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Nutritional and aroma improvement of gluten-free bread: is bee pollen effective?

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ABSTRACT

The impact of the addition of bee pollen (BP) (1, 2, 3, 4, and 5%) on the nutritional, bioactive and aroma composition of technologically viable gluten-free (GF) breads has been studied. The content of some macro-constituents (such as protein and lipids), minerals (K, Ca, Mg, Fe, Cu, Mn), and phytochemicals (carotenoids), the composition of biologically active compounds (soluble, insoluble and bioaccessible polyphenols), the antioxidant activity and the aroma profile of breads were investigated. The incorporation of BP into GF breads increased proteins, minerals, soluble and bioaccessible polyphenols, total carotenoids, and antiradical activity at almost all levels (2%–5%). Improvements in the phenolics composition of the resulting breads, as evidenced by a decrease in the insoluble/soluble polyphenols ratio (from 8.7 in the control to 4.2 in the breads with 4% and 5% of BP) and a concomitant increase in the polyphenol bioacessibility (36% rise in the bread with 5% of BP) in comparison to the control were also observed. BP did not affect the lipids content and the mineral composition, except for K and Ca. 48 volatile compounds were found in the examined breads, but only 5, namely pyrazinamide, 5-methyl-2-furaldehyde, 2-acetylfuran, furfural, 2-pentyl-furan, seemed to increase according to BP supplementation.

1. Introduction

The prevalence of celiac disease in Western countries is around 1% of the general population (WGO, 2018). People suffering from this chronic intestinal malabsorption should life-long exclude gluten by their diet (Naik, Seidner, & Adams, 2018). Unfortunately, many of the GF products have a lower quality, with respect to the gluten-containing counterparts, especially in terms of nutritional value and sensory attributes (Foschia, Horstmann, Arendt, & Zannini, 2016). On a nutritional basis, GF breads lack of macro and micronutrients (protein, iron and calcium, vitamins), thus celiac people have nutritional deficiencies (Conte, Fadda, Dabrińska, & Krupa-Kozak, 2019). Research for improving the nutritional quality of GF breads has focused its attention on the use of different nutrient-dense raw materials like amaranth, buckwheat, quinoa and teff flours (Collar, Conte, Fadda, & Piga, 2015; Haghayegh & Ataye Salehi, 2017; Marti et al., 2017; Turkut, Cakmak, Kumcuoglu, & Tavman, 2016), as well as dairy products (Krupa-Kozak, Wronkowska, Soral-Śmietana, Troszyńska, & Sadowska, 2009), and other various sources, such as dried fruits, nuts, almonds, sunflower seeds, pumpkin, dried oyster mushroom, and pomegranate seed powder (Bourekoua et al., 2018; Regula & Kedzior, 2017). Another critical factor that lowers GF breads quality is the aromatic profile, as the consumers detect not only unpleasant flavours imparted by the ingredients used, but also an aromatic composition weaker than that of the traditional wheat bread (Pico, Bernal, & Gómez, 2017).

In this context, due to its unique composition in both nourishing substances and biologically active compounds, the use of a natural apicultural product like bee pollen (BP) may provide a new perspective in the improvement of the overall quality of GF breads. In fact, BP can be considered as a complete food particularly rich in proteins, especially in the form of essential amino acids, which can serve as a nutritional source and enter the Maillard reaction with consequent aroma improvement of the final products. It also contains microconstituents, such as vitamins (B-complex, tocopherol, and folic acid) and minerals (K, Ca, Mg, Fe, Cu) (Denisow & Denisow-Pietrzyk, 2016). This natural product, being also rich in phytochemicals, including phenolic acids, flavonoids and carotenoids, which are usually linked to the reduced risks of several chronic disease, may also be useful in the development of functional GF breads able to provide health benefits beyond the primary nutritional value (Li et al., 2018). Moreover, as reported in our previous study, BP was successfully employed as techno-functional and sensory improver in the development of GF breads (Conte, Del Caro, Balestra, Piga, & Fadda, 2018). The authors found that not only different technological parameters (specific volume, texture, colour, and

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crumb grain), but also the firming rate and the sensory attributes (especially odour and flavour) of the resulting breads were improved by the addition of BP, even at high levels of supplementation (4–5%).

The aim of the present research was to study the nutritional properties and the aroma profile of GF breads samples formulated with different levels of pollen supplementation (1, 2, 3, 4, and 5%). Special emphasis will also be placed on relevant phenolic compounds fractions and antioxidant activity of fortified GF breads.

2. Materials and methods

2.1. Chemicals

Analytical grade methanol, acetone, petroleum ether (40–60 °C), ethyl ether, hydrogen peroxide (30%), nitric acid, sulphuric acid (96%), boric acid, hydrochloric acid (37%), trichloroacetic acid (pure), sodium chloride, sodium hydroxide (40%), sodium sulphate, sodium carbonate, potassium chloride, copper sulphate, and Ca, Mg, K, Mn, Fe, Cu standard solutions were purchased from Carlo Erba Reagents (Cornaredo, MI, Italy). Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, Folin Ciocalteu's phenol reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH●), and β-carotene (USP) reference standard, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cholic acid (99%) and deoxycholic acid (99% extra pure) sodium salts were purchased from Acros Organics (Thermo Fisher Scientific, NJ, USA). Pure and ultrapure water were obtained with a Water Purification System (Human Corporation, Korea).

