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Quality of bee pollen submitted to drying, pasteurization, and high-pressure processing – A comparative approach

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ABSTRACT

Bee Pollen is a valuable and useful natural food product that can be used for different purposes, among which medical ones. This matrix is deemed a superfood because of its chemical composition, which is rich in nutrients and possesses significant bioactivities, including antioxidant and microbiological properties. Nevertheless, the storage conditions and processing methods must be optimized to maintain their properties and maximise their application. This work investigates the best bee pollen conservation process and its impact on individual constituents. Monofloral bee pollen was analysed for 30 and 60 days after three different storage processes (drying, pasteurization, and high-pressure pasteurization). The results showed a decrease mainly in fatty acid and amino acid content for the dried samples. The best results were obtained with high-pressure pasteurization, maintaining the proteins, amino acids and lipids characteristics of pollen and the lowest microbial contamination.

1. Introduction

Bee pollen is a natural food product collected by honeybees from flowering plants. It is a mixture of flower pollen, nectar, enzymes, honey, wax, and bee secretions. It is considered a superfood due to its rich nutritional composition and has several beneficial properties, including antioxidant, anti-inflammatory, antibacterial, anti-cancerogenic and anti-inflammatory (Estevinho et al., 2012; Khalifa et al., 2021; Komosinska-Vassev et al., 2015). This matrix has essential nutrients such as proteins, lipids, carbohydrates, fibers, minerals and vitamins. The BP contains the most important amino and fatty acids in the human diet (Thakur & Nanda, 2020). Concerning the BP nutrients, carbohydrates contribute to around 67% of the total pollen dry weight (Li et al., 2018). Moreover, its chemical composition depends on some factors, notably the botanical and geographical origins (Gonçalves, Estevinho, Pereira, Sousa, & Anjos, 2018).

The polyphenolic compounds, mainly flavonoids and derivatives of phenolic acids, are responsible for various biological activities as, for instance, the antioxidant capacity (Carpes, 2009; Feas, Vazquez-Tato, Estevinho, Seijas, & Iglesias, 2012; Harif Fadzilah, Jaapar, Jajuli, & Wan Omar, 2017).

Most of the products available in the market are dried at no more than 40 °C, ensuring long-term stability and safety by reducing microbial load and hazard. However, this processing method may potentially affect bee pollen nutritional value, sensory quality, vitamins stability and polyphenols content (Anjos, Paula, Delgado, & Estevinho, 2019; Estevinho, Dias, & Anjos, 2019; Mauriello, De Prisco, Di Prisco, La Storia, & Caprio, 2017). Alternatively, freezing or lyophilized BP may be used to preserve its sensorial and nutritional value.

Applying High-Pressure Pasteurization (HPP) technology involves subjecting food products to pressures ranging from 100 to 600 MPa. By doing so, this technology effectively eliminates microorganisms, making

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the food product just as safe as thermally pasteurized food, and extending its shelf lifetime (Amsasekar, Mor, Kishore, Singh, & Sid, 2022) more than other methods, inactivating oxidative enzymes and food pathogens yet preserving the natural characteristics of food products (Tsai, Cheng, Chen, & Wang, 2018).

The application of pressure in HPP may be performed uniformly throughout the food product, not influenced by the size, shape or food composition (Ramaswamy, Zaman, & Smith, 2008). This technique has been used in several food products to increase storage time and improve quality, reducing the processing temperature (Carrapiso, Martillanes, Delgado-Adámez, & Ramírez, 2022; Lou et al., 2022; Yagiz et al., 2009). HPP is a more natural and environmentally friendly technique compared to traditional pasteurization, given the high temperature used in the process.

In this context, this study aimed to compare BP quality submitted to three different conservation process methods (oven dry, pasteurization and HPP) during 60 days of storage. As far as we know, this is the first work that studied the performance of HPP applied to bee pollen.

2. Material and methods

2.1. Samples and experimental design

The BP samples used in this work were collected directly from three beekeepers in the North Region of Portugal (3 Kg of BP, harvested in the "Terras do Barroso" region [composite sample]) and stored in the dark at room temperature (±15 °C) until further processing (that occurred in up to 24 h). After homogenization, the fresh composite sample (3 Kg) was divided into three sub-samples (1 Kg) that were submitted to three different processing protocols: dehydrated bee pollen - D; pasteurized dehydrated bee pollen - P; HPP dehydrated bee pollen - HPP). The drying process (D) was performed at room temperature in an electric oven (ESA 1368, Sercon) with forced air circulation at 42 °C until a moisture value of around 6–8% was achieved. The pasteurization process (P) was carried out at ambient pressure (0.1 MPa) and temperature at 70 °C for 15 min, using a thermostatic water bath (Julabo SW23). The samples were fully immersed into the water bath and then immediately placed in ice-cooled water (4 °C) to cool down for 10 min and then stored at −20 °C. Concerning the HPP treatment, the samples were moistened with peptone water as a neutral reference medium (1.5 g/mL) - bee-pollen paste. Then, paste samples were poured into polyethylene bags, heat-sealed (MULTIVAC Thermosealer, Switzerland), and placed in hermetically sealed sterile vials. The HPP treatment was performed in an industrial hydrostatic pressurisation unit (Wave 6000/135. NC Hyperbaric, Burgos, Spain). The samples were pressurized at 400 MPa, at room temperature (18–22 °C), for 15 min. The time needed to achieve the treatment pressure was approximately 4 min, and the decompressing was instantaneous.

Subsequently, each of the sub-samples was again divided into three parts:

1. Dehydrated bee pollen analysed at time zero (D0); dehydrated bee pollen analysed at 30 days (D30) and dehydrated bee pollen analysed after 60 days (D60);
2. Dehydrated pasteurized pollen was analysed at time zero (P0); pasteurized dehydrated bee pollen was analysed after 30 days (P30 days), and pasteurized bee pollen was analysed at 60 days (P60);
3. HPP-dehydrated bee pollen was analysed at time zero (HPP0); HPP dehydrated pollen was analysed at 30 days (HPP30), and HPP-dehydrated BP was analysed at 60 days (HPP60).

2.2. Evaluation of the botanical origin

To identify the pollen types, the BP samples underwent acetolysis according to the method proposed by Campos et al. (Campos et al., 2021). After acetolysis, slides were prepared using glycerinated gelatin for analysis by optical microscopy. The identification of pollen grains was carried out by comparison with the existing pollen collection at the Polytechnic Institute of Bragança and with the literature on this subject (Roubik & P., 1992)- The classification system adopted for the family level was APG IV (2016).

