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Isolation of Lignin-Degrading Bacteria from Different Sources and Testing of Their Ligninolytic Activities

Farklı Kaynaklardan Lignin Parçalayıcı Bakterilerin İzolasyonu ve Lignolitik Aktivitelerinin Test Edilmesi

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Abstract

Nine lignin-degrading bacteria were isolated from petroleum-contaminated soil and animal manure samples and characterized by 16S rRNA sequence analysis. Three isolates were identified as *Enterobacter cancerogenus*, two as *Enterobacter ludwigii*, one as *Citrobacter sedlakii*, one as *Citrobacter farmeri*, one as *Klebsiella pneumoniae*, and one as *Citrobacter murliniae*. These bacteria used ligno sulphate as the sole carbon source but did not utilize kraft lignin (KL) as the sole source of carbon and energy. For this reason, basic nutrients, such as 1.0% glucose (w/v) and 0.5% peptone (w/v), were used as additional carbon and nitrogen sources to stimulate bacterial growth for KL decolorization. Under these conditions, the isolates *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter ludwigii* L4, *Enterobacter cancerogenus* PT21, *Citrobacter farmari* PT22, *Citrobacter sedlakii* PT41, *Klebsiella pneumoniae* G1, and *Citrobacter murliniae* C1 degraded kraft lignin by 37 %, 14

Özet

Petrol bulaşmış toprak ve gübre örneklerinden dokuz adet lignin parçalayan bakteri izole edilmiş ve 16S rRNA analizleri yapılmıştır. Bu izolatlardan üç tanesi *Enterobacter cancerogenus*, iki tanesi *Enterobacter ludwigii*, bir tanesi *Citrobacter sedlakii*, bir tanesi *Citrobacter farmeri*, bir tanesi *Klebsiella pneumoniae*, biri *Citrobacter muryliniae* olarak tanımlanmıştır. İzolatlar, tek karbon kaynağı olarak ligno sülfat kullanırken, tek karbon ve enerji kaynağı olarak kraft lignini (KL) kullanmamışlardır. Bu nedenle kraft lignin parçalanmasında bakteri çoğalmasını teşvik etmek için ilave karbon ve azot kaynakları olarak % 1.0 glukoz (a / h) ve % 0.5 pepton (a / a) gibi ek karbon ve azot kaynakları kullanılmıştır. Bu koşullar altında, *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter ludwigii* L4, *Enterobacter cancerogenus* PT21, *Citrobacter farmari* PT22, *Citrobacter sedlakii* PT41, *Klebsiella pneumoniae* G1 ve *Citrobacter murliniae* C1 izolatları ile kraft lignini sırasıyla

%, 20%, 43%, 48%, 51%, 28%, 60%, and 99%, respectively. Remazol Brilliant Blue R (RBBR) decolorization potentials of the isolates were determined. The isolates were decolorized RBBR by 20-90 % , respectively.

Keywords: Lignin, Bacteria, 16S rRNA, Decolorization

% 37,% 14,% 20,% 43,% 48,% 51,% 28, %60 ve% 99 oranında parçalamışlardır. İzolatların ayrıca Remazol Brilliant Blue R (RBBR) dekolorizasyon oranı analiz edilmiştir ve sırasıyla % 20-90 oranında RBRR dekolorizasyonu tespit edilmiştir.

Anahtar kelimeler: Lignin, Bakteri, 16S rRNA, Dekolorizasyon

Abbreviations: KL:Kraft Lignin, LiP: Lignin peroxidase, MnP: Manganese peroxidase

1. INTRODUCTION

The aromatic polymer lignin is well known for its resistance to microbial degradation because of its high molecular weight and the presence of various biologically stable carbon-to-carbon and ether linkages. The microorganisms that degrade plant lignin through an oxidative process are fungi (Tien & Kirk, 1983), actinomycetes (Hernandez et al., 2001), and to a lesser extent, bacteria (Trojanowski et al., 1977). Among them, white rot fungi have attracted widespread attention because of their powerful lignin-degrading enzymatic systems (Hatakka, 1994; Janusz et al., 2017). However, the use of fungi in industrial applications is not feasible due to the structural hindrance caused by fungal filaments; the requirement of particular culture conditions, such as humidity, aeration, temperature, and pH, that are not compatible with industrial processing environments; the requirement of a long lag period, which thus results in very slow lignin degradation; the need for additional food sources, such as glucose and nitrogen, to support the fungal growth; and the instability of fungi in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and high lignin concentrations (Bholay et al., 2012). In contrast, bacteria are worth studying for their ligninolytic potential due to their immense environmental adaptability and biochemical versatility (Chandra et al., 2007).

The ligninolytic system is an extracellular enzymatic complex that includes peroxidases, laccases, and oxidases responsible for the production of extracellular hydrogen peroxide (H_2O_2)(Ruiz-Duenas & Martinez, 2009). Subject to the species, strains and culture conditions those enzymes display differential characteristics (Kirk & Farrel, 1987). Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, cosmetic, and other industries (Maciel et al., 2010). The enzymes reported to be involved in bacterial lignin degradation are laccases, manganese peroxidase, lignin peroxidase, glutathione S-transferases, ring-cleaving dioxygenases (Masai et al., 2003; Allocati et al., 2009), monooxygenases, and phenol oxidases (Perestelo et al., 1989). Such enzymes are also involved in degradation of polycyclic aromatic hydrocarbons (PAHs), which have the same structural properties and resistance to microbial degradation as lignin (Allocati et al., 2009; Perestelo et al., 1989).

Dyes and dyestuff are also used in the textile, cosmetic, pharmaceutical, and leather industries but are of primary importance to textile manufacturing, the wastewater of which may contain a variety of these pollutants (McKay, 1979). The release of dyes has caused concern because of their environmental and health effects. Bioremediation is still seen as an attractive solution due to its reputation as a low-cost and environmentally friendly technology compared to chemical and physical treatment processes (Mc Mullan et al., 2001). As mentioned above, ligninolytic enzymes are used in dye decolorization; however, although bacteria are more efficient than fungi, the latter are widely used. For this reason, there is still a need for a continued search for more efficient ligninolytic bacterial strains for bioremediation and other applications (Raj et al., 2007). Therefore, in the present study, we isolated several bacterial strains which have a great lignin-degrading potential from fuel-contaminated soil and manure and we evaluated their dye decolorization and bioremediation capacity. The aim of this study is to determine the usability of isolated bacteria for various industries. Considering that alternative ways are being sought for mechanical and chemical methods used in waste dye decolorization and paper production, this study will contribute to the fulfillment of these needs.

2. MATERIALS AND METHODS

2.1. Isolation of Lignin-Degrading Bacteria

The contaminated soil and manure samples were collected with polyethelene bags from different areas in Arsin (Trabzon) were combined with lignin medium [(l-1): 5 g lignosulphonate, 9 g NaCl at pH 7.0] in a 250-ml Erlenmayer flask and then shaken at 37°C for 2 days to enrich the culture. After 2 days, the samples were inoculated into lignin agar plates that contained (l-1): 5 g lignin, 9 g NaCl, and 17 g agar at pH 7.0. This medium was used for screening the lignin-degrading bacteria. The best lignin-degrading colonies were selected according to their colony morphology and their growth potential on lignin agar. Cells of ligninolytic strains were Gram-stained using the method of Dussault (1955) and the Gram type was also determined using the KOH test (Powers, 1995). Cell morphology was examined by using phase-contrast microscopy (Nikon Eclipse E600; Olympus) on an exponentially growing liquid culture.

2.2. DNA Isolation and 16S rRNA Gene Sequence Analysis

The 16S rRNA genes were selectively amplified from purifed genomic DNA (according to the users manuel of Promega Wizard® Genomic DNA Purification Kit) with the use of oligonucleotide primers designed to anneal to conserved positions in the 3’ and 5’ regions of the bacterial 16S rRNA genes. The forward primer, UNI16S-L(5’-ATTCTAGAGTTTGATCATGGCTTCA), corresponded to positions 11 to 26 in Escherichia coli 16S rRNA, and the reverse primer, UNI16S-R (5’-ATGGTACCGTGTGACGGGCGGTGTTGTA), corresponded to the complement of positions

1411 to 1393 in Escherichia coli 16S rRNA (Somogyi, 1952). PCR reaction conditions were carried out according to Beffa et al. (1996), and the PCR product was cloned into the pGEM-T vector system. After the PCR amplification and the cloning of the 16S rRNA genes of our isolates, the 16S rRNA sequences were determined with the use of an Applied Biosystems 373A DNA sequencer with an ABI PRISM cycle sequencing kit (Macrogen, Holland). Sequences consisting of about 1400 nt of 16S rRNA genes were determined and compared with those in the EzTaxon database. The 16S rRNA gene sequences of the species most closely related to our isolates were retrieved from the database. The retrieved sequences were aligned with the use of the Clustal X program (Thompson et al., 1997) and manually edited. Phylogenetic trees were constructed by the neighbor-joining method with the use of the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007).

2.3. Enzyme Assays

For enzyme assays bacterial supernatant was used. The isolates were grown in lignin medium for seven days at 37°C and 120 rpm. Then the cultures were centrifuged at 10000 rpm for 10 minutes and the bacterial supernatant was used as enzyme solution.

Laccase activity was determined by the oxidation of 2,2'azinobis - (3,ethylbenzthiazoline-6-sulphonate) (ABTS). The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer (pH 5.0), and a suitable amount of enzyme. The oxidation of ABTS was followed by an absorbance increase at 420 nm (More et al., 2011).

Manganese peroxidase (MnP) activity was measured by monitoring the formation of Mn(III)-malonate complex at 270 nm. The reaction mixtures contained MnSO4 (0.2 mM), H202 (0.1 mM), and enzyme in 50 mM sodium malonate (pH 4.5) (Paice et al., 1993).

Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm. The reaction mixtures contained 50 mM Na tartrate buffer (pH 2.5 or 4.5), 0.1 mM H202, and 2 mM veratryl alcohol in a 1.0-ml reaction volume (Tien and Kirk, 1983).

2.4. The Ratio of Lignosulphonate Degradation

The percentage of lignosulphonate degradation of the isolates was tested in a 500-ml erlenmayer flask with 100 ml of lignin degradation medium (LDM) consisting of (l-1): 1 g KH_2PO_4, 4 g $NaHPO_4$, 0.2 g NaCl, 0.2 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2$, 2 g yeast extract, and 5 g lignin. The sterilized medium was inoculated with 0.5 ml of a 24-h culture of bacteria in nutrient broth. Controls consisting of uninoculated flasks were also prepared for comparison. The cultures were incubated for 1 week at 120 rpm and 37°C (El-Gammal et al., 1997).

After 1 week, the cultures were sterilized and passed through filter paper, the weight of which had been previously determined. After

filtration, the papers were dried in Pasteur's furnace to determine the residual lignin ratio. The lignin degradation ratio of different isolates was expressed according to the following equation:

$$\text{Degradation (\%)} = \frac{L_I - L_F}{L_I} \times 100$$

where L_I = initial amount of lignin, and L_F = the amount of lignin after 1 week of culture.

2.5. Kraft Lignin Decolorization

Biodegradation experiments were carried out in 250-ml Erlenmayer flasks containing 100 ml of sterile mineral salt medium (MSM, g l-1: 2.4 g Na_2HPO_4, 2 g K_2HPO_4, 0.1 g NH_4NO_3, 0.01 g $MgSO_4$, 0.01 g $CaCl_2$, 10 g D-glucose, 5 g peptone. The trace element solution (1 ml l–1, pH 7.6) contained 2 g l–1 kraft lignin. The sterilized medium was inoculated with 0.25 ml of a 24-h culture of bacteria in nutrient broth. The Control and inoculated erlenmayer flasks were incubated for six days in a rotary shaker at 120 rpm under aerobic conditions at 30°C (Raj et al., 2006). The biodegradation of kraft lignin was determined in terms of the loss of total kraft lignin from the treated sample. Uninoculated (control) and bacteria-inoculated samples (30 ml) were centrifuged at 8,000 g for 15 min to remove biomass. Supernatants were acidified to pH 1–2 with concentrated HCl. The precipitate was collected in tared centrifuge tubes by centrifugation at 8,000 g for 30 min, dried at 60 ± 5°C for 48 h, and then weighed. The kraft lignin loss (%) in the sample treated with bacteria was determined as dry weight (assuming kraft lignin present in identical volume of control as 100%).

2.6. Ligninolytic Activity Tests

Isolates were subjected to different plate assays for screening of several lignin-degrading enzymes (laccase and peroxidase (LiP and MnP). An extracellular peroxidase (LiP and MnP) test was done according to a modification of the method proposed by Rayner & Boddy (1988). The bacteria were grown on NA at 37°C for 2 days. The addition of equal parts of 0.4% (v/v) H_2O_2 and 1% pyrogallol in water gave a yellow-brown color to colonies with peroxidase activity.

Laccase activity was determined with the use of ABTS agar. This colorless agar medium turns green due to the oxidation of ABTS (2,2'azino-bis(3-ethylbenzthiazoline-6-sulphonate) to ABTS-azine in the presence of laccase. For preparation of ABTS agar LME basal medium (g-1 in distilled water: 1 g KH_2PO_4, 0.5 g $C_4H_{12}N_2O_6$, 0.5 g $MgS0_4$•$7H_20$, 0.01 g Yeast Extract, 0.001 g $CuS0_4.5H_2O$, 0.001 g $Fe_2(S0_4)_3$, 0.01 g $CaCl_2$•$2H_2O$, 0.001 g $MnS0_4.H_2O$) medium supplemented with 0.1 % w/v ABTS and 1.6 % w/v agar and autoclave. To every 100 ml of the growth medium prepared, 1 ml of a separately sterilized 20 % (w/v) aqueous glucose solution was aseptically added. The production of laccase was shown by the formation of a green color in the growth medium.

