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Microbiological and parasitological quality of honey produced in İstanbul

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Abstract: In this study, 500 honey samples (with combs) were collected directly from the hives in various parts of İstanbul. The presence and the interactions of different microbiological and parasitological parameters (coliforms, *Escherichia coli*, *Staphylococcus aureus*, *Ascosphaera apis*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Paenibacillus larvae*, *Melissococcus pluton*, and *Nosema* spp.) were investigated. According to the results, 80 samples (16%) tested positive for coliforms, 18 samples (3.6%) for *E. coli*, 67 samples (13.4%) for *S. aureus*, 51 samples (10.2%) for *A. apis*, 22 samples (4.4%) for *A. flavus*, 32 samples (6.4%) for *A. fumigatus*, 16 samples (3.2%) for *P. larvae*, 29 samples (5.8%) for *M. pluton*, and 39 samples (7.8%) for *Nosema* spp. The results showed that there were significant correlations among all the binary relationships of microbiological parameters (coliforms, *E. coli*, *S. aureus*, *P. larvae*, and *M. pluton*), while no interactions were detected among the parasitological parameters (*A. apis*, *A. flavus*, *A. fumigatus*, and *Nosema* spp.). Additionally, no significant relations were determined between parasitological and microbiological parameters.

Key words: Honey, foodborne pathogens, foodborne parasites

1. Introduction

Honey contains approximately 80% carbohydrates (35% glucose, 40% fructose, and 5% sucrose) and 20% water, serving as an excellent source of energy. It also contains more than 180 substances, including amino acids, vitamins, minerals, enzymes, organic acids, and phenol compounds (1). Honey contains some nonhydrogen peroxide-based phenolic compounds, like benzoic acid and flavonoids, as well as a minimal amount of foodborne pathogens. However, the antimicrobial activity of honey is not sufficient because of low water activity.

Honey has several sources of microbiological and parasitological contamination. Primary sources include pollen, the digestive tracts of honey bees, dust, air, soil, and nectar, and these are somewhat difficult to eliminate. On the other hand, secondary sources, due to honey handlers and processing, are easier to control by the application of good manufacturing practices.

The major microbiological and parasitological contaminants of honey include molds and yeasts, as well the spores of *Bacillus* spp., *Clostridium* spp., *Escherichia coli*, *Staphylococcus aureus*, *Ascosphaera apis*, *Nosema* spp., *Aspergillus flavus*, and *Aspergillus fumigatus*, their counts being indicative of honey's commercial quality and safety (2). The Codex Alimentarius Standard (3) for honey quality includes several chemical and physical parameters. However, EU legislation lacks specifications concerning microbiological or parasitological contamination in honey, as well as the hygiene of the product. In spite of various studies about the physicochemical features of honey (4,5), microbiological contamination has not been extensively investigated.

İstanbul generates more than 1% of the total honey production of Turkey with about 60,000 active hives. These hive numbers increase in the summer season due to the arrival of nomad beekeepers to obtain chestnut and sunflower honey. This situation exacerbates the existing risks of food; bee-originated pathogens and fungal agents can be transferred to the honey and may eventually risk the consumers' health. The aim of this study was to investigate the microbiological and parasitological quality of honey samples in İstanbul.

2. Materials and methods

2.1. Sample collection

A total of 500 honey samples (with combs) were collected directly from the hives in various parts of İstanbul (Table 1). The collected samples were put in sterile plastic bags

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Table 1. Sampling program and detailed data about the collected honey.

District	Region	Sample	Number of collected samples
Avcılar	European zone of İstanbul	Honey (with comb)	50
Büyükçekmece	European zone of İstanbul	Honey (with comb)	50
Çatalca	European zone of İstanbul	Honey (with comb)	50
Eyüp	European zone of İstanbul	Honey (with comb)	50
Silivri	European zone of İstanbul	Honey (with comb)	50
Beykoz	Anatolian zone of İstanbul	Honey (with comb)	50
Kartal	Anatolian zone of İstanbul	Honey (with comb)	50
Sultanbeyli	Anatolian zone of İstanbul	Honey (with comb)	50
Şile	Anatolian zone of İstanbul	Honey (with comb)	50
Tuzla	Anatolian zone of İstanbul	Honey (with comb)	50
Total			500

and they were transported to the laboratory in cold boxes at 4 °C.

2.2. Microbiological analysis

Coliforms, *E. coli*, *S. aureus*, *Melissococcus pluton*, and *Paenibacillus larvae* levels were determined for each honey sample. Microbiological analyses were performed according to standards set by the Food and Drug Administration Bacteriological Analytical Manual (6).