2.3. Chemicals and reagents

The following compounds were used as standards: gallic acid (1-Hidrate) from Panreac (99%, Spain) and quercetin from Sigma-Aldrich (>95%, Belgium). Regarding solvents, ethanol was acquired from Panreac (HPLC quality, Spain, 99.9%) and petroleum ether from Chem-Lab (40–60 a.r., Zedelgem). Other reagents were sodium carbonate from Merck (Germany), Folin-Ciocalteu reagent from Panreac (Spain, d = 1.234), aluminium chloride from Merck (Germany), sodium acetate from PronalyśAR (99.5%, England), peptone water and PCA from Panreac (Spain), rose bengal and baird-parker broth from VWR (prolabo chemicals, Geldenaaksebaan), egg yolk tellurite from Himedia (India) and differential reinforced clostridial broth from Liochem (Italy).

2.4. Analytical methods

All analytic determinations were made in triplicate.

2.4.1. pH

The pH values of the samples were measured using a digital pH meter (Mettler Toledo, Switzerland) according to the described by Campos et al. (2021). Calibration was performed with two standard buffer solutions (4 and 7 pH). The measurements were performed at room temperature (around 25 °C).

2.4.2. Moisture content

Moisture determination was determined by the standard method AOAC Official Method 934.01 (AOAC 934.01, 2005), direct measurement, where the water content is determined by removing moisture and then by measuring weight loss, using a vacuum oven (VWR Venti-Line, EC) at 105 °C. The water content was determined using the equation:

$$\%moisture = \frac{mi - mf}{mi - mc} \times 100$$

where *mi* is the initial mass of the crucible and sample, *mf* crucible mass with sample after drying, and *mc* empty crucible dough.

2.4.3. Total lipid content

Total Lipid content (TLC) was determined according to the Soxhlet extractor behrotest (behr Labor-Technik, Germany) with petroleum ether as the solvent (Campos et al., 2021). The solvent is heated up to 60 °C and allowed to evaporate, leaving behind the extracted lipids in the thimble. This process is repeated until a constant weight is obtained. The weight of the extracted lipids is then divided by the weight of the initial sample and multiplied by 100 to determine the percentage of TLC. This methodology is a traditional technique for extracting lipids in foods, where they know weight sample is dried and placed in a porous thimble. The Soxhlet apparatus comprises three compartments, flask, extraction chamber, and condenser.

2.4.4. Total nitrogen-Protein content

Nitrogen determination was performed by the Kjeldahl method (Velp Scientifica UDK139, semi-automatic distillation unit, Italy), which is based on the analysis of the total nitrogen content in the samples using 6.25 value (N × 6.25) as a conversion factor to obtain the value into protein (Campos et al., 2021).

2.4.5. Ash content

The method used to determine the ash content was calcination at

600 °C in an oven (Lindberg, Watertown, Wisconsin) according to the standard methods for pollen research (Campos et al., 2021).

The total ash content, expressed as the percentage of residue left after sample calcification (%), was calculated from the following equation:

$$\%Ash = \frac{mi - mf}{mi - mc} \times 100$$

where *mi* is the initial mass of the crucible and sample, *mf* is the crucible mass with ash, and *mc* is the empty crucible mass.

2.4.6. Fatty acids

The methyl esters were analysed by gas chromatography (DANI GC 1000) equipped with a split/spitless injector. The components were separated on a capillary column (Zebron, ZB-FAME), with 3 m long, 5 m of pre-column, an internal diameter of 0.25 mm, and a film thickness of 0.20 μm. The initial temperature of the column was 100 °C, held for 2 min, then a 10 °C/min ramp to 140 °C, 3 °C/min ramp to 190 °C, 30 °C/min ramp to 260 °C and held for 2 min. The injector temperature was 250 °C, and the detector temperature was 260 °C. The carrier gas (hydrogen) flow rate was 1.1 mL/min, measured at 100 °C, the split injector (1:50) was carried at 250 °C, and the volume of the sample injector was 1 μL. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using Clarity v.4.0.00.681 (DataApex) and expressed in relative percentage of each fatty acid. Two replicates were done for each sample.

2.4.7. Determination of the total phenolic and flavonoid content

Total phenolic content (TPC) was determined using microplates according to the methodology described by Campos et al. (Campos et al., 2021). Aliquots of 20 μL of the sample were mixed with 100 μL of 1:4 diluted Folin-Ciocalteu reagent and stirred for 60 s in a 96-well flat-bottomed microplate. The mixture was allowed to stand for 4 min; then, 75 μL of sodium carbonate solution (100 g/L) was added, and the mixture was stirred for 1 min. After two h in the dark at room temperature, the absorbance was quantified at 750 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, Finland). Gallic acid (GA) was used as a calibration standard. The results were expressed as mg gallic acid equivalent (GAE) per g of sample. Total flavonoid content (TFC) was determined by colorimetric assay with an aluminium chloride (Sembiring, Elya, & Sauriasari, 2017).

2.4.8. Amino acid

The amino acid composition was determined by an amino acid analyser S433 amino acid analyser (Sykam GmbH, Germany) following the standard method (AOAC, 1990). After hydrolysis in 6 N HCl for 24 h at 110 °C in a nitrogen atmosphere, the samples were concentrated in a rotary evaporator. The concentrated samples were reconstituted with the dilution buffer provided by the manufacturer (0.12 N, pH 2.20) and analysed.

3. Microbiological determinations

3.1. Sample preparation

An aliquot of each sample (10 g) was aseptically taken and homogenized using a Stomacher (Seward Stomacher 400, England) for 2 min with 90 mL of sterile peptone water. Decimal serial dilutions were prepared from this homogeneous in the same sterile diluents (1:10, w/w).

3.2. Enumeration of the total mesophilic microorganisms

The aerobic mesophilic microorganisms were counted by incorporating 1 mL of each dilution into standard Plate Count Agar (PCA), as recommended in NP-3788 (2002) – Portuguese Legislation. The incubation was carried out in an oven at 30 °C and 72 h. Microbial counts were expressed as colony-forming units per gram of sample (CFU/g).

3.3. Enumeration of yeast and moulds

Moulds and yeasts enumeration was made in Rose Bengal medium, which was incubated at 25 °C for 48 h for yeasts and five days for moulds. Microbial counts were expressed as colony-forming units per gram of sample (CFU/g).