2.2. Raw materials

Commercial rice flour, corn starch, guar gum and *Psyllium* fiber were obtained from Chimab Campodarsego (PD, Italy). Multi-floral dry BP (Apicoltura Piana, Bologna, Italy), fresh compressed yeast, salt and sugar were purchased from a local supermarket. Sunflower oil was from Carapelli Firenze (Italy).

2.3. Breadmaking process

All ingredients used to make both control and fortified breads were based on 100 g of flour/starch (50% rice flour and 50% corn starch) and 90 mL of water (26 °C). The other ingredients were: 6% sunflower oil, 3% yeast, 3% sucrose, 1.8% sodium chloride, 1.5% guar gum, and 1.5% *Psyllium* fiber. BP was incorporated into the basic formula at 5 different levels of supplementation (from 1% to 5%), as follows: Control (0%), Pol1%, Pol2%, Pol3%, Pol4%, Pol5%. For the preparation of the pollen-enriched samples, BP (in the form of pollen grains) was dissolved in an aliquot of water before adding the other premixed dry ingredients. GF breads were prepared using a straight-dough breadmaking process according to the procedure previously described by Conte et al. (2018).

2.4. Chemical and mineral composition of bee pollen and breads

Moisture and ash content of BP and GF breads were determined according to the Official Standard Methods AACC 44–15.02 and 08–01.01, respectively (AACC, 2005). Protein content was estimated by the Kjeldahl method using 6.25 as conversion factor from nitrogen to protein. The total lipid content was determined by gravimetric analysis: samples were extracted in a Soxhlet apparatus during a 6-h period using petroleum ether as solvent. Total carbohydrates were calculated by indirect determination as 100 − (Moisture + Protein + Lipids + Ash) (FAO, 2003). Three repetitions for each sample were made.

The mineral composition was analysed using Flame Atomic Absorption Spectroscopy (AAnalyst 200, PerkinElmer, CT, USA) according to AOAC official methods (AOAC, 1997). Quantification of three macro- (Ca, Mg, and K) and microelements (Mn, Fe, and Cu) was calculated using calibration curves with $R^2 > 0.998$. The test was performed in duplicate and the results were expressed as mg per 100 g of bread (BP) dry matter (d.m.).

2.5. Determination of polyphenol fractions and antioxidant activity of bee pollen and breads

Soluble and insoluble phenolic fractions were measured according to the procedures previously described by Vitali, Dragojević and Sebečić (2009) with some modifications. Briefly, 1 g of ground sample was extracted twice (room temperature for 2 h) using 4 mL of a solution of 37% hydrochloric acid/methanol/water (1/80/10, v/v/v). The supernatants were collected, filtered and used for the determination of the soluble polyphenols. Sample residues from free phenolics fraction were then digested in a shaking water bath (85 °C for 20 h) using 5 mL of a solution of methanol/concentrated sulphuric acid (10:1, v/v) for the determination of the insoluble fraction. The obtained extracts were spectrophotometrically (Spectrophotometer, Hewlett–Packard, PaloAlto, California) analysed at 750 nm using the Folin-Ciocalteau method (Singleton, Orthofer, & Lamuela-Raventós, 1998). To estimate the bioaccessible polyphenol fraction, BP and GF breads were subjected to an "*in vitro*" enzymatic digestion that simulates the conditions in the gastrointestinal tract following the procedure previously described by Glahn, Lee, Yeung, Goldman, and Miller (1998) and adapted for breads by Angioloni and Collar (2011). In brief, 1 g of each ground sample was firstly incubated with 0.5 mL of pepsin and 10 mL of distilled water in a shaking water bath for 1 h at 37 °C. To accurately mimic the gastric conditions, pH was adjusted to 2 using 5 M hydrochloric acid solution. The simulation of the gastric digestion was interrupted by adding sodium hydrogen carbonate (1 M) until the pH 7.2 was reached. Then, 2.5 mL of bile/pancreatin solution and 2.5 mL of sodium chloride/potassium chloride solution were added to the samples to simulate the intestinal digestion (2.5 h at room temperature). After protein's removal by addition of trichloroacetic acid (20% v/v), aliquots of 2 mL (0.5 mL for BP) of the digested extracts were mixed with 0.5 mL of Folin-Ciocalteau reagent, 10 mL of sodium carbonate (7.5%) and adjusted to 25 mL with distilled water. The mixture was incubated in the dark for 1 h at room temperature, before measuring the absorbance at 750 nm. For all the polyphenols fractions, calibration curves were made using gallic acid and the results (mean of three replicates) were expressed as g of gallic acid equivalent per 100 g of bread (or BP) d.m.

The free radical scavenging activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method according to Collar, Jiménez, Conte, and Fadda (2014) with some modification. In brief, aliquots of 0.3 mL of organic extracts (0.1 mL for BP) were added to 2.7 mL (2.9 mL for BP) of 0.0634 μmol/mL DPPH methanol solution and the absorbance was read at 515 nm at 1 min and every 5–10 min until the plateau was reached (60 min). The test was performed in triplicate and plots of μmol DPPH vs time (min) were drawn. The antiradical activity (AR) was calculated using the following equation: $AR = [(DPPH_{initial} - DPPH_{plateau}) \times 100]/DPPH_{initial}$.