2.7. Utilization of Lignin Monomers

To monitor the growth on lignin monomers, a mineral medium (MM: g-1 in distilled water: 1.55

g KH_2PO_4, 0.85 g $NaH_2PO_4.2H_2O$, 2 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2.6H_2O$, 10 mg EDTA and 5 ml trace element solution) was supplemented with 5 mM lignin monomers (vanilic acid, vanillin, 4-hydroxybenzoic acid, syringic acid, phenol, veratryl alcohol, guaiacol) as the sole carbon and energy source. Growth was considered positive if observed after successive transfers to fresh medium (Bogdan et al., 2018).

2.8. Decolorization of Azo, Triphenylmethane and Heterocyclic Dyes

The decolorization of dyes was monitored at the maximum visible absorbance of each dye. Unless otherwise stated, the reaction mixture consisted of 80 µM of dye (see Table 1), 0.1 U of lignin peroxidase, and 0.4 mM of H_2O_2 in 50 mM sodium tartrate in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2, and absorbance was measured 15 min after the initiation (Ollikka, 1993).

Table 1. Conditions for dye decolorization by lignin preoxidase

Dye	Absorbance maximum
BPB	590
Methylene blue	662
Methyl orange	502
Toluidine blue	625
Malachite green	620

2.9. Dye Decolorization Determination

The dye decolorization by the isolated microorganisms was tested in 250 ml erlenmayer flasks with 25 ml of basal liquid medium (BLM) consisting of (l−1): 10 g glucose, 5 g peptone, 2 g yeast extract, and 70 ml of trace element solution (in g l-1: 1.5 g nitrilacetic acid at 99%, 3 g $MgSO_4•7H_2O$, 0.5 g $MnSO_4•H_2O$, 1 g NaCl, 0.1 g $FeSO_4•7H_2O$, 0.1 g $CoSO_4$, 0.1 g $CaCl_2•2H_2O$, 0.1 g $ZnSO_4•7H_2O$, 0.01 g $CuSO_4•5H_2O$, 0.01 g $AlK(SO_4)_2•12H_2O$, 0.01 g H_3BO_3, and 0.01 g $Na_2MoO_4•2H_2O$) supplemented with 200 mg l-1 of Remazol Brilliant Blue R (RBBR). The sterilized medium was inoculated with 0.25 ml of a 24-h culture of bacteria in nutrient broth. Controls consisting of uninoculated flasks were also prepared for comparison. Triplicate flasks were used for each isolate and control. The cultures were incubated for 7 days at 120 rpm and 37° C and then centrifuged at 13,000×g for 5 min at 4° C. The cell-free supernatant color was read with a spectrophotometer at the maximum absorbance spectra (λmax) of the dyes used, i.e., 595 nm for rbbr. the uninoculated dye-free medium was used as blank. the uninoculated dye-containing controls were used as reference to correct abiotic color disappearance (Chantarasiri and Boontanom, 2017). The decolorization efficiency of different isolates was expressed according to the following equation:

$$\text{decolorization}(\%) = \frac{A_{\lambda},\text{initial} - A_{\lambda},\text{final}}{A_{\lambda},\text{initial}} \times 100$$

where aλ, initial = initial absorbance; and aλ, final = absorbance after 7 days of culture.

3. RESULTS AND DISCUSSION

3.1. Ligninolytic Microorganisms

As a result of isolation and screening, nine lignin-degrading bacteria were selected on the basis of their growth degree on lignin agar. The medium contained lignin as the sole carbon source. The isolates designated as L1, L2, L3, L4, PT21, PT22, PT41, G1, and C1 used lignin as the carbon source and developed colonies within 24 h of incubation. The cells of the strains were gram-negative and rod-shaped. The isolate G1 had a bacterial capsule. On the agar plates, all isolates had white and mucoid colonies.

3.2. 16S rRNA Gene Sequence Analysis

A total of 1400 nucleotides of the 16S rRNA from 9 isolates were aligned and compared to sequences of related bacteria. A phylogenetic tree was constructed with the use of the neighbor-joining method (Figure 1). On the basis of the 16S rRNA gene sequence analysis, the isolates were identified as members of the Enterobacteriaceae family. Five of them belong to the genus of Enterobacter, three to the genus Citrobacter, and one to the genus Klebsiella. The accession numbers of the 16S rRNA gene sequences of the 9 isolates had been assigned by GenBank (Tables 2 and 3). According to the data obtained, the isolates L1, L2, and PT21 are different strains of Enterobacter cancerogenus; L3 and L4 are different strains of Enterobacter ludwigii; PT22 is a strain of Citrobacter farmeri C1 is a strain of Citrobacter murliniae; PT41 is a strain of Citrobacter sedlakii and G1 is a strain of Klebsiella pneumoniae.

Table 2. The percentage similarity of 16S rRNA of *Enterobacter* species and the isolates

	L1	L2	L3	L4	PT21
Enterobacter cancerogenus	99	98,8	96,6	98,9	98,7
Enterobacter asburiae	98,6	98,2	96,5	98,4	98
Enterobacter ludwigii	98,5	98	97,3	99,7	97,8
Enterobacter cowanii	97	98,1	95,7	97,7	98,2

Table 3. The percentage similarity of 16S rRNA of *Citrobacter* and *Klebsiella* species and the isolates

	PT22	PT41	C1	G1
Citrobacter sedlakii	97,2	99,2		
Citrobacter farmeri	99,2	98,1		
Citrobacter rodentium	97,8	98,4		
Citrobacter murliniae			97,4	
Klebsiella pneumoniae				99,8
Klebsiella variicola				99
Klebsiella granulomatis				98

3.3. Lignin Degradation

Figure 2 shows the rate of lignosulphonate degradation after one week of incubation. As shown in the figure, the lignin degradation of the isolates is considerably high. Lignins are very complex natural polymers with many random couplings, but their exact chemical structure is not known. The physical and chemical properties of lignin differ depending on the extraction technology. For example, whereas lignosulphonates are hydrophilic (dissolve in water), kraft lignins are hydrophobic (do not dissolve in water). However, the kraft lignin is very important in the paper and pulp industry. Thus, the kraft lignin degradation of the isolates was investigated in this study. The isolates do not

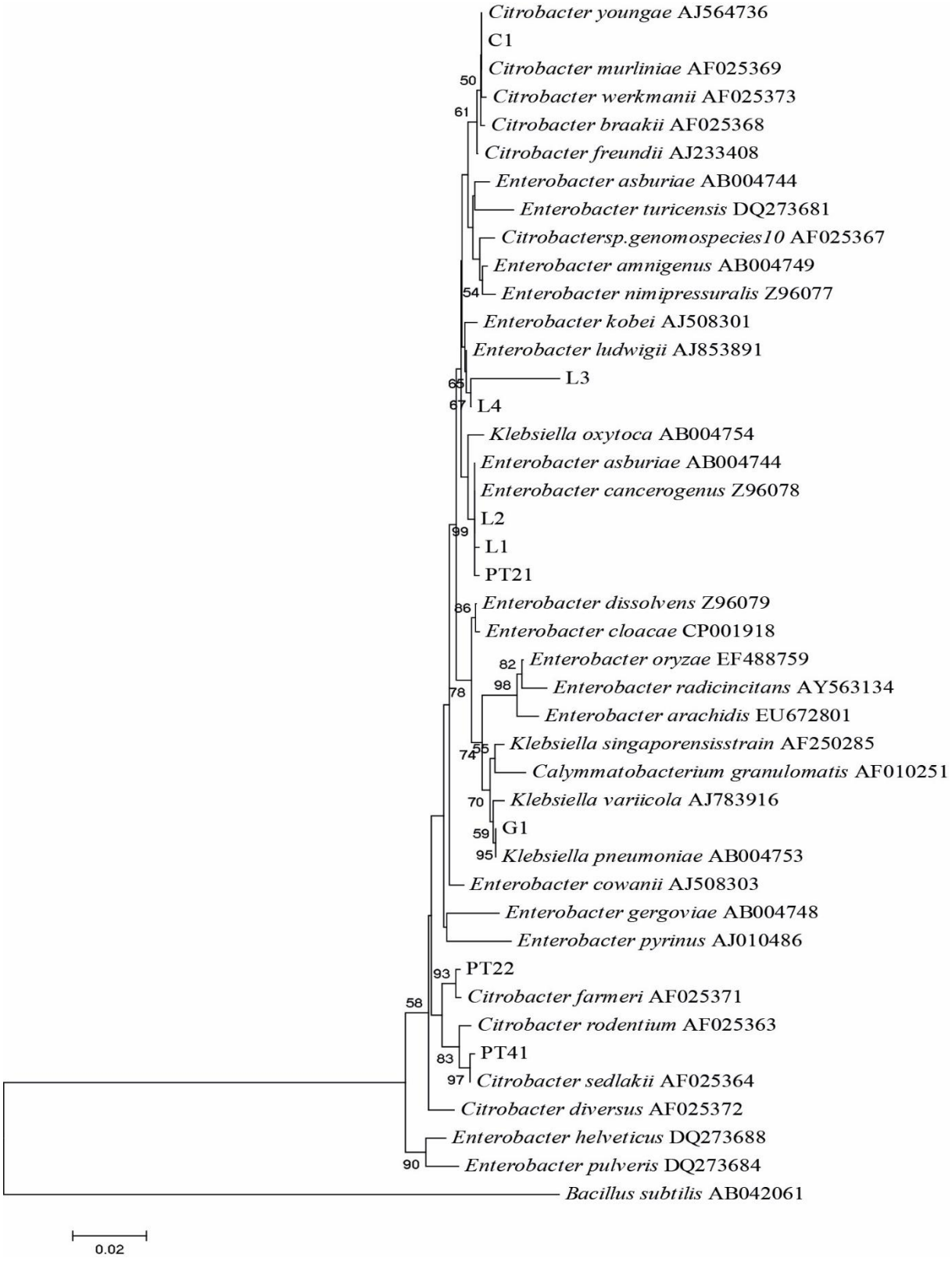

Figure 1. Dendrogram estimated phylogenetic relationship on the basis of 16S rRNA gene sequence data of the bacteria isolated from different sources and some reference strains, using the neighbor-joining method. The accession numbers are given in parentheses. The scala bar represents 2% divergence.

use kraft lignin as the sole carbon source. For this reason, basic nutrients, such as 1.0% (w/v) glucose and 0.5% (w/v) peptone, were used as additional carbon and nitrogen sources to stimulate bacterial growth for KL decolorization.

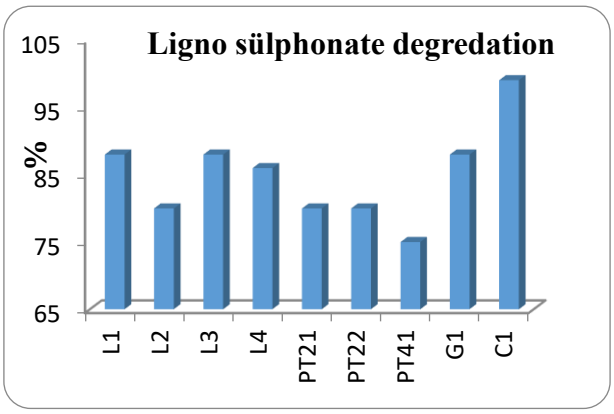

Figure 2. The percentage of lignin biodegradation

Figure 3 provides the data obtained from the kraft lignin biodegradation. The results are in accordance with those obtained by El-Hanafy et al. (2008) but are much better because the KL concentration is higher. El-Hanafy et al. (2008) found that the isolated strains BahHAE3 and BahHAE8 reached a maximum lignin degradation of 76.3% and 67.1%, respectively, on the sixth day. The lignin concentration in our study (2 g lignin/l) is much higher than the 0.7 g used by Chandra et al. (2007), who reported that *Paenibacillus sp.*, *Aneurinibacillus aneurinilyticus*, and *Bacillus sp.* achieved lignin degradation rates of 37, 33, and 30%, respectively, during the incubation period.

Deschamps et al. (1980) used an industrial kraft lignin (1 g lignin/l) as the sole carbon source in their study. They found that Aeromonas sp. degraded 98% of the kraft lignin after 5 days of incubation. The differences in the lignin degradation rates obtained between previous studies could be explained by the variations in bacterial strains, incubation conditions, and lignin structures used.

The results of the current study support the finding that many bacterial strains degrade and assimilate lignin (Chandra et al., 2007; Bal et al., 1989; Nishimura et al., 2006; Odier et al., 1981; Pometto et al., 1986). Furthermore, the capability of the soil-isolated bacteria to effectively degrade and assimilate lignin as the sole carbon source complied with the findings of Morii et al. (1995), who reported that three bacterial species isolated from compost soil, namely, Azotobacter, Bacillus megatarium, and Serretia marcescens, are capable of degrading lignin. Regarding the Enterobacter species isolated in the current study, many studies have pointed out the ability of the species to degrade lignin (Chandra et al., 2007; Morii et al., 1995).