3.4. Enumeration of total coliforms and Escherichia coli

Enumeration of coliforms and Escherichia coli was done using the SimPlate CEc-CI method using multiple test mediums (BioControl System) as described by De-Melo et al. (A.A.M. De-Melo, Estevinho, & Almeida-Muradian, 2015). The SimPlates were stacked and stored at 37 ± 1 °C for 24–28 h. Wells were counted positive for total coliforms based on the colour change and positive for *E. coli* based on color change and fluorescence under UV light (365 nm). The coliform and *E. coli* populations were determined based on the number of positive wells correlated with the SimPlate conversion table. The results are expressed in colony-forming units per gram for the sample (CFU/g).

3.5. Determination of sulphite-reducing clostridium spores

For sulphite-reducing clostridia counting, aliquots of 10, 5, 1 and 0.1 mL of the initial suspension were added to an empty tube, thermally treated at 80 °C for 10 min and covered with Differential Reinforced Clostridial Broth which was then incubated at 37 °C for five days. At the end, the black colonies were counted. The results were expressed as the presence of Sulphite-reducing clostridia in a 0.01 g (ISO 15213, 2003). Results were expressed in colony-forming units per gram (CFU/g).

3.6. Determination of S. Aureus

The quantification was performed according to the protocol of NP 4400-1 (2002). Serial dilutions of the sample were inoculated in Baird-Parker Broth with Egg Yolk Tellurite (Himedia) for 24 h at 37 °C. After, 3–5 characteristic colonies were selected to testify to the presence of coagulase and catalase in rabbit plasma. Microbial counts were expressed as colony-forming units per gram of sample (CFU/g).

3.7. Detection of Salmonella sp.

The detection of *Salmonella* sp. in the samples was carried out using the immunodiffusion 1–2 test, which is a patented single-use test for *Salmonella* used by analysts worldwide and recognized as the AOAC Official Method 989.13-1998 (AOAC 989.13-1998, 2000). Rapid and reliable results are obtained 16–20 h after pre-enrichment in buffered peptone water (25 g of sample was weighed and dissolved in 225 mL of peptone water and incubated at 37 °C for 24 h). The results are interpreted visually by observing the development of an immunoband, a characteristic immobilization pattern of cells.

3.8. Enterobacteriaceae counts

For detection and quantification of the total Enterobacteriaceae populations, the SimPlate Entero-CI method was used ISO 21528-2 (ISO 21528-2, 2004). It is based on Binary Detection Technology (BDT) which equates the presence of total Enterobacteriaceae to the presence of a colour change in the medium. The medium/sample mixture is dispensed into a SimPlate® device and incubated for 24–28 h. The total Enterobacteriaceae count was determined by counting the wells with changed colour and referring to the SimPlate conversion table. The results are expressed in colony-forming units per gram for the sample

(CFU/g).

4. Results and discussion

4.1. Botanical origin

Table 1 summarizes the percentage of pollen grains belonging to each botanical family identified. The BP samples were classified as monofloral, *Cistus ladanifer* being the predominant genus (94.51 ± 2.22%). Other genera appear in the sample, namely *Cytisus scoparius*, *Echium vulgare*, and *Erica umbellata* but with percentages lower than 2.50%.

4.2. Chemical and nutritional characterisation

The average values of the chemical and nutritional composition (expressed on a dry weight basis) observed for the BP samples at different conservation techniques and for different storage times are represented in Table 2.

Moisture content is an essential parameter because it is related to the BP shelf lifetime. Higher moisture content, as a consequence of inadequate storage, is an important parameter. The presence of excess water can lead to the growth of spoilage microorganisms, such as bacteria, fungi, and yeasts, which can make bee pollen unsafe for human consumption (Anjos et al., 2019).

The moisture content of the sample under analysis ranged between 5.59% and 8.09% (Table 2); all values were within the defined by the standards. The observed variability is mainly explained by the different thermic treatments (Table 2), with a significantly higher moisture content observed for Pasteurized BP because the moisture content remained practically unchanged during storage. During the pasteurization process, a higher temperature (70 °C) was applied compared to the other techniques (42 °C for dried and 18–22 °C for HPP processes). The formation of a drier surface layer during the pasteurization process may make it difficult to remove the internal water. Therefore, a higher final moisture content is registered. Indeed, pasteurization does not typically affect a product's water content or solute concentration, so it does not typically affect water activity. However, it is worth noting that some types of pasteurization methods, such as ultra-high temperature (UHT) pasteurization, can involve more extreme temperature and pressure conditions that may affect the water activity of a product.

Regarding the pH and ash values, the variability observed for the thermic treatment and storage time was not significant as expected (Table 2). The analysed BP was acidic for the pH, ranging between 3.28 and 3.48. Similar results of pH were previously observed by other authors in dried BP Fields (Feas et al., 2012; Thakur & Nanda, 2020). The mean values found for ash content in the BP sample ranged between 2.94 and 4.15 % (Table 2) and are similar to those reported in the literature (Anjos et al., 2019; Feas et al., 2012; Thakur & Nanda, 2020). Regarding *Cistus* BP Nogueira et al. (Nogueira, Iglesias, Feás, & Estevinho, 2012) found, in samples with a higher amount of cistus pollen (than 84%), values of 2.86% and 2.79% for the TLC. However, a study based on Cistus BP from Italy plotted lower values for TLC (1.9%) (Sagona et al., 2017). These differences can be related to geographical origin and other external factors. The *taxa* are also a critical key factor influencing the chemical composition. For instance, bee pollen from Brassicaceae may have values of TLC between 6.56% and 10.99% (Thakur & Nanda, 2020). In this investigation, the storage time can explain 83% of the variation observed on TLC (Table 2). The temperature used in the dried process (42 °C) is near the lipase activation temperature (around 40 °C), where some lipids are hydrolysate to fatty acids. During pasteurisation, the temperature used is higher than that of lipase activation. Even for HPP, where the temperature was around 18 and 22 °C, a decrease in the TLC during the storage time was verified. These values align with the data provided by other authors for dried and fresh bee pollen (Anjos et al., 2019; Estevinho et al., 2019; Liolios, Tananaki, Kanelis, Rodopoulou, & Argena, 2022).

