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Bee bread preservation methods: physical, chemical and microbial stability throughout storage

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Abstract

Bee bread is an important beehive product, and it is growing in commercial interest due to its high nutritional value and richness in bioactive compounds, and it is thus considered as a human functional food. The rich composition of bee bread with different nutrients can result in microbial growth and spoilage, especially when handling practices are not approprite. This work intends to evaluate the impact of different preservation methods - freezing, room temperature, oven drying at 30 °C, and freeze drying - on bee bread physicochemical characteristics and microbial stability, during a storage period of six months. Samples were analysed before the application of the treatments, immediately after the application of the treatments (T0), and after one (T1), three (T3) and six (T6) months of storage. For each time point of analysis, the samples were analyzed for several parameters: microbiological (aerobic mesophiles, lactic acid bacteria, yeasts, moulds, and coliforms), physical-chemical (pH, water activity, moisture content, proteint content, lipid content and ash) and bioactive (total phenolic compounds and antioxidant activity).

The techniques of oven-drying and freeze-drying were the most effective in reducing the initial moisture content of bee bread from 16.5% to 9.9% and 11.0%, respectively. These reductions were also evident in bee bread water activity, which was reduced from 0.57 to approximately 0.36, for both preservation techniques. Overall, the various preservation treatments significantly affected the nutritional composition of bee bread. This was particularly evident when room temperature drying was used, causing an increase of 80% on bee bread total lipid content, after a storage period of 3 months. The total phenolic content was also influenced by the preservation treatment applied to bee bread, particularly for freeze-drying, which caused a reduction of approximately 40%, after 1 month of storage, reflected on an increase of 50% for EC_{50} value, evidencing a decrease in the bee bread antioxidant activity that continued during the whole 6 month storage period.

The results obtained for microbial analysis showed that the frozen samples have the highest microbial loads. The preservation at room temperature without previous treatment presented similar or better preservation efficiency compared to oven drying and freeze drying. No significant differences were observed between these treatments in most situations, except for the yeast counts, which the results presented a significant differences between all the treatment througout storage.

Overall, each preservation technique acted differently on the various parameters over time. From a chemical perspective, after six months of storage there was no significant difference between treatments. From a microbial point of view, freezing was the least adequate preservation technique, while the remaining preservation techniques showed similar good product stability. Given the results obtained in the present study, preservation at room temperature without any previous treatment seems to be an adequate technique for the preservation of bee bread under the tested conditions.

Further studies are required to elucidate the effect of different levels of pH and water activity on the stability of non-treated bee bread.

Keywords: bee products; preservation methods; shelf-life

Resumo

O pão de abelha é um importante produto da colmeia, e apresenta crescente interesse comercial devido à sua riqueza nutricional e elevada bioatividade, sendo por isso considerado um alimento funcional. A riqueza nutricional do pão de abelha pode resultar em crescimento microbiano e consequente deterioração, especialmente quando as práticas de manipulação são inadequadas.

O presente trabalho tem como objetivo a avaliação do impacto de vários métodos de preservação – congelação a -20 ºC, conservação à temperatura ambiente, secagem em estufa a 30 ºC, e liofilização – nas características fisico-químicas e estabilidade microbiana, durante um período de armazenamento de seis meses. Amostras de pão de abelha foram analisadas antes da aplicação dos tratamentos, imediatamente após a aplicação (T0), e após um (T1), três (T3) e 6 (T6) meses de armazenamento. Em cada tempo de análise, foram avaliados os seguinte parâmetros: microbiológicos (mesófilos aeróbios, bactérias do ácido lático, leveduras, bolores, coliformes), físico-químicos (pH, atividade de água, conteúdo em água, conteúdo proteico e lipídico, cinzas), e bioativos (compostos fenólicos totais e atividade antioxidante).

As técnicas de secagem em estufa e liofilização foram os mais eficientes na redução do conteúdo em água de 16.5% para 9.9% e 11.0%, respetivamente. A atividade de água sofre redução de 0.57 para 0.36. Em geral, os vários tratamentosafetaram de forma significativa a composição nutricional do pão de abelha. Na conservação à temperatura ambiente, verificou-se um aumento de 80% no conteúdo lipídico das amostras após três meses de conservação. O conteúdo fenólico total foi igualmente afetado, em particular no tratamento por liofilização, que conduziu a uma redução de 40% após um mês de armazenamento e consequente aumento de 50% do valor EC_{50}, evidenciando uma redução da atividade antioxidante do pão de abelha ao longo dos seis meses de conservação.

Os resultados obtidos na análise microbiana mostraram que as amostras congeladas apresentaram as mais elevadas cargas microbianas. A preservação à temperatura ambiente sem qualquer tratamento prévio apresentou uma eficiência de conservação equivalente ou melhor do que a secagem em estufa e a liofilização. Não se registaram diferenças significativas entre tratamentos, exceto no caso das leveduras onde se verificou que houve diferenças significativas entre os tratamentos ao longo do armazenamento.

Em geral, as técnicas de conservação tiveram diferentes efeitos sobre o pão de abelha nos vários parâmetros e ao longo do tempo. No entanto, não foram demonstradas diferenças significativas entre tratamentos para os parâmetros químicos após seis meses de conservação. Em termos microbiológicos, a congelação mostrou ser o método de preservação menos adequado, enquanto os restantes tratamentos demonstraram ser equivalentes na manutenção da estabilidade microbiológica do produto. Em face dos resultados obtidos, e considerando as condições testadas, a conservação do pão de abelha à temperatura ambiente sem tratamento prévio parece ser um método adequado. Será no entanto importante o desenvolvimento de estudos complementares que permitam elucidar o efeito de diferentes níveis de pH, atividade de água e qualidade microbiana do produto na estabilidade de pão de abelha conservado à temperatura ambiente sem tratamento prévio.

Palavras-chave: produtos da abelha; métodos de preservação; tempo de prateleira.

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1. Introduction

1.1 Framework

Nowadays, man is more and more interested in substances of natural origins. This interest also applies to beekeeping products because of their extensive, nutritional and powerful healing properties. Therefore, bee products are widely used in human diet and medicine, and as a consequence, it is a growing business for the beekeeping industry.

Honey, propolis, pollen, royal jelly and bee venom are the most known and used beeproducts due to their powerful properties and their content in bioactive molecules (Martinello and Mutinelli, 2021), and several researches have been devoted to the study of the chemical composition and the therapeutic properties and effects of these products (Fratini *et al.,* 2016; Zhou *et al.,* 2015). However, bee bread, which is also a bee product, remains unexplored by many beekeepers due the difficulty of its extraction (Bakour *et al*., 2019).

Bee bread is considered as functional food due to its high nutritional value and to the bioactive compounds it contains. In fact, this product is very rich in proteins, essential amino acids, simple sugars and Ω fatty acids (Mărgăoan, 2019). These characteristics strengthen immunity and keep the body healthy, and due to these properties bee bread is used for apitherapeutic aims (Mărgăoan, 2019). Bee bread is increasingly applied as a health food and medicine due to its biological properties such as hypolipidaemic, hepatoprotective, anti-inflammatory and immunomodulatory effects, it helps also to improve visual activity (Khalifa *et al.,* 2019).

Numerous studies are currently available on the chemical and bioactive properties of bee bread, as can be confirmed by the recent reviews on this subject (Kieliszek *et al*., 2018; Khalifa *et al*., 2019). On the opposite side, little is known about the microbiological stability and safety of this product. In fact, besides the chemical and bioactive properties, the acknowledgement of the microbial composition of this food product is mostly directed to long term microbial succession and nutrient conversion or "pre-digestion" of the raw material, bee pollen (Anderson *et al.,* 2014).

Safety is a fundamental issue of every food for the protection of human health. To the best of our knowledge, no research related to microbiological hazards and storage-related degradation has been reported for bee bread. Due to the significant impact that bee bread is gaining in the field of human nutrition, the assessment of its microbiological aspects and the establishment of safe preservation methods is of paramount importance for the assurance of consumer's safety and satisfaction. For the establishment of appropriate preservation methods, microbial safety and stability must be verified, but chemical and bioactive properties must also be retained.

1.2 Objectives

Due to the important impact that this product is gaining and the lack of information and research on the most appropriate way to preserve its safety and stability, the aim of this work is to evaluate the impact of different preservation methods on the microbial, physical and chemical stability of bee bread throughout six months of storage.

This work was developed in the framework of the project "DivInA: DIVersificação e INovação na Produção Apícola" (Diversification and Innovation in Apiculture Production).

2. Literature Review

2.1 Definition of bee bread

Pollen is the main food for bees, followed by honey. It is in the form of microscopic oval to spherical grains, which correspond to the male reproductive cells of the flower. These grains are located at the anthers of the flower. In addition to water, when the bee forages a flower, it collects two foods: nectar, which is converted to honey and is used as a source of carbohydrates, and pollen, as a source of protein, minerals, and fats (Hoover and Ovinge, 2018).). The bee mixes the pollen with flower nectar or honey and saliva, using her back legs on which there are pollen baskets (**Figure 1**) (Komosinska-Vassev et al., 2015).

