2 <sup>a</sup>	7.9 ±1.3 <sup>a</sup>	7.6 ±1.4 <sup>a</sup>	9.0 ±0.2 <sup>a</sup>
Cu	0.9 ±0.0 <sup>c</sup>	0.9 ±0.0 <sup>c</sup>	0.8 ±0.0 <sup>c</sup>	1.2 ±0.1 <sup>ba</sup>	1.0 ±0.0 <sup>bc</sup>	1.3 ±0.0 <sup>a</sup>
Cd	0.004 ±0.0 <sup>c</sup>	< 0.003*	0.006 ±0.0 <sup>a</sup>	< 0.003*	0.003 ±0.0 <sup>d</sup>	0.005 ±0.0 <sup>b</sup>
Pb	< 0.04* ±0.0 <sup>c</sup>	< 0.04* ±0.0 <sup>c</sup>	< 0.04* ±0.0 <sup>a</sup>	< 0.04* ±0.0 <sup>a</sup>	< 0.04* ±0.0 <sup>d</sup>	< 0.04* ±0.0 <sup>b</sup>
Soluble sugar						
Fructose	17.9 ±0.2 <sup>c</sup>	22.0 ±0.7 <sup>b</sup>	22.7 ±0.4 <sup>ab</sup>	23.5 ±0.4 <sup>a</sup>	21.9 ±0.3 <sup>b</sup>	22.6 ±0.6 <sup>b</sup>
Glucose	13.6 ±0.1 <sup>c</sup>	16.2 ±0.1 <sup>b</sup>	17.5 ±0.1 <sup>a</sup>	12.4 ±0.3 <sup>d</sup>	12.2 ±0.2 <sup>de</sup>	12.0 ±0.4 <sup>e</sup>
Sucrose	ND	3.3 ±0.2 <sup>b</sup>	11.2 ±0.1 <sup>a</sup>	ND	ND	ND
Maltulose	2.3 ±0.5 <sup>b</sup>	0.6 ±0.0 <sup>c</sup>	ND	2.3 ±0.2 <sup>b</sup>	2.2 ±0.0 <sup>b</sup>	3.5 ±0.3 <sup>a</sup>
Maltose	1.1 ±0.4 <sup>c</sup>	0.6 ±0.0 <sup>d</sup>	ND	1.7 ±0.1 <sup>b</sup>	1.2 ±0.1 <sup>c</sup>	2.9 ±0.2 <sup>a</sup>
Total	34.9	42.7	51.4	39.9	37.5	41.0

Results are expressed on the dry weight of bee pollen (BP) and bee bread (BB). Each value is the mean ± SD, n = 3. In each row, different superscript letters means significantly different (p < 0.05). \* Below the quantification limit.

carotenoids (Conte et al., 2017).

### 3.4. Mineral composition

Table 4 shows the mineral content for BP and BB. The main element in both bee products was potassium: an average of 467.3±5.8 mg.100 g<sup>-1</sup>, for BP, and 584.1±1.4 mg.100 g<sup>-1</sup>, for BB. Magnesium, calcium, and iron were the next minerals, present in both bee products, but at a concentration level 10 times less than potassium. Compared to BP samples, magnesium and calcium were found at a higher level in BB, while the iron was at a lower concentration. Sodium, manganese, and copper were the elements found also in all samples but generally at concentrations lower than 10 mg.100 g<sup>-1</sup>. Likewise, the study conducted by Liolios et al. (2019) on the mineral content of 90 BP samples harvested from different regions of Greece, confirmed the dominance of

potassium, calcium, and magnesium elements. Moreover, Yang et al. (2013), Bakour et al. (2019), and Mayda et al. (2020) findings were similar to our results for manganese, potassium, calcium, and magnesium elements, with some slightly higher/lower levels in the sodium, iron, and copper concentrations. These differences could be attributed to the fact that the individual minerals strongly depend on the botanical origin of the plant, the soil, and geographical conditions (Liolios et al., 2019; Mayda et al., 2020).