In this study, the protein content decreased during storage for all different thermic treatments. That variance can be explained by 24.2% by storage time and 22.4% by the thermic treatment. Proteins are composed of long chains of amino acids that can denature influenced by different factors, namely, food processing temperature and storage time (Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998; Szczesna, 2006) which could be the cause. Proteins' denaturation represents a structural change in the molecules and the loss of the protein's function due to any physical or chemical factor, such as high temperature or pH changes. According to these authors, the elevation of the temperature of a matrix causes the rotating, twisting and bending of bonds and functional groups within the molecule. When individual hydrogen bonds are ruptured, those nearby are more susceptible to break, and the process is generally considered irreversible. Such a process may continue during storage and may lead to changes in the solubility and digestibility of the proteins, which can affect their nutritional and functional quality. To minimize protein denaturation in BP, storing it in a cool, dry place and using it within a reasonable time frame is recommended.

BP mainly comprises carbohydrates, including long-chain compounds and free sugars such as fructose and glucose (Li et al., 2018). During the storage processes, the long carbohydrate chains may hydrolysate, increasing the percentage of free sugars. As a result, in this study, the content of carbohydrates increased over the treatment time and explained 56% of the total variation observed.

4.3. TPF and TFC

TPC and TFC variations are highly significant for the different thermal processing, explaining 81% and 65% of the total variance, respectively (Table 2). Regarding the TPC, the values ranging from 7.8 mg GAE/g of BP (for P60) and 30.3 mg GAE/g of BP (for HPP0) and for TFC, the observed values raging between 2.17 mg QE/g of BP (for P60) and 4.05 mg QE/g of BP (for D0). The values of TPC and TFC are similar to those observed by other authors (Carpes, Begnini, Alencar, & Masson, 2007; Dimcheva & Karsheva, 2018; Harif Fadzilah et al., 2017). Regarding the TPC, the content is significantly higher for HPP when compared to the D and P processes. It was also observed that the decrease of the TPC and TFC during the storage period is lower for the HPP than those observed for D and P. Considering the results plotted in Table 2, the best conservation technique seems to be the HPP.

4.4. Fatty acids profile

Table 3 summarizes the mean values of the fatty acids observed for the BP samples submitted to different technological processes and storage times. Only the fatty acids palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2n6), α-linolenic acid (C18:3n3) present highly significant differences regarding the technological processes and the storage time do not present significant variation.

The α-linolenic acid (C18:3n3) was dominant in BP samples with values from 32.8 g/100 g for dried bee pollen (sample D0) to values of 15.0 g/100 g for pasteurized bee pollen (sample P60), followed by palmitic acid (C16:0) with a value ranging between 21.8 g/100 g to15.6 g/100 g for D60 and P60 respectively. The lowest fatty acids observed in BP are C20:0, C12:0 and C18:1n7. Sinapis BP Field also reports a higher quantity of α-linolenic acid (Szczesna, 2006). The differences observed

Table 1
Frequency of each plant genus in the BP sample analysed.

Species	Mean ± SD (%)	Max - Min (%)
Cistus ladanifer L.	94.51 ± 2.22	97.54–92.31
Cytisus scoparius	1.75 ± 0.70	2.37–0.78
Echium vulgare	2.18 ± 0.73	3.01–1.23
Erica umbellata L.	1.38 ± 0.72	2.31 – 0.54

Table 2
Physicochemical values and component variance analysis of BP samples dried and frozen (mean ± standard deviation).

	Dried			Pasteurized			High Pressure Processing			Variance Source			
	0 days	30 days	60 days	0 days	30 days	60 days	0 days	30 days	60 days	Time (T)	DM	TxDM	Error
Moisture (%)	6.06 ± 0.16^{a}	5.80 ± 0.16^{a}	5.59 ± 0.33^{a}	8.09 ± 0.19^{c}	7.68 ± 0.32^{bc}	7.45 ± 0.49^{b}	6.23 ± 0.38^{a}	5.89 ± 0.17^{a}	5.66 ± 0.15^{a}	4.9*	86.0***	ns	9.1
pH	3.45 ± 0.09^{a}	3.47 ± 0.30^{a}	3.43 ± 0.24^{a}	3.41 ± 0.05^{a}	3.43 ± 0.24^{a}	3.48 ± 0.32^{a}	3.28 ± 0.20^{a}	3.31 ± 0.19^{a}	3.29 ± 0.09^{a}	ns	ns	ns	–
Protein (%)	26.4 ± 0.9^{b}	25.8 ± 1.8^{ab}	22.6 ± 2.3^{ab}	23.1 ± 2.1^{ab}	22.2 ± 3.1^{ab}	19.5 ± 0.6^{a}	25.4 ± 0.7^{ab}	24.5 ± 1.0^{ab}	22.4 ± 3.2^{ab}	24.2*	22.4*	ns	53.5
TLC (%)	4.70 ± 0.22^{b}	3.97 ± 0.23^{c}	3.05 ± 0.59^{a}	4.67 ± 0.54^{b}	3.56 ± 0.48^{c}	2.64 ± 0.38^{a}	4.69 ± 0.33^{b}	3.28 ± 0.33^{a}	2.92 ± 0.59^{a}	82.8***	6.2**	ns	11
CH (%)	59.12 ± 0.82^{a}	60.96 ± 1.93^{a}	65.25 ± 2.07^{b}	60.90 ± 2.27^{a}	62.12 ± 3.98^{a}	66.04 ± 1.49^{b}	60.31 ± 0.57^{a}	63.05 ± 1.61^{a}	65.72 ± 4.19^{b}	55.7***	ns	ns	44.3
Energy (Kcal/100 g)	385.14 ± 0.91^{d}	381.63 ± 1.13 ^{cd}	377.97 ± 4.92^{c}	380.89 ± 1.27 ^{cd}	372.16 ± 0.91^{ab}	368.06 ± 4.61^{a}	383.49 ± 3.80^{d}	377.57 ± 2.53^{c}	376.49 ± 2.46^{bc}	45.7***	34.9***	ns	19.4
Ash (%)	3.62 ± 0.27^{a}	3.60 ± 0.21^{a}	3.62 ± 0.27^{a}	2.94 ± 0.14^{a}	4.15 ± 0.44^{a}	4.15 ± 0.44^{a}	3.53 ± 0.34^{a}	3.53 ± 0.44^{a}	3.53 ± 0.44^{a}	ns	ns	ns	–
TPC (GAE/g)	19.3 ± 1.0^{c}	15.0 ± 2.6^{b}	11.6 ± 1.8^{ab}	13.6 ± 0.8^{b}	9.0 ± 1.0^{a}	7.8 ± 0.8^{a}	30.3 ± 1.4^{d}	28.3 ± 1.8^{d}	21.9 ± 3.0^{c}	13.9***	81.0***	ns	5.1
TFC(QE/g)	4.05 ± 0.50^{b}	2.57 ± 0.40^{a}	2.21 ± 0.33^{a}	3.22 ± 0.43^{b}	2.67 ± 0.32^{a}	2.17 ± 0.42^{a}	3.32 ± 0.58^{b}	2.81 ± 0.19^{a}	2.33 ± 0.38^{a}	ns	65.2***	ns	34.8