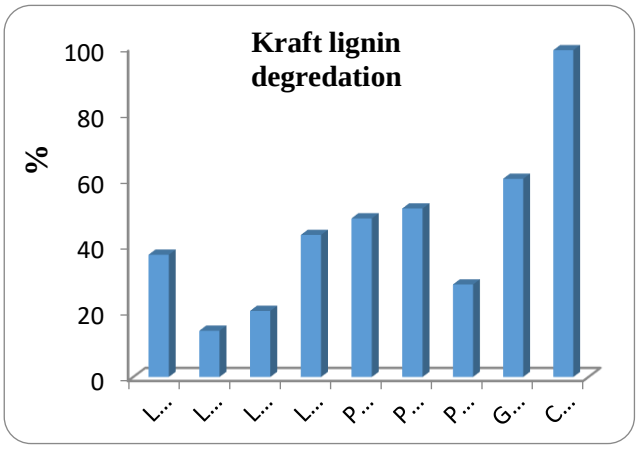

Figure 3. The percentage of kraft lignin biodegradation

3.4. Ligninolytic Enzymes of The Isolates

In this study, all the isolates showed extracellular peroxidase activity. To determine whether the peroxidase was MnP or LiP, enzyme assays were done. With the use of spectrophotometric methods, we determined that the isolates showed lignin peroxidase activity. Only the C1 isolate showed manganese peroxidase activity.

According to the ABTS agar laccase test, only the isolate PT41 showed laccase activity. As a result, the isolated bacterium Citrobacter sedlakii PT41 contained both laccase and lignin peroxidase. Previous studies have reported that the Bacillus species contains laccase (Canas et al., 2007; Reiss et al., 2011; Franc et al., 2001); in addition, Oliviere at al. (2009) found that Bacillus pumilus and Paenibacillus sp. contain manganese peroxidase. The Streptomyces species has also been found to contain lignin peroxidase (Gottschalk et al., 1999; Nascimento & Silva, 2008).

3.5. Utilization of Lignin Monomers

There is a wide spectrum of lignin monomers that could be used to stimulate the growth of the isolates *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter cancerogenus* PT21, and *Citrobacter sedlakii* PT41 (Table 4). In this study, these isolates used all of the tested monomers in their growth. C1 was the only isolate that did not grow on vanillic acid; neither did it grow on vanillin and 4-hydroxybenzoic acid. *Enterobacter ludwigii* L4, *Citrobacter farmeri* PT22, and *Klebsiella pneumoniae* G1 also did not grow on vanillin. Remarkably, the isolated strains have the ability to oxidize aromatic alcohols or aldehydes to their corresponding carboxylic acid form. Bandounas et al. (2011) found that the spectrum of lignin monomers that could be utilized for growth was relatively limited for all of the isolates and that the alcoholic forms of the aromatic monomers (veratryl alcohol and guaiacol) were not metabolized by any of the isolates. In contrast, the isolates in this study metabolized the aromatic monomers well.

3.6. Decolorization of Azo, Triphenylmethane and Heterocyclic Dyes

In this study, we examined the decolorization of several dyes by crude lignin peroxidase. Only the lignin peroxidase obtained from *Klebsiella pneumoniae* G1 decolorized dyes significantly. It decolorized BPB, methylene blue, methyl orange, and toloudine blue by 70%, 40%, 19%, and 58%, respectively (Figure 4). These results are in accordance with those obtained by Ollikka et al. (1993), who tested the dye decolorization of Phanerochaete chrysosporium and found that the best decolorization (93%) was obtained for bromophenol blue, as shown in this study. Ollikka et al. found that the decolorization ability of the enzymes was increased when veratryl alcohol was present in the reaction mixtures. In the present study, veratryl alcohol was not used in the reaction mixtures. Taking into consideration the previous findings, we can predict that the

Table 4 : Growth of bacterial isolates on lignin monomers

Aromatic compound	L1	L2	L3	L4	PT21	PT22	PT41	G1	C1
Vanilic acid	+	+	+	+	+	+	+	+	-
Vanilin	+	+	+	-	+	-	+	-	-
4-Hydroxybenzoic acid	+	+	+	+	+	-	+	+	-
Syringic acid	+	+	+	+	+	+	+	+	+
Phenol	+	+	+	+	+	+	+	+	+
Veratryl alcohol	+	+	+	+	+	+	+	+	+
Guaiacol	+	+	+	+	+	+	+	+	+

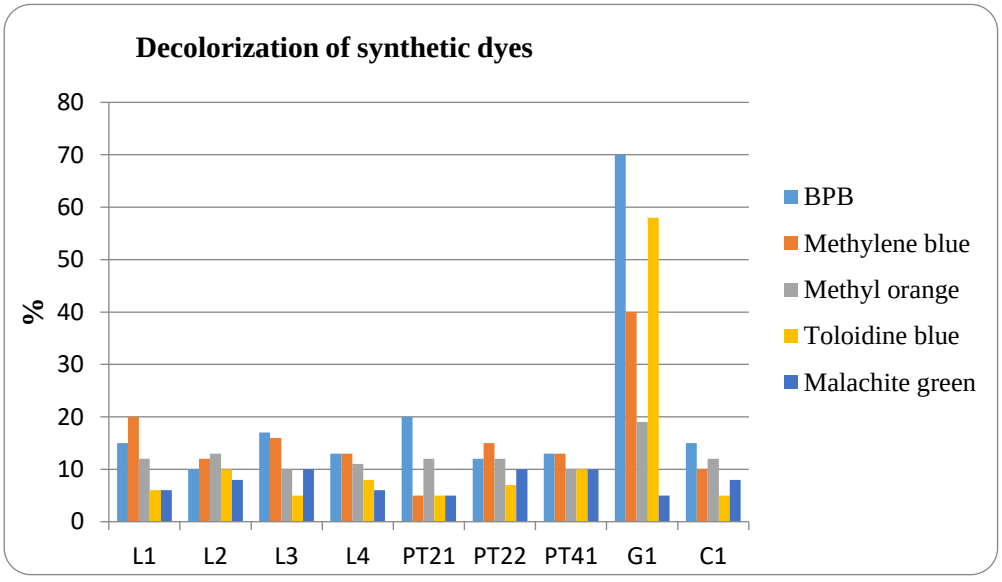

Figure 4. The percentage of dye decolorization

decolorization capacity of *Klebsiella pneumoniae* G1 lignin peroxidase will be increased.

3.7. Dye Decolorization Studies

The nine isolates were further tested by measuring their efficiency of RBBR decolorization (Figure 5). All of the strains were able to decolorize RBBR to some extent, and four of them achieved a 90% decrease in dye color. In the present study, this showed that the ability to decolorize RBBR dye is correlated to ligninase production. Previous works also found a correlation between decolorization of polymeric dyes and lignin degradation activity among several wood- or soil-inhabiting fungi (Falcon et al., 1995; Pasti and Crawford, 1991). However, there are very few studies about such correlation among bacteria. In this research, we showed that some bacteria have as much ability to degrade polymeric dyes as fungi.

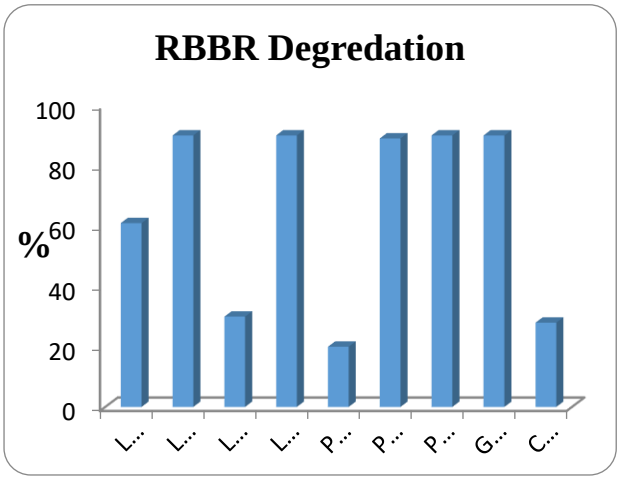

Figure 5. The percentage of RBBR degradation

M. J. Lopez et al. (2006) tested the dye decolorization of bacteria and fungi that they had isolated, and they found that RBBR was difficult to decolorize. Twenty-five strains showed some decolorization of RBBR, but the efficiency was around 15–30% except for three mesophilic fungi, whose decolorization rates were greater than 50%. Rodriguez et al. (1999) found that Pleurotus ostreatus strains exhibited high laccase and manganese peroxidase activity and decolorized different industrial dyes very efficiently. An earlier study by Özsoy et al. (2005) revealed that F. trogii could effectively decolorize RBBR dye by up to 90% within 24 h. They used an initial dye concentration of about 10-50 mg/l, compared to the RBBR concentration of 200 mg/l used in this study. Kiran et al. (2019) found that when synthetic dye wastewater concentration was increased from 0.01-0.05%, the percent decolourization decreased from 80.93% to 24.705%. Taking into consideration these previous findings and the dye concentration, we can say that our isolates were more effective at dye decolorization.

In conclusion, many lignin-degrading organisms are reported in this study, as are their lignin degradation and dye decolorization rates. The isolation and identification of environmentally friendly bacteria for lignin degradation have become essential because all previous researchers concentrated on the use of fungal treatments. The importance of ligninolytic bacteria has increased because lignin-degrading bacteria have a wider tolerance for temperature differences, pH variations, and oxygen limitation compared to fungi. This study found that the strains *Klebsiella pneumoniae* G1 and C1 have a strong potential for KL degradation. In particular,

the *Klebsiella pneumoniae* G1 strain is very effective in RBBR decolorization. Additional studies have to be made to determine the optimum conditions (nutrients, temperature, etc.) for maximum lignin degradation and dye decolorization by pure or mixed cultures of the strains. In addition, ligninolytic enzymes from these strains could be extracted and evaluated.

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Antioxidant Capacity of a Bee Pollen Sample Obtained from Giresun, Turkey

Giresun, Türkiye'den Elde Edilen Arı Poleni Örneğinin Antioksidan Kapasitesi

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Abstract

This study was related to evaluating the antioxidant of one pollen sample which was collected from Giresun, Turkey. The antioxidant properties of this sample were determined to use total phenolic contents (TPCs), total flavonoid contents (TFCs), proanthocyanidin contents (PCs), and ferric reducing antioxidant power (FRAP) assays. The result of the total phenolic content was 6.33±0.11 mg GAE/ g sample, total flavonoid content was 1.88±0.11 mg QE/g sample, proanthocyanidin content was 0.86±0.00 mg CE/g sample, FRAP was also 72.38±0.21 µmoL $FeSO_4.7H_2O$/g, respectively. The obtaining results revealed that the pollen could be seen as a valuable natural source thanks to its significant antioxidant capacity.

Keywords: Bee-pollen, Antioxidant, Total phenolic content, Total flavonoid content, Proanthocyanidin content

Özet

Bu çalışma, Türkiye'nin Giresun ilinden temin edilen bir arı poleni örneğinin antioksidan değerlendirilmesi ile ilgilidir. Çalışılan polenin antioksidan özelliği, toplam fenolik madde (TPC), toplam flavonoid madde (TFC), proantosiyanidin içeriği (PC) ve demir indirgeyici antioksidan güç (FRAP) metotları kullanılarak tayin edilmiştir. Sonuçlar sırasıyla, toplam fenolik içeriği 6,33±0,11 mg GAE/g, toplam flavonoid içeriği 1,88±0,11 mg QE/g, proantosiyanidin içeriği 0,86±0,00 mg CE/g ve FRAP metodu da 72,38±0,21 µmoL $FeSO_4.7H_2O$/g idi. Elde edilen sonuçlar, önemli antioksidan kapasitesinden ötürü arı poleninin değerli bir doğal kaynak olarak görülebileceğini ortaya koymuştur.

Anahtar kelimeler: Arı poleni, Antioksidan, Toplam fenolik içerik, Toplam flavonoid içerik, Proantosiyanidin içerik

Abbreviations: TPC, Total phenolic content; GAE, Gallic acid equivalent; TFC, Total flavonoid content; PC Proanthocyanidin content; FRAP, Ferric reducing antioxidant power

1. INTRODUCTION

Turkey is one of the leading countries in terms of the apiculture, which is an occupation with bee products such as honey, beebread, bee venom, bee pollen, propolis, and royal jelly, thanks to its wonderful geographical location. Also, these products have opened a new road with the name of apitherapy which is the science of prolonging, sustaining, and retaining health (Komosinska-Vassev, Olczyk, Kaźmierczak, Mencner, & Olczyk, 2015; Pasupuleti, Sammugam, Ramesh, &

Gan, 2017; Sahin, 2016). Especially, bee-pollen collected by honey bees is one of these products and acts as a natural warrior against some diseases. This warrior characteristic of bee pollen comes from some bioactive compounds such as polyphenol substances which are responsible for the potent antioxidants.

In the literature, there have been so much considerable researches on the biological activity of bee pollen. But, it could be seen as limited in terms of the location because it was emphasized as Giresun, Turkey. Hence, the aim of the current study is to determine the antioxidant activity of a local bee pollen sample supplied from Giresun, Turkey with some different assays.

2. MATERIALS AND METHODS

2.1. Sample Collection

Pollen sample was supplied from a beekeeper at the season of 2019 in Giresun, Turkey. The sample was stored at +4°C in dark plastic containers until analyzed on the instruments.

2.2. Extraction Procedure

For all experiments, approximately 10 g of pollen sample was extracted with 50 mL methanol in a flask with a condenser at 60ºC in 6 h. Extract was subsequently filtered to remove particles, and the final volume was determined.

