



Thermal stability of nutraceuticals in bread enriched with bee products

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ABSTRACT

Over the last years, the rise of disease cases caused by different reasons have enforced people to live healthier and give attention to a diet with a significant effect on body health. Thus, there is a considerable interest in developing healthy food products. Bee pollen and bee bread (fermented bee pollen) are apicultural products composed mainly of flower pollen from plant species, and used in both traditional medicine and human nutrition for thousands of years due to its nutritional and physiological properties and recognized therapeutic and disease-preventing functions. Both bee pollen and bee bread are natural sources of carbohydrates, crude fibers, proteins and lipids as well as minor components such as phenolic compounds.

In the last few years, research has focused on the study of novel raw materials and natural compounds that could be included in bread formulations in order to enhance health-promoting properties and the overall quality since bread is a common food all over the world in the daily diet. Thus, given the nutritional characteristics of bee pollen and bee bread, its application on breads has potential to enable the obtention of a healthier product as well as widen the possible applications of both bee pollen and bee bread.

In this study, enriched bread samples with bee pollen and bee bread have been evaluated for nutritional properties such as protein, fat, sugars and, according to the results, enriched breads presented an increase in these parameters compared to traditional bread. In addition, the enrichment of breads with both bee products also showed an increase in their total phenolic and flavonoid contents, which was reflected by a significant increase in the antioxidant activity. The addition of these two products also affected properties such as water content, odor, colour, and overall appearance in bread, reflecting those differences in the physical properties of bread such as specific volume, colour, and texture. The change in the appearance of enriched breads also created differences in terms of consumer preferences. Sensory evaluation revealed, among all bread samples, the preference for bread enriched with 3% of bee bread, followed by 1% bee bread. The same pattern was observed for pollen enriched bread's. Besides, the microbial activity tests showed that the use of both bee products had no major negative effect on food safety in breads.

Consequently, this study shows that bee pollen and bee bread products have potential for usage in functional foods, with no modification in the basic properties of the enriched product.

Keywords: Bread; bee pollen; bee bread; functional foods; physico-chemical properties

RESUMO

Nos últimos anos, o aumento de casos de doenças com causas diversas obrigou as pessoas a viver de maneira mais saudável e prestar mais atenção a uma dieta que tenha um impacto significativo na saúde. Assim, existe um interesse considerável no desenvolvimento de produtos alimentares saudáveis. O pólen apícola e o pão de abelha (pólen apícola fermentado) são produtos apícolas compostos principalmente por pólen com origem em plantas de diversas espécies, sendo usados na medicina tradicional e na nutrição humana há milhares de anos devido às suas propriedades nutricionais e fisiológicas, bem como pelas propriedades terapêuticas na prevenção de certas doenças. Ambos os produtos são fontes naturais de hidratos de carbono, fibras, proteínas e lipídios, além de outros compostos minoritários como os compostos fenólicos.

Nos últimos anos, observa-se um atenção focada na pesquisa de novas matérias-primas e compostos naturais com potencial para incluir em formulações de panificação, a fim de aumentar as propriedades promotoras da saúde e no geral a sua qualidade, uma vez que na dieta diária, o pão é um alimento comum. Considerando as características nutricionais do pólen e do pão de abelha, a sua aplicação na panificação reveste-se de elevado potencial podendo contribuir para a obtenção de um produto mais saudável, ao mesmo tempo que alargar as possíveis utilizações destes produtos da colmeia.

Neste estudo, avaliou-se a qualidade de pão enriquecido com pólen apícola e pão de abelha através da identificação dos parâmetros nutricionais tais como proteínas, gorduras e açúcares, verificando-se um incremento desses parâmetros em relação ao pão tradicional. Paralelamente, o enriquecimento dos pães resultou num aumento dos teores de compostos fenólicos e flavonóides, o que se refletiu num aumento significativo da atividade antioxidante. A adição destes dois ingredientes funcionais afetou também o teor de água, o odor, a cor e aparência geral do pão, refletindo-se nas propriedades físicas do pão, como volume específico, cor e textura.

A mudança na aparência dos pães enriquecidos também foi apreciada pelos consumidor, verificando-se pelo painel de provadores que o pão mais apreciado foi o pão enriquecido com 3% de pão de abelha, seguindo-se o enriquecido com 1%. Para o pão enriquecido com pólen verificou-se também o mesmo padrão de preferência em função da percentagem. Os testes de atividade microbiana realizados mostraram que o uso de ambos produtos apícolas não induz efeitos negativos na segurança alimentar dos pães.

Consequentemente, este estudo demonstra que produtos com pólen apícola e o pão de abelha apresentam potencial para uso como ingrediente em alimentos funcionais, melhorando as suas características sem comprometer as propriedades básicas.

Palavras-chave: pão, pólen apícola, pão de abelha, alimentos funcionais, propriedades físico-químicas

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TABLE OF CONTENTS

ABSTRACT	ii
RESUMO	iv
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
CHAPTER 1	1
INTRODUCTION	1
1.1. Apiculture and hive products	2
1.2. Bee pollen and bee bread	3
1.2.1. Botanical origin	5
1.2.2. Physico-chemical properties and nutrition values	5
1.2.2.1 Water content	6
1.2.2.2. Proteins and amino acids	6
1.2.2.3. Total carbohydrates	7
1.2.2.4. Lipids	7
1.2.2.5. Polyphenols	8
1.2.2.6. Vitamins	8
1.2.2.7. Minerals	9
1.2.3. Functional properties and usage	9
1.2.3.1. Antimicrobial activity	10
1.2.3.2. Antioxidant activity	10
1.2.3.3. Anti-inflammatory activity	11
1.2.3.4. Anti-inflammatory activity	11
1.2.3.5. Anti-allergic activity	11
1.2.3.6. Anti- atherosclerosis activity	12
1.3. Functional foods	12
2.4. Aim and objectives	13

CHAPTER 2.....	14
MATERIALS AND METHODS	14
2.1. Chemicals and reagents.....	15
2.2. Bee pollen and bee bread samples	15
2.3. Palynological analysis.....	16
2.4. Nutritional analysis	16
2.4.1. Water content	16
2.4.2. Ash content.....	16
2.4.3. Protein content	16
2.4.4. Lipid content	16
2.4.5. Fiber content.....	17
2.4.6. Total carbohydrates and energy	17
2.4.7. Sugar profile analysis.....	17
2.5. Phenolic compounds	18
2.5.1. Extraction	18
2.5.2. Total phenolic compounds	18
2.5.3. Total flavonoid content	19
2.6. Antioxidant activity	19
2.6.1. DPPH free radical scavenging activity	19
2.6.2. Reducing power activity	19
2.7. Phenolic composition analysis by LC-ESI-MS ⁿ	20
2.8. Enriched breads with bee pollen and bee bread	21
2.8.1. Bread preparation	21
2.9. Bread characterization	21
2.9.1. Specific volume.....	21
2.9.2. Colour.....	22
2.9.3. Texture Profile Analysis (TPA)	22
2.10. Microbial analysis.....	22
2.10.1. Sampling plan.....	22
2.10.2. Preparation of samples	23
2.10.3. Total viable counts (TVC)	23
2.10.4. Yeasts and moulds (Y&M); DRBC (ISO 21527-1/2:2008).....	23
2.10.5. Spores of sulfite-reducing clostridia (SRC)	24

2.11. Sensory evaluation.....	24
2.12. Statistical analysis.....	25
CHAPTER 3.....	26
RESULTS AND DISCUSSION.....	26
3.1. Palynological analysis.....	27
3.2. Nutritional analysis.....	28
3.2.1. Water content.....	31
3.2.2. Ash content.....	31
3.2.3. Lipid content.....	32
3.2.4. Protein content.....	32
3.2.5. Fiber content.....	33
3.2.6. Total carbohydrate and energy.....	33
3.2.7. Sugar profile.....	34
3.3. Total phenolic and flavonoid content.....	35
3.4. Antioxidant activity.....	37
3.5. Bioactive compounds profile.....	38
3.6. Bread properties.....	48
3.6.1. Specific volume.....	48
3.6.2. Colour analysis.....	48
3.6.3. Texture profile analysis.....	52
3.7. Microbial analysis.....	55
3.7.1. Total viable counts, PCA, pour plate (ISO 4833-2:2013).....	55
3.7.2. Yeasts and Moulds (Y&M); DRBC (ISO 21527-1/2:2008).....	56
3.7.3. Spores of sulfite-reducing clostridia (SRC) (adapted from ISO 15213:2003)	57
3.8. Sensory evaluation.....	59
CHAPTER 4.....	61
CONCLUSIONS AND FUTURE PERSPECTIVES.....	61
4.1. Conclusion.....	62
4.2. Future perspective.....	63
REFERENCES.....	64

LIST OF TABLES

Table 1. Bee pollen/bread composition and nutritional requirements.	6
Table 2. Ingredients used in bread and their proportions.	21
Table 3. Relative frequency of the main pollen types (%)* found in the bee pollen and bee bread samples.	28
Table 4. Nutritional parameters of enriched breads and crude ingredients (bee pollen and bee bread, flour and yeast) (% of dry weight).	30
Table 5. Sugar profile for flour, yeast, raw bee pollen and bee bread samples with control and fortified bread samples (in g/100 g).	35
Table 6. Phenolic and phenylamide compounds of the raw bee pollen (mg/g).	40
Table 7. Phenolic and phenylamide compounds of the enriched bread with bee pollen (mg/g).	42
Table 8. Phenolic and phenylamide compounds of the raw bee bread (mg/g).	45
Table 9. Phenolic and phenylamide compounds of the enriched bread with bee bread (mg/g).	47
Table 10. Crust and crumb colour of control and enriched bread samples.	51
Table 11. Crumb texture parameters of enriched bread samples.	54
Table 12. Crust texture parameters of enriched bread samples.	54
Table 13. Total viable counts (log ₁₀ CFU/g) in bee pollen and bee bread added breads, in raw dough (immediately before cooking), cooked bread (immediately after cooking), and 3 and 5 days after being cooked.	56
Table 14. Yeast counts (log ₁₀ CFU/g) in bee pollen and bee bread added breads, in raw dough (immediately before cooking), cooked bread (immediately after cooking), and 3 day and 5 days after being cooked.	57
Table 15. Spores of sulfite-reducing Clostridia (SRC) counts (log ₁₀ CFU/g) in bee pollen (BP) and bee bread (BB) added breads, in raw dough (immediately before	

cooking), cooked bread (immediately after cooking), and 3 and 5 days after being cooked.
..... 58

LIST OF FIGURES

Figure 1. General view of bee pollen (A) and bee bread (B).	4
Figure 2. Potential therapeutic properties of bee pollen and bee bread, and their bioactive components.....	10
Figure 3. A) Bee pollen and B) bee bread sample.....	15
Figure 4. Microscopic view of some pollen types seen in bee pollen and bee bread samples. <i>Castanea sativa</i> (A); <i>Plantago</i> sp. (B); <i>Rubus</i> sp. (C); <i>Crepis capillaris</i> (D); <i>Cytisus striatus</i> (E); and <i>Jasione montana</i> (F).	27
Figure 5. Total phenolic (TPC) (A) and total flavonoid content (TPC) (B) of raw bee pollen and bee bread.	36
Figure 6. (A) Total phenolic (TPC) and (B) total flavonoid content (TPC) of enriched breads with bee pollen and bee bread.....	36
Figure 7. Antioxidant capacities of raw bee pollen and bee bread: (A) DPPH radical scavenging activity and (B) reducing power activity.	37
Figure 8. Antioxidant capacities of enriched breads with bee pollen and bee bread: (A) DPPH radical scavenging activity and (B) reducing power activity.	38
Figure 9. Specific volume of the bread samples.....	48
Figure 10. Images of baked sample slices of control and functionalized breads with bee pollen and bee bread.	50
Figure 11. A) Total viable counts (TVC) of bee pollen added bread samples, B) Total viable counts (TVC) of bee bread added bread samples, C) Yeast counts of bee pollen added bread samples, D) Yeast counts of bee bread added bread samples, E) Spores of sulfite-reducing clostridia (SRC) counts of bee pollen added bread samples, F) Spores of sulfite-reducing clostridia (SRC) counts of bee bread added bread samples.	59
Figure 12. Sensor analysis profile of enriched bread with bee pollen and control group.	60

Figure 13. Sensor analysis profile of enriched bread with bee bread and control group.
..... 60

LIST OF ABBREVIATIONS

AACC	American Association for Clinical Chemistry
AOAC	Association of Official Agricultural Chemists
aw	Water activity
BB	Bee bread
BC	Before Christ
BP	Bee pollen
CFU	Colony-forming unit
CID	Collision-induced dissociation
CNBP	Chronic non-bacterial prostatitis
CPPS	Chronic pelvic pain syndrome
DAD	Diode-array detection
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRBC	Dichloran rose bengal chloramphenicol
EC ₅₀	Half maximal effective concentration
ESI-MS	Electrospray ionisation-mass spectrometry
GA	Gallic acid
GAE	Gallic acid equivalent
HPLC	High-performance liquid chromatography
ISA	Iron sulphite agar
ISO	International Organization for Standardization
kcal	Kilocalorie
LC	Liquid chromatography
LOQ	Limit of quantification
<i>m/z</i>	Mass-to-charge ratio
NF	Nuclear factor
PCA	Plate count agar
PP	Parallelepiped
PW	Peptone water

QE	Quercetin equivalent
RI	Refractive index
SRC	Spores of sulfite-reducing clostridia
TFC	Total flavonoid content
TNF	Tumor necrosis factor
TPA	Texture profile analysis
TPC	Total phenolic content
TVC	Total viable counts
UPLC	Ultra performance liquid chromatography
WHO	World health organization
Y&M	Yeasts and Moulds

CHAPTER 1

INTRODUCTION

1.1. Apiculture and hive products

Apiculture (beekeeping) is one of the oldest hunter-gatherer examples in human history [1]. It is estimated that the history of mankind and bees dates back to 7000 BC. In present, we have a lot of historical and even ancient information about bees and honey. In rock drawings in Europe, Asia, and Australia, in many civilizations such as ancient Egypt, Mesopotamia, and Ancient Greece, bee products have been used especially, honey. When a king or a general dies in a war, they used honey or beeswax in order to preserve the body. In Ancient Egypt and Sumer, they offer honey to their gods as worship and used honey for healing with different formulas [2, 3]. Apiculture, is an activity tendency and colonization of bees in return to harvest. This return is usually honey, but some other bee products (e.g bee pollen, bee bread, royal jelly, beeswax, bee poison or propolis) or bees can be (e.g queenbees or colonies for pollination) [4]. With the help of technology, it can be said bee products have enormous nutritional value. Bee products are rather precious for human consumption due to its diverse effects on health. They mostly characterized by antimicrobial activity, expediting tissue regeneration, immune-stimulating effect [5, 6], increase the adenosine triphosphate level [7].

Honey is one of the oldest food and the best known product of bees that mainly contains fructose and glucose sugars but also comprises proteins, minerals, vitamins, phenolic compounds which are beneficial for health [8]. Honey bees visit hundreds of flowers and collect flower nectars to make honey [9]. Vitamins like B₃, B₅, folic acid, aroma, flavor and color of the honey is depends substantially on the plant origin and geographical region [10, 11]. Today, there is an increasing demand for using honey with the aim of its healing properties. Honey is utilized for its therapeutic purposes, for skin infections by virtue of ulcers, wounds, burns and bedsores [12]. Honey has an inhibitory effect property on not only bacteria but viruses and parasites [13]. Furthermore, honey has antioxidant activity and its antioxidant property depends on the vegetal source of nectar, seasonal and environmental factors [14]. Antioxidant properties of honey provided flavonoids (apigenin, pinobanksin, pinocembrin, kaempferol, etc.), vitamins (thiamine, riboflavin, α -tocopherol, ascorbic acid, etc.), enzymes (glucose oxidase, catalase, peroxidase, etc.) and compounds such as organic acids. Also, honey has been reported to activate the immune system and to protect against cancer and metastasis [15].

Royal jelly is a bee product that produced by 5-15 days old worker bees secretions from their hypopharyngeal and mandibular glands [16]. In the early stages of

their life, all of the larvae feed with royal jelly, after 2 days, worker bees continue to feed with honey and pollen mixture while the queen bee continues to feed on royal jelly in her lifetime. Nutrients in royal jelly and time of exposure make queen bee fertile on the contrary of workers and live longer (4-5 years) and larger comparing to worker bees [17]. Royal jelly is nowadays used in diet due to its high nutritional value.

Bee venom was used as a traditional medication for different occasions like back pain, rheumatism [18], it has both anti-arthritis and anti-rheumatic arthritis effect [19], anti-asthmatic [20], anti-cancer [21], suppressing effect angiogenesis and tumor angiogenesis [22], pain relief action [23], there are also researches that bee venom therapy can be use to cure Parkinson's disease [18], and Alzheimer's disease [24].

Propolis is a complex mixture of mainly beeswax, honey and resin that bees collect from trees and bushes and use as a construction and also protection material for their nests [25, 26]. Propolis is used for protection of bee colonies from diseases and microbial infections, provide hive walls and combs put larvae to protect by covering with propolis and to supply a hygienic environment, and to inhibit infections spreading from dead insects inside the beehive [27]. The use of propolis dates back to the very old times. While Ancient Egyptians mummify their dead, Greek and Roman doctors used propolis for antiseptic and wound healing. Mentioned products also known with its antimicrobial [26], antioxidant [26], anti-viral, anti-inflammatory, and anti-protozoal activities [28].

Another bee product called beeswax is synthesized by bees and used to raise offspring and to build honeycombs as a primary structure material, for the storage of honey and pollen [3]. Beeswax is used since ancient times by humans for different purposes, e.g from building firearms to illumination or as a religious symbol [3]. But in the present, it is used especially in the cosmetics sector to develop different products (e.g, sunscreens) [29].

1.2. Bee pollen and bee bread

Pollen, as Ancient Egyptians called 'life-giving dust', used as medicinal purposes even in 1200 [30]. Pollens are carried by the wind or insects, this is called pollination. Honey bees it has pollen from plants in their pollen basket, and they agglutinate it with honey, nectar and secretion enzymes thus it becomes bee pollen [31]. Bee pollen is significantly important for bees and their nutrition in regard to survival of bee colony, by reason of only protein resource of bees [7]. It has carbohydrates, proteins, lipids, crude

fibers, all of the 20 proteinogenic amino acids [32], polyphenols [33, 34], and different affects on health such as fighting against cancer [35], antiprostatitis [36], lowering heart diseases [37], liver and gut improvement [36]. It has been used in ancient times like honey but for newly times, human started to use bee pollen after Second World War because of its nutritional value [36].