CH – Carbohydrates; TLC- total lipid content; TPC – Total Phenolic Content; TFC – Total Flavonoid Content; D - dehydrated bee pollen; P-pasteurized dehydrated bee pollen; HPP - HPP dehydrated bee pollen n.s. for $p > 0.05$; *$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$; ns – non-significative.

in the fatty acid concentration may be related to the structural changes induced by the thermal processing (van Dooremalen & Ellers, 2010), probably the production of peroxides.

Another critical parameter is the protein content and its preservation, once BP is mainly consumed due to it and to the free amino acids, which are fundamental in the diet for good health. In the samples under analysis, 18 free amino acids were found. Glutamine (Glu) was the major one, with an average of 23.21 mg/g of BP in the HPP sample, 20.38 mg/g of BP in the dried sample and 20.30 mg/g of BP in the pasteurized samples (considering the average of the three storage periods). The second major one was 19.12 mg/g of BP in the HPP sample, 17.94 mg/g of BP in the dried sample, and 12.01 mg/g of BP in the pasteurized samples (also considering the three storage periods) (Table 3). Quite similar results were observed for *Cistus ladanifer* Spanish BP. However, in this case, the Pro was the most abundant, with an average of 23.91 mg/g of BP, and Glu accounted for 16.09 mg/g of BP (Paramás, Bárez, Marcos, García-Villanova, & Sánchez, 2006). Leucine (Leu) and glycine (Gly) also presented important amounts, with 15.51 mg/g of BP and 11.27 mg/g of BP, respectively.

The amino acids that were presented in lower concentration in BP correspond to tryptophan (Trp), methionine (Met) and hydroxyproline (Hyp) with a concentration of 1.55 mg/g, 1.95 mg/g and 2.16 mg/g, respectively.

Regarding Table 3, it is vital to note that for the three conservation processes evaluated (D, P or HPP), the high significance of the final amino acids quantity in BP explains from 64.1% to 98.5% of the total variance observed, respectively, for Hyp and Glu. The lower values were consistently observed in BP pasteurized and the higher ones for the samples submitted to HPP.

4.5. Microbiological characterisation

The values obtained for the microbiological parameters are summarized in Table 4. The total count of aerobic mesophilic microorganisms ranged between 3.82 ± 0.03 CFU/gr and undetected (ND), while the total count of molds and yeasts ranged between 3.81 ± 0.04 CFU/gr and ND. Notably, these microorganisms are considered non-pathogenic. These values are identical to those reported in the literature for dehydrated Portuguese pollen (Anjos et al., 2019) and for dehydrated and freeze-dried Brazilian pollen (Adriane Alexandre Machado De-Melo et al., 2016), suggesting that the analyzed raw material was handled correctly.

Concerning the microorganisms with low to moderate health risks (indicators of possible fecal contamination), *Escherichia coli* was absent in all the samples. Total coliforms were only present in two and at very low levels.

Bacillus cereus ranged between 1.49 ± 0.18 CFU/gr and 1.11 ± 0.08 CFU/gr. The evaluated samples did not detect coagulase-positive Staphylococcus, Salmonella sp. and sulfite-reducing clostridium spores. These results are very relevant because these microorganisms are potentially pathogenic, being public health hazards.

From a microbiological-quality point of view, the conservation processes may be graded as follows: dehydration < pasteurization < and high-pressure pasteurization. In addition, the microbiological parameters quantified in the dehydrated pollen did not vary significantly during storage. Regarding the pasteurization and high-pressure pasteurization processes, although there were no significant differences between time 0 and 30 days, a very significant decrease was observed after two months of storage. *Bacillus cereus* showed a peculiar behavior since no significant differences were observed during storage, regardless of the preservation process used. This occurrence may be related to the fact that these microorganisms form spores, which makes them more resistant to "stress" conditions.

4.6. Overall analysis

The overall analysis of the results indicates that its bee pollen microbial load was particularly affected by the preservation process, being the HPP method the most effective. To enhance the relative variation of pollen analytical parameters contents, a global analysis was done using a cluster heat map to better understand the response of the different conservation techniques.

The heat map (Fig. 1) was generated from the content of different analytical parameters measured for dried, pasteurized and HPP-treated pollen stored at 0, 30 and 60 days. Blue colors identify a positive association between analyte levels and modalities, whereas red colors represent a negative correlation. More intense colors represent a higher correlation observed.

Concerning Fig. 1A, the heat map clustered the pollen samples based on their conservation techniques (D, P and HPP). Furthermore, the heat map can divide the pollen's chemical composition into 2 clusters, one composed of the ash and pH and another one by the P, TPC, TFC and Fat.

Table 3
Fatty acids, amino acids profile and component variance analysis of BP oven dried, pasteurized and submitted to HPP.

