Quality Assurance and Safety of Crops & Foods, 2019; 11 (6): 571-576

Wageningen Academic Publishers

Identification of moulds from Croatian honey

M. Kiš[1*], S. Furmeg[1], V.J. Tkalec[1], M. Zadravec[2], M. Benić[2], J. Sokolović[1] and D. Majnarić[1]

[1]Veterinary Centre Križevci, Croatian Veterinary Institute, Ivana Z. Dijankovečkog 10, 48260 Križevci, Croatia; [2]Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia; kis.vzk@veinst.hr

Received: 8 January 2019 / Accepted: 2 August 2019

RESEARCH ARTICLE

Abstract

Assessment of microbiological quality of honey is very important, because of its use as a supplement in the human diet. Fungal contamination of food poses a challenge to public health and global food security. Although honey is known for its antifungal activity, there are not many species of fungi that have been tested. In this study, 30 honey samples collected in various parts of Croatia during the period of 2012-2017 were tested for the presence of yeasts and moulds through mycological analysis. The mycological identification showed that out of nine genera recovered, *Cladosporium* was the most frequent genus, followed by *Penicillium* and *Alternaria*, being found in 47.37% (9 samples), 10.53% (2 samples) and 10.53% (2 samples). In addition, genera of *Mucor, Aureobasidium, Acremonium, Botrytis, Stachybotrys* and *Paecylomyces* were isolated in 5.26% samples. The results showed that most of the moulds identified in this study are commonly found in honey.

Keywords: honey, microbiology analysis, fungal contamination, moulds, mycobiota

1. Introduction

Honey is considered to be a relatively safe foodstuff due to its compositional properties. It is a complex mixture of carbohydrates and water, with small amounts of organic acids, amino acids, proteins, minerals, lipids and vitamins (White *et al.,* 1975). The natural ingredients present in honey are responsible for its broad-spectrum of antimicrobial (antibacterial, antifungal and antiviral) properties (Almasaudi *et al.,* 2017). Presence of specific bacteriostatic and bactericidal compounds (hydrogen peroxide, lysozyme, phenolic acids, antioxidants, terpenes, volatile substances) alongside with high sugar concentration, acidity and low water activity in honey, creates a medium that inhibits the growth and replication of pathogenic bacteria and food spoiler microorganisms (Salgado Silva *et al.,* 2017). Natural honey exhibits a large variation in the antimicrobial activity because the composition of active components in plants is subjected to changes and depends on various factors (i.e. season, chemotype, climatic conditions). Some species of microorganisms can survive and influence the stability of honey and its hygienic quality. Introduction of microorganisms into the food chain during honey production could derive from two sources of contamination: primary sources include pollen, digestive tract of honeybees, dust, air, nectar and soil; while secondary (after-harvest) sources arise from honey manipulation by people and include processing plants, appliances, food handlers, air and cross-contamination. The presence of microorganisms deriving from primary sources of contamination is very difficult to control, while secondary sources can be controlled by the application of good manufacturing practices (Grabowski and Klein, 2015; Salgado Silva *et al.,* 2017).

Many studies dealing with food safety of honey concentrate mostly on the major bacterial contamination, while reporting on minor risks from yeasts and moulds. Still, honey is a suitable medium for proliferation of yeasts and moulds because it is a rich source of free amino acids, sugars and minerals, particularly if the product is improperly handled and stored during production. Yeasts and moulds presence in honey is common and unavoidable, since they are also widespread in the environment of the hive and bees collect them together with the nectar. Moulds with thermal resistant spores have a great capacity of surviving and can be introduced into the honey by man, through dust, water installations, containers and also by primary sources of

contamination since they are associated with the intestinal contents of bees, the hive and the environment in which bees forage (Kačaniova *et al.,* 2009). Common microflora associated with honeybees and their products are moulds of the genera *Penicillium, Mucor* and *Aspergillus.* Growth of moulds and osmotolerant yeasts largely depends on environmental factors, with water activity and temperature being the most important environmental determinants. Only honeys containing less than 17% water are regarded as safe (Bogdanov and Martin, 2002). When the water content surpasses 17%, the products stability depends on the microbial content and honey is susceptible to fermentation, while honey with over 19% moisture is very likely to ferment (Snowdon and Cliver, 1996).

Many authors have reported studies on honey, some based on physicochemical and microbiological characteristics (Kiš *et al.,* 2018), the chemical identification of certain compounds in honey from different sources and regions (Bilandžić *et al.,* 2011) and, in recent years, studies on the antimicrobial properties of honey (Almasaudi *et al.,* 2017). The scientific literature about the occurrence of moulds in bee products is mostly related to microscopic fungi isolated from bee pollen (Kostić *et al.,* 2017, 2019; Petrović *et al.,* 2014), while data on mycobiota present in honey are scarce and often ignored by the EU legislation. Therefore, the main goal of this study was to investigate honey produced in Croatia from the mycological point of view, and thus contribute towards the knowledge on the genera of moulds present in honey.