On the other hand, the elemental analysis also revealed the presence of toxic elements, in both bee products, nevertheless at very low levels. The highest cadmium concentration was detected for BP-A3 and BB-A3, with 0.006±0.0 mg.100 g<sup>-1</sup> and 0.005±0.0 mg.100 g<sup>-1</sup>, respectively. For lead, the concentration was lower than the quantification limit. The presence of these elements in the tested samples was clearly at a low level compared to the results obtained by Yang et al. (2013) and Bakour et al. (2019).

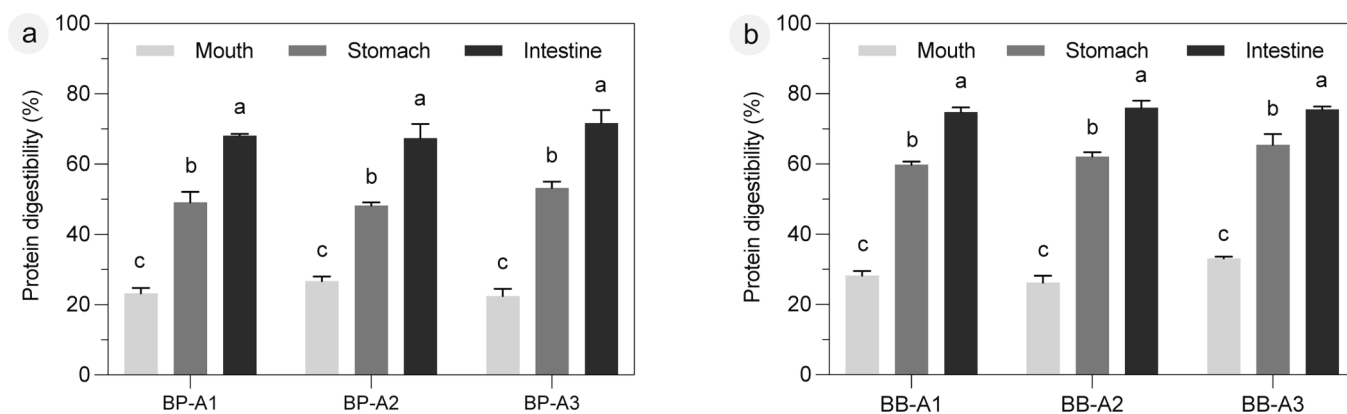
### 3.5. Soluble sugar profile

The soluble sugar profiles extracted from raw BP and BB are given in Table 4. Accordingly, fructose and glucose were the main sugars detected in all tested samples, with values ranging from 17.9±0.2 to 23.5±0.4 g.100 g<sup>-1</sup> and from 12.0±0.4 to 17.5±0.1 g.100 g<sup>-1</sup>, respectively. The average fructose/glucose (1.86) ratio appears to be greater in BB samples compared to BP (1.32). BP samples had a sucrose level ranging from 3.3±0.2 to 11.2±0.1 g.100 g<sup>-1</sup>, whereas no sucrose was detected in the BB samples. This absence could be explained by the break-down of sucrose into glucose and fructose molecules and the consumption of glucose by the metabolic processes of lactic acid bacteria, which is most likely present in BB (Bakour et al., 2019; Kaškonienė et al., 2020). Besides, maltose and maltulose, other disaccharides present in BP and BB, were found in lower amounts in the raw samples comparing to the other sugars. Previous studies reported a similar sugar profile in BP and BB, highlighting the higher composition of fructose and glucose, followed by other sugars such as sucrose and maltose (Bakour et al., 2019; Mărgăoan et al., 2012).

### 3.6. Protein digestibility

Proteins are the second major component of BP and BB. Even though, both products are characterized by an high protein content (10–40 % dw) (Aylanc et al., 2021a; Campos et al., 2015), which is known not to be completely digested in the digestive tract because of the multi-layered pollen wall (Kaškonienė et al., 2020; Kieliszek et al., 2018). Thus, it is important to reveal the true capacity of the protein to be absorbed in the intestinal lumen at the in vitro level and to understand its digestibility rate. Taking into consideration this, we evaluated BP and BB in terms of protein digestibility rates.

