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Does Dry or Fresh Bee Bread Contain Clinically Significant, and Antimicrobial Agents Resistant Microorganisms?

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ABSTRACT

Bee bread is fermented and naturally preserved pollen that is enriched with digestive enzymes and organic acids from both honey and the salivary gland secretions of honeybees. As yet, there is insufficient information concerning which bacteria and yeasts are involved in the fermentation. This study seeks to determine the contents of microorganisms in fresh and dry bee bread samples and to ascertain the antimicrobial resistance of these isolated microorganisms. Fresh and dry bee bread samples were obtained from 8 different colonies that were cultivated in suitable medium to reproduction the aerobic microorganisms, anaerobic microorganisms, and fungi. The isolated strains in bee bread samples were identified by conventional and MALDI-TOF MS methods. The minimal inhibitory concentrations (MIC) of the antimicrobial agents for strains were determined according to the Clinical and Laboratory Standards Institute (CLSI). The 34 strains were isolated from fresh bee bread samples. There were no microrganisms reproduced in the dried bee bread samples. The 34 isolated strains were; *Aspergillus* spp. (12), *Rhizopus oryzae* (6), *Mucor circinelloides* (1), *Bipolaris* (2), *Trichoderma* (3), *Paecilomyces variotii* (1), *Penicillium chrysogenum* (1), *Kodamaea ohmeri* (1), *Bacillus altitudinis/pumilus* (3), *Bacillus licheniformis* (1), *B. megaterium* (1), *Micrococcus luteus* (1) and *Serratia marcescens* (1). The MIC values of itraconazole (IT), voriconazole, anidulafungin (AND), and caspofungin (CS) for *Mucor* and *Rhizopus* strains were higher (≥32 µg/mL), with the exception of amphamphotericin B posaconazole. All antifungal agents had lower MIC values compared to the *Aspergillus, Bipolaris, P. variotii*, and *K. ohmeri* strains. The *trichoderma* strains had low MIC values (≤0.50 µg/mL), with the exception of IT. The *P. chrysogenum* strains were found to have low MIC value (≤0.25 µg/mL) compared to posaconazole, AND, and CS. In this study, there were no microorganisms reproduce in the dried bee bread samples stored under suitable conditions. In addition, it was concluded that yeast, mold, and bacteria isolated in fresh bee bread samples may be resistant to antibiotics and antifungal drugs.

Keywords: Perga, Bacteria, Yeast, Molds, Antimicrobial susceptibility

Introduction

Honeybees use bee bread rather than honey and pollen for their nutrition. Because both nectar and pollen undergo some biochemical changes before being consumed by honey bees. While the pollen brought to the hive by the bees is filled into the honeycomb cells, honey, organic acids in the salivary gland secretions of the bees and digestive enzymes are added to the pollen (Deveza et al. 2015). Then, lactic acid fermentation caused by *Lactobacillus* bacteria takes place under anaerobic conditions. An important reason for fermentation is the dissolution of the outer layer (exine) of the pollen and the easy absorption of the nutrients in the pollen interior. Thus, the fermentation process serves not only to preserve the pollen content but also to form new compounds. During fermentation, bee pollen proteins are broken down into peptides and amino acids. DeGrandi - Hoffman (2013) reported that the protein concentration of pollen is higher than that of bee bread, while the amino acid concentration is lower. In another study, the lactic acid concentration in bee bread was found to be 6 times higher than in pollen (Nagai et al. 2005). It is also reported that bee bread contains vitamin K, which is not found in fresh pollen and is richer in B vitamins (Gilliam 1979a,b).

Bee bread has higher nutritional value, better digestibility, and richer chemical composition than bee-collected pollen (Habryka et al. 2016). Bee bread contains carbohydrates (24-34%), proteins (14-37%), lipids (6-13%), and other nutrients such as minerals and vitamins (Stanciu et al. 2009; Tomas et al. 2017; Kieliszek et al. 2018; Belina-Aldemita et al. 2019). In addition, it provides the essential amino acids humans cannot synthesize (Bonvehi & Escola 1997; Human & Nicolson 2006). However, the chemical composition of bee bread varies depending on the botanical origin, geographical location, climatic condition, soil type, beekeepers' activities, or storage treatments in commercial production (Pascoal et al. 2014; Ares et al. 2018). Since it contains easily digestible sugars, oil, mineral components, and higher free amino acids compared to bee pollen, it has a higher bioavailability and is easily digestible (Nagai et al. 2005). The antibacterial and antioxidant activity, immune system benefits, intestinal regulator, anti-fatigue, lipid regulator in blood and tissue, and antiaging effects of bee bread has been discussed in various studies (Villanueva et al. 2002; Nogueira et al. 2012; Kaplan et al. 2016; Urcan et al. 2018; Bakour et al. 2019).

