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Lyophilized bee pollen extract: A natural antioxidant source to prevent lipid oxidation in refrigerated sausages

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A B S T R A C T

Bee pollen, a honeybee product, offers an alternative approach to preventing the oxidative deterioration in meat products. The aim of this study was to evaluate antioxidant properties of lyophilized bee pollen extract (LBP), to determine the phenolic profile by liquid chromatography, and to evaluate the effect of LBP on the oxidative stability of pork meat sausage. The sausages were evaluated for lipid oxidation on the day of their production and every five days during 30 days of storage at 4 °C by thiobarbituric acid reactive substances (TBARS). High concentrations of total phenolic compounds with antioxidant activity were detected in LBP (19.69 mg GAE/g: Gallic Acid Equivalent, EC_{50}: 0.97 mg/mL respectively). The kaempferol was the majority compound (0.68 mg/g). The TBARS values increased over time with an average of 1.29 at 4.22 mg malonaldehyde/kg meat at the beginning and end of the experiment, respectively. Treatment with LBP showed lower ($P < 0.05$) TBARS values during any day of storage than the control and sodium erythorbate (SE) treatments. The LBP extract exhibited strong anti-oxidative effects in pork sausage, probably due to high antioxidant activity and the presence of the phenolic compounds in bee pollen; which has potential to be used in pork sausage.

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1. Introduction

Foods that contain vegetable or animal fat may suffer rancidity when exposed to oxygen, heat, moisture or action of enzymes. The meat processing industry and derivatives constantly face the complexity of food processing where the products are vulnerable to lipid oxidation (Falowo, Fayemi, & Muchenje, 2014; Georgantelis, Blekas, Katikou, Ambrosiadis, & Fletouris, 2007). Lipid oxidation is one of the main reactions that can occur in meat products during their production, storage and distribution. This reaction generates compounds like aldehydes and derivatives, such as malonaldehyde, that can be harmful to health. Malonaldehyde is the major product of lipid peroxidation, and has been used as an index of marker of oxidative stress in meat products (Nakamura, Watanabe, Miyake, Kohno, & Osawa, 2003; Fisch, Bohm, Wright, & Koning, 2003; Guyon, Meynier, & Lamballerie, 2016).

Synthetic antioxidants, like butylated hydroxyanisole (BHA), butylated hydroxytoleune (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG), have been used to inhibit lipid oxidation in the food industry (Mohdaly, Sahan, Mahmoud, Ramadan, & Smetanska, 2010; Shah, Don Bosco, & Mir, 2014). However, the toxicity of some of these compounds and the growing consumer demand for healthier products has stimulated the food industry to use natural products as additives in processed foods (Haščík et al., 2011; Shahidi & Zhong, 2010). The natural antioxidants are capable of preserving food against undesirable changes caused by oxidation since many herbs, plants, fruits and apicultural products have antioxidant and antimicrobial properties (Ito et al., 1986; Estévez, Ramírez, Ventanas, & Cava, 2007; Jayathilakan, Sharma, Radhakrishna, & Bawa, 2007; Karre, Lopez, & Getty, 2013). There

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is growing health concern in the discovery of new biological molecules with eminent potential for industrial applications in replacement of synthetic compounds (Melo et al., 2015; Souza et al., 2014; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez, 2011).

Bee pollen is an agglomerate of flower pollen from various botanical sources, which are collected by the bees and mixed with nectar and honeybee salivary substances, carried out by worker bees and collected at the hive's entrance (Komosinska-Vassev, Olczyk, Kafmierczak, Mencner, & Olczyk, 2015; Melo & Almeida-Muradian, 2010). The bee pollen consists of substances that are nutritionally essential, such as antioxidant vitamins (Arruda, Pereira, Freitas, Barth, & Almeida-Muradian, 2013; Campos et al., 2008), as well as polyphenols (Estevinho, Rodrigues, Pereira, & Feás, 2012; Leja, Mareczek, Wyzgolik, Klepacz-Baniak, & Czekonska, 2007).

The antioxidant activity of bee pollen has been recognized as a free radical scavenger and as lipid peroxidation inhibitor (Carpes et al., 2013; Krystyjan, Gumul, Ziobro, & Korus, 2015; Čuboň et al., 2013). Now, it is recognized that bee pollen has a wide range of biological properties, such as anti-inflammatory (Maruyama, Sakamoto, Araki, & Hara, 2010), antimutagenic (Tohamy, Abdella, Ahmed, & Ahmed, 2014) and antimicrobial (Morais, Moreira, Feás, & Estevinho, 2011; Pascoal, Rodrigues, Teixeira, Feás, & Estevinho, 2014) activities.

The aim of this study was to evaluate antioxidant properties and the phenolic profile by the liquid chromatography of LBP, and its effect on pork sausage in order to prevent lipid oxidation during processing and storage.

2. Material and methods

2.1. Sample

A bee pollen sample was provided by Breyer & Cia Ldta of União da Vitória – PR, Brazil. The bee pollen, collected by local beekeepers during spring, came from hives of *Apis mellifera* L bees.

