TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 838-844

doi:10.3906/vet-2001-20

Investigation of propolis in terms of hygienic quality, some pathogenic bacteria and *Nosema spp*.

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Received: 06.01.2020 • **Accepted/Published Online:** 26.05.2020 • **Final Version:** 18.08.2020

Abstract: Propolis collected from plants by honeybees is used for the construction and protection of hives. In addition, propolis has been used in the treatment of many diseases since ancient times because of its antimicrobial, antiseptic, antiinflammatory and antioxidant properties. Despite all these positive health effects, propolis can be microbiologically risky for many reasons such as environmental contamination and insufficient personnel hygiene. Therefore, chemical, physical, antimicrobial properties as well as microbiological properties are among the parameters to be investigated. This study aims to explore propolis's initial bacteriological and parasitological flora using 5 different parameters (total coliform group, *Escherichia coli*, *Staphylococcus aureus*, *Clostridium botulinum* and *Nosema* spp.). For this purpose, 100 propolis samples produced in Bolu and its districts were collected. Another objective is to determine the bacteriological/parasitological risk factors and contamination ways of the aforementioned pathogens in the propolis producing process. According to the results, 14 (14%) of total coliform group, 5 (5%) *E. coli,* 38 (38%) *S. aureus,* 11 (11%) *C. botulinum* and 8 (8%) *Nosema spp.* were found to be positive. The data obtained shows that propolis can be contaminated with some microorganisms and parasites during both production and collection processes.

Key words: Propolis, pathogenic bacteria, *nosema*, contamination sources

1. Introduction

Propolis (bee glue) is defined as the general name of the resinous substances that are collected from various flowers/plants by bees. In Greek, pro means "defense" and polis means city; therefore, propolis can be defined as a hive city and/or city of bees [1]. Propolis is used for instruction, caring, and protection of the hives [2]. Propolis is also a natural bee product that is used against pathogens in both human and veterinary medicine [3,4,5]. Ancient Romans and Egyptians widely used propolis for medication [6]. Propolis has also antiseptic, antiinflammatory, antimycotic, anticancer, antioxidant, antibacterial, antifungal, antiviral, and antiprotozoal properties [3,7,8]. Additionally, it is used widely for the treatment of mouth diseases, heart diseases, and protection of diabetes, nondegenerative diseases, and some cancer types [9,10,11]. Because of the common properties mentioned above, propolis is still being used as a mouth washing agent, throat drops, dietary supplement, and cosmetics/haemopoietic agent alone or with foods and/or drugs at present [6,12].

Propolis may have various colors, odors, components, and efficiency due to being collected from diversified trees and/or shrubs [4,13]. A bioactive fragment of propolis consists of flavonoids, phenolic components, esters, and terpenoids [7,14]. Propolis products may also have structural differences due to different geographic regions. This situation may affect the quality and medical usage of propolis; therefore, chemical and microbiological analyses definitely must apply to all propolis products to protect public health [4,8].

Worker bees produce propolis by mixing the materials they obtain from trees such as pine, oak, eucalyptus, and some herbaceous plants with pollen and enzymes in their mouths and store them in various places in the hive [15,16]. Bees store propolis behind the bottom board, the frame edges, and the entry hole in the hive. Afterwards, propolis is collected by beekeepers to be processed or used in raw form. Propolis, which can be exposed to many sources of contamination during both production and collection, may be a carrier for some microbial or parasitological factors.

Escherichia coli (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and coliform analysis must be applied to propolis for determining microbiological quality and to expose

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staff hygiene [17]. As is known, *S. aureus* is an important pathogen due to its toxins, antibiotic resistance, and invasion properties [18]. Furthermore, for being an indicator microorganism of fecal contamination, *E. coli* analysis must not be ruled out for propolis products as for all kinds of foods or food supplements [19].

Clostridium botulinum (*C. botulinum*) is a toxic and infectious microorganism and its toxins are defined as a paralytic cocktail for the hosts. *C. botulinum* spores may contaminate propolis via dust in the air, gastrointestinal systems of the bees, pollens, legs of the bees, and contaminated bee foods. Thus, *C. botulinum* analysis of propolis is important for the protection of consumer health [20].