The fact that fresh bee-collected pollen contains 21-30% water causes the development of microorganisms and the deterioration of the pollen. For this reason, it is recommended to dry it at the appropriate temperature (40 °C) and store it in a deep freezer to preserve its biological activity (Barene et al. 2015). Bee bread is more acidic than pollen and is more durable because it is a fermented product. Therefore, bee bread and pollen products should be dried, and water activity should be reduced to prevent microbial growth. In the studies carried out to date, *Candida parapsilosis, Cryptococcus neoformans, Pichia dispore, Saccharomyces heterogenicus, Torulopsis etchellsii, Torulopsis magnoliae, Torulopsis stellata, Zygosaccharomyces bailii, Bacillus subtilis, B. pumilis*, *B. licheniformis,* Penicillium spp. *Assporiformis cladosporioides,* and *Scopulariopsis brevicaulis* are among the microorganisms isolated from bee bread (Egorova 1971; Gilliam et al. 1989; Sinpoo et al. 2017). Although the fresh form of Bee bread, which is a fermented product, is the best way of consumption. It is necessary to find out the microbial content of both fresh and stored forms of bee bread.

This study seeks to determine the contents of microorganisms in fresh and dry bee bread samples, and to identify the antimicrobial resistance of these isolated microorganisms.

2. Material and Methods

2. 1. Bee bread samples

Bee bread samples were obtained from bee colonies in Erciyes University Agricultural Research Center and Erciyes Technopark (Nutral Therapy, Co), Kayseri. Fresh bee bread samples collected from 8 different colonies were brought to the laboratory under hygienic conditions. Half of each sample (one colony) was divided into two. The first half (fresh bee bread) was analyzed as soon as collected, and the second half (stored bee bread) was kept in a deep freezer for three months after being dried at 40 °C (Figure 1). Afterwards all samples were analyzed.

Figure 1- Bee bread samples

2. 2. Microbiological analysis

A microbiological analysis of each bee bread (fresh and stored) was performed according to the method described by Gilliam et al. (1989). Each sample was divided into four sub-samples of approximately 1 g and studied in 4 replicates. Each of these four replicates was homogenized in 2.5 mL of sterile 0.85% NaCl on a glass shredder, taking care of sterilization at each stage of the study. The homogenates (100 µl) were incubated in Sabouraud's dextrose agar (SD, Oxoid, UK) medium with and without antibiotics (cycloheximide and chloramphenicol) in incubators adjusted to 37 °C and 25 °C to determine the fungi contents of samples. When yeast and mold colonies appeared, their purity was checked.

The identification of yeasts were performed according to phenotypical methods with macroscopic morphology on SDA, microscopic morphology on corn meal agar, germ tube test, growth ability at 37 ºC, urea hydrolysis, and a carbohydrate assimilation test using API 20C AUX (BioMérieux, France) kits.

Mold identification was performed according to phenotypical methods with macroscopic morphology on SDA, microscopic morphology on corn flour agar and potato dextrose agar, growth ability at 37 ºC, and sensitivity to cycloheximide.

Molecular identification of all isolates at the species and genus level was performed with MALDI-TOF MS (VITEK® MS, BioMérieux, Marcy l'Etoile, Made by France) (Mutlu Sariguzel et al. 2016). The strains were tested by depositing one colony on a steel MALDI target slide by loop and a drop of formic acid was placed on the slide. The spot was dried and then overlaid with 1 µl MALDI matrix solution (VITEK MS-CHCA) and air-dried. For the mold colony, the colony was mixed with 70% alcohol. After centrifugation at 10000 rpm for 2 minutes, the upper liquid was poured out, 40 microliters of 70% formic acid and 40 microliters of 99% acetonitrile was added to the bottom sediment and 1 microliter of the top liquid was added after centrifugation at 10000 rpm for 2 minutes. The top liquid was dropped into the slide well and after a steel MALDI target slide dried, the Matrix was dropped. The prepared slide was inserted into the VITEK®MS system. Identification of yeast, mold and bacteria was analysed using the VITEK® MS database. The peaks from these spectra were compared to the characteristic pattern for a particular species, genus or family of microorganisms and this allowed for organism identification. The quality control strains were *C. albicans* ATCC 90028. *E. coli* ATCC 8739 was used to calibrate the instrument for each run (Mutlu Sariguzel et al. 2016).

To investigate aerobic and anaerobic bacteria microorganisms, 100 µl of homogenate was inoculated on 5% sheep blood agar (Oxoid, UK), chocolate agar, MacConkey agar (Oxoid, UK), Schaedler +5% sheep blood agar (BioMérieux, Marcy, France). Schaedler Neo Vanco +5% sheep blood agar (BioMérieux, Marcy, France) followed by plates were incubated under aerobic and anaerobic conditions in 37 °C and 25 °C incubators. The bacterial isolates were identified using conventional microbiological methods (Gram stain, oxidase, catalase, aerotolerance testing), automated systems with VITEK2 cards (BioMérieux, France), and MALDI-TOF MS (VITEK® MS, BioMérieux, Marcy l'Etoile, France).