Fig. 1 shows the protein digestibility rates of BP and BB obtained



**Fig. 1.** Digestibility rates of total protein in (a) bee pollen (BP) and (b) bee bread (BB) at each digestion phase. Within each group (BP and BB samples), different letters mean significantly different ( $p < 0.05$ ).

after different phases (oral, gastric, and intestinal) of a simulated gastrointestinal digestion. The percentage of digestible protein in BP samples after the oral phase had an average value of 24 %, reaching 69 % at the end of digestion. Although there were some differences in digestibility among BP samples in each phase, this was not statistically significant, in opposition to the significant differences found when comparing between oral, gastric, and intestinal phases. One of the possible reasons for the continuous increase in protein digestible after each digestion phase may be the sustained release of the pollen grains' inner content, which acts as a natural porous capsule under simulated physiological conditions. In a previous study, Benavides-Guevara et al. (2017) found that pollen pre-treatment with the protease enzyme increased the digestibility of the total protein in BP from 62 % to 84 %. In other studies with different food matrices, it has been reported that the rate of digestible protein exceed 80 % (whey protein:casein) (Nguyen et al., 2015). Partial low digestibility of BP and BB compared to other food matrices may be related to the resistance of strong pollen walls called exine (sporopollenin-based) and intine (cellulose/pectin-based) to digestive enzymes and conditions.

On the other hand, the average protein digestibility rates of BB samples were found to be 29 %, 63 %, and 76 %, respectively, for the oral, gastric, and intestinal phases. The protein digestibility trend of the samples in the digestive tract was similar to BP, with statistical significant differences ( $p < 0.05$ ) between phases. Besides, it was possible to observe that, for each phase, BB was a more digestible product compared to BP. This behaviour sets on the fact that BB, as a fermented product, incurs both chemical and physical changes as a consequence of bacterial activity throughout the fermentation (Aylanc et al., 2021a), increasing the bioaccessibility of the pollen grain content. Di-Cagno et al. (2019) applied a simulated gastrointestinal model to test the protein digestibility of fresh BP and artificial fermented BP (a product obtained by a biotechnological process), and according to the reported findings, the post-digestion protein digestibility was 62 % for fresh BP and 75 % for fermented BB, which is in accordance with the presently reported values.

### 3.7. Soluble sugar digestibility

Fig. 2 shows the evolution of the soluble sugar content of samples throughout the digestion process. Enzymatic hydrolysis occurred at different rates depending on the type of soluble sugars and the digestion phase. Fructose showed a continuously increasing digestibility rate after each digestion phase in all samples, except for BP-A3 and BB-A3. This difference in BP-A3 and BB-A3 was probably related to the slowing down of the released fructose from the samples or the hydrolysis of the existing fructose in the ambient. The glucose, another sugar present in

large quantities in both bee products, following fructose, exhibited an increasing trend of digestibility. At the end of digestion, fructose reached the highest digestibility rate of 19 % (BP-A1, Fig. 2a) and 21 % (BB-A2, Fig. 2e) among BP and BB samples, respectively. On the other hand, the maximum digestibility rate of glucose was 37 % (BP-A1, Fig. 2a) and 45 % (BB-A3, Fig. 2f) for BP and BB, respectively. These findings demonstrated that the digestible rate of glucose was higher than fructose. This may be due to their stability at different pH or synergies with other compounds depending on the chemical composition (Shallenberger & Mattick, 1983). Besides, BB was more digestible for both sugars than BP, with statistical significance (typically  $p < 0.05$ ). Another important point is that the presence of a high digestible glucose level does not necessarily mean that there is a high rate of free glucose in the samples, since additional released of glucose could also occur as a result of the breaking of the glycoside bonds in molecules such as sucrose ( $\alpha$ -1,2 glycoside bonds) or maltose ( $\alpha$ -1,4 glycoside bonds) due to the activity of the digestive enzymes. Ferreira-Lazarte et al. (2017) investigated the degree of hydrolysis of dietary sugars in the oral, gastric, and intestinal phases by applying an in vitro digestion model and concluded that the digestibility of carbohydrates increased after each digestion phase. Similar data was seen in the results found by Mishra & Monro (2012).