	Dried			Pasteurized			High Pressure Processing			Variance Source			
	0 days	30 days	60 days	0 days	30 days	60 days	0 days	30 days	60 days	Time (T)	DM	TxDM	R
Fatty acids (g/100 g)													
C12:0	0.46 ± 0.10	0.45 ± 0.15	0.46 ± 0.05	0.47 ± 0.12	0.46 ± 0.01	0.46 ± 0.05	0.45 ± 0.10	0.44 ± 0.06	0.44 ± 0.07	ns	ns	ns	–
C14:0	1.02 ± 0.20	1.00 ± 0.24	1.10 ± 0.18	1.05 ± 0.33	1.07 ± 0.14	1.06 ± 0.23	1.09 ± 0.27	0.97 ± 0.23	1.00 ± 0.32	ns	ns	ns	–
C16:0	20.5 ± 1.1^{b}	21.7 ± 1.0^{b}	21.8 ± 2.5^{b}	16.4 ± 0.7^{a}	15.7 ± 0.2^{a}	15.6 ± 1.0^{a}	19.9 ± 2.0^{b}	19.2 ± 2.2^{b}	19.2 ± 3.0^{b}	ns	60.5***	ns	39.5
C18:0	1.58 ± 0.23^{b}	1.27 ± 0.14^{b}	1.25 ± 0.15^{b}	0.87 ± 0.23^{a}	0.76 ± 0.20^{a}	0.79 ± 0.18^{a}	1.60 ± 0.32^{b}	1.59 ± 0.16^{b}	1.60 ± 0.29^{b}	ns	68.4***	ns	31.6
C20:0	0.36 ± 0.10	0.31 ± 0.14	0.32 ± 0.07	0.37 ± 0.10	0.30 ± 0.04	0.30 ± 0.04	0.36 ± 0.09	0.37 ± 0.06	0.35 ± 0.08	ns	ns	ns	–
C22:0	0.17 ± 0.02	0.16 ± 0.03	0.16 ± 0.04	0.13 ± 0.04	0.11 ± 0.03	0.12 ± 0.04	0.16 ± 0.06	0.16 ± 0.01	0.17 ± 0.01	ns	ns	ns	–
C18:1n9	7.12 ± 0.80	7.06 ± 0.26	7.03 ± 0.04	7.05 ± 0.81	6.95 ± 0.66	6. 95 ± 0.66	7.12 ± 0.63	7.07 ± 0.37	7.07 ± 0.37	ns	ns	ns	–
C18:1n7	0.55 ± 0.07	0.53 ± 0.05	0.55 ± 0.03	0.57 ± 0.07	0.56 ± 0.07	0.56 ± 0.08	0.58 ± 0.10	0.56 ± 0.09	0.57 ± 0.05	ns	ns	ns	–
C18:2n6	20.6 ± 2.0^{c}	20.4 ± 1.1^{c}	20.5 ± 2.1^{c}	9.0 ± 1.1^{a}	9.0 ± 1.1^{a}	9.0 ± 0.7^{a}	18.0 ± 2.4^{b}	17.8 ± 0.3^{b}	17.8 ± 1.4^{b}	ns	90.8***	ns	9.2
C18:3n3	32.8 ± 2.6^{c}	31.8 ± 3.7^{c}	30.6 ± 3.7^{c}	15.1 ± 1.0^{a}	15.2 ± 0.8^{a}	15.0 ± 1.2^{a}	23.5 ± 1.6^{b}	29.7 ± 2.4^{b}	30.3 ± 0.7^{b}	ns	90.7***	ns	9.3
Amino acids (mg/g)													
Phe	6.87 ± 0.27^{c}	6.83 ± 0.22^{c}	6.66 ± 0.25^{c}	5.34 ± 0.31^{b}	5.37 ± 0.46^{b}	4.72 ± 0.28^{a}	7.56 ± 0.27^{d}	7.53 ± 0.06^{d}	7.27 ± 0.15^{d}	2.1*	90.7***	ns	7.2
Trp	1.55 ± 0.17^{b}	1.53 ± 0.10^{b}	1.39 ± 0.11^{b}	1.01 ± 0.10^{a}	0.95 ± 0.09^{a}	0.86 ± 0.10^{a}	1.50 ± 0.18^{b}	1.42 ± 0.10^{b}	1.30 ± 0.04^{b}	ns	80.7***	ns	19.3
Pro	18.08 ± 0.45^{b}	18.04 ± 0.25^{b}	17.71 ± 0.27^{b}	12.09 ± 0.28^{a}	12.20 ± 0.61^{a}	11.75 ± 0.44^{a}	19.23 ± 0.40^{c}	19.27 ± 1.06^{c}	18.87 ± 1.12^{c}	ns	96.1***	ns	3.9
Leu	15.51 ± 0.61^{b}	15.41 ± 0.31^{b}	14.46 ± 0.41^{b}	11.49 ± 0.38^{a}	11.42 ± 0.52^{a}	10.78 ± 0.47^{a}	16.85 ± 0.34^{c}	16.73 ± 0.60^{c}	16.63 ± 1.04^{c}	ns	94.4***	ns	5.6
Met	1.95 ± 0.29^{b}	2.00 ± 0.15^{b}	1.76 ± 0.18^{b}	1.02 ± 0.17^{a}	0.99 ± 0.19^{a}	0.89 ± 0.11^{a}	1.60 ± 0.32^{b}	1.65 ± 0.47^{b}	1.57 ± 0.38^{b}	ns	65.6***	ns	34.4
Ile	8.09 ± 0.35^{b}	8.03 ± 0.36^{b}	7.64 ± 0.41^{b}	6.78 ± 0.29^{a}	6.70 ± 0.52^{a}	6.37 ± 0.65^{a}	10.29 ± 0.39^{c}	10.28 ± 0.42^{c}	9.92 ± 0.33^{c}	ns	92.1***	ns	7.9
His	6.41 ± 0.28^{b}	6.45 ± 0.29^{b}	6.14 ± 0.11^{b}	4.29 ± 0.21^{a}	4.43 ± 0.44^{a}	3.94 ± 0.05^{a}	6.50 ± 0.36^{b}	6.47 ± 0.45^{b}	6.40 ± 0.27^{b}	ns	91.9***	ns	8.1
Thr	7.12 ± 0.80^{b}	7.13 ± 0.77^{b}	6.54 ± 0.46^{b}	5.65 ± 0.43^{a}	5.66 ± 0.29^{a}	5.01 ± 0.16^{a}	7.17 ± 0.43^{b}	7.10 ± 0.32^{b}	6.76 ± 0.29^{b}	ns	67.2***	ns	32.3
Ala	6.02 ± 0.18^{c}	5.95 ± 0.29^{bc}	5.36 ± 0.14^{b}	3.70 ± 0.21^{a}	3.72 ± 0.22^{a}	3.14 ± 0.25^{a}	7.30 ± 0.35^{d}	7.26 ± 0.54^{d}	7.15 ± 0.06^{d}	1.4*	95.4***	ns	3.2
Asp	19.1 ± 0.3^{b}	18.6 ± 0.6^{b}	17.83 ± 0.22^{b}	9.74 ± 1.42^{a}	9.63 ± 1.00^{a}	8.74 ± 0.68^{a}	20.74 ± 1.44^{c}	20.71 ± 1.78^{c}	20.27 ± 0.13^{c}	ns	95.8***	ns	4.2
Glu	20.64 ± 0.91^{b}	20.65 ± 0.89^{b}	19.85 ± 0.77^{b}	12.09 ± 0.34^{a}	12.11 ± 0.49^{a}	11.68 ± 0.45^{a}	23.25 ± 0.38^{c}	23.34 ± 0.23^{c}	23.04 ± 0.35^{c}	ns	98.5***	ns	1.5
Gly	11.27 ± 0.58^{bc}	11.21 ± 0.49^{bc}	10.58 ± 0.36^{b}	6.85 ± 0.43^{a}	6.82 ± 0.24^{a}	6.21 ± 0.08^{a}	12.79 ± 0.51^{d}	12.70 ± 0.81^{d}	11.70 ± 0.53^{c}	1.7*	94.6***	ns	3.7
Val	7.76 ± 0.28^{d}	7.74 ± 0.36^{d}	7.17 ± 0.07^{c}	4.93 ± 0.22^{b}	4.90 ± 0.27^{b}	4.30 ± 0.10^{a}	9.02 ± 0.33^{e}	9.06 ± 0.24^{e}	8.73 ± 0.11^{e}	1.5**	96.6***	ns	1.8
Hyp	2.16 ± 0.21^{a}	2.13 ± 0.12^{a}	1.77 ± 0.16^{ad}	1.37 ± 0.18^{bc}	1.38 ± 0.21^{bcd}	1.04 ± 0.06^{b}	2.05 ± 0.24^{a}	2.08 ± 0.21^{a}	1.55 ± 0.27 ^{cd}	19.7**	64.1***	ns	22.3
Ser	8.12 ± 0.24^{d}	8.12 ± 0.24^{d}	7.68 ± 0.29^{c}	5.16 ± 0.31^{b}	5.20 ± 0.29^{b}	4.15 ± 0.06^{a}	8.10 ± 0.16^{d}	8.06 ± 0.19^{d}	7.48 ± 0.10^{c}	4.4***	93.3***	ns	2.2
Tyr	3.45 ± 0.19^{c}	3.46 ± 0.47^{c}	2.88 ± 0.24^{b}	2.15 ± 0.20^{a}	2.17 ± 0.27^{a}	1.79 ± 0.19^{a}	3.25 ± 0.36^{bc}	3.27 ± 0.17^{bc}	2.74 ± 0.23^{b}	11.0**	70.6***	ns	18.4
Arg	8.64 ± 0.29^{d}	8.63 ± 0.45^{d}	7.85 ± 0.13^{c}	6.04 ± 0.45^{b}	6.06 ± 0.13^{b}	5.28 ± 0.38^{a}	8.56 ± 0.11^{d}	8.61 ± 0.29^{d}	8.22 ± 0.25 ^{cd}	4.8*	90.8***	ns	5.4
Lys	12.69 ± 0.28^{c}	12.70 ± 0.38^{c}	11.23 ± 0.54^{b}	8.51 ± 0.67^{a}	8.53 ± 0.57^{a}	7.73 ± 0.32^{a}	13.60 ± 0.33^{d}	13.63 ± 0.21^{d}	12.74 ± 0.26 ^{cd}	4.4***	92.1***	ns	3.5