2. 3. Antimicrobial susceptibility tests of strains

2. 3. 1. Antifungal susceptibility tests of strains

The gradient diffusion test (E-test strips) and broth microdilution method were used for antifungal susceptibility testing. The MIC values of itraconazole (IT), voriconazole (VO), amphotericin B (AP), fluconazole (FLU) and ketoconazole (KTZ) (Sigma Chemical Co, St. Louis, USA) were determined using the the broth microdilution method. The MIC values of posaconazole (POS), anidulafungin (AND), and caspofungin (CS) were determined via the gradient diffusion test (Etest^{R}, bioMerieux, Marcy Etoile, France). FLU and KTZ susceptibility were investigated only in yeast strains. The *in vitro* efficacy of antifungal drugs has been studied according to the recommendation of the CLSI M27-S4 for yeast and CLSI M38-A2 for molds (Clinical and Laboratory Standards Institute 2008; 2017).

1. 3. 1. 1. Broth microdilution method

RPMI 1640 medium with 34.53 g MOPS (3-N-morpholinepropanesulfonic acid) (PanReac & AppliChem, USA) containing L-glutamine, free of sodium bicarbonate and phenol red (Sigma-Aldrich, UK) was used for the broth microdilution method. Two-fold dilutions of drugs were made and dispensed into 96-well flat-bottom plates at concentrations ranging from 64-0.125 µg/mL for FLU, 16-0.03 µg/mL for AP, IT, VO, and KTZ. These plates were incubated at 35 °C for 24-48 hours. The fungal inoculum was prepared from a 24-hour culture of SDA (Oxoid, UK) incubated at 35 °C, and mold suspensions were prepared from well-spored cultures grown on potato dextrose agar and adjusted spectrophotometrically to a turbidity.

The MIC of the antifungal agents used were determined according to the CLSI recommendations.

2. 3. 1. 2. Gradient diffusion test (E-test strips)

The suspension of fungal strain (0.5 MacFarland) was spread on Mueller-Hinton agar (BD Diagnostics, France), and POS, AND, and CS MIC gradient E-test strips were applied. All media were then incubated at 37 °C. The diameters of the inhibition zones were measured after 24-48 hours of incubation.

2. 3. 2. Antibacterial susceptibility tests of strains

2. 3. 2. 1. Disk diffusion method

In vitro, antibiotic susceptibility patterns of bacterial isolates were determined by the disk diffusion method, and the results were interpreted according to CLSI (Clinical and Laboratory Standards Institute, 2015). Sterile swabs were dipped in bacterial suspensions (0.5 MacFarland) and plated on Müller Hinton agar. Antibiotic discs were placed on the plate, and all media were incubated at 37 °C. The diameters of the inhibition zones were measured after 24 hours of incubation. The antibiotics were investigated: penicillin (1 µg/mL), ampicillin (2 µg/mL), amoxicillin-clavulanic acid (20/10 µg/mL), levofloxacin (5 µg/mL), erythromycin (15 µg/mL), clindamycin (2 µg/mL), co-trimoxazole, cefoxitin (30 µg/mL), ceftriaxone (30 µg/mL), cefuroxime (15 µg/mL), gentamicin (10 µg/mL), vancomycin (30 µg/mL), meropenem (1 µg/mL). Meropenem has been studied only for gram-negative strains. All discs are Oxoid, a UK brand.

2. 4. Quality control strains

C. albicans ATCC 90028. and *E. coli* ATCC 25922 were used as quality control strains.

3. Results

All 34 microorganisms, 7 bacteria, 1 yeast, and 26 molds, were isolated from fresh bee bread samples (n=8). Anaerobic microorganisms were not detected in the samples. Bacteria, yeast, and mold did not grow in stored bee bread samples (n=8). Bacteria, yeast, and mold species isolated from fresh bee bread are shown in Table 1. Bee bread samples are shown in Figure 1. The number of strains isolated from bee bread samples collected from different colonies is shown in Figure 2a. *A. niger* colonies is shown in Figure 2b.

Molds isolated from fresh bee bread were *Aspergillus niger* (5), *Aspergillus fumigatus* (1), *Aspergillus nidulans* (2), *Aspergillus terreus* (2), *Aspergillus flavus* (2), *Rhizopus oryzae* (6), *Mucor circinelloides* (1), Bipolaris spp. (2), Trichoderma spp. (3), *Paecilomyces variotii* (1), *Penicillium chrysogenum* (1); yeast: *Kodamaea ohmeri* (1); bacteria: *Bacillus altitudinis/pumilus* (3), *Bacillus licheniformis* (1), *Bacillus megaterium* (1), *Micrococcus luteus* (1) and a yeast; *Serratia marcescens* (1). Figure 3 shows the MALDI-TOF MS protein spectra of *A. fumigatus* strain.