One of the most important parasitological diseases of the bees is nosemosis and the spores of *Nosema* generally contaminates honey/bees by various insects, flowers, pollens, contaminated bees, and feces of the bees, or contaminated water sources [21]. Nosemosis causes colony losses due to bee deaths and queen failure. Also, *Nosema* is one of the factors that cause colony collapse disorder (CCD) and it can leave the hive open to other pathogens as a result of immune system suppression [22, 23].

Bolu is an important district because of its honey production capacity and geographical region. Although Bolu is included in the West Black Sea Region of Turkish Republic, it is also very close to both the Marmara and Middle Anatolian regions. Therefore, the district is affected by the geographical features of both regions, and honey producers all over Turkey may visit the Bolu district seasonally for its suitable natural structure for honey production. Because of the reasons above, this study aims to expose the microbiological quality and contamination profile of the propolis originating from the Bolu district.

2. Materials and methods

One hundred samples of propolis (n = 100) (50 g) were collected from different fixed comb and active beehives in Bolu. Propolis samples were obtained at different times under hygienic conditions. Samples taken from raw propolis material sticking to the flight hole and the frames in hives were examined. Propolis was delivered aseptically to the laboratory in a cool box at less than 4 °C.

2.1. Sample preparation

Ten g of each propolis sample were aseptically taken and homogenized with 90 mL of saline water. Serial decimal dilutions were then prepared from this initial homogenate in the same chilled sterile diluents.

2.2.Bacteriological and parasitological analysis

Samples were analyzed for their microbiological quality and safety as well as the prevalence of selected bacterial pathogens.

S. aureus strains were isolated using Baird–Parker agar with 5% egg yolk tellurite emulsion and incubated at 37 °C for 24–48 h. Typical colonies (i.e. gray to jet-black, surrounded by opaque zone and frequently with an outer clear zone) were transferred to DNase (deoksiribonukleik) agar to determine DNase activity and incubated at 37 °C for 24 h. After incubation, 1 N hydrochloric acid was poured on the plates, and colonies with clear color were considered DNase positive. Positive colonies were confirmed by coagulase tests [24].

Total coliform bacteria were detected using violet red bile agar (VRBA). The samples were plated on VRBA then overlaid with 8-10 mL of melted, cooled VRBA and incubated at 35 °C for 18–24 h. Purple–red colonies that are 0.5 mm or larger in diameter and surrounded by a zone of precipitated bile acids were counted [25].

E. coli were isolated using tryptone bile X-glucuronide agar and incubated at 44 °C for 24 h. Typical colonies were confirmed by 4-methylumbelliferyl-β-D-glucuronide (MUG) test, which is based on the enzymatic activity of β-glucuronides [26].

C. botulinum were isolated using cooked meat medium and trypticase-peptone-glucose-yeast extract broth for 5–10 days at 35 °C and 28 °C, respectively. After the colonies were confirmed, positive strains were plated on egg yolk agar and grown anaerobically for 48 h at 35 °C [27].

Nosema spp. spore were detected under a microscope. A 1 mL sample and 1 mL distilled H_2O were mixed and counted in a hemocytometer (Neubauer chamber) by microscopic method [28].

2.3. Statistical analysis

Kendall's tau–b correlation coefficient was used to compare the correlations between bacteriological and parasitological parameters.

3. Results and discussion

Propolis, which has high bioactive properties and antioxidant activities, has been considered as a therapeutic agent from ancient times [29]. Although many studies have been conducted of the antimicrobial, antioxidant, and chemical composition of propolis, studies of the microbiological properties of propolis have not been encountered.

The aim of this study was to expose the microbiological contamination for some important food and bee borne pathogens profile of propolis originated from the Bolu district, which is one of the most important honey production regions of the Turkish Republic. Propolis may also be contaminated by bee equipment and during packaging, transportation, and sales periods secondarily. From this point of view, the 100 propolis samples that were collected from the Bolu district were analyzed for

coliforms, *E. coli*, *S. aureus*, and *C. botulinum*. According to the results, 14 samples (14%) for coliforms, 5 samples (5%) for *E. coli*, 38 samples (38%) for *S. aureus*, 11 samples (11%) for *C. botulinum*, and 8 samples (8%) for *Nosema spp*. were evaluated as positive. The results of statistical analysis are given in Table 1, and distribution of analysis results of the Bolu districts are given in Table 2.