2.3. Total Phenolic Contents (TPCs)

TPCs were determined using the Folin-Ciocalteau procedure with gallic acid as standard (Singleton & Rossi, 1965). For this, 680 µL distilled water, 20 µL methanolic extract of pollen and 400 µL of 0.2 N Folin-Ciocalteu were mixed and then vortexed. After 2 min, 400 µL Na_2CO_3 (10%) was added, the mixture was incubated with intermittent shaking for 2 h at room tempature. Absorbance was measured at 760 nm and TPC concentration was calculated as mg of gallic acid equivalents per gram of g sample.

2.4. Total Flavonoid Contents (TFCs)

Total flavonoid contents were determined by aluminium chloride colorimetric assay which was read at 415 nm. Quercetin was used as the standard for the preparation of the calibration curve. The final results were expressed as mg of quercetin equivalents (QE) per g pollen sample (Fukumoto & Mazza, 2000).

2.5. Proanthocyanidin Content (Condensed Tannins)

Proanthocyanidin contents (condensed tannins) of the analyzed sample were determined with a spectrophotometric assay previously described by Julkunen-Tiitto, (1985). This method is also known as vanillin method. To apply this assay, various concentrations of 25 µL methanolic pollen extracts, 750 µL of 4% vanillin dissolved in methanol and also 375 µL of concentrated HCl were mixed. After 20 min incubation, the absorbance of this mixing was measured at 500 nm. Catechin was used to establish the standard curve (0.02–1 mg/mL). The results were expressed as mg catechin equivalent to (CE)/g pollen sample.

2.6. Ferric Reducing Power (Frap)

For this antioxidant test, a freshly prepared FRAP reagent was used, which the preparing methodology of this reagent was detailed given by a previous study (Benzie & Strain, 1996). Briefly, 3 mL freshly FRAP reagent and 100 µL of methanolic sample was mixed and incubated in 4 min at 37 °C, and the absorbance was read at 595 nm. FRAP values were expressed as µmol $FeSO_4.7H_2O$ equivalent of g pollen sample.

3. RESULT AND DISCUSSION

Flower pollen is a product hosted in the anthers of flowers. It is produced from the male organs to fertilize the female organs (Orzáez Villanueva, Díaz Marquina, Bravo Serrano, & Blazquez Abellán, 2002). The vary of botanical and geographic origins is responsible for the differentiation of the flower pollen's composition (Aličić, Šubarić, Jašić, Pašalić, & Ačkar, 2014). Honey bees agglutinate the different pollen grains on the hind legs and they carry to their hive by mixing their secretions and also a small percentage of nectar (Bilisik, Cakmak, Bicakci, & Malyer, 2008). The general composition of bee pollen is carbohydrates (13-55%), crude fibers (0.3-20 %), proteins (10-40 %), and lipids (1-10%), respectively (Villanueva et al., 2002). Besides these major components, many minor ones are seen such as minerals and trace elements, vitamins and carotenoids, phenolic compounds, sterols, and terpenes (Bogdanov, 2017). Especially, polyphenol substances mainly phenolic acids and flavonoids play an important role in the biological processes such as antioxidant, antiaging, anticarcinogen, antiinflammatory etc. (Aličić et al., 2014; Can et al., 2015; Zillich, Schweiggert-Weisz, Eisner, & Kerscher, 2015). Here, the current study was based on phenolic compounds related to the antioxidant characterization. It can be seen many assays to determine the antioxidant capacity according to the literature. When compared to each other, the total capacity assays summarized by total phenolic contents (TPCs) and total flavonoid contents (TFCs) give a more general idea for antioxidant properties than other specific methods. Proanthocyanidin contents and ferric reducing power (FRAP) just like emphasized by this work can be seen as a more specific methodology. As showed in Table 1**,** the antioxidant data were given.

Table 1. Applied assays for analyzed pollen

Assays	Units	Result*
Total Phenolic Content	(mg GAE/g sample)	6.33±0.11
Total Flavonoid Content	(mg QE/g sample)	1.88±0.02
Proanthocyanidin Contents	(mg CE/g sample)	0.86±0.00
FRAP	(µmol $FeSO_4.7H_2O$/g sample)	72.38±0.21

*In all results given, analyses were performed in triplicate and given with the standard deviation.

Briefly, the result of total phenolic content (TPC) of pollen sample was determined as 6.33±0.11 mg GAE/ g pollen, total flavonoid content was 1.88±0.11 mg QE/g sample, proanthocyanidin content was 0.86±0.00 mg CE/g sample, finally, FRAP was 72.38±0.21 µmoL $FeSO_4.7H_2O$/g. There was a limitation to our study in terms of sample numbers. It was studied and evaluated with one sample. It is well-known that it assumed as a

fixed nearly the same properties in all bee pollen samples however it was absolutely avoided generalization in the evaluation.

According to the current results, our data were actually lower than previous studies (Harif Fadzilah, Jaapar, Jajuli, & Wan Omar, 2017; Vasconcelos, Duarte, Gomes, da Silva, & López, 2017). Vasconcelos et al. (2017) publish a study to assess the influence of botanical origin on the physicochemical composition and antioxidant potential of pollen samples from *Apis mellifera* from the meso-regions of Brazil during a dry season. They found that the phenolic constituents of pollens were exactly affected by the season. In that study, although there were high results in terms of total phenolic, the lower ones were also recorded as 7.57±2.45. Hence, this result was nearly similar to the current study.

Aličić et al., (2014) reported that only flavonoids of a certain structure and particularly hydroxyl position in the molecule, determine antioxidant properties. Generally, the qualitative and quantitative of flavonoids depend on the differentiation of floral and location situations just as all phenolic compounds (Can et al., 2015). Because of these explanations, the having of the lower total flavonoid degree of the analyzed sample was seemed as normal. That meant our sample did not include specific flavonoid compound, it needed to achieve some advanced analysis just like chromatographic determination to support our result.

There has not been any condensed tannins are a group of the member of the polymeric flavonoid molecules that are found in higher plant species (Robbins, Bavage, Strudwicke, & Morris, 1998). Also they responsible for many bioactivities such as antitumor, antimicrobial, and antioxidant (Yılmaz, Yıldız, Kılıç, & Can, 2017). So, it was a reality that 0.86 mg catechin equivalent per one gram sample was so precious result for us. It has not been met with any study which includes proanthocyanidin contents of bee pollen in the literature. For this reason, there was a limitation to compare our results with any study.

FRAP method is based on electron transfer and regarded as accurate markers of total antioxidant power because total reducing power is defined as the sum of the reducing powers of the individual compounds contained in a particular sample (Aliyazicioglu et al., 2016). Ulusoy & Kolayli, (2014) studied some Anzer bee pollen, they found the FRAP value range from 11.77 to 105.06 µmol Trolox/g pollen. Another study from Yıldız et al., (2013) revealed that a pollen sample supplied from Zonguldak was 82.31 ± 2.41 mM Trolox/g DW. It was seen that the units were different according to the previous studies, but Saral, Kiliçarslan, Şahin, Yildiz & Dinçer (2019) utilized the $FeSO_4.7H_2O$ as the standard just like us and supported our results ranged from 8.69-84.89 µmol $FeSO_4.7H_2O$/g sample.

4. CONCLUSION

According to these results, different antioxidant assays are very important to show the bioactivity degrees of any natural compounds. Here was a pollen sample study in terms of antioxidant capacity, it was found as a moderate when compared with literature. Because all bee products -bee pollen is one of them- show variability thanks to different reasons such as climatic, seasonal, botanical origin etc. Our result demonstrated that all bee-pollens whatever types can be helpful for human consumption and health owing to its antioxidant capacity.

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Phenolic Composition of Pine (Pinus spp.) Honey from Turkey

Türkiye'den Elde Edilen Çam (Pinus spp.) Balının Fenolik Bileşimi

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Abstract

In this study, the phenolic component of pine honey which is a secretion honey type were studied. Total polyphenol content and total flavanoid contents of the pine honeys were ranged from 30 to 52 mg GAE/100 g and 0.86 to 1.58 mg QE/100 g, respectively. The phenolic composition of the honeys were analyzed by HPLC-UV assay with C_{18} column. The honey was found to be rich in protocatehuic acid, chrysin, caffeic acid phenyl ester, p-OH benzoic acid, catechine, luteolin and gallic acid.

Keywords: Honey, Pine, Phenolic Component, Anatolia, Turkey

Özet

Bu çalışmada, bir salgı balı olan çam balının fenolik bileşenleri incelendi. Balların toplam polifenol içeriği ve toplam flavanoid içeriği sırasıyla 30 ile 52 mg GAE / 100 g ve 0.86 ile 1.58 mg QE / 100 g arasında olduğu görüldü. Balların fenolik bileşimi, HPLC-UV ile analiz edildi. Balların protokatekuik asit, krisin, kafeik asit fenil ester, p-OH benzoik asit, kateşin, luteolin ve gallik asit bakımından zengin olduğu bulundu.

Anahtar kelimeler: Bal, Çam, Fenolik Bileşen, Anadolu, Türkiye

Abbreviations: TPC, Total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalent; QE, quercetin equivalents.

1. INTRODUCTION

According to the sources of produced honey, there are two different types of honey as blossom and secretion. Blossom (flower) honeys are collected by honey bees (Apis mellifera) from flower nectars, and are the most produced honey species in the world. Secretion honeys are secreted not from flowers of plants, but only from leaves and trees with sugar containing stem. Honey bees are generally produced honey in two different ways. One of them is the production from the insects that live on trees as parasites such as pine honey. The other way is the secreted from the trees by sweating depending on the weather such as oak and cedar honeys (Kara, Can & Kolaylı, 2019;

Kolayli, Can, Çakir, Okan & Yildiz, 2018; Özkök & Silici, 2017). While pine honey is the most produced in Turkey, other Mediterranean countries such as Greece, Italy, Spain, and Portugal are also less produced. It has a relatively dark colored, turbid appearance, and characteristic smell and aroma. Also, pine honey is not easily crystallized (Can et al. 2015).

Polyphenols are secondary metabolites of plants and have many biological values such as anti-oxidant, anti-microbial, anti-inflammatory, antiviral, anti-repellent and anti-tumoral etc. (Bahramsoltani, et al. 2019; Joseph, Edirisinghe & Burton-Freeman, 2016). Polyphenols are the most important secondary metabolites of honeys and they are also responsible many characteristic features of honeys such as color, aroma, taste and biological activity.

In the literature, the studies with the polyphenols of pine honey are very limited. The aim of this study was determined the phenolic composition and phenolic markers of the pine honeys from Mugla region of Turkey.

2. MATERIAL AND METHODS

Ten pine honey samples were collected in Mugla around of near the Aegean Sea of Turkey in October, 2018. Physicochemical properties (moisture content, optical rotation, pH) and phenolic compounds and contents of honey samples were determined.

2.1. Physicochemical Properties

Moisture contents of these samples were measured by refractometer method (Atago, Tokyo, Japan). Optical rotation of the samples were measured by polarimetry (BetaPPP7, England).

2.2. Preparation of samples extraction for antioxidant analysis and phenolic compounds

For determination of total phenolic content, ethanolic extracts of honey samples were used. 10 g of honey was extracted using 90 % ethanol solvent for 24 hours at room temperature using a shaker (Heidolph Promax 2020, Schwabach, Germany). After incubation, extracts were filtered with Whatmann No: 4 filter paper and stored at 4ºC for further analysis. Extracts were divided into two parts. The first part for antioxidant tests and the second part are for phenolic component analysis.

2.3. Total Phenolic Content Determination

Total phenolic content was measured by Folin Ciocalteu method (Singleton, Orthofer & Lamuela-Raventós, 1999). Firstly, 680 µL pure water, 20 µL of honey ethanolic extract and 400µL 0.5 N Folin–Ciocalteu reagent were mixed, and vortexed. Then, 400 µL of Na2CO3 (10 %) was added into the tubes. After vortexing, the mixture was incubated for 2 h at 20°C with shaker. For calibration curve of gallic acid standard, different concentrations of gallic acid solution were prepared with the same analysis procedure. The reaction using the intensity of the blue color at 760 nm in spectrophotometer was read and the results

were expressed as mg gallic acid equivalent (GAE) / 100 g. All the measurements were performed in triplicate.

2.4. Total Flavonoid Determination

For determination of total flavonoid substance, Fukumoto and Mazza (2000)' method was used with using quercetin standard (Fukumoto and Mazza, 2000). Firstly, 0.5 mL of the samples, 0.10 mL of 10 % Al $(NO_3)_3$ and 0.10 mL of 1 M $NH_4.CH_3COO$ was added to reaction mixture. This mixture was incubated at room temperature for 40 min and the absorbance was measured against a blank at 415 nm. Quercetin (0.03125-1 mg/mL) was used as a standard to obtain the calibration curve. The total flavonoid content (TFC) was calculated as mg of quercetin equivalents (QE)/100 g honey.

2.5. Analysis of Phenolic Components by RP-HPLC-UV

Phenolic composition of the honey was determined in RP-HPLC-UV. For this purpose, a calibration curve was prepared in the study using 19 phenolic standards and phenolic compositions of the samples were determined according to these curve values. Gallic acid, syringic acid, p-OH benzoic acid, ferulic acid, caffeic acid, t-cinnamic acid, p-coumaric acid, catechin, epicatechin, rutin, daidzein, myricetin, luteolin, hesperetin, chrysin, pinocembrin, protocatechuic acid, caffeic acid phenethyl ester, resveratrol were used as phenolic standards.

