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Microbial contamination of bee pollen and impact of preservation methods

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Aknowlegements

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Abstract

Since ancient times, bee pollen has gained reputation as an important source of energy and beneficial substances from the nutritional and health points of view. However, in recent years this natural product has gained much more interest, largely consumed and used in increasing quantities as a food. Due to the nutrient content of bee pollen, a variety of spoilage microorganisms can grow, especially when handling practices are not appropriate. Taking into account human safety, the production of high quality products is of paramount importance.

This work intended to: i) characterise Portuguese bee pollen in terms of microbial loads and potential hazards based on established analytical methods; ii) evaluate the impact of different preservation methods (oven drying at 35 ºC, 40 ºC, and 45 ºC, freezing, and freeze-drying) on the microbial stability of bee pollen. For bee pollen characterisation, the studied microorganisms were aerobic mesophiles (AM), lactic acid bacteria (LAB), yeasts and moulds (Y&M), coliforms, *Escherichia coli*, *Salmonella* and sulphite-reducing *Clostridium.* For the impact of the different preservation methods, AM, LAB, and Y&M were enumerated after one month, three months and six months of storage.

Our results showed that bee pollen has a poor microbial quality, and the loads differ between regions. *E. coli* and *Salmonella* were absent in all the samples. Our results showed that freezing and freeze drying techniques present the highest microbial levels, while the oven drying technique presents the lowest microbial loads throughout time, without a significant difference between the different temperatures tested.

There is the need to adopt appropriate preservation practices of Portuguese bee pollen to prevent possible contamination by equipment or handling.

Resumo

Desde a antiguidade, o pólen tem ganho reputação como uma importante fonte de energia e de substâncias benéficas do ponto de vista nutricional e de saúde. No entanto, mais recentemente, este produto natural tem vindo a ganhar bastante interesse, sendo largamente consumido e utilizado como alimento em quantidades crescentes. Devido à sua riqueza nutricional, diversos microrganismos de deterioração podem desenvolver-se, especialmente quando as práticas de manuseamento não são as mais adequadas. Assim, para garantir a segurança alimentar, é fundamental a obtenção de produtos de elevada qualidade.

Com este trabalho pretendeu-se: i) caracterizar o pólen apícola português no que respeita à sua carga microbiana e potenciais riscos, utilizando métodos analíticos estabelecidos; ii) avaliar o impacto da aplicação de diferentes métodos de preservação (secagem em estufa a 35 ºC, 40 ºC e 45 ºC, congelação e liofilização) na estabilidade microbiana do pólen. Para a caracterização do pólen apícola, os microrganismos estudados foram os mesófilos aeróbicos (AM), bactérias do ácido láctico (LAB), leveduras e bolores (Y&M), coliformes, *Escherichia coli*, *Salmonella* e *Clostridium* sulfito-redutores (SRC). Para a avaliação do impacto dos diferentes métodos de preservação procedeu-se à enumeração de AM, LAB e Y&M após um, três e seis meses de armazenamento.

Os resultados obtidos mostraram que o pólen estudado possui má qualidade microbiológica e que as cargas microbianas diferem de acordo com a região de captura. Não foram detetadas *E. coli* e *Salmonella* nas amostras analisadas. Relativamente aos processos de preservação, os resultados evidenciaram a presença de maiores níveis de microrganismos nas amostras preservadas por congelação e liofilização, enquanto as amostras secadas em estufa apresentaram as menores cargas microbianas ao longo do tempo de armazenamento, sem diferença significativa entre as três temperaturas testadas.

Existe a necessidade de adoptar práticas de preservação adequadas que permitam prevenir a possibilidade de contaminação do pólen português através do equipamento ou manipulação.

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1. Introduction

1.1. Framework

Bee pollen has a considerable potential for being largely consumed and used in increasing quantities as food and, as a consequence, it is a growing business for the beekeeping industry. Bee pollen has a long and well documented past throughout human history. In the ancient Egypt, pollen was described as "a life-giving dust", and considered the only perfectly complete food, although its nutritional value and chemical composition was still a mystery. Nowadays pollen is considered a human functional food and is promoted as a healthy food with a wide range of nutritional and therapeutic properties associated with its chemical composition, particularly as a rich source of free amino acids (Saa-Otero et al., 2000).

Natural products have received much attention from medical scientists because of their potential nutritional and medical applications. Bee pollen is one of the most useful therapeutic products favoured by consumers as an alternative drug, a valuable dietary supplement, and a source of nourishing substances and energy. It has been observed that bee pollen exhibits many important biological activities, such as antimicrobial, immunostimulating, antioxidant, and hepatoprotective (Komosinska-Vassev et al., 2015). Other reported biological activities include the ability to normalize wound healing and to enhance growth performance, immunity responses, and blood variables (Abdelnour et al., 2018)

Taking into account the food safety aspects little is known on bee pollen safety related to microbiological hazards. Therefore, the assessment of its microbiological aspects and the establishment of guidelines for microbiological standards are of paramount importance for the assurance of human safety. The microbiological safety is particularly influenced by the moisture content of the bee pollen after being collected. Bee pollen may contain about 20-30 g water per 100 g, making it an ideal culture medium for microorganisms like bacteria, yeast, fungi, and mites (Postupolski et al., 1999). Therefore, the bee pollen is submitted to preservation methods, such as oven drying or freezing, before commercialization.

1.2. Objectives

The influence of the preservation methods applied to bee pollen on its microbial stability is scarcely documented. Thus, this work aimed to:

1) Characterize Portuguese bee pollen in terms of microbial loads and potential hazards based on established analytical methods, and
2) Evaluate the impact of different preservation methods (oven drying at 35 ºC, 40 ºC, and 45 ºC, freezing, and freeze-drying) on the microbial stability of bee pollen.

This work was developed under the framework of the project "DivInA: Diversification and Innovation in the beekeeping production".

2. Literature Review

2.1. Pollen

Pollen, which plays a crucial role in the plant world, is a mass of microspores in a seed plant appearing usually as a fine dust. This fine to coarse powdery substance is situated in the anthers of the higher flowering plants, representing the male portion of the reproductive process in plants and trees (**Figure 2.1**).

Figure 2.1 Collected bee pollen (Source: Bogdanov, 2016).

Pollen consists in male microgametophytes, which produce male gametes (sperm cells). In plants, they are used for transferring haploid male genetic material from the anther of a single flower to the stigma of another in cross-pollination, this important process is known as fertilization. Although too small to be seen individually, pollen grains can be seen by the naked eye in large quantities. Each pollen grain is a minute body of varying shapes and structure formed in the male structures of seed-bearing plants. Depending on its botanical origin, the size and shape of the pollen grains can vary, as evidenced in **Figure 2.2**.

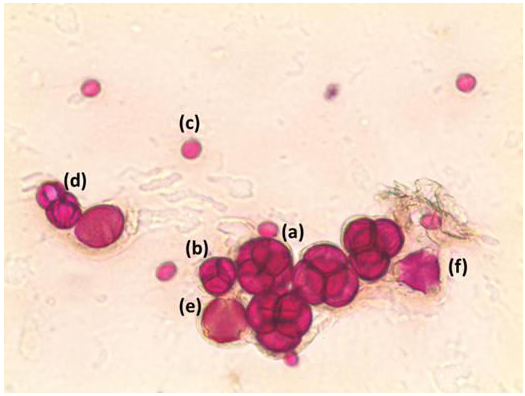

Figure 2.2 Pollen grains from distinct botanical origins evidencing different shapes: (a) *Erica umbellata*; (b) *Erica arborea*; (c) *Castanea sativa*; (d) *Rubus* sp.; (e) *Cytisus* sp.; (f) *Crataegus* sp. (Source: Caveiro, 2017)

Their weight is equal to a dozen or several dozens of micrograms. The majority of pollens consist of single grains that are sometimes joined with two or more grains (Shubharani *et al.*, 2013). Pollen grains consist of three distinct parts. The central cytoplasmic part is the source of nuclei responsible for fertilization. The other parts constituting the wall of the grain are an inner layer, the intine, and an outer layer, the exine. The intine consists, at least in part, of cellulose or hemicellulose while the exine is very resistant to disintegration and deterioration, highly sophisticated and waterproof (Bogdanov, 2016).

2.2. Bee pollen

The transfer of pollen grains to the female reproductive structure is called pollination. To create a new seed, pollen is swirling in the air and on the legs of insects so that they can join the female part of the plant. The importance of bee pollination for ecology and agriculture is immense. In fact, bees pollinate around 40000 plant species. When honeybees visit blossomed flowers and touch the stamen, its body is covered with pollen dust. Using hind legs, the bee compresses the pollen into the pollen basket and with a secretion from its mouth, the pollen cling together in the basket. This secretion used for moistening the pollen contains different enzymes such as amylase and catalase (Bogdanov, 2016). While honey is the energy source of the bee colony, pollen is a very important factor for the development of the colony for producing brood, presenting the necessary food: proteins, lipids and minerals (Keller *et al.*, 2005b).

On average, bees need to visit approximately 200 different flowers to collect 8 mg of pollen. Pollen is placed in cells in the hive after being removed from the rear legs by

a spike on the mid legs. Often the head is used to pack the pollen in the cell. In the hive, the workers add more nectar and glandular secretions to the pollen, which then undergoes lactic acid fermentation and mix freshly collected pollen with some nectar before packing it into their corbiculae. To ensure the long-term survival of a colony and to maintain its productivity, an adequate pollen supply will be essential as the main source of important nutrients, such as proteins, minerals, fats, amongst other important substances for the bees (Keller *et al.*, 2005a).

The pollen basket, which is brought to the hive, usually consists of the pollen from one plant. However, it sometimes happens that the bees collect pollen from many different plant species (Komosinska-Vassev *et al.*, 2015). Different investigations show that the most important pollen sources are plants occurring at high densities either naturally or due to cultivation: white and red clover (*Trifolium repens* and *T. pratense*), corn (*Zea mays*), rape (*Brassica napus*) and sunflower (*Helianthus sp*.) (Shubharani *et al.*, 2013). Bees use olfactory and visual cues, to detect and to discriminate between different pollen sources. Wild-foraging bees seem to preferentially collect higher-quality pollen (based on protein content) they appeared to go after the taste of the sucrose-laced pollen (Muth *et al.*, 2016). There are many factors affecting the gathering of the pollen amount and the foraging behaviour of the bees such as, the abundance of pollen, weather conditions and the nutritional need of the colony.

2.3. Bee pollen collection

Pollen is collected using pollen traps, which are devices that are adapted on the beehive structure. Although there are different trap designs, all of them operate based on the same basic principle: scraping pollen off the bee's legs as they enter the hive. **Figure 2.3** shows an individual bottom pollen trap and a hive with a bottom pollen trap adapted on its structure. Each trap is composed of two basic elements: a grid through which pollen-carrying bees must crawl to separate the pollen pellets from the bees' legs, and a container to store these pellets.

Figure 2.3 Bottom pollen trap (on the left) and hive with bottom pollen trap (on the right) (Adapted from Somerville, 2012).

The honeybee colonies may vary in the average size of the workers or may collect a different spectrum of pollen types. Therefore, the accurate estimates of the actual quantity of pollen collected by a colony are virtually impossible (Bogdanov, 2016). Beekeepers provide quantitative estimates of the pollen harvest of a colony, but the percentage of pollen retained in a trap may be quite variable. More than 50 kg of pollen is estimated to be retained in the traps. In different studies the amount of pollen was determined in different locations in Europe and the USA, The available estimates range between 5.6 kg and 222 kg, This study shows that in California the amount of pollen in traps is 40.4 kg, however in Europe it varied between 1.4 and 9.2 kg (Pernal and Currie, 2001).

2.4. Chemical composition of bee pollen

Bee pollen is considered as a quite varied plant product rich in biologically active substances. In the group of basic chemical substances, there are proteins and free amino acids, carbohydrates, including reducing sugars, lipids and free fatty acids, phenolic compounds, enzymes, and coenzymes, as well as vitamins and minerals, such as calcium, magnesium, phosphorous, and potassium (Campos *et al.*, 2008; Komosinska-Vassev *et al.*, 2015). As evidenced in **Table 2.1**, the chemical composition of bee pollen is highly variable. This is also evidenced in a study by Komosinska-Vassev *et al.* (2015), which reported varying amounts of protein, ranging from 2.9% to 33.5%, depending on the bee pollen botanical origin.

Table 2.1 Chemical composition of bee pollen (Adapted from Campos *et al.*, 2008)

Main Components	**Content Minimum – Maximum (g/100g dry weight)**
Proteins	10-40
Lipids	1-13
Total carbohydrates	13-55
Dietary fibre, pectin	0.3-20
Ash	2-6
Undetermined	2-5
Mineral Components	**Content Minimum – Maximum (mg/kg dry weight)**
Potassium	4000-20000
Magnesium	200-3000
Calcium	200-3000
Phosphorus	800-6000
Iron	11-170
Zink	30-250
Copper	2-16
Manganese	20-110

The human body cannot synthesize nine of the essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), and they must be included in the diets. In fact, bee pollen is also considered an excellent source of these amino acids, comprising from 35 to 49% of the total amino acid content. Amongst the more than 16 essential and non-essential amino acids that have been found in bee pollen, the most abundant are glutamic acid, aspartic acid, proline, leucine and lysine (Taha *et al.*, 2019).

Considering lipids, bee pollen is particularly rich in essential fatty acids (EFA´s), namely linoleic, γ-linoleic, and archaic, which together comprised 0.4%. Phospholipids amount to 1.5%, while phytosterols, especially *p*-sitosterol, are present in the amount of 1.1% (Guine, 2015).

