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Original Article

MICROBIOLOGICAL CONTAMINATION OF HONEYS FROM DIFFERENT SOURCES IN TURKEY

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Abstract

Honey samples obtained by beekeepers taken from thirty-five different apiaries in Turkey's region of Marmara were investigated for the presence of microorganism. Each honey sample was examined for the number of total aerobic mesofilic bacteria, coliforms, moulds and yeasts and the presence of *Salmonella* spp., *Clostridium botulinum,* along with other aerobic bacteriae such as *Paenibacillus larvae* and *Melissococcus plutonius.* In total, fifty-four honey samples of different botanical origins including unifloral (Umbelliferae, Leguminosae, Trifolium, Onobrychis), multifloral and chestnut were evaluated in the means of microbiological properties. Microorganisms were isolated in twenty-eight samples (60.86%) of pure cultures and eighteen samples (39.13%) of mixed cultures. On the other hand, no microorganisms were isolated in eight samples. *Bacillus* spp, *Corynebacterium* spp., *Streptococcus* spp., *Staphylococcus* spp. *C. albicans* and *Penicillium* spp. were isolated and identified in other honey samples. The bacteria counts were 4x102 -1.4x103 cfu/g for aerobic mesofilic bacteria and 1-185 cfu/g for the fungi. The application of sanitary practices (hand washing, avoidance of sneezing or coughing, etc.) may be effective in controlling contamination by microorganisms. On the other hand, air, equipment and dust may be contributing causes of microbiological contamination. Therefore it is important to take precautions in order to avoid such contamination, even though such factors are often difficult to control.

Keywords: bacteria, fungi, honey, Turkey

INTRODUCTION

Honey is a natural food mainly composed of a complex mixture of carbohydrates and other such minor substances as organic acids, amino acids, proteins, vitamins, minerals and lipids (Finola, Lasagno, & Marioli, 2007). It is also a combination of fructose (38.4%), glucose (30.3%), sucrose (1.3%) and other kinds of carbohydrates (12%) with a water content of about 17.2% (White, Subers, & Schepartz, 1963). Due to its nutritive, therapeutic and dietetic properties honey is widely used in many areas of the food industry (Vica et al., 2009). Its low water content prevents bacterial reproduction and multiplication and so causes a low probability of pathogen presence (Snowdon & Cliver, 1996).

Despite the numerous inhibiting factors, some microorganisms are still able to survive in honey and may be transmitted to consumers (Sinacori et al., 2014). Honey may be contaminated by microorganisms through primary and secondary sources. Primary sources include pollen, honey-bee digestive tracts , dust, air, soil and nectar which are very difficult to control in natural conditions. (Snowdon & Cliver, 1996; Finola, Lasagno, & Marioli, 2007). The secondary sources (after-harvest) - air, food handlers, cross-contamination, equipment and buildings can be controlled by fine manufacturing practices (Snowdon & Cliver, 1996).

Such microorganisms as yeasts and spore-forming bacteria commonly found in honey are not considered to be dangerous for human health. Instead, the presence of coliforms or yeasts are indicatives of the sanitary or commercial quality of honey. However, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum* may cause illness in humans (Iurlina & Fritz, 2005; Sinacori et al., 2014). *Acinetobacter, Bacillus,*

Clostridium, Corynebacterium, Pseudomonas, Psychrobacter and *Vagococcus* are bacteria commonly found in soil (Snowdon & Cliver, 1996; Sinacori et al., 2014). The microbial contaminants taken into consideration are aerobic mesophiles, moulds, yeasts, fecal coliforms, sulphite reducing Clostridia and Salmonella (Tudor et al., 2011). Penicillium and Mucor are the microorganisms which usually exist in honey (Kacainova et al., 2009). In this study, the microbiological contamination and microbial loads in in fifty-four honey samples are investigated to determine the microbiological contamination and presence of aerobic mesofilic bacteria, moulds, yeasts, fecal coliforms, sulphite reducing Clostridia and Salmonella.

MATERIAL AND METHODS

Honey Samples

Honey samples obtained by beekeepers directly from thirty-five different apiaries in Turkey's Marmara region were investigated to examine the presence of microorganisms. Samples for microbiological analysis were taken from different plants in July, August and September 2014. The standard plate count method was used for culturing and isolating the different microorganisms. Blood agar, MYPGP agar, *Paenibacillus larvae* agar (PLA), XLD, Violet Red Bile (VRB) agar, cooked-meat medium agar were used as the medium for bacteria culturing while Saboraud Dextrose Agar was used to grow fungi . All samples were counted in terms of anaerobic/aerobic mesophilic bacteria, molds and yeasts.