C12:0 – Lauric acid; C14:0 – Myristic acid; C16:0 – Palmitic acid; C18:0 – Stearic acid; C20:0 – Arachidic acid; C22:0 – Behenic acid; C18:1n9 – Elaidic acid; C18:1n7 – Oleic acid; C18:2n6 – Linoleic acid; C18:3n3 – Linolenic acid; Phe – Phenylalanine; Trp – Triptophan; Pro – Proline; Leu – Leucine; Met – Methionine; Ile – Isoleucine; His – Histidine; Thr – Threonine; Ala – Alanine; Asp - Asparagine; Glu – Glutamine; Gly – Glycine; Val – Valine; Hyp – Hydroxyproline; Ser – Serine; Tyr – Tyrosine; Arg – Arginine; Lys – Lysine; n.s. for $p > 0.05$; *$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$; ns – non significative.

This last cluster is composed of parameters that are higher influenced by temperature, and as aforementioned, the pasteurization is more damaging to the final quality of the matrix. The variation of storage time is another crucial point, especially for the pasteurized and dried bee pollen. The storage time in the HPP treatment has a low influence on the product's final quality.

Regarding Fig. 1B, the HM cluster also corresponds to the conservation techniques' samples. It is possible to see a higher separation from the pasteurized pollen (with low quality) from the other conservation methods. Concerning the fatty acids, three clusters were identified. The first one comprises the amino acids C12:0, C14:0, C18:1n9; C18:1n7 and C20:0, which do not present significant differences according to the conservation techniques and storage time. The second cluster is composed of the C16:0, C18:2n6, C18:3n3 and C18:0

Table 4
Antimicrobial bioactivity activity of the of BP oven dried, pasteurized and submitted to HPP.

Microbial parameters (CFU/gr)	Dried			Pasteurized			HPP		
	0 days	30 days	60 days	0 days	30 days	60 days	0 days	30 days	60 days
CTBAMT	3.60 ± 0.01^{e}	3.82 ± 0.03^{f}	3.68 ± 0.09^{ef}	1.98 ± 0.14^{c}	1.92 ± 0.11^{c}	1.27 ± 0.14^{b}	2.33 ± 0.11^{d}	2.42 ± 0.04^{d}	nd
CTBL	3.81 ± 0.04^{d}	3.81 ± 0.04^{d}	3.81 ± 0.04^{d}	1.67 ± 0.20^{c}	1.63 ± 0.39^{c}	1.12 ± 0.17^{b}	1.17 ± 0.12^{b}	1.31 ± 0.18^{b}	nd
CE	2.64 ± 0.24^{c}	2.85 ± 0.08^{c}	2.85 ± 0.08^{c}	1.40 ± 0.29^{b}	1.28 ± 0.22^{b}	1.03 ± 0.04^{b}	1.14 ± 0.10^{b}	1.13 ± 0.18^{b}	nd
CCT	1.54 ± 0.38^{a}	nd	nd	nd	Nd	nd	nd	1.07 ± 0.06^{b}	nd
C *E. coli*	nd	nd	nd	nd	Nd	nd	nd	nd	nd
C *B. cereus*	1.49 ± 0.18^{a}	1.20 ± 0.04^{a}	1.20 ± 0.04^{a}	1.38 ± 0.23^{a}	1.34 ± 0.07^{a}	1.10 ± 1.17^{a}	1.11 ± 0.08^{a}	1.30 ± 0.11^{a}	1.17 ± 0.16^{a}
C *S. aureus*	nd	nd	nd	nd	Nd	nd	nd	nd	nd

nd- not detected; CTBAMT - Total Aerobic Mesophilic Bacteria Count; CTBL - Total mould and yeast count; CE- *Enterobacteriaceae* count; CCT - Total coliform count; C E. coli - *Escherichia coli* count; C *B. cereus* - *Bacillus cereus* count; C *S. aureus* - *Staphylococcus aureus* count; CECSR - Spore count of sulphite-reducing *clostridia*; *Salmonella* sp (absent in 25 g); n.s. for $p > 0.05$; *$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$; ns – non significative.