Moreover, pollen is quite a significant source of vitamins, such as provitamin A, vitamins B, D, and E. Mineral elements are also present in bee pollen, namely macroelements such as calcium, phosphorus, magnesium, sodium, and potassium,

besides microelements such as iron, copper, zinc, manganese, silicon, and selenium (Komosinska-Vassev *et al.*, 2015).

2.5. Properties and usages of bee pollen

Bee pollen is a natural honeybee product marketed for its nutritive properties, it is appreciated by consumers and used as supplements to achieve certain health effects. For thousands of years, medicinal properties of bee pollen have been used (Xi *et al.*, 2018), and in recent times, the Federal Ministry of Health in Germany recognizes bee pollen as a medicine (Salles *et al.*, 2014). Several biological properties have been detected in many studies, and introduced bee pollen as a pharmaceutical aid and in clinical practice. In fact, promising results show that bee pollen may offer certain benefits, as it demonstrates a series of actions such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-fungicidal, hepatoprotective, and anti-atherosclerotic activities capable of modifying or regulating immune functions (Xi *et al.*, 2018). Experimental pharmacological animal studies have shown that bee pollen has a hypolipidemic activity decreasing the content of plasma total lipids and triacylglycerols, and in people with near-sightedness caused by clogged arteries and with a high level of LDL cholesterol, bee pollen extracts may lower their level (Kasianenko *et al.*, 2011).

Moreover, pollen given to older people allows both the inhibition of the atherosclerotic changes of blood vessels and improvement of cerebral blood flow. This natural product has a high anti-inflammatory activity, as it packs several compounds that can reduce inflammation and swelling, including the antioxidant quercetin, which lowers the production of inflammatory omega-6 fatty acids, such as arachidonic acid. Its magnitude is compared to such nonsteroidal anti-inflammatory drugs as naproxen, analgin, phenylbutazone, or indomethacin (Pascoal *et al.*, 2014).

Bee pollen has also been proposed as a valuable dietary supplement. Experimental studies show that mice and rats fed with pollen showed a higher vitamin C and magnesium content in thymus, heart muscle, and skeletal muscles as well as a higher hemoglobin content and greater number of red blood cells when compared to animals given standard feed (Oliveira *et al.*, 2009). Moreover, it is characterized by the great potential for development in the cosmetics field. It may effectively enhance protective mechanisms against skin aging and dryness, ultraviolet B radiation, oxidative damage,

inflammation and melanogenesis, which are involved in a wide range of negative effects on human skin (Xi *et al.*, 2018).

2.6. Microbiological and safety aspects of bee pollen

Bee pollen is often reported as the hive product least influenced by contaminants from beekeeping manipulation. However, bee pollen can be polluted by air contaminants, or by contamination of floral pollen grains on the plant or by bees (De-Melo *et al.*, 2015). Pollen, bacteria, fungi, and several other contaminants all coexist in the aerospace. A broad range of pollinators harbours parasites and deformed wing virus, and floral transmission may be a likely source of these pathogens (Evison *et al.*, 2012). Bees dampen the pollen with nectar and lug them in corbiculaes on their rearmost legs during transport; thus, bee pollen is more vulnerable to microbial contamination. Sanitary conditions around the hive, human handling operations such as harvesting, drying, packaging, and storage also have strong implications on pollen contamination (Serra-Bonvehí and Escolà-Jordà, 1997).

To meet the high demand for bee-pollen and to guarantee a safe use in human consumption, permanent controls and diagnostics of the microbiological quality and safety of bee pollen are essential. As previously presented, many works describe the chemical composition of bee pollen in different countries. On the other hand, little attention has been dedicated to the bee pollen microbial contamination and microbiological risks related to its human consumption. Given the requirements for the sanitary quality of food, and because bee pollen is considered a food item, the microbiological safety is the main quality criterion of this product, especially the absence of pathogenic bacteria and fungi, following the legislation applied for food (EC, 2005).

Some countries have established official quality and identity standards of bee pollen such as Argentina, Brazil, Bulgaria, Poland and Switzerland (Campos *et al.*, 2008), but no specific international legislation on the chemical and microbiological quality and safety of this food product has been set. On the basis of a comprehensive analysis of various studies from different countries, an international proposal for the quality criteria for dry bee pollen used for human nutrition was put forward by Campos *et al.* (2008), and is presented in **Table 2.2**.

Table 2.2 Quality and safety criteria for bee pollen, as proposed by Campos *et al.* (2008).

Microbiological criteria	**Limits**
Salmonella	Absent / 10 g
Staphylococcus aureus	Absent / 1 g
Enterobacteriaceae	Max. 100/g
Escherichia coli	Absent / g
Total aerobic plate count	<100 000/g
Moulds and yeasts	< 50 000/g
Chemical contaminants	**Limits**
Aflatoxin B1	Max. 2 µg/kg
Aflatoxin B1+B2+G1+G2	Max. 4 µg/kg
Cloramphenicol (CAP)	absent
Nitrofuran metabolites	absent
Sulfonamides	absent
Heavy metal Pb	Max 0.5 mg/kg
Heavy metal Hg	Max 0.01 mg /kg
Heavy metal Cd	Max 0.03 mg/kg
Radioactivity (Cs-134 and Cs-137)	<600 Bq / kg

A study aiming to assess the microbiological parameters of Brazilian bee pollen showed that *Salmonella* sp.*,* sulfite-reducing clostridia, faecal coliforms, *Escherichia coli*, *Staphylococcus aureus*, yeast, and moulds were absent in all samples. However, the mesophilic aerobic microorganisms were present in all the analysed pollen samples and with high variability in concentrations (85±63 cfu·g^{-1} and 443±142 cfu·g^{-1}) (Bárbara *et al.*, 2015). In other works, for example, Coronel et al. (2004) detected contaminations by faecal coliforms in twenty-three bee pollen samples and Estevinho *et al.* (2012) detected yeasts and moulds in 60% of pollen samples which suggest that microbial contaminations are possible and mainly associated with the pollens' processing.

The levels of microbial contamination of bee pollen in different countries are presented in **Table 2.3**. The aerobic mesophiles are the most abundant microorganisms, while all samples showed negative results for *E. coli, Salmonella* and sulphite-reducing clostridia.

Table 2.3 Microbiological characteristics of bee pollen in different countries.

Country of origin	Type of processing	Aerobic mesophile (cfu.g^{-1})	Yeasts and moulds (cfu.g^{-1})	Enterobacteriaceae (cfu.g^{-1})	Coliforms (cfu.g^{-1})	Lactic acid bacteria (cfu.g^{-1})	Coagulase-positive *Staphylococcus* (cfu.g^{-1})	Reference
Portugal	Dried pollen	n.d. – 2.8×10^3	n.d. – 2.7×10^3	n.d.	n.d.	n.d.	n.d.	Estevinho *et al.*, 2012
Spain	Commercial pollen	n.d. – 8.7×10^3	n.d. – 9.4×10^2	n.d.	n.d.	n.d.	n.d.	Nogueira *et al.*, 2012
Bulgaria	Fresh pollen	n.d. – 4.3×10^5	n.d. – 37×10^3	n.d. – 1.2×10^4	n.d.	n.d. – 1.2×10^3	n.d. – 1.0×10^3	Beev *et al.*, 2018
	Dried pollen	n.d. – 6.7×10^4	n.d. – 1.1×10^4	n.d. – 3.8×10^3	n.d.	n.d. – 5.4×10^3	n.d. – 2.0×10^2	
Brazil	Dehydrated pollen	n.d. – 1.0×10^4	n.d. – 7.7×10^3	n.d.	n.d. – 2.8×10^3	n.d.	n.d.	De-Melo *et al.*, 2015)

n.d.: not detected.

The findings of these studies raise questions on the quantitative and qualitative difference of the microbiological contamination of pollens in several countries, which suggest that several factors can influence the origin of contamination and its load in pollen such as floral diversity, the region and climatic conditions, processing and packaging, pollen trap system used, and storage conditions (Deveza *et al.*, 2015). These significant differences between results explain the difficulty to propose general quality criteria and the absence of a worldwide pollen standard. The main function of the standard is to establish the parameters and technical requirements for food safety of products and processes, certification of the industry organisation, protection of their products against any technical restrictions, and compliance to market demands (Camargo, 2008). However, bee pollen registers a lack of regulation of the microbiological quality with weak terms of legislation (Deveza *et al.*, 2015).

It is clear that there is a considerable lack of knowledge in this field. Therefore, the production of low-quality products like bee pollen can arise. The growing concern with the sanitary quality of food suggests the need to revise the legislation for this product and increase the surveillance on the quality of pollen for human consumption. Also, to minimise risks to human health, the process of bee pollen production should be reviewed, from collection to storage as suggested by several authors (Cast, 2003; Rodriguez *et al.*, 2008; Deveza *et al.*, 2015).

2.7. Bee pollen preservation

When the grains of bee pollen remain in the collection traps fixed at the hive entrance, they are in contact with air, dust and other dirt. Additionally, the high humidity and temperature, together with an adequate chemical composition, makes it an ideal matrix for bacterial and fungal colonization and growth (Kačániová *et al.*, 2014; Mauriello *et al.*, 2017), simultaneously leading to the occurrence of chemical and enzymatic reactions (Campos *et al.*, 2008). Pollen collection, manipulation, and processing, as well as inadequate storage can favour the spoilage by some microorganisms.

After collection, the bee pollen can exhibit moisture contents higher than 18%, which can be favourable for microorganism growth, with consequent fermentation processes, potential development of pathogenic bacteria or production of microbial toxic secondary metabolites. This necessarily leads to a decrease in the shelf life of this product or to safety problems. As such, bee pollen moisture content is a parameter of quality for this product, and several countries have established minimal requirements for dried pollen: Argentina (max. 8%), Brazil: (max. 4%), Bulgaria (max. 10%), Poland (max. 6%), and Switzerland (max. 6%) (Melo and Almeida-Muradian, 2011).

Therefore, the use of preservation techniques that allow an increase in bee pollen shelf life and safety is a generalised practice before commercialisation. Oven drying is the preservation technique most frequently used in commercial bee pollen, because of the reasonable process time, better sanitary conditions, and control of the drying conditions (Ortiz *et al.*, 2011). Freeze-drying is another preservation technique, which has been increasingly used mainly due to its ability to preserve the biological properties of various food products (Ciurzyńska and Lenart, 2011).

For preservation of a maximum quality, the pollen is best dried in an electric oven, where humidity can continuously escape and the water content should be reduced to approximately 6 g water per 100 g pollen (Bogdanov, 2016). In Poland, the effect of different methods of preservation (freezing, drying at about 40 ºC and freeze-drying) on selected parameters attributed to the biological quality of bee pollen were tested (Bogdanov, 2016). In order to maintain the nutritional value of bee pollen, the drying process should be conducted at relatively low temperatures, not exceeding 45 ºC. The better alternative is to use freeze-drying to preserve the chemical and the biological

properties, while the freezing technique should be recommended when the preservation of the pollen load for nutrition or therapeutic purposes is important.

Microbiologically, several investigations showed that fresh pollen revealed the highest levels of contamination and, differently from freezing, the dehydration procedure completely affected all microbial communities of bee pollen (Mauriello *et al.*, 2017). A study on chestnut bee pollen and willow pollen processed by different treatments (conventional, freeze- and microwave assisted drying) confirmed that the conventional drying of chestnut pollen significantly reduced the abundance of aerobic mesophilic bacteria and the contamination by *enterobacteria* and yeasts. The microwave-assisted drying reduced aerobic spore forming bacteria, while all preservation treatments strongly decreased coagulase-positive staphylococci. None of the preservation techniques allowed the reduction of moulds contamination and the abundance of sulphite-reducing clostridia (Palla *et al.*, 2018) .

A study on the microbiological quality of dehydrated bee-pollen produced in Brazil showed that the highest observed counts are for aerobic mesophilic, which ranged from <10 to 1.12×10^3 CFU.g^{-1}, and for yeast and moulds, which ranged from <10 to 7.7×10^3 CFU.g^{-1}. All samples were negative for sulphite-reducing *Clostridium* spores, *Salmonella*, coagulase-positive *Staphylococcus* and *Escherichia coli* (De-Melo *et al.*, 2015). These levels of contamination are a reflection of the initial microbiological load of fresh bee-pollen, since dehydration is not able to kill already existing microbes. This is particularly important for fungi, for which spores are highly resistant to dehydration. Toxigenic fungi present in pollen can grow and produce mycotoxins if the period between harvest and dehydration is too long. Furthermore, these toxins remain in the bee pollen even after heat exposure during drying. Numerous spoilage yeasts have also been frequently found in commercial samples of dehydrated bee pollen commercialized in Portugal and Spain (Nogueira *et al.*, 2012). Therefore, the process of dehydration requires caution: this step should be performed in a facility with controlled temperature and immediately after collection using good quality fresh pollen.

Mauriello *et al.* (2017) microbiologically characterised bee pollen collected from the Vesuvius area using three different traps. It was reported that the same microbiological qualitative parameter has a similar trend in all types of traps and that fresh bee pollen is highly contaminated by *Enterobacteriaceae*, while frozen samples

were negative for presence of *Salmonella* spp. even when it was present in the fresh sample indicating that freezing affected *Salmonella* spp. viability. Moreover, it was evidenced that dried pollen showed the lowest microbial contamination and no pathogens were detected after the drying process and during the storage.