The yeast fungus *Kodamaea ohmeri* was isolated and obtained from only one of the eight colonies. *Rhizopus oryzae,* which is the most common mold fungus, was isolated from six different colonies. and *Aspergillus niger* was isolated from 5 different colonies. The majority of microorganisms were isolated from fresh bee bread of colony 1, including seven molds (3 *Aspergillus* spp., *Rhizopus*, *Mucor, Bipolaris* spp., *Trichoderma* spp.), a yeast (*Kodamaea ohmeri*), and one bacteria (*Serratia marcescens*). In addition, three *Aspergillus* spp., one Rhizopus, and one *Bacillus altitudinis/pumilus* strains were isolated in colony 5. In colonies 2 and 3, four different microorganisms as mold and bacteria were isolated. In the other colonies, three different microorganisms as mold and bacteria were isolated (Table 1).

Bacillus altitudinis/pumilus strains were isolated from colonies 2, 5, and 7. *Bacillus licheniformis* was isolated from colony 2, and *Bacillus megaterium* from colony 4. *Micrococcus luteus* strain was only isolated from colony 3.

All *Aspergillus* and *Bipolaris* strains had low MIC values for all tested antifungal drugs. *Trichoderma* strains isolated from colonies 1 and 2 had low MIC values, with the exception of ITR. The *Paecilomyces variotii* strain isolated from colony 3 had low MIC values for other antifungal drugs, except CS and VO (8 µg/mL).

The MIC values of antifungal drugs compared to the *Mucor* and *Rhizopus* strains were determined to be high, with the exception of AP-POS (MIC ranges 2-0.125 µg/mL). For the *Penicillium chrysogenum* strain isolated from colony 2, the MIC values of POS, AND, and CS were found to be low (MIC range 0.008-0.25 µg/mL)

Kodamaea ohmeri was sensitive to all the antifungal drugs studied, including KTZ and FLU. The FLU and KTZ MIC values for the *Kodamaea ohmer* strain were 8 µg/mL and 0.064 µg/mL, respectively. Table 2 shows the Minimal Inhibitor Concentrations (µg/mL) for six antifungal agents of fungal strains isolated from fresh bee bread samples.

Table 1- Bacteria, yeast and mold species isolated from fresh bee bread samples collected from eight different colonies

Microorganisms	*Colonies*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Molds								
Aspergillus fumigatus	+							
Aspergillus niger	+		+		+	+		+
Aspergillus nidulans	+						+	
Aspergillus terreus				+	+			
Aspergillus flavus					+			+
Rhizopus oryzae	+		+		+	+	+	+
Mucor circinelloides	+							
Bipolaris spp.	+			+				
Trichoderma spp	+	+				+		
Paecilomyces variotii			+					
Penicillium chrysogenum		+						
Yeast								
Kodamaea ohmeri	+							
Bacteria								
Bacillus altitudinis/pumilus		+			+		+	
Bacillus licheniformis		+						
Bacillus megaterium				+				
Micrococcus luteus			+					
Serratia marcescens	+							

+: There was growth

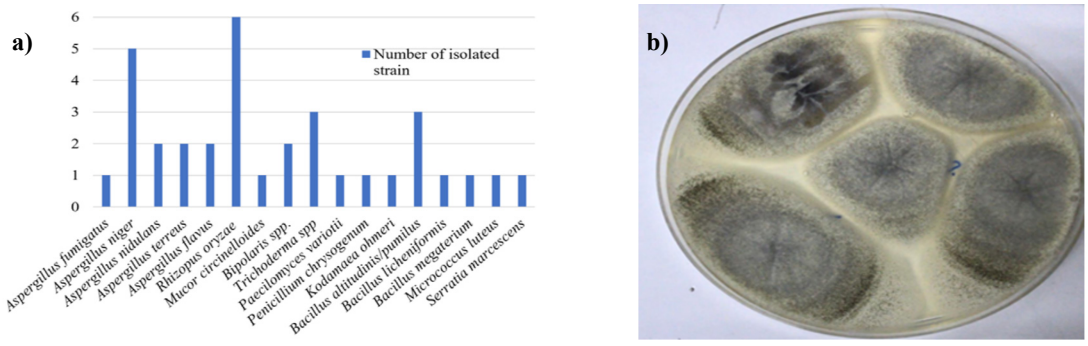

Figure 2- a)- The number of isolated strains from bee bread samples collected from different colonies, b) *A. niger* colonies