For this purpose, 25 g of honey samples was aseptically transferred into a sterile bag containing 225 mL of physiological saline water and was homogenized for 3 min (Stomacher BA 6021). The mixtures were diluted to different concentrations for analysis. Following homogenization, 10-fold serial dilutions were made in sterile maximum recovery diluents and then inoculated on to specific culture media.

Coliforms were isolated by surface plating on violet red bile agar (Merck 1.01406). Plates were incubated at 37 °C for 24 h (6). *E. coli* were examined by surface plating on TBX Agar (Merck 1.16122). Plates were incubated at 44 °C for 24 h for enumeration (6). S. *aureus* was determined by surface plating on Baird Parker Agar (Merck 1.05406) supplemented with egg yolk–tellurite emulsion (Merck 1.03785). Spread plates were incubated at 35 °C for 46–48 h. Colonies with typical S. *aureus* morphology were examined microscopically following Gram staining and tested for catalase and coagulase activity (6). For determining *P. larvae* and *M. pluton*, homogenized and diluted samples were placed in a water bath at 80 °C for 30 min. They were then centrifuged for 20 min at 2500 rpm to condense the spores. After removing the supernatant, the agglutinated part was transported to tryptic soy broth (TSB; Merck 1.05459). TSB was incubated at 36 °C for 48 h in 10% CO_2-included media. Broths with turbidity were considered suspicious. Loopful samples were inoculated to blood agar base (Merck 1.10886; with 5% sheep blood) from the suspicious broths and they were incubated at 36 °C for 72 h in 10% CO_2-included media. Gram staining was performed for the grown colonies and gram-positive bacteria were evaluated as *P. larvae* and *M. pluton*. The Holst milk test was used to differentiate 2 agents from each other (7).

2.3. Parasitological analyses

A. apis, *A. flavus*, *A. fumigatus*, and *Nosema* spp. were determined for each honey sample. *A. apis* was isolated by surface plating on rose Bengal chloramphenicol agar (Merck 1.00466), Czapek Dox agar (Merck 105460), and malt extract agar (Merck 1.05398), separately. Plates were incubated for 7 days at room temperature (25 °C). At the end of the incubation period, white, cotton-like colonies of 5–7 cm in diameter were evaluated as suspected colonies. Additionally, microscopic examinations were applied to the suspected colonies for verification (8). For this purpose, 25 g of honey samples was aseptically transferred to a sterile bag containing 225 mL of physiological saline water and was homogenized for 3 min (Stomacher BA 6021). Following homogenization, 10-fold serial dilutions were made in sterile maximum recovery diluents and inoculated on to specific culture media.

A. flavus was examined by surface plating on Czapek Dox agar and *Aspergillus* differentiation agar base (Oxoid CM0731) separately. Plates were incubated for 7 days at room temperature (25 °C). As a result, yellowish-green colonies of 5–7 cm in diameter on *Aspergillus* differentiation

agar and green colonies with white centers on Czapek Dox agar were evaluated as suspected colonies. Microscopic examination was used for verification. Sabouraud 4% dextrose agar (Merck 1.05438) was used for *A. fumigatus*. Plates were incubated for 7 days at room temperature (25 °C). For the identification of colonies that had significant growth, morphological and microscopic examinations were performed (6). Microscopic examination was applied for the identification of *Nosema* spp. After homogenization, 1 mL of distilled water was added to a 1-mL honey sample. A Neubauer instrument (Marienfeld C964130) was used for identification. The Neubauer LAM has 4 zones on it, and each zone consists of 16 squares. The volume of every zone is 0.1 µL. After counting the spores in the given 4 zones, the average number of spores for every zone was calculated. Thus, the number of spores at 0.1 µL was obtained. The obtained number was then multiplied by 10^4 to get the number of spores in 1 mL (7).

2.4. Statistical analysis

Kendall's Tau b relationship method (9) was used to determine the binary relationship of each parameter. Kendal's Tau b relationship is a relationship method, using 2 × 2 tables, for when the data are not regular for all the samples. In our study, because the data distributions were nonnormal, Kendall's Tau b relationship method was used.

3. Results

In this study, 80 samples tested positive (16%) for coliforms, 18 samples (3.6%) for *E. coli*, 67 samples (13.4%) for *S. aureus*, 51 samples (10.2%) for *A. apis*, 22 samples (4.4%) for *A. flavus*, 32 samples (6.4%) for *A. fumigatus*, 16 samples (3.2%) for *P. larvae*, 29 samples (5.8%) for *M. pluton*, and 39 samples (7.8%) for *Nosema* spp. (Table 2). The results showed that there were significant correlations among all the binary relationships of microbiological parameters (coliforms, *E. coli*, *S. aureus*, *P. larvae*, and *M. pluton*), while no interactions were detected among the parasitological parameters (*A. apis*, *A. flavus*, *A. fumigatus* and *Nosema* spp.).