The bees move from flower to flower to harvest pollen, and with this will also disseminate pollen and ensure plant reproduction. Once their baskets are full, they go to the hive to feed the colony. During one trip, the two balls of pollen are the result of around 80 visits to different flowers, and each gather between 100,000 and 5,000,000 pollen grains for a weight of 4 to 10 mg per ball.

Figure 1. Bee with its full pollen baskets (Rathbone, 2010).

Once arrived at the hive, the pollen balls mixed with flower nectar or honey and saliva are transmitted to workers that will transform them into bee bread (**Figure 2**), which is pollen preserved through natural fermentation. The bees fill the mixture into honeycomb cells for ¾ of the cell volume, then the residual volume is filled with a thin layer of honey or propolis to protect the pollen mass from oxygen (Barene *et al.*, 2014). At this stage, the bee bread is mainly composed of pollen, honey, and secretions of bees' salivary glands (Vasquez and Olofsson, 2009; Barajas *et al*., 2011).

Figure 2. Bee bread in honeycomb cells (A) and after extraction (B) (Milojkovic, 2018).

An anaerobic lactic fermentation process takes place, thanks to the enzymes of the salivary secretions of the workers (Mărgăoan *et al.*, 2019). Three microorganisms are involved in these transformations (**Figure 3**).

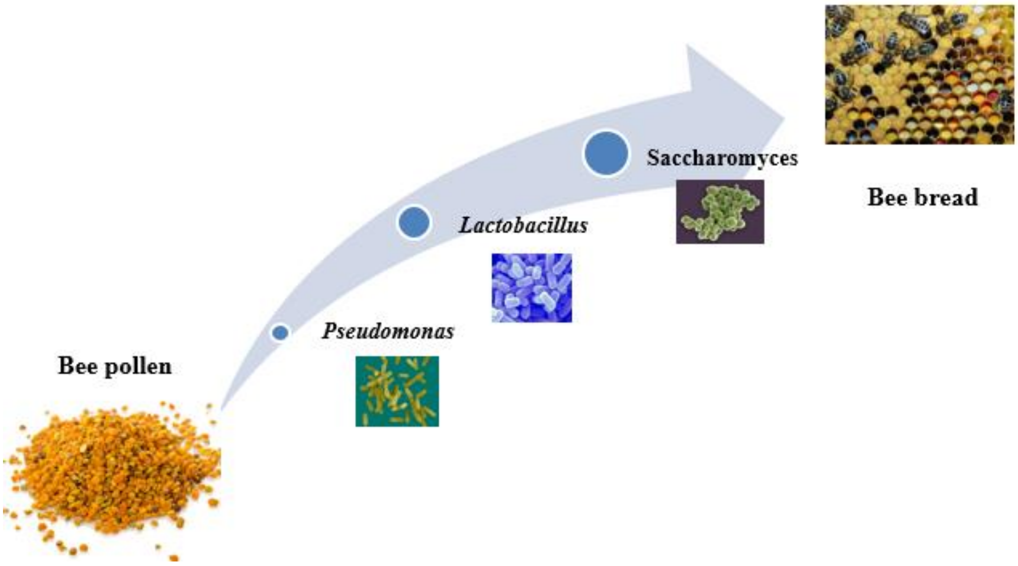

Figure 3. Fermentation of bee pollen into bee bread

First of all *Pseudomonas* spp. grow and consume oxygen before suffocating, thus creating the anaerobic environment. *Lactobacillus* spp. take over by fermenting carbohydrates into lactic acid, which will destroy the germination capacity of the pollen. Finally *Saccharomyces* spp.

metabolize the remaining carbohydrates. All these steps lead to increased acidity, nutritional value and pollen assimilation (Aosan, 2015).

2.2 Chemical composition of bee bread

The chemical composition of bee bread, which is close to that of the pollen from which it originates, is complex and variable, as shown in **Table 1**, and it depends on the predominant floral sources. It is recognized that bee bread is rich in protein, carbohydrates, and lipids, containing also other nutrients like minerals and vitamins. Bee bread can provide the essential amino acids which cannot be synthetized by humans, such as methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, and tryptophan (Komosinska-Vassev, 2015; Mohammad *et al*., 2020).

Besides the proteins, and the other components, bee bread is supplemented by a wide variety of enzymes. It contains different enzyme groups, such as acid phosphatase, leucine aminopeptidase, and β-glucosidase, that hydrolyse carbohydrates, cellobiose, salicin, amygdalin, and gentibiose; N-acetyl-β-glucosaminidase hydrolyses chitin; β-galactosidase hydrolyses lactose, and α-glucosidase hydrolyses sucrose, maltose, trehalose, and melizitose (Khalifa *et al.*, 2019).

Bee bread also contains the following substances: flavonoids, polyphenols, phytosterols, vitamins C, B_2, (riboflavin), B_3 (nicotinic acid), B_5 (pantothenic acid), B_7 (biotin), B_9 (folic acid), E, K, and P (rutin). It is rich in pigments such as carotenes and anthocyanins, and contains more than 25 minerals, including oligo elements like iron, calcium, magnesium, phosphorus, potassium, copper, zinc and selenium (**Table 1**) (Khalifa *et al.*, 2019).

The amount of lactic acid is about six times higher in bee bread than in pollen, the degree of acidity is higher, and consequently the pH is lower. The pH values can vary between 7.2, for fresh pollen, and 4.2, for bee bread because of lactic acid formation (Isidorov *et al*., 2009). This acidity allows better conservation of bee bread, by slowing the growth of moulds and other microorganisms.

Table 1. Chemical and nutritional composition of bee bread (Khalifa *et al.*, 2019).

Parameter	Composition (%)
Water content	5.91
Total lipid content	7.79
Total protein	20
Total carbohydrates	24-35
	Fructose 18.95
	Glucose 11.54
Fatty acids	Medium-chain saturated fatty acids (C_{10}-C_{18})
	Long-chain saturated fatty acids (C_{20}-C_{24})
Enzymes	Saccharase, amylase, phosphatases
Magnesium	0.061
Sodium	0.014
Zinc	0.003
Manganese	0.002
Phosphorus	0.251

2.3 Properties and use

Due to its complex composition, bee bread has been reported to have several biological properties. Bee bread is considered to have a significant antioxidant activity, based on its richness in phenolic compounds, which are known to be the most important natural antioxidants responsible for removing overproduced free radicals that damage molecules on DNA, protein, and lipids. The antioxydant activity of bee bread depends on the year of pollen collection, botanical origin, and storage period (Nakajima *et al.,* 2009; Mohammad *et al.,* 2021). Antimicrobial activity against bacteria, yeasts and virus has been reported to be among the biological properties of bee bread also due to its content of phenolic compounds (Yildirim *et al.*, 2016; Veiga *et al.*, 2017; Mohammad *et al.*, 2021). This natural product also shows anti-inflammatory activity, due to the ability of phenolic compounds to inhibit the release of histamine and reduce prostaglandin synthesis (Rimbach *et al.,* 2017; Mohammad *et al.*, 2021), anticancer (Liu *et al.,* 2016), antinociceptive (the ability to stop the pain), and antidiabetic properties (Mohammad *et al.*, 2021).

Bee bread has a positive effect on the immune and antioxidant systems of healthy people and also improves concentration and memory. It can be used during increased mental or physical effort because it provides a lot of energy, and it has a significant calming effect, thus being beneficial in case of depression, cerebral cortex inflammation and psychiatric and nervous disorders (Aosan, 2015), probably due to its high vitamin B content (Mutsaers et al., 2005). Bee bread has an effect in regulating the digestive system, as it aids in the treatment of disorders such as colitis, constipation and diarrhea, it stimulates the appetite and is a beneficial fortifier for the old people and convalescents adults (Mutsaers *et al*., 2005).

Also, it lowers cholesterol and establishes the balance between good and bad cholesterol, improves lipid balance and improves the functioning of the liver and gall as well as blood pressure (Kieliszek *et al*., 2018). Due to its high concentrations of vitamin P, which is found only in bee bread at such high concentration, it has a special significance for improving the condition of blood vessels, regulates their permeability and tightens the walls of the capillaries (Milojkovic, 2018). In addition, it is proved that bee bread has an anti-aging and anti-anemic activities because of its rich antioxidant properties (Kieliszek *et al.*, 2018). It is also advisable in the same way as honey to avoid prostate problems in adults (Mutsaers *et al*., 2005).

2.4 The microbiology of bee bread

The transformation of pollen into bee bread is the result of microbial action, mainly a lactic acid fermentaion, carried out by bacteria and yeasts (Anderson *et al*., 2014; Aosan, 2015; Cagno *et al*., 2019). However, the biochemical changes happening to pollen during its collection and storage by bees are not clearly understood and relatively little is known about the microbiology of bee bread. To study the microbiological quality of bee bread, it is necessary to know the microorganisms involved in the transformation of pollen into bee bread.