2. Materials and methods

Sample collection

During the routine microbiological analysis of honey samples according to Croatian National Guidelines on microbiological criteria for foodstuffs (Croatian Regulation, 2011) samples that were incompatible according to quantitative criterion for yeasts and moulds (maximum permissible concentration <10 cfu/g) were singled out. The research was carried out on 30 honey samples (Table 1) collected from manufacturers in various parts of Croatia during the period of 2012-2017. Honey samples are stemming from different botanical origin which was assigned according to beekeepers' declaration: floral (6 samples), black locust (*Robinia pseudoacacia* L., 10 samples), chestnut (*Castanea sativa* Mill., 5 samples), rapeseed (*Brassica napus* L., 4 samples), lime (*Tilia* spp., 3 samples) and meadow (2 samples). Until the analysis, honey samples were stored under controlled conditions, in the dark at room temperature.

Microbiological analysis

Microbiological analysis was performed by means of standard methods for isolation and identification of microorganisms according to requirements of ISO/FDIS 21527-2 (2008). For each honey sample, 10 g of sample was homogenised into 90 ml of saline solution (NaCl, 8.5 g/l). A serial dilution method was done and 1 ml of the first and second decimal dilution was transferred on Dichloran 18% mass fraction glycerol agar plates (DG18; Biokar, Beauvais, France) intended for the enumeration of viable osmophilic yeasts and xerophilic moulds in food or animal feed products with a water activity of less than or equal to 0.95 by a colony count technique. Moulds and yeasts were enumerated after seven days of incubation at 25±1 °C. Average number of colonies, multiplied by the dilution factor, was considered for the counting of moulds and yeasts colonies. For moulds identification to the genera level, Czapek yeast extract and malt extract agar nutrition medium (Biokar, Beauvais, France) were used according to Pitt and Hocking (2009) by defining their macroscopic and microscopic morphological characteristics.

Statistical analysis

Statistical analysis was carried out using Stata 13.1 statistical package (Stata Corp., College Station, TX, USA). Contamination occurrence of yeasts and moulds and their loads were compared between years and types of honey using Fisher Exact test and Kruskall-Wallis test.

3. Results and discussion

The levels of yeasts and moulds contamination are shown in Table 1. Number of yeasts in all samples ranged from 0.00 to 3.11 log cfu/g of sample, with an average of 1.58 log cfu/g. In this study, 73.33% of samples were contaminated with yeasts. Rapeseed honey showed a rather high number of yeast count in comparison to other honey types from this study. All the rapeseed honey samples were found to be microbiologically incompatible because of the yeast contamination, with an average count of 2.99 log cfu/g. In this study, the highest mould count was detected in meadow honey (2.26 log cfu/g) while the average mould count of samples was 1.53 log cfu/g, which is higher than average number of moulds from commercial and apiary honeys from Slovakia (1.2×10^{-1} cfu/g) reported by Kačaniova *et al.* (2012). Gradvol *et al.* (2015) reported similar numbers for moulds and yeasts as in this study, while investigating different types of Croatian honey. In a study done by Rozanska (2011), total number of yeasts and moulds was the highest in a sample of lime honey from Poland, with the value of 8.0×10^{4} cfu/g. Erkan *et al.* (2015) investigated commercial honey samples from Turkey and found that the maximum yeast count was 1.4×10^{5} cfu/g with mean yeast count 5.4×10^{4} cfu/g. In this

Table 1. Levels of yeasts and moulds contamination in tested honey samples during 2012-2017.

Sample	Honey type	Year of production	Yeasts contamination (log cfu/g)	Moulds contamination (log cfu/g)
1	Black locust	2012	1.43	1.65
2	Black locust	2012	0.00	1.26
3	Black locust	2012	1.95	1.26
4	Black locust	2014	2.04	0.00
5	Black locust	2016	1.56	0.00
6	Black locust	2016	1.26	2.00
7	Black locust	2017	2.34	1.43
8	Black locust	2017	1.43	1.26
9	Black locust	2017	1.91	0.00
10	Black locust	2017	2.62	0.00
11	Floral	2012	0.00	1.26
12	Floral	2012	0.00	1.26
13	Floral	2012	2.58	1.43
14	Floral	2013	2.00	1.56
15	Floral	2017	2.91	0.00
16	Floral	2017	3.04	0.00
17	Chestnut	2012	0.00	1.56
18	Chestnut	2013	0.00	1.26
19	Chestnut	2015	0.00	1.26
20	Chestnut	2017	0.00	1.43
21	Chestnut	2017	1.26	0.00
22	Rapeseed	2017	3.00	0.00
23	Rapeseed	2017	2.92	0.00
24	Rapeseed	2017	2.94	0.00
25	Rapeseed	2017	3.11	0.00
26	Lime	2012	2.07	1.26
27	Lime	2013	1.43	0.00
28	Lime	2014	2.00	0.00
29	Meadow	2015	1.56	0.00
30	Meadow	2016	0.00	2.26