Method of determining the botanical origin of honey samples

For melissopalynological analysis, 10 g of honey was taken from each of the samples, and Lycopodium spore tablets were added to them which are known to contain a set number of spores (18,583 for one tablet of the batch). Samples were prepared to the method by Louveaux, Maurizio, & Vorwohl (1978) and stained with glycerine gelatine colored with basic fuchsine, and the slides were analyzed by light microscopy. At least 500 Lycopodium spores and correspondent pollen grains were counted. Absolute pollen content of the 10 g sample was derived from the ratio of the total pollen counted to the number of Lycopodium spores counted during the pollen analysis (Gunes et al., 2017).

Total anaerobic/aerobic mesofilic bacteria and yeast count

10 g from each sample were homogenized with 90 ml of sterile phosphate-buffered saline solution. Plate count agar was used for the enumeration of total aerobic mesophilic bacteria and incubated at 37°C for 24-28 h. Violet red bile Agar (VRB) was used for the enumeration of total coliforms.

10 g of honey was taken from the surface of the container and diluted in 90 ml of phosphate buffer, pH 5.3, and 0.1 g of agar (10^{-1} dilution). A series of dilutions (10^{-2}, 10^{-3}) were then obtained from these solutions. One milliliter of each of these dilutions (10^{-1}, 10^{-2}, 10^{-3}) were then mixed in Petri dishes with 12 ml of culture medium (pH 3.5) containing yeast extracts, glucose, minerals and chloramphenicol (10 mg/ml). Finally, they were incubated at 25°C for five days. The experiments were carried out in duplicate (Finola, Lasagno, & Marioli, 2007).

Bacteria identification

The honey samples were processed according to Gilmore, Link & Fell (2010) for detecting *P. larvae*, and *M. plutonius*. The samples were heated to 35°C in a water bath prior to mixing and analysis. 1 ml were taken from honey samples and diluted 1:2 (w/v) with phosphate-bufffered saline. The samples were then centrifuged at 3.000 x g for 45 min. The suspension was heated to 80°C for 15 min and 100 µl spread onto PLA mediums. The plates were incubated under microaerophilic conditions at 37°C for up to seven days. The honey samples were diluted 1:2 (w/v) with phosphate-bufffered saline and the suspension was inoculated onto MYPGP medium for *Melissococcus plutonius*. The plates were incubated under aerobic conditions at 37°C for up to seven days (Gilmore, Link, & Fell, 2010). *P. larvae* and *M. plutonius* were the presumptive colonies that had been initially identified by their appearences on MYPGP and PLA agar, catalase test, Gram

and spores staining.

For pre-enrichment, 25 g honey was added to 225 ml of lactose broth and the cultures were incubated at 37°C for 24 h for isolation of *Salmonella* spp. The enrichment was performed in tetrathionate broth and incubated at 37°C for 24 h. The isolations were cultured onto XLD agar at 37°C for 24 h (Iurlina & Fritz, 2005) .

Clostridium spp. were tested by the method modified by Küplülü et al. (2006). 25 g of honey were diluted in 100 ml sterile distilled water with 1% Tween 80 and homogenized. The solution was held in a 65°C water bath for 30 min then centrifuged for 30 min at 9000 × *g*. The precipitates were transferred to 9 ml of cooked meat medium, which was then incubated anaerobically with the AnaeroGen AN25 system at 30°C for 72 h (Küplülü et al., 2006).

Blood agar was used for the examination of other microorganisms. The plates were incubated under aerobic condition at 37°C for to one to three. All plates were controlled on a daily basis in case of bacterial growth. The isolates were examined by light microscopy following Gram and carbol fuchsin stain and catalase test, then identified with BBL crystal system (BBL Crystal Enteric/Nonfermenter ID, BBL ANR ID and Gram Positive ID Kits -Becton Dickinson and Company, USA)

Yeast identification

The Saboraud dextrose agar was used for the detection of fungi and the incubation period at 22°C lasted five days (Joseph et al., 2007). 10 g honey samples were homogenized in 90 ml sterile phosphate buffered saline (Iurlina & Fritz, 2005). Colonies were identified by their morphological appearances in their medium.

RESULTS

Fifty-four honey samples from different botanical origins were analyzed for the detection of microorganism contamination. Forty-six honey samples (85.18%) were determined to be positive for microorganisms presence.

The botanical origin of honey samples

Fifty-four honey samples from different botanical origins including unifloral (Umbellif-

Table 1.