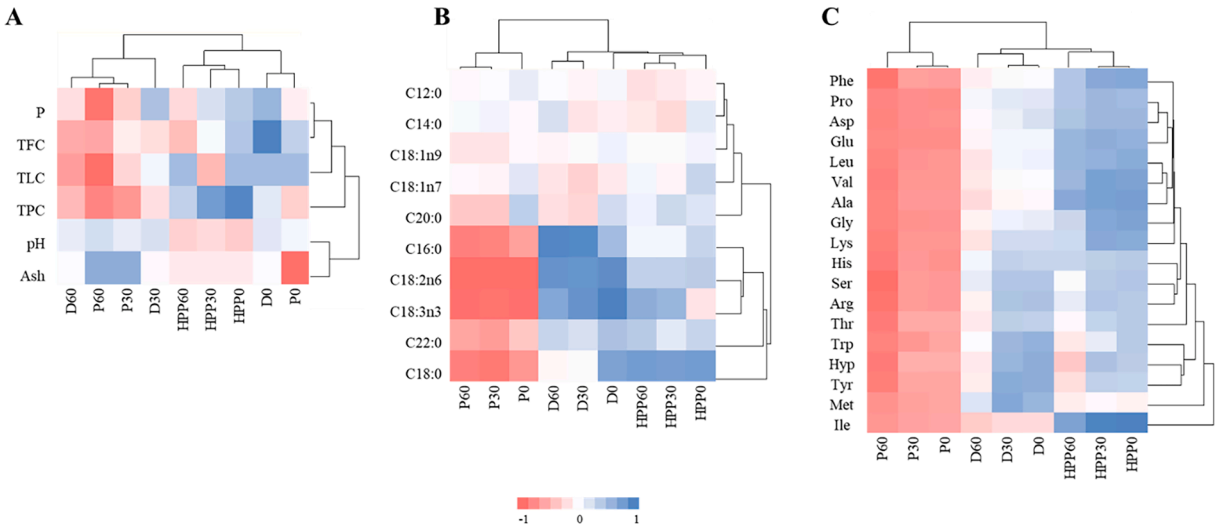

Fig. 1. Heat maps plotting clusters of contents of chemical analytical parameters (A), fatty acids (B) and aminoacids (C) of the pollen samples on the plane of the two main dendrograms. Phe – Phenylalanine; Trp – Triptophan; Pro – Proline; Leu – Leucine; Met – Methionine; Ile – Isoleucine; His – Histidine; Thr – Threonine; Ala – Alanine; Asp - Asparagine; Glu – Glutamine; Gly – Glycine; Val – Valine; Hyp – Hydroxyproline; Ser – Serine; Tyr – Tyrosine; Arg – Arginine; Lys – Lysine.

and is related to the fatty acids significantly affected by the conservation techniques. In this last case, as undermentioned, a lower quality was observed for the pasteurized pollen. This differentiation corroborates the previous data analysis.

Concerning Fig. 1C, the heat map clustered the BP samples based on their conservation techniques. Relating to different amino acids (Fig. 1C), it is possible to find three different clusters that are very well differentiated from each other and linked to the different conservation techniques. Fen, Pro, AAsp, AGlu, Leu, van, Ala, Gli and Lis comprise the first cluster very well linked to the conservation techniques, where the higher amount of these amino acids was observed for the bee pollen samples conserved with HPP and the lower for the pasteurized one. The second cluster was formed with Hist, Ser, Arg, Treo, Trip, Hyd and Tir. Several amino acids have been founded in BP, essential and non-essential from different botanical origins (Nicolson & Human, 2013; Taha, Al-Kahtani, & Taha, 2019). Cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine threonine, tryptophan, tyrosine and valine must be included in the diets because they cannot be synthesized by human body (FAO, 2007). BP is an important optional source of those essential amino acids, and its preservation in the product is crucial for a good product to be marketed. This work proves that their richness is influenced by the thermal treatment they are subjected to during storage and pretreatments. For all analytical parameters studied, the amino acids are the most sensible to the effect of the conservation techniques.

HPP was the preservation methodology that had the most reduced effect on pollen nutritional quality. In addition, it was also the one that induced the most marked decrease in the microbiota of this product.

5. Conclusion

When considering processing methods for bee pollen preservation, High-Pressure Pasteurization (HPP) has demonstrated superior results in maintaining the product's nutritional value. HPP does not result in the loss or degradation of proteins, amino acids, or lipids - the main nutrients of interest. However, the storage period significantly affects the fatty acid content, which is more pronounced than the effect of the preservation process itself. In addition, the conservation technique employed has a significant impact on the amount of phenylalanine, alanine, glycine, valine, hydroxyproline, serine, tyrosine, arginine, and lysine in bee pollen, with HPP producing the best results, followed by drying and pasteurization. The results obtained for the microbiota of the samples according to the conservation methods reveal that the High-Pressure Pasteurization method showed better stability during storage. The cluster heat map was utilized to enhance the global analysis of all collected data. This analysis provided an overview of the outcomes

obtained for dried, pasteurized, and HPP-treated pollen stored for 0, 30, and 60 days. The generated profile confirmed that HPP treatment resulted in the least loss or degradation of the matrix, indicating it as the better choice for producing a safer and superior quality product for consumers.

CRediT authorship contribution statement

Ofélia Anjos: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Statistical analysis. **Natália Seixas:** Formal analysis, Investigation. **Carlos A.L. Antunes:** Formal analysis. **Maria Graça Campos:** Writing – original draft. **Vanessa Paula:** Formal analysis, Investigation. **Letícia M. Estevinho:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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