2.6. Determination of carotenoid content

The extraction procedure was made according to the method suggested by Zuluaga et al. (2016) with slight modifications. Briefly, 10 g of each GF ground sample (5 g of BP) was extracted four times with 25 mL of acetone using an Ultra-Turrax T25 (IKA Labortechnik, Janke & Kunkel, Staufen, Germany) in an ice water bath for 1 min. The obtained extracts were vacuum filtered (Whatman filter paper Grade 4, Fisher Scientific, MA, USA) and gradually added, together with 20 mL of sodium chloride solution (100 g/L), to 50 mL of ethyl ether in a separation funnel. Then, the ether phase containing the pigments was recovered, washed three times with 20 mL of sodium sulphate (20 g/L), and evaporated to dryness in a rotavapor (Laborota400, Heidolph Instrument, Schwabach, Germany) at 30 °C. The residue was dissolved in acetone to a final volume of 100 mL and spectrophotometrically measured at

450 nm. Calibration curve was made using β-carotene as standard and the results (mean of three replicates) were expressed as mg of β-carotene per kg of bread (or BP) d.m.

2.7. Determination of volatile compounds

Determination of volatile compounds was carried out on freshly prepared breads 2 h after baking.

Headspace Solid-Phase Microextraction (HS-SPME): 7 ml of saturated aqueous NaCl solution were added to 3 g of homogenized crushed crust and crumb (1:1 w/w, the crust was defined as the part of the bread within 1 cm from the surface) in a SPME vial (20 mL, 75.5 × 22.5 mm), which was tightly closed with a septum and allowed to equilibrate for 5 min at 60 °C under agitation (250 rpm). A preconditioned 100 μm (Polydimethylsiloxane/Divinylbenzene/Carboxen)-coated fiber 50/30 Stableflex (Supelco, Sigma Aldrich, St. Louis, Mo., USA) was then exposed to the headspace. Based on a previous optimization, the extraction time was fixed to 30 min. All experiments were carried out under constant agitation. After the extraction, the fiber was desorbed for 2 min into the injector, operating at 250 °C in a splitless injection mode.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis: the qualitative analysis was performed using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MS detector according to Petretto et al. (2016). The chromatographic separation was performed on a WF-WAX capillary column (60 m × 0.25 mm i.d., 0.5 μm film thickness) using the following temperature program: 40 °C for 4 min, gradually increased to 150 °C at 5.0 °C/min, held at this temperature for 3 min, and heated again to 240 °C at 10 °C/min, and finally held for 12 min. Helium was used as the carrier gas at a constant flow of 1 mL/min for both columns. Data were analysed using a MassHunter Workstation B.06.00 SP1. Identification of the individual components was made by comparison with co-injected pure compounds and matching the MS fragmentation patterns and retention indexes with the built-in libraries, literature data, or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 Agilent Technologies).

2.8. Statistical analysis

The experimental data were submitted to one-way analysis of variance (ANOVA) using the Statistica 10.0 software (StatSoft, Inc., Tulsa, OK, USA). Fisher's least significant differences (LSD) test was applied to know the difference between each pair of means with 95% confidence. Pearson correlation analysis for relationships between some selected parameters was also used.

3. Results and discussion

3.1. Chemical and mineral composition of bee pollen and breads

Although the recognized beneficial effects derived from a strict adherence to a GF diet, the unsatisfactory nutritional value of many GF foods can be still considered a priority issue.

In the present study, when comparing the control bread to the pollen-enriched samples, significant differences in terms of macro- and microconstituents composition were observed (Table 1). All fortified breads, except for sample Pol1%, showed moisture values significantly lower than the control. As expected, as the addition of a dry and hygroscopic ingredient - as BP is - was increased, the moisture content of the supplemented samples decreased, suggesting a possible effect of the pollen on the water absorption capacity of the resulting breads. However, no significant differences were observed among the 2, 3, 4 and 5% fortified samples, thus indicating that increasing levels of pollen supplementation did not changed the moisture levels of the final products. All pollen-enriched GF breads showed a higher protein value, with respect to the control sample (Table 1). In particular, the higher the BP addition, the higher the protein content of the resulting breads, with the sample Pol5% showing the highest value (4.44 ± 0.01 g/100 g bread, d.m.). These results were in line, or even slightly higher - as for Pol3%, Pol4%, Pol5% samples - with those reported by Cornicelli, Saba, Machello, Silano, and Neuhold (2018) for 40 different types of GF breads currently available on the Italian market (mean value: 4.29 ± 1.80 g/100 g). On the contrary, the addition of BP appears not to have any influence on the lipid content of the fortified breads, which values ranged from 1.75 ± 0.00 to 1.85 ± 0.01 g/100 g of bread, d.m.