The ethanolic extracts of honey samples were evaporated until dryness in a rotary evaporator at 40° C. The residue was dissolved in 15 mL acidified distilled water (pH 2). Liquid–liquid extraction was carried out with 5×3 mL diethyl ether and 5×3 mL ethyl acetate, consecutively (Kim, Tsao, Yang & Cui, 2006). Both diethyl ether and ethyl acetate phases were pooled and evaporated by rotary evaporation (IKA-Werke, Staufen, Germany) at 40° C. The pellet was suspended in 2 mL ethanol, filtered with syringe filters (RC membrane, 0.45 µm), and injected to HPLC.

HPLC (EliteLaChrom Hitachi, Japan) with UV detector was used for analysis and conducted by reverse phase C18 column (150 mm x4.6 mm, 5 µm; Fortis). It was carried out by applying a gradient program with acetonitrile, water and acetic acid. (Malkoç, Çakır, Kara, Can & Kolaylı, 2019b).

For HPLC analyses, the mobile phase (A) and (B) consisted of 2% acetic acid in water and acetonitrile: water (70:30), respectively. The sample injection volume was 20 µL, the column temperature was 30° C and the flow rate was 0.75 mL / min. Using of the programmed solvent began with a linear gradient held at 95% A for 3 min, decreasing to 80% A at 10 min, 60% A at 20 min, 20% A at 30 min and finally 95% A at 50 min. Three injections were used for each sample. All calibration values for phenolic components are between 0.998 and 0.999 (Çakir, Şirin, Kolayli & Can, 2018).

3. RESULTS AND DISCUSSION

Specific optical rotation, moisture content, and pH value of the samples was given in Table 1. Optical rotation value is an important distinguishing feature for flowers and honey (Dinkov, 2003; Serrano, Rodríguez, Moreno & Rincón, 2019). Honeydew (such as pine and oak honey etc.) optical rotation is dextrorotary, while blossom honey is laevorotary (Cavrar, Yıldız, Sahin, Karahalil & Kolayli, 2013). It was determined that the optical rotation values of the studied honeys were ranged from 0.90 to 2.50. As a matter of fact, the positive rotation values of the studied honeys confirm that all honeys are secretion honeys.

Table 1. Physicochemical analyses of Pine honey from Mugla region.

	Min	Max	Mean
Specific Optic rotation $[\alpha]^{20}$	0,560	2.80	1,48±0,76
Moisture (%)	14.00	20.30	17.50±2.60
Ph	3.98	5.40	4.58±0.70

It was determined that the moisture amount of the honeys varied between 15% and 20.30% and the average value was 17.40%. The moisture values are found suitable with the recommended amount of water in the honey codex (Bogdanov et al, 1999).

It was determined that the pH values of the studied honeys were ranged from 3.98 to 5.58 and all the honeys had acidic medium. Moreover, the current values found were similar to the honey codex (Bogdanov et al, 1999). Acidic properties of honey earns honey a stronger antibacterial effect and bacteria cannot survive at this pH. It has been reported using capillary electrophoretic technique that gluconic acid, formic, malic, citric and succinic acids are major acids of honey (Kaygusuz et al. 2016). However, there are phenolic acids in honey, which is higher than these organic acids, and gallic acid, benzoic acid, p-OH benzoic acid, coumaric acid, syringic acid, valinic acid, ferulic acid are important sources of honey (Can et al. 2015; Ertürk, Şahin, Kolaylı & Ayvaz, 2014; Kolaylı et al. 2018).

The biological activity of honey, it consists of phenolic acids and flavonoids. Phenolic content of honey varies according to flora and geographical origin. In this study, total polyphenol values of pine honey were measured spectrophotometrically, and the results were given in Table 2. In general, honeys consist of between 20 and 150 mg gallic acid/100 g depending on honey species. Total polyphenol contents of the samples were found between 24.60 and 68.20 mg GAE/100 g of the samples and the mean value was 46.30 mg GAE/100g. All phenolic compounds in honey are measured by the total polyphenol method and phenolic acids, flavonoids, stilbenes, tannins are included in this family (Can, Baltaş, Keskin, Yıldız & Kolaylı, 2017). Total flavonoid contents of the honey samples were changed from 0.80 to 2.10 mg QE/100 g. The flavonoids contents of pine honeys were indicated nearly high and the other study findings confirmed this situation (Can et al, 2015; Kolaylı, Baltas, Sahin & Karaoglu, 2017).

Table 2. Total phenolic contents of Pine honey from Mugla region.

	Min	Max	Mean
Total phenolic content (mg GAE/100g)	24.60	68.20	46.30±10.30
Total flavonoid content (mg QE/100 g)	0.80	2.10	1.46±0.78

Phenolic profile of the pine honey was determined using nineteen polyphenolic standards with high pressure liquid chromatography (HPLC-UV) (Malkoç, Kara, Özkök, Ertürk & Kolaylı, 2019a). The results were summarized in Table 3. Protocatechuic acid was the major phenolic compound of the studied phenolic compounds, and chyrisin, p-OH benzoic acid and catechin are followed them. Haroun et al. (2012) reported that Turkish honeydew honeys (pine and oak) have been shown to contain protocatechuic acid in the range of 1639 to 5986 µg/kg honey. In the current study, protocatechuic acid was identified as the major component. The presence of protocatechuic acid as a major ingredient in pine honey might be considered to use as a characteristic indicator of honey's origin. The concentrations of protocatechuic acid 480.20 µg/kg honey for pine honeys. Another study was found concentrations of protocatechuic acid ranged from 3058 to 5967 µg/kg honey for pine honeys (Spilioti et al., 2014). Chrysin was observed to be main flavonoid in pine honeys. Chrysin content was found 210.30 µg/100 g in pine honey. In this study was determined that phenolic components were detected in different proportions in pine honey.

Table 3. Phenolic profiles analyses in HPLC-UV of Mugla pine honey (µg/100g)

Phenolic acids	
Gallic acid	33.20±5.80
Protocatechuic acid	480.20±105.30
p-OH Benzoic acid	98.45±22.08
Caffeic acid	28.60±12.56
Syringic acid	24.10±6.20
p-Coumaric acid	17.80±10.20
Ferulic acid	40.66±4.05
t-Cinnamic acid	-
Flavonoids	
Catechin	80.20±14.04
Epicatechin	22.07±2.80
Rutin	-
Myricetin	-
Daidzein	-
Resveratrol	-
Luteolin	38.50±12.40
Hesperetin	18.06±3.40
Chrysin	210.30±56.07
Pinocembrin	33.60±4.80
Caffeic acid phenethyl ester	24.50±8.40

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Royal Jelly: Proteins and Peptides

Arı Sütü: Proteinleri ve Peptidleri

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Abstract

Royal jelly is secreted by the hypopharyngeal and mandibular glands of 5-15 days worker honeybees (*Apis mellifera*). Royal jelly is a thick and milky bee product with high nutritional value. Besides the nutritional functions on queen and worker larvaes, it also has a very important role on the specific functions of queen bees. Due to its important biological properties, royal jelly has been used in the pharmaceutical, food and cosmetics industry especially for the last 50-60 years as a functional and nutraceutical food. The studies have shown that royal jelly has antioxidant, antidiabetic, antitumor, neurotrophic, antimicrobial, anti-inflammatory, hypotensive, hepatoprotective, antihypercholesterolemic, hypoglycaemic properties and effects on the reproductive system and fertility. These activities are attributed to the bioactive components it contains such as major royal jelly proteins (MRJPs), jelleins and royalisin peptides and 10-hydroxy-2-decenoic acid (10-HDA). Especially MRJPs are considered as an important factor in the development of queen bees. The aim of this study is to summarize and update physicochemical and bioactive properties of royal jelly, as well as characterization and functions of royal jelly proteins (RJPs) and peptides.

Keywords: Royal Jelly, Bioactive Properties, Major Royal Jelly Proteins, Peptides, Characterization, Apitherapy

Özet

Arı sütü 5-15 günlük genç işçi arıların üst çene (mandibular) ve boğaz bezlerinden (hipofaringeal) salgılanmaktadır. Arı sütü yüksek besin değeri ile yoğun ve süt kıvamında bir arı ürünüdür. Kraliçe ve işçi larvaları üzerindeki beslenme fonksiyonlarının yanı sıra, kraliçe arıların özel fonksiyonları üzerinde de çok önemli rolü vardır. Özellikle son 50-60 yıldır önemli biyolojik özellikleri nedeniyle arı sütü fonksiyonel ve nutrasötik bir gıda olarak ilaç, gıda ve kozmetik endüstrisinde kullanılmaktadır. Çalışmalar, arı sütünün antioksidan, antidiyabetik, antitümör, nörotrofik, antimikrobiyal, antienflamatuar, hipotansif, hepatoprotektif, antihiperkolesterolemik, hipoglisemik özellikleri ve üreme sistemi ve doğurganlık üzerine etkileri olduğunu göstermektedir. Bu aktiviteler, arı sütünün içerdiği majör arı sütü proteinleri, jellein ve royalisin peptidleri ile 10-hidroksi-2-dekenoik asit (10-HDA) gibi biyoaktif bileşenlere atfedilir. Özellikle majör arı sütü proteinleri (MRJPs) bal kraliçe arıların gelişiminde önemli bir faktör olarak kabul edilir. Bu çalışmanın amacı, arı sütünün fizikokimyasal ve biyoaktif özellikleri ile arı sütü protein ve peptidlerinin karakterizasyon ve fonksiyonlarını özetlemek ve güncellemektir.

Anahtar kelimeler: Arı Sütü, Biyoaktif Özellikler, Majör Arı Sütü Proteinleri, Peptidler, Karakterizasyon, Apiterapi

Abbreviations: MRJPs, Major Royal Jelly Proteins; Royal Jelly Proteins (RJPs); 10-Hydroxy-2-Decenoic acid (10-HDA)

1. INTRODUCTION

The importance of royal jelly from bee products was recognized in the 1600s and it was called "Royal Jelly" which means perfect food in English (Akyol & Baran, 2015). However, especially in the last fifty-sixty years its commercial production and consumption has increased. The royal jelly is secreted from the mandibular and hypopharyngeal glands of 5-15 days worker bees (*Apis mellifera* L.) (Balkanska, Zhelyazkova & Ignatova, 2012). This secretion is produced by the ingestion of pollen and nectar in the digestive organs of the worker bee. Royal jelly is not only nutrient for the growth of the honeybee larvae, but it is also necessary for the queen bees to feed and maintain its functions. In addition, royal jelly has great importance in the caste differentiation of these species. All larvae are fed with only royal jelly throughout the first 3 days, while larvae that will be queen are fed with only royal jelly in all larval and adult periods (Balkanska & Kashamov, 2011; Kolayli et al., 2016; Šimúth, 2001). Although having the same genetic structure, larvae fed by royal jelly continuously develop as queen bees; larvae that feed on royal jelly only in the first 3 days of the larval period and then a mixture of honey and pollen as worker bees (Akyol & Baran, 2015). Thereafter, a honeybee queen lives for 4 to 5 years, and a worker bee approximately 6 to 8 weeks (Moselhy Fawzy & Kamel, 2013).

Royal jelly is a dense and milky homogeneous substance with a density of 1.1 g / mL and partially soluble in water (Ramadan & Al-Ghamdi, 2012). It is highly acidic having the pH 3.4–4.5 (Popesco, Marghitasl & Dezmireand, 2008). It has a sharp phenolic smell and characteristic sour-bitter taste. Its colour is slightly beige and yellowish-whitish and darkens during storage (Shirzad, Kordyazdi, Shahinfard & Nikokar, 2013).

The water content of royal jelly is between 60-70% and the water activity (aw) is above 0.92. Addition, royall jelly consists of proteins (9–18 %), carbohydrates (10–16 %), fats (3–8 %), small amounts of mineral matter, polyphenols and vitamins (Sabatini, Marcazzan, Caboni, Bogdanov & Almeida-Muradian, 2009; Xue, Wu, & Wang, 2017). Table 1 shows the vitamin and mineral content of royal jelly (Benfenati, Sabatini & Nanetti, 1986; Maghsoudlou, Mahoonak, Mohebodini & Toldra, 2019) In addition, the chemical composition of royal jelly varies depending on a lot of factors: seasons and ecological conditions, race and caste of the honey bee, harvest time of royal jelly, methods of sampling and analysis used (Maghsoudlou et al., 2019; Ramanathan, Nair & Sugunan, 2018).