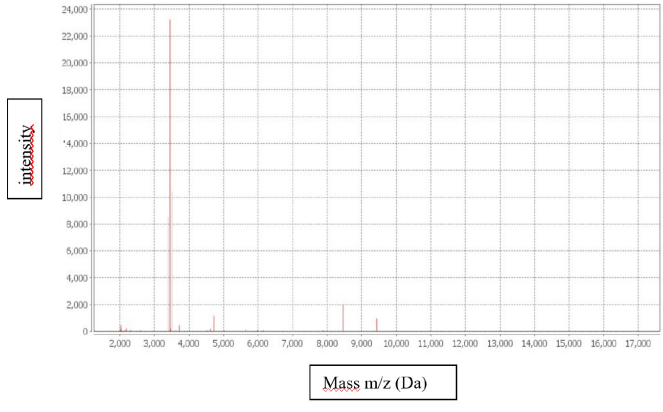

Figure 3- MALDI-TOF MS protein spectra of *A. fumigatus* strain

The epidemiological value (ECV) for six antifungal drugs (IT, VO, POS, AND, AP and CS) of the *Aspergillus* strains were found to be below the values determined according to CLSI. However, the six antifungal drugs' MIC values of the *Mucor* and *Rhizopus* strains were found to be higher than the *Aspergillus* strains. The *Mucor circinelloides* strain isolated from colony 1 was found to be 0.125 µg/mL for AP, but high MIC values were determined for other antifungal drugs. The *Rhizopus oryzae* strains were found to have high MIC values for other antifungals with the exception of AP and POS. The MICs (µg/mL) for IT, VO, POS, AND, AP, and CS of fungal strains isolated from bee bread samples are shown in Table 2.

Serratia marcescens was observed to be sensitive to ceftriaxone, meropenem, gentamicin and cotrimoxazole. The *Bacillus* spp. and *Micrococcus luteus* strains do not have susceptibility limit values determined in CLSI, only the zone diameters of antibiotics are given (Table 3).

4. Discussion

There is a heterogeneous microorganism community consisting of bacteria, yeast and molds in the ripening stage of bee bread (Haydak 1958; Di Cagno et al. 2019; Disayathanoowat et al. 2012). During intense incubation activities, bee bread is consumed in a few days, while the surplus can be stored in the honeycomb cells for several months (vanEngelsdorp et al. 2009; Podriznik & Bozic 2015). However, consumption of 21-day-old bee bread compared to 14-day-old bee bread has been reported to cause harm to the colony health and gut microbiome (Maes et al. 2016).

Table 2- Minimal inhibitor concentrations (µg/mL) for six antifungal agents of fungal strains isolated from fresh bee bread samples

Colonies	*Microorganisms*	*IT*	*VO*	*POS*	*AND*	*AP*	*CS*
		µg/mL					
1	*Aspergillus fumigatus*	0.50	0.125	0.064	0.002	0.25	0.016
	Aspergillus niger comp.	0.50	0.064	0.125	0.002	0.25	0.004
	Aspergillus nidulans	0.125	0.004	0.016	0.032	1	0.125
	Rhizopus oryzae comp.	>32	>32	2	>32	2	>32
	Mucor circinelloides	>32	>32	2	>32	0.125	>32
	Bipolaris spp.	0.50	1	0.002	0.002	0.125	0.016
	Trichoderma spp.	4	0.25	0.50	0.004	0.016	0.064
	Kodamaea ohmeri	0.25	0.064	0.032	0.25	0.008	0.50
2	*Trichoderma* spp	>32	0.25	0.50	0.008	0.25	0.032
	Penicillium chrysogenum	>32	>32	0.25	0.002	12	0.008
3	*Aspergillus niger com.*	0.5	0.50	0.25	0.002	0.25	0.006
	Rhizopus oryzae com.	>32	32	2	>32	1	>32
	Paecilomyces variotii	0.016	8	0.032	0.002	0.064	8
4	*Aspergillus terreus*	0.064	0.125	0.016	0.008	0.5	0.5
	Bipolaris spp.	0.008	0.064	0.002	0.008	0.008	0.002
5	*Aspergillus niger comp.*	0.50	0.125	0.064	0.002	0.25	0.004
	Aspergillus terreus	0.064	0.125	0.016	0.008	0.50	0.50
	Aspergillus flavus	0.125	0.125	0.064	0.002	1	0.064
	Rhizopus oryzae comp.	>32	>32	2	>32	2	>32
6	*Aspergillus niger comp.*	1	0.25	0.002	0.002	0.125	0.008
	Rhizopus oryzae comp.	>32	>32	0.50	>32	2	>32
	Trichoderma spp	32	0.25	0.50	0.002	0.125	0.064
7	*Aspergillus nidulans*	0.125	0.008	0.002	0.002	0.25	0.002
	Rhizopus oryzae comp.	>32	>32	0.50	>32	1.5	>32
8	*Aspergillus niger comp.*	1	0.025	0.008	0.002	0.25	0.002
	Aspergillus flavus	0.50	0.125	0.064	0.002	0.50	0.032
	Rhizopus oryzae comp.	>32	>32	0.50	>32	1	>32

In our study, molds were found to be the most frequently detected microorganism in bee bread samples. Molds are multicellular fungi that are common in nature on air, soil, water and organic materials and form mycelium. Molds have low water activity and optimum breeding temperatures are between 22-32 °C. Although the optimum pH requirements for molds vary between 5-6, some species of molds can grow in highly acidic environments such as pH 2.5. Molds have become industrially important due to both positive and negative changes in foods. The *Aspergillus* species are the most common molds and are used in the production of citric and gluconic acid from sucrose, as well as in the production of essential enzymes for the food industry such as amylase and pectinase. The *Aspergillus* species are used in the production of lipase and protease enzymes and in the ripening of cheeses in fermented food production. Although the pathogenic and toxic effects of molds in foods are negligible, some molds are important because they make mycotoxins and are pathogenic. The *Aspergillus flavus* and *A. parasiticus* species produce a deadly mycotoxin known as aflatoxin, which is a health risk. Mucor is a class of mold that is abundant in soil, plants, rotting fruits and vegetables. *Rhizopus oryzae* is used in the production of prebiotic and probiotic fermented soy products and lipase enzyme production (Matthews et al. 2017).