The addition of BP increased the total ash content of the GF breads, but only when supplementation levels were equal to/or higher than 2% (Table 1). Moreover, analysis of the mineral composition evidenced that the amounts of the most of the macro- and microelements analysed (such as Mg, Fe, Cu, and Mn) were comparable for all the experimental breads, including the control sample. In particular, the percentages of the BP used were not able to produce significant changes in the Mg, Mn, Fe, and Cu content of the fortified breads, even if a slight increase in the Mg value can be observed, especially at the medium-high levels of supplementation. The addition of BP seemed to be effective only in two of the six measured minerals, namely K and Ca. However, even if the K content of the fortified breads (68.0–82.5 mg/100 g, d.m.) was significantly higher than that of the control (64.0 mg/100 g, d.m.) at all percentages, a significant increase in the Ca content was only observed in those samples prepared with the highest levels of pollen (Pol4% and Pol5%) (Table 1). In particular, when compared to the control bread, the GF samples Pol4% and Pol5% showed an increment in K and Ca content of 23–29% and 34–37%, respectively. It is well known from literature that BP, which composition widely varies depending on the plant origins, is rich in micronutrients, especially K, Mg and Ca that constitute about 60% (400–2000 mg/100 g, d.m.), 20% (20–300 mg/100 g, d.m.), and 10% (20–300 mg/100 g, d.m.) of its total mineral content, respectively (Campos et al., 2008). According to this data and considering that the investigated pollen contained about 417.5 mg/100 g of K, 51.3 mg/100 g of Mg and 55.8 mg/100 g of Ca, and only 3.05, 0.55, and 0.75 mg/100 g of Mn, Fe, and Cu, respectively, it can be hypothesized that the content of Mn, Cu, and Fe of the BP was not enough to produce significant changes in the amount of these trace elements in all the fortified breads in comparison to the control.

3.2. Polyphenol fractions and antioxidant activity of bee pollen and breads

The addition to bread formulations of natural compounds with bioactive substances, such as polyphenols, may have beneficial effects on the quality parameters (colour, flavour, astringency, bitterness, oxidative stability) of the final products, but also on the potential health-promoting effects that they could exert on the human body (Pandey & Rizvi, 2009). For these reasons, BP, which is known to be a rich source of phenolic compounds with proven antioxidant effects, was selected as a promising health-promoting supplement in the development of functional GF breads. Table 2 summarizes the results of soluble, insoluble and bioaccessible polyphenols fractions in both BP and GF breads. The total amount of polyphenols found in the investigated pollen was 2872.8 ± 131.6 mg of GAE/100 g d.m with a soluble, insoluble and bioavailable fractions that accounted for 66.8%, 33.2% and 48.4% of the total phenolic content, respectively. The polyphenol concentration obtained in this study falls within the range previously recorded by other authors in pollen extracts from different origins (Pascoal, Rodrigues, Teixeira, Feás, & Estevinho, 2014). As reported in Table 2, both soluble and insoluble polyphenol fractions of the fortified breads were significantly affected by the addition of BP, which effect was more pronounced at increasing percentages of supplementation. In particular, the greatest polyphenol values were observed in the bread samples prepared with the highest levels of additional pollen (Pol4% and Pol5%). In all experimental breads, as opposite to that observed in the BP, the content of the insoluble polyphenol fraction was higher than that recorded for the soluble phenolic compounds. These findings were perfectly in line with those observed in cereals and cereal-based baked

Table 1
Proximate chemical and nutritional composition of multi-floral dry bee pollen, gluten-free control and fortified breads (g/100 g d.m.).

Characteristics	Samples[a]						
	Control	Pol1%	Pol2%	Pol3%	Pol4%	Pol5%	Bee Pollen
Moisture	41.2 ± 0.4^{a}	41.5 ± 0.0^{a}	39.4 ± 0.3^{b}	39.1 ± 0.2^{b}	39.1 ± 0.5^{b}	39.0 ± 0.2^{b}	–
Lipids	1.2 ± 0.1^{a}	1.1 ± 0.0^{a}	1.5 ± 0.1^{a}	1.5 ± 0.2^{a}	1.4 ± 0.1^{a}	1.4 ± 0.2^{a}	3.6 ± 0.1
Protein[b]	3.90 ± 0.02^{e}	4.08 ± 0.02^{d}	4.18 ± 0.00^{c}	4.37 ± 0.02^{b}	4.41 ± 0.02^{ab}	4.44 ± 0.01^{a}	16.87 ± 0.02
Ash	1.77 ± 0.03^{de}	1.75 ± 0.00^{e}	1.80 ± 0.00^{cd}	1.82 ± 0.01^{bc}	1.82 ± 0.01^{bc}	1.85 ± 0.01^{a}	1.58 ± 0.01
TC[c]	51.9 ± 0.5	51.5 ± 0.1	53.1 ± 0.4	53.3 ± 0.4	53.2 ± 0.7	53.3 ± 0.0	–
*Macroelements (mg/*100 g d.m.*)*							
Ca	8.8 ± 1.8^{b}	8.8 ± 0.4^{b}	9.0 ± 0.0^{b}	9.8 ± 0.4^{b}	11.8 ± 0.4^{a}	12.0 ± 0.0^{a}	55.8 ± 1.1
Mg	14.5 ± 0.7^{a}	14.9 ± 0.1^{a}	15.0 ± 0.0^{a}	15.5 ± 0.7^{a}	15.5 ± 0.7^{a}	17.5 ± 2.1^{a}	51.3 ± 1.8
K	64.0 ± 1.3^{f}	68.0 ± 1.4^{e}	70.5 ± 0.7^{d}	74.0 ± 0.0^{c}	79.0 ± 0.0^{b}	82.5 ± 0.7^{a}	417.5 ± 0.0
*Microelements (mg/*100 g d.m.*)*							
Mn	0.52 ± 0.0^{a}	0.50 ± 0.0^{a}	0.60 ± 0.0^{a}	0.6 ± 0.0^{a}	0.60 ± 0.01^{a}	0.60 ± 0.0^{a}	3.05 ± 0.07
Fe	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.55 ± 0.07
Cu	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.20 ± 0.0^{a}	0.75 ± 0.07