The transformation process of bee pollen is done by a succession of microorganisms in partially superimposed steps (Aosan, 2015; Khalifa *et al*., 2019). The first step, which lasts for approximately 12 hours (Khalifa *et al.*, 2019), is carried out by a mix of bacteria and yeasts, dominated by *Pseudomonas*, which consume the stock oxygen present in pollen (Aosan, 2015). Oxygen consumption leads to asphyxiation and disappearance of these microorganisms. In the second stage, under anaerobic conditions, *Streptpcoccus* and *Lactobacillus* start to grow, using carbohydrates as a source of energy and producing lactic acid. The concentration of lactic acid gradually increases to 3%, lowering the pH of the composition, which contributes to the preservation of the bee bread. The third step corresponds to growth of yeasts of the genus *Saccharomyces*, which use the remaining carbohydrates to induce the final fermentation of pollen and finalize its transformation into bee bread. The alternation between aerobic and anaerobic is made by the thin film of honey that covers the socket. This film prevents consumption of the air by bacteria. At the end of the transformation process, the bee bread is exposed to the surrounding air. When the three stages of microbial transformation in the combs end after 15 days, the pH of bee bread is approximately 4, and the microbial loads are highly reduced when compared with its raw material, bee pollen (Aosan, 2015).

In fact, the transformation of bee pollen into bee bread seems to be related with pollen preservation (storage) more than nutrient conversion which means transformation into more nutritious food, and comparing to other plant material involving microbial digestion or extensive fermentation, bee bread contains very few microbes (Anderson *et al.*, 2014). In a study on the evolution of microbial loads and diversity in hive-stored bee pollen (throughout fermentation into bee bread), Anderson *et al.* (2014) reported a decrease in the absolute number of bacteria in bee bread in the first 96 hours of fermentation to values below 2.5 x 10^3 CFU/g in more than 62% of the analysed samples, indicating that it is not a suitable medium for microbial growth.

Even so, contamination with several types of microbes other than those involved in fermention can occur. High levels of microbial contamination can be a result of poor bee pollen quality, extrinsic factors like wind, dew, rain splash, sprinkler irrigation splash and drip (De-Melo *et al*., 2015), or low hygiene during hive manipulation. Jaya *et al.* (2020) reported mean total viable counts in bee bread from different species of honey bee (*Apis cerana, Trigona sp.* and *Apis mellifera*) varying from 5.58±0.38 to 7.76±0.25 log10 CFU/g, and yeasts varying between 8.67 ±0.03 and 5.25±0.06 log10 CFU/g. This study also concluded that the bee bread produced by the three different honeybee species showed a poor microbiological quality, in comparison with the pollen, which is the raw material of bee bread. The most common fungi contaminating bee bread are reported to be *Cladosporium*, followed by *Aspergillus* and *Penicillium* (Sinpoo *et al.*, 2017; Disayathanoowat *et al*., 2020). The genera *Mucor* and *Pestalotiopsis* have been isolated in new bee pollen but disappeared after six weeks of storage in the hive (Disayathanoowat *et al.*, 2020). The major genera of yeast that have been found in pollen and bee bread are *Candida* spp., *Cryptococcus* spp., *Kloeckera* spp., *Metschnikowia* spp., and *Rhodotorula* spp. (Gilliam, 1979).

2.5 Preservation of bee bread

According to the Institute of Food Science and Technology, the shelf life is the period of time during which the food product will remain safe, retaining its desired sensory, chemical, physical, microbiological, and functional characteristics, and, where appropriate, comply with any label declaration of nutrition data, when stored under the recommended conditions (Zweep, 2018). The shelf life can be affected by several factors such as product formulation, raw material quality, pH, a_w, storage conditions (temperature and relative humidity), packaging, and consumer handling. So the effect of these factors on the growth of specific microorganisms must be considered (Everis, 2019). Knowing that the quality of food is very important and can affect consumer's behavior, the development of new production technologies and storage conditions is the basis for global market evolution, so the produced food will be free of risks (Kieliszek *et al.*, 2018).

Given the requirements of food safety and knowing that bee bread is gaining more and more attention as a food, its chemical stability and microbiological safety are important quality criteria of this product. In order to increase bee bread shelf life, adequate preservation methods should be developed to obtain high microbiological quality of the bee bread, while retaining its physico-chemical and bioactive properties.

According to the literature, bee bread can last for several months if subjected to two weeks of fermentation in the hive under certain moisture and temperature (35-36 °C) conditions. After this natural fermentation process, the high levels of lactic acid and other metabolites preserve the bee bread and prevent its deterioration (Vásquez and Olofsson, 2009). But even after the fermentation is completed, inapropriate moisture content and a_w values can endanger bee bread safety and stability. A high value of a_w causes proliferation of microorganisms such as deteriorating or pathogenic bacteria and fungi (Mathlouthi, 2001), and it can also affect other aspects of food safety and quality.

Studies by Mohammad *et al.* (2020) showed that *Heterotrigona itama* bee bread had a moisture content between 11.1 and 12.5% and that dried *Tetragonula biroi* bee bread presented a moisture value of 14.2 to 16.7%, moisture contents can go up to 53.4% (Rebelo *et al.*, 2016). Even though moisture content is a common way of measuring food hydration, a_w is a better indicator of food spoilage in some food items, like those rich is osmoactive compounds such as sugars and salts, since these will determine the level of water available for microbial metabolism. Values of a_w higher than 0.6 are considered favourable to fungal growth, while pathogenic bacteria will grow at a_w values higher than 0.85 (Mohammad *et al.*, 2020). Mohammad et al. (2020) reported that a_w of fresh bee bread of *H. itama* was in the range of 0.729 to 0.852. In Brazil, a_w of fresh bee bread from *Melipona* spp. achieved values ranging from 0.85 to 0.93 (Rebelo *et al.*, 2016; Alves *et al.*, 2018). These studies evidenced that fresh bee bread has a high a_w, which increases the chances for growth of both beneficial and pathogenic bacteria, yeasts or molds. Also, this may contribute to the presence of mycotoxins like aflatoxins which has been reported in multiple types of pollen (Kostic *et al*., 2019). Therefore, preservation methods that decrease a_w below the level at which microbial growth occurs (>0.60) can help extend the shelf life of bee bread (Adams *et al*., 2016).

The information related with the effect of preservation methods on bee bread microbial, bioactive and nutritional stability is scarce. In a rare study on the subject, Combey (2017) tested three preservation methods - refrigeration, freezing and oven-drying - of bee bread to assess the best method of preserving bee bread. According to this study, the moisture content of bee bread samples submitted to these processes were very different. The oven dried bee bread had a moisture content of 6.5%, while the moisture contents for the frozen and refrigerated samples were 25% and 15%, respectively. In terms of bacterial counts, the dried bee bread had the lowest bacterial counts (1.8 x 10^7 CFU/g), while the other treatmeants had a two-fold level of contamination. This study did not report the microbial loads throughout storage.

Regarding the physico-chemical properties, the same study by Combey (2017) evidenced that the frozen and refrigerated bee bread kept the same color as the fresh bee bread, but the oven drying method changed its color to brown. Regarding the taste, no noticeable changes were registered after the application of the various preservative methods.

No other studies where found on the application of preservation methods to bee bread, and on the effect of those methods on its microbial and physico-chemical stability. No studies have been found on that effect throughout long-term storage. If bee pollen is considered, the most used preservation technique is oven drying because of the reasonable process time, better sanitary conditions and better control of the drying conditions and the preservation of a maximum quality (Bogdanov, 2015; Mekki, 2019). To maintain the nutritional properties of bee pollen, this technique should be conducted at low temperature, not exceeding 45 °C. Mekki (2019) and Lema *et al*. (2019) did not find significant differences between different drying temperatures (ranging from 35 to 45 ºC) on the microbial stability and nutritional value of bee pollen, respectively. However, the study performed by Lema *et al*. (2019) evidenced that oven drying performed at 45 °C, caused a significant increase in the bee pollen reducing power. This behaviour was also registered for bee pollen freeze-drying (Lema *et al*., 2019), which is a preservation technique that has been increasingly used mainly due its ability to preserve the chemical and biological properties of various food products (Ciurzyńska and Lenart, 2011). However, Mekki (2019) observed that microbial stability was not optimal when bee pollen was preserved through freeze-drying.

Despite the importance that bee bread has been gaining, there are not many studies focussed on the impact of preservation methods on the chemical and microbiological characteristics of bee bread during storage. Therefore, more research must be carried out in order to understand how physicochemical characteristics and microbial stability are influenced by the application of different preservation methods, and subsequently establish the adequate preservation techniques for bee bread.