Although it is indicated that propolis has an inhibitive effect on many various microorganisms under in vitro conditions, many scientists revealed that propolis has significant inhibitive effects on gram positive bacteria, while it does not have a wide effect on gram negative microorganisms [30,31]. It has been reported that propolis ethanol extract has a high antibacterial effect against gram positive cocci (*S. aureus*), but it has a low effect against gram negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) [32,33,34].

There is no research on propolis contamination with coliform and *E. coli*. However, since our study is also a honey product, we have tried to compare our results with the results in honey. Iurlina and Fritz exposed that the honeys that are sold in the Argentina market were positive for fecal coliforms [35]. Saha et al. determined 8% prevalence of *E. coli* in honey samples [36]. Our results are similar to the aforementioned researchers' studies. Probable reasons for our results may originate from microbiological pollution from different environmental sources like staff, soil, surfaces, and equipment and secondary contaminations by pathogens to the honey products. Coliforms, *E. coli*, and its serovars are not

Table 1. Demonstration of dual relationships in terms of bacteriological and parasitological parameters by Kendall's tau–b correlation analysis.

Parameter	Correlation coefficient / Significance	Total coliform bacteria	*E. coli*	*S. aureus*	*C. botulinum*	*Nosema spp.*
Total coliform bacteria	Correlation coefficient	1.000	0.377	0.231	0.478	0.477
	Sig. (2-tailed)	--------	**0.000***	**0.003***	**0.005***	**0.000***
E. coli	Correlation coefficient	0.292	1.000	0.366	0.813	0.481
	Sig. (2-tailed)	**0.000***	--------	**0.004***	**0.000***	**0.000***
S. aureus	Correlation coefficient	0.804	0.495	1.000	0.848	0.276
	Sig. (2-tailed)	0.199	0.528	--------	0.721	0.186
C. botulinum	Correlation coefficient	0.722	0.599	0.182	1.000	0.191
	Sig. (2-tailed)	**0.005***	**0.000***	**0.000***	--------	0.444
Nosema spp.	Correlation coefficient	0.443	0.138	0.785	0.092	1.000
	Sig. (2-tailed)	0.490	0.602	0.229	0.158	--------

*There is a statistically significant and positive correlation between the parameters written in bold black.

Table 2. Distribution of microbiological and parasitological analysis results of the Bolu district.

Parameter / District	Total coliform bacteria	*E. coli*	*S. aureus*	*C. botulinum*	*Nosema spp.*
Bolu center	2	1	4	2	1
Gerede	-	-	3	1	-
Göynük	1	-	3	-	1
Kıbrıscık	2	2	5	2	1
Mengen	2	-	4	1	1
Mudurnu	-	-	4	1	-
Seben	3	1	7	2	2
Yeniçağa	4	1	8	2	2
Total	**14**	**5**	**38**	**11**	**8**

existent in honey under normal conditions. However, the mentioned microorganisms can survive in honey if they contaminate the hives by primary and/or secondary sources. The incidence of *E. coli* and coliforms in honey or honey products may increase or decrease due to various environmental parameters [37].

Unfortunately, there are limited studies about both the inhibitory effect and contamination profile of propolis. Despite these limited medical literatures indicate the high–level inhibitory effect of propolis against *S. aureus* [30,38], our results differ from studies that were performed. According to the results of the study, the number of *S. aureus* positive samples were 38 (38%) and it was thought that this high rate of *S. aureus* contamination originated from staff hands and/or hand contaminated equipment. One of these presumptive reasons for contamination may also be welded from the contents of propolis due to different environmental conditions. Because of the lack of studies of the microbiological quality of propolis we cannot compare our results with any other research about *S. aureus.* Not only propolis but also honey can be contaminated with *S. aureus* because of insufficient hygienic conditions. Adebayo and Banjo isolated *S. aureus* from honey samples in Nigeria and Dümen et al. investigated a 13.4% prevalence of *S. aureus* in honey samples in Turkey [39,40].