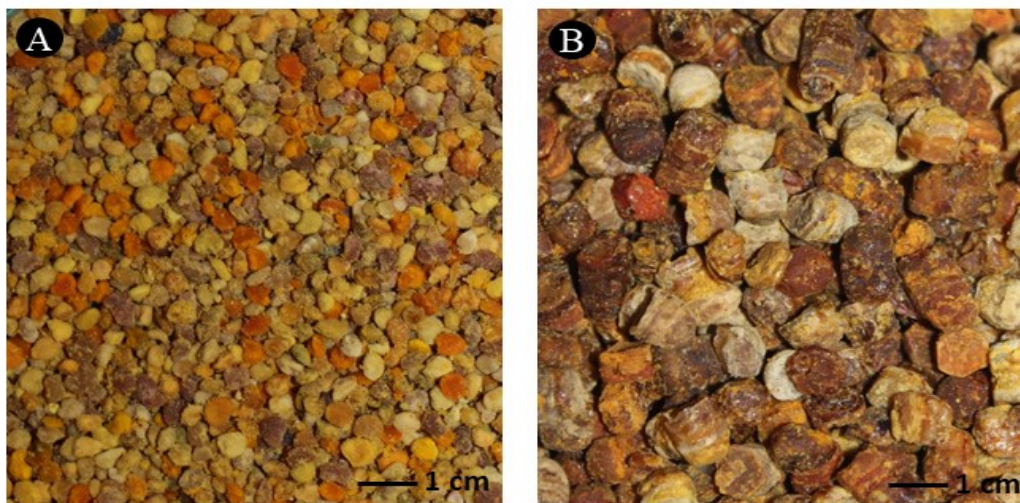


Figure 1. General view of bee pollen (A) and bee bread (B).

Bee bread is also a bee hive product, basically composition at the beginning is same with bee pollen, honey, nectar and digestive enzymes, but bee bread subject to lactic acid fermentation in the honey comb, with that process bees preserves the products and it gains more nutritional value [30]. With his process outer layer of pollen broke down, bee bread is more bioavailable [38]. Composition of bee bread shows a trifle difference from pollen, for instance, acidity is quite higher in bee bread depending on the presence of lactic acid and a high amount of vitamin K [6]. Bee bread also has essential amino acids [39], minerals, fatty acids [38], carotenoids [7]. The protein content is higher in pollen comparing to bee bread on the other hand bee bread has higher amino acid content [7]. On the contrary to their benefits, they are not in vast amounts. Both bee pollen and bee bread are important for bees and therefore colonies because of their nutritional content. As in old times, all these products attract humans' attention because of the present and possible benefits today, since they contain many of the compounds used in the interactions required in the human body. Parallel to that, people aware of the importance of diet on a daily basis day after day, and they tend to change their daily nutrition by improving them with natural products. Bee products such as bee pollen and bee bread can play a pioneering role in this field. Since pollen and bee bread obtained small quantities

and precious, it would be more rational to use it as food additive. Bread plays a key role in daily basis nutrition in nearly every country and it studied the most [40].

1.2.1. Botanical origin

Bee pollen is comprised of flower pollens (male reproductive cells) collected by bees and unite with their secretions while bee pollen loads comprise of agglutination of pollen by bees. Bee pollens have a different color, appearance, and chemical composition. Monofloral pollen loads contain pollen grains from a unique plant species, whereas hetero floral pollen loads contain more than one plant species [41].

It is really important to determine the botanical origin of bee pollen. Because people who have especially suffer from hay-fever can show allergic reaction to bee pollen bee bread and other bee products as well in case of consumption [42]. Determining the botanical origin of bee pollen is also used to determine the commercial quality and biological activity of bee pollen and bee bread [42]. Origin of bee pollen; have an effect on physicochemical properties such as color, water activity, sugar content, biochemical properties such as total phenolic and phenolic composition, amino acid, lipid, and protein content, and biological properties such as antioxidant, anti-radical and microbiological activity [27].

1.2.2. Physico-chemical properties and nutrition values

Lately, there is an increasing demand for natural products, especially for bee products. Bee pollen and bee bread are used for different purposes due to their nutrition and medical properties. It is determined pollens from different plant species have more than 250 substances with different biological activities [43]. These substances include proteins, amino acids, carbohydrates, lipids, fatty acids, flavonoids, enzymes, vitamins, and micronutrients. These micro and macronutrients will be discussed in detail afterwards.

There are six components required for the metabolic functioning of humans and animals. Three of these, namely carbohydrate, fat, and protein, provide energy to the living thing, while the other three are vitamins, minerals, and water do not provide energy but yet are important for living health indeed. The examined bee products, defined by their high nutritional value that provides benefits for health. Bee pollen and bee bread can be an ideal natural supplementary food that provides energy, repair tissues, and boosts

the immune system by virtue of simple sugars, high content of amino acids, monounsaturated fatty acids [39]. Nutritional values of bee pollen and bee bread are given in Table 1.

Table 1. Bee pollen/bread composition and nutritional requirements.

Main components	Bee pollen	Bee bread	% RDI^a for 15 g	References
Carbohydrates	15-60%	24-35%	5-22%	[44], [45], [42]
Proteins	10-40%	14-37%	1-4.6%	[46], [42], [47]
Lipids	1-13%	6-13%	0.1-4%	[42], [48]
Flavonoids	0.2-2.5%	Nd	0.03%	[43], [49]
Vitamin	0.02-0.7%	Nd	2-70%	[50], [42], [7]
Nucleic acid	0.6-4.8%	Nd	-	[51]

^a Campos et al. [42]; Required Daily Intake (RDI) requirements are according to Reports of the Scientific Committee for Food, 2010, average RDI values have been assumed.

^b no data.

1.2.2.1 Water content

The water content of bee pollen and bee bread is a factor that has an affect on other components' quantities, the water content of bee pollen and bee bread is a factor has affect on other components' quantities, furthermore markedly important for the activity of microorganisms and storage conditions [7]. The water content of bee bread is similar to bee pollen and constitutes about 6% of dry weight [48].

1.2.2.2. Proteins and amino acids

Consumption of both bee pollen and proteins that form an important part of bee bread has various benefits on human health and well-being. It has other positive effects on the organism due to important biological activities such as anti-bacterial, antioxidant, immunostimulant, antithrombotic and anti-inflammatory [52]. Proteins, which are important components of bee pollen, contain as much protein as expected (10 to 40% by weight of dry pollen). Bee pollen is characterized by very high protein content, but this ratio is strongly dependent on the plant origin of the pollen, for example, bee pollen of pine origin contains 7% protein, while date palm origin bee pollen contains 40% protein [53]. In addition, protein content affects the taste value of pollen. Bee pollen includes protein fractions such as albumins (35%), globulins (19%), glutelins (19%), prolamins (22%), and other proteins (including enzymes —5.3%) [51].

On the other hand, another protein-rich bee product bee bread contains approximately 20% protein [48]. The protein content of the bee bread may be different from the bee pollen. This difference is due to the reduction of certain proteins into amino acids by enzymes during the conversion of bee pollen into bee bread by the fermentation process [7]. Bee bread contains relatively low protein content than bee pollen, however, proteins of bee bread is more digestible. Because the exine layer can break and it releases pollen content during the fermentation process [27]. Besides, bee pollen and bee bread are rich in amino acids. Protein concentration in bee pollen is higher than protein concentration of bee bread whereas in most of the cases amino acid content is higher than bee pollen and bee bread contains all essential amino acids [48, 53]. In the bee pollen, only about 1/10 of the total protein comes from free amino acids, and the amino acid, such as proline, glutamic acid, and aspartic acid, constitutes the main amino acids found in the pollen [32, 54].

1.2.2.3. Total carbohydrates

Carbohydrates play numerous important roles in living beings. Monosaccharides are the main resource of metabolism while if polysaccharides stores energy and act as structural components. Carbohydrates are the main components present in bee pollen and bee bread. Carbohydrates in dry weight of bee pollen and bee bread respectively on average 15-60% and 24-35% [48, 55]. The carbohydrate content of both products depends on botanical origin, factors such as processing and pollen collection. The carbohydrate content of both products depends on botanical origin, factors such as processing and pollen collection. In both bee pollen and bee bread, fructose, glucose, and sucrose contain about 90% of all low molecular sugars, while the proportion of different sugars varies from plant to plant [48, 54]. These products also contain other carbohydrates such as polysaccharides, oligosaccharides, and dietary fiber. Oligosaccharides and polysaccharides are also important substances that help regulate various biological functions in bee pollen. Furthermore, these compounds are considered to be characteristic markers for distinguishing the botanical origin of bee pollen [32].

1.2.2.4. Lipids

Bee pollen lipid content is various and changes between 1-13%, and the bee pollen contains mainly polar and neutral oils and a small amount of fatty acids, sterines,

and hydrocarbons [54]. There are significant differences in lipid content and composition of bee pollen depending on plant origin. According to the researches, there are a total of 20 fatty acids in bee pollen: fatty acid (C18: 3) and fatty acid (C16: 0) are the main fatty acids [32]. Bee pollen frequently has unsaturated fatty acids. The bee pollen contains saturated fatty acids such as myristic acid, stearic acid, and palmitic acid, as well as unsaturated fatty acids such as oleic acid, linoleic acid, and α -linolenic acid, and the total content of saturated and unsaturated fatty acids is 62% and 38%, respectively [56, 57]. Fatty acids of bee pollen vary depending on the type of pollen. In addition, bee pollen contains the following essential fatty acids: linoleic acid, γ -linolenic acid and arachidonic acid [43]. The lipid content of the bee bread is about 7% and strongly depends on the plant origin of the pollen. Bee bread contains the same fatty acids as bee pollen, but both have a lower fatty acid content than fresh bee pollen collected by bees [48].

1.2.2.5. Polyphenols

Polyphenols are secondary metabolites found in plants, therefore they abundantly present in plant originated bee pollen and bee bread as well [58]. They have different actions such as protection of plants against diseases, pathogens, UV. They are usually indicated in two groups as flavonoids and phenolic acids. The molecule of phenolic acid consists of an aromatic ring and a carboxyl group, it is likely to distinguish benzoic acids, phenylacetic acids, and cinnamic acids while the cinnamic and benzoic acids have the significant importance. Flavonoids are the most substantial group of polyphenols present in bee pollen and bee bread and they are characterized by diphenylpropan ring system. Flavons, flavonols, flavanons, flavanes, anthocyanins, isoflavons, and chalcones are seven groups of flavonoids [59]. Flavonoid in pollen include kaempferol, quercetin, and isorhamnetin content is 0.2–2.5% and chlorogenic acid in phenolic acids with the range of 0.2% [7]. They are important for human health with the action of preventing asthma, cancer, diabetes, cardiovascular problems, and aging [60] yet mammalian cells can not synthesized them.

1.2.2.6. Vitamins

Vitamins are a diverse group of organic compounds with varying biochemical roles that serve as regulators of mineral metabolism or cell and tissue growth and differentiation, and some vitamins are precursors of enzyme cofactors [55]. Vitamins

show different biological functions (e.g antioxidant activity) and chemical compositions. Bee pollen (approximately 0.7%) and bee bread are rich in vitamins and vitamin content depends on the origin of the pollen as in other components [7]. Bee pollen contains different vitamins, mainly vitamin B: ascorbic acid (C), β -Carotene (provitamin A), tocopherol (vitamin E), niacin (B3), pyridoxine (B6), thiamin (B1), riboflavin (B2), folic acid and biotin (H) [54]. Bee pollen also contains polyphenolic compounds, flavonoids, and phenolic acids, and the content of the polyphenols in bee pollen (2-5% w/w) varies according to the type of plant. Bee bread also contains the following vitamins: vitamins C, B, K, P, and E [48]. Depending on the season of the year, the content of certain vitamins varies among different types of pollen. The concentration of vitamins naturally found in bee pollen and bee bread is difficult to predict because of the presence of many factors or the complexity of the matrix.

1.2.2.7. Minerals

Bee pollen and bee bread are a rich source of minerals such as potassium, phosphorus, magnesium, calcium, sodium, sulfur, iron, copper, manganese, zinc, chromium, nickel and selenium [61]. The mineral content of these bee products may vary depending on the type of pollen. The presence of sufficient macro and microelements in human beings is very important for the proper conduct of many different metabolic processes. Minerals are necessary for the proper regulation of metabolic pathways and physiological processes [62]. Adequate intake is essential for the maintenance of homeostasis, cell protection, functionality, and health. The main mineral in bee pollen is potassium (about 60% of the total mineral content) and magnesium is present in about 20%, sodium and calcium 10% in bee pollen [54].

1.2.3. Functional properties and usage

Apicultural products have been used in phytotherapy for a long time and also in diet due to their positive health effects. Today comes into prominence because of the presence of bioactive compounds associated with the beneficial properties of bee products on health. In particular, bee pollen and bee bread stand out as a functional food for human consumption due to the high compound content that has health-promoting effects such as essential amino acids, antioxidants, vitamins, and lipids.

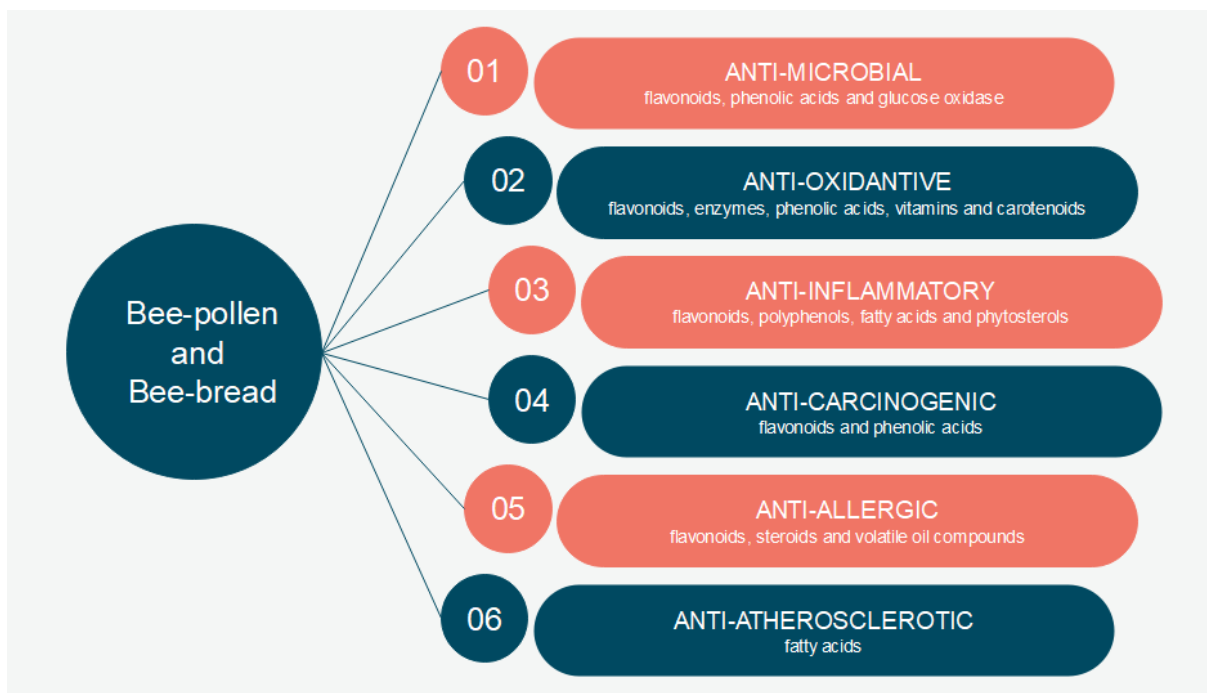


Figure 2. Potential therapeutic properties of bee pollen and bee bread, and their bioactive components.

1.2.3.1. Antimicrobial activity

The antibacterial effect of pollen is probably related to the property of glucose oxidase enzyme, an enzyme produced by honey bees, and is added to pollen when bee pollen granules are formed [54]. Another microbiological activity of bee pollen and bee bread has been shown to be associated with phenolic acids and flavonoids [63]. These products not only have antibacterial activity but also have anti-fungal and anti-viral activity, for example in the study showed antifungal effects on different microscopic fungal and yeast species (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Geotrichum candidum*, and *Rhodotorula mucilaginosa*) of bee pollen [64]. Antibacterial activity of bee pollen against 13 different pathogenic bacteria was investigated and reported to be effective against all bacterias [65]. In addition, the antibacterial effect of bee bread against both gram-positive and gram-negative bacteria was investigated and it was shown that bee bread was effective against both bacterial species [66].

1.2.3.2. Antioxidant activity

Oxidative stress results from an increase in reactive oxygen species in the cells. Reactive oxygen species are produced by both exogenous and endogenous factors.

Increased levels of reactive oxygen species contribute to the cell membrane or DNA damage and cause a variety of cellular responses that cause chronic inflammation [67]. Reactive oxygen species play a role in the development of numerous diseases such as cardiovascular, diabetes, degenerative and neoplastic disorders [68]. The antioxidant effects of bee pollen and bee bread may depend on the activity of antioxidant enzymes as well as the content of plant metabolites such as phenolic substances, carotenoids, vitamins, and glutathione. Flavonoids are the most abundant and most studied polyphenol class of low molecular weight. The free radicals of flavonoids present in bee pollen prevent reactive oxygen species from being cleansed and thus become mutagenized [69].

1.2.3.3. Anti-inflammatory activity

Inflammation is a physiological response to tissue or cell damage. Previous studies have shown that bee pollen and bee bread have anti-inflammatory activity due to their abundant polyphenolic compounds and have a positive effect on many cells (i.e. macrophages, T cells, B cells, NK cells, hepatocytes, mast cells, basophils, neutrophils, eosinophils) [69-71]. In previous studies, bee pollen relieves chronic non-bacterial prostatitis / chronic pelvic pain syndrome (CNBP / CPPS) [72], local pain relief and prevention of platelet aggregation [73], as well as flavoring agents of bee pollen (e.g anethole) are considered to be a potent inhibitor of tumor factor necrosis factor (TNF) induced nuclear factor (NF) - κ B activation [74].

1.2.3.4. Anti-inflammatory activity

Some studies have shown that bee pollen has more or less anti-mutagenic properties in certain types of cancer [29, 75]. Anticarcinogenic activities result from their antioxidant properties, i.e, suppression or inactivation of the formation of oxygen reactive species [69, 76]. Inhibition effects of bee pollen extracts on different cell lines such as K - 562 cells, leukemia cells, or prostate cancer PC - 3 cells have been reported [77, 78]. In another study, bee bread was found to show toxicity on different human tumor cells (MCF-7, NCI-H460, HeLa and HepG2) [79].