2.2. Chemicals

Chlorogenic acid, *trans*-cinnamic acid, gallic acid, ferulic acid, vanillic acid, caffeic acid, coumaric acid, salicylic acid, rutin, kaempferol, quercetin, myricetin, *trans*-resveratrol, DPPH (2,2-Diphenyl-1-picryl-hydrazyl), ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), Folin–Ciocalteu phenol reagents, Trolox, linoleic acid, β-carotene, and polyoxyethylene sorbitan monopalmitate (Tween 40) were obtained from Sigma–Aldrich (Sternheim, Germany). Aluminum chloride, sodium carbonate, sodium erythorbate (SE), potassium acetate, ethanol, ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid (TBA) and chloroform were purchased from Vetec (São Paulo, Brazil) and their purities were all over 99%. All reagents used were of analytical grade.

2.3. Scanning electron microscope (SEM)

The pollen loads (2 g) were grouped into subsamples according to their coloring. Each subsample was metallized with gold and analyzed by scanning electronic microscopy (SEM) in Digital Scanning Microscope DSM 940 A (Zeiss Co.). The images were captured with voltage acceleration of 15 kV, flow of 1750 mA and observed with 500–1000 X magnifications.

2.4. Preparation of lyophilized bee pollen extract (LBP)

Samples containing 10 g of bee pollen (dry basis) were subjected to the extraction process with 100 mL of ethanol solution (800 mL/L) in a shaker (SL 222, São Paulo, Brazil) at 40 °C for 60 min at a stirring rate of 150 rpm. The extract was filtered through qualitativefilter paper, and the supernatants were evaporated in a rotary evaporator (Fisatom 802, São Paulo, Brazil) (vacuum pressure of 600 mm Hg and 40 °C) until completely dry and lyophilized (Liotop L101, São Carlos, Brazil).

2.5. Total phenolic compounds (TPC) and total flavonoid compounds (TFC)

TPC were quantified by the Folin-Ciocalteu method described by Singleton, Orthofer, and Lamuela (1999), using gallic acid as the standard. 500 μL of the extract was mixed with 2.5 mL of Folin-Ciocalteu and 2 mL of sodium carbonate 40 g/L (v/v). After 2 h in darkness, the absorbance of the extract was measured at 764 nm in spectrophotometer (UV–VIS Bel Photonics 2000 Piracicaba, Brazil). The results were expressed as mg GAE/100 g of sample (GAE: gallic acid equivalent, $y = 43.259x+3.335$, $R^2 = 0.997$).

The TFC was quantified by the colorimetric method with aluminum chloride, following methodology by Park, Koo, Sato, and Contado (1995). An aliquot of 0.5 mL of the each extract was mixed with 4.3 mL of ethanol 800 g/L, 0.1 mL of $Al(NO_3)_3$ 100 g/L and 0.1 mL of 1 M potassium acetate. After 40 min at room temperature, the absorbance was measured at 415 nm in a spectrophotometer. Results were interpolated with the quercetin calibration curve and presented in mg of QE/g of sample (QE: quercetin equivalent, $y = 76.259x- 2.589$, $R^2 = 0.998$). All the assays were carried out in triplicate.

2.6. Preliminary chromatography analysis of LBP

The LBP was analyzed through high performance liquid chromatography in a Dionex Ultimate 3000 (Dionex, Idstein, Germany) chromatograph equipped with RP-18 Acclaim® 120 column (4.6 mm × 250 mm × 5 μm) and a photodiode array detector (HPLC/PDA). The LBP was filtered through at 0.22 μm filter (Millipore) and aliquots of 10 μL at 0.1 g/mL concentration were injected and the detection was determined at 280, 320 and 370 nm. The mobile phase consisted of a linear gradient of phosphoric acid: water (1:100) (solvent A) and methanol (solvent B) starting with 5% B and increasing to 100% B (45 min), keeping at 100% B for 5 min and decreasing to 5% B (60–70 min) with a solvent flow rate of 1 mL/min. The polyphenols were identified based on their retention times, UV–Vis absorption pattern, and the peak purity confirmed by diode array detector (DAD) and software Chromeleon. The quantification was performed by external standardization (Haminiuk et al., 2011). The following authentic standards of phenolic acids and flavonoids were examined: ferulic acid, gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, *trans*-cinnamic acid, syringic acid, kaempferol, myricetin, rutin, and quercetin. The content of bioactive compounds in the sample was expressed as mg/g. Determination by HPLC was also performed in triplicate.

2.7. Activity antioxidant

2.7.1. ABTS (2,-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) assay

The ABTS method was performed as described by Re et al. (1999). The stock solutions included 7.4 mM $ABTS^{•+}$ and 2.6 mM potassium persulfate. The solution was prepared by mixing 1 mL $ABTS^{•+}$ solution with 60 mL ethanol to obtain an absorbance of 0.70

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