3. Materials and methods

3.1 Sampling

Bee bread was collected by beekeepers in the spring of 2020 in the northeast region of Portugal. Bee bread was then extracted manually from the frozen hive and the externel wax was removed using a scalpel (**Figure 4**), homogenized, air-dried and stored at -20 °C for further use. Bee bread was then divided into aliquots and submitted to different preservation processes, described in section 3.2. Samples were then submitted to physico-chemical and microbial analysis at five different time points: immediately before being processed (before); immediately after being processed (T0), and after 1 month (T1), 3 months (T3) and 6 months (T6) of storage.

Figure 4. Removing of bee wax from bee bread.

3.2 Preservation methods

Bee bread was divided into four portions of 250 grams, and each portion was submitted to one of the following preservation techniques:

- Freezing at -20 °C: bee bread was stored at -20 °C in a domestic freezer;
- Freeze-drying: bee bread was submitted to freeze-drying (Zirbus Lyophilizer Vaco 10-II-D) and stored at room temperature (RT; between 20-25 ºC);
- Oven drying: bee bread was dried in an oven (Climacell, MMM Group, model CLC-B2V-M/CLC111-TV), at 30 °C for 24h and stored at RT.
- Preservation at room temperature: bee bread was stored at RT.

After applying the preservation methods each sample was divided into three portions of 15 g and stored in 50 mL Falcon® tubes for further analysis (**Figure 5)**.

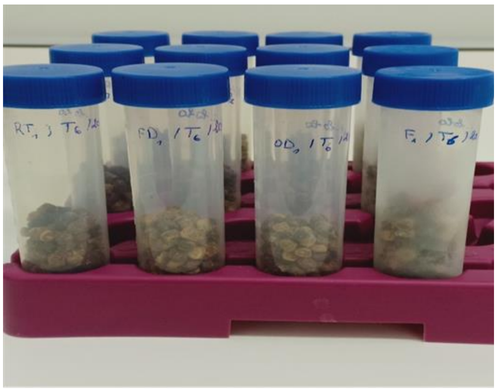

Figure 5. Bee bread samples stored after the application of preservation methods.

3.3 Physico-chemical analyses

3.3.1 Water content

Water content was evaluated by drying approximately 2 g of bee bread, at 105 °C for 2 hours, in a Memmert Basic UNB-500. The analysis was performed in triplicate. The water content was then calculated and expressed as percentage (%), according to the following equation:

Water content (%) = (M0+M1-M2)/M1×100

where:

M0 = tare weight of the container (g)

M1 = original test sample weight (g)

M2 = weight after drying (g) (container + test sample after drying)

3.3.2 Water activity

The values of a_w in bee bread samples were measured using a Dew Point Water Activity Meter 4TE (**Figure 6)**. The value of a_w is obtained by the determination of the precise dewpoint temperature of the sample and then translated into water activity. The analysis was carried out in triplicate.

Figure 6. Water activity meter.

3.3.3 Ash content

The ash content was evaluated by incineration of approximately 2 g of bee bread, at 550 °C for 4 hours, according to the AOAC (1995) procedures. The analysis was performed in triplicate.

3.3.4 Lipid content

The lipid content was determined using a Soxhlet apparatus (**Figure 7**) for the extraction of approximately 2 g of bee bread with diethyl ether, duringfor 4 hours. The diethyl ether extract was evaporated at room temperature to dryness. After this, the residue was weighted and the lipid content was expressed as a mass percentage (Almeida-Muradian *et al.*, 2005). The analysis was performed in triplicate.

Figure 7. Soxhlet apparatus used for the determination of lipid content.

3.3.5 Protein content

The protein content was determined through the evaluation of the total nitrogen content, using the Kjeldahl method, after applying an adequate conversion factor. Briefly, approximately 2 grams of bee bread were digested through a conventional acid hydrolysis, using a copper catalyst, followed by neutralization and titration of the ammonia liberated. For the conversion of nitrogen levels to proteins, the factor 5.6 was used (Rabie *et al.*, 1983). The analysis was performed in triplicate.

3.3.6 Total carbohydrates and energy

The value of total carbohydrates was evaluated by difference, using the expression:

Total carbohydrates = 100 − (g ashes + g proteins + g lipids). And the energy content of bee bread was estimated by equation, using the Atwater system:

Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g lipids) (Merill and Watt, 1955).

3.3.7 Phenolic compounds analysis

3.3.7.1 Extraction of phenolic compounds

For the extraction of the phenolic compounds, 2 g of bee bread sample were mixed with 20 mL of 80% ethanol/water, at room temperature. After 6 hours, the mixture was filtered by gravity using filter paper, and the residue was re-extracted in the same conditions. After the second extraction, the solutions were mixed, freeze dried ,and stored at -20 °C for further use.

3.3.7.2 Total phenolic compounds content

The total phenolic content was analyzed by the method of Folin-Ciocateu using gallic acid as standard (Falcão *et al.*, 2013). Briefly, 0.5 ml of ethanolic extract (1 mg/mL) was mixed with 0.25 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of saturated sodium carbonate was added and then deionized water was added to adjust the final volume to 5 mL. The solution was then heated for 10 min at 70 °C, cooled in the dark for 30 min, and the absorbance was measured at 760 nm (Analytikijena 200–2004 spectrophotometer, Analytik Jena, Jena, Germany). For the quantification, a calibration curve of gallic acid was prepared with solutions in the range of 0.03–0.5 mg/mL ($y = 9.85x + 0.07$; $R^2 = 0.992$). The results were expressed as mg of GAE (gallic acid equivalent) per g of extract. The assays were run in triplicate.

3.3.8 Antioxidant activity

The antioxidant activity of the ethanolic extracts previously obtained, as described in section 3.3.7.1, was evaluated using the 2,2-diphenyl-1-pycrylhydrazyl (DPPH) method, according to the procedure described by Brand-Williams (1995), with slight modifications.

An aliquot (0.03 mL) of bee bread extract solution, with concentrations from 0.03 to 1.7 mg/mL, was added to 0.15 mL of DPPH (25 mg/L, in 80% ethanol/water), prepared daily, then the plate was incubated in the dark for 45 minutes at room temperature and the absorbance was measured at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.).

The percentage of radical inhibition was calculated as follow:

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

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

3.4 Microbial analysis

3.4.1 Sample preparation

Samples were prepared following the procedure described in ISO 6887-1:2003 (ISO, 2003a). Under sterile conditions, 2.5 grams of each bee bread sample were homogenized in 22.5 mL of sterilized buffered peptone water. The mixture was placed into sterile bags and homogenized in a Stomacher for 1 min. Decimal dilutions were made using the same diluent.

3.4.2 Enumeration of mesophiles

The enumeration of aerobic mesophiles was performed as described in the ISO 4833:2003 (ISO, 2003b). One mL of each decimal dilution was inoculated in 9 cm Petri dishes containing 15 mL of Plate Count Agar (PCA, HiMedia), in duplicate, using the pour plate technique, and incubated at 30 °C for 72 hours. All colonies were counted. Microbial counts were expressed as colony-forming units per gram of bee bread (CFU/g) using the formula:

$$N = \Sigma C / [V * (n_1 + 0.1 n_2) * d] \text{ CFU/g}$$

where:

ΣC = sum of colonies counted in all countable plates
V = volume of inoculum inoculated in each plate
n1 = number of plates on which the first dilution was counted
n2 = number of plates on which the second dilution was counted
d = dilution from which the first counts were obtained

3.4.3 Enumeration of lactic acid bacteria

The enumeration of lactic acid bacteria was performed according to the method described in the ISO 15214:1998 (ISO, 1998). One mL of each decimal dilution was inoculated in 9 cm Petri dishes containing 15 mL of Man Rogosa Sharp Agar (MRS, BioLife), in duplicate, using the pour plate technique, and then incubated at 37 °C for 72 hours. Colonies were counted using the formula, and were expressed as CFU/g.

3.4.4 Enumeration of coliforms

Coliform counts were performed as described in the ISO 4832:2006 (ISO, 2006). One mL of test sample from each decimal dilution was inoculated in 9 cm Petri dishes containing 15 mL of Violet Red Bile Lactose Agar (VRBLA), in duplicate), using the pour plate technique. After incubation at 37 °C for 48 hours, colonies were counted using the formula and were expressed as CFU/g.

3.4.5 Enumeration of yeasts and molds

Yeasts and molds were counted as described in the ISO 21527-2:2008 (ISO, 2008). Two hundred μL of each dilution were inoculated by the spread plate technique, in duplicate, onto a 9 cm Petri dish containing 15 mL of Dichloran Glycerol 18% agar (DG18, HiMedia). Petri dishes were incubated at 25 °C for 5 days. Yeast and mould colonies were counted separately, after three and five days of incubation, respectively, using the formula, and were expressed as CFU/g.