1.2.3.5. Anti-allergic activity

Although pollen is known to be allergic in most cases, the anti-allergic effect of pollen has been reported [59]. Extract treatment rich in phenolic compounds can prevent

allergic reactions [32]. It protects the organism, by inhibiting the histamine-releasing that is responsible for the degranulation of mast cells to any allergic reaction [80].

1.2.3.6. Anti- atherosclerosis activity

Atherosclerosis is one of the important health problems that causes death in developed countries [54]. Studies have shown that bee pollen and bee bread have hypolipidemic activity by reducing the content of total lipids, triacylglycerol, and cholesterol. Intake of bee pollen and bee bread in patients with heart conditions can lower the blood viscosity which reduces atherosclerotic plaque formation [61].

1.3. Functional foods

In recent years, people are more aware of the importance of the relationship between diet and health, hence, they have turned their consumption preferences to more functional foods in terms of nutritions. Studies showed consumers have different reasons to consume functional foods like desire to have a healthy lifestyle, protection from diseases, increasing quality of life [81]. Functional food is nutrient enriched with specific nutrients hence has high nutritional or physiological benefits to the consumer [82]. Consumer profiles also vary according to demands for their necessities. It is important to be part of the consumer's daily intake and the provision of their demand in this regard bread is a good example to follow and modifications, since it is used frequently all along the countries and it is an important part of the daily diet. In the previous studies, bread has enriched with different substances like fruits, nuts, seeds [83], *Brassica* species for its antioxidant, anti-inflammatory properties [84], iron enriched for iron deficiency anaemia [85], carob legume to increase traditional breads' protein content [86], bee pollen to increase the nutritional value of gluten-free bread and use a substitute to eliminate the other used gluten-free substances undesirable properties [40].

The antimicrobial effect of substance in a food is also important, as mentioned in the antimicrobial activity section before, in the previous studies, the antimicrobial effect of bee pollen and bee bread has been studied, results are promising in terms of antibiotic-resistance bacteria, and not only for the pharmaceutical industry but for functional food consumers and producers as well.

2.4. Aim and objectives

This study has attempted to investigate the bee pollen and bee bread samples as a natural added ingredient in order to boost and improve the nutritional value of traditional bread, designing a healthy bread. For that, different amounts of multifloral bee pollen and bee bread were added in the bread manufacturing under different conditions. The physicochemical properties as well as the thermal stability of the main nutraceuticals were evaluated and compared at different stages, from the raw materials and after cooking through:

- Water content, ash content, protein content, lipids, fiber content, carbohydrate contents analyzed by AOAC procedure;
- Phenolic compounds analysis through UPLC-ESI-MSⁿ.
- Bread parameters evaluation such as: specific volume, texture, crumb grain, color, and leavening properties.
- Microbiological analysis.
- Evaluation of sensorial parameters through desirable flavour and visual appeal using a sensorial panel.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals and reagents

Ethanol, methanol, sodium phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), potassium ferrocyanide ($\text{C}_6\text{FeK}_4\text{N}_6 \cdot 3\text{H}_2\text{O}$), trichloroacetic acid ($\text{C}_2\text{HCl}_3\text{O}_2$), acetonitrile ($\text{C}_2\text{H}_3\text{N}$), formic acid (CH_2O_2), sulfuric acid (H_2SO_4), diethyl ether ($\text{C}_4\text{H}_{10}\text{O}$), sodium hydroxide (NaOH), hydrochloric acid (HCl), petroleum ether, and gallic acid were purchased from Fisher Scientific (Pittsburgh, PA).

Folin-Ciocalteu's reagent, Kjeldahl catalyst tablets, and acetic acid glacial (CH_3COOH) were purchased from Panreac Applichem (Barcelona, Spain). Iron(III) chloride (FeCl_3), and aluminum chloride (AlCl_3) were purchased from Acros Organics (Pittsburgh, PA).

2,2-diphenyl-1-picrylhydrazyl (DPPH) (D913-2), quercetin, *p*-coumaric acid, chrysin, fructose, glucose, saccharose, trehalose, turanose, maltulose, and maltose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol was purchased from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system (TGI pure system, Houston, TX, USA).

2.2. Bee pollen and bee bread samples

This study was conducted with bee pollen and bee bread collected in the northeast region of Portugal, during spring 2019. Bee pollen was collected through bee pollen traps, while bee bread was removed from the inside of the comb manually. Both bee pollen and bee bread were crushed, homogenized, lyophilized, and maintained in a desiccator. For the following analysis, raw bee pollen and bee bread samples were named as BP and BB, respectively.

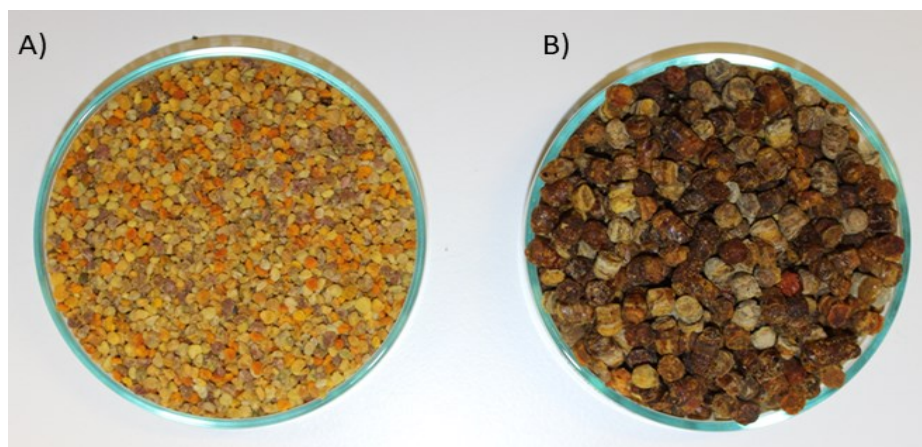


Figure 3. A) Bee pollen and B) bee bread sample.

2.3. Palynological analysis

About 1 gram of BP and BB samples previously mixed and homogenized were placed in a vial with distilled water, allowed to soften slightly and vigorously vortexed. Then, 200 µl of the mixture was removed from the mixture and centrifuged for 5 min at 1000 rpm. After the centrifugation, the pellet was exposed to acetolysis in accordance to the procedure of Louveaux et al [87], with the recommended modifications from W. Von der Ohe et al. [88]. Counts and identifications were implemented in an optical microscope. More than 1,200 grains per preparation were counted, attending the criteria of Vergeron [89]. This work was made in collaboration with *LabApis^{UTAD}*.

2.4. Nutritional analysis

2.4.1. Water content

The water content was determined in 2 g of sample using, the PMB Moisture Analyzer (Kingston, Milton Keynes, U.K.) The analysis was performed in triplicates.

2.4.2. Ash content

The ash content of the samples was estimated through the incineration of the samples (2 g) in a muffle (Optic Ivymen System) at 550 ± 5 °C according to the AOAC 923.05 Method [90]. The analysis was performed in triplicate.

2.4.3. Protein content

The protein content estimation was performed through Kjeldahl nitrogen determination using a copper catalyst in 1 g of of each sample [91], in Kjeldahl steam distillation unit (Pro-Nitro A, Selecta, Spain) following the AOAC 920.87 Method. For the conversion of nitrogen levels to protein, the factor 6.25 was used. The analysis was performed in triplicates.

2.4.4. Lipid content

For the lipid content, 2 g of sample was treated with petroleum ether in a Soxhlet apparatus for 4 hours. The petroleum ether extract was evaporated under reduced pressure to dryness, the residues were weighed and the lipid content was expressed as a mass percentage. The analysis was performed in triplicates [92].

2.4.5. Fiber content

Total dietary fiber content was performed using the fiber assay kit (Megazyme K-TDFR-200A, Wicklow, Ireland) following the AOAC 991.43 method [93]. Briefly, 1 g of dried sample was subjected to 12.5 mL of phosphate buffer, and then 12.5 μ L of α -amylase solution was added and kept at 100 °C for 30 min. After this process, the samples were allowed to cool to room temperature and the pH was adjusted to 7.5 with 2.5 mL 0.275 N NaOH solution. Then 25 μ L of protease solution was added and left for 30 min incubation at 60 °C. The samples were allowed to cool again and then adjusted to pH 4.5 with 2.5 mL 0.325 N HCl solution. Then, 50 μ L of amyloglucosidase solution was added and incubated at 60 °C for 30 min. After incubation, the samples were allowed to cool. The sample was then precipitated with 95% EtOH (40 mL) preheated to 60 °C. After filtering the samples, 0.1 g of celite and 10 mL of 95% EtOH were added sequentially. Following this procedure, the samples were washed successively with three 5 mL portions of 78% EtOH, two 2.5 mL portions of 95% EtOH and two 2.5 mL portions of acetone. The sample was dried overnight in a 105 °C air oven. The crucible and celite weights were then removed to determine the weight of the samples. Then, the residue from the sample was analyzed by AOAC 920.87 method using N x 6.25 as the conversion factor. The second residual sample was burned at 525 °C for 5 h and cooled in a desiccator. Crucible and celite weights were removed to determine ash.

2.4.6. Total carbohydrates and energy

The total carbohydrate and energy values of the samples were calculated using the following equations [92]:

$$\text{Total carbohydrates} = 100 - (\text{g ashes} + \text{g proteins} + \text{g lipids})$$

$$\text{Energetic value (kcal)} = 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g lipids}).$$

2.4.7. Sugar profile analysis

The sugar extraction procedure used was done accordingly to the previously described method [92]. Sugars were 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) a degasser (Smartline 5000), an autosampler (Jasco AS-2057) and a RI detector (Knauer Smartline 2300). Data

acquisition and remote control of the HPLC system was done by ClarityChrom software (Knauer, Berlin, Germany). The chromatographic separation was obtained with a column Eurospher 100-5 NH2 (4.6 × 250 mm, 5 mm, Knauer) kept at 30 °C. The mobile phase was composed of acetonitrile/water, 80:20 (v/v) at a flow rate of 1.5 mL min⁻¹.

The sugar profile was obtained comparing the retention times of standard individual sugars solutions. The quantitative assays were achieved using external calibration in a concentration range of 0.4 to 40 mg/mL, based on the following equations: fructose ($y = 1,7219x + 0,4791$; $R^2 = 0,998$), glucose ($y = 1,5499x + 0,4068$; $R^2 = 0,999$), saccharose ($y = 1,8431x + 0,3705$; $R^2 = 0,999$), trehalose ($y = 1,7372x + 0,1899$; $R^2 = 0,999$), turanose ($y = 1,4418x - 0,1113$; $R^2 = 0,999$), maltulose ($y = 1,3132x + 0,012$; $R^2 = 0,998$) and maltose ($y = 1,1554x + 0,0555$; $R^2 = 0,993$). The assay was performed in triplicate and the results were expressed as g/100 g.

2.5. Phenolic compounds

2.5.1. Extraction

For extraction, 2 g of the raw material and enriched breads samples were mixed with 40 ml of 80% ethanol/water, and stirred in magnetic stirrer for 6 hours at room temperature. The resulting mixture was filtered and the residue was re-extracted in the same conditions. After the second extraction, the solutions were combined, freeze-dried and stored -20°C [92].

2.5.2. Total phenolic compounds

The total phenolic content was determined by using the Folin-Ciocateau method [92], and gallic acid (GA) used as standard. An aliquot of 500 µL of 1 mg/mL ethanolic extract was mixed with 250 µL of Folin–Ciocalteu reagent. After 3 min, 1 mL of 20% sodium carbonate solution (Na₂CO₃) was added and the volume adjusted to 5000 µL with deionized water. The solution was heated for 10 minutes at 70°C, and after cooled in the dark. After 30 min, the absorbance was measured at 760 nm (Analytikjena 200–2004 spectrophotometer, Analytik Jena, Jena, Germany). The results were expressed as mg of GAE (gallic acid equivalent) per g dry weight sample. The assays were run in triplicates.

2.5.3. Total flavonoid content

The flavonoid content was determined through the method previously described [94]. An aliquot of 200 μL of 5 mg/mL ethanolic extract was mixed with 200 μL aluminum chloride solution (2% of AlCl_3 in 5% acetic/methanol), and the total volume completed to 5000 μL with 5% glacial acetic acid in methanol. After 30 min, the absorbance of the solution was measured at 415 nm. The results were expressed as mg of QE (quercetin equivalent) per g dry weight sample. All the determinations were performed in triplicates.

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging activity

DPPH free radical scavenging activities of crude and cooked samples from enriched breads were performed according to Brand-Williams, Cuvelier, and Berset (1995) with minor modifications. An aliquot (30 μL) of extract solution, with concentrations from 0.03-0.43 mg/mL, was added to 150 μL of DPPH 25 mg/L in 80% ethanol/water (the DPPH solution must be prepared daily). The absorbance was read at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.). The percentage of radical inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_{DPPH} - A_{Sample}) / A_{DPPH}] \times 100$$

The amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC_{50}) was achieved plotting the inhibition percentage against the extract concentration.

2.6.2. Reducing power activity

The Fe(III) reducing power activities of extracts was performed according to previously described method [95]. 0.25 mL ethanolic extract (1 mg/mL) of bee pollen was mixed with 1.25 mL phosphate buffer (0.2 mol/L, pH 6.6) and 1.25 mL 1% potassium ferricyanide, respectively. The mixture was incubated in a water bath at 50 $^{\circ}\text{C}$ for 20 min. Then 1.25 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm (Centurion K2R series) for 10 min. 1.25 mL of the mixture was taken, followed by 1.25 mL of distilled water and 0.25 mL of 0.1% FeCl_3 , and the absorbance was read at 700 nm. The same process was done for bee bread extract and digested samples. The

results were expressed as mg of GAE (gallic acid equivalent) per g dry weight sample. The assays were run in triplicates.

2.7. Phenolic composition analysis by LC-ESI-MSⁿ

For the analysis, 20 mg of the previously extracted samples were dissolved in EtOH/H₂O (80:20, v/v, 2 mL). The samples were filtered through a 0.45 µm membrane filter and kept at -20 °C until analysis.

A Dionex UltiMate 3000 ultra-pressure liquid chromatography instrument connected to a diode array and attached to a mass detector was used for LC/DAD/ESI-MSⁿ analyses (Thermo Fisher Scientific, San Jose, CA, USA). The analysis was conducted on a Macherey-Nagel Nucleosil C18 column (250 mm × 4 mm id; particle diameter of 5 µm, endcapped) and its temperature kept constant at 30°C. The conditions applied in the liquid chromatography were based on a previous work [96]; the flow rate was 1 mL/min, and the injection volume was 10 µL. The final spectra data were accumulated in the wavelength interval of 190-600 nm.

The LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, CA, USA) equipped with an ESI source was set in the negative ion mode with the following parameters: source voltage 5 kV; tube lens voltage, -20 V; capillary voltage, -65 V; capillary temperature, 325°C; and sheath and auxiliary gas flow (N₂) 50 and 10 (arbitrary units), respectively.

Mass spectra was acquired in a full range of 100–1000 *m/z*. For the fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID). The normalized collision energy of CID cell was set at 35 (arbitrary units). All data acquisition was gathered using the Xcalibur® software (Thermo Fisher Scientific, San Jose, CA, USA). The elucidation of the phenolic compounds was achieved by comparison of their chromatographic behaviour, UV spectra and MS information, to those of reference compounds. When standards were not available, the structural information was confirmed with UV data combined with MS fragmentation patterns previously reported in the literature.

Quantification was achieved using calibration curves for *p*-coumaric acid (0.00925-0.4 mg/mL; $y = 1.9 \times 10^7x - 12927$; $R^2 = 9.957$), quercetin (0.037-1.6 mg/mL; $y = 4 \times 10^6x - 10216$; $R^2 = 9.970$), kaempferol (0.037-1.6 mg/mL; $y = 4.3 \times 10^6x - 13567$; $R^2 = 9.981$), chrysin (0.0185-0.8 mg/mL; $y = 1.2 \times 10^7x - 51265$; $R^2 = 9.999$), and

naringenin (0.0185-0.8 mg/mL; $y = 8 \times 10^6x - 10998$; $R^2 = 9.976$). All compounds were quantified using the calibration curve of the structurally closest standard, and the final result was given in equivalent terms. Each value resulted from three different assays and is expressed as mg/g of sample.

2.8. Enriched breads with bee pollen and bee bread

2.8.1. Bread preparation

Breads were prepared using home-making bread machine (Tefal bread maker XXL, Windsor, Berkshire, UK). The selected bread making program included dough preparation (2 h 20 min) and baking (20 min). After baking, every bread was cooled at room temperature during 120 min before further analyses. All the ingredients were provided by a local bakery Delícias from Bragança, Portugal. The breads were prepared following the bakery recipe, with the proportions of each ingredient listed in Table 2. Three different bread formulations were produced for each type of bee product with addition of 3 different levels (1.0%, 3.0%, 5.0%) of supplementation flour basis. For the control bread, no extract was added.

Table 2. Ingredients used in bread and their proportions.

Ingredients (flour-based+ substitute)	Control	Bee pollen (1%)	Bee pollen (3%)	Bee pollen (5%)	Bee bread (1%)	Bee bread (3%)	Bee bread (5%)
Flour	520 g	514.8 g	504.4 g	494 g	514.8 g	504.4 g	494 g
Water	300 mL	300 mL	300 mL	300 mL	300 mL	300 mL	300 mL
Yeast	5 g	5 g	5 g	5 g	5 g	5 g	5 g
Salt	1.5 g	1.5 g	1.5 g	1.5 g	1.5 g	1.5 g	1.5 g
Substitute	-	5.2 g	15.6 g	26 g	5.2 g	15.6 g	26 g

2.9. Bread characterization

2.9.1. Specific volume

The specific volume of bread samples was measured according to the AACC with some modifications by following the rapeseed displacement method [97]. The rapeseed grains were placed inside the parallelepiped (PP) until it covered the PP, then weighed the quantity of the rapeseed on a scale. The rapeseed inside the PP was displaced to another container, the bread was placed inside the PP and covered with the rapeseed. The following equation applied after:

$\text{Vol (bread)} = \text{Vol (PP)} \times ((\text{weight of the rapeseed "inside" the PP}) / (\text{weight of the rapeseed that enters the PP}))$

2.9.2. Colour

The colour of bread samples were analysed with a portable colorimeter CR400 from Konica Minolta (Chiyoda, Toko, Japan). For the analysis, three breads slices of every formulation were cut and the measurements were performed in three different points on the bread crumb and side crust. The colour was recorded in the CIElab system, which consists of a lightness component (L^*) and two chromatic components, where the a^* value represents green ($-a$) to red ($+a$) and the b^* value represents blue ($-b$) to yellow ($+b$) colors [98]. The colorimeter was calibrated using a standard white plate as a blank.