4. Discussion

Coliforms and *E. coli* are indicators of fecal contamination and poor hygienic conditions in foods. Gomes et al. (10) isolated *Salmonella* spp., coliforms, and *E. coli* in Portugal at a rate of 34%. Kokubo et al. (11) analyzed 70 honey samples and isolated coliforms at a rate of 95.7%. Unfortunately, the studies about coliforms and *E. coli* in honey are limited in Turkey. However, our findings are parallel to the aforementioned studies of Kokubo et al. and Gomes et al. Additionally, it was determined that coliforms and *E. coli* had significant positive correlations with *S aureus*, *P. larvae*, and *M. pluton*. In this study, coliforms ranged from 1.2×10^1 to 6.2×10^3 cfu/g and *E. coli* ranged from $<10^1$ to 3.4×10^2 cfu/g. The results may be related to fecal contamination and environmental conditions.

S. aureus is the causative agent of the numerous outbreaks of foodborne disease worldwide. Toxification generally takes place by intake of enterotoxins via the alimentary canal. In this study, *S. aureus* ranged from $<10^1$ to 1.1×10^4 cfu/g. According to the results, 67 samples (13.4%) tested positive for *S. aureus*; of those samples, 28 (5.8%) had higher than the acceptable amounts. Dixon (12) indicated that *S. aureus* is destroyed in honey. On the other hand, Packer et al. (13) reported that the consistency of honey does not inhibit nor slow down the pathogenicity of *S. aureus*. Our results are similar to the findings of Packer et al. The results may be due to contamination of *S. aureus* to the hives via equipment and/or hands of honey handlers.

Table 2. Number of positive samples according to the chosen parameters.

Parameter	Sample	Positive sample number
Coliforms	Honey (with comb)	80 (16%)
Escherichia coli	Honey (with comb)	18 (3.6%)
Staphylococcus aureus	Honey (with comb)	67 (13.4%)
Ascosphaera apis	Honey (with comb)	51 (10.2%)
Aspergillus flavus	Honey (with comb)	22 (4.4%)
Aspergillus fumigatus	Honey (with comb)	32 (6.4%)
Paenibacillus larvae	Honey (with comb)	16 (3.2%)
Melissococcus pluton	Honey (with comb)	29 (5.8%)
Nosema spp.	Honey (with comb)	39 (7.8%)

Table 3. Binary correlations of the parameters from collected honey samples by using Kendall' s tau b method (*: P < 0.05, NS: not significant).

Parameters and significance	Coliforms	*E. coli*	*S. aureus*	*A. apis*	*A. flavus*	*A. fumigatus*	*P. larvae*	*M. pluton*	*Nosema* spp.
Coliforms	-----	*	*	NS	NS	NS	*	*	NS
E. coli	*	-----	*	NS	NS	NS	NS	NS	NS
S. aureus	*	*	-----	NS	NS	NS	*	*	NS
A. apis	NS	NS	NS	-----	NS	NS	NS	NS	NS
A. flavus	NS	NS	NS	NS	-----	NS	NS	NS	NS
A. fumigatus	NS	NS	NS	NS	NS	-----	NS	NS	NS
P. larvae	*	*	*	NS	NS	NS	-----	*	NS
M. pluton	*	*	*	NS	NS	NS	*	-----	NS
Nosema spp.	NS	NS	NS	NS	NS	NS	NS	NS	-----

A. flavus and *A. fumigatus* cause stonebrood infections in larvae and adult honeybees. The agents may contaminate humans via honey and cause dysentery-like infections and hepatic cancers (14). According to our results, 4.4% of the samples were positive for *A. flavus* and 6.4% of the samples were positive for *A. fumigatus*. All the positive samples were beyond the acceptable limits. On the other hand, a study that had lower results than ours was done by Kırpık et al. (15), (1.5% for both *A. flavus* and *A. fumigatus* in 167 hives in Kars), and they reported that *A. flavus* and *A. fumigatus* were the most common pathogen fungi for honey bees. There were no binary correlations for both of the agents with other analyzed parameters. Because of the mycotoxin-producing feature of *A. flavus* and *A. fumigatus* and the lack of treatment of the infected hives, maximum care must be taken (16).