Table 1. Vitamin and mineral content of royal jelly

Minerals and Vitamins	Royal Jelly (mg/100 g)
Potassium	200-1000
Magnesium	20-100
Iron	1-11
Zink	0.7-8
Copper	0.33-1.6
B1 (Thiamin)	0.1-1.7
B2 (Riboflavin)	0.5-2.5
B3 (Niacin)	4.5-19
B5 (Pantothenic acid)	3.6-23
B6 (Pyridoxin)	0.2-5.5
H (Biotin)	0.15-0.55
Pantothenic Acid	0.2-0.25
Nicotinic Acid	0.4-0.48
Folic Acid	0.01-0.06
Inositol	0.1-0.12

Royal jelly is one of the most interesting food among functional and nutraceutical foods. The

studies have shown that royal jelly has antioxidant (Guo et al., 2008; Nagai, Sakai, Inoue, Inoue & Suzuki, 2001; Kolayli et al., 2016), antidiabetic (Dania et al., 2008; Maleki et al., 2019; Pourmoradian, Mahdavi, Mobasseri, Faramarzi & Mobasseri, 2014), antitumor (Kimura, 2008; Nakaya et al., 2007; Premratanachai & Chanchao, 2014), neurotrophic (Furakawa, 2008; Hattori, Nomoto, Fukumitsu, Mishima & Furukawa, 2007; Mannoor, Tsukamoto, Watanabe, Yamaguchi, K., & Sato, 2008), antimicrobial (Bărnuţiu, Mărghitaş, Dezmirean, Mihai & Bobis, 2011; Bílikova, Huang, Lin, Šimuth & Peng, 2015; Coutinho, Karibasappa & Mehta, 2018), anti-inflammatory (Aslan & Aksoy, 2015; Karaca et al. 2012; Kohno et al., 2004), hypotensive (Nagai, Inoue, Suzuki & Nagashima., 2008; Pan et. al., 2019; Takaki-Doi, Hashimoto, Yamamura & Kamei, 2009), hepatoprotective (Almeer et al., 2018; Chen, Fang & Wang, 2020; Kanbur et al., 2009; Ghanbari, Nejati & Azadbakht, 2015;), antihypercholesterolemic (Chiu et al., 2017; Guo et al., 2007; Kamakura, Moriyama & Sakaki, 2006), hypoglycaemic (Fujii et al., 1990),andanti-aging activities (Han et al., 2011; Qiu et al., 2019; Pasupuleti, Sammugam, Ramesh & Gan, 2017) and effects on the reproductive system and fertility (Eshtiyaghi, Deldar, Pirsaraei & Shohreh, 2016; Husein & Haddad, 2006; Yang et al., 2012). These activities are mainly attributed to the its bioactive components such as fatty acids, proteins and phenolic compounds (Ramadan & Al-Ghamdi, 2012). One of the major bioactive components is the major royal jelly proteins (MRJP), which are considered to be an important factor in the development of the honey bee queen (Tamura et al., 2009).

2. ROYAL JELLY PROTEINS AND PEPTIDES

Major Royal Jelly Proteins (MRJPs), also named as apalbumins, constitute 82–90% of royal jelly proteins. In the royal jelly protein (RJP) family, there are nine different members, MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8 and MRJP9, which are encoded by nine different genes (Buttstedt, Moritz & Erler, 2014; Nozaki, Tamura, Ito, Moriyama, Yamaguchi & Kono, 2012; Schmitzova et. al., 1998). Among the RJPs, MRJP 1-5 are the most abundant proteins typifying 90% of RJPs, and have essential nutritional function. The remaining content of RJPs composes of small proteins, peptides, free amino acids, enzymes such as endopeptidase and exopeptidase (Maqsoudlou et al., 2018). The members of the MRJP family comprise high amounts of essential amino acids necessary to feed both queen bees and all larvae. It is thought to be responsible for the specific physiological roles of royal jelly especially in the development of queen honey bees (Ahmad, Campos, Fratini, Altaye & Li, 2020). Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are most common essential amino acids in MRJPs. The essential amino acid contents of MRJPs are given in Table 2 (Ramanathan et al., 2018).

Table 2. Essential amino acid contents of MRJPs

MRJPs	Amino Acid Contents
MRJP1	48 %
MRJP2	47 %
MRJP3	39.3 %
MRJP4	44.5 %
MRJP5	51.4 %
MRJP6	42 %
MRJP7	48.3 %
MRJP8	49.5 %
MRJP9	47.3 %

MRJPs contain 400-578 amino acids. Their theoretical molecular weights are from 45 to 68 kDa, isoelectric points change from 4.85 to 6.50 (Table 3) (Buttstedt et al., 2014).

Table 3. Molecular characteristics of honeybee MRJPs

MRJPs	Amino Acids	Moleculer Weights (kDa)	IsoelectricPoints (pI)
1	413	46.86	5.03
2	435	49.15	6.65
3	524	59.49	6.50
4	444	50.67	5.74
5	578	68.13	5.95
6	417	47.58	6.01
7	426	48.66	4.85
8	400	45.06	5.81
9	403	46.27	8.62

However, the studies indicate that molecular weight and isoelectric points of RJPs, which are used as very significant parameters in identifying proteins, depend on the types and genetic diversityof honey bees, post translational changes such as glycosylation and phosphorylation, and proteolysis reactions that occur during storage of royal jelly (Ohashi et. al., 1997; Qu et al., 2008; Santos et. al, 2005; Schmitzova et. al., 1998; Zhang et al., 2012).

2.1. MRJP1

Among the major royal jelly proteins, MRJP1, the first identified protein, is the most abundant glycoprotein (Tian et al., 2018). It is also known by different names such as apalbumin and royalactin (Ramanathan et. al., 2018). MRJP1 exists in different forms, including monomers, oligomers and water-soluble forms. The monomeric forms of MRJP1 have an important role in the queen determiner (Foret et al., 2012; Kamakura, 2011). Oligomeric forms of MRJP1 combine with fatty acids to demonstrate the ability to form spontaneously in aqueous solutions (Šimúth, 2001). Studies have determined that MRJP1 is a weak acidic glycoprotein and its isoelectric point is ranged from 4.23 to 6.3 (Cruz et al., 2011; Santos et. al., 2005; Tamura et. al., 2009). The molecular sizes of MRJP1 monomers were changed from 48.81 to 57 kDa (Kamakura, 2011; Kamakura, Suenobu & Fukushima, 2001; Majtán, Kováčová, Bíliková & Šimúth, 2006; Santos et al., 2005; Schmitzova et al., 1998). The molecular sizes of MRJP1 oligomers were found to be between 280-450 kDa including apisimin molecule (Bilikova et al., 2002; Kamakura, 2011; Mandacaru et al., 2017; Nozaki et al., 2012; Ramadan ve Al-Ghamdi, 2012). Table 4 shows the molecular sizes of MRJPs and protein purification and characterization methods used by various researchers.

It has been determined that MRJPI and its degradation products ensueing during storage, especially at storage temperatures above 4 °C,

can be used to evaluate the freshness of royal jelly (Kamakura, Fukuda, Fukushima & Yonekura, 2011; Shen et al., 2015).

2.2. MRJP2

MRJP2 is a basic protein with molecular weights between 49-72 kDa (Bilikova et. al., 2002; Imjongjirak, Klinbunga & Sittipraneed, 2005; Nozaki et. al., 2012; Scarselli et. al., 2005; Schmitzova et. al., 1998; Šimuth, Bíliková & Kovácová, 2003) and isoelectric points ranging from 4.92-8.3 (Bilikova et. al., 2002; Santos et. al., 2005).

2.3. MRJP3

MRJP3, unlike other MRJPs, has 14-30 pentapeptides repeating in its structure. Isoelectric points vary between 7.05 and 8.04 and molecular weights range from 55 to 87 kDa (Kubo et al.,1996; Šimuth et al., 2003; Santos et al., 2005; Scarselli et al., 2005).

2.4. MRJP4

MRJP4 that provides nutrient components to royal jelly is determined to be approximately 60 kDa (Schmitzova et. al., 1998; Sano et al., 2004; Li, Wang, Zhang & Pan, 2007). Isoelectric pointof MRJP4 has been reported to be between 6.28 and 6.48 (Li et al., 2007).

2.5. MRJP5

The most important feature of MRJP5 is that there is a wide repeating region of 174 amino acids between 367 and 540 amino acid residues (Qu et al., 2008). Li et al. (2007) and Santos et al. (2005) have been reported that the molecular weights of MRJP5 vary between 74.89-79.87 and 79.075-79.471, respectively. In additon, isoelectric points were determined between 6.34 and 6.80 by Santos et al. (2005). MRJPs 2-5 contain glycoproteins of size 49, 60-70, 60 and 80 kDa, respectively (Schonleben et al., 2007; Tamura et al., 2009). Studies show that the isoelectric points vary between 4.93 and 8.3 due to post-transitional changes in MRJP2-MRJP5 (Li et al., 2007; Schönleben, Sickmann, Mueller & Reinders, 2007; Tamura et al., 2009). MRJPs6-9 do not have nutritional function on honey bees.

2.6. Peptides

In addition to MRJPs, royal jelly comprises a low variety of small proteins, including bioactive peptides (Jamnik, Raspor & Javornik, 2012). Most of the peptides in royal jelly are formed by proteolytic hidrolysis from major royal jelly proteins (Schönleben et al., 2007). A lot of this peptides show antioxidant activity in different levels (Ramadan & Al-Ghamdi, 2012). In addition, peptides having antimicrobial properties such as royalicin, jelleins and apisimin have also been identified. Royalisin and jelleins secreted into royal jelly by worker bees provide multiple microbial protections (Fontana et al., 2004).

Royalisin is an antimicrobial peptide isolated from royal jelly and has a molecular weight of 5523 Da. It is stable at low pH and high temperature due to its disulfide bonds in its structure (Barnuţiu et al., 2011). Royalisin prevents microbial contamination in royal jelly for Gram-positive bacteria, especially (Fontana et al., 2004).

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Jelleins are short chain peptides of royal jelly with antimicrobial activity. The jelleins consist of 8-9 amino acid residues. A number of peptides called Jellein-I, Jellein-II, Jellein-III and Jellein-IV have been identified (Ramanathan et al., 2018). Fontana et al. (2004) reported that Jelleines-I and III are effective against Gram-positive and Gram-negative bacteria and yeast. However, Jellein-IV has no antimicrobial activity.

2.7. Isolation and Characterization of Proteins

In researches on royal jelly proteins, for isolation and characterization of proteins have used chromatographic methods, particularly gel filtration chromatography, ion exchange chromatography and high pressure liquid chromatography (HPLC). In chromatographic methods, separation is based on the adsorption, dispersion (partition), ion exchange, affinity or differences in molecular weights of molecules. High performance liquid chromatography (HPLC) is a column chromatography method widely used for the purification, identification, qualitative and quantitative analysis of compounds. The chromatographic methods can be used alone or in combination with other methods depending on the properties of the proteins (Coskun, 2016; Sugecti, Imalı & Kocer, 2018).

Among electropohretic methods, sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) method is the most widely used gel electrophoretic method for protein characterization. SDS-PAGE seperates proteins based on their rate of movement to molecular weights in an applied electric field (Hu et al., 2017). Another method is two-dimensional polyacrylamide gel (2D-PAGE) electrophoresis method which is developed in addition to SDS-PAGE method. It provides seperation based on not only molecular weights, but also isolelectric points (Issaq & Veenstra, 2008). These methods are used in combination with mass spectrophotometry (MS) or gel-free proteomics for identification purposes (Maghsoudlou et al., 2019).

Blotting (western blot, dot blot, northern blot) methods are based on absorbing into the membrane of a targeted protein in a complex

protein separated or not separated by electrophoresis and immunochemical determination of the protein or proteins in the membrane (Burgess, 2009).

In addition for the protein purificitaion and characterization advanced molecular methods such as circular dichroism, N-terminal amino acid sequence, MALDI-TOF methods are also used.

Table 4. Molecular weights for MRJP1-MRJP9 with protein characterization methods

	Molecular Weights (kDa)	**Methods**	**Referance**
MRJP 1	55	Affinity Chromatography, SDS-PAGE Electrophoresis, Western Blotting, N-Terminal Amino Acid Sequence	Majtán et al. (2006)
MRJP 1	57	Native-PAGE and SDS-PAGE Electrophoresis, Diethylaminoethyl (DEAE)-Colon Chromatography, Gel Filtration Colon Chromatography	Kamakura et al. (2001)
MRJP1 oligomer	290 (55 kDa MRJP1 monomer and 5 kDa Apisimin)	Size-Exclusion HPLC, Two Dimensional(2-DE), SDS-PAGE and Native-PAGE Electrophoresis, MALDI-TOF MS Analysis	Tamura et al. (2009)
MRJP1 oligomer and monomer	420 55	Ultracentrifugation, SDS-PAGEElectrophoresis, Size-Exclusion ColonChromatography, Immunoblotting, N-Terminal Amino Acid Sequence	Šimúth (2001)
MRJP 2	49	DEAE-Cellulose ColonChromatography, SDS-PAGE Electrophoresis, N-Terminal AminoAcid Sequence, Dotblot, Northern blot	Schmitzova et al. (1998)
MRJP 2	52	Ultracentrifugation, Size-Exclusion HPLC, Two Dimensional(2-DE) and SDS-PAGE Electrophoresis	Nozaki et. al. (2012)
MRJP 3	55	Two Dimensional (2-DE) Electrophoresis, MALDI-TOF MS Analyses	Scarselli et al. (2005)
MRJP 3	64	SDS-PAGE Electrophoresis, Reverse Phase HPLC, Immunoblotting	Kubo et al. (1996)
MRJP 4	60.71–61.73	Two Dimensional (2-DE) Electrophoresis, MALDI-TOF MS Analyses	Li et al. (2007)
MRJP 5	74.89–79.87	DEAE-Cellulose ColonChromatography, SDS-PAGE Electrophoresis, N-Terminal AminoAcid Sequence, Dotblot, Northern blot	Schmitzova et al. (1998)
MRJP 6	47.5		Buttstedt, Moriz & Erler (2014)
MRJP 7	48.06		Buttstedt, Moriz & Erler (2014)
MRJP 8	45.06		Buttstedt, Moriz & Erler (2014)
MRJP 9	46.27		Buttstedt, Moriz & Erler (2014)

3. CONCLUSION

Royal jelly is a natural bee product with bioactive compounds. It has wide variety of therapeutic properties used from ancient times until today. In addition to being nutritious for bees, it has different functions such as growing and reproduction, caste differentiation, providing antimicrobial protection and extending the life of the queen bee, regulating physiological and temporal mechanisms. Major royal jelly proteins, the main proteins of royal jelly, are thought to be responsible for this multiple functions of royal jelly. In recent years, royal jelly is one of the most drawing commercial bee products in various industry such as food, medicine, neuturatic and cosmetic industries. However, present studies are not sufficient, because there is great potential for applications in the neutrotherapeutic and food sciences for this product.