[a] Mean values ± standard deviation. Within rows, values (n = 3 for moisture, fat, protein and ash; n = 2 for macro- and microelements) with the same letter do not differ significantly from each other according to LSD test ($p < 0.05$).
[b] Conversion factor from N to protein = 6.25.
[c] TC: total carbohydrates calculated by indirect determination: TC = 100 – (Moisture + Lipids + Protein + Ash).

products by other authors(Saura-Calixto, Serrano, & Goñi, 2007; Vitali, Dragojević, & Šebečić, 2009). In the present study, however, an increase in the level of BP supplementation caused a progressive decrease in the insoluble/soluble polyphenols average ratio of the resulting breads (Table 2), indicating a significant increase in the extractable polyphenols fraction and, in turn, an effective enrichment of the final products.

To assess which proportion of the total polyphenols could be effectively digested, adsorbed and metabolized by the human body, thus exerting its biological activity, the polyphenol bioaccessibility of both control and fortified samples was also analysed. As in the case of the soluble and insoluble fractions, bioavailable polyphenol content (mg of GAE/100 g bread, d.m.) of the pollen-enriched breads, which ranged from 189.1 mg (Pol1%) to 239.9 mg (Pol5%), was significantly higher than that of the control (184.3 mg), with the only exception of the sample prepared with the lowest level of additional pollen (Pol1%). Furthermore, all the fortified breads showed an increment, from 3% (Pol1%) to 36% (Pol5%), in polyphenol bioaccessibility with respect to the control. To the best of our knowledge, this is the first study related to the fortification of GF breads with the use of BP, but also the first time in which the polyphenols fractions of GF bakery products was analysed. For both these reasons, the comparison of the obtained data with literature is difficult.

It is well established that several phenolic compounds, such as phenolic acids and flavonoids (usually found in large amount in BP), due to their ability to neutralize free radicals, could exhibit a strong antioxidant potential (Kostić et al., 2019). Results, which correspond to the remaining unreacted μmol of DPPH● after 60 min of reaction, were reported in Fig. 1. All the fortified breads, except for sample Pol1%, showed an antioxidant activity higher than the control (Table 2). In particular, the greatest reduction of the DPPH radical concentration was observed in the samples Pol4% (53.1%) and Pol5% (53.4%) prepared with the highest amounts of additional pollen, closely followed by those samples obtained with medium supplementation levels (Pol2%-Pol3%: 48.9%). This enhancement effect of BP incorporation was in line with the same effect previously described for the polyphenol fractions. Furthermore, considering that, as reported by other authors, the antiradical activity is often related with the total polyphenol content (Krystyjan, Gumul, Ziobro, & Korus, 2015; Vitali et al., 2009), associations between antiradical activity and phenolics fractions were analysed by using Pearson correlations. Values of correlation coefficients (r) revealed that higher value of antiradical activity corresponded to larger amounts of soluble ($r = 0.91$; $p < 0.01$), insoluble ($r = 0.83$; $p < 0.01$), and bioaccessible ($r = 0.90$; $p < 0.01$) polyphenols fractions. Similarly, a good correlation was also found between antioxidant activity and increased amounts of additional BP in GF bread formulations ($r = 0.90$; $p < 0.01$).

3.3. Carotenoids content

Carotenoids are other bioactive substances of BP with proven biological functions (such as provitamin A and antioxidant activity) (Zuluaga et al., 2016). Although a wide range of carotenoids content previously reported in the literature for BPs from different origins, data

Table 2
Polyphenol fractions and antioxidant activity of multi-floral dry bee pollen, gluten-free control and fortified breads.

Samples[a]	Polyphenol fractions (mg GAE/100 g d.m.)						Antioxidant activity (%)
	Soluble	Insoluble	IP/SP	Total	Bioaccessible	Δ bioaccessibility (%)	
Control	29.6 ± 0.8^{e}	257.5 ± 11.3^{c}	8.7	287.0 ± 11.2^{d}	184.3 ± 0.6^{e}	–	42.3
Pol1%	41.9 ± 2.3^{d}	263.9 ± 9.7^{c}	6.3	305.8 ± 12.7^{d}	189.1 ± 1.7^{e}	3	43.0
Pol2%	52.8 ± 1.6^{c}	275.5 ± 8.7^{c}	5.2	328.4 ± 7.0^{c}	202.2 ± 4.4^{d}	13	48.9
Pol3%	63.9 ± 1.1^{b}	320.8 ± 5.9^{b}	5.0	384.6 ± 4.6^{b}	213.9 ± 2.4^{c}	22	48.9
Pol4%	82.7 ± 0.4^{a}	347.5 ± 7.3^{a}	4.2	430.2 ± 6.8^{a}	228.4 ± 3.3^{b}	29	53.1
Pol5%	84.1 ± 2.6^{a}	358.0 ± 5.6^{a}	4.2	442.1 ± 9.3^{a}	239.9 ± 5.5^{a}	36	53.5
Bee Pollen	1918.7 ± 55.5	954.2 ± 76.1	–	2872.8 ± 131.6	1390.1 ± 8.1	–	51.2*