In one of the earliest studies on the microbiological content of bee bread, Burnside reported that some molds (*Cladosporium, Mucor, Penicillia* and *Aspergillus*) were found in the honeycombs and in the pollen stored in the hive (Burnside 1929). *Penicillium* was found to be the most common mold, *Aspergillus* less common and *Mucor* spp. was not isolated in their study. Yoder et al. (2013) reported that *Aspergillus, Penicillium, Rhizopus* and *Cladosporium* were the most commonly isolated strains in bee bread. Also, the researchers reported that it was found the presence of molds such as *Absidia, Alternaria, Aureobasidium, Bipolaris, Fusarium, Geotrichum, Mucor, Nigrospora, Paecilomyces, Scopulariopsis* and *Trichoderma*. In our study, while the *Aspergillus* species was the most frequently isolated in fresh bee bread, followed by *Rhizopus* spp. A study, it was reported that molds such as *Cladosporium, Aspergillus* and *Penicillium* were found in pollen but disappeared after six weeks of storage in the hive (Sinpoo et al. 2017).

In this study, only one yeast was isolated from the bee bread samples. Gilliam (1979 a) reported that yeast strains isolated from bee bread were less than pollen due to the different chemical and physical properties of bee products. Since the acidic character of bee bread creates a suitable environment for the development of yeast, yeast is crucial in transforming pollen into bee bread. The pH of the environment is important in the transformation of pollen into bee bread. Yeasts are the ones that provide this acidic environment. At the same time, yeasts play a role in the synthesis of B vitamins found in bee bread. It has been reported that bacteria such as lactic acid bacteria found in bee bread benefit from amino acids and vitamins produced by yeast strains in bee bread (Egorova 1971). It has been reported that different genus and types of yeasts were isolated in different studies investigating microorganisms in bee bread (Gilliam et al. 1977; Čadež et al. 2015). To our knowledge, *Kodamaea ohmeri* was first isolated from bee bread in our study, and this strain was found to be susceptible (a low MIC value) to all antifungals, including FLU (8 µg/mL) and KTZ (0.064 µg/mL). In a study done in 2020, a yeast isolation protocol was developed in pollen samples stored with bees from two apiaries in Belgium. Yeast isolates

Table 3- Zone diameters for various antibiotics of *Bacillus* spp. and *Micrococcus luteus* strains

	≤10 mm	***11-24 mm***	***≥25 mm***
Bacillus altitudinis/pumilus	Clindamycin	Vancomycin Cefuroksim Ceftriaxone	Penicillin Erythromycin, Co-Trimoxazole, Levofloxacin, Ampicillin, Cefoxitin, Amoxicillin, Gentamicin
Bacillus licheniformis	Penicillin Clindamycin	Vancomycin Cefuroksim Ceftriaxone Ampicillin	Erythromycin, Co-trimoxazole, Levofloxacin, Cefoxitin, Amoxicillin, Gentamicin,
Bacillus megateriumw	Clindamycin	Vancomycin Penicillin Ampicillin	Erythromycin, Co-trimoxazole, Cefoxitin, Amoxicillin, Gentamicin, Ceftriaxone,
Micrococcus luteus	-	Vancomycin	Erythromycin, Co-trimoxazole, Cefoxitin, Amoxicillin, Gentamicin, Ceftriaxone, Ampicillin, Ceftriaxone, Clindamycin

were grouped according to their macro and micromorphology and representative isolates were identified using DNA sequences. Most of the 252 isolates identified were found to belong to the genera Starmerella, Metschnikowia, and Zygosaccharomyces. According to the results of this study, Detry et al. (2020) reported that high yeast abundance in fresh bee bread decreased rapidly with storage time, Candida species were dominant in fresh bee bread, and Zygosaccharomyces members were mostly isolated from aged bee bread.