3.5 Statistical analysis

The statistical software IBM SPSS Statistics version 22 was used for data analysis. Levene's test verified the homogeneity of the variances. As the data followed a normal distribution, analyses of variance (ANOVA) were carried out to evaluate if there were significant differences ($p < 0.05$) between samples. ANOVA was applied when homogeneity of variances was observed. Additionally, significant Post-Hoc analyses were performed by the Tukey HSD test.

4. Results and discussion

In this study bee bread samples were subjected to different preservation methods. Physico-chemical parameters and microbial loads were analyzed and recorded before applying the various preservation techniques and monitored over time after 1, 3, and 6 months. The statistical analyses related with the following results are presented in **Appendix 1**.

The results of the analyzed parameters before applying the preservation methods are presented in the table below (**Table 2**).

Table 2. Physico-chemical and microbial results before applying preservation methods

Parameters	Average ± SD
Water activity	0.57 ± 0.0
Moisture content (%)	16.55 ±0.08
pH	3.72 ±0.03
Total protein (% Dry basis)	27.55 ±1.21
Total lipid (% Dry basis)	2.70 ±0.28
Ash content (% Dry basis)	2.95 ±0.19
Carbohydrates (% dry basis)	85.43 ±3.25
Total phenolics (mg/g extract)	5.75 ±0.67
EC50 (mg/ml)	0.07 ±0.0
Total viable counts (Log10 CFU/g)	2.22 ±0.16
Lactic acid bacteria (Log10 CFU/g)	1.59 ±0.0
yeasts(Log10 CFU/g)	3.36 ±0.04
Molds (Log10 CFU/g)	3.11 ±0.22
Coliforms (Log10 CFU/g)	2.40 ±0.50

4.1 Physico-chemical analysis

The preservation and the shelf life of bee bread can be influenced by several parameters including water activity and moisture content. Therefore, to maintain the microbial, chemical and nutritional stability of bee bread, where there is a high risk of bacterial growth and the possibility of initiating chemical and enzymatic reactions, it is important to control these two parameters. The results obtained for moisture content and a_w are shown in **Figure 8.**

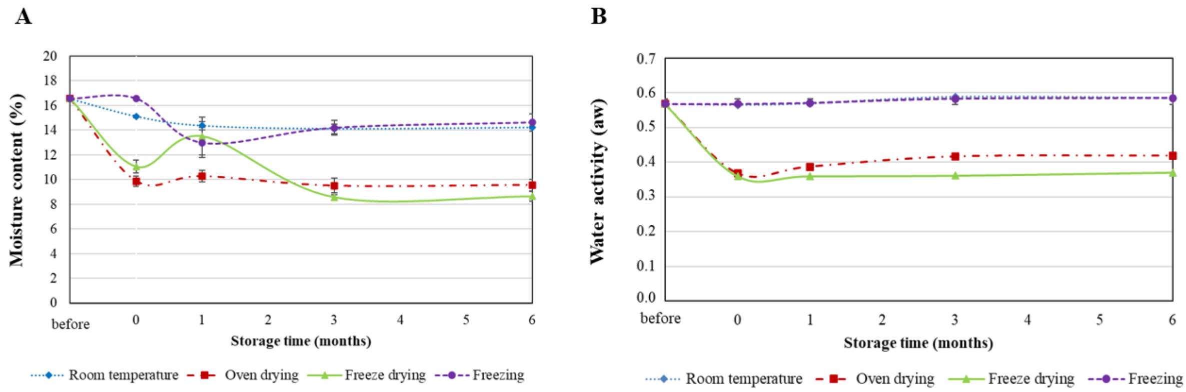

Figure 8. Moisture content (**A**) and water activity (**B**) obtained before and after the application of different preservation methods at different storage time points (0, 1, 3 and 6 months).

Moisture content showed a significant difference between before applying the treatment and after for all the techniques except for the frozen samples. For the a_w there was no significant difference between the frozen samples and the samples stored at room temperature. There was a significant difference also between all the treatments in all the storage period (six months) for both parameters. Despite that the statistical analysis showed a significant difference for the frozen samples and the samples stored at room temperature for the moisture content and a_w, there was no variation throughout time. But as expected, there was decrease in the a_w and moisture content of the oven dried and freeze-dried samples (**Figure 8**), even though there was no significant difference concerning moisture content in the oven dried samples throughout storage period and a_w in the freeze-dried samples. The highest value of moisture content after six months of storage was registered for the frozen samples (14.62 %). For the a_w, the highest value was registered for both frozen samples and samples stored at room temperature with a value of 0.58. It must be noted that the a_w for the frozen samples might show some deviation to the reality because this parameter is lowered by freezing, and we cannot exclude the possibility that the frozen samples thawed before analysis. Yeasts and molds usually thrive when the value of a_w is more than 0.61, while for bacterial growth the a_w should be greater than 0.91 (Anjos *et al.*, 2019). Lowering the a_w in order to prevent microbial growth, as well as chemical and enzymatic reactions, is the basis of preservation. Under this specific aspect, all preservation methods applied to the samples seem to be adequate for bee bread preservation.

All the analyzed bee bread samples had acidic pH, as shown in **Figure 9**, ranging between 3.48 and 4.06, without significant differences between treatments. Freeze dried samples were the only ones that showed a significant variation in the pH from 1 to 3 months. The low pH, combined with reduced a_w values could inhibit the microbial growth and decrease the problems with texture and stability, thus increasing the shelf life of bee bread (Anjos *et al.*, 2019).

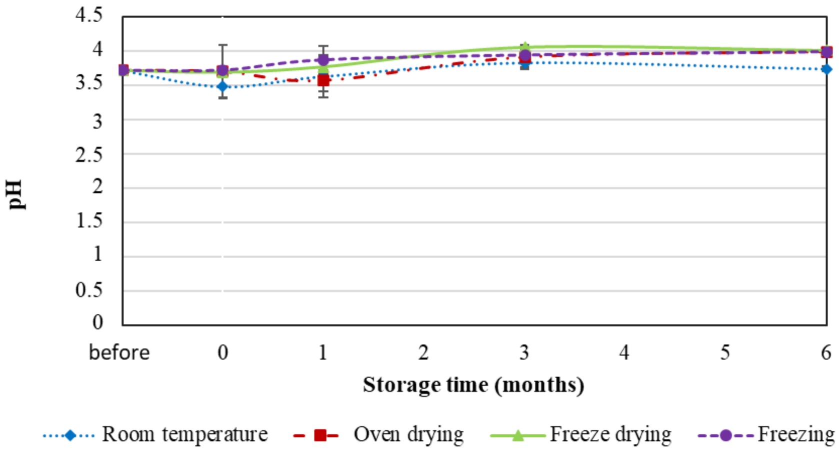

Figure 9. pH obtained before and after the application of different preservation methods at different storage time points (0, 1, 3 and 6 months).

Results obtained for protein, lipid, ash, and carbohydrate contents are shown in **Figures 10 A, B, C,** and **D**, respectively.

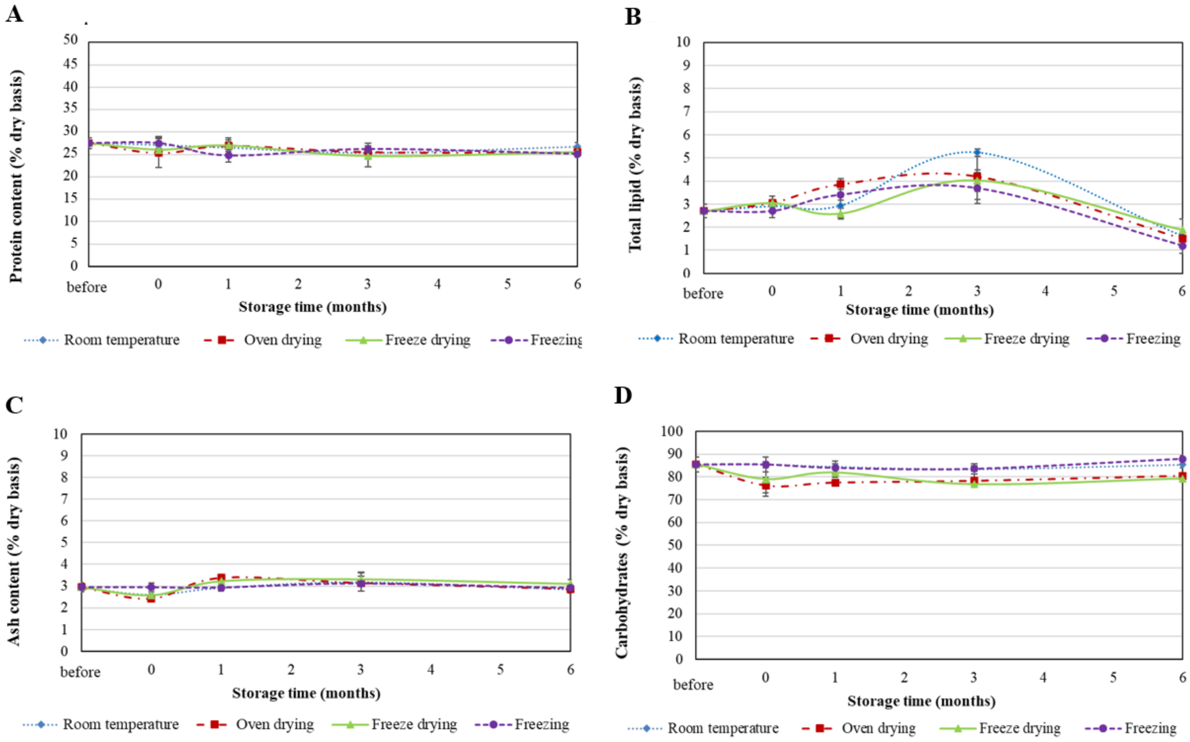

Figure 10. Protein content (**A**), total lipid content (**B**), ash content (**C**) and carbohydrates content (**D**) obtained before and after the application of different preservation methods at different storage time points (0, 1, 3 and 6 months).