According to all these studies, it can be expected that microbiological analysis of fresh pollen reveals high levels of contamination. The presence of some contaminants such as bacteria and fungi is relevant and a high health risk could be associated to the consumption of fresh bee pollen. Same assumptions are also applicable to consumption of frozen bee pollen, which shows that a more comprehensive microbiological risk assessment is required, while dried pollen remains the safest form of consumption.

3. Materials and methods

3.1. Microbial quality of pollen

3.1.1. Sampling

Forty-three bee pollen samples were collected by beekeepers between May 2018 and May 2019 in different apiaries located in the North - Bragança (*n* = 05) and Vila Real (*n* = 14) - and Centre of Portugal – Leiria (*n* = 06), Lisboa (*n* = 05), Nisa (*n* = 08), Portalegre (*n* = 04) (**Figure 3.1**). Samples were coded and delivered to Centro de Investigação de Montanha (CIMO, Bragança, Portugal, where they were stored in the dark at room temperature (±20 °C). The study was conducted in the Microbiology Laboratory of CIMO.

Figure 3.1 Place of origin of bee pollen samples.

3.1.2. Moisture content

Moisture content was evaluated according to the methodology described by Almeida-Muradian *et al.*, (2012). Approximately two grams of sample were used for drying at 105 ºC, during 90 minutes, in a Memmert Basic UNB-500. The moisture content was calculated and expressed as percentage (%), according to the following equation:

$$\text{Moisture content (\%, wet weight basis)} = \frac{M_0 + M_1 - M_2}{M_1} \times 100$$

where:

M_0 = tare weight of the container (g)
M_1 = original test sample weight (g)
M_2 = weight after drying (g) (container + test sample after drying).

3.1.3. Microbiological analysis

3.1.3.1. Sample preparation

In a biological safety cabinet, five grams of each bee pollen sample were homogenised in 45 mL of sterilised buffered peptone water solvent (HiMedia, Mumbai, India). The portion was placed aseptically into sterile bags and homogenised in a Stomacher VWR (Seward type 400, Italy) for 1 min. Decimal dilutions were made using the same diluent.

3.1.3.2. Enumeration of aerobic mesophiles

The enumeration of aerobic mesophiles (AM) was performed using 3M™ Petrifilm™ Aerobic Count (AC) Plates, which is a ready culture medium system that facilitates colony enumeration. One mL of each dilution was inoculated in the Petrifilm (in duplicate) and incubated at 30 ± 1 °C for 48 h ± 3 hours. All red colonies regardless of size or intensity were counted to define the number of microorganisms present in the test sample. Microbial counts were expressed as colony-forming units per gram of bee pollen (CFU/g) using the formula:

$$\text{CFU g}^{-1} = \sum C / [V * (n_1 + 0.1n_2) * d]$$

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.

3.1.3.3. Enumeration of coliforms and *E. coli*

The enumeration of coliforms was performed using 3M™ Petrifilm™ Coliform count (CC) Plates. One mL of the decimal dilutions was inoculated in the Petrifilm (in duplicate) and incubated at 37 ± 1°C for 24 h ± 3 hours. Confirmed coliforms are red colonies with associated gas bubbles (**Figure 3.2**). Only the positive results for coliforms were confirmed for *E. coli* enumeration using 3M™ Petrifilm™ Select *E. coli* Count (SEC) Plates. All green to blue-green colonies in SEC plates were counted to enumerate *E. coli* present in the test sample. Microbial counts were expressed as colony-forming units per gram of bee pollen (CFU/g).

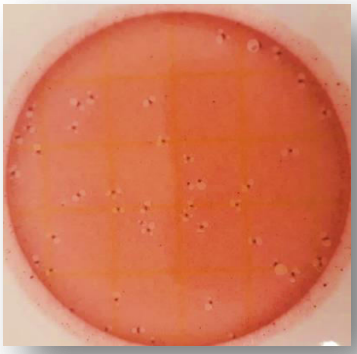

Figure 3.2 3M™ Petrifilm™ Coliform count (CC) Plate with coliform colonies.

3.1.3.4. Enumeration of lactic acid bacteria

Lactic acid bacteria (LAB) count was performed as described in the ISO 15214:1998. One mL of test sample from each decimal dilution was inoculated using the pour plate technique in Man Rogosa Sharp (MRS Agar, Biolife, Italy) plates (in duplicate). After incubation at 37 ˚C, colonies were counted under subdued light using

an automatic colony-counting equipment (SELECTA – Digital S) (**Figure 3.3**). Microbial counts were expressed as colony-forming units per gram of bee pollen (CFU/g).

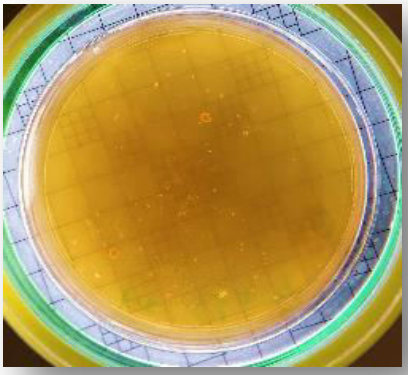

Figure 3.3 Petri dish with lactic acid bacteria colonies.

3.1.3.5. Enumeration of yeasts and moulds

Yeasts and moulds were enumerated as described in the ISO 21527-2:2008. 0.2 mL of each dilution were inoculated by the spread plate technique onto a plate containing DG18 (Himedia Mumbai, India). Petri dishes were incubated at 25 °C for 5 days. Yeasts and moulds were counted under subdued light using automatic colony-counting equipment (SELECTA – Digital S) (**Figure 3.4**). Microbial counts were expressed as colony-forming units per gram of bee pollen (CFU/g).

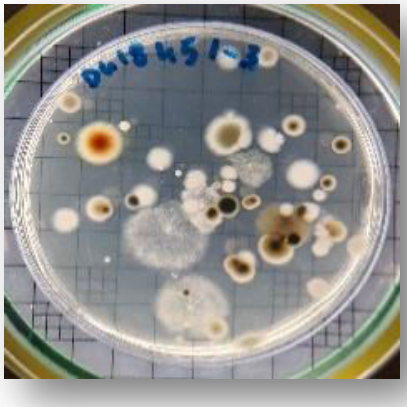

Figure 3.4 Petri dish with moulds and yeasts growth.

3.1.3.6. Enumeration of spores of sulphite-reducing clostridia

Sulphite-reducing clostridia (SRC) were counted as described in the ISO 15213:2003. Five mL of the initial dilution were added to a sterilised test tube, thermally treated at 80 °C for 10 min and covered with approximately 35 mL of ISA medium (iron sulphite agar; HiMedia, Mumbai, India). After the medium had solidified, a cover layer was added to reduce the contact with oxygen. The incubation occurred at 30 °C for 48 h. Tubes with characteristic black colonies were considered positive (**Figure 3.5**). The results were expressed as colony-forming units per gram of bee pollen (CFU/g). Whenever less than 4 black colonies were detected in the tube, the results were expressed as < 4 CFU/g, as described in the ISO 7218:2007.

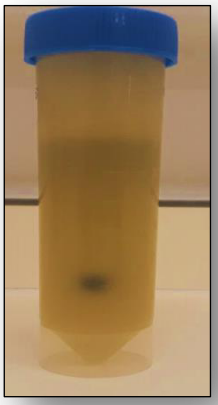

Figure 3.5 Tube with a black colony of sulphite-reducing clostridia.

3.1.3.7. Detection of *Salmonella* sp.

The detection of *Salmonella* sp. was performed using the 1–2 TEST kit (BioControl; AOAC, 2000) (**Figure 3.6**), prepared according to the manufacturer's instructions. A 100 µL aliquot of the initial suspension was added to the kit and incubated for 14–30 h at 37 ± 2 °C. Results for *Salmonella* sp. were considered positive upon the formation of a white, U-shaped or meniscus-shaped band. Results were expressed as presence or absence of *Salmonella*.

Figure 3.6 1–2 TEST kit (BioControl).

3.2.Impact of preservation methods on microbial contamination

3.2.1. Pollen harvest

For the preservation tests, 1.5 kg of pollen were collected by beekeepers from June to July and kept frozen (at -20 °C) until use. Bee pollen was thoroughly homogenised and divided into 5 aliquots of 300 g each. Each aliquot was submitted to a different preservation process (**Figure 3.7**).

Figure 3.7 Aliquots of bee pollen submitted to a different preservation process.

3.2.2. Preservation methods

Pollen aliquots were submitted to the following preservation techniques:

1) Freezing at -20 °C: Bee pollen was stored at -20 °C in a home-type freezer.
2) Freeze-drying: Bee pollen was freeze-dried in a freeze dryer (Zirbus Lyophilizer Vaco 10-II-D).

3) Oven drying: Bee pollen drying was made in an oven drier (Climacell, MMM Group, model CLC-B2V-M/CLC111-TV) with hot air without recirculation under the following conditions:

3.1) 35 °C for 15 hours.

3.2) 40 °C for 7 hours and 30 minutes.

3.3) 45 °C for 5 hours and 50 minutes.

3.2.3. Sampling time points

Samples were analysed for moisture content and microbial loads 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.

3.2.4. Moisture content

The moisture content was determined according to the methodology described in section 3.1.2.

3.2.5. Microbiological analysis

3.2.5.1. Sample preparation

Aliquots of 5 g of each preservation method were prepared for microbiological analysis as described in section 3.1.3.1. Analyses were made in triplicate.

3.2.5.2. Enumeration of aerobic mesophiles

The enumeration of aerobic mesophiles was made as described in the ISO 4833:2003. One mL of each decimal dilution was inoculated by the pour plate technique into 20 mL of standard Plate Count Agar (PCA; Himedia, Mumbai, India). After incubation at 30 °C for 48 h, colonies were counted under subdued light using an automatic colony-counting equipment (SELECTA – Digital S) (**Figure 3.8**). Microbial counts were expressed as colony-forming units per gram of bee pollen (CFU/g).

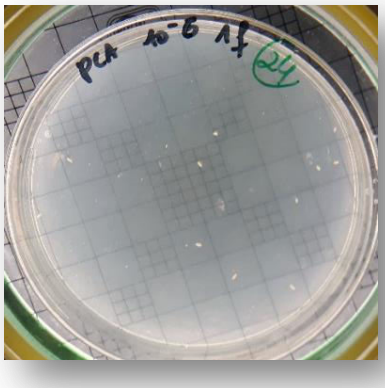

Figure 3.8 Petri dish with aerobic mesophilic colonies.

3.2.5.3. Enumeration of lactic acid bacteria

The enumeration of lactic acid bacteria was performed according the methodologies described in the section 3.1.3.4.

3.2.5.4. Enumeration of yeast and moulds

The enumeration of yeasts and moulds was performed as described in the section 3.1.3.5.

3.3. Statistical analysis

Data from plates (counts CFU g^{-1}) were transformed to log10 values (LOG_{10} CFU g^{-1}). As a first approach, an overall descriptive analysis was performed, by calculating averages and standard deviation. The existence of association between the variables *microbial loads* and *moisture content* was analysed using the Pearson correlation and adopting the coefficient of correlation (*r*). To study the overall data's variability, principal component analysis (PCA) was used to generate a new set of orthogonal variables (linear combinations of the original variables). Kruskal-Wallis test was used whenever there was homogeneity of variances (homoscedastic variables) and normality. All statistical studies were performed using the open source statistical program R (Merril and Halverson, 2002) version 1.0.6. and adopting 5% ($p < 0.05$) as a significance level.

4. Results and discussion

4.1.Microbial Quality

In this study, the moisture content of the bee pollen samples collected from different locations in Portugal was determined, and is presented in **Table 4.1**.

Table 4.1 Moisture content (% average ± standard deviation) of bee pollen samples collected from different locations in Portugal.

Region	**number of samples**	**moisture content (%) ± SD**
Bragança	n = 05	12.8 ± 2.59
Leiria	n = 06	24.7 ± 6.45
Lisboa	n = 05	29.8 ± 7.87
Nisa	n = 08	18.38 ± 5.76
Portalegre	n = 04	19.72 ± 7.14
Vila Real	n = 14	18.82 ± 3.17

Microbial quality and safety parameters were studied on bee pollen collected from different locations in Portugal. The microbial loads of the analysed parameters are presented in **Figure 4.1**.