2.9.3. Texture Profile Analysis (TPA)

For the texture analysis of the bread samples a Stable Micro Systems (Vienna Court, Godalming, UK) TA.XT Plus Texture Analyzer have used by following the described method by Carocho et al. [98] with a 5 kg load cell. A P/35 aluminum cylinder probe (35mm diameter) was used to performed a texture profile analysis (TPA) which is a typical test that simulates the chewing of the human mouth by performing two compressions of the matrix. Results from the analysis were processed via a macro in order to reach different dimensions of texture, like hardness, adhesiveness, springiness, cohesiveness, chewiness, and resilience. The results then analyzed through the Exponent program.

2.10. Microbial analysis

2.10.1. Sampling plan

For the microbial analysis of breads enriched with bee bread and bee pollen, samples were analysed from the raw dough (after the mixing process of the bread in the bread machine, before cooking), and from the cooked breads at three time points: immediately after cooking, 3 and 5 days after cooking. Samples were analysed in triplicate (from three different bread-making processes), and two repetitions were performed, in a total of 6 samples per treatment.

2.10.2. Preparation of samples

The preparation of samples for analysis followed the procedure described in ISO 6887-1:1999 (ISO, 1999) [99]. Samples were taken from different parts of the dough and breads under sterile conditions. Ten g of sample were weighed into a stomacher bag and 90 mL of peptone water (PW) were added. The samples were homogenized in a stomacher (Seward type 400, Italy), for 60 seconds at 300 units. Serial decimal dilutions were prepared: 1 mL of the initial suspension (10^{-1}) was diluted in 9 mL of PW (in 15 mL Falcons) to obtain 10^{-2} ; 1 mL of 10^{-2} into a new falcon containing 9 mL of PW to obtain 10^{-3} , and so forth.

2.10.3. Total viable counts (TVC)

The determination of Total Viable Counts (TVC) followed the procedure described in ISO 4833-1:2013 (ISO, 2013) [100]. Dilutions were inoculated in Plate Count Agar (PCA; BRAND, COUNTRY) by the pour plate technique, in duplicate: 1 mL of each suspension was pipetted into a plate and 15 mL of melted PCA were poured into the plate, homogenized and left to solidify. Plates were incubated at 30 °C for 72 h, in reversed position. Colonies were counted in the plates having between 15 and 300 colonies. The results were expressed as colony-forming units per gram of sample (CFU/g), using formula in below, as described in ISO 7218:2007 (ISO, 2007) [101]. The limit of quantification (LOQ) was 10 CFU/g.

$$N = \frac{\sum C}{V \times (n_1 + 0,1.n_2) \times d} \text{CFU/g}$$

where:

$\sum C$ = sum of colonies counted in all countable plates;

V = volume of inoculum inoculated in each plate;

n_1 = number of plates on which the first dilution was counted;

n_2 = number of plates on which the second dilution was counted;

d = dilution from which the first counts were obtained.

2.10.4. Yeasts and moulds (Y&M); DRBC (ISO 21527-1/2:2008)

The determination of Yeasts and Moulds (Y&M) followed the procedure described in ISO 21527:2008 (ISO, 2008) [102]. Dilutions were inoculated in DRBC (samples with $a_w > 0.95$) by the spread plate technique, in duplicate: 0.2 mL of suspension

was pipetted onto a plate containing 15 mL of the medium and spread with a disposable spreader, incubated at 25 °C for 5 days, in the upright position. Yeasts and moulds colonies counted separately after 2 and 5 days of incubation, respectively, in the plates having less than 150 colonies. Colonies were counted in the plates having between 15 and 150 colonies. The results were expressed as CFU/g, using formula given above. The LOQ was 50 CFU/g.

2.10.5. Spores of sulfite-reducing clostridia (SRC)

The determination of spores of sulfite-reducing clostridia (SRC) followed the procedure described in ISO 15213:2003 (ISO, 2003) [103], with some modifications. 5 mL of the initial suspension (10^{-1}) were transferred into a 50 mL Falcon (in duplicate) and dipped in a water bath at 80 °C for 10 min (counting from the moment the sample reaches 80 °C. Another Falcon with 5 mL of PW and a thermometer was used for temperature control.). After the heat-treatment, the suspension was immediately cooled in a cold water bath and 25 mL of Iron Sulphite Agar (ISA, HiMedia, Mumbai, India) were poured into the Falcon tube. When the medium was solid, the tube was filled with ISA to create anaerobiosis. The tubes were incubated at 30 °C for 24 h to 48 h (final counting at 48 h). The number of black spots obtained in the tubes was counted. The results were expressed as CFU/g, using formula given above. The LOQ was 2 CFU/g.

2.11. Sensory evaluation

An attribute sensory test using the hedonic rating to measure consumer responses to specific attributes was performed. This test was used to determine the reasons for the preference and rejection of the bread samples by assessing individual attributes. A panel composed of 15 consumers (4 men and 11 women ranging in age from 20 to 50 years) was asked to evaluate the seven samples and to rate their preference using a 9-point hedonic scale (1 = extremely dislike; 9 = extremely like). The attributes rated were the following: colour, taste, smell, texture, mouthfeel, and overall acceptance, and data were recorded at the same time by testers. Enriched bread samples were prepared the same day of the sensory evaluation. Once the loaves had cooled, the pieces of bread were cut in equal size, with a slice thickness of 3 cm for the tests, and they were all packed and a special code was given to each bread, in order to prevent the effect on users decision and to have objective results.

2.12. Statistical analysis

All analyzes were performed in triplicate and all data were denoted as mean \pm standard deviation (SD). The obtained data was analyzed using GraphPad Prism version 8 (San Diego, CA, USA). One-way analysis of variance and Tukey's multiple comparison test was conducted to see whether there is a statistical significance. $p < 0.05$ was considered as significant. Also, Pearson's correlation coefficients were calculated to ascertain the relationship between the tested parameters.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Palynological analysis

The identification of botanical origin was determined by pollen profile analysis. A total of 31 pollen types classified as dominant pollen, accompanying pollen, important pollen, and rare pollen were identified at the species, genus, or family level in BP and BB samples. All samples were classified as heterofloral due to the absence of any taxa with a relative frequency greater than 80% [54].

The relative predominance of the main pollen types found in the bee pollen and bee bread samples are shown in Table 3. For pollen analysis, in case of a pollen samples ratio 45% or above that pollen was named dominant, between 15- 45% accessory, and between 3-15% named isolated [104]. As can be seen in the table, dominant pollens were not detected in both samples, whereas *Cytisus striatus* was detected as accessory pollen for bee pollen, with a relative predominance of 20.7% and *Castanea sativa* pollen ratio was found to be 38.8% in the bee bread sample. The results are in accordance with previous studies, confirming that the pollen samples in bee pollen and bee bread samples were collected from the flora of northeastern Portugal [92].

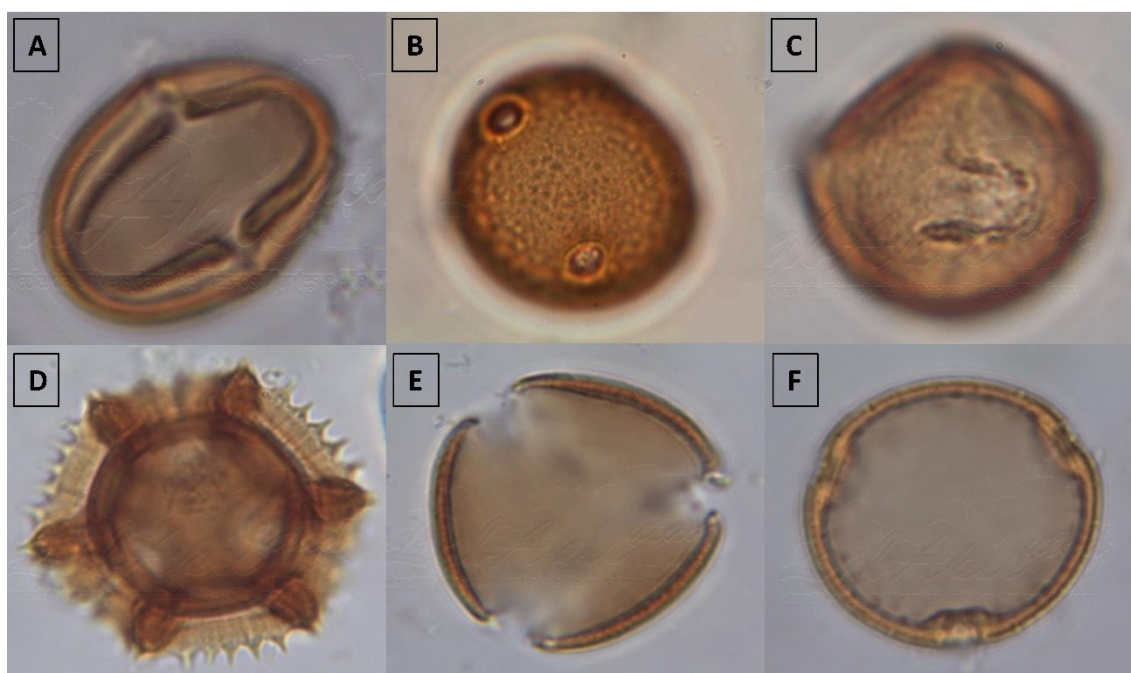


Figure 4. Microscopic view of some pollen types seen in bee pollen and bee bread samples. *Castanea sativa* (A); *Plantago* sp. (B); *Rubus* sp. (C); *Crepis capillaris* (D); *Cytisus striatus* (E); and *Jasione montana* (F).

Table 3. Relative frequency of the main pollen types (%)* found in the bee pollen and bee bread samples.

Family	Pollen type	Bee Pollen (%)	Bee Bread (%)
Aceraceae	<i>Acer</i>	0.4	-
Amaranthaceae	<i>Chenopodium</i> sp.	1.7	-
Apiaceae	<i>Thapsia vilosa</i>	0.8	0.1
Asteraceae	<i>Centaurea</i> sp.	0.1	1.4
	<i>Anthemis arvensis</i>	0.4	-
	<i>Crepis capillaris</i>	17.0	3.9
Boraginaceae	<i>Echium</i> sp.	0.6	2.6
Brassicaceae	<i>Raphanus raphanistrum</i>	0.9	4.3
Campanulaceae	<i>Jasione montana</i>	-	6.4
Cistaceae	<i>Cistus ladanifer</i>	0.4	0.2
	<i>Cistus</i> sp.	0.4	-
Crassulaceae	<i>Sedum</i> sp.	-	2.3
Ericaceae	<i>Erica</i> sp.	11.3	-
Fabaceae	<i>Lotus</i> sp.	-	0.2
	<i>Cytisus striatus</i>	20.7	6.9
	<i>Trifolium</i> sp.	0.3	3.7
	<i>Vicia</i> sp.	-	0.6
Fagaceae	<i>Castanea sativa</i>	-	38.7
	<i>Quercus</i> sp.	1.4	0.2
Lamiaceae	<i>Lavandula</i> sp.	-	0.6
	<i>Mentha</i>	0.1	0.2
	<i>Thymus</i> sp.	-	0.7
Liliaceae	-	1.7	4.5
Myrtaceae	<i>Eucalyptus</i> sp.	4.7	-
Papaveraceae	-	3.1	-
Plantaginaceae	<i>Plantago</i> sp.	8.5	1.9
Polygonaceae	<i>Rumex acetosa</i>	0.9	-
Rosaceae	-	2.6	-
	<i>Rubus</i> sp.	2.8	17.6
Salicaceae	<i>Salix</i> sp.	9.2	1.3
Vitaceae	<i>Vitis vinifera</i>	5.0	-
Classification		Heterofloral	Heterofloral

* > 45%: Dominant pollen; 16–45%: accompanying pollen; 3–15%: important pollen; < 3%: rare pollen.

3.2. Nutritional analysis

In the course of this work, traditional breads were enriched with different proportions of BP and BB raw samples. In this context, different nutritional parameters, such as moisture content, ash, lipids, proteins, fibers, and sugars profile, as well as total carbohydrate and energetic value were identified to determine the nutritional properties

of raw BP, BB and breads enriched with both bee products. The obtained results are given in Table 4.

The nutritional properties of bee products, and in this particular case, bee pollen and bee bread, are known and confirmed by previous studies to have high nutritional properties: bee pollen contains high levels of carbohydrates, protein and lipids while bee bread is known to have even higher nutritional value than bee pollen [105]. The use of bee products for human consumption is recommended due to their potential to compensate several nutritional deficiencies [106]. In order to observe a more precise effect of bee pollen and bee bread, the nutritional value of the flour was also evaluated, since the major component of the breads was flour.

Table 4. Nutritional parameters of enriched breads and crude ingredients (bee pollen and bee bread, flour and yeast) (% of dry weight).

Sample	Water content (%)	Ash content (%)	Lipid content (%)	Protein content (%)	Fiber content (%)	Total carbohydrates (%)	Energy(kcal/100)
Flour	3.8 ± 0.2 ^b	0.7 ± 0.0 ^b	1.8 ± 0.5 ^b	10.9 ± 1.15 ^b	5.1 ± 1.1 ^a	86.6 ± 1.3 ^b	406.5 ± 2.5 ^b
Yeast	9.2 ± 0.7 ^c	5.9 ± 0.1 ^c	1.2 ± 0.1 ^b	44.5 ± 0.46 ^c	12.7 ± 0.7 ^b	48.4 ± 0.4 ^c	381.8 ± 0.8 ^c
BP	14.9 ± 0.4 ^a	3.1 ± 0.1 ^a	5.7 ± 0.6 ^a	22.5 ± 0.96 ^a	6.0 ± 1.3 ^a	68.7 ± 1.5 ^a	415.8 ± 2.8 ^a
BB	13.0 ± 0.5 ^a	3.1 ± 0.0 ^a	5.4 ± 0.2 ^a	22.3 ± 0.61 ^a	4.2 ± 0.5 ^a	69.2 ± 0.5 ^a	414.3 ± 0.9 ^a
Control	34.6 ± 2.9 ^a	0.3 ± 0.1 ^a	0.1 ± 0.0 ^a	10.5 ± 0.2 ^a	1.3 ± 0.0 ^a	88.3 ± 0.9 ^a	451.1 ± 1.4 ^a
BP 1%	37.0 ± 0.4 ^a	0.4 ± 0.2 ^a	0.2 ± 0.1 ^{a,db}	11.9 ± 0.6 ^b	1.1 ± 1.2 ^a	87.7 ± 0.6 ^{a,d}	458.1 ± 3.8 ^b
BP 3%	37.4 ± 2.0 ^a	0.7 ± 0.3 ^b	0.4 ± 0.1 ^{b,e}	12.4 ± 0.6 ^b	1.9 ± 0.8 ^a	86.4 ± 0.9 ^b	459.3 ± 2.2 ^{b,e}
BP 5%	37.6 ± 0.4 ^a	0.5 ± 0.1 ^a	0.7 ± 0.2 ^c	12.7 ± 0.1 ^b	3.0 ± 0.6 ^{b,a}	86.7 ± 0.6 ^b	461.2 ± 0.9 ^{b,e}
BB 1%	37.6 ± 3.0 ^a	0.3 ± 0.1 ^a	0.1 ± 0.1 ^a	12.3 ± 0.1 ^b	1.7 ± 0.1 ^a	88.3 ± 1.6 ^{a,e}	459.9 ± 0.5 ^{b,c}
BB 3%	37.9 ± 2.5 ^a	0.3 ± 0.0 ^a	0.3 ± 0.4 ^b	12.6 ± 0.4 ^b	1.3 ± 0.2 ^a	87.1 ± 0.4 ^{c,db,e}	461.6 ± 2.2 ^{b,c}
BB 5%	38.4 ± 2.7 ^a	0.4 ± 0.1 ^a	0.5 ± 0.1 ^{c,c}	12.8 ± 0.2 ^b	0.7 ± 0.0 ^a	86.8 ± 0.3 ^{c,d,b}	462.4 ± 0.5 ^c

Results are expressed on the dry weight, except water content. Each value is the mean ± standard deviation, n = 3. In each column (for raw and cooked samples), different superscript letters (a–e) means significantly different (p < 0.05).

3.2.1. Water content

Water content is an important parameter for bee pollen and bee bread in terms of biological activity and also in the determination of other components in bee pollen and bee bread. A high level of water content can lead to the growth of microorganisms and thus resulting in the product degradation [107]. Some countries have established minimal requirements for the water content of commercial bee pollen, which should be no more than 6-8 % [54]. The obtained results from BP and BB, which ranged the 13-15%, were higher than the presented values, which might be the result of using different bee pollen and bee bread mixtures or water absorption capacity of bee pollen and its fermented derivative bee bread [108].

Amongst the bread samples, the lowest value was obtained from the control bread with 34.6 ± 2.9 % value, and the highest values for the enriched bread samples were 37.6 ± 0.4 % for BP5% and 38.4 ± 2.7 % for BB5%. In a previous study of gluten-free bread made with pollen, it was shown that as the pollen ratio increased, the moisture content decreased [109], however contrarily, according to our results, with the increase of the BP and BB add percentage, the moisture content of the breads also increased.

3.2.2. Ash content

The ash content is generally associated with the inorganic content of the product and can present different values depending on plant soil differences [110]. In this study, the obtained results for both raw BP and BB were 3.1 %, which is in accordance with the previously pollen quality criteria that established a limit of 6% [54]. In studies conducted with pollen collected from different countries, Portuguese bee pollen showed results between 1.7 ± 0.3 % and 2.4 ± 0.4 [110], and for bee bread from Portugal values were between 2.1 ± 0.0 % and 4.1 ± 0.0 % [92].

In all bread samples, ash content was found to decrease comparing to raw samples, which was expected due to other substance content in bread. In our results, an increase in ash content was observed in all bread samples compared to the control bread, being the difference not statistically significant. Previous studies concerning the development of functional bread associates the ash content with overall acceptance and total colour difference [111].