While studies on the microbiological analysis of bee bread are limited, it is clear that there is an relationship between bacteria, yeast, and molds. This study detected only 7 bacteria (5 Bacillus species) in the bee bread samples Vasquez and Olofsson reported that the lack of pathogenic microorganisms in bee bread may be due to the accumulation of various metabolites during fermentation by lactic acid bacteria. These metabolites reduce the number of microorganisms by lowering the pH of the environment (Vasquez & Olofsson 2009). The group of microorganisms remaining in the finished product are Gram-positive aerobic bacilli and some molds that can survive in adverse environmental conditions (Audisio et al. 2005). Some lactic acid bacteria produce hydrogen peroxide at a concentration that inhibits the growth of many pathogenic microorganisms (Bang et al. 2003). DeGrandi-Hoffman et al. (2013) reported that bee bread contains bactericidal compounds, as well as carbohydrates and lactic acid, that are effective in reducing the growth of microorganisms, which include mold, and spoilage bacteria. *Bifidobacterium* and *Lactobacillus* inhibitor the growth of *Staphylococcus aureus,* Gram-negative bacteria like *Listeria monocytogenes, Escherichia coli,* and *Camplylobacter jejuni*, rods like *Salmonella, Shigella, Vibrio,* and *Klebisella* species and yeast like *Candida albicans.* Lowering the pH value during fermentation is attributed to the activity of lactic acid bacteria that are introduced into the pollen from the gastrointestinal tract of the bees. Moreover, it has been reported that lactic acid bacteria isolated from the honeybee digestive tract inhibit the growth of the pathogen *Paenibacillus larvae* (Vásquez et al. 2012). The reason why no pathogenic microorganisms were detected in bee bread is thought to be a result of the accumulation of various metabolites by the fermentation process carried out by lactic acid bacteria. These metabolites reduce the number of microorganisms by lowering the pH of the environment (Vásquez & Olofsson 2009). In addition, some lactic acid bacteria are reported to produce hydrogen peroxide at concentrations that inhibit the growth of some pathogenic species (Bang et al. 2003). After the fermentation of the pollen, *Bacillus* spp. spores and fungi have been the dominant microorganism group in the pollen (Gilliam 1979 a,b). Fungal growth causes degradation of the outer wall of the pollen, potentially changing the nutritional value of the pollen (Gilliam 1979a). In addition, bacteria and fungi isolated from pollen contribute to the stabilization and transformation of pollen by producing enzymes, vitamins, antibacterial substances, organic acids, and lipids (Anderson et al. 2014). Bee pollen and bee bread inhibit the growth of antibiotic-resistant microorganisms. This effect is higher in gram positive bacteria than in Gram-negative bacteria. In studies with ethanol extract of pollen, it has been shown that it is effective against many bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Paenibacillus larvae* (Carpes et al. 2007; Fatrcovaa-Sramkovaa et al. 2016). Lactic acid bacteria accepted in GRAS status gain energy with the transformation of saccharides in the environment with typical fermentation metabolism, and was produced bacteriocins and metabolic products such as organic acids, diacetyl, acetoin, acetaldehyde (Forsgren et al. 2010; Wasko et al. 2012).

Bobis et al. (2010) reported that raw pollen contains 5.10^5 cfu/g of aerobic mesophilic microorganisms and about 1.10^2 cfu/g yeast and mold. A 2015 study by De-Melo et al. (2015) demonstrated mesophilic bacteria's presence at a level of <10 to 1.1 10^4 cfu/g in dried Brazilian pollen. Nogueira et al. (2012) showed that psychrophilic bacteria and bacilli, respectively, ranged from <10 to 1.1 103 cfu/g, <10 to 2.8 103 cfu/g, while the number of yeasts and molds in pollen ranged from <10 to 7.6 103 cfu/g. *Zygosaccharomyces rouxii* yeast was found in commercial samples of dried bee pollen from Portugal and Spain (Nogueira et al. 2012). Deveza et al. (2015) showed the presence of *Aspergillus* and *Cladosporium*, among the most common molds, in Brazilian bee pollen.

Pollen contamination by microorganisms is deppendent on the harvesting practices, cleaning, drying, and storage of the crop as well as its nutrient composition. As yet, there are no international microbiological limits on bee bread. In addition, according to International Honey Commission (IHC) and quality control standards, acceptable microorganism load in pollen can be <10 cfu/g for aerobic microorganism, <5 104 cfu/g for yeast and mold, and max 1 cfu/g for *Enterobacteriaceae*. Pollen should not contain *E. coli, Salmonella* spp. and *Staphylococcus aureus* (Campos et al. 2008; De-Melo et al. 2015).

In our study, mold was isolated in all bee bread samples. Detry et al. (2020) found mold and yeast in their study on bee pollen in 50% of 28 samples. Mold fungi such as *Aspergillus, Penicillium, Rhizopus,* and *Mucor* are inflammatory pathogens, especially in immunocompromised individuals, and can potentially cause severe diseases. *Aspergillus* spp. affects patients with asthma, cystic fibrosis, sinusitis, and acute invasive aspergillosis in some cancer patients and chemotherapy patients (Chen et al. 2015). *Rhizopus* and *Mucor* are found in soil and decayed plant and organic material. The routes of transmission to humans are respiratory, digestive, and cutaneous. Thrombus formed due to vascular invasion in immunocompromised individuals causes distal necrosis and infarcts. Since these strains are resistant to antifungals, they are difficult to treat, and because deep debridement is required in their treatment, patients experience tissue and organ losses such as the eyes and nose (Garcia-Hermoso et al. 2015).