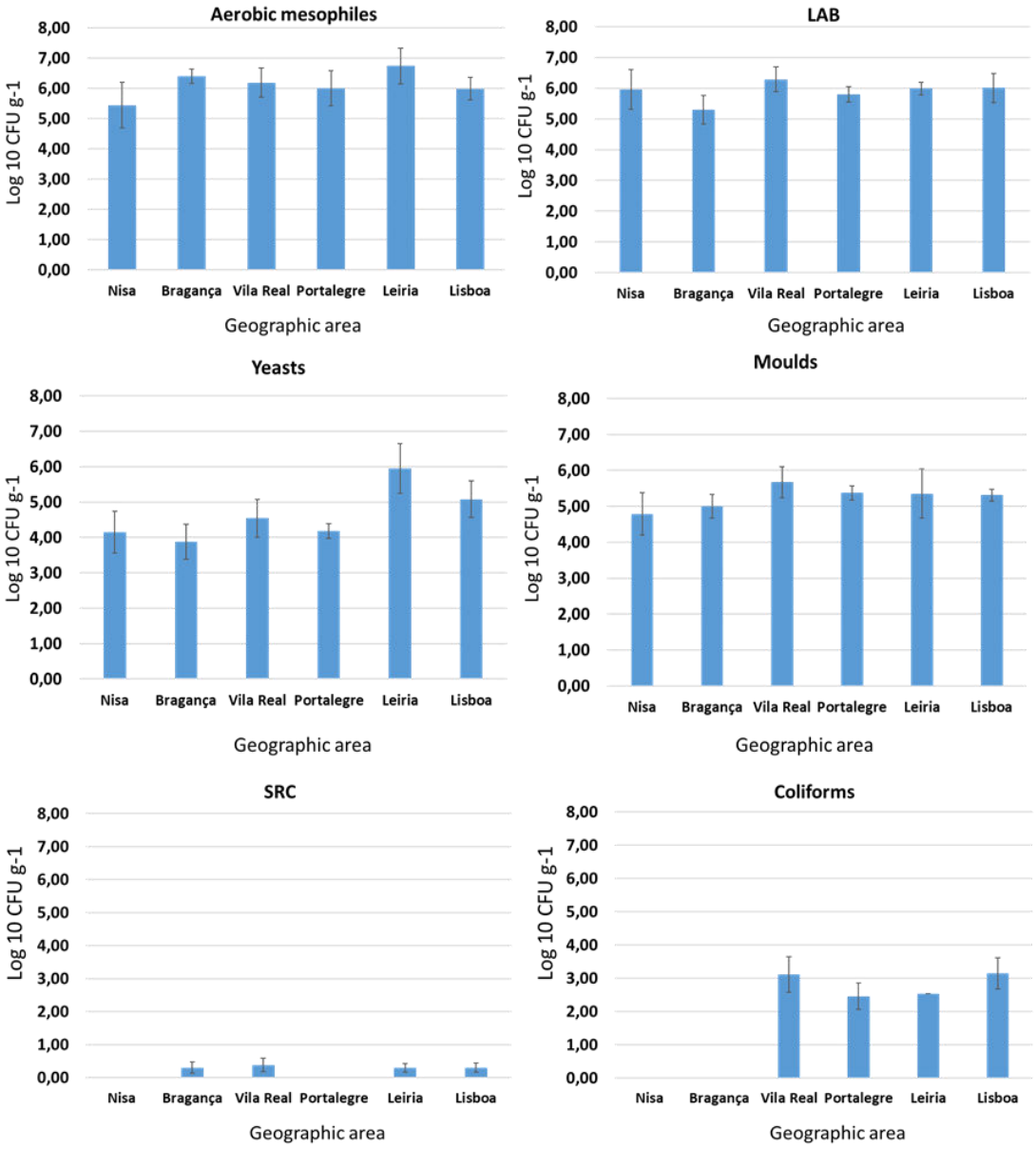

Figure 4.1 Microbial loads (LOG_{10} CFU g^{-1}) of aerobic mesophiles, lactic acid bacteria (LAB), yeasts, moulds, coliforms and sulphite reducing clostridia (SRC); of pollen samples from Bragança, Leiria, Lisboa, Nisa, Portalegre and Vila Real.

The change in abundance of microbial parameter, the rate of their change and contribution according the different locations is presented in **Figure 4.2**.

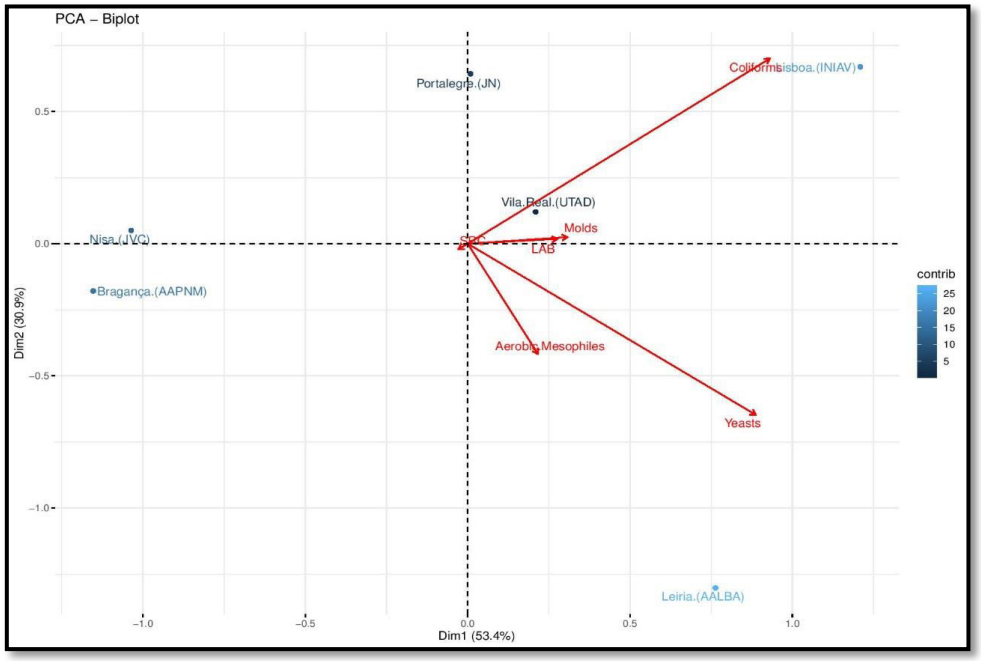

Figure 4.2 Principal Component Analysis (PCA) biplot describing the change in abundance of microbial parameter and the rate of their change and contribution according to the different set locations; the direction of arrows describes the greatest change in abundance and the length of arrows is related to the rate of change.

Since there are no official quality and identity standards of Portuguese bee pollen, our results were compared with standards of other countries and with findings of other studies aimed to evaluate the microbial bee pollen quality. Aerobic mesophiles, yeasts, and moulds are the general quality parameters investigated in this study. Considering the different locations, aerobic mesophiles ranged from 5.44 to 6.7 LOG_{10} CFU g^{-1}, yeasts from 3.87 to 5.96 LOG_{10} CFU g^{-1} and moulds from 4.79 to 5.67 LOG_{10} CFU g^{-1}. Bee pollen samples analysed in this study seem to have higher levels than those reported by others. Hani *et al.* (2012) found that aerobic mesophiles ranged from 3.67 to 5.8 LOG_{10} CFU g^{-1} in Algerian bee pollen, while an average of 5.6 LOG_{10} CFU g^{-1} was found in Bulgarian bee pollen (Beev *et al.*, 2018). Regarding yeasts and moulds, our levels were higher than those reported by Hervatin (2009) in Brazilian bee pollen, with

an average of 4 LOG_{10} CFU g^{-1}. LAB were also detected at levels ranging from 5.30 to 6.29 LOG_{10} CFU g^{-1}.

The sanitary quality parameters assessed in the present study were coliforms and *E. coli*, while the safety parameters were sulphite reducing clostridia (SRC) and *Salmonella* sp. The average of coliforms ranged from 3.11 to 3.15 LOG_{10} CFU g^{-1} and of SRC was 0.30 to 3.38 LOG_{10} CFU g^{-1}. No *E. coli* or *Salmonella* were detected in the samples. Our findings are in agreement with another Portuguese study in which no *Salmonella* or *E.coli* were detected (Estevinho *et al.*, 2012). Coliforms showed lower numbers when compared with the levels reported by Hani *et al.* (2012) and De-Melo *et al.* (2015), where the presence of coliforms in analysed samples were 4 LOG_{10} CFU g^{-1} and 3.44 LOG_{10} CFU g^{-1}, respectively. For sulphite reducing clostridia, our findings are in contrast with results reported by other authors. As a matter of fact, SRC were absent in the studies of Coronel *et al.* (2004), De-Melo *et al.* (2016), Estevinho *et al.* (2012), and Nogueira *et al.* (2012). Our results showed that the analysed bee pollens are not within the limits established in the article 785 of the Argentinian code for bee pollen, that sets the maximum value of aerobic mesophiles at 5.17 LOG_{10} CFU g^{-1}, and the maximum value for yeasts and moulds at 2 LOG_{10} CFU g^{-1} (Argentina, 1990). It is confirmed that, as a food, bee pollen is highly vulnerable to microbial contamination, as previously referred by Beev *et al.* (2018). The contamination of bee pollen could result from air contaminants that potentially contain bacteria and fungi, from contamination of floral pollen grains on the plant, and from bees activities (De-Melo *et al.*, 2015). Indeed, the inside of the anther pollen is sterile, thus its microbial contamination can be attributed to plant materials, environmental conditions, insects, humans and their agricultural devices (Gonzalez *et al.*, 2005). Sanitary conditions around the hive, human handling operations such as harvesting, packaging, and storage also have strong implications on bee pollen contamination (Serra *et al.*, 1997).

In Portugal there is an absence of regulation about microbiological quality for bee pollen, making it imperative to establish quality standards for beekeeping products. Regional differences for Portuguese pollen in terms of microbial abundance and the rate of its change were also studied in the present work. Aerobic mesophiles and yeasts presented the highest change in abundance for Leiria, with a higher contribution of yeasts. Coliforms presented the highest change in abundance for Vila Real and Lisboa with a high rate of change. LAB and moulds are more abundant in Vila real and Lisboa with a low rate of change. Our study showed a difference in the microbial levels in the

different locations of Portugal. Our results of Portuguese bee pollen, when compared with the different observations of foreign bee pollen reported by other authors, showed that microbial contamination differ quantitatively and qualitatively from one country to another. Hence microbial levels change over the geographic areas.

In Portugal, the weather differs according to the geographic area. In the north the climate is cool and rainy, however moving south it becomes gradually warmer and sunnier. It is clear that pollen contamination change over the environmental factors such as humidity, temperature and precipitation amount (Hani *et al.*, 2012). Our findings are in agreement with Hani *et al.* (2012), who studied the pollen microbial levels from different locations with different weather, and reported that precipitation and relative humidity are two principal factors that affect the pollen contamination and correlate positively with microbial contamination. In addition, collected pollen from locations that have a higher percentage of precipitation and relative humidity, gave the highest viable count of microorganisms. The pollen structure is different from a region to another in terms of botanical origin, chemical and nutritional composition (Estevinho *et al.*, 2012). The latter reported that the variability in bee pollen composition is explained by season, environmental conditions, and different localities. The same authors also studied the relationship between the pollen composition and microbial contamination. The results showed that different nutritional compositions are interestingly related to microbial numbers with a verified positive correlation. Therefore, taking into account human safety, more studies are needed to understand the microbial content of bee pollen from Portugal, and it is important and imperative to establish at least a national microbiological quality parameter and standard processing protocols.

4.2. Preservation methods

In this study bee pollen samples were submitted to different preservation techniques. Mmoisture content and the microbial loads were subsequently analysed and monitored over time after 1, 3, and 6 months. The moisture content values obtained after the application of the various preservation methods are presented in **Table 4.2**.

Table 4.2 Moisture content (%) obtained after the application of different preservation method at different storage time points (0, 1, 3 and 6 months).

Preservation method	0 month	1 month	3 months	6 months
Freeze drying	5.79 ± 0.15	5.37 ± 0.07	5.51 ± 0.37	5.34 ± 0.11
Oven drying at 35ºC	9.62 ± 0.17	9.786 ± 0.08	9.43 ± 0.12	11.17 ± 2.57
Oven drying at 40ºC	9.81 ± 0.14	9.23 ± 0.05	9.60 ± 0.07	9.67 ± 0.08
Oven drying at 45ºC	10.13 ± 0.12	9.68 ± 0.13	10.01 ± 0.31	10.09 ± 0.09
Frozen	13.79 ± 0.08	13.13 ± 0.10	12.79 ± 0.13	12.16 ± 0.36

The loads of aerobic mesophiles, LAB, yeasts and moulds obtained are presented in **Figures 4.3**, **4.4**, **4.5** and **4.6**, respectively.

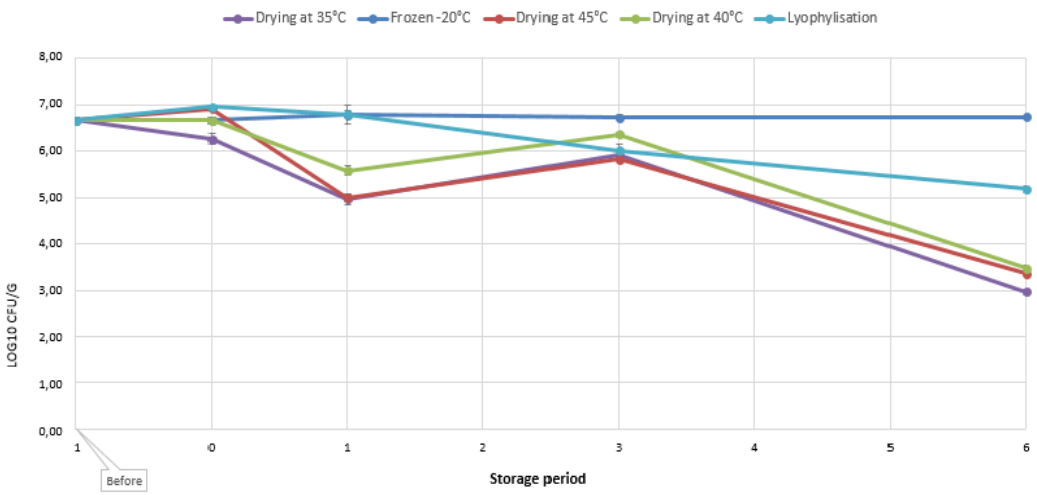

Figure 4.3 Microbial loads of aerobic mesophiles submitted to different preservation methods at different periods of storage (Before treatment, 0, 1, 3, and 6 months). The standard deviation is shown as error bars.