3.2.3. Lipid content

According to the results given in Table 4, the lipid content for BP and BB samples were 5.7 % and 5.4 %, with no statistically significant ($p < 0.05$) difference between them. Previously, quality criteria established that the fat content of pollen should be not less than 1.5%, being the samples under study within that range [54]. According to Estevinho et al. [110] the lipid content of bee pollen samples collected from the north region of Portugal varied between 2.3-3.3%, and in a previous study by Tomas et al. [92] bee bread rate was stated as 4 g/100 to 17 g/100.

Our results showed that the bread supplementation with bee pollen and bee bread increased the lipid content. The highest lipid content was observed in BP5% (0.7%) and BB5% (0.5%), as expected, while control bread has showed the lowest result ($0.1 \pm 0.0\%$). The lipid content of the flour was determined to be $1.8 \pm 0.5\%$. On the other hand, the addition of bee pollen and bee bread in different ratios in enriched breads were showed a proportional increase in lipids. From highest to lowest, values were aligned: BP5% > BB5% > BP3% > BB3% > BP1% > BB1% > Control. In a previous study conducted with different ratios of bee pollen, bread samples showed slightly higher values than control bread, therefore, it may be said the raw and cooked lipid content of the samples were quite similar to our results.

3.2.4. Protein content

Bee pollen and bee bread are characterized by high protein content. In this study, samples consisting of different bee pollen and bee bread samples and breads enriched with these products were analyzed in terms of total protein content, and the obtained results are given in Table 4.

The protein content of raw BP was $22.5 \pm 1.0\%$ and BB $22.3 \pm 0.6\%$. The results are in accordance with the previously proposed quality standards that established a total protein content in pollen not less than 15% [54]. The presence of bee pollen increased the protein value of the functional breads and increased compatibly with the ratio of bee pollen. Amongst the breads enriched with bee pollen, the highest value was observed at BP5% ($12.7 \pm 0.1\%$) as expected, the same tendency was also observed in the breads enriched with bee bread BB5% ($12.8 \pm 0.2\%$). On a study conducted by Conte et al. [109], an increase in the protein content in gluten-free breads fortified with pollen was

observed. Similarly, other previously studies, found that yogurt supplemented with bee pollen also showed an increase of the protein content [112].

3.2.5. Fiber content

The content in fiber for the raw samples can be seen in Table 4, with the highest value found for BP, with $6.0 \pm 1.3\%$, followed by BB with $4.2 \pm 0.5\%$. On the study conducted by Herbert and Shimanuki [113], fiber content of bee pollen collected from different USA geographical regions ranged between 5.8-17.7 g/100 g.

Almost all of the bread samples showed higher fiber value comparing to control which was $1.3 \pm 0.0 \%$. For the fortified breads with bee pollen and bee bread, BP5% presented the highest for fibers with a value of $3.0 \pm 0.6\%$, followed by BP3% with a value of $1.9 \pm 0.8\%$.

There are some studies showing the fiber content of different breads and the one with refined wheat bread gave values of 2.7 g/100 g [114]. Therefore, it can be said that the addition of bee products particularly bee pollen and bee bread may increase the fiber content in a traditional bread, even applied in low percentages.

3.2.6. Total carbohydrate and energy

Both bee pollen and bee bread are acknowledged with their high carbohydrate content [92, 115]. Carbohydrates content in pollen are due mainly to the polysaccharides cell wall material in pollen, and in the case of bee bread, due to the honey addition during the storage process [116]. Also, previous studies found that the total carbohydrates content in the pollen may be linked to the plant from which the bees collect the pollen, and to its geographical origin [90].

Our results for raw materials were $68.7 \pm 1.5 \%$ for BP and $69.2 \pm 0.6 \%$ for BB. According to Estevinho et al. [110], the content of carbohydrates in pollen from Portugal ranged from 61 to 71 %. In the study conducted by Tomas et al., the carbohydrate content of bee bread from Portugal were found between $58 \pm 0.0 \%$ and 78 ± 0.0 [92].

For bread samples, control bread showed the highest value comparing to the fortified breads with $88.3 \pm 0.9 \%$. As bee pollen and bee bread ratio increased, a compatible decrease was observed in total carbohydrate ratios. BP5% fortified bread showed $86.7 \pm 0.6 \%$ and BB5%, $86.8 \pm 0.3 \%$ which were the lowest values amongst the others. In a previous study of Mounjouenpou et al. [116] functional breads prepared with

cereal brans, values differed between 80.1 % and 85.0 % for carbohydrate content, which was found to be higher in control bread when compared with functional breads. As the carbohydrate ratio of flour was 86.6 ± 1.3 %, the fortification with BP and BB may have caused a decrease in the carbohydrate content in the functional breads.

The energetic value of the food is related to its nutritional value [92]. Our analyses showed higher energetic values for both bee pollen and bee bread comparing to previous studies [115]. The energy value of the raw BP sample was found to be 415.8 ± 2.8 kcal/100 g, while the BB value was found to be 414.3 ± 0.9 kcal/100 g. Estevinho et al. [110] pollen studies presented values between 400.7 ± 0.4 kcal/100 g and 411.8 ± 0.6 kcal/100 g for Portuguese bee pollen, while Ferreira et al. [117] reported a energy value of 396.2 ± 0.4 kcal/100 g for Moroccan bee bread.

BP and BB used as flour reinforcement also increased the energy value of breads. Among the fortified breads, the lowest value was found to be BP1% with 458.07 ± 3.8 kcal/100 g as expected, while the highest value was found in BB5% with 462.4 ± 0.5 kcal/100 g value. Decreasing the flour by the time of increasing the flour substitute, may have caused the increase in the energetic value.

3.2.7. Sugar profile

Sugar profile of raw BP, BB and fortified breads is given in Table 5. The proposed quality standard for pollen established a sugar's content of not less than 40 g/100g of pollen [118]. According to the results, BP presented a high content on fructose (18.9 g/100 g), followed by glucose (15.5 g/100 g) and lastly, sucrose (9.4 g/100 g), while is the same pattern, BB showed a high content in fructose (25.0 g/100 g), followed by glucose (13.6g/100 g), and then maltulose (2.7 g/100 g). The reason behind the difference found in sucrose content when comparing BP and BB is correlated to BB lactic acid fermentation [119]. As reported by Bertoneclet al. [120], sugars found in pollen from Slovenia follow this order, glucose (28.5 ± 1.6), fructose (27.8 ± 1.3). Ferreira et al. [117], revealed a sugar content in Morocco bee bread such as fructose (11.8 g/100 g), glucose (5.7 g/100 g), and trealose (0.9 g/100 g) [121]. According to our records, the used yeast contained 14.0 g/100g of turanose, 1.2 g/100g of glucose, while in the flour no sugars were detected.

After the cooking process of breads, turanose was found as the major sugar for BP1% and BP3% fortified bread samples, except for sample BP5% and BB5%, which in

addition to turanose, also presented fructose. The pattern was followed in the breads enriched with bee bread. The increase in the turanose content after the cooking process can be correlated to yeast proliferation during fermentation, because the turanose content of raw yeast is much higher than flour, BP, and BB. Degradation of sugars may be linked due to non-enzymatic browning Maillard reaction were sugars react with amino acids [122].

Table 5. Sugar profile for flour, yeast, raw bee pollen and bee bread samples with control and fortified bread samples (in g/100 g).

Sample	Fructose	Glucose	Turanose	Maltulose	Maltose	Sacarose
Flour	-	-	-	-	-	-
Yeast	-	1.2 ± 0.2 ^a	14.0 ± 0.5 ^a	-	-	-
BP	18.9 ± 1.1 ^a	15.6 ± 1.0 ^b	2.3 ± 0.2 ^b	1.6 ± 0.1 ^a	1.1 ± 0.3 ^a	9.4 ± 0.5 ^a
BB	25.0 ± 0.0 ^b	13.6 ± 0.0 ^c	2.8 ± 0.4 ^b	2.9 ± 0.1 ^b	1.4 ± 0.2 ^a	-
Control	-	-	30.0 ± 0.7 ^{a,c}	-	-	-
BP1%	-	-	30.6 ± 0.8 ^{a,c}	-	-	-
BP3%	-	-	28.0 ± 1.0 ^a	-	-	-
BP5%	1.1 ± 0.5 ^a	-	24.4 ± 1.1 ^{b,d}	-	-	-
BB1%	-	-	31.6 ± 0.6 ^c	-	-	-
BB3%	-	-	23.1 ± 0.1 ^d	-	-	-
BB5%	1.9 ± 0.4 ^b	-	16.8 ± 0.8 ^e	-	-	-

Results are expressed on the dry weight of sample. Each value is the mean ±SD, n = 3. In each column (for raw and cooked sample), different superscript letters (a–e) means significantly different (p < 0.05). – : not detected.

3.3. Total phenolic and flavonoid content

Bee pollen and bee bread are characterized by their antioxidant properties, which are correlated with phenolic substances in their composition [123]. Phenolics are neutralizers of active oxygen species constituents recommended as health-promoting agents present highly in plant kingdom [124]. The antioxidant properties showed dependence on the plant origin, but also on the geographical origin and even on the collection time [123].

According to our results for raw material (Figure 5), BB showed the highest total phenolic content with 12.6 ± 1.1 mg GAE/g, followed by BP with a content of 6.8 ± 0.1 mg GAE/g. In a previous study, a high content in phenolic compounds was reported,

ranging from 13 mg GAE/g to 20 mg GAE/g, while bee bread ranged from 14 ± 1 up to 84 ± 1 mg GAE/g [92], which are in accordance with our results [123].

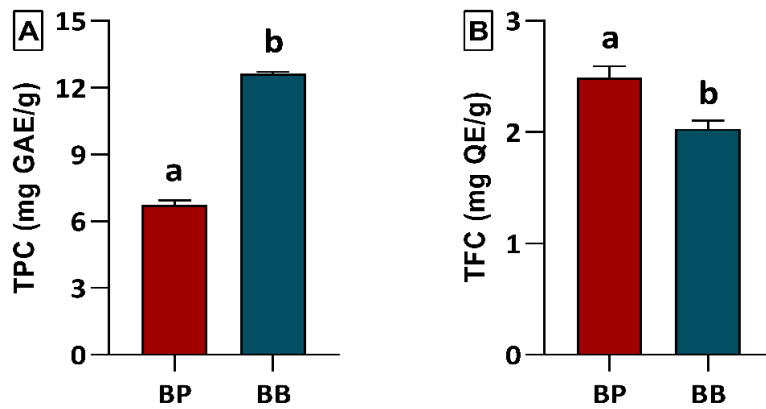


Figure 5. Total phenolic (TPC) (A) and total flavonoid content (TFC) (B) of raw bee pollen and bee bread.

When it comes to breads (Figure 6), control (0.1 ± 0.0 mg GAE/g) showed the lowest total phenolic content as expected. When functional breads enriched with bee pollen and bee bread were compared, the highest rate was shown for BB5% with 0.5 ± 0.0 mg GAE/g, followed by BP5% with 0.4 ± 0.0 mg GAE/g, which in accordance with previous studies where an increase in the phenolic content of bee products fortified was observed [125]. Although phenolic compounds were present in the enriched bread, a decrease in content was observed when comparing with the raw samples, which can be attributed to the application of heat during bread-making process.

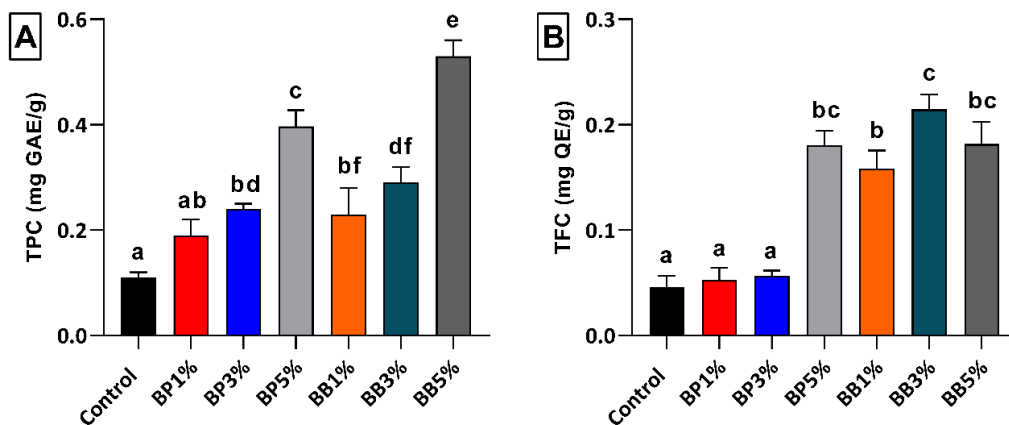


Figure 6. (A) Total phenolic (TPC) and (B) total flavonoid content (TFC) of enriched breads with bee pollen and bee bread.

On the other hand, for total flavonoid content in raw material, BP (2.5 ± 0.4 mg QE/g) showed a slightly high content of flavonoids when comparing to BB (2.0 ± 0.2 mg

QE/g). As reported by earlier studies, the flavonoid content of bee pollen ranged from 4.5 mg CAE/g to 7.1 mg CAE/g [126], and bee bread samples from Portugal ranged from 11 ± 1 mg QE/g to 91 ± 2 mg QE/g [92].

The total flavonoid content was higher in the enriched breads when comparing with the control (0.1 ± 0.0 mg QE/g). As for TPC, similar pattern was observed, where the increase of the percentage of bee product increased the TFC. The highest value detected in BB3% with 0.2 ± 0.0 mg QE/g followed by BB5% with a value of 0.2 ± 0.0 mg QE/g. Although the BB5% sample contained more BB, it displayed a lower value in terms of total flavonoid content. This may be due to the degradation of flavonoid compounds in the BB5% sample with temperature during cooking.

3.4. Antioxidant activity

The antioxidant capacities of the raw BP, BB samples, as well as the enriched breads were measured by two different assays: DPPH radical scavenging and reducing power activity. The obtained results are given in Figures 7 and 8.

The radical scavenging activity of the BP sample was 0.20 ± 0.0 mg/mL, while for BB it was 0.19 ± 0.0 mg/mL, with no statistically significant difference between them ($p > 0.05$). The results for reducing power of raw BP and BB were 5.0 ± 0.1 mg GAE/g and 2.7 ± 0.2 mg GAE/g, respectively. These results are in agreement with previously reported studies for bee pollen and bee bread [92, 127] which means addition of bee products namely bee pollen and bee bread may increase the antioxidant activity of a bread which has traditional recipe.

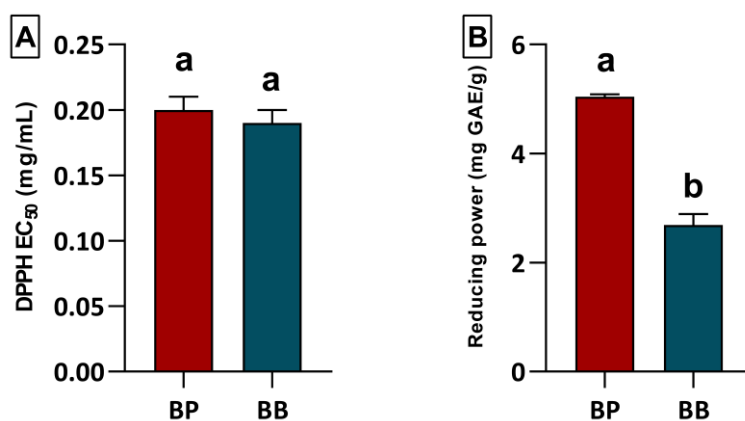


Figure 7. Antioxidant capacities of raw bee pollen and bee bread: (A) DPPH radical scavenging activity and (B) reducing power activity.

Addition of BP and BB to bread at different ratios resulted in a significant increase in radical scavenging and reducing power activities compared to the control group. According to the results, while the DPPH radical scavenging of the control bread was 0.81 ± 0.0 mg/mL, the addition of BP changed the results to 0.47 ± 0.0 mg/mL, 0.40 ± 0.0 mg/mL and 0.33 ± 0.0 mg/mL for samples BP1%, BP3%, and BP5%, respectively. For the breads enriched with BB, radical scavenging values were found as 0.55 ± 0.0 mg/mL (BB1%), 0.47 ± 0.0 mg/mL (BB3%) and 0.31 ± 0.0 mg/mL (BB5%). Differences for the results between control and enriched breads were noticeable and these results were statistically significant. Reducing power values of bread samples enriched with BP ranged from 0.2 ± 0.0 mg GAE/g to 0.3 ± 0.0 mg GAE/g, while BB enriched bread results ranged from 0.1 ± 0.0 mg GAE/g to 0.3 ± 0.0 mg GAE/g, both showing higher reducing power values than the control. Furthermore, in general, BP and BB enriched bread samples showed quite close reducing power activity profile to each other.

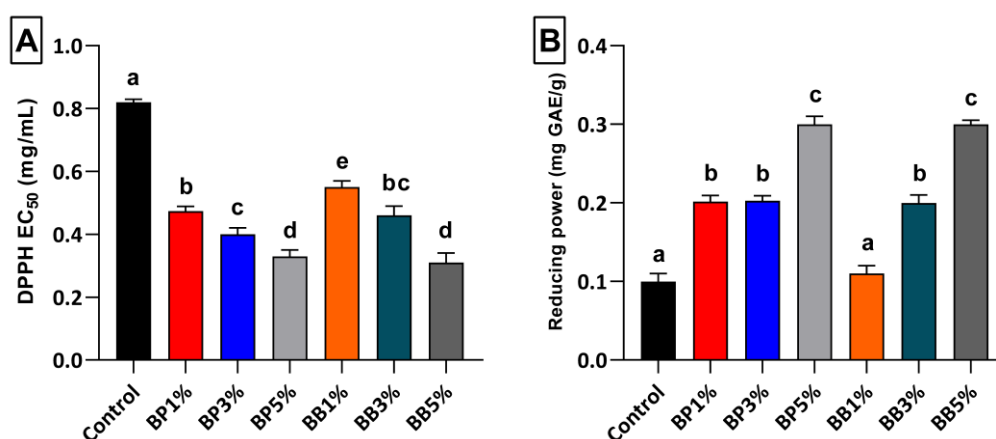


Figure 8. Antioxidant capacities of enriched breads with bee pollen and bee bread: (A) DPPH radical scavenging activity and (B) reducing power activity.

3.5. Bioactive compounds profile

The phenolic composition of raw BP, BB and enriched bread samples were characterized by LC/DAD/ESI-MSⁿ, in the negative ion mode. The chromatographic profile recorded at 280 nm allowed the identification and quantification of bioactive compounds, which included phenolic compounds, mostly flavonols, but also phenylamides (Table 6 and 7).