The protein content is an important nutritional characteristic of bee bread and the values reported in different studies can vary, depending on the plant source (Mohammad *et al.*, 2020). In the present study, the average protein content of bee bread samples ranged between 24.6 g/100 g (dw) and 27.6 g/100 g (dw), as presented in the **Figure 10A**. This is in accordance with the values reported by Urcan *et al.* (2017), where values ranging from 14.1 to 37.3 g/100 g (dry basis) were registered. After applying the different preservation methods, a decrease, although not statistically significant, between 2-3% was registered.

Similarly, to protein, the lipid content of bee bread can be affected by the bee pollen lipid composition. Lipids contain important molecules for human nutrition, such as essential fatty acids and important antioxidant compounds (Mărgăoan *et al.*, 2014; Arien *et al.*, 2015). Therefore, it is important that the preservation methods used for long-term storage do not negatively affect the bee bread lipid profile. In all samples, there were no significant differences in the total lipid content before and after applying the preservation methods (**Figure 10B**). Nevertheless, the results showed a significant increase, between 32-80%, in the lipid content for all the preservation methods until the third month of storage. The highest value of lipid content was reached for the samples stored at room temperature at the third month of storage with a value of 5.24 %. After the third month of storage, the lipid content decreased significantly for all treatments until 6 months, but without a significant difference between treatments. The lowest value (1.19 %) occurred in the frozen bee bread samples.

The amount of ash represent the mineral content, which can be affected by the geographical conditions, soil characteristics, origin, and also by the collection and cleaning processes (Tomás *et al.*, 2017; Urcan *et al.*, 2017). The ash content of the frozen samples was kept stable throughout the period of storage, as evidenced in **Figure 10C**. On the other hand, all the other samples showed a similar trend for the variation of the ash content over the 6 months storage. Oven dried and freeze-dried samples had significant changes in the ash content throughout the storage period (six months). And both samples showed a significant decrease right after the treatment. After one month of storage, the samples showed a significant increase in ash content. After six months of storage, the results showed no significant difference between treatments.

The carbohydrates, which include polysaccharides such as starch and various cell wall components, are the main components of bee pollen, comprising up to 55% (Campos *et al.*, 2008). For bee bread, the content is higher probably due to the addition of honey during the production process by bees, but it varies very widely depending on the origin of the pollen. This is in agreement with the results previously reported by Tomás *et al.* (2017) in which the carbohydrates found in bee bread samples ranged between 58 -78 %, and confirmed by our results, where the carbohydrates value before applying the preservation treatments was 85.3 % (**Figure 10D**). The oven dried and freeze-dried samples showed a significant difference before and after the application of the treatments, however there were no significant difference registered for all the treatments throughout storage period.

Bee bread is a rich source of phenolic compounds which determine its biological activity. It was reported that the total phenolic content can be highly affected by bee pollen origin and the storage methods (Canale *et al.*, 2016; Castagna *et al.*, 2020). **Figure 11** presents the results obtained for total phenolics content and antioxidant activity of the samples submitted to the different treatments.

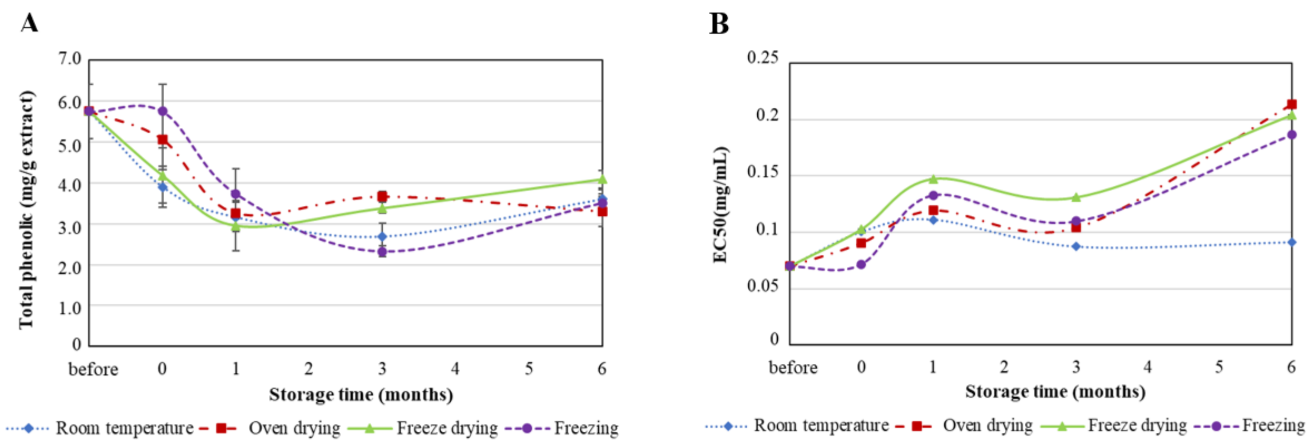

Figure 11. Total phenolics content, expressed as mg GAE/g extract (**A**) and antioxidant activity (**B**), expressed as EC_{50} value (mg/mL) evaluated through the DPPH method.

Before applying the preservation methods, the total phenolic content of bee bread samples was 5.75 mg/g extract, then the results showed a significant decrease after treatment for freeze dried samples and samples stored at room temperature to a value of 4.18 mg/g extract and 4.90 mg/g extract respectively, as can be observed in **Figure 9A**. All the treatments induced a decrease in the total phenolic compounds throughout the six months of storage. Right after the treatments and after one month of storage, the highest value was for the frozen samples (3.73 mg/g extract), and it decreased to be the lowest value at 3 months of storage (2.32 mg/g extract). This decrease can be explained by the fact that freezing causes cell breakage and allow enzymatic reactions; thus, phenolic compounds can degrade during the storage and more extensively when thawing during analyses (Rabie *et al.*, 2015). At one month of storage the lowest value was registered for the freeze-dried samples with value (2.96 mg/g extract) and started to increase to be the highest value after six months of storage (4.09 mg/g extract), this increase is probably due to a treatment-dependent liberation of the insoluble fraction of polyphenols (Antonella *et al.*, 2020). At three months of storage, oven dried bee bread had the highest value (3.67 mg/g extract) despite the significant decrease which can be explained by the degradation of polyphenols promoted by the drying process via enzymatic and non-enzymatic oxidation of phenolic compounds (Abhay *et al.*, 2016). After six months of storage there was no significant difference between oven dried, frozen and room temperature stored samples.

Bee bread antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The results showed a significant difference between treatments during all the storage period (six months) and also a significant difference among every treatment during the storage period. During the first 3 months of storage, the freeze-dried samples showed the highest EC_{50} values, as shown in **Figure 11B**, reflecting a lower antioxidant activity, which can be explained by the relatively low phenolic content found during this period of storage. Several studies have shown that antioxydant activity is strongly related to total phenolics compounds (Kalt *et al.*, 1999; Gheldof and Engeseth 2002), and polyphenolics contribute substantially to the antioxydant capacity of many small fruits (Kalt *et al.*, 2001) The DPPH result for freeze dried, oven dried and frozen bee bread samples continued to increase until the sixth month of storage with a significant difference, confirming the results found for total phenolic content. The samples preserved at room temperature showed the best scavenging effect from the first month until the sixth month of storage, suggesting that this preservation technique should be considered when the maintenance of the bee bread biological activity is an important issue. Interestingly, an increase in the EC_{50} values was registered for freezing, freeze-drying and oven-drying, after 6 months of storage. However, this reduction in the antioxidant activity is not supported by the variation observed for the total phenolic compounds content, which increased. This could be explained by the dynamic metabolic processes, which result in the degradation and/or biosynthesis of phenolic compounds with distinct antioxidant activity (Ghirardello *et al.*, 2016). Therefore, a more in-depth research must be performed in order to clarify, understand and explain the changes in the profile of the phenolic compounds throughout long-term storage.