It has been reported that there was a significant difference in antifungal susceptibility between the strains isolated from patients and the strains isolated from the Aspergillus medium (Sabino et al. 2016). However, in our study, the epidemiological threshold value (ECV) of *Aspergillus* strains for six antifungal drugs (IT, VO, POS, AND, AP and CS) was found below the values determined by CLSI. The MIC values of the *Mucor* and *Rhizopus* strains for six antifungal drugs were found to be higher than those of the *Aspergillus* strains. While the AP value for the *Mucor circinelloides* strain isolated from colony 1 was 0.125 µg/mL, high MIC values were detected for the other antifungal agents. We found that the *Rhizopus oryzae* complex strains have high MIC values for other antifungals compared to AP and POS. The results of our study are similar to the antifungal susceptibility of the *Mucor* and *Rhizopus* strains, which were isolated from clinical samples of immunocompromised patients and considered infectious agents (Sağıroğlu et al. 2019). The *Bipolaris* strains were found to have low MIC values for all antifungal drugs. The *Trichoderma* spp. strains were found to have low MIC values for other antifungal drugs except for IT. 8 µg/mL, VO and CS MIC values were found for *Paecilomyces variotii*, but the MIC values of other antifungal drugs were below 0.064 µg/mL. IT ve VO MIC values for *Penicillium chrysogenum* was >32 µg/mL, AP MIC value was 12 µg/mL, but the MIC values of the POS, AND, and CS drugs were below 0.25 µg/mL (Table 2).

This study isolated five *Bacillus* strains from four fresh bee bread samples. The colony-isolated *Bacillus* strains were identified as *Bacillus alttitudinis/pumilus, Bacillus licheniformis,* and *Bacillus megaterium*. The sensitivity limit values for CLSI were not determined according to CLSI criteria, only zone diameters were given and no sensitivity-resistance distinction was made. The susceptibility of the *Bacillus alttitudinis/pumilus* strains isolated from three different colonies was the same. The Food and Drug Administration states that *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* are safe to use as probiotics (Salminen et al. 1998; Schallmey et al. 2004). Gilliam et al. (1979 b) reported that *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* were isolated from bee bread stored in honeycomb cells for periods of one, three, and six weeks. Bee bread is used more and more as a food supplement due to its beneficial effects on health and probiotic properties, and in the treatment of infectious diseases due to its immune system strengthening properties.

In this study, *M. luteus* and *S. marcescens* were isolated from fresh bee bread samples, but they were not detected in stored bee bread. The sensitivity breakpoints for *M. luteus* were not determined according to CLSI criteria, only zone diameters are given (Table 3). *S. marcescens* was found susceptible to CRO, MER, CN, SXT, and resistant to AMP and CXM, according to CLSI criteria. Disayathanoowat et al. (2012) investigated bacterial and fungal communities in corbicular pollen and colony-preserved bee bread of two commercial honeybees (*Apis mellifera* and *Apis cerana*) in China. During pH reduction in bee bread stored in the hive, they observed that the bacterial population (*Enterobacteriaceae bacterium*) decreased with both traditional culture methods and next-generation sequencing. The fungal population, however, remained stable (especially *Cladosporium*) and filamentous fungi had the potential to inhibit the growth of both common/contaminant bacteria and pathogens by releasing organic acids. Our study results were consistent with Disayathanoowat's study; for fresh bee bread samples, the bacterial population in our study is very low compared to the fungi.

As a result, bee bread can be easily contaminated with mold spores, considered opportunistic pathogens, by environmental factors (climate, temperature, oxygen level) in which the hive is located. For this reason, it is necessary to prevent contamination during the production and storage of bee bread samples and to eliminate the conditions that create an environment for the growth of fungi and other microorganisms. Therefore, it is recommended to store bee bread in a deep freezer or by drying.

5. Conclusions

Most molds, a few yeasts, and bacteria were isolated from fresh bee bread samples in this study. The clinically important *Aspergillus* species were the most common mold species detected in all but one of the samples (colony 2). To increase the quality and safety of all bee products, not just bee bread, the optimization of hygienic procedures throughout the production chain should be ensured. Hygiene is essential to reduce the risk of food spoilage and accompanying disease and poisoning.

It was determined that the yeast, mold, and bacteria isolated may be resistant to antibiotics and antifungal drugs. Another striking point in our study, the bee bread sample stored under appropriate conditions such as keeping in a freezer for maturation and reducing the microorganism load was the absence of yeast, mold, and other microorganisms.

The development of new technologies in food production, processing, and storage conditions are critical elements in the global market. Systems for the control of production practices are provided by continuous monitoring for consumer protection against microbiological risks that may occur at all stages of bee bread production.

Data availability: Data are available on request due to privacy or other restrictions.

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