The analytical approach used in this work allowed the identification of 23 and 20 different bioactive compounds in BP and BB samples, respectively. In both bee products, flavonol derivatives such as quercetin, kaempferol, isorhamnetin and herbacetin

glycosides were the main compounds. Also, phenylamides were another family of bioactive compounds found in samples and at higher concentrations than the total amount of phenolic compounds. Previously, studies demonstrated that both phenolic compounds and phenylamides exist in both bee products [79, 128, 129].

Table 6. Phenolic and phenylamide compounds of the raw bee pollen (mg/g).

Peak	tr (min)	λ_{\max} (nm)	[M-H] ⁻ m/z	MS ⁿ (% base peak)	Proposed compound	Raw BP
1	7.54	257, 353	625	MS ² : 301 (100), 300 (99), 445 (85), 271 (18)	Quercetin- <i>O</i> -diglucoside ^{a,c,h}	0.07 ± 0.0
3	8.50	272, 326sh, 353sh	639	MS ² : 315(100), 477(70); MS ³ : 300, 301, 287	Methyl herbacetin- <i>O</i> -dihexoside ^{a,c,d}	0.16 ± 0.0
4	9.68	265, 348	609	MS ² : 285 (100), 429 (49)	Kaempferol- <i>O</i> -dihexoside ^{a,d}	0.02 ± 0.0
5	11.62	255, 353	609	MS ² : 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin- <i>O</i> -pentosyl-hexoside ^{a,e}	0.13 ± 0.0
6	12.13	265, 348	593	MS ² : 284 (100), 285 (73), 429 (93)	Kaempferol- <i>O</i> -rutinoside ^{a,b,f}	0.02 ± 0.0
8	12.59	256, 354	463	MS ² : 301 (100)	Quercetin-3- <i>O</i> -glucoside ^{a,b,f}	0.02 ± 0.0
9	13.38	267, 347	593	MS ² : 285 (100)	Kaempferol- <i>O</i> -hexosyl-deoxyhesoside ^{a,k}	0.02 ± 0.0
11	14.22	254, 347	447	MS ² : 301 (100)	Quercetin-3- <i>O</i> -rhamnoside ^{a,b,e}	0.49 ± 0.0
12	12.49	255, 354	563	MS ² : 519 (100); MS ³ : 315 (100)	Isorhamnetin-3- <i>O</i> -malonyl glucoside ^{a,h}	0.06 ± 0.0
13	14.83	277, 311	301	MS ² : 283 (100), 286 (40)	Hesperetin ^{a,b}	0.03 ± 0.0
14	15.46	255, 355	563	MS ² : 283 (100), 286 (40); MS ³ : 315 (100)	Isorhamnetin- <i>O</i> -malonyl-hexoside ^{a,i}	0.01 ± 0.0
15	16.22	264, 341	431	MS ² : 285 (100)	Kaempferol-3- <i>O</i> -rhamnoside ^{a,c}	0.04 ± 0.0
16	16.73	295, 230	630	MS ² : 468 (100), 494 (100), 358 (7); MS ³ : 315 (100)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ^{7/0} -tricaFFEoylspermidine ^{a,d,g}	0.81 ± 0.1
17	18.27	299, 308	478	MS ² : 358 (100), 332 (12), 145 (5)	<i>N</i> ¹ -acetyl- <i>N</i> ⁵ , <i>N</i> ^{7/0} - <i>di</i> - <i>p</i> -coumaroylspermidine ^{a,f}	0.37 ± 0.0
18	18.99	296, 314	614	MS ² : 478 (100), 479 (24), 468 (20); MS ³ : 342 (100), 332 (75)	<i>N</i> ¹ - <i>p</i> -coumaroyl- <i>N</i> ⁵ , <i>N</i> ^{7/0} -dicaFFEoylspermidine ^{a,e}	0.07 ± 0.0
19	19.46	295, 311	614	MS ² : 494 (25), 478 (100), 452 (71), 358 (22)	<i>N</i> ¹ - <i>p</i> -coumaroyl- <i>N</i> ⁵ , <i>N</i> ^{7/0} -dicaFFEoylspermidine (isomer) ^{a,e}	1.06 ± 0.0
20	21.36	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ^{7/0} - <i>tri</i> - <i>p</i> -coumaroylspermidine ^{a,e}	0.34 ± 0.0
21	22.37	294, 309	598	MS ² : 462 (100), 478 (39), 452 (34), 342 (14)	<i>N</i> ¹ , <i>N</i> ⁵ - <i>di</i> - <i>p</i> -coumaroyl- <i>N</i> ^{7/0} -caFFEoylspermidine ^{a,e}	0.84 ± 0.0
22	22.95	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ^{7/0} - <i>tri</i> - <i>p</i> -coumaroylspermidine ^{a,e}	1.96 ± 0.1
23	24.34	292, 305	582	MS ² : 342 (100), 436 (9), 462 (100)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ^{7/0} - <i>tri</i> - <i>p</i> -coumaroylspermidine (isomer) ^{a,e}	0.39 ± 0.0

24	26.70	295, 305	582	MS ² : 342 (100), 436 (9), 462 (100)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ - <i>tri-p</i> -coumaroylspermidine (isomer) ^{a,e}	3.44 ± 0.2
25	27.19	270	785	MS ² : 665 (100), 545 (14), 639 (13); MS ³ : 545 (100), 546 (33)	Tetracoumaroyl spermine ^{a,l}	0.29 ± 0.0
26	27.75	295, 308	612	MS ² : 492 (100); MS ³ : 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine ^{a,l}	0.37 ± 0.0
27	28.81	280, 307sh	785	MS ² : 545 (14), 639 (13), 665 (100); MS ³ : 545 (100), 546 (33)	Tetracoumaroyl spermine (isomer) ^{a,l}	2.01 ± 0.1
28	30.53	289, 306sh	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer) ^{a,l}	0.56 ± 0.1
29	32.19	293, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer) ^{a,l}	1.05 ± 0.1
30	34.25	299, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer) ^{a,l}	1.26 ± 0.0
The total amount of phenolic compounds						
The total amount of phenylamides						
						1.1
						14.8

Confirmed with: ^a MSⁿ fragmentation; ^b Standard; References: ^c [130]; ^d [131]; ^e [132]; ^f [133]; ^g [134]; ^h [135]; ⁱ [136]; ^k [137]; ^l [138]. Values expressed as mg of each compound/g sample. ND = not detected.

Table 7. Phenolic and phenylamide compounds of the enriched bread with bee pollen (mg/g).

Peak	tr (min)	λ_{\max} (nm)	[M-H] ⁻ <i>m/z</i>	MS ⁿ (% base peak)	Proposed compound	BP1%	BP3%	BP5%
1	14.22	254, 347	447	MS ² : 301 (100)	Quercetin-3-O-rhamnoside	0.01 ± 0.0	0.09 ± 0.0	0.10 ± 0.0
2	16.73	295, 230	630	MS ² : 468 (100), 494 (100), 358 (7); MS ³ : 315 (100)	N ¹ , N ⁵ , N ¹⁰ -tricaFFEoylspermidine	0.01 ± 0.0	0.04 ± 0.0	0.06 ± 0.0
3	19.46	295, 311	614	MS ² : 494 (25), 478 (100), 452 (71), 358 (22)	N ¹ - <i>p</i> -coumaroyl-N ⁵ , N ¹⁰ -dicaFFEoylspermidine (isomer)	0.01 ± 0.0	0.02 ± 0.0	0.10 ± 0.0
4	22.37	294, 309	598	MS ² : 462 (100), 478 (39), 452 (34), 342 (14)	N ¹ , N ⁵ - <i>di-p</i> -coumaroyl-N ¹⁰ -caFFEoylspermidine	0.01 ± 0.0	0.01 ± 0.0	0.08 ± 0.0
5	22.95	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p</i> -coumaroylspermidine	0.05 ± 0.0	0.09 ± 0.0	0.18 ± 0.0
6	24.34	292, 305	582	MS ² : 342 (100), 436 (9), 462 (100)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p</i> -coumaroylspermidine (isomer)	0.02 ± 0.0	0.04 ± 0.0	ND
7	26.70	295, 305	582	MS ² : 342 (100), 436 (9), 462 (100)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p</i> -coumaroylspermidine (isomer)	0.15 ± 0.0	0.10 ± 0.0	0.37 ± 0.0
8	27.19	270	785	MS ² : 665 (100), 545 (14), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine	0.07 ± 0.0	0.02 ± 0.0	0.01 ± 0.0
9	27.75	295, 308	612	MS ² : 492 (100); MS ³ : 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine	0.01 ± 0.0	ND	0.04 ± 0.0
10	28.81	280, 307sh	785	MS ² : 545 (14), 639 (13), 665 (100); MS ³ : 545 (100), 546 (33)	Tetracoumaroyl spermine (isomer)	0.00 ± 0.0	0.11 ± 0.0	0.19 ± 0.0
11	30.53	289, 306sh	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer)	0.02 ± 0.0	0.03 ± 0.0	0.07 ± 0.0
12	32.19	293, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer)	ND	0.04 ± 0.0	0.13 ± 0.0
13	34.25	299, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer)	ND	ND	0.12 ± 0.0
The total amount of phenolic compounds								
The total amount of phenylamides						0.01	0.09	0.10
						0.36	0.49	1.35

Values expressed as mg of each compound/g sample. ND = not detected.

Within the phenolic compounds, quercetin-*O*-diglucoside (m/z 625), quercetin-3-*O*-glucoside (m/z 463) and quercetin-3-*O*-rhamnoside (m/z 447), were detected in BP in higher concentrations than other flavonols, while methyl herbacetin-*O*-dihexoside (m/z 639), methyl herbacetin-*O*-rutinoside (m/z 623) and methyl herbacetin-3-*O*-hexoside (m/z 477) were detected in higher concentrations in the BB sample. Concerning the BP enriched breads, only quercetin-3-*O*-rhamnoside was detected, with values of 0.01 ± 0.0 mg/g (BP1%), 0.09 ± 0.0 mg/g (BP3%) and 0.10 ± 0.0 mg/g (BP5%). On the other hand, no phenolic compound was identified in breads with BB. The presence or absence of phenolic compounds in breads with BP and BB could be attributed to their degradation by temperature during baking [139]. Another important point regarding this situation is that phenolic compounds were found in raw samples at lower concentrations than other bioactive compounds, which were affected by proportion of BP and BB added to breads. In a previous study, it was reported that biscuits fortified with bee pollen had a significant reduction in phenolic compound after baking [140]. Phenylamides, another group that constitutes an important part of the bioactive compounds found in BP and BB, are plant secondary metabolites, which can be found in different parts of the plants such as pollen and seeds, and are associated with diverse functions such as protection against environmental stresses, sporopollenin formation, pollen protection against UV or pollination [141]. Hydroxycinnamic acids like *p*-coumaroyl, caffeoyl and feruloyl, are able to conjugate with phenylamide compounds such as spermidine or spermine. The formation of the amide linkage between a phenylamide and phenolic acid can occur at different positions: N^1 , N^5 and N^{10} positions [142]. Previously, was found that phenylamides are abundant compounds within Asteraceae, Fabaceae and Rosaceae plant families, the main pollen types found in the samples under study [129, 143]. Phenylamides were determined in both raw ingredients and cooked samples. Regarding the BP sample, the total amount of phenylamides was 14.8 mg/g. In particular, N^1 , N^5 , N^{10} -tri-*p*-coumaroylspermidine (m/z 582) and its isomer (m/z 582), N^1 -*p*-coumaroyl- N^5 , N^{10} -dicaffeoylspermidine (m/z 614) and tetracoumaroyl spermine (m/z 785) were detected at high concentrations in the BP. Also, in the BP enriched breads, these compounds were the main compounds observed. In the BP enriched breads, with the increase of percentage of BP added, an increase in the total amount of phenylamides was observed, presenting the following values, 0.36 mg/g (BP1%), 0.49 mg/g (BP3%) and 1.35 mg/g (BP5%). On the other hand, for the bee bread sample, the total amount of phenylamides was 6.8 mg/g, which was lower when comparing to bee pollen. Similar to BP, phenylamide compounds

such as N^1 , N^5 , N^{10} -tri-caffeoylspermidine (m/z 630), N^1 , N^5 , N^{10} -tri-p-coumaroylspermidine (m/z 582) and tetracoumaroyl spermine (m/z 785) were detected at high concentrations in both BB and BB enriched breads. Although the total amount of bioactive compound of BP was higher than the BB, when comparing with the enriched breads, the total amount of bioactive compounds was higher in breads with BB, with the exception of BB1%. This can be explained by the fact that bioactive compounds are affected by temperature and/or microbial activity during the fermentation of the dough.

Table 8. Phenolic and phenylamide compounds of the raw bee bread (mg/g).

Peak	tr (min)	λ_{max} (nm)	[M-H] ⁻ m/z	MS ⁿ (% base peak)	Proposed compound	Raw BB
1	8.51	272, 353sh	639	MS ² : 271 (10), 300 (34), 315 (91), 459 (100), 477 (11), 624 (20)	Methyl herbacetin- <i>O</i> -dihexoside ^{a,c,d}	0.26 ± 0.0
2	10.78	272, 353sh	623	MS ² : 299 (61), 300 (38), 314 (100), 315 (69), 459 (86), 477 (19)	Methyl herbacetin- <i>O</i> -rutinoside ^{a,e}	0.04 ± 0.0
3	11.73	255, 353	609	MS ² : 315 (100), 314 (47), 459 (51), 300 (20)	Isorhamnetin- <i>O</i> -pentosyl-hexoside ^{a,e}	0.07 ± 0.0
4	12.15	266, 349	593	MS ² : 284 (94), 285 (57), 431 (100), 447 (20)	Kaempferol-3- <i>O</i> -rutinoside ^{a,b,f}	0.01 ± 0.0
5	12.33		623	–	Quercetin derivative ^a	0.07 ± 0.0
6	12.64	256, 354	463	MS ² : 301 (100)	Quercetin-3- <i>O</i> -glucoside ^{a,b,f}	0.05 ± 0.0
7	13.64	270	477	MS ² : 315 (100), 462 (42), 300 (14); MS ³ : 300 (100)	Methyl herbacetin-3- <i>O</i> -hexoside ^{a,c}	0.03 ± 0.0
8	14.1	265, 347	447	MS ² : 285 (100), 284 (80)	Kaempferol- <i>O</i> -hexoside ^{a,i}	0.07 ± 0.0
9	14.37	254, 355	477	MS ² : 314 (100), 315 (45)	Isorhamnetin- <i>O</i> -hexoside ^{a,i}	0.04 ± 0.0
10	15.04	265, 345	533	MS ² : 489 (100); MS ³ : 285 (100)	Kaempferol- <i>O</i> -malonyl-hexoside ^{a,h}	0.04 ± 0.0
11	15.83	295, 315	630	MS ² : 468 (100), 494 (84), 358 (7); MS ³ : 332 (100)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ - <i>tri</i> -caffeoylspermidine ^{a,d,g}	0.18 ± 0.0
12	16.83	296, 319	630	MS ² : 468 (100), 494 (86), 358 (7); MS ³ : 332 (100)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ - <i>tri</i> -caffeoylspermidine ^{a,d,g}	1.24 ± 0.2
13	18.18	295, 311	614	MS ² : 494 (25), 478 (100), 452 (69), 358 (20)	<i>N</i> ¹ - <i>p</i> -coumaroyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicafeoylspermidine ^{a,c}	0.05 ± 0.0
14	18.8	295, 311	614	MS ² : 478 (100), 468 (20), 452 (68), 342(5)	<i>N</i> ¹ - <i>p</i> -coumaroyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicafeoylspermidine (isomer) ^{a,c}	0.15 ± 0.0
15	19.45	295, 311	614	MS ² : 494 (24), 478 (100), 452 (76), 358 (22)	<i>N</i> ¹ - <i>p</i> -coumaroyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicafeoylspermidine (isomer) ^{a,c}	0.18 ± 0.0
16	20.14	295, 318	644	MS ² : 358 (8), 482 (75), 508 (100); MS ³ : 332 (27), 358 (100), 372 (49)	<i>N</i> ¹ -feruloyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicafeoylspermidine (isomer) ^{a,c}	0.06 ± 0.0
17	20.46	295, 310	598	MS ² : 478 (46), 462 (100), 452 (46), 342 (14)	<i>N</i> ¹ , <i>N</i> ⁵ - <i>di</i> - <i>p</i> -coumaroyl- <i>N</i> ¹⁰ -caffeoylspermidine ^{a,c}	0.04 ± 0.0
18	21.38	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ - <i>tri</i> - <i>p</i> -coumaroylspermidine ^{a,c}	0.42 ± 0.0

19	22.35	296, 310	598	MS ² : 462 (100), 452 (42), 478 (41), 342 (14)	N ¹ , N ⁵ - <i>di-p-coumaroyl-N^{1/0}-caffeoyl</i> spermidine (isomer) ^{a,d,g}	0.45 ± 0.0
20	22.94	295, 310	582	MS ² : 462 (100), 436 (9), 342(7)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p-coumaroyl</i> spermidine ^{a,e}	0.83 ± 0.0
21	24.34	295, 310	582	MS ² : 462 (100), 436 (9), 342 (6)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p-coumaroyl</i> spermidine ^{a,e}	0.76 ± 0.0
22	25.3	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p-coumaroyl</i> spermidine ^{a,e}	0.25 ± 0.0
23	26.71	296, 310	582	MS ² : 462 (100), 436 (10), 342 (7)	N ¹ , N ⁵ , N ¹⁰ - <i>tri-p-coumaroyl</i> spermidine ^{a,e}	1.90 ± 0.0
24	27.77	295, 308	612	MS ² : 492 (100); MS ³ : 372 (100), 449 (24)	Feruloyl dicoumaroyl spermidine ^{a,j}	0.02 ± 0.0
25	28.8	280, 307sh	785	MS ² : 665 (100), 666 (27), 545 (14); MS ³ : 545 (100), 546 (20)	Tetracoumaroyl spermine ^{a,j}	0.17 ± 0.0
26	34.24	299, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer) ^{a,j}	0.11 ± 0.0
The total amount of phenolic compounds						
The total amount of phenylamides						
						0.7
						6.8

Confirmed with: ^a MSⁿ fragmentation; ^b Standard; References: ^c [130]; ^d [131]; ^e [132]; ^f [133]; ^g [134]; ^h [135]; ⁱ [137]; ^j [138]. Values expressed as mg of each compound/g sample. ND = not detected.