P. larvae is the causative agent of American foulbrood disease. American foulbrood is a quite contagious and infectious disease. The agent can survive in soil and in the hives for up to 60 years (17). According to our results, *P. larvae* ranged from 1.2×10^1 to 1×10^2 cfu/g. Özkırım and Keskin (18) isolated *P. larvae* from hives in Ankara. *P. larvae* was correlated with all the analyzed microbiological parameters statistically at the P < 0.005 level. The effects of *P. larvae* on human health are still unknown and the related studies are very limited. However, the most recent studies suggested that different proteins are released from the enzymes and cell membrane of *P. larvae* in the virulence mechanism. The most famous of these proteins is enolase, a functional protein, which works in glycolytic activities with various cytoplasmic enzymes (19). Enolase may be the key biological structure in the positive correlation of *P. larvae* and other pathogens.

M. pluton is the primary agent of European foulbrood disease. McKee et al. (20) point out that the agents are transported to the hives by the feet of adult honeybees. The agents colonize in the hives asymptomatically in the early period of the contamination while the honeybees spread *M. pluton* via feces in this period. *M. pluton* ranged from $<10^1$ to 1.3×10^2 cfu/g and according to our results, *M. pluton* was positively correlated with *E. coli* statistically at the P < 0.005 level. Zeybek (21) indicated that one of the most important sources of *M. pluton* is old basic honeycombs. It is thought that one of the possible causes of this correlation is the fecal cross-contamination to worker honeybees of *E. coli* from the fields and usage of old basic honeycombs. The correlation between *M. pluton* and *S. aureus* may be due to poor hygienic conditions of honey handlers and/or contaminated environments.

Nosema spp. causes dysentery-like symptoms in honeybees. The agent also decreases honey yield and terminates the hives. The contamination often occurs via feces found on the antennae of the bees. *Nosema* spp. was detected in 39 samples (7.8%) in our study and ranged from 1 to 5 parasites for each positive sample. Şimşek (22) detected nosematosis in honey samples at a rate of 8.77% in a study performed in Elazığ. In another study, 217 hives were investigated for nosematosis and 24% of the hives tested positive in the southern Marmara region (23). It was demonstrated that *Nosema* spp. could exist in a comb including honey samples. *Nosema* spp. was not correlated with any other analyzed parameters according to the statistical analysis. These results showed that *Nosema* spp. may survive in honey without being affected by any other pathogens or parasites. Applying good manufacturing

practice and using correct hygienic precautions would be good ways to eliminate *Nosema* spp. from both the honey and the hives.

A. apis may commonly be found in soil, plants, hives, stored honey, combs, and pollens, as well as in the gastrointestinal system and on the bodies of adult honeybees (24). In this study, *A. apis* was isolated from 51 samples (10.2%). Easy contamination from agent to pollen and honey is a risk factor for honey consumers. Another important point is the high endurance property of the agent to environmental conditions. In environmental conditions, the spores of the agent may be infective for up to 15 years. In pollens, *A. apis* remains dormant for at least 12 months. Furthermore, it can survive for up to 5 days at –16 °C and for up to 1 year at 12 °C. Because of its high endurance, the equipment used in beekeeping, hives, pollens, and honey is classified in the primary risk group (25). In a study performed in Ankara in 2002, Özkırım and Keskin stated that chalk brood disease was discovered in 6 hives out of 156 (3.84%). In 2003, Çakmak et al. (23) investigated 217 hives located in 22 different districts and the incidence of *A. apis* was reported as 26%. In the literature, there are few studies about the effects of *A. apis* on human health. However, because *A. apis* is a fungal agent, it may have negative effects on the metabolism of the human liver. Insufficient information about the pathogenicity of the agent to human health is an important risk factor. Additionally, senseless and widespread antibiotic applications also cause the hives to be predisposed to fungal agents, as *A. apis* breaks down the microbiological flora of both hives and honeybees. With this situation at hand, honey consumers may be confronted with more resistant *A. apis* species and pesticide residues in honey and honeycombs.

Turkey is the fourth biggest honey producer in the world. However, exportation and supplementation of honey to internal markets is not at expected levels. This situation causes honey to be an expensive food. Honeybee- and honey-originated pathogens and parasites terminate the bee colonies, significantly decrease the honey yield, and threaten the health of honey consumers. Unfortunately, there are limited studies about the honeybee- and honey-originated pathogens and parasites in Turkey. The studies about determining the microbiological and parasitological risk factors in both hives and honey would decrease the incidence of the potential diseases. Furthermore, it is thought that continuous education programs concerning good manufacturing processes and decontamination rules to honey handlers, applied by related official governmental institutes, would help to increase the honey yield of Turkey. Finally, further studies that investigate the genetic mechanism of important honey/bee/hive pathogens and parasites would be very important for understanding the interactions among the pathogens and parasites and to protect honey consumers' health.

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