Sucrose was observed only in the samples BP-A2 and BP-A3, Fig. 2b and 2c, respectively. At the end of the gastric phase, sucrose in the BP-A3 was completely hydrolysed to its monomers namely glucose and fructose, but sucrose released from the BP-A2 continued and was hydrolysed at the end of digestion. The digestion of sucrose in the gastric phase can be attributed to the acidic ambient of the stomach breaking the glycoside bonds between the monomer units in the structure of the molecule, rather than digestive enzymes (Shallenberger & Mattick, 1983). Also, the hydrolysis of sucrose into its monomers may have contributed significantly to the continued increase in the digestibility of fructose and glucose.

Regarding maltose and maltulose, two disaccharides detected in BP and BB samples, after the gastric phase, the rate of digestible maltose was 46 % for BP-A1 and showed an average value of 29 % for BB samples. The highest digestibility score of maltose was 82 % in the BP-A2 at the end of the digestion, whereas that score was 83 % in the BB-A1, Fig. 2b and d, respectively. Maltulose showed a slower and low digestibility rate: in BP samples was completely vanished after the intestinal phase, whereas the highest digestibility rate, among BB samples, was found to be 19 % (BB-A2, Fig. 2e) after the gastric phase and 6 % at the end of digestion. These results are in accordance with previous data revealed by Ferreira-Lazarte et al. (2017), who reported that maltose is more rapidly and highly digestible than other carbohydrates following an in vitro digestion model.