4.2 Microbial analysis

The loads of aerobic mesophiles, LAB, yeasts, molds and coliforms are presented in **Figure 12**.

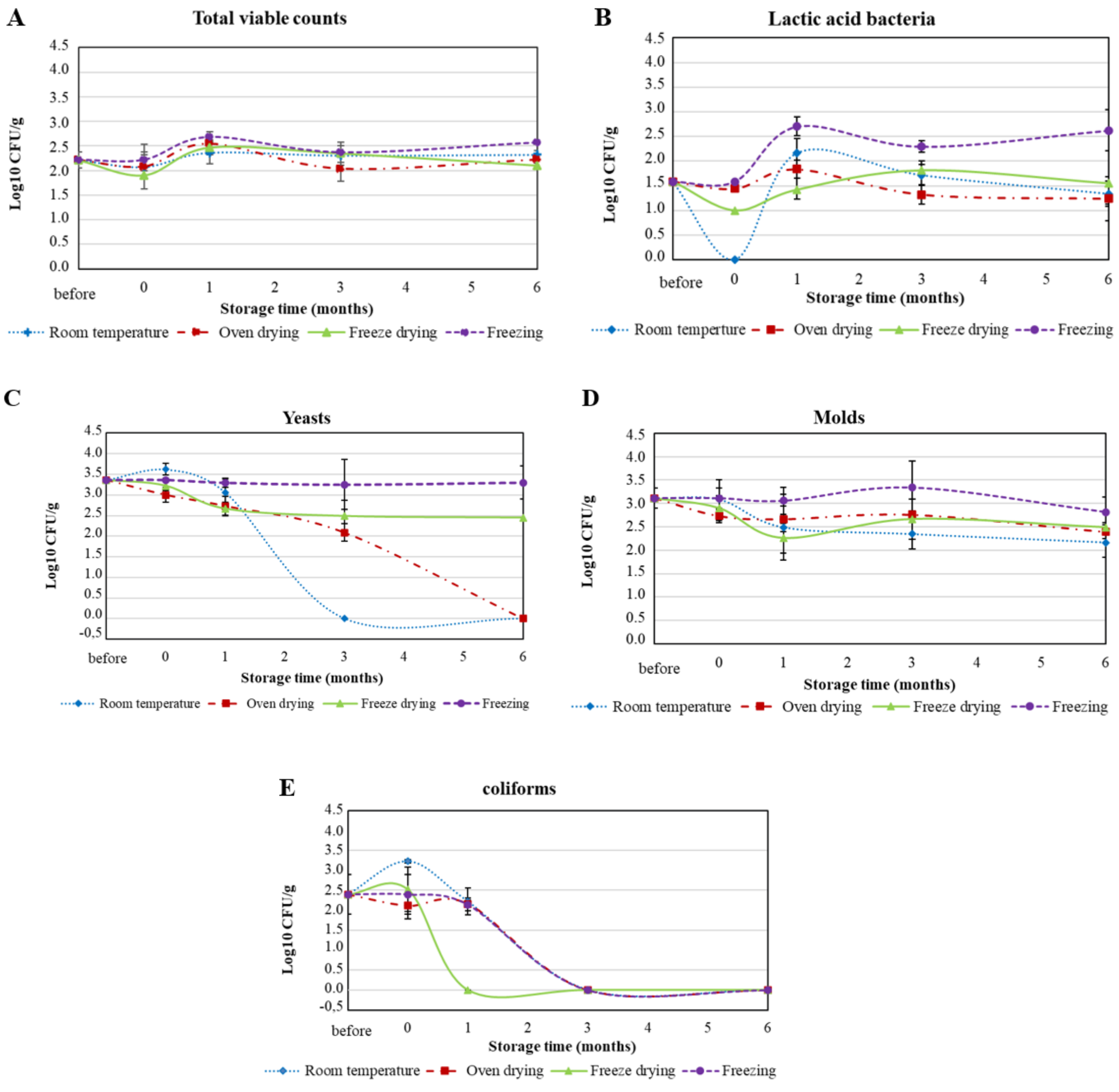

Figure 12. Microbial loads of bee bread samples submitted to different preservation methods, throughout different periods of storage (Before treatment, 0, 1, 3, and 6 months): Total viable counts (aerobic mesophiles) (A), Lactic Acid Bacteria (B), Yeasts (C), Molds (D) and Coliforms (E).

Aerobic mesophiles are indicators of the hygienic quality of food or raw materials, the production conditions, processing, and storage conditions (de Arruda *et al*. 2017). High levels of these microbes in the food product can endanger its commercial quality and shelf life and can limit the efficacy of the preservation methods. The results of aerobic mesophiles showed low levels of aerobic mesophiles, and no significant difference was observed before and after applying the treatments, as well as between treatments. A stable trend was observed throughout time of storage for over dried and room temperature samples, but frozen and freeze-dried samples showed a significant increase in the aerobic mesophile loads between months three and six of storage. At six months of storage, the lowest level was registered for the freeze-dried samples with a value of 2.11 log_{10} CFU/g, while the highest was observed for frozen samples (2.57 log_{10} CFU/g).

The bee bread is a result of lactic acid fermentation of bee pollen; therefore, the analysis of LAB is important in our study. The results showed a significant difference before and after treatments for the freeze-dried samples and those stored at room temperature, but these differences can be due to the low levels detected, which can introduce higher bias to the results. During the storage period of bee bread samples, the frozen bee bread samples showed significantly higher values than the remaining samples. Oven dried samples were kept stable without significant difference during the six months of storage, and registered the lowest value after six months, with no significant differences when compared with room temperature and freeze-dried samples. Comparing our results to the results founds by (Mekki, 2019), they showed the same trends after six months of storage, with a highest value for the frozen bee pollen and the lowest one registered for the oven dried samples.

Regarding yeast counts, oven dried samples and samples at room temperature registered a significant reduction throughout storage, while frozen samples remained stable during all the storage period. Freeze-dried samples showed a significant decrease during the first month of storage and continued in a stable trend until the sixth month. Yeasts found in foods are usually associated with fermentation processes, so their control throughout storage is mandatory to maintain the commercial quality of the product. Our results show that all treatments are adequate for controlling yeast development, but oven drying and simple room temperature storage seem to be the best treatment and storage conditions, since they induce a significant reduction of these microorganisms.

In terms of mold counts, all the samples showed a similar trend during all the storage period, without significant differences between treatments. Also, there was no significant difference throughout time for each treatment. Molds are important contaminants in food products, as they can grow and produce mycotoxins even at low levels of a_w and pH. For this reason, their control during bee bread storage is mandatory. Under this perspective, all preservation methods are able to control mold growth, with room temperature showing the lowest mold loads after six months of storage. As for other microbial groups, freezing seems to be the worst preservation method. Coliform contamination is an indicator of the hygienic conditions of the manufacturing process (de Arruda *et al.*, 2017). Therefore, it is necessary to analyze it to ensure the effectiveness of the preservation methods for safety reasons.

For all the sampling times except time 0 for yeasts and coliforms, the frozen bee bread had the highest microbial counts. Freezing is the method mostly used by beekeepers to preserve bee products, including bee bread. This method acts by cold and by turning the available water into ice crystals, which stops the microbial activity and growth, but it does not kill the microorganisms, so they will survive the freezing treatment and grow again once the product is thawed. Bacteria have a considerable variation in the ability to resist freezing treatment, with Gram positive bacteria being more resistant than Gram negative bacteria (Rahman and Velez-Ruiz, 2007). This can explain the fact that the frozen samples have the highest microbial levels. However, there is still no regulation and standards established for microbiological quality control for bee bread. When comparing with similar studies on bee pollen, other authors reported that most of microbiological indicators of frozen pollen were still detected after four and six months of storage (Mauriello *et al.*, 2017; Mekki, 2019).

Overall, the results of microbial loads of freeze-dried samples, except for the coliforms, showed a stable trend during all the storage period although it presented the lowest value for a_w at all sampling points. Freeze drying is a drying method based on the dehydration by sublimation of frozen product (Oyinloye and Yoon, 2020), its performance can explain the stability of microorganisms in freeze dried samples. Studies proved that 100% cell viability of Gram negative and Gram-positive microorganisms is possible using lyophilization techniques. As a matter of fact, freeze drying can preserve the biological and chemical properties of various food products that can improve the microbial viability (Morgan *et al.*, 2006). Also, freeze drying can be used to preserve bioactive molecules like DNA, enzymes, and proteins, ant it is an effective technique for long-term living systems such as cells, bacteria, and fungi (Nireesha *et al.*, 2013).