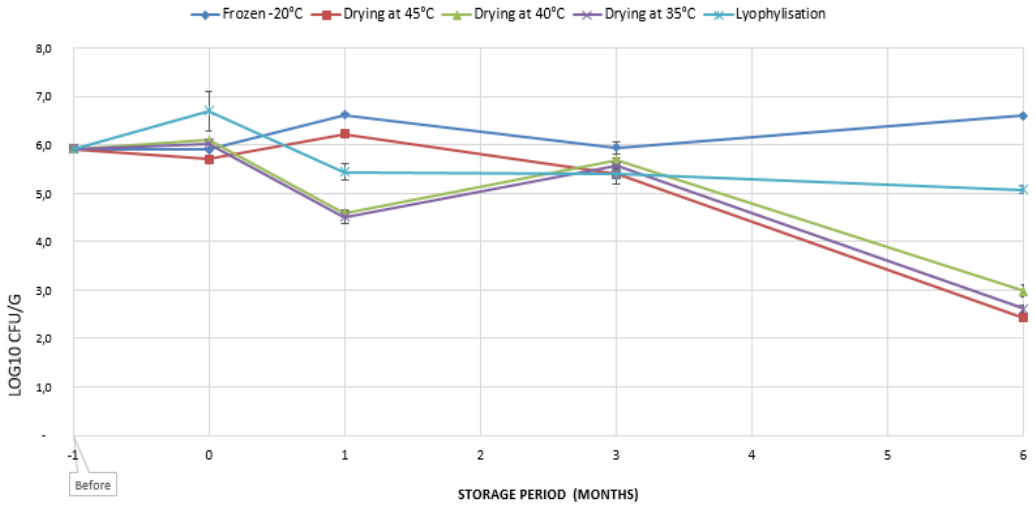

Figure 4.4 Microbial loads of lactic acid bacteria submitted to different preservation methods at different periods of storage (Before treatment, 0, 1, 3, and 6 months). The standard deviation is shown as error bars.

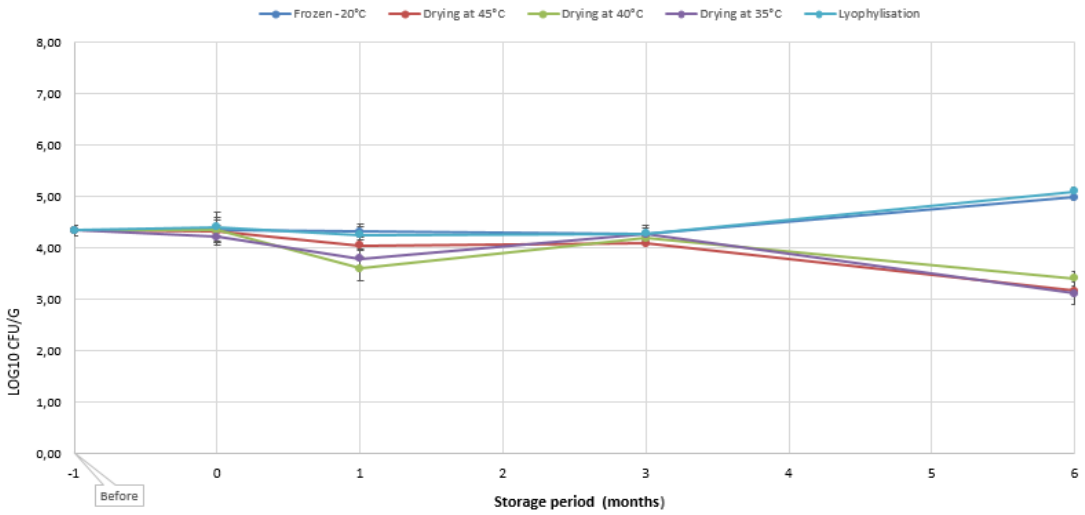

Figure 4.5 Microbial loads of yeasts submitted to different preservation methods at different periods of storage (Before treatment, 0, 1, 3, and 6 months). The standard deviation is shown as error bars.

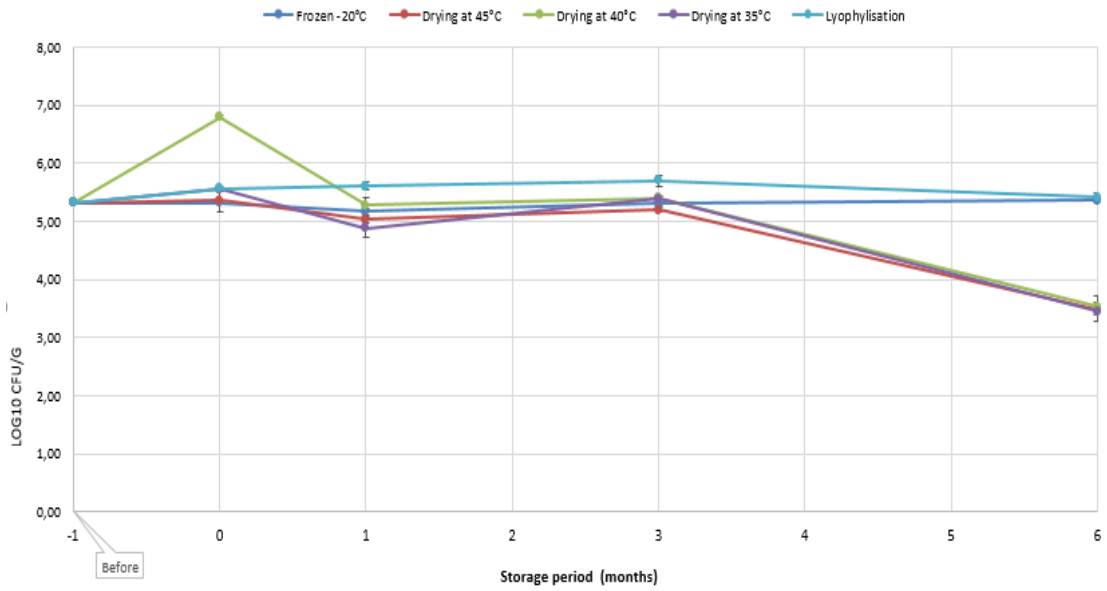

Figure 4.6 Microbial loads of moulds submitted to different preservation methods at different periods of storage (Before treatment, 0, 1, 3, and 6 months). The standard deviation is shown as error bas.

During all the storage periods, the highest counts for bacteria were observed in frozen bee pollen samples: the average of aerobic mesophiles and of LAB after six months of storage was 6.74 LOG_{10} CFU g^{-1}, and 6.7 LOG_{10} CFU g^{-1}, respectively **(Figures 4.3** and **4.4)**. Aerobic mesophiles are not within the limits established by the Argentinian code (max ± 5.17 LOG_{10} CFU g^{-1}). Knowing that freezing is one of the most used methods to preserve food, this technique turns the available water in food into solid ice crystals which inhibits the growth of microorganisms and stops their multiplication. However, freezing reduces the microbial activity but does not kill them (Solomon and Obioha, 2017). Also, it has been proven that Gram-positive bacteria are resistant to freezing and storage at sub-zero temperatures (Dimitraki *et al.*, 2007). In another study, Hervatin *et al.* (2012) highlighted the presence of pathogens in fresh and frozen Brazilian bee pollen. The comparison of our results of bacteria loads by analysing the frozen samples at different storage times showed no significant difference ($p > 0.05$) throughout time. Our findings are in agreement with results reported by other authors. As a matter of fact, Mauriello *et al.* (2017) found that most of the microbiological indicators of pollen quality analysed were still detected after four months of frozen storage. Analysed samples after the same storage period showed an unchanged load for total aerobic mesophiles, and LAB population persisted in samples after the frozen storage period.

Differently from freezing, freeze drying is a drying process in which water is removed from the sample by sublimation (Wu *et al.*, 2018). Although freeze dried samples showed the lowest value for moisture content at all storage periods with an average of 5.50%, there was no significant difference between microbial loads found in frozen samples and samples treated with freeze drying. Noteworthy, in all time points, the load of yeasts and moulds found in treated samples with freeze drying **(Figures 4.5** and **4.6)** is high when comparing with loads of analysed samples treated with different preservation methods. Moulds load of freeze dried bee pollen is significant different from moulds load of oven dried bee pollen at 35 °C after one month of storage ($p < 0.05$), same significant difference between analysed samples with freeze drying and oven drying at 45°C after three months of storage ($p < 0.05$), Also for yeasts, their load in freeze drying samples is significant different from their load in oven drying samples at 35°C after six months of storage ($p < 0.05$).

Our findings are in agreement with results reported by other authors, according to a Slovakian study on microbial properties of bee pollen used in human nutrition, the levels of microscopic fungi found in treated samples with freeze drying are higher than the levels found in frozen and dried samples at 35°C (Mauriello *et al.*, 2017). The stability of these microoganisms in treated bee pollen with freeze drying can be explained by the performance of this technique in keeping a good stability for microorganism. As a matter of fact, studies on the freeze drying performance showed that freeze drying is the preferred method to preserve microorganisms for culture collection because it offers the best survivability (Morgan *et al.*, 2006), it has been proved that freeze drying can preserve bioactive molecules (DNA, enzymes, and proteins) and it is a successful application for long-term living systems like cells, also for proliferating bacteria and fungi (Nireesha *et al.*, 2013). Our findings can also be explained by the freeze drying process technique and its ability to preserve the biological and chemical properties of various food product that can enhance the microbial viability (Ciurzyńska and Lenart, 2011). Indeed, the food itself may influence the survivability positively such as by offering protective compounds and structures (Morgan *et al.*, 2006). The matrix of bee pollen is an ideal culture medium for microorganisms (Hani et al., 2012), sucrose and other sucrose, polypeptides, polyalcohol, amino acids, glycerol, and carboxylic acids have been shown to increase the survivability of microorganism during the drying process (Morgan *et al.*, 2006).

Therefore, one may expect a great viability in the survival of microorganisms during the freeze drying of bee pollen due to their structure and composition.

Overall, the comparison of microbial loads showed that after six months of storage, freezing and freeze drying techniques presented the highest levels of all detected microorganisms, while oven drying presented the lowest levels specially for oven drying at 35 °C. After six months of storage, results of microbial loads of treated samples with oven drying at 35 °C showed a significant difference when compared with those treated with freezing for aerobic mesophiles ($p < 0.05$), and with freeze drying for yeasts ($p < 0.05$). There is also a significant difference for LAB loads between results of treated samples with oven drying at 45 °C and freezing ($p < 0.05$), However, there is no statistically significant difference between the registered results of microbial levels that were submitted to oven drying at different temperatures and were analysed at different period times of storage. According to our viable count of bacteria, yeast and moulds, there are no remarkable differences in the values during the different months of storage. Noteworthy, oven drying at 40 °C present the highest levels for bacteria. After six months of storage, aerobic mesophiles are less present in oven dried samples at 35 °C (3.96 LOG_{10} CFU g^{-1}), while LAB are less present in oven drying samples at 45 °C (2.4 LOG_{10} CFU g^{-1}). The yeasts values ranged from 3.12 to 3.41 LOG_{10} CFU g^{-1} and moulds ranged from 2.4 to 3.0 LOG_{10} CFU g^{-1}. The values of aerobic mesophiles, yeasts, and moulds are within the Argentinian code set for bee pollen.

To the extent of our knowledge, no previous study aiming to compare the microbiological characterisation of treated bee pollen with oven drying at different temperatures is available and, in general, no previous study characterising the microbial stability and survivability of oven drying treated bee pollen at different periods of storage. Nevertheless, a Slovakian study about microbial properties in frozen, freeze dried, and oven dried at 35 °C bee pollen confirms our findings about microbial loads for aerobic mesophiles and moulds that remain less than those of freeze dried and frozen bee pollen (Mauriello *et al.*, 2017). This study does not report the contamination with LAB and yeasts (Mauriello *et al.*, 2017). The studies about inactivation of microorganisms during drying processes are still limited. Most of the studies on dehydration mechanisms of inactivation and survivability of microorganisms during heat and drying have been studied in the field of preservation of microbial cultures

(Morgan *et al.*, 2006). There is also a lack of information concerning the survivability during drying at different temperatures of complex solid matrices such as solid foodstuffs like bee pollen (Smelt *et al.*, 2014). During drying process, the different cellular components may be affected by the dehydration of bacteria. The removal of water can induce DNA and RNA break down, protein denaturation, cytoplasmic membrane alteration and cell wall damage. Furthermore during drying the concentration of acids and toxic compounds in the cell is increased with the risk of oxidation reactions occurrence cells (Lievense *et al.*, 1994). The viability loss was mainly due to the damage to the cell membrane and proteins (Ananta et al., 2005). Temperature is one of the most important factors that can affect microorganisms during the drying process (Chávez and Ledeboer, 2007).