Origins of honey samples

Botanical origin of honey samples	Period of honey extraction
Multifloral (1)	July 2014
Castanea (2)	July 2014
Multifloral (3)	July 2014
Multifloral (4)	July 2014
Umbelliferae (5)	July 2014
Multifloral (6)	July 2014
Multifloral (7)	July 2014
Leguminosae (8)	July 2014
Trifolium (9)	July 2014
Multifloral (10)	August 2014
Castanea (11)	August 2014
Multifloral (12)	August 2014
Multifloral (13)	August 2014
Multifloral (14)	September 2014
Multifloral (15)	September 2014
Onobrychis (16)	September 2014
Multifloral (17)	September 2014
Multifloral (18)	September 2014
Trifolium (19)	July 2014
Multifloral (20)	July 2014
Multifloral (21)	July 2014
Multifloral (22)	July 2014
Umbelliferae (23)	August 2014
Multifloral (24)	August 2014
Multifloral (25)	August 2014
Multifloral (26)	August 2014
Multifloral (27)	August 2014
Castanea (28)	August 2014
Leguminosae (29)	September 2014
Multifloral (30)	September 2014
Multifloral (31)	September 2014
Multifloral (32)	September 2014
Multifloral (33)	September 2014
Castanea (34)	July 2014
Multifloral (35)	July 2014
Multifloral (36)	July 2014
Multifloral (37)	July 2014
Multifloral (38)	August 2014
Castanea (39)	August 2014
Multifloral (40)	August 2014
Multifloral (41)	August 2014
Castanea (42)	August 2014
Multifloral (43)	August 2014
Multifloral (44)	August 2014
Castanea (45)	August 2014
Multifloral (46)	August 2014

Table 2.

The levels of microbial contamination of honey samples

Botanical origin of honey samples	Aerobic mesophiles CFU/g	Moulds and yeasts CFU/g	Fecal Coliforms CFU/g	*Clostridium* spp. CFU/g	*Salmonella* spp. CFU/g
Multifloral (1)	*< 10*	Negative	Negative	Negative	Negative
Castanea (2)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (3)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (4)	*< 10*	Negative	Negative	Negative	Negative
Umbelliferae (5)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (6)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (7)	$2x10^2$	Negative	Negative	Negative	Negative
Leguminosae (8)	*< 10*	Negative	Negative	Negative	Negative
Trifolium (9)	$2x10^2$	Negative	Negative	Negative	Negative
Multifloral (10)	*< 10*	Negative	Negative	Negative	Negative
Castanea (11)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (12)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (13)	$2x10^2$	1	Negative	Negative	Negative
Multifloral (14)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (15)	*< 10*	Negative	Negative	Negative	Negative
Onobrychis (16)	*< 10*	3	Negative	Negative	Negative
Multifloral (17)	$2x10^2$	Negative	Negative	Negative	Negative
Multifloral (18)	*< 10*	Negative	Negative	Negative	Negative
Trifolium (19)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (20)	$4x10^2$	Negative	Negative	Negative	Negative
Multifloral (21)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (22)	$1.4x10^3$	1	Negative	Negative	Negative
Umbelliferae (23)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (24)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (25)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (26)	$2x10^2$	1	Negative	Negative	Negative
Multifloral (27)	*< 10*	185	Negative	Negative	Negative
Castanea (28)	*< 10*	Negative	Negative	Negative	Negative
Leguminosae (29)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (30)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (31)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (32)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (33)	*< 10*	Negative	Negative	Negative	Negative
Castanea (34)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (35)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (36)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (37)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (38)	*< 10*	Negative	Negative	Negative	Negative
Castanea (39)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (40)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (41)	*< 10*	Negative	Negative	Negative	Negative
Castanea (42)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (43)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (44)	*< 10*	Negative	Negative	Negative	Negative
Castanea (45)	*< 10*	Negative	Negative	Negative	Negative
Multifloral (46)	*< 10*	Negative	Negative	Negative	Negative

erae, Leguminosae, Trifolium, Onobrychis), multifloral and chestnut types were evaluated for their microbiological properties (Tab. 1).

Total anaerobic/aerobic mesofilic bacteria and yeast count

The total count of aerobic mesophilic varied between $4x10^2$ - 1.4 $x10^3$ cfu/g. while mold and yeasts numbers varied between 1-185 cfu/g. in honey samples. Fecal coliforms were not detected (Tab. 2).

Bacteria identification

Only one bacteria species of out of twenty-eight honey samples (60.86%) was detected, but eighteen samples (39.13%) were contaminated with several bacterial species. Out of thirty-one samples, *Bacillus* spp. (*B. cereus, B. licheniformis, B. subtilis, B. brevis, B. pumilus* and *B. sphaericus)* were isolated. *C. jeikum, C. aquaticum* and *C. renale* were isolated from seventeen samples. *S. epidermidis and S. pasteuri* were isolated from fifteen samples*. S. salivarius* was isolated from only one sample (Tab. 3). None of the fifty-four honey samples contained *E. coli, Salmonella spp., Clostridium spp. P. larvae* and *M. plutonius.*

Yeast identification

Penicillium spp. and *Candida albicans* were detected in the samples as well. These microorganisms were isolated from all but eight samples. The microorganism enumeration was highest in the samples collected in July 2014 in comparison to the other months.