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The Presence and Distribution of Nosemosis Disease in Turkey

Türkiye'de Nosemosis Hastalığı'nın Varlığı ve Dağıtımı

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Abstract

Nosemosis is one of the most important bee diseases causing economic losses in beekeeping, which is one of the significant reasons for Colony Collapse Disorder (CCD) in the world. *Nosemaapis* and *Nosemaceranae*, the microsporidian species, are the main causative agents of nosemosis in honey bees worldwide. This disease causes digestive system disorders, a decrease in the average life of bees and colony losses. In this review, the general characteristics of nosemosis disease, and information about the situation in Turkey are given.

Keywords: Honey bees, Microsporidia, Nosemosis, *Nosema apis*, *Nosema ceranae*, Turkey

Özet

Nosemosis hastalığı dunyada önemli ekonomik kayıplara neden olan Koloni Çökme Bozukluğu'nun en önemli nedenlerinden biri olarak kabul edilen arı hastalıklarından birisidir. *Nosemaapis* ve *Nosemaceranae*, (microsporidia türleri) nosemosis hastalığının iki etkenidir. Bu hastalık arıların sindirim sistemi bozukluklarına, ortalama ömrünün azalmasına ve koloni kayıplarına neden olur. Bu derleme makalesinde nosemosis hastalığının genel karakteristik özellikleri ve Türkiye'deki durumu hakkında detaylı bilgiler verilmektedir.

Anahtar kelimeler: Bal arısı, Microsporidia, Nosemosis, *Nosema apis*, *Nosema ceranae*, Türkiye

Abbreviations: CCD, Colony Collapse Disorder

1. INTRODUCTION

Turkey has great potential for beekeeping with some features such as climate and wealth of flora. The Beekeeping sector increases the importance of the economy due to these positive factors day by day. According to TUIK, 2017 dates, while Turkey placed in the second row after China with 5 million beehives, placed in the third row after China and Argentina with 82.003 tone honey production. Besides; while China placed in the first row with 46,4 kg, Turkey placed in the sixth row with 17,6 kg according to yield per colony (Tosun, 2012). The contradiction between hives number and honey production is due to bee diseases and pests prevalence which decreases the honey and larvae production, causing bee losses in winter and slow colony development in spring (Dagaroglu, 1999; Tosun &Yaman, 2016).

2. NOSEMOSIS

Microsporidia are obligate intracellular pathogens with a wide range of hosts that are nature infecting all animal phyla commonly insects and other invertebrates (Chen et al., 2009a; Higes, Martin& Meana, 2006). The Phylum Microsporidia has 200 genera and more than 1300 species (Becnel, Takvorian & Cali, 2014). Nosemosis is one of the most important diseases causing economic losses in beekeeping, which is one of the significant reasons for Colony Collapse Disorder (CCD) in the world (Cox-foxter et al., 2007; Paxton, 2010).

Nosema apis and *Nosema ceranae*, the microsporidian species, are the main causative agents of nosemosis in honey bees worldwide (Chen et al., 2009b; Higes et al., 2006; Paxton, 2010; Williams, Shafer, Rogers, Shutler & Stewart, 2008a). *N. apis* was the historic species infecting *Apis mellifera* (Hymonoptera: Apidae) honey bees. However, probably early in this century, *N. ceranae* became an invasive parasite of *A. mellifera*, transferring from Asian honey bees *Apis cerana* (Chen & Wang, 2007; Fries, Martín, Meana, García-Palencia & Higes, 2006; Higes et al., 2006; Huang, Jiang,). In addition, two species can co-infect honey bees which results in the more virulent infection on the host (Paxton, Klee, Korpella & Fries, 2007). These disease factors cause infection in adult bees' intestines, decrease bee life and decrease the honey production capacity of honey bees (Malone & Gatehouse, 1998).

Studies carried out are that *N. ceranae* causes a high rate of colony loss along with severe disease symptoms, unlike *N. apis* (Paxton, 2010). Martín- Hernández et al. (2009) showed that the honey bee individuals infected by *N. ceranae* are able to multiply and spread more rapidly than *N. apis* in suitable environmental conditions. In addition, it has been determined that *N. ceranae* causes nutritional stress in worker bees and causes more deaths (Mayack & Naug, 2009; Naug & Gibbs, 2009; OIE, 2008). Studies on the distribution and environmental resistance of *N. ceranae* show how different it is from *N. apis*. (Fries, 2010).

The characteristic stage of nosemosis infection is the spore stage. The spore contains taxonomically important structures such as polar filament, polaroplast, nuclei, and posterior vacuole. Huang et al. (2007) reported that polar filament forms 20 - 23 spirals in nosemosis spores and polar filament consists of 4 layers and other characteristic factors belong to a typical nosema. Chen et al. (2009a) reported that *N. cerenea* created 18-21 polar filament coils. In Huang (2012) study, the number of coils of the polar filament in *N. apis* spores is 27-30; Higes et al. (2006) and Fries, Feng, Silva, Slemenda &Pieniazek (1996) reported that *N. apis* spores formed 30 polar filament coils, *N. ceranae* spores formed 20-23 polar filament coils. Suwannapong, Maksong, Seanbualuang & Benbow (2010) reported exospore thickness on the sports wall as 25 - 50 nm. Chen & Huang (2010) say that the differences between *N. apis* and *N. ceranae* are

limited by their size and number of polar filament coils. Likewise, Huang (2012) and Brenna Traver, Matthew, Williams, Richard & Fell (2012) reported in their study that nosemosis disease factors were similar except for the number of polar filaments. The development stages of *N. apis* and *N. ceranae* pathogens in host tissues are the same (Fries, 2010; Higes, Garcia-Palencia, Martín-Hernández & Meana, 2007; Chen vd., 2009a). Unlike this, Huang (2012) reported in his study that there may be morphological differences between vegetative stages. Both disease factors are similar in terms of sports morphology, the most important difference in the sport's internal structure is the difference between the number of rings made by the polar filament and the size of the spore. These differences are based on the fact that *N. ceranaae* sports size and the number of polar filament coils are relatively smaller than *N. apis*, but these differences are not sufficient for the characterization of these two disease factors at the species level.

2.1. Symptoms and Tissue Pathogeny

Nosemosis disease has few external symptoms (Bailey, 1967; OIE, 2008; Whitaker, Szalanski & Kence, 2011). The only external symptom that is difficult to detect is behavioral changes. Campbell, Kessler, Mayack & Naug (2010) reported that infected young bees exhibit behaviors of mature bees. The external symptoms of *N. apis* and *N. ceranae* pathogens, which are the two factors of Nosemosis disease, are not very different from each other (Huang, 2012). As the symptoms of this disease, especially in the first months of spring, findings such as the presence of brown stools in front of the hives and honeycombs, the presence of diseased or dead adults at the entrance of the hive, separation of the wings, swelling of the abdomen, not flying and crawling on the ground are accepted (Bailey, 1967; Uygur & Giriskin, 2008; OIE, 2008). *N. ceranae* shows fewer symptoms than *N. apis* pathogen. Therefore, it is very difficult to detect. These external symptoms are evaluated as a preliminary finding and give clues about the presence of the disease.

Light microscopy studies are carried out by examining the fresh tissues dissected directly and comparing the morphological differences in the tissues where the infection is found. Intestinal lumen and epithelium, which is yellow and white and light brown in places in a healthy host, is mildly white or off-white with nosemosis disease, and it is more swollen than healthy intestine (Tosun, 2012). It is determined by the fact that spore structures, which are the characteristic life stage of the microspore pathogen in the tissues of the host, break the light in their way and have a wide oval structure with approximately the same shape and dimensions.

Spores belonging to nosemosis pathogen, morphologically thin oval - shaped small and spore ends are seen as sharp and symmetry. Tosun (2012) determined that *N. ceranae* was 4.9 x 2.83 µm in fresh samples and 4.41 x 2.47 µm in dyed samples. Huang et al. (2007) measured the length of *N. ceranae* spores as 4.5 x 2.4 µm. Chen

et al. (2009a) reported that *N. ceranae* spores are 3.9 - 5.3 µm length and 2.0 - 2.5 µm width. The World Animal Health Organization OIE (2008) reported that *N. apis* spores were 5 - 7 µm length and 3 - 4 µm width and declared with these measurements *N. apis* spores bigger than the *N. ceranae*. Although there are records in the literature that *N. ceranae* spores are smaller than *N. apis* spores as the spore morphology of these two disease factors, the differences that these two disease factors show morphologically are insufficient to distinguish between these two species (Chen & Huang, 2010; Higes et al., 2006, 2007; Fries, 2010).

It is known that nosemosis infection intensely infects the intestinal tissue of honey bees and vegetative stages of the microspore pathogen occur in the intestinal tissue (Fries, 2010; Higes et al., 2006). Chen et al. (2009a) reported that nosemosis infection intensely infects the intestine and body cavity. Martín-Hernańdez et al. (2009) reported that both disease factors did not cause infection in Malpighian tubes and muscle tissues. Besides this information, there are reports in the literature that nosemosis spores of honey bees are detected in salivary glands and secretion cells and Malpighian tubes, adipose tissue and muscle tissue by various methods (Chen et al., 2009a; Klee et al., 2007; Somerville & Hornitzky, 2007). *N. ceranae*spores spread faster in host tissues than *N. apis* spores (Paxton et al., 2007; Martín-Hernańdez et al., 2009). Huang (2012) reported that nosemosis disease factors were similar in terms of tissues infected in the host.

Light and electron microscopy studies are sufficient for the detection of *Nosema* microsporidium, which is the cause of nosemosis infection in honey bees, at the level of genus (Chen et al., 2009a; Fries, 2010; Higes et al., 2007; Huang et al., 2007). This is the most important reason why it is thought that the only cause of nosemosis disease in honey bees in Europe and Asia for many years is *N. apis*. Studies in recent years have been carried out with molecular techniques and the presence of a second disease factor has been determined. It was revealed that *N. apis* records, which were previously defined by light and electron microscopy with the developed molecular techniques, were *N. ceranae*. Molecular characterization is required to determine which nosemosis disease is caused by these two factors in honey bees (Bourgeois, Rinderer, Beaman & Danka, 2010; Higes et al., 2006, 2007; Huang et al., 2007; Klee et al., 2007; OIE, 2008). Almost all of the studies on nosemosis infections detected in Turkey is the light microscopeespecially until 2010.

2.2. Transmission

It is known that the stools in front of the hive caused by the infected bees and the death of infected bees near the hive play an important role in the spread of nosemosis. In many studies, it has been reported that healthy worker bees make direct contact with *Nosema* spores while working to clean the feces in the flying board in front of

the hive. Also, there are many reports that *Nosema* spores are transported to other individuals in the hive after the contact of pollen bees with feces in front of the hive and infected bees (Brenna et al., 2012; Chen & Huang, 2010; Fries, 2010). Fries (1993) reported that feeding and defecation played an effective role in the spread of infection by *N. apis*, and again, Fries (2010) reported that the factor in the spread of *N. ceranae* in hives is unknown. Fenoy, Rueda, Higes, Martín-Hernandez & del Aguila (2009) reported that the honey wax melt in beekeeping and reused in the new season retain the infectivity of the pathogen spores and infect clean hives in the new honey season. With the precautions to be taken, the ways of infection can be cut and the speed of infection can be controlled.

2.3. Presence in the Honey Bee Colony

The number of detailed studies that determine which individuals in the colony occurred in infection studies conducted in Turkey is quite limited. Only Tosun (2012) determined that while worker bees were infected with *N. ceranae* infection, it was not found in male and queen bees. Chen et al. (2009b) and Somerville & Hornitzky (2007) said that nosemosis infection can cause infection in male bees as well as worker and queen bees, but the presence of infection in the colony individuals has not been reported in either study. Besides, Webster, Pomper, Hunt, Thacker & Jones (2004) detected the infection only in worker and queen bees, which are female individuals. Czekońska (2000) detected nosemosis infection only in female individuals. In the experimental study he conducted in the same study, he proved that the infection was transmitted from queen bees to worker bees. Webster, Thacker, Pomper, Lowe & Hunt (2008) in their study, nosemosis spores do not have vertical transmission like other microsporidia; reported no nosemosis in eggs, larvae, and pupae developing in infected queens. Martín-Hernańdez et al. (2009) reported that *N. ceranae* infection is more deadly than *N. apis* infection in worker bees. Malone, Gatehouse & Tregidga (2001) investigated the presence of *Nosema* infection in terms of the number of spores in beehives and bees in charge of collecting pollen and stated that nosemosis infection is different. Also, Brenna et al. (2012) stated that it did not show a significant difference.