For all preservation processes, our study has shown that the microbiological behaviour differs according to the time of analysis and preservation method. After six months of storage, there is a decrease in bacterial levels for all treatments except freezing, which increased even if without a significant difference ($p > 0.05$). Yeast and moulds levels decreased in oven dried samples at different temperatures with a significant difference in oven dried samples at 35 °C ($p < 0.05$), and with a significant difference for moulds in treated samples with oven drying at 40 °C ($p < 0.05$). In contrast, yeast and moulds levels increased in frozen samples, and freeze drying samples after six months of storage with a significant difference for moulds ($p < 0.05$). Thus, there is a variability in the microbiological behaviour and in the survivability of the investigated microorganisms. 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 (Lievense *et al.*, 1994; Smelt *et al.*, 2014). Hence, one may expect a different survivability during thermal drying process for bee pollen. The variability in microbial levels at different period of storage of treated samples can also be explained by the different stresses applied on microorganisms, to which they can behave and respond differently. The decreasing of water content and water activity is not the only stress applied to the microorganism. Depending on the submitted preservation method, microorganisms may be exposed to different temperatures. The presence of several stresses at the same time makes the interpretation of microbial stability and survivability difficult because these stresses could act synergistically or antagonistically. For example, during oven drying

thermal inactivation and dehydration inactivation of the present microorganisms may occur simultaneously. However, a reduced water activity could enhance heat resistance of microorganisms so the dehydration processes may act competitively with the heat processes, and similar antagonistic effects could be observed for other stress combinations (Lievense *et al.*, 1994; Smelt *et al.*, 2014; Roopesh *et al.*, 2016).

Overall, freezing and freeze drying were not efficient techniques to preserve hygienic quality of bee pollen. Furthermore, freeze drying is considered as one of the most expensive techniques with a high energy consumption and high operational costs. Indeed, comparing freeze drying with oven drying the cost of freeze drying is 4-8 times higher and the basic energy for removing 1kg of water is almost the double (Flink, 1977). Oven drying can be a treatment which protect bee pollen from spreading and allows the most decreasing number of microorganisms. Since the advantages of oven drying often result in the saving of time or money, the most suitable process between the oven drying treatments at the different temperature depends on different factors as reasonable process time, better sanitary conditions and control of the drying conditions. In addition, to determine which particular oven drying temperature for bee pollen preservation, attention must be focused not only on the contribution of the process to reduce or eliminate microorganisms, but also to its effect on the preservation of chemical, physical and nutritional properties. Information is still scarce and more studies are needed on the effect of preservation technologies on the microbial stability, and survivability during and after preservation process.

Considering the results obtained in the present study, drying the bee pollen at a temperature between 35 and 45 ºC seems to be the most effective method to reduce the viability of microorganisms, at least at a medium term (6 months). This method seems to be the one causing the highest negative impact on the survivability of the microbial contaminants. However, we must be aware of the possibility that contaminants are kept in the pollen under the status of viable but non cultivable (VBNC). This would mean that, even though we are not able to account for them as live microbes, they are still viable and will be able to grow if the appropriate conditions (e.g. high humidity) become available.

5. Conclusions

Our study showed that the loads of microorganisms used as general quality parameters aerobic mesophiles, LAB, yeasts and moulds seem to be high and are not within the limits established for the Argentinian code set for bee pollen. Sanitary parameters gave negative results for *E. coli* and *Salmonella* in all the analysed samples. However, SRC and coliforms were present. Our findings showed that bee pollen is as vulnerable to microbial contamination as any other food item. Microbial loads were found to differ between regions. Leiria presented the highest contamination and Nisa the lowest.

Bee pollen continues to be used without the existence of official bee pollen quality standards, which propose that contamination must be avoided and controlled during production and handling practices through quality assurance measures such as good agricultural and collection practices (GACP). Furthermore, more studies about hygienic and sanitary conditions in beekeeping products are necessary to provide more information to national regulatory agencies in order to establish microbiological standards for bee pollen.

Concerning bee pollen preservation, our results showed that freezing, freeze drying and oven drying have different impacts on bee pollen microbial stability. Oven drying at a temperature between 35 and 45 ºC seems to be the most adequate method of preservation, in terms of microbial stability, at least for a preservation period of 6 months.

Overall, the impact of preservation methods technologies in relation with microbial inactivation in bee pollen needs to be more studied, in order to validate an effective food safety management system.

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APPENDIX

False: $p > 0.05$
True: $p < 0.05$

Significant difference between microbial loads for each parameter at time 0

```
###Aerobic mesophiles
> kruskal.test(data.0$Aerobic.mesophiles, data.0$Treatment)

     Kruskal-Wallis rank sum test

data:  data.0$Aerobic.mesophiles and data.0$Treatment
Kruskal-Wallis chi-squared = 14.56, df = 5, p-value = 0.01242

> kruskalmc(data.0$Aerobic.mesophiles, data.0$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                            obs.dif critical.dif difference
before-drying.35C          5.833333     12.79424      FALSE
before-drying.40C          0.500000     12.79424      FALSE
before-drying.45C          6.500000     12.79424      FALSE
before-freeze              0.000000     12.79424      FALSE
before-lyophylization      8.833333     12.79424      FALSE
drying.35C-drying.40C      6.333333     12.79424      FALSE
drying.35C-drying.45C     12.333333     12.79424      FALSE
drying.35C-freeze          5.833333     12.79424      FALSE
drying.35C-lyophylization 14.666667     12.79424       TRUE
drying.40C-drying.45C      6.000000     12.79424      FALSE
drying.40C-freeze          0.500000     12.79424      FALSE
drying.40C-lyophylization  8.333333     12.79424      FALSE
drying.45C-freeze          6.500000     12.79424      FALSE
drying.45C-lyophylization  2.333333     12.79424      FALSE
freeze-lyophylization      8.833333     12.79424      FALSE

###LAB
> kruskal.test(data.0$LAB, data.0$Treatment)

     Kruskal-Wallis rank sum test

data:  data.0$LAB and data.0$Treatment
Kruskal-Wallis chi-squared = 14.966, df = 5, p-value = 0.01051

> kruskalmc(data.0$LAB, data.0$Treatment)
Multiple comparison test after Kruskal-Wallis
```

```
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C              8.5     12.79424      FALSE
before-drying.40C              5.5     12.79424      FALSE
before-drying.45C             11.5     12.79424      FALSE
before-freeze                  0.0     12.79424      FALSE
before-lyophylization          1.5     12.79424      FALSE
drying.35C-drying.40C          3.0     12.79424      FALSE
drying.35C-drying.45C          3.0     12.79424      FALSE
drying.35C-freeze              8.5     12.79424      FALSE
drying.35C-lyophylization     10.0     12.79424      FALSE
drying.40C-drying.45C          6.0     12.79424      FALSE
drying.40C-freeze              5.5     12.79424      FALSE
drying.40C-lyophylization      7.0     12.79424      FALSE
drying.45C-freeze             11.5     12.79424      FALSE
drying.45C-lyophylization     13.0     12.79424       TRUE
freeze-lyophylization          1.5     12.79424      FALSE

###Yeasts
> kruskal.test(data.0$Yeasts, data.0$Treatment)

     Kruskal-Wallis rank sum test

data:  data.0$Yeasts and data.0$Treatment
Kruskal-Wallis chi-squared = 2.0568, df = 5, p-value = 0.8412

> kruskalmc(data.0$Yeasts, data.0$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                             obs.dif critical.dif difference
before-drying.35C          3.8333333     12.79424      FALSE
before-drying.40C          0.3333333     12.79424      FALSE
before-drying.45C          0.6666667     12.79424      FALSE
before-freeze              0.0000000     12.79424      FALSE
before-lyophylization      2.1666667     12.79424      FALSE
drying.35C-drying.40C      4.1666667     12.79424      FALSE
drying.35C-drying.45C      3.1666667     12.79424      FALSE
drying.35C-freeze          3.8333333     12.79424      FALSE
drying.35C-lyophylization 6.0000000     12.79424      FALSE
drying.40C-drying.45C      1.0000000     12.79424      FALSE
drying.40C-freeze          0.3333333     12.79424      FALSE
drying.40C-lyophylization 1.8333333     12.79424      FALSE
drying.45C-freeze          0.6666667     12.79424      FALSE
drying.45C-lyophylization 2.8333333     12.79424      FALSE
freeze-lyophylization      2.1666667     12.79424      FALSE

###Molds
> kruskal.test(data.0$Molds, data.0$Treatment)

     Kruskal-Wallis rank sum test

data:  data.0$Molds and data.0$Treatment
Kruskal-Wallis chi-squared = 14.244, df = 5, p-value = 0.01413
```

```
> kruskalmc(data.0$Molds, data.0$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         7.166667     12.79424      FALSE
before-drying.40C        12.500000     12.79424      FALSE
before-drying.45C         1.833333     12.79424      FALSE
before-freeze             0.000000     12.79424      FALSE
before-lyophylization     8.500000     12.79424      FALSE
drying.35C-drying.40C     5.333333     12.79424      FALSE
drying.35C-drying.45C     5.333333     12.79424      FALSE
drying.35C-freeze         7.166667     12.79424      FALSE
drying.35C-lyophylization 1.333333     12.79424      FALSE
drying.40C-drying.45C    10.666667     12.79424      FALSE
drying.40C-freeze        12.500000     12.79424      FALSE
drying.40C-lyophylization 4.000000     12.79424      FALSE
drying.45C-freeze         1.833333     12.79424      FALSE
drying.45C-lyophylization 6.666667     12.79424      FALSE
freeze-lyophylization     8.500000     12.79424      FALSE
```

Significant difference between microbial loads for each parameter at time 1

```
###Aerobic mesophiles
> kruskal.test(data.1$Aerobic.mesophiles, data.1$Treatment)

     Kruskal-Wallis rank sum test

data:  data.1$Aerobic.mesophiles and data.1$Treatment
Kruskal-Wallis chi-squared = 15.363, df = 5, p-value = 0.00892

> kruskalmc(data.1$Aerobic.mesophiles, data.1$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         9.000000     12.79424      FALSE
before-drying.40C         3.666667     12.79424      FALSE
before-drying.45C         7.333333     12.79424      FALSE
before-freeze             2.666667     12.79424      FALSE
before-lyophylization     4.333333     12.79424      FALSE
drying.35C-drying.40C     5.333333     12.79424      FALSE
drying.35C-drying.45C     1.666667     12.79424      FALSE
drying.35C-freeze        11.666667     12.79424      FALSE
drying.35C-lyophylization 13.333333     12.79424       TRUE
drying.40C-drying.45C     3.666667     12.79424      FALSE
drying.40C-freeze         6.333333     12.79424      FALSE
drying.40C-lyophylization 8.000000     12.79424      FALSE
drying.45C-freeze        10.000000     12.79424      FALSE
drying.45C-lyophylization 11.666667     12.79424      FALSE
freeze-lyophylization     1.666667     12.79424      FALSE

###LAB
> kruskal.test(data.1$LAB, data.1$Treatment)
```

```
     Kruskal-Wallis rank sum test

data:  data.1$LAB and data.1$Treatment
Kruskal-Wallis chi-squared = 15.494, df = 5, p-value = 0.008448

> kruskalmc(data.1$LAB, data.1$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         11.833333     12.79424      FALSE
before-drying.40C         10.166667     12.79424      FALSE
before-drying.45C          3.000000     12.79424      FALSE
before-freeze              1.500000     12.79424      FALSE
before-lyophylization      6.500000     12.79424      FALSE
drying.35C-drying.40C      1.666667     12.79424      FALSE
drying.35C-drying.45C      8.833333     12.79424      FALSE
drying.35C-freeze         13.333333     12.79424       TRUE
drying.35C-lyophylization  5.333333     12.79424      FALSE
drying.40C-drying.45C      7.166667     12.79424      FALSE
drying.40C-freeze         11.666667     12.79424      FALSE
drying.40C-lyophylization  3.666667     12.79424      FALSE
drying.45C-freeze          4.500000     12.79424      FALSE
drying.45C-lyophylization  3.500000     12.79424      FALSE
freeze-lyophylization      8.000000     12.79424      FALSE

###Yeasts
> kruskal.test(data.1$Yeasts, data.1$Treatment)

     Kruskal-Wallis rank sum test

data:  data.1$Yeasts and data.1$Treatment
Kruskal-Wallis chi-squared = 13.91, df = 5, p-value = 0.01619

> kruskalmc(data.1$Yeasts, data.1$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                             obs.dif critical.dif difference
before-drying.35C         10.1666667     12.79424      FALSE
before-drying.40C         12.1666667     12.79424      FALSE
before-drying.45C          6.6666667     12.79424      FALSE
before-freeze              0.6666667     12.79424      FALSE
before-lyophylization      2.3333333     12.79424      FALSE
drying.35C-drying.40C      2.0000000     12.79424      FALSE
drying.35C-drying.45C      3.5000000     12.79424      FALSE
drying.35C-freeze          9.5000000     12.79424      FALSE
drying.35C-lyophylization  7.8333333     12.79424      FALSE
drying.40C-drying.45C      5.5000000     12.79424      FALSE
drying.40C-freeze         11.5000000     12.79424      FALSE
drying.40C-lyophylization  9.8333333     12.79424      FALSE
drying.45C-freeze          6.0000000     12.79424      FALSE
drying.45C-lyophylization  4.3333333     12.79424      FALSE
freeze-lyophylization      1.6666667     12.79424      FALSE
```

```
###Molds
> kruskal.test(data.1$Molds, data.1$Treatment)

      Kruskal-Wallis rank sum test

data:  data.1$Molds and data.1$Treatment
Kruskal-Wallis chi-squared = 15.67, df = 5, p-value = 0.007853

> kruskalmc(data.1$Molds, data.1$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         10.500000     12.79424      FALSE
before-drying.40C          1.333333     12.79424      FALSE
before-drying.45C          8.500000     12.79424      FALSE
before-freeze              4.666667     12.79424      FALSE
before-lyophylization      4.000000     12.79424      FALSE
drying.35C-drying.40C      9.166667     12.79424      FALSE
drying.35C-drying.45C      2.000000     12.79424      FALSE
drying.35C-freeze          5.833333     12.79424      FALSE
drying.35C-lyophylization 14.500000     12.79424       TRUE
drying.40C-drying.45C      7.166667     12.79424      FALSE
drying.40C-freeze          3.333333     12.79424      FALSE
drying.40C-lyophylization  5.333333     12.79424      FALSE
drying.45C-freeze          3.833333     12.79424      FALSE
drying.45C-lyophylization 12.500000     12.79424      FALSE
freeze-lyophylization      8.666667     12.79424      FALSE
```