Table 9. Phenolic and phenylamide compounds of the enriched bread with bee bread (mg/g).

Peak	tr (min)	λ_{\max} (nm)	[M-H] ⁻ m/z	MS ⁿ (% base peak)	Proposed compound	BB1%	BB3%	BB5%
1	15.83	295, 315	630	MS ² : 468 (100), 494 (84), 358 (7); MS ³ : 332 (100)	N ⁷ , N ⁵ , N ¹⁰ -tri-caffeoylspermidine	0.01 ± 0.0	0.11 ± 0.0	ND
2	16.83	296, 319	630	MS ² : 468 (100), 494 (86), 358 (7); MS ³ : 332 (100)	N ⁷ , N ⁵ , N ¹⁰ -tri-caffeoylspermidine	0.03 ± 0.0	0.34 ± 0.0	0.36 ± 0.0
3	18.8	295, 311	614	MS ² : 478 (100), 468 (20), 452 (68), 342(5)	N ⁷ -p-coumaroyl-N ⁵ , N ¹⁰ -dicafeoylspermidine (isomer)	0.00 ± 0.0	0.10 ± 0.0	0.08 ± 0.0
4	19.45	295, 311	614	MS ² : 494 (24), 478 (100), 452 (76), 358 (22)	N ⁷ -p-coumaroyl-N ⁵ , N ¹⁰ -dicafeoylspermidine (isomer)	0.00 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
5	20.14	295, 318	644	MS ² : 358 (8), 482 (75), 508 (100); MS ³ : 332 (27), 358 (100), 372 (49)	N ⁷ -feruloyl-N ⁵ , N ¹⁰ -dicafeoylspermidine (isomer)	ND	ND	0.01 ± 0.0
6	21.38	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	N ⁷ , N ⁵ , N ¹⁰ -tri-p-coumaroylspermidine	ND	0.13 ± 0.0	0.15 ± 0.0
7	22.35	296, 310	598	MS ² : 462 (100), 452 (42), 478 (41), 342 (14)	N ⁷ , N ⁵ -di-p-coumaroyl-N ¹⁰ -cafeoylspermidine (isomer)	0.02 ± 0.0	0.19 ± 0.0	0.31 ± 0.0
8	22.94	295, 310	582	MS ² : 462 (100), 436 (9), 342(7)	N ⁷ , N ⁵ , N ¹⁰ -tri-p-coumaroylspermidine	0.03 ± 0.0	0.44 ± 0.0	0.46 ± 0.0
9	24.34	295, 310	582	MS ² : 462 (100), 436 (9), 342 (6)	N ⁷ , N ⁵ , N ¹⁰ -tri-p-coumaroylspermidine	0.04 ± 0.0	0.41 ± 0.0	0.49 ± 0.0
10	25.3	295, 310	582	MS ² : 462 (100), 436 (9), 342 (7)	N ⁷ , N ⁵ , N ¹⁰ -tri-p-coumaroylspermidine	0.01 ± 0.0	0.23 ± 0.0	0.35 ± 0.0
11	26.71	296, 310	582	MS ² : 462 (100), 436 (10), 342 (7)	N ⁷ , N ⁵ , N ¹⁰ -tri-p-coumaroylspermidine	0.15 ± 0.0	1.85 ± 0.1	1.69 ± 0.0
12	28.8	280, 307sh	785	MS ² : 665 (100), 666 (27), 545 (14); MS ³ : 545 (100), 546 (20)	Tetracoumaroyl spermine	ND	0.06 ± 0.0	0.04 ± 0.0
13	34.24	299, 310	785	MS ² : 665 (100), 545 (13), 639 (13); MS ³ : 545 (100)	Tetracoumaroyl spermine (isomer)	0.00 ± 0.0	0.09 ± 0.0	0.07 ± 0.0
The total amount of phenolic compounds						0.00	0.00	0.00
The total amount of phenylamides						0.30	3.97	4.02

Values expressed as mg of each compound/g sample. ND = not detected.

3.6. Bread properties

3.6.1. Specific volume

Specific volume is one of the parameters that significantly affect bread quality and strongly influence consumer's choice, since from an economic standpoint, a high ratio of volume per weight is requested [144, 145]. Highest result for specific volume was observed in BB1% (5.23 mL/g) closer to the control (5.39 mL/g). Generally, BB bread samples showed a specific volume higher when comparing to the BP bread samples, but the difference among the breads may not be enough for a significant distinction. Specific volume values for all breads were the following: Control (5.4 ± 0.3) > BB1% (5.2 ± 0.5) > BB3% (5.2 ± 0.0) > BB5% (5.1 ± 0.3) > BP3% (5.1 ± 0.1) > BP5% (4.9 ± 0.1) > BP1 (4.9 ± 0.0).

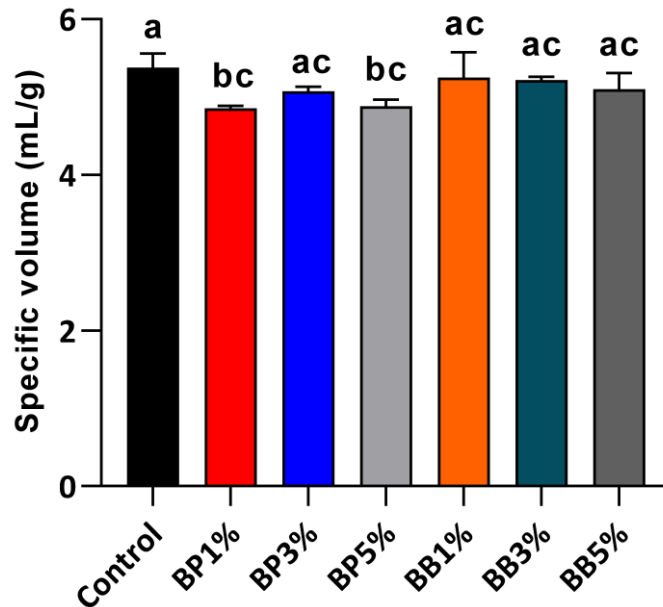


Figure 9. Specific volume of the bread samples

3.6.2. Colour analysis

Colour formation in bread is called browning and is attributed to non-enzymatic browning reaction, which includes caramelization and Maillard reactions, during the bread-making process [146]. Complex reactions like caramelization come into existence by heating the carbohydrates [147], while the Maillard reactions occurs between the carbonyl group of reducing carbohydrates and the amino group of free aminoacids. In both the Maillard and caramelization reactions, highly UV-absorbing and colorless

compounds are formed at intermediate stages, whereas the brown polymers are formed at final stages [148]. Temperature, moisture, and sugar structures are the factors influencing the Maillard reaction in bread [149]. In some studies, the light color of bread was defined as an undesirable feature [97].

Indeed, as the ratio of bee pollen and bee bread was increased, the colors of the breads became darker, breads enriched with bee pollen dominated a relatively yellowish color, while bee bread added breads became more brownish.

The CIELAB colour system was applied to characterize the enriched bread colour, independent of the observer. The CIELAB system is an approximately uniform three-dimensional colour scale, extensively used to evaluate food colours. In this scale, the coordinate L^* measures the degree of lightness, and a^* e b^* are the horizontal axes defining green/red (-60/60) and blue/yellow (-60/60) chromaticity, respectively [150].

Observing the colour results (Table 10), while a decrease in the L^* value was observed in the crusts of pollen enriched breads, a^* and b^* values increased. The same pattern was shown by the bee bread enriched breads. For the crumb colour of bee pollen enriched breads, a decrease of the L^* and a^* values was observed from the control bread to BP5%, while the b^* value increased. For bee bread enriched breads, the crumb colour analysis showed a decrease in the L^* values, while both a^* and b^* values increased (Figure 10, Table 10). The obtained results are in accordance with previous studies which were focused on functional bread enriched with bee pollen [97]. Yellow-brownish colour of bee pollen and bee bread is essentially related to the accumulation of carotenoids [151], which may have had a role in the final colour presented by the breads. In terms of consumer preferences, the changing in the bread colour can be attributed as a desirable result for enriched bread samples.



Figure 10. Images of baked sample slices of control and functionalized breads with bee pollen and bee bread.

As can be seen in the Figure 10, and mentioned before in the above, control breads for both trials looks traditional-white bread, but when it comes to BP1%, BP3%, BP5% colour became yellowish, and for bee bread added breads, BB1%, BB3%, BB5% respectively colour became brown. It is clear on the samples that the color is homogeneously distributed along the different places of the bread slice. This situation may have been ensured by the substitute bee pollen and bee bread to be mixed with flour when dry.

Table 10. Crust and crumb colour of control and enriched bread samples.

Crust Colour			Crumb Colour				
Sample	L^*	a^*	b^*	Sample	L^*	a^*	b^*
Control	73.1 ± 5.5 ^a	3.4 ± 2.0 ^a	24.3 ± 2.9 ^a	Control	68.4 ± 2.4 ^a	-0.7 ± 0.2 ^a	15.9 ± 1.2 ^a
BP1%	73.2 ± 4.9 ^a	3.0 ± 1.5 ^a	25.4 ± 3.1 ^a	BP1%	63.8 ± 2.6 ^a	-1.3 ± 0.7 ^a	20.4 ± 1.4 ^a
BP3%	69.6 ± 5.8 ^a	6.1 ± 2.4 ^a	29.5 ± 4.6 ^a	BP3%	61.1 ± 1.9 ^a	-2.0 ± 0.2 ^a	25.78 ± 1.2 ^a
BP5%	61.5 ± 4.1 ^a	5.1 ± 4.6 ^a	32.5 ± 2.0 ^a	BP5%	58.2 ± 2.4 ^{b,c}	-1.3 ± 0.9 ^{b,c}	31.7 ± 1.4 ^{b,c}
BB1%	73.1 ± 5.5 ^a	3.8 ± 1.1 ^a	25.3 ± 3.0 ^a	BB1%	62.4 ± 3.4 ^a	-0.5 ± 0.3 ^a	20.9 ± 0.8 ^a
BB3%	69.0 ± 2.5 ^a	5.6 ± 7.7 ^a	27.0 ± 2.9 ^a	BB3%	55.7 ± 2.7 ^{b,a}	0.4 ± 0.4 ^{b,a}	24.5 ± 1.1 ^{b,a}
BB5%	63.0 ± 7.4 ^a	7.4 ± 9.3 ^a	30.5 ± 1.7 ^a	BB5%	53.8 ± 2.5 ^a	1.5 ± 0.4 ^a	27.5 ± 1.3 ^a

Each value in is the mean ±SD, n = 3. In each column, different superscript letters (a–c) means significantly different (p < 0.05).

3.6.3. Texture profile analysis

Texture profile analysis is a double compression test used to determine the textural properties of products from different industries such as food, pharmaceuticals, cosmetics, personal care products, among others. For this test, the breads were sliced to 3 cm in thickness, and each bread sample repeated 3 times. The results for the bread crumb and crust texture analysis are given in Table 11 and 12, respectively.

The TPA parameters, such as hardness, adhesiveness, resilience, cohesiveness, springiness, gumminess, chewiness properties of bread samples have been evaluated and compared among each other with the control group bread.

Hardness, is defined as the force that the teeth have to apply on the food, and is measured in grams [98]. Regarding the crumb hardness of the bread samples, BP3% bread has the highest value, followed by BB3% while the less hardness value was detected in BB1% and control samples. For the crust hardness texture profile of the bread samples, the results showed that BP3% has the highest value for hardness followed by BP5%, while BB1% and control have the lowest values respectively. For the hardness of both crust and bread crumb properties, the obtained results were statistically significant. Other previous studies found that the moisture content can be related to the hardness of the bread [152]. Also, the type of flour, the percentage of bee pollen added and bee bread in different proportions can affect the hardness parameter of breads. Also, previous studies showed significant correlation between bread texture and protein content [152].

Adhesiveness is defined as the capacity that food has to adhere to the teeth while chewing and is expressed in negative values due to the measuring force being applied from bottom to top in the texturometer [153]. The obtained adhesiveness values were low, equal or above 0.2 g.sec, which is in accordance with previously results and to the fact that breads are known by their low adhesiveness. Comparing the adhesiveness of the enriched breads with control, with the increase of the percentage of bee pollen and bee bread added to breads, a decrease was observed in the adhesiveness values.

Springiness can be defined by the rate at which a deformed food reverts to the undeformed state after removing the deforming force, being measured in percentage [98]. For the springiness parameter of the enriched bread samples, the highest values were observed for BP1% and BB1% respectively. Springiness is a desired property for bakery products like muffins and bread [154]. Enriched bread samples have been tested with force and speed in the texturometer, in order to see the resilience response of the bread in

terms of its texture. Cohesiveness can be considered as the resistance of a food to two consecutive compressions while chewiness might be considered as the energy to the food chewing [98].

For crust hardness, BP3% enriched bread was the hardest bread followed by BP5%. BB1% and BB5% breads shown the lowest results respectively for adhesiveness parameter. Analysing the crust springiness of the enriched breads, BP5%, and BB5% samples have shown the highest values. An increase of resilience and springiness may reflect a higher specific volume value for the bread since it was reported in a previous work that springiness might associate with aeration and elasticity of a product [154]. Resilience is similar to springiness, although it measures both the speed and forces involved in the recovery of a food when a deforming force is removed, being also measured in percentage. As can be seen in Table 12, in general, all bee pollen and bee bread enriched bread samples showed higher resilience than control bread.

Table 11. Crumb texture parameters of enriched bread samples.

Samples	Hardness (g)	Adhesiveness (g.sec)	Resilience (%)	Cohesion (%)	Springiness (%)	Chewiness
Control	1249.3 ± 39.5 ^a	0.1 ± 0.2 ^a	47.4 ± 0.0 ^{a,c}	0.8 ± 0.0 ^a	173.9 ± 24.9 ^a	1275.9 ± 116.8 ^a
BP 1%	1419.6 ± 55.3 ^{a,c,e}	-0.1 ± 0.1 ^b	51.3 ± 2.3 ^a	0.8 ± 0.0 ^a	161.3 ± 17.1 ^b	1851.3 ± 22.9 ^{b,c}
BP 3%	2786.4 ± 83.4 ^b	-0.1 ± 0.2 ^b	38.6 ± 4.1 ^{b,c}	0.7 ± 0.0 ^a	98.9 ± 5.0 ^c	1890.9 ± 108.3 ^c
BP 5%	1903.4 ± 174.1 ^{c,d,e}	-0.3 ± 0.4 ^c	47.1 ± 6.0 ^{a,c}	0.8 ± 0.1 ^a	97.4 ± 2.3 ^c	1374.0 ± 137.9 ^a
BB 1%	913.9 ± 82.3 ^a	0.2 ± 0.2 ^d	54.3 ± 3.5 ^a	0.9 ± 0.0 ^a	141.8 ± 8.9 ^d	1194.9 ± 71.6 ^a
BB 3%	2029.6 ± 133.4 ^{d,f}	-0.1 ± 0.2 ^b	46.1 ± 4.6 ^{a,b}	0.8 ± 0.1 ^a	97.0 ± 1.4 ^c	1459.0 ± 171.6 ^a
BB 5%	1844.9 ± 49.2 ^{c,f}	0.1 ± 0.1 ^a	47.7 ± 3.6 ^{a,c}	0.8 ± 0.0 ^a	103.8 ± 4.1 ^c	1519.1 ± 15.4 ^a

Each value in is the mean ±SD, n = 3. In each column, different superscript letters (a–f) means significantly different (p < 0.05).

Table 12. Crust texture parameters of enriched bread samples.

Samples	Hardness (g)	Adhesiveness (g.sec)	Resilience (%)	Cohesion (%)	Springiness (%)	Chewiness
Control	1204.8 ± 131.7 ^{a,d}	-146.9 ± 0.2 ^a	25.7 ± 1.5 ^{a,c}	0.6 ± 0.0 ^a	87.4 ± 0.2 ^a	592.2 ± 145.1 ^a
BP 1%	1482.9 ± 169.3 ^{a,e}	-253.3 ± 0.9 ^a	29.5 ± 2.4 ^{c,d}	0.6 ± 0.0 ^a	87.9 ± 4.7 ^a	788.5 ± 62.8 ^b
BP 3%	2767.4 ± 50.2 ^b	-158.6 ± 1.0 ^a	23.7 ± 3.1 ^{a,d,e}	0.5 ± 0.0 ^a	87.1 ± 4.9 ^a	1326.6 ± 10.4 ^{c,d}
BP 5%	2485.6 ± 96.7 ^b	-0.3 ± 0.1 ^b	33.0 ± 0.6 ^{b,c}	0.6 ± 0.1 ^a	91.3 ± 0.8 ^a	1397.2 ± 58.4 ^d
BB 1%	994.4 ± 75.4 ^{c,d}	-426.1 ± 0.1 ^c	33.4 ± 6.6 ^c	0.7 ± 0.1 ^a	88.3 ± 3.3 ^a	595.1 ± 64.0 ^a
BB 3%	1525.2 ± 8.6 ^c	-0.4 ± 0.3 ^{d,b}	25.5 ± 4.3 ^{c,e}	0.5 ± 0.1 ^a	87.3 ± 1.6 ^a	552.1 ± 18.6 ^a
BB 5%	1664.1 ± 34.1 ^c	-255.2 ± 0.1 ^a	29.6 ± 2.6 ^{c,e}	0.6 ± 0.0 ^a	88.6 ± 2.2 ^a	1382.5 ± 13.2 ^{c,d}

Each value in is the mean ±SD, n = 3. In each column, different superscript letters (a–e) means significantly different (p < 0.05).

3.7. Microbial analysis

Bacillus subtilis is also one of the microorganisms present in bee pollen and bee bread, and this may negatively affect the spoilage property of breads due to its presence. Apprehensions for the negative effects of the *Bacillus subtilis* bacteria on important criteria such as the shelf life of conventional wheat bread were indicated and activity of this microorganism has been tried to reduce [155].

3.7.1. Total viable counts, PCA, pour plate (ISO 4833-2:2013)

Total viable count (TVC), estimates the population of live microbial load for food quality assurance [156]. According to WHO Standard (1994), the acceptable range of TVC is 2.0×10^5 CFU/g [156].