GAE: gallic acid equivalent; IP/PS: insoluble polyphenols/soluble polyphenols ratio.
[a] Mean values ± standard deviation. Within columns, values (mean of three replicates) with the same letter do not differ significantly from each other according to LSD test ($p < 0.05$).
* Corresponding to 2 mg of bee pollen (36 mg of bread), which consumed these percentages when 0,17 μmol of DPPH are available to react.

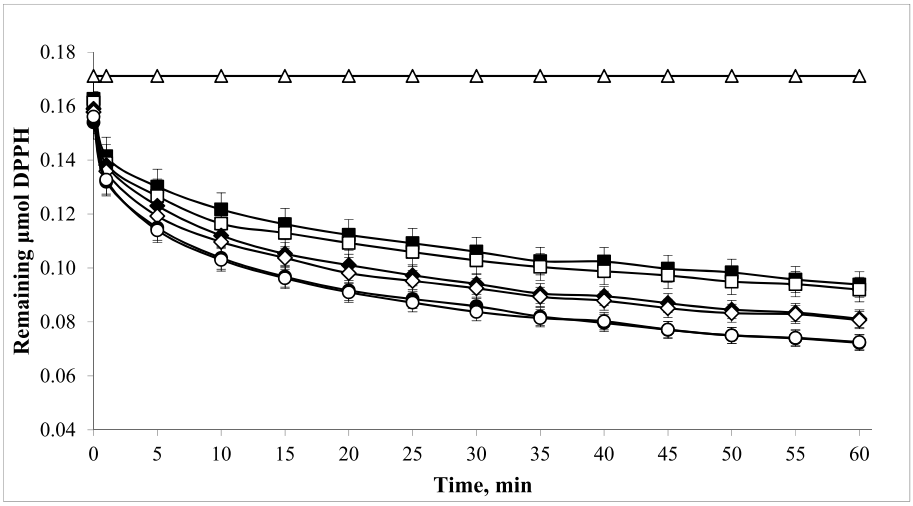

Fig. 1. Time evolution of the DPPH curves in methanol of organic extracts from gluten-free control and fortified bread. DPPH (Δ), Control (■), Pol1% (□), Pol2% (♦), Pol3% (◊), Pol4% (●), Pol 5% (○).

Table 3
Carotenoid content of both bee pollen and gluten-free breads.

Samples[a]	Carotenoids (mg β-carotene/kg d.m.)
Control	0.78 ± 0.01[e]
Pol1%	1.77 ± 0.04[d]
Pol2%	2.60 ± 0.29[c]
Pol3%	3.36 ± 0.16[b]
Pol4%	5.44 ± 0.01[a]
Pol5%	5.44 ± 0.01[a]
Bee Pollen	88.72 ± 2.45

[a] Mean values ± standard deviation. Within columns, values (mean of three replicates) with the same letter do not differ significantly from each other according to LSD test ($p < 0.05$).

obtained in this study (88.72 mg β-carotene/kg d.m.) are consistent with those observed by Almeida-Muradian, Pamplona, Coimbra, and Barth (2005) (average value: 82.54 μg/g; range: traces-489.2 μg/g) and Pereira De Melo and De Almeida-Muradian (2010) (average value: 98.15 μg/g; range: 25.34–268.5 μg/g). Despite a likely carotenoid degradation that may occur during the mixing and baking phases (Hidalgo, Brandolini, & Pompei, 2010), the addition of BP seemed to be effective in improving the carotenoid content of the GF breads (Table 3). In fact, the incorporation of increasing percentages of the experimental BP was followed by a concurrent increase in the pigment concentration of the resulting breads. As expected, the greatest values were observed in the samples Pol4% and Pol5% (5.44 mg for both breads) that contained up to 8-fold more carotenoids than the unfortified bread (0.78 mg). However, a significant enhancement effect was also observed in those breads prepared with the lowest level of BP, which showed a total amount of carotenoids 2.3-fold higher than that of the control.