2.4. Management

If nosemosis infection is not controlled, it may cause colonies to collapse, especially if the queen bee gets infected (Higes et al., 2008; Martín-Hernańdez, Meana, Prieto, Salvador, Garrido-Bailón & Higes, 2007). Today, the fight against this disease is mostly done in the form of chemical control. Fumagilin-B® (Medivet Pharmaceuticals Ltd.) is used extensively in the fight against nosemosis infection (Williams, Sampson, Shutler & Rogers, 2008b; Bourgeois et al., 2010; Fries, 2010). In addition, physical combat techniques, which are not preferred by beekeepers due to the difficulty of implementation and the need for intense labor, have the potential to be used in combating this disease. For example, the treatment of hive

materials with a temperature of 24 hours at 49 ºC ensures that *N. apis* infection is eliminated (Malone et al., 2001). Nosemosis disease can be easily detected by careful monitoring of symptoms. The most important of these symptoms is the presence of feces in front of the hive. Beekeepers can control the presence of the disease by taking the necessary precautions when they detect these external symptoms. Especially the humidity increases the amount of infection. Controlling the moisture in the hives by the beekeepers will affect the existence of the disease and the disease can be taken under control.

Besides the chemicals, which are widely used to control the infection, it is effective in reducing the presence of nosemosis infection in the measures taken by the beekeepers with their own experience. Among these, the methods used to decrease the moisture content in the hive come first. In addition, it prevents the spread of a possible nosemosis infection in the collection of the dead in front of the hive and cleaning the feces in front of the hive. The presence of *Nosema* spores can be reduced with the method of sterilization for flame cleaning in the hive for spring cleaning in the hive or during the storage process.

2.5. Nosemosis in Turkey

In 1986, the first identification of *N. apis* infection was done in laboratory of "Turkiye Kalkınma Vakfı Arı Hastalıkları" (Tutkun &Inci 1992). In 1988, atotal of 15600 worker bees from 312 apiaries were inspected on light microscopy and the averageinfection rate was reported as 26.4% by Kutlu& Kaftanoglu (1990). In this study, reported that *N. apis* was found in Mugla (31.3%), Adana (29.8%), Dalaman (29.6%), Aydın (28,.6%), Datca (25.7%), Milas (25.0%), Fethiye (23.8%), Koycegiz (23.3%) and Marmaris (20.5%) respectively.

Between 1988 and 1989, Basar (1990) investigated *N. apis* infections of honey bees in Trakya region, Mugla and Istanbul provinces. A total of 9590 worker bees from 126 hives were examined on light microscopy by Basar (1990). The intensity of *Nosema* spores per bee was between 0.5 million and 16 million and the maximum level of infection was reached at spring and winter in the same study. Additionally, the highestintensity ofinfectionwas reported in Trakya region. In another study, Keskin, Basar & Saracbası (1996) examined 7820 honey beein the sameyear (1988-1989) and in the same localities (Trakya regions, Mugla and Istanbul provinces) with Basar (1990). Additionally, Keskin et al. (1996) reported that the highest density of *Nosema* infection was observed from April to November.

In 1999, Ozbilgin, Alatas, Balkan, Ozturk & Karaca (1999) reported that*Nosema*infection ratewas 2% for the Aegean Region of Turkey.

In 2001, the nosemosis infection research reported by Ozkırım &Keskin (2001) in Anzer locality has been regarded as one of the most important studies for Turkey. Because the "Anzer honey" which is produced in Anzer locality of Rize province is the most famous and expensive

in Turkey. In that study, Ozkırım & Keskin (2001) reported that *N. apis* infection was observed on light microscopy in Anzer, but they did not report infection rate in their study. In another study, Aydın, Gulegen &Cetinbas (2001a, 2001b) found that the prevalence of *N. apis* spores was 26.4% in Bursa province, and 26.25% in the South Marmara Region of Turkey. Additionally, Cengiz & Genc (2001) reported that nosemosis infection rate was 4.48% in Erzurum according to a survey conducted. In another study conductedin the same year, the prevalence of nosemosis infection was reported as 4% in the center of Elazıg, 4% in Baskil and 10% Sivrice localities in a study conducted in Elazig province by Simsek, Dilgin & Gultekin (2001). Kutlu & Gazioglu (2008) reported that a total of 47 of 122 hives were infected with nosemosis which infection rates varied from 52.9% to 25% and the average contamination rate 38.5% in Bingol provinces.

In 2002, nosemosis infection rate was reported for the Black Sea Region of Turkey in beekeeping apiaries was 30.95% (Yasar, Guler, Yesiltas, Bulut & Gokce, 2002). The presence of nosemosis infection was determined by Aydın, Cakmak, Gulegen & Korkut (2003) with a survey conducted with 50 beekeepers in the Bursa and Yalova provinces of South Marmara Region in March 2002.

In 2003, Cakmak, Aydın, Seven & Korkut (2003a) and Cakmak, Aydın & Gulegen (2003b) reported nosemosis infection rate as 24% in 217 hives in the South Marmara Region. In another study, Kutlu & Ekmen (2003) inspected 1220 worker bees from 122 hives in Bingol provinces and reported that nosemosis infection rate was between 25% and 52.9% (average 38.5%) in 2003.

In 2004, Topcu & Aslan (2004) observed *N. apis* infection in 54 of 343 (15.74%) honey bee hives which were examined in terms of nosemosis in the Kars province. In the same study, nosemosis infection rates were reported as 28.0% in Kagızman, 20.69% in Selim, 18.56% in Kars Center, 18.33% in Susuz, 15.79% in Digor, 13.04% in Arpacay, and 6.82% in Akyaka localities, and also no infection was recorded in Sarıkamıs locality by Topcu and Aslan (2004). Additionally, *N. apis*infection was found at the highest levelinMay-June in Kars (Topcu & Aslan 2004).

Furthermore, from the year 2002 to 2004,the percentage of Nosema infection wasreported as8.77% inElazıg province by Simsek (2005).

In 2005, Aydin, Cakmak, Gulegen &Wells (2005) reported that Nosema infection rate was identified on light microscopy as 60% of the apiaries sampled from seven regions in Turkey. Marmara and Black Sea Regions have higher infection rates than other regions in Turkey. There was no infection in the Southeast Anatolia Region. Additionally, the temperature was a significant factor in the presence of nosemosis disease. And also rainfall and humidity factors are more effective than temperature factors on nosemosis infection (Aydin et al., 2005). In

another study, the presence of nosemosis without specifying the species name was reported as an average of 6.5% in Edirne, Tekirdag, Kırklareli, Istanbul and Canakkale provinces in Trakya and Marmara Regions by Sıralı & Dogaroglu (2005). Soysal & Gurcan (2005) reported that 9% of beekeepers had apiaries infected with Nosema disease in their questionnaire study in Tekirdag in 2005.

In 2007, nosemosis infection rates varied from 25% to 54.16% (average rate of 42.45%) in 68 of 147 apiaries that reported by Kutlu & Gazioglu (2008). Besides reported that nosemosis illness showed an increase of 10.25% in 2007 compared to 2001.

Between the years 2003 and 2007, Giray, Kence, Oskay, Doke & Kence (2010) reported that Nosema infection *(N. apis* or *N. ceranae* is not specified) was accounted for 9% of colony losses among all causes in Turkey especially from 2006 to 2007.

In 2009, the queen honey beesinfected with*Nosema*sp. was reported for the first time by Muz & Muz (2009) in Hatay. Yalcınkaya, Keskin & Ozkırım(2009) investigate 3880 adult honeybee from Adana province and 3520 adult honeybee from Hatay province, and published nosemosis (without the name ofthe species) infection as 12.97% in 2009. Gul & Kutlu (2009) investigated the presence of Nosema disease in six localities in Bingöl province and reported *Nosema* infection rate as 8.41% in 2009.

Between the years 2007 and 2009, the first study aboutthe moleculardiagnosis of nosemosis was reported by Muz, Girisgin, Muz & Aydın (2010). In that study, Muz et al. (2010) reported that Hatay province had 89% *N. cerana* and 11% *N. apis* infections, in addition to the Marmara region were found to be 84% *N. cerana* and 16% *N. apis* infections.

From the year 2010, many scientists have begun to use molecular techniques to determine the factor (*N. apis* or *N. ceranae*) that causes nosemosis disease in Turkey. As mentionedabove, the first*N. ceranae*infection in honey bees in Turkey was detected from thespecimenscollected from the Marmara region between the years2007 and 2009by Muzet al.(2010). Utuk, Piskin & Kurt (2010) reported the presence of *N.ceranae*infection in Giresun and Sivas provinces in 2010. In that study, the infection rate was not reported butthe existence of *N. ceranae* was mentioned (Utuk et al. 2010). In the same year, Whitakeret al.(2011)reported the distribution of*N. ceranae* fromTurkey for the first time. The percentage ofnosemosis diseasewasdetermined as8.3% in Turkey by Whitakeret al.(2011) in 2010. In the same study, Whitakeret al.(2011)determined that the percentage ofinfection caused by*N. apis* was 4.7% in 4 of 20 provinces (Sivas, Izmir, Bitlis and Gaziantep), whilethe percentage ofinfection caused by*N. ceranae* was3.5% in 3 of 20 provinces (Artvin Hatay and Mugla) in Turkey in 2010. Any nosemosis infection was not observed in Gokceada locality in Canakkale province, Kırklareli, Bursa, Sakarya, Duzce, Giresun,

Ankara, Gaziantep, Adıyaman, Diyarbakır Batman, Sırnak and Erzincan provinces in 2010.

From 2006 to 2011, Utuk, Piskin, Girisgin, Selcuk & Aydın (2016) reported that *N. apis* infection as 6.25% in Cankırı province and 93.75% in Ankara, Bursa, Erzurum, Kayseri, Mugla, and Zonguldak provinces for Turkey.

From 2009 to 2011, *N. ceranae* was determined as the only factor of nosemosis in the Eastern Black Sea Region of Turkey with molecular techniques by Tosun (2012). A total of 5330 dead worker bees, 559 dead male bees and 4 dead queen bees collected from 20 different localities in Artvin, Rize, Trabzon, Giresun, Ordu, Gumushane and Bayburt provinces were examined for nosemosis and only worker bees were observed to be infected (Tosun, 2012). *N. ceranae* infection rates were reported as 4.72%, 15.28% and 21.23% in 2009, 2010 and 2011, respectively, the total infection was 20.59% and highest infection rates were observed in June and July (Tosun, 2012). Also, Tosun & Yaman (2016) reported that *N. ceranae* infectionwas affected bychangingtemperature and humidityfactors around the hives. Additionally, the humidity was more effective than the temperature factor on *N. ceranae* infection.

Between 2010 and 2011, Muz, Solmaz, Yaman & Karakavuk (2012) determined 10% of Nosema disease ofhives in winteringseason in Hatay province.

Between 2011 and 2012 Ivgın Tunca, Oskay, Gosterit & Tekin (2016) reported that *N. ceranae* infection observed in Izmir, Aydın, Mugla, Tekirdag, Kirklareli, Zonguldak, Artvin Isparta, Adana and Kırsehir range of 8.8-100% rates. The main point is that the article all samples were negative for *N. apis*.

In 2015 *N. ceranae* infection was reported with 3.28% in Ordu province by Guner, Erturk & Yaman (2019). Additionally, Oguz Karapınar, Dincer & Deger (2017) reported Nosema spp. Infection rate as 32.5% in Van Province.

From 2009 to 2016 Ozkırım, Shiesser & Keskin (2019) made research on the presence of nosemosis infection in 72 provinces of Turkey. They found three types of infection such as single *N. apis* infection, single *N. ceranae* infection and mixed infection with both species. *N. apis* infection rates reported as 16.3% in 2009, 8.8% in 2010, 21.7% in 2011, 29.2% in 2012, 20.5% in 2013 19.7% in 2014, 22.5% in 2015 and 22.3% in 2016. For *N. ceranae* 63.4% in 2009, 72.6% in 2010, 32.3% in 2011, 26.8% in 2012, 33.1% in 2013 34.2% in 2014, 28.5% in 2015 and 31.9% in 2016 rates were reported. Additionally, co-infection with both species 20.3% in 2009, 18.6% in 2010, 46% in 2011, 44% in 2012, 46.4% in 2013 46.1% in 2014, 49% in 2015 and 45.8% in 2016 reported in that study. According to the data in that study, especially winter conditions changed the rates of nosema infection levels in colonies.

3. RESULTS AND DISCUSSION

Turkey has a geographical location that connects Asia to Europe. Trade and globalization play an

important role in the rapid spread of nosemosis infection all over the world. In bees with nosemosis infection, the appearance is quite similar to the two disease factors in external symptoms such as intestinal and abdominal changes. The development stages of *N. apis* and *N. ceranae* pathogens in host tissues are the same. The differences between *N. apis* and *N.ceranae* are limited by the size of spores and the number of polar filament rings. Although there are records in the literature that *N. ceranae* spores are smaller than *N. apis* spores as the spore morphology of these two disease factors, the differences that these two disease factors show morphologically are not sufficient to characterize at the species level.

Light and electron microscopy studies are sufficient for the detection of *Nosema* microsporidium in the genus level, which is the cause of nosemosis infection in honey bees. For nosemosis, molecular characterization is required to determine to differentiate the disease factor in species level. In Turkey, this disease was mostly determined by looking at the spore morphology by light microscopy. In most of these studies, while there was no emphasis on the disease factor, only a few studies were accepted as *N. apis*. There are very few studies on whether the cause of nosemosis disease occurring in bee colonies in different regions of our country is *N. apis* or *N. ceranae.*

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