Another microbiological parameter that was analyzed in our study was *C. botulinum* and, according to the results, 11 propolis samples (11%) were evaluated as *C. botulinum* positive. The most critical clinical cases originated by the agent via bee products is "infant botulism". Although the risk factors in infant botulism are quite multifarious, honey and infant formulas contaminated by the babies are the main causes [41]. Information about propolis is inadequate in world literature unfortunately. Du et al. investigated 152 honey samples and they determined that 2 of these samples were positive for *C. botulinum* [42]. Nevas et al. also analyzed 190 honey samples and they indicated that 20 of the total samples were positive for *C. botulinum.* [43]. In the other study, 216 of 1168 samples collected in relation to honey, and pollen, hives, and bees were found to be positive [44]. In Turkey, Küplülü et al. determined that 6 honey samples out of 48 (12.5%), Koluman et al. indicated that 19 samples out of 250 (7.6%), and Gücükoğlu et al. found that 4 samples out of 150 (2.6%) tested positive for *C. botulinum* [45,46,47]. In Lithuania and Poland, Wojtacka et al. analyzed 48 and 102 honey samples respectively, and 30 samples (60%) in Lithuania and 22 samples (21.6%) in Poland were identified as *C. botulinum* positive [48,49]. A study of the detection of infant botulism in honey and honey products shows that not only honey but also many honey products such as pollen, bees, beeswax, and feeding sugar also are *C. botulinum* positive [50]. If it is looked closely, all the aforementioned studies are about honey and the studies about the existence of *C. botulinum* in propolis are almost absent in medical literature. As in the world, there is not a study of infant botulism and propolis in our country, unfortunately. However, according to our results, it is considered that there may be a lot of infant botulism cases that cannot be diagnosed, understood, detected, and/or hospitalized in our country. Also, our results show that *C. botulinum*, which causes infant botulism, can be transmitted to humans not only with honey but also with the consumption of propolis. In this case, it is necessary to pay attention to the consumption of honey especially under the age of 1, as well as honey products such as propolis.

Another analyzed parameter in the study was *Nosema spp*. and 8 propolis samples (8%) were evaluated as positive. *Nosema* disease shows up itself by contamination of *Nosema apis* and/or *Nosema ceranae* in adult honeybees and the infection is generally called as nosemosis [51,52]. Nosemosis may cause a decrease in colony efficiency and productivity, and an increase in colony losses. In our study, species differences in *Nosema spp*. were ignored and the samples that were contaminated by one of the aforesaid species were evaluated as nosemosis positive. There is not a study in world medical literature on the existence of *Nosema spp.* in propolis. All the studies of *Nosema spp.* incidence are in honeybees and honey.

In a study that was done in the Elazığ district, the prevalence of nosemosis was determined at 8.77% while it was about 24% in the Southern Marmara District of the Turkish Republic [53,54]. According to the various studies, the general prevalence of nosemosis in different districts are as follows: 15.7% in Kars and 10% in Hatay [55,56]. Nosemosis is also a widespread parasite throughout the world as in Turkey and causes serious hive losses. Traver and Fell analyzed 293 hives and determined a 37.5% prevalence of nosemosis in Virginia, USA [57]. Varis et al. studied 39 hives and found 11 hives as nosemosis positive in Finland, while Chauzat et al. said that the prevalence of *N. ceranae* in France is about 65.6% [58,59]. During the *Nosema spp*. analysis, we also aimed to see adult spores of *Varroa* and *Malpighamoeba* parasites. However, since no positive findings were found in the analysis results, no extra information was given about these parasites.

Propolis is an important bee product because of its positive effects on the human immune system, nutritional features, and high energy potential. Honey and propolis being produced in the Turkish Republic are qualified because of the country's ecosystem and herbal fauna. Both in our country and in the world, the studies of honey products including foodborne pathogens, viruses, parasites, and the risk factors that threat consumer health are limited. When the data obtained in our study were

evaluated, it was seen that the hygienic quality of propolis, which is an important honey product, was changed as a result of primary or secondary contamination. In order to obtain a more hygienic product, special attention should be paid especially to the equipment, packaging materials, personnel hygiene, production, and sales conditions should be improved, and hygiene training should be given to beekeepers.

Our study is an important one that reveals the microbiological and parasitological profile of the propolis samples, but it is thought that further study is needed. It is concluded revealing correlations of the propolis pathogens each other, exposing the contamination ways, determining the behaviors and molecular genetic structures of the propolis contaminants would be useful to increase the exportation, develop food security systems for bee products, and protect public health.

Acknowledgment
This study was supported by the Research Fund of the University of İstanbul, İstanbul, Turkey (Project No: 51329).

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