Significant difference between microbial loads for each parameter at time 3

```
###Aerobic mesophiles
> kruskal.test(data.3$Aerobic.mesophiles, data.3$Treatment)

      Kruskal-Wallis rank sum test

data:  data.3$Aerobic.mesophiles and data.3$Treatment
Kruskal-Wallis chi-squared = 14.863, df = 5, p-value = 0.01096

> kruskalmc(data.3$Aerobic.mesophiles, data.3$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         10.000000     12.79424      FALSE
before-drying.40C          3.666667     12.79424      FALSE
before-drying.45C         11.000000     12.79424      FALSE
before-freeze              1.666667     12.79424      FALSE
before-lyophylization      8.000000     12.79424      FALSE
drying.35C-drying.40C      6.333333     12.79424      FALSE
drying.35C-drying.45C      1.000000     12.79424      FALSE
drying.35C-freeze         11.666667     12.79424      FALSE
```

```
drying.35C-lyophylization  2.000000     12.79424      FALSE
drying.40C-drying.45C      7.333333     12.79424      FALSE
drying.40C-freeze          5.333333     12.79424      FALSE
drying.40C-lyophylization  4.333333     12.79424      FALSE
drying.45C-freeze         12.666667     12.79424      FALSE
drying.45C-lyophylization  3.000000     12.79424      FALSE
freeze-lyophylization      9.666667     12.79424      FALSE

###LAB
> kruskal.test(data.3$LAB, data.3$Treatment)

     Kruskal-Wallis rank sum test

data:  data.3$LAB and data.3$Treatment
Kruskal-Wallis chi-squared = 15.455, df = 5, p-value = 0.008587

> kruskalmc(data.3$LAB, data.3$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                          obs.dif critical.dif difference
before-drying.35C        9.833333     12.79424      FALSE
before-drying.40C        6.000000     12.79424      FALSE
before-drying.45C       12.833333     12.79424       TRUE
before-freeze            3.000000     12.79424      FALSE
before-lyophylization   13.333333     12.79424       TRUE
drying.35C-drying.40C    3.833333     12.79424      FALSE
drying.35C-drying.45C    3.000000     12.79424      FALSE
drying.35C-freeze        6.833333     12.79424      FALSE
drying.35C-lyophylization  3.500000     12.79424      FALSE
drying.40C-drying.45C    6.833333     12.79424      FALSE
drying.40C-freeze        3.000000     12.79424      FALSE
drying.40C-lyophylization  7.333333     12.79424      FALSE
drying.45C-freeze        9.833333     12.79424      FALSE
drying.45C-lyophylization  0.500000     12.79424      FALSE
freeze-lyophylization   10.333333     12.79424      FALSE

###Yeasts
> kruskal.test(data.3$Yeasts, data.3$Treatment)

     Kruskal-Wallis rank sum test

data:  data.3$Yeasts and data.3$Treatment
Kruskal-Wallis chi-squared = 9.4102, df = 5, p-value = 0.09378

> kruskalmc(data.3$Yeasts, data.3$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                          obs.dif critical.dif difference
before-drying.35C        3.666667     12.79424      FALSE
before-drying.40C        7.166667     12.79424      FALSE
before-drying.45C       11.500000     12.79424      FALSE
before-freeze            2.333333     12.79424      FALSE
before-lyophylization    2.333333     12.79424      FALSE
drying.35C-drying.40C    3.500000     12.79424      FALSE
```

```
drying.35C-drying.45C      7.833333     12.79424      FALSE
drying.35C-freeze          1.333333     12.79424      FALSE
drying.35C-lyophylization  1.333333     12.79424      FALSE
drying.40C-drying.45C      4.333333     12.79424      FALSE
drying.40C-freeze          4.833333     12.79424      FALSE
drying.40C-lyophylization  4.833333     12.79424      FALSE
drying.45C-freeze          9.166667     12.79424      FALSE
drying.45C-lyophylization  9.166667     12.79424      FALSE
freeze-lyophylization      0.000000     12.79424      FALSE

###Molds
> kruskal.test(data.3$Molds, data.3$Treatment)

     Kruskal-Wallis rank sum test

data:  data.3$Molds and data.3$Treatment
Kruskal-Wallis chi-squared = 15.045, df = 5, p-value = 0.01017

> kruskalmc(data.3$Molds, data.3$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         4.166667     12.79424      FALSE
before-drying.40C         5.166667     12.79424      FALSE
before-drying.45C         5.500000     12.79424      FALSE
before-freeze             1.333333     12.79424      FALSE
before-lyophylization     9.500000     12.79424      FALSE
drying.35C-drying.40C     1.000000     12.79424      FALSE
drying.35C-drying.45C     9.666667     12.79424      FALSE
drying.35C-freeze         5.500000     12.79424      FALSE
drying.35C-lyophylization 5.333333     12.79424      FALSE
drying.40C-drying.45C    10.666667     12.79424      FALSE
drying.40C-freeze         6.500000     12.79424      FALSE
drying.40C-lyophylization 4.333333     12.79424      FALSE
drying.45C-freeze         4.166667     12.79424      FALSE
drying.45C-lyophylization 15.000000    12.79424       TRUE
freeze-lyophylization    10.833333     12.79424      FALSE
```

Significant difference between microbial loads for each parameter at time 6

```
###Aerobic mesophiles
> kruskal.test(data.6$Aerobic.mesophiles, data.6$Treatment)

     Kruskal-Wallis rank sum test

data:  data.6$Aerobic.mesophiles and data.6$Treatment
Kruskal-Wallis chi-squared = 16.366, df = 5, p-value = 0.005872
```

```
> kruskalmc(data.6$Aerobic.mesophiles, data.6$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                          obs.dif critical.dif difference
before-drying.35C            12.5     12.79424      FALSE
before-drying.40C             6.5     12.79424      FALSE
before-drying.45C             9.5     12.79424      FALSE
before-freeze                 2.0     12.79424      FALSE
before-lyophylization         3.5     12.79424      FALSE
drying.35C-drying.40C         6.0     12.79424      FALSE
drying.35C-drying.45C         3.0     12.79424      FALSE
drying.35C-freeze            14.5     12.79424       TRUE
drying.35C-lyophylization     9.0     12.79424      FALSE
drying.40C-drying.45C         3.0     12.79424      FALSE
drying.40C-freeze             8.5     12.79424      FALSE
drying.40C-lyophylization     3.0     12.79424      FALSE
drying.45C-freeze            11.5     12.79424      FALSE
drying.45C-lyophylization     6.0     12.79424      FALSE
freeze-lyophylization         5.5     12.79424      FALSE


###LAB
> kruskal.test(data.6$LAB, data.6$Treatment)

      Kruskal-Wallis rank sum test

data:  data.6$LAB and data.6$Treatment
Kruskal-Wallis chi-squared = 16.249, df = 5, p-value = 0.006168

> kruskalmc(data.6$LAB, data.6$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                           obs.dif critical.dif difference
before-drying.35C         9.833333     12.79424      FALSE
before-drying.40C         6.833333     12.79424      FALSE
before-drying.45C        12.833333     12.79424       TRUE
before-freeze             1.333333     12.79424      FALSE
before-lyophylization     3.833333     12.79424      FALSE
drying.35C-drying.40C     3.000000     12.79424      FALSE
drying.35C-drying.45C     3.000000     12.79424      FALSE
drying.35C-freeze        11.166667     12.79424      FALSE
drying.35C-lyophylization 6.000000     12.79424      FALSE
drying.40C-drying.45C     6.000000     12.79424      FALSE
drying.40C-freeze         8.166667     12.79424      FALSE
drying.40C-lyophylization 3.000000     12.79424      FALSE
drying.45C-freeze        14.166667     12.79424       TRUE
drying.45C-lyophylization 9.000000     12.79424      FALSE
freeze-lyophylization     5.166667     12.79424      FALSE

###Yeasts
> kruskal.test(data.6$Yeasts, data.6$Treatment)

      Kruskal-Wallis rank sum test
```

```
data:  data.6$Yeasts and data.6$Treatment
Kruskal-Wallis chi-squared = 15.491, df = 5, p-value = 0.008459

> kruskalmc(data.6$Yeasts, data.6$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                            obs.dif critical.dif difference
before-drying.35C          8.166667     12.79424      FALSE
before-drying.40C          3.666667     12.79424      FALSE
before-drying.45C          6.166667     12.79424      FALSE
before-freeze              3.666667     12.79424      FALSE
before-lyophylization      5.333333     12.79424      FALSE
drying.35C-drying.40C      4.500000     12.79424      FALSE
drying.35C-drying.45C      2.000000     12.79424      FALSE
drying.35C-freeze         11.833333     12.79424      FALSE
drying.35C-lyophylization 13.500000     12.79424       TRUE
drying.40C-drying.45C      2.500000     12.79424      FALSE
drying.40C-freeze          7.333333     12.79424      FALSE
drying.40C-lyophylization  9.000000     12.79424      FALSE
drying.45C-freeze          9.833333     12.79424      FALSE
drying.45C-lyophylization 11.500000     12.79424      FALSE
freeze-lyophylization      1.666667     12.79424      FALSE

###Molds
> kruskal.test(data.6$Molds, data.6$Treatment)

     Kruskal-Wallis rank sum test

data:  data.6$Molds and data.6$Treatment
Kruskal-Wallis chi-squared = 14.299, df = 5, p-value = 0.01382

>
> #kruskal with postdoc
> kruskalmc(data.6$Molds, data.6$Treatment)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
                              obs.dif critical.dif difference
before-drying.35C          7.0000000     12.79424      FALSE
before-drying.40C          5.1666667     12.79424      FALSE
before-drying.45C          7.3333333     12.79424      FALSE
before-freeze              2.8333333     12.79424      FALSE
before-lyophylization      4.6666667     12.79424      FALSE
drying.35C-drying.40C      1.8333333     12.79424      FALSE
drying.35C-drying.45C      0.3333333     12.79424      FALSE
drying.35C-freeze          9.8333333     12.79424      FALSE
drying.35C-lyophylization 11.6666667     12.79424      FALSE
drying.40C-drying.45C      2.1666667     12.79424      FALSE
drying.40C-freeze          8.0000000     12.79424      FALSE
drying.40C-lyophylization  9.8333333     12.79424      FALSE
drying.45C-freeze         10.1666667     12.79424      FALSE
drying.45C-lyophylization 12.0000000     12.79424      FALSE
freeze-lyophylization      1.8333333     12.79424      FALSE
```

Significant difference between microbial loads at different storage periods of frozen bee pollen:

```
###Aerobic mesophiles
> kruskal.test(data.F$Aerobic.mesophiles, data.F$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.F$Aerobic.mesophiles and data.F$Storage.period
Kruskal-Wallis chi-squared = 3.8565, df = 4, p-value = 0.4258

> kruskalmc(data.F$Aerobic.mesophiles, data.F$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0 0.0000000     10.24984      FALSE
-1-1 5.3333333     10.24984      FALSE
-1-3 3.5000000     10.24984      FALSE
-1-6 4.5000000     10.24984      FALSE
0-1  5.3333333     10.24984      FALSE
0-3  3.5000000     10.24984      FALSE
0-6  4.5000000     10.24984      FALSE
1-3  1.8333333     10.24984      FALSE
1-6  0.8333333     10.24984      FALSE
3-6  1.0000000     10.24984      FALSE

###LAB
> kruskal.test(data.F$LAB, data.F$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.F$LAB and data.F$Storage.period
Kruskal-Wallis chi-squared = 7.6709, df = 4, p-value = 0.1044

> kruskalmc(data.F$LAB, data.F$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0 0.0000000     10.24984      FALSE
-1-1 2.5000000     10.24984      FALSE
-1-3 6.3333333     10.24984      FALSE
-1-6 2.1666667     10.24984      FALSE
0-1  2.5000000     10.24984      FALSE
0-3  6.3333333     10.24984      FALSE
0-6  2.1666667     10.24984      FALSE
1-3  8.8333333     10.24984      FALSE
1-6  0.3333333     10.24984      FALSE
3-6  8.5000000     10.24984      FALSE

###Yeasts
> kruskal.test(data.F$Yeasts, data.F$Storage.period)
```

```
     Kruskal-Wallis rank sum test

data:  data.F$Yeasts and data.F$Storage.period
Kruskal-Wallis chi-squared = 7.8175, df = 4, p-value = 0.0985

> kruskalmc(data.F$Yeasts, data.F$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0     0.0     10.24984      FALSE
-1-1     1.0     10.24984      FALSE
-1-3     3.0     10.24984      FALSE
-1-6     6.5     10.24984      FALSE
0-1      1.0     10.24984      FALSE
0-3      3.0     10.24984      FALSE
0-6      6.5     10.24984      FALSE
1-3      2.0     10.24984      FALSE
1-6      7.5     10.24984      FALSE
3-6      9.5     10.24984      FALSE

###Molds
> kruskal.test(data.F$Molds, data.F$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.F$Molds and data.F$Storage.period
Kruskal-Wallis chi-squared = 9.8462, df = 4, p-value = 0.0431

> kruskalmc(data.F$Molds, data.F$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       0     10.24984      FALSE
-1-1       7     10.24984      FALSE
-1-3       2     10.24984      FALSE
-1-6       4     10.24984      FALSE
0-1        7     10.24984      FALSE
0-3        2     10.24984      FALSE
0-6        4     10.24984      FALSE
1-3        5     10.24984      FALSE
1-6       11     10.24984       TRUE
3-6        6     10.24984      FALSE
```