3.4. Volatile compounds

After the analysis of the full scan GC/MS chromatogram, 48 compounds were tentatively identified in the headspace of the GF breads. As reported in Table 4, all the classes of compounds, which are commonly identified in GF bread, were detected. Specifically, the headspace of the analysed samples contained several pyrazine derivatives, such as dimethyl-pyrazines or ethyl-methyl-pyrazines, which origin could be linked to the Maillard reaction. Besides to nitrogen-containing compounds, the Maillard reaction also arise several furans that were detected as well in the experimental samples. Recently Pico, Bernal, et al. (2017) and Pico, Hansen, et al. (2017), in studying the aromatic profile of several GF breads obtained from flours and starches of different origins, selected a list of the main volatiles identified in all GF samples, including compounds deriving from fermentation process, lipid oxidation, and Strecker degradation. The main bulk of components included in such list (ethanol, hexanal, 1-pentanol, 2-heptanal, 2,4-decadienal, acetaldehyde, 2-methylbutanal, 2,3-butanedione, acetic acid, furfural, and furfuryl alcohol) was also detected in the GF breads analysed in the present study. It is worth underlining that the above-mentioned volatiles have been frequently found also in gluten-containing breads, as recently reported by Pasqualone, Caponio, Pagani, Summo, and Paradiso (2019) for durum wheat bread.

Data on volatile compounds are reported in Table 4. Results showed that the addition of BP seemed to affect specific reaction pathways involved in the production of some furan derivatives, which followed the general trend reported in Fig. 2. The amount of such compounds in the headspace increased according to the pollen addition, suggesting that the components contained in the investigated pollen drove the pathways involved in their formation. The production of those furans could mainly be linked to the dehydration pathway of the 1-amino-1-deoxy-2-ketose in the Maillard reaction. However, some furan derivatives could also come from other chemical reactions, such as caramelization of sugars, which generate furfural, 5-methyl-2-furaldheyde or furfuryl alcohol (Ait Ameur, Rega, Giampaoli, Trystram, & Birlouez-Aragon, 2008), as well as lipid oxidation and thermal degradation of ascorbic acid (Rannou, Laroque, Renault, Prost, & Sérot, 2016). It should be pointed out that some of the identified furans, which content increased at increasing levels of additional BP, have been associated with pleasant sensory characteristics, such as caramel (furfural), balsamic and cinnamon (2-acetylfuran), and fruit and floral aroma (2-pentylfuran) (Pico, Hansen, & Petersen, 2017), suggesting that the addition of BP could be responsible for the improved flavour and aroma previously observed by Conte et al. (2018) for the investigated GF breads. As opposite, the heterocyclic compounds like pyrazines, which derived from the fission step in the Maillard reaction, do not seem to have been affected by the pollen supplementation.

4. Conclusions

The present study has revealed, for the first time, that the incorporation of increasing percentages of BP (from 1% to 5%) could be an effective way to obtain GF breads with improved nutritional, bioactive and aroma composition. Almost all pollen-enriched breads (from 2% to 5%) exhibited higher values of protein and ash content. On the contrary, the additional BP appears not to have any influence on both lipid content and mineral composition of the resulting breads, except for K and Ca elements. In addition to an increase in the total polyphenols content, all fortified breads (except Pol1%) also exhibited a

Table 4
Volatile organic compounds tentatively identified in gluten-free control and fortified breads using HS-SPME coupled with GC-MS technique. Results are expressed as chromatogram peak area [$\times 10^6$].