While no significant difference was observed in all raw dough samples for TVC, values ranged from $7.9 \pm 0.1 \log_{10}$ CFU/g to $7.6 \pm 0.1 \log_{10}$ CFU/g among bee pollen added doughs, from all the baked breads enriched with bee products, the highest microbial activity was observed at BP1% with $3.2 \pm 0.1 \log_{10}$ CFU/g. As seen in Table 13, when the breads with bee pollen added are compared with the control bread, the ones with high concentrations are statistically close to each other (BP3%, BP5%), and those with low concentrations are statistically close to each other (Control, BP1%). For bee bread added breads the highest microbial count has been observed in BB1% with $7.8 \pm 0.1 \log_{10}$ CFU/g value, and after the cooking process, there was no significant microbial activity observed in performed tests. Our results showed that raw dough samples have relatively higher microbial activity but, microbial counts have a limit after they are cooked, what can be a result of the high heat during the cooking process.

According to Hani et al. [157], the total aerobic count for pollen was $5.8 \log_{10}$ CFU/g and the anaerobic count was $5.8 \log_{10}$ CFU/g, in another study conducted with bee pollen formulated food, bee pollen has shown inhibition over the microbial growth [158], and for bee bread total viable count was quite similar to our results with the value of $7.8 \pm 0.3 \log$ CFU/g [159]. Normally bee pollen and bee bread are considered as risk products for foods but the cooking process and applied heat may increase the safety properties of both bee products.

Table 13. Total viable counts (log₁₀ CFU/g) in bee pollen and bee bread added breads, in raw dough (immediately before cooking), cooked bread (immediately after cooking), and 3 and 5 days after being cooked.

Samples	Raw dough	Cooked	3 days	5 days
Control BP	7.89 ± 0.14 ^{Aa}	3.14 ± 0.29 ^{Aa}	2.98 ± 0.05 ^{Aa}	<1* ^{Aa}
BP 1%	7.88 ± 0.11 ^{Aac}	3.18 ± 0.14 ^{Ba}	2.38 ± 0.49 ^{Bb}	<1* ^{Aa}
BP 3%	7.66 ± 0.12 ^{Bbd}	2.47 ± 0.34 ^{Cc}	2.45 ± 0.52 ^{Bb}	<1* ^{Aa}
BP 5%	7.70 ± 0.10 ^{Bbc}	1.38 ± 0.46 ^{Dd}	<1* ^{Cc}	<1* ^{Aa}
Control BB	7.73 ± 0.28 ^{ABcde}	<1*	<1*	<1*
BB 1%	7.83 ± 0.10 ^{Aac}	<1*	<1*	<1*
BB 3%	7.75 ± 0.12 ^{ACad}	<1*	<1*	<1*
BB 5%	7.59 ± 0.07 ^{BCbd}	<1*	<1*	<1*

Results are expressed on the dry weight of bee pollen and bee bread. In each column, different superscript letters (a–e) mean significant differences ($p < 0.05$) the lowercase letters represent the statistical differences between all parameters, while the uppercase letters represent the statistical difference between BP control and bee pollen added breads, BB control and bee bread added breads. * limit of detection. BP: bee pollen; BB: bee bread; BP1%: ratio of 1% added bee pollen to total flour amount; BP3%: ratio of 3% added bee pollen to total flour amount; BP5%: ratio of 5% added bee pollen to total flour amount; BB1% ratio of 1% added bee bread to total flour amount; BB3%: ratio of 3% added bee bread to total flour amount; BB5%: ratio of 5% added bee bread to total flour amount.

3.7.2. Yeasts and Moulds (Y&M); DRBC (ISO 21527-1/2:2008)

Dichloran rose bengal chloramphenicol (DRBC) is a selective medium for the counting of moulds and yeasts in foods, restricts the size and height of mould colonies thus preventing overgrowth of luxuriant species and assisting accurate counting of colonies. The addition of chloramphenicol and the reduced pH of 5.6 serves to prevent the growth of most bacteria [160].

The results obtained for the total yeast count were considered as the highest 7.5 ± 0.3 log CFU/g for raw dough containing bee pollen (BP1%), and this number has shown a decrease significantly for baked bread (Table 14). For the yeast count of pollen 5.6 log CFU/g reported by Hani et al. [157]. The obtained results in this study are similar to those previously reported for bee pollen [161]. In a previous study, the total yeast count counted for bee bread was determined as 8.7 ± 0.0 log CFU/g [159]. For our results of the bee bread enriched bread samples, BB1% and BB5% were the two highest values but the same pattern have been observed in yeast count, values have been decreased in bee bread added breads dramatically when doughs were cooked. These results of decrease may have

reflected the high heating process and the use of heat against the spoilage caused by yeasts may have increase safety for foods.

Table 14. Yeast counts (log₁₀ CFU/g) in bee pollen and bee bread added breads, in raw dough (immediately before cooking), cooked bread (immediately after cooking), and 3 day and 5 days after being cooked.

Samples	Raw dough	Cooked	3 days after	5 days after
Control BP	7.34 ± 0.21 ^{Aa}	<1.7*	<1.7*	<1.7*
BP 1%	7.50 ± 0.25 ^{Bb}	<1.7*	<1.7*	<1.7*
BP 3%	6.87 ± 0.17 ^{Cc}	<1.7*	<1.7*	<1.7*
BP 5%	6.71 ± 0.09 ^{Dd}	<1.7*	<1.7*	<1.7*
Control BB	7.81 ± 0.10 ^{Ae}	<1.7*	<1.7*	<1.7*
BB 1%	7.83 ± 0.15 ^{Ae}	<1.7*	<1.7*	<1.7*
BB 3%	7.82 ± 0.13 ^{Ae}	<1.7*	<1.7*	<1.7*
BB 5%	7.83 ± 0.11 ^{Ae}	<1.7*	<1.7*	<1.7*

Results are expressed on the dry weight of bee pollen and bee bread. In each column, different superscript letters (a–e) mean significant differences ($p < 0.05$) the lowercase letters represent the statistical differences between all parameters, while the uppercase letters represent the statistical difference between BP control and bee pollen added breads, BB control and bee bread added breads. * limit of detection. BP: bee pollen; BB: bee bread; BP1%: ratio of 1% added bee pollen to total flour amount; BP3%: ratio of 3% added bee pollen to total flour amount; BP5%: ratio of 5% added bee pollen to total flour amount; BB1% ratio of 1% added bee bread to total flour amount; BB3%: ratio of 3% added bee bread to total flour amount; BB5%: ratio of 5% added bee bread to total flour amount.

3.7.3. Spores of sulfite-reducing clostridia (SRC) (adapted from ISO 15213:2003)

Clostridium perfringens are anaerobic, spore-forming pathogenic bacteria and produce enterotoxins that cause diarrhea in humans. Its spores may resist the baking process, germinate in the food and, if the amount of bacteria is high enough, a significant part survives the stomach acid [162]. In our results (Table 15), raw dough contained sulfite-reducing *Clostridia*, however, in the cooked samples a significant reduction was observed. *Clostridia*, which was detected in raw samples in small amounts, was not detected in cooked samples, the reason for this decrease may be due to the high temperature.

Table 15. Spores of sulfite-reducing Clostridia (SRC) counts (log₁₀ CFU/g) in bee pollen (BP) and bee bread (BB) added breads, in raw dough (immediately before cooking), cooked bread (immediately after cooking), and 3 and 5 days after being cooked.

Samples	Raw dough	Cooked	3 days after	5 days after
Control BP	0.30 ± 0.30 ^{Aa}	<0.3*	<0.3*	<0.3*
BP 1%	0.46 ± 0.15 ^{Bb}	<0.3*	<0.3*	<0.3*
BP 3%	0.00 ± 0.00 ^{Cc}	<0.3*	<0.3*	<0.3*
BP 5%	0.59 ± 0.11 ^{Dd}	<0.3*	<0.3*	<0.3*
Control BB	0.17 ± 0.29 ^{Aa}	<0.3*	<0.3*	<0.3*
BB 1%	0.05 ± 0.12 ^{Bc}	<0.3*	<0.3*	<0.3*
BB 3%	0.05 ± 0.12 ^{Bc}	<0.3*	<0.3*	<0.3*
BB 5%	0.00 ± 0.00 ^{Cc}	<0.3*	<0.3*	<0.3*

Results are expressed on the dry weight of bee pollen and bee bread. In each column, different superscript letters (a–e) mean significant differences ($p < 0.05$) the lowercase letters represent the statistical differences between all parameters, while the uppercase letters represent the statistical difference between BP control and bee pollen added breads, BB control and bee bread added breads. * limit of detection. BP: bee pollen; BB: bee bread; BP1%: ratio of 1% added bee pollen to total flour amount; BP3%: ratio of 3% added bee pollen to total flour amount; BP5%: ratio of 5% added bee pollen to total flour amount; BB1% ratio of 1% added bee bread to total flour amount; BB3%: ratio of 3% added bee bread to total flour amount; BB5%: ratio of 5% added bee bread to total flour amount.

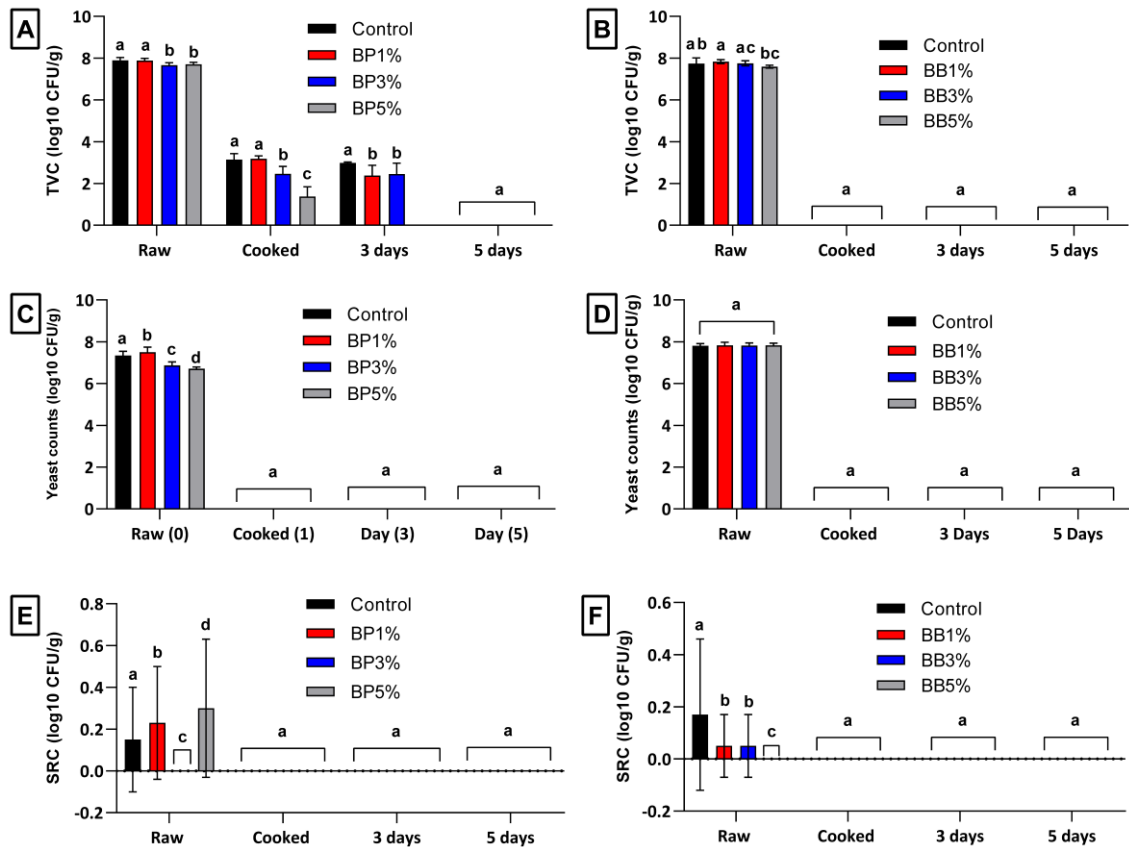


Figure 11. A) Total viable counts (TVC) of bee pollen added bread samples, B) Total viable counts (TVC) of bee bread added bread samples, C) Yeast counts of bee pollen added bread samples, D) Yeast counts of bee bread added bread samples, E) Spores of sulfite-reducing clostridia (SRC) counts of bee pollen added bread samples, F) Spores of sulfite-reducing clostridia (SRC) counts of bee bread added bread samples.

3.8. Sensory evaluation

According to the obtained results, Figure 12 and 13, the most appreciated bread on taste was BB3%, followed by BB1%. For the bread fortified with bee pollen, the preference was BP3%. It worth to mentioned that the control bread received the lowest evaluation for taste. For the mouthfeel, the highest evaluation belongs to BB5% followed by BB3%. Regarding the overall acceptance when each group was compared with its own control, for bread enriched with bee pollen the following pattern was observed: BP3% > BP1% > Control > BP5%. For the breads enriched with bee bread the following results were observed; BB3% > BB5% > BB1 > Control. The reason why the enriched breads with BB were more liked may be the sweetness due to the honey constituent in bee bread. Our results confirm the same aptitude was other sensory analysis in bee pollen breads.

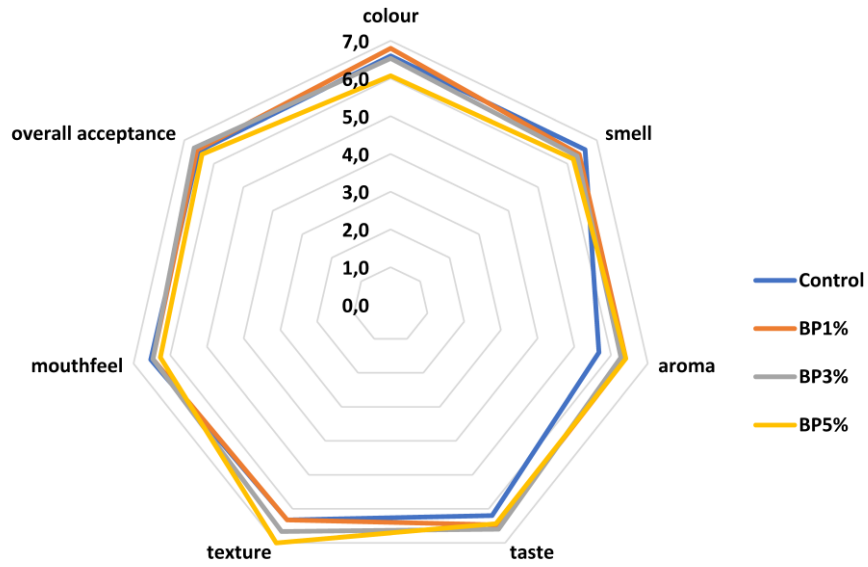


Figure 12. Sensor analysis profile of enriched bread with bee pollen and control group.

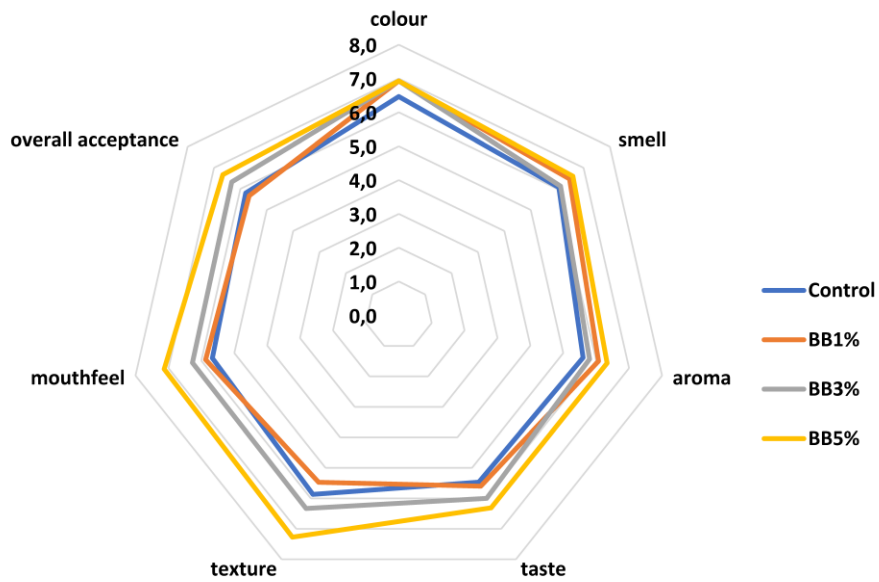


Figure 13. Sensor analysis profile of enriched bread with bee bread and control group.

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. Conclusion

The aim of this work was to test the physicochemical properties of bee pollen and bee bread products, which are characterized by high nutritional value, as a traditional bread strengthening agent and to give a new impulse to functional food production. Also, the quality parameters related to the thermostability of potentially strong functional breads, were evaluated. Both raw bee products were quantitatively evaluated through different physico-chemical parameters, such as moisture content, ash, protein, lipid contents, phenolic compounds and antioxidant properties. After enriching the breads with different percentages of bee products, the physico-chemical parameters were also evaluated after the cooking and the results compared to a control group.

While properties such as protein, lipid, moisture, and energy values have been increased as expected and desired in fortified breads, a decrease in carbohydrate ratio was observed. According to the results of antioxidant tests, it was also observed that the antioxidant properties of fortified breads increased slightly compared to the control bread, accordingly, it can be said that fortified breads have a higher antioxidant activity. For the phenolic compounds, raw bee pollen has quercetin-*O*-diglucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside, but when it comes to bread samples enriched with bee pollen, we only detected quercetin-3-*O*-rhamnoside the reason for that may be increased temperature during cooking process.

As mentioned earlier, these products also affected the moisture content in breads, the moisture content and the type of flour used also had an effect over the texture and specific volume of the breads, and the fortified breads showed better texture and specific volume values compared to the comparison group. In addition, the color of our breads, can be said to have the desired characteristics, as the dark color is a more desirable feature as stated before. The sensory evaluation test revealed the fortified bread scores were very promising in order for people to take these breads into their daily diet. One of the potential limitations in bee pollen and bee bread is the bacteria and yeast present in these products and their effects over the product quality. However, according to the microbial activity test results, these products have not negatively affected the bread quality to a large extent, this may be due to the high temperature during the baking process.

4.2. Future perspective

Although the main objectives were achieved in this study, some recommendations for future research are given below:

- Since studies on these products are very limited in the literature, more research is essential.
- As the ratio of products increases, nutritional values have increased, so higher rates can be used in future studies.
- As a separate approach, these products can be mixed in order to complement each other due to the nutritional values of the two products.
- Gastrointestinal simulation tests can be performed to evaluate the bioavailability of nutritional products on humans.
- The use of these products only as enhancers on bread can only give an idea over breads but not give detailed information about their interactions with other products, hence other products may need to test.
- No products other than basic ingredients for bread such as flour, salt, yeast, and water were used in breads, and apart from the use of simple and basic products, the effects of using excess ingredients over these bee products are unknown.

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