Significant difference between microbial loads at different storage periods of freeze dried bee pollen:

```
###Aerobic mesophiles
> kruskal.test(data.L$Aerobic.mesophiles, data.L$Storage.period)

     Kruskal-Wallis rank sum test
```

```
data:  data.L$Aerobic.mesophiles and data.L$Storage.period
Kruskal-Wallis chi-squared = 12.9, df = 4, p-value = 0.01177

> kruskalmc(data.L$Aerobic.mesophiles, data.L$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       5     10.24984      FALSE
-1-1       4     10.24984      FALSE
-1-3       3     10.24984      FALSE
-1-6       6     10.24984      FALSE
0-1        1     10.24984      FALSE
0-3        8     10.24984      FALSE
0-6       11     10.24984       TRUE
1-3        7     10.24984      FALSE
1-6       10     10.24984      FALSE
3-6        3     10.24984      FALSE

###LAB
> kruskal.test(data.L$LAB, data.L$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.L$LAB and data.L$Storage.period
Kruskal-Wallis chi-squared = 12.233, df = 4, p-value = 0.0157

> kruskalmc(data.L$LAB, data.L$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
        obs.dif critical.dif difference
-1-0  1.0000000     10.24984      FALSE
-1-1  5.3333333     10.24984      FALSE
-1-3  5.6666667     10.24984      FALSE
-1-6 10.0000000     10.24984      FALSE
0-1   6.3333333     10.24984      FALSE
0-3   6.6666667     10.24984      FALSE
0-6  11.0000000     10.24984       TRUE
1-3   0.3333333     10.24984      FALSE
1-6   4.6666667     10.24984      FALSE
3-6   4.3333333     10.24984      FALSE

###Yeasts
> kruskal.test(data.L$Yeasts, data.L$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.L$Yeasts and data.L$Storage.period
Kruskal-Wallis chi-squared = 7.6493, df = 4, p-value = 0.1053

> kruskalmc(data.L$Yeasts, data.L$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
```

```
-1-0 1.1666667     10.24984      FALSE
-1-1 2.0000000     10.24984      FALSE
-1-3 1.1666667     10.24984      FALSE
-1-6 7.0000000     10.24984      FALSE
0-1  3.1666667     10.24984      FALSE
0-3  2.3333333     10.24984      FALSE
0-6  5.8333333     10.24984      FALSE
1-3  0.8333333     10.24984      FALSE
1-6  9.0000000     10.24984      FALSE
3-6  8.1666667     10.24984      FALSE

###Molds
> kruskal.test(data.L$Molds, data.L$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.L$Molds and data.L$Storage.period
Kruskal-Wallis chi-squared = 12.135, df = 4, p-value = 0.01638

> kruskalmc(data.L$Molds, data.L$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0  6.833333     10.24984      FALSE
-1-1  8.500000     10.24984      FALSE
-1-3 11.166667     10.24984       TRUE
-1-6  2.666667     10.24984      FALSE
0-1   1.666667     10.24984      FALSE
0-3   4.333333     10.24984      FALSE
0-6   4.166667     10.24984      FALSE
1-3   2.666667     10.24984      FALSE
1-6   5.833333     10.24984      FALSE
3-6   8.500000     10.24984      FALSE
```

Significant difference between microbial loads of oven dried at 35°C bee pollen at different storage periods:

```
###Aerobic mesophiles
> kruskal.test(data.35$Aerobic.mesophiles,
data.35$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.35$Aerobic.mesophiles and data.35$Storage.period
Kruskal-Wallis chi-squared = 13.524, df = 4, p-value = 0.008979

> kruskalmc(data.35$Aerobic.mesophiles, data.35$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       3     10.24984      FALSE
-1-1       9     10.24984      FALSE
```

```
-1-3       6     10.24984      FALSE
-1-6      12     10.24984       TRUE
0-1        6     10.24984      FALSE
0-3        3     10.24984      FALSE
0-6        9     10.24984      FALSE
1-3        3     10.24984      FALSE
1-6        3     10.24984      FALSE
3-6        6     10.24984      FALSE

###LAB
> kruskal.test(data.35$LAB, data.35$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.35$LAB and data.35$Storage.period
Kruskal-Wallis chi-squared = 13.622, df = 4, p-value = 0.008606

> kruskalmc(data.35$LAB, data.35$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       3     10.24984      FALSE
-1-1       9     10.24984   FALSE
-1-3       6     10.24984      FALSE
-1-6      12     10.24984       TRUE
0-1        6     10.24984      FALSE
0-3        3     10.24984      FALSE
0-6        9     10.24984      FALSE
1-3        3     10.24984      FALSE
1-6        3     10.24984      FALSE
3-6        6     10.24984      FALSE

###Yeasts
> kruskal.test(data.35$Yeasts, data.35$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.35$Yeasts and data.35$Storage.period
Kruskal-Wallis chi-squared = 11.511, df = 4, p-value = 0.02138

> kruskalmc(data.35$Yeasts, data.35$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0  2.833333     10.24984      FALSE
-1-1  7.500000     10.24984      FALSE
-1-3  1.666667     10.24984      FALSE
-1-6 10.500000     10.24984       TRUE
0-1   4.666667     10.24984      FALSE
0-3   1.166667     10.24984      FALSE
0-6   7.666667     10.24984      FALSE
1-3   5.833333     10.24984      FALSE
1-6   3.000000     10.24984      FALSE
3-6   8.833333     10.24984      FALSE
```

```
###Molds
> kruskal.test(data.35$Molds, data.35$Storage.period)

      Kruskal-Wallis rank sum test

data:  data.35$Molds and data.35$Storage.period
Kruskal-Wallis chi-squared = 13.281, df = 4, p-value = 0.009982

> kruskalmc(data.35$Molds, data.35$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0  5.666667     10.24984      FALSE
-1-1  3.333333     10.24984      FALSE
-1-3  2.333333     10.24984      FALSE
-1-6  6.333333     10.24984      FALSE
0-1   9.000000     10.24984      FALSE
0-3   3.333333     10.24984      FALSE
0-6  12.000000     10.24984       TRUE
1-3   5.666667     10.24984      FALSE
1-6   3.000000     10.24984      FALSE
3-6   8.666667     10.24984      FALSE
```

Significant difference between microbial loads of oven dried at 45°C bee pollen at different storage periods:

```
###Aerobic mesophiles
> kruskal.test(data.45$Aerobic.mesophiles,
data.45$Storage.period)

      Kruskal-Wallis rank sum test

data:  data.45$Aerobic.mesophiles and data.45$Storage.period
Kruskal-Wallis chi-squared = 13.524, df = 4, p-value = 0.008979

> kruskalmc(data.45$Aerobic.mesophiles, data.45$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       3     10.24984      FALSE
-1-1       6     10.24984      FALSE
-1-3       3     10.24984      FALSE
-1-6       9     10.24984      FALSE
0-1        9     10.24984      FALSE
0-3        6     10.24984      FALSE
0-6       12     10.24984       TRUE
1-3        3     10.24984      FALSE
1-6        3     10.24984      FALSE
3-6        6     10.24984      FALSE
```

```
###LAB
> kruskal.test(data.45$LAB, data.45$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.45$LAB and data.45$Storage.period
Kruskal-Wallis chi-squared = 13.196, df = 4, p-value = 0.01036

> kruskalmc(data.45$LAB, data.45$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0     5.5     10.24984      FALSE
-1-1     2.0     10.24984      FALSE
-1-3     8.5     10.24984      FALSE
-1-6    11.5     10.24984       TRUE
0-1      3.5     10.24984      FALSE
0-3      3.0     10.24984      FALSE
0-6      6.0     10.24984      FALSE
1-3      6.5     10.24984      FALSE
1-6      9.5     10.24984      FALSE
3-6      3.0     10.24984      FALSE

###Yeasts
> kruskal.test(data.45$LAB, data.45$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.45$LAB and data.45$Storage.period
Kruskal-Wallis chi-squared = 13.196, df = 4, p-value = 0.01036

> kruskalmc(data.45$LAB, data.45$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0     5.5     10.24984      FALSE
-1-1     2.0     10.24984      FALSE
-1-3     8.5     10.24984      FALSE
-1-6    11.5     10.24984       TRUE
0-1      3.5     10.24984      FALSE
0-3      3.0     10.24984      FALSE
0-6      6.0     10.24984      FALSE
1-3      6.5     10.24984      FALSE
1-6      9.5     10.24984      FALSE
3-6      3.0     10.24984      FALSE

###Molds
> kruskal.test(data.45$Molds, data.45$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.45$Molds and data.45$Storage.period
Kruskal-Wallis chi-squared = 11.901, df = 4, p-value = 0.0181
```

```
> kruskalmc(data.45$Molds, data.45$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
        obs.dif critical.dif difference
-1-0  0.1666667     10.24984      FALSE
-1-1  7.0000000     10.24984      FALSE
-1-3  3.1666667     10.24984      FALSE
-1-6 10.0000000     10.24984      FALSE
0-1   7.1666667     10.24984      FALSE
0-3   3.3333333     10.24984      FALSE
0-6  10.1666667     10.24984      FALSE
1-3   3.8333333     10.24984      FALSE
1-6   3.0000000     10.24984      FALSE
3-6   6.8333333     10.24984      FALSE
```

Significant difference between microbial loads of oven dried at 40°C bee pollen at different storage periods:

```
###Aerobic mesophiles
> kruskal.test(data.40$Aerobic.mesophiles,
data.40$Storage.period)

      Kruskal-Wallis rank sum test

data:  data.40$Aerobic.mesophiles and data.40$Storage.period
Kruskal-Wallis chi-squared = 12.856, df = 4, p-value = 0.012

> kruskalmc(data.40$Aerobic.mesophiles, data.40$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
        obs.dif critical.dif difference
-1-0  0.3333333     10.24984      FALSE
-1-1  7.3333333     10.24984      FALSE
-1-3  4.3333333     10.24984      FALSE
-1-6 10.3333333     10.24984       TRUE
0-1   7.6666667     10.24984      FALSE
0-3   4.6666667     10.24984      FALSE
0-6  10.6666667     10.24984       TRUE
1-3   3.0000000     10.24984      FALSE
1-6   3.0000000     10.24984      FALSE
3-6   6.0000000     10.24984      FALSE

###LAB
> kruskal.test(data.40$LAB, data.40$Storage.period)

      Kruskal-Wallis rank sum test

data:  data.40$LAB and data.40$Storage.period
Kruskal-Wallis chi-squared = 13.524, df = 4, p-value = 0.008979

> kruskalmc(data.40$LAB, data.40$Storage.period)
Multiple comparison test after Kruskal-Wallis
```

```
p.value: 0.05
Comparisons
     obs.dif critical.dif difference
-1-0       3     10.24984      FALSE
-1-1       9     10.24984      FALSE
-1-3       6     10.24984      FALSE
-1-6      12     10.24984       TRUE
0-1        6     10.24984      FALSE
0-3        3     10.24984      FALSE
0-6        9     10.24984      FALSE
1-3        3     10.24984      FALSE
1-6        3     10.24984      FALSE
3-6        6     10.24984      FALSE

###Yeasts
> kruskal.test(data.40$Yeasts, data.40$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.40$Yeasts and data.40$Storage.period
Kruskal-Wallis chi-squared = 11.212, df = 4, p-value = 0.02428

> kruskalmc(data.40$Yeasts, data.40$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
       obs.dif critical.dif difference
-1-0 0.1666667     10.24984      FALSE
-1-1 7.6666667     10.24984      FALSE
-1-3 2.8333333     10.24984      FALSE
-1-6 9.3333333     10.24984      FALSE
0-1  7.5000000     10.24984      FALSE
0-3  2.6666667     10.24984      FALSE
0-6  9.1666667     10.24984      FALSE
1-3  4.8333333     10.24984      FALSE
1-6  1.6666667     10.24984      FALSE
3-6  6.5000000     10.24984      FALSE

###Molds
> kruskal.test(data.40$Molds, data.40$Storage.period)

     Kruskal-Wallis rank sum test

data:  data.40$Molds and data.40$Storage.period
Kruskal-Wallis chi-squared = 12.076, df = 4, p-value = 0.01679

> kruskalmc(data.40$Molds, data.40$Storage.period)
Multiple comparison test after Kruskal-Wallis
p.value: 0.05
Comparisons
        obs.dif critical.dif difference
-1-0  7.0000000     10.24984      FALSE
-1-1  0.3333333     10.24984      FALSE
-1-3  3.3333333     10.24984      FALSE
-1-6  5.0000000     10.24984      FALSE
0-1   7.3333333     10.24984      FALSE
```

```
0-3   3.6666667     10.24984      FALSE
0-6  12.0000000     10.24984       TRUE
1-3   3.6666667     10.24984      FALSE
1-6   4.6666667     10.24984      FALSE
3-6   8.3333333     10.24984      FALSE
```

Correlation between moisture content and microbial parameters:

```
Correlation (pearson)
Positive value: there is a positive correlation
Negative value: there is no correlation

#Moisture / Aerobic mesophiles
-0.15

#Moisture / Coliforms
-0.17

#Moisture / LAB
0.43

#Moisture / Yeasts
0.36

#Moisture / Moulds
0.18

#Moisture / SRC
0.01
```