Volatile compounds	Samples						RI
	Control	Pol1%	Pol2%	Pol3%	Pol4%	Pol5%	
Aldehydes							
Acetaldheyde	0.23	0.23	0.52	0.22	0.42	0.29	712
2-Methyl-propanal	7.93	10.70	7.06	6.45	1.51	8.66	821
2-Methyl-butanal	3.67	4.89	5.89	3.41	nd	4.92	922
3-Methyl-butanal	2.21	3.77	5.16	1.81	4.33	4.14	925
Hexanal	1.12	1.06	0.83	0.75	0.97	1.16	1090
(E)-2-Octenal	1.72	1.75	1.05	1.07	1.14	1.30	1453
Benzaldehyde	4.54	5.13	5.55	4.39	5.73	5.80	1566
(E,E)-2,4-Decadienal	8.53	10.79	9.00	8.91	9.16	6.92	1848
Esters							
Ethyl acetate	0.16	0.32	nd	0.23	0.28	0.34	896
Ethyl octanoate	2.06	2.46	3.76	0.63	1.83	2.31	1443
Ethyl decanoate	1.19	1.72	1.96	1.61	1.87	1.67	1653
Ethyl dodecanoate	1.49	1.58	3.51	1.28	1.54	1.26	1856
Alcohols							
Ethanol	38.16	45.90	35.66	36.44	43.12	37.61	940
1-pentanol	5.76	6.49	3.18	3.88	3.84	2.99	1213
Phenylethyl Alcohol	14.19	16.46	14.40	15.08	14.44	12.58	1950
Ketones							
2-Butanone	0.27	0.26	0.46	0.27	0.33	0.33	910
2,3-Butanedione	nd	1.04	1.25	0.66	1.24	1.14	986
2,3-Pentadienone	nd	1.51	1.04	1.05	0.84	0.92	1066
2-Heptanone	0.91	1.40	0.75	0.44	nd	1.59	1192
1-Hydroxy-2-propanone	0.52	0.57	0.76	1.04	0.88	0.95	1330
Furans							
2-Methyl-furan	nd	0.22	0.15	0.02	0.2	0.32	877
3-Methyl-furan	0.18	0.20	0.09	0.16	0.20	0.19	904
2,3,5-Trimethyl-furan	nd	0.19	0.13	0.05	0.25	0.37	1064
2-Pentyl-furan	3.13	3.53	3.23	2.48	3.69	5.04	1236
2-Methyltetrahydrofuran-3-one	nd	nd	0.70	0.65	0.84	0.83	1284
Furfural	17.60	33.69	43.99	34.44	58.11	57.94	1491
2–Acetylfuran	nd	2.23	2.86	2.77	4.28	4.41	1538
Furfuryl alcohol	6.33	9.52	7.17	9.41	8.88	8.46	1679
5-Methyl-2-furaldehyde	1.78	5.20	6.01	6.61	12.96	11.87	1608
Nitrogen-containing derivatives							
Methyl pyrazine	5.03	5.71	5.32	4.95	5.45	4.75	1289
1H-Pyrrole (not identified isomer)	nd	0.32	0.44	0.44	0.48	0.43	1318
Dimethyl-pyrazine (not identified isomer)	4.49	3.89	2.39	2.27	2.32	2.54	1347
Dimethyl-pyrazine (not identified isomer)	3.47	3.62	3.71	3.13	3.81	3.80	1352
Ethyl-pyrazine	2.10	2.99	1.68	1.96	2.09	1.64	1358
2,3-Dimethyl-pyrazine	0.87	1.18	0.76	0.77	0.87	0.76	1373
2-Ethyl-6-methyl-pyrazine	4.86	5.98	3.48	4.06	5.13	4.55	1409
2-Ethyl-5-methyl-pyrazine	0.85	nd	0.56	0.83	0.91	nd	1416
2-Ethyl-3-methyl-pyrazine	1.51	1.96	1.06	1.21	1.44	1.10	1429
2-Ethenyl-6-methyl-pyrazine	1.08	0.66	0.58	0.41	0.92	nd	1519
Pyrazinamide	1.05	2.38	2.93	2.19	3.02	3.49	1754
2-Acetyl-1-methylpyrrole	0.39	0.74	0.66	1.00	1.25	0.90	2016
Others							
n-Octane	0.22	0.25	nd	0.20	0.23	0.22	799
Disulfide dimethyl	0.32	0.30	0.48	0.45	0.55	0.52	1085
Limonene	2.12	2.60	3.20	2.76	2.44	1.56	1204
3-carene	0.40	0.49	0.52	0.54	0.60	nd	1254
Styrene	0.42	0.50	0.45	0.32	0.28	0.36	1274
Acetic acid	0.71	0.81	0.55	1.62	1.53	1.07	1469
2-Methyl-propanoic acid	0.69	1.03	0.93	0.92	0.65	1.05	1586

RI: linear retention indexes in a 60 m VF-WAX capillary column.

better polyphenol composition, showing an increment in the soluble fraction higher than that of the insoluble one. Moreover, a significant increase in the polyphenols bioaccessibility, carotenoid content, and antioxidant activity was observed in the pollen-enriched breads, revealing their full potential as health-promoting and/or health-preventing foods, especially at the highest levels of BP supplementation (4% and 5%). Furthermore, the increased content of some furans, which are usually associated with pleasant aroma properties, seemed to improve the aromatic composition of the fortified breads. The obtained results evidenced that BP, being a good source of nutrients, in particular protein and minerals, as well as phytochemicals and biologically active compounds with proven antioxidant activity, could be an effective supplement in the development of functional GF breads.

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Declaration of competing interest

The authors declare that there are no conflicts of interest regarding

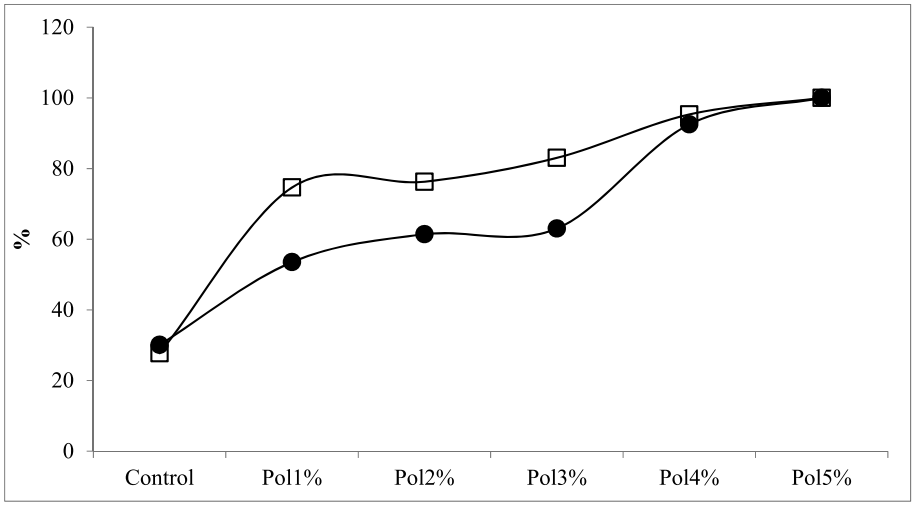

Fig. 2. Relative percent variation of absolute peak area of selected volatile compounds according to pollen addition in gluten-free breads. Pyrazinamide (□), 2-Acetylfuran (▲).

the publication of this paper.

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