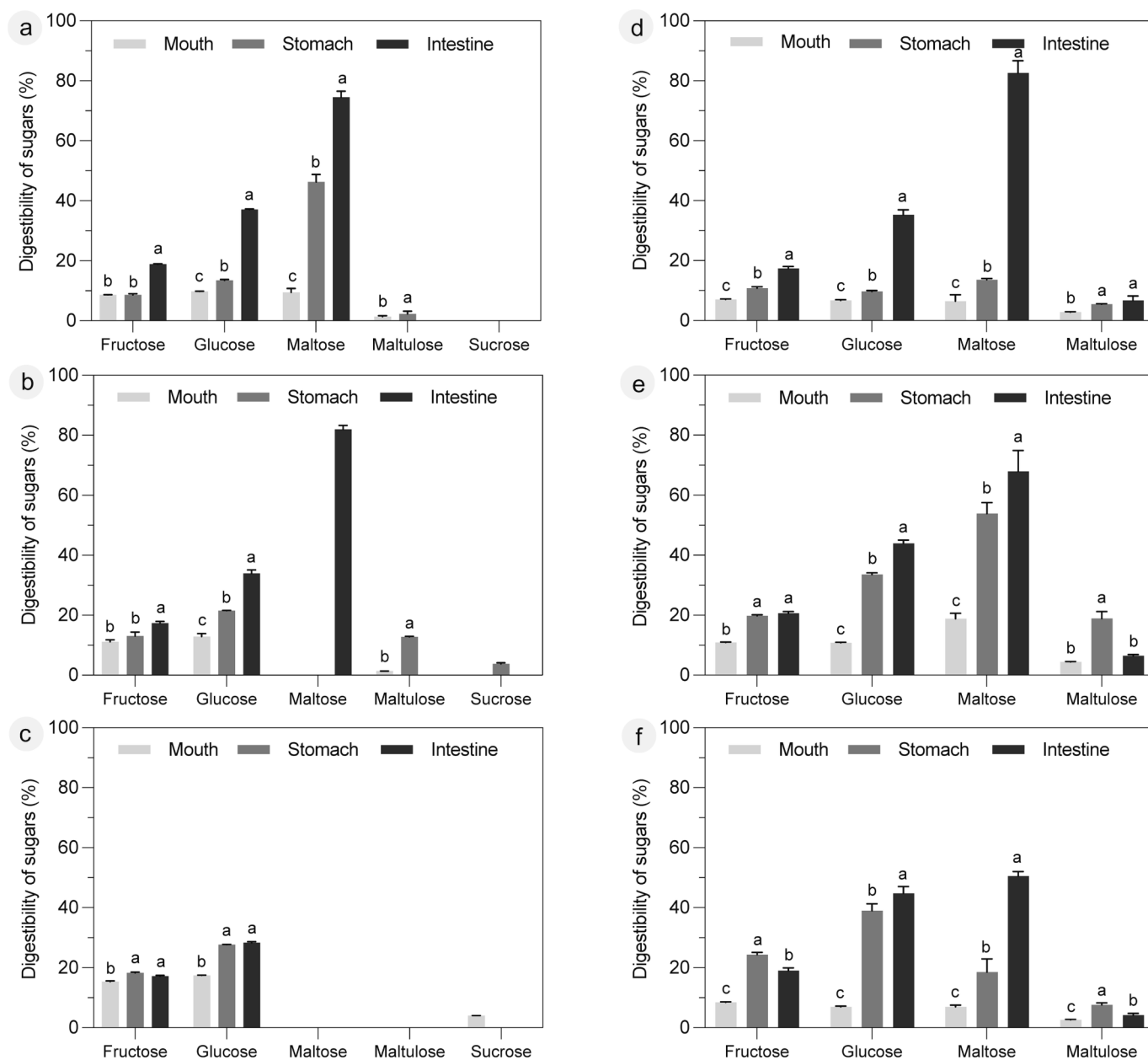


Fig. 2. Digestibility rates of individual soluble sugars in bee pollen (BP) and BB samples at each digestion stage: (a) BP-A1, (b) BP-A2 and (c) BP-A3, (d) BB-A1, (e) BB-A2 and (f) BB-A3. Within each group (soluble sugars), different letters mean significantly different ( $p < 0.05$ ).

#### 4. Conclusion

Here, we evaluated the chemical composition and nutritional value of BP and BB samples with different botanical origins, and tried to understand the digestibility degrees of protein and individual soluble sugars of these bee products under a simulated *in vitro* human gastrointestinal digestion model. Although the nutritional composition of both bee products vary somewhat depending on the botanical origin of the pollen, they proved to be good sources of proteins (15.1–21.5 g $\cdot$ 100 g $^{-1}$ ), fibre (3.8–5.8 g $\cdot$ 100 g $^{-1}$ ), carbohydrates (64.5–68.4 g $\cdot$ 100 g $^{-1}$ ), different fatty acids, amino acids, including 8 essential and 10 non-essential, as well as minerals such as potassium, calcium, magnesium and iron. In regard to the macronutrient digestibility, BB exhibited a more digestible profile in protein than BP, with an average of 76 %, together with a higher ratio in digestibility of individual soluble sugars, for most cases.

Altogether, the present study's findings can contribute to baseline

scientific knowledge for evaluating the nutritional value and digestibility of BP and BB and serve as a reference for establishing quality standards for them. However, to achieve a global overview of the potential of these bee products as functional foods or functional food ingredients it will be also important to evaluate the impact of the digestion on small molecules such as the amino acids and lipids.

#### CRediT authorship contribution statement

**Volkan Aylanc:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Soraia I. Falcão:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Miguel Vilas-Boas:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Miguel Vilas-Boas reports financial support was provided by Foundation for Science and Technology. Volkan Aylanc reports financial support was provided by Foundation for Science and Technology. Soraia I. Falcão reports financial support was provided by Foundation for Science and Technology. Miguel Vilas-Boas reports financial support was provided by Programa Apícola Nacional 2020–2022 (National Beekeeping Program). Miguel Vilas-Boas reports financial support was provided by European Regional Development Fund (ERDF).

## Data availability

Data will be made available on request.

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