study, mean yeast contamination level is lower than those reported by Erkan *et al.* (2015).

Of the 30 honey samples analysed in this study, 56.66% were contaminated with moulds and 30.00% were contaminated with both moulds and yeasts. Observed difference between mould and yeast occurrence is statistically significant (P=0.002). It was found that yeast contamination is statistically related with origin of honey (P=0.031), but not with the year of production (P=0.230). Opposite of that, mould contamination is statistically related with the year of production (P=0.003), but not with the honey type (P=0.209). Yeast and mould loads are expressed as binary variable and as a log cfu/g and checked for association with the year of production and origin using Fisher Exact and Kruskall-Wallis test (Table 2). Observed differences of mould load between years and honey types were not statistically different. Yeast load differed statistically between honey types (P=0.0073), but not between years (P=0.0741).

Table 2. Observed differences in yeast and mould load.

		Yeasts	
		No	Yes
Moulds	No	0	14
	Yes	8	8
Total		8	22

High yeast counts can be a good indicator of low microbiological quality of honey. Osmophilic yeasts are usually the cause of spoilage of high-sugar foods like honey. Their development is promoted by higher water activity in conditions of low pH and high sucrose concentrations. In favourable conditions, yeasts in honey can grow to very high numbers and ferment honey by converting glucose

and fructose into alcohol and carbon dioxide. Because of the increased rate of fermentation and higher acidity, those samples are no longer palatable (Grabowski and Klein, 2015). Possible sources of yeasts in honey include nectar, the body of the bee, hive, apiary soil and equipment used during processing. According to Kačaniova *et al.* (2009), hive presents an ultimate reservoir for yeasts in honey with working bees distributing yeasts to the nectar as it is collected.

Results obtained with regard to the occurrence of different genera of moulds in honey samples and the number of positive samples is shown in Table 3. In this study, from 30 honey samples, 19 isolates of moulds were recovered. According to the mycological analysis, nine genera of moulds were determined: *Cladosporium, Penicillium, Alternaria, Mucor, Aureobasidium, Acremonium, Botrytis, Stachybotrys* and *Paecylomyces.* Examination of microscopic fungi from honey samples in earlier articles (Felšociova *et al.,* 2012; Kačaniova *et al.,* 2012; Sinacori *et al.,* 2014) showed a great diversity in the presence of different genera of moulds which was also confirmed by the results of this study. Stressing conditions of honey are highly selective and specific environment conditions together with variations in honey composition (depending on the floral source) can affect the mycological profile of honey. Sinacori *et al.* (2014) analysed 38 samples of floral honey and honeydew from different botanical and geographical origin and identified 17 species of moulds, belonging to eight different genera. In this study the predominant moulds were from the genera *Cladosporium* (47.37%), followed by *Penicillium* (10.53%) and *Alternaria* (10.53%). These genera of moulds are considered to be common contaminants of bee products, as reported in literature (Gonzalez *et al.,* 2005; Kačaniova *et al.,* 2012; Kostić *et al.,* 2017), along with the *Aspergillus* genus whose presence was not detected in the samples analysed by this study. Felšociova *et al.* (2012) found that moulds belonging to the genus *Penicillium* were detected in 66% of honey samples, with *Penicillium chrysogenum* being the most encountered species.