On the other hand, oven dried samples and those stored at room temperature showed an effective performance for all the analysed microbes. To the extent of our knowledge, the studies about the preservation of bee bread are extremely limited. Comparing our results with the results found by Mekki (2019) on bee pollen, oven dried bee pollen also registered the lowest microbial values. The information about the survivability during drying process at different temperatures of complex solid foodstuffs such as bee pollen and bee bread is scarce (Smelt *et al.,* 2014). During drying, the dehydration of microbial cells may affect the different cellular components, and the removal of water can cause DNA and RNA break down, protein denaturation, cytoplasmic membrane alteration and cell damage. Also, drying process induce the increase of the concentration of acids and toxic compounds in the cell with the risk of oxidation reactions occurrence in cells (Lievense *et al.*, 1994).

It is well known that microorganisms differ in sensitivity during their submission to preservation methods specially to heating and drying, and their prior growth conditions also influence this sensitivity. Also, microorganisms are more resistant to dry heat than to wet heat (Smelt *et al.,* 2014). Therefore, it is expected to have different behavior during thermal drying process for bee bread. But the sensitivity and variability of microorganism at different storage time of treated bee bread samples can be explained also by the multiple stresses applied on these microorganisms. Temperature changing is not the only stress that has been applied to microorganisms. The decreasing of water content and water activity and the low pH value can affect the microbial growth and survival. The presence of several constraints at the same time could act synergistically or antagonistically. On the one side, reduced water activity could increase heat resistance of microorganisms so that dehydration processes can act competitively with heat process, but similar antagonistic effects could be observed for other stresses combinations (Lievense *et al.*, 1994; Smelt *et al.*, 2014). For example, low pH can turn microbial cells more sensitive to dehydration.

Under the tested conditions, freezing and freeze drying were not considered efficient in maintaining the microbial, chemical, and nutritional quality of bee bread. Also, freeze drying is among the most expensive techniques with a high energy consumption and high operational costs. Oven drying seems to be a good choice to preserve bee bread, and it is a gain in time and money compared to freeze drying, which has costs 4-8 times higher. The samples stored at room temperature showed good microbial stability, and the chemical, physical and nutritional properties were maintained. Therefore, it seems to be a good choice that can be used by beekeepers without any costs compared to the other techniques.

5. Conclusions

Our study showed that the preservation methods and storage period influence the nutritional composition of bee bread. Lipids, ashes, carbohydrates, total phenolic compounds, and antioxidant activity were highly influenced by the preservation techniques and storage conditions, but these methods did not have a significant impact on protein content. We can conclude that storage at room temperature without the application of any preservation technique can be an adequate method to preserve the nutritional quality of bee bread.

Concerning the impact of preservation methods on the microbial stability of bee bread, our study showed that all the used techniques have different impact on bee bread stability. in all the analysis performed frozen samples registred the highest values of microbial loads. Freezing and freeze drying were not considered efficient in maintaining the microbial quality of bee bread. Oven drying and room temperature storage showed the best results and with no significant differences between the two techniques in most cases, so we can conclude that oven drying and room temperature storage are the most adequate methods in terms of microbial stability for a preservation period of six months.

So, considering the physico-chemical and microbial stability of the bee bread, as well as the economic costs and the energy expenditure, a regular storage at room temperature without the application of any preservation technique seems to be a good way of preserving bee bread, and a cost-effective alternative to the commonly used oven drying and freezing techniques.

Further studies are required to elucidate the effect of different levels of pH and water activity on the stability of non-treated bee bread.

6. Perspectives

Based on the work presented in this thesis we give some recommendations for future research:

- Knowing that the susceptibility to microbial deterioration is highly dependent on the pH, moisture content, and water activity of bee bread at the moment of collection, further studies are required to evaluate the impact of different levels of pH, moisture content, and water activity on the shelf-life of bee bread;

- Also, since the botanical origin of pollen used by the bees to produce bee bread has an significant influence on the chemical composition of this hive product, it would be important to assess how this can influence the impact of the various preservation methods on the nutritional parameters and microbial loads throughout the storage period;

- Phenolic compounds can play an important role in the bioactive properties of bee bread, such as antioxidant activity. The results presented in our work showed that, except for bee bread preserved by oven drying, after 3 months of storage, an increase in the total phenolic compounds content, accompanied by a decrease in the antioxidant activity, was registered. Altough this result could be explained by a possible loss of the antioxidant activity of phenolic compounds, the profiling of the various types of phenolic compounds throughout storage could provide a more in-depth vision of the impact of the various preservation methods on these compounds.

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Appendix 1

Results of the analyses of variance by ANOVA, followed by the Tukey HSD Post-Hoc test. Significant differences between samples were established for $p < 0.05$.

* Different lowercase letters in the same line indicate significant differences ($p < 0.05$) between preservation treatment. Different uppercase letters on the same column indicate significant differences ($p < 0.05$) between storage times.

aw	FR	RT	OD	FD	p
T0	aA	aA	bA	cA	0,000
T1	aAB	aA	bAB	bA	0,000
T3	aBC	aB	bB	cA	0,000
T6	Ac	aB	bB	cA	0,000
p	0.011	0,000	< 0.004	> 0.112	

Moisture	FR	RT	OD	FD	p
T0	aA	bA	cA	dA	0.000
T1	abB	bB	aA	abA	< 0,013
T3	aB	aB	bA	bB	0.000
T6	aB	aB	bA	bB	0.000
p	< 0.001	< 0.007	> 0.292	<0,023	

pH	FR	RT	OD	FD	p
T0	aA	aA	aA	aA	> 0.518
T1	aA	aA	aA	aA	> 0.376
T3	aA	aA	aA	aB	> 0.122
T6	aA	bA	aA	aB	0,000
p	> 0.073	> 0.159	> 0.168	< 0.002	

PROTEIN	FR	RT	OD	FD	p
T0	aA	aA	aA	aA	> 0.520
T1	aA	aA	aA	aA	>0,271
T3	aA	aA	aA	aA	> 0,615
T6	aA	aA	aA	aA	>0,246
p	> 0.065	> 0.445	> 0.730	>0,303	

LIPID	FR	RT	OD	FD	p
T0	aA	aA	aA	aA	> 0.230
T1	abA	aA	bA	aAC	< 0.009
T3	aA	aB	aA	aB	> 0.087
T6	aB	aC	aB	aC	> 0.153
p	0.000	0.000	< 0.002	< 0.001	

ASH	FR	RT	OD	FD	p
T0	aA	abA	bA	abA	< 0.023
T1	aA	aA	bB	abB	< 0.005
T3	aA	aA	aBC	aB	> 0.881
T6	aA	aA	aC	aB	> 0.273
p	> 0.699	> 0.079	0.000	< 0.017	

CARBS	FR	RT	OD	FD	p
T0	aA	aA	bA	bA	< 0.006
T1	aA	aA	bA	abA	< 0.006
T3	aA	aA	bA	bA	< 0.004
T6	aA	aA	bA	bA	0.000
p	> 0.176	> 0.254	> 0.298	>0,073	

T.PHENOL	FR	RT	OD	FD	p
T0	aA	bA	bA	bA	< 0.004
T1	aB	Aab	aB	aB	> 0.225
T3	aC	abB	cB	bcB	< 0.003
T6	abB	abAB	aB	bA	> 0.037
p	0.000	< 0,017	< 0,005	< 0.002	

DPPH	FR	RT	OD	FD	p
T0	aA	bA	cA	dA	0.000
T1	aB	bB	cB	dB	0.000
T3	aC	bC	cC	dC	0.000
T6	aD	bD	cD	dD	0.000
p	0.000	0.000	0.000	0.000	

TVC	FR	RT	OD	FD	p
T0	aA*	aA	aA	aA	> 0.562
T1	aB	aA	aA	aB	> 0.145
T3	aAB	aA	aA	aB	> 0.200
T6	aB	bA	bcA	cC	0.000
p	< 0.015	> 0.346	> 0.151	0	

LAB	FR	RT	OD	FD	p
T0	aA	bA	aA	bA	0.000
T1	aA	aB	aA	aB	> 0.119
T3	aA	bcBC	cA	bB	< 0.002
T6	aA	bC	bA	bB	0.000
p	> 0.104	0,000	> 0.082	<0,007	

Yeasts	FR	RT	OD	FD	p
T0	acA	aA	bA	bcA	0,001
T1	aA	abB	bA	bB	> 0,029
T3	aA	bC	cB	acB	0.000
T6	aA	bC	Bc	cB	0.000
p	> 0,986	0.000	0.000	< 0.008	

Molds	FR	RT	OD	FD	p
T0	aA	aA	aA	aA	> 0.373
T1	aA	aA	aA	aA	> 0.225
T3	aA	aA	aA	aA	> 0.075
T6	aA	aA	aA	aA	> 0.062
p	> 0.429	> 0.164	> 0.116	> 0.142	

Coliforms	FR	RT	OD	FD	p
T0	abA	bA	aA	abA	0,03
T1	aA	aB	aA	bB	0,000
T3	-B	-C	-B	-B	-
T6	-B	-C	-B	-B	-
p	0,000	0,000	0,000	0,000	