DISCUSSION

The total count of aerobic mesophilic varied between $4x10^2$ - 1.4 $x10^3$ cfu/g. while mold and yeasts numbers varied between 1-185 cfu/g. in honey samples. Fecal coliforms were not detected. Variations in enumeration of microorganisms may be related to the type of the sample, the freshness of honey and the time of harvest. It was detected that honey samples which were collected in July 2014 are contaminated at the highest level.

Tysett et al. (1970) reported that the total count of aerobic mesophilic obtained from 175 commercial honey samples were 227 cfu/g in France which is similar to the current research. To compare this study's results with those of Tysset & Rosseau (1981), they found a mean value for viable counts of 227 cfu/g, with values that varied from 3 to 9500 cfu/g. while the results of this study were lower. Nakano & Sakaguchi (1991) tested 270 honey samples from Japan, and reported a mean aerobic viable count of 83 cfu/g. Piana et al. (1991) determined 1-350 cfu/g counts of osmophilic fungi. Tysset & Rosseau (1981) reported that the counts of molds and yeasts varied from 0 to 2500 cfu/g. Nakano & Sakaguchi (1991) found that the

Table 3.

The microorganisms identified in honey samples

The species of microorganisms	The number of honey samples in which the strains were identified
Corynebacterium jeikum	*14 (20.28%)*
Bacillus brevis *Staphylococcus epidermidis*	10 (14.49%)
Bacillus subtilis	8 (11.59%)
Bacillus cereus	6 (8.69%)
Staphylococcus pasteuri	5 (7.24%)
Candida albicans	4 (5.79%)
Bacillus licheniformis	*3 (4.34%)*
Corynebacterium aquaticum *Bacillus sphaericus* *Bacillus pumilus*	2 (2.89%)
Corynebacterium renale *Streptococcus salivarius* *Penicillium* spp.	1 (1.44%)
Total	69

yeast count varied from 0 to 300 cfu/g as well. In this study, the numbers of yeasts and mould were found to be similar to prior studies.

Iurlina & Fritz (2005) reported that fecal coliforms, *E. coli, Salmonella* spp., *Shigella* spp., and *Clostridium* spp. were not detected but *P. larvae* subsp. *larvae*, *B. cereus, B. pumilus* and *B. laterosporus* were found in some samples.

Sadık & Ali (2012) reported that total coliforms *E .coli, Salmonella* spp., *Shigella* spp.,*P. larvae* and *Clostridium* spp. were not detected but *B. licheniformis, B. wakoensis, B. subtilis, B. atrophaeus, B. sonorrensis, B. spizizenii, B. vallismortis, B. alcolophilus, B. murimartini, B. horti* and *A. niger* were found for all of the current samples were negative for sanitary quality (fecal coliforms) and safety (sulphite-reducing Clostridia and Salmonella).

Sinacori et al. (2014) reported that, they had found thirteen species of bacteria, five species of yeasts and seventeen species of filamentous fungi; the species that had been isolated most frequently were *Bacillus amyloliquefaciens*, *Zygosaccharomyces mellis* and *Aspergillus niger*.

Joseph et al. (2007) reported that they had detected *Bacillus* spp., *Candida* spp., *Aspergillus* spp. *Geotrichum* spp. and *Rhizopus* spp. in their samples.

Omafuvbe (2009) reported that Bacillus species had been detected and identified as *B. cereus, B. megaterium, B. polymyxa, B. licheniformis, B. firmus* and *B. pumilus*.

Snowdon & Cliver (1996) have performed studies on the survival of some Salmonella species or other vegetative pathogenic microorganisms which are normally not present in honey. Piana et al. (1991), Delmas, Vidon & Sebald (1994) reported that *C. perfringens* and *C. botulinum* were not found in any samples. The current results align with these studies.

The bacterial spores, particularly *Bacillus* spp. are regularly found in honey; Clostridial spores are also found, but less frequently. Since bacterial replication does not occur in honey, no vegetative forms of disease-causing bacteria species have been found in samples. The high numbers of vegetative bacteria indicate recent contamination from a secondary source.

We found in our research that isolated microorganisms originated from human, animal and environmental factors, which was similar the results by Tysett & Rosseau (1981).

A lack of hygiene is the main cause of honey contamination, and it is necessary to place importance on manufacturing practices to control the presence of microorganisms in honey. Microorganisms can be controlled by using such sanitary practices as hand washing, avoidance of sneezing and coughing, while the control of air, equipment and dust is equally important but often more difficult.

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