Great majority of moulds isolated from honey samples in this study represents the fungal group of saprophytic microorganisms that inhabit organic residues of plants and soil, indicating their origin from environment. The study of Kačaniova *et al.* (2009) characterised microbial transit among the honey-bee gastrointestinal tract microflora, beehive environment and honey. Their study showed that the primary sources of microbial community present in honey are the beehive environment and digestive tract of bees, mainly due to microorganisms naturally present in dust, air and flowers. Genera *Botrytis* and *Alternaria* are common plant pathogens on agricultural and forest plants, while *Acremonium* and *Aureobasidium* are saprophytes isolated from plants, soil, wood, and indoor air environment. *Cladosporium cladosporioides* and *Alternaria tenuissima* are often associated with intestines of honeybees and *Paecylomyces* genus is entomopathogenic fungus isolated from insects (Gilliam, 1997). The most representative genera of moulds from beehive environment are *Aspergillus, Cladosporium* and *Penicillium,* which are also likely to occur in honey and other bee products. This was also confirmed by the results of mycological analysis of bee pollen, where *Aspergillus, Alternaria* and *Penicillium* were the predominant genera of moulds (Petrović *et al.,* 2014). It is likely that the majority of isolated moulds from contaminated honey samples in this study derive from the primary sources. Exception may be the presence of *Stachybotrys* sp. found in one sample. Some species of this genus are common in soil, decaying plant material and wild fruits, but some are associated with poor indoor air quality which arises after fungal growth on water-damaged building materials. The presence of some genera of moulds in bee products presents a particular problem for their safe use in the human diet since they can produce extremely dangerous mycotoxins as a part of their metabolism. *Stachybotrys*

Table 3. Occurrence frequency of identified genera of moulds in different types of honey.

Genera of moulds	Number of mould isolates in different types of honey							
	Black locust	Floral	Chestnut	Rapeseed	Lime	Meadow	∑	Frequency [%]
Cladosporium sp.	+ (5)	+ (2)	—	—	+ (1)	+ (1)	9	47.37
Penicillium sp.	+ (1)	+ (1)	—	—	—	—	2	10.53
Alternaria sp.	—	+ (1)	+ (1)	—	—	—	2	10.53
Mucor sp.	+ (1)	—	—	—	—	—	1	5.26
Aureobasidium sp.	+ (1)	—	—	—	—	—	1	5.26
Acremonium sp.	—	—	+ (1)	—	—	—	1	5.26
Botrytis sp.	—	—	+ (1)	—	—	—	1	5.26
Stachybotrys sp.	+ (1)	—	—	—	—	—	1	5.26
Paecylomyces sp.	—	—	+ (1)	—	—	—	1	5.26
∑	9	4	4	0	1	1	19	

chartarum produces several mycotoxins (highly toxic macrocyclic trichothecenes and related trichoverroids) as well as immunosuppressants and endothelin receptor antagonists and it is known for its harmful effects on animal and human health (Li and Yang, 2005). The presence of aflatoxin B1 was confirmed in all investigated samples of bee pollen collected in Serbia (Kostić *et al.,* 2017; Petrović *et al.,* 2014). Obtained results indicate that during manipulation, harvesting and manufacturing of honeybee pollen and other bee products, the implementation of good manufacturing practices must be applied in order to provide natural and safe product without the risk on human health. Prevention of moisture re-absorption and the general improvement of processing plant and storage facilities are highly recommended as a protection against mould deterioration of bee products. According to the literature data, necessary conditions for the presence of moulds in honey do not imply the presence of mycotoxins. Favourable conditions that enable mycotoxin synthesis (adequate temperature, pH, relative humidity, water activity values, and substrate composition) are not achieved during any phase of honey collection or production (Kostić *et al.,* 2019; Salgado Silva *et al.*, 2017). Even when honey is contaminated with *Aspergillus flavus,* as highly toxigenic fungi, conditions for aflatoxin production in honey are inappropriate (Kačaniova *et al.,* 2012). However, many of the fungi isolates from honey are considered to be potential mycotoxin producers. *Penicillium* isolates from honey proved to be producers of significant mycotoxins including citrinin, cyclopiazionic acid, griseofulvin, patulin, penitrem A and roquefortin C (Kačaniova *et al.*, 2012).

4. Conclusions

Although honey is considered to be a relatively safe foodstuff with antifungal properties, the literature data reveals the presence of some mycotoxin-producing fungi genera in honey which indicates the need for regular analysis of honey microbiological characteristics. Out of 30 analysed samples, incompatible according to the Croatian Regulation, results of this study revealed 56.66% of the samples contaminated with moulds, 73.33% samples contaminated with yeasts, with observed high yeast and mould counts in certain honey samples. Fungal colonisation and contamination of stored honey can cause a decline in nutritional value over time, reduces shelf life, affects palatability and indicates low microbiological quality of honey. Therefore, the implementation of good manufacturing practices in all stages of honey production could reduce the risk of possible contamination and ensure the high quality of the product. Further mould identification carried in this study revealed nine genera of moulds: *Cladosporium, Penicillium, Alternaria, Mucor, Aureobasidium, Acremonium, Botrytis, Stachybotrys* and *Paecylomyces*. Considering that the majority of moulds identified in this study are commonly found in the intestinal contents of bees, hive and environment in which bees forage, it can be concluded that the moulds in honey samples from this study derive mainly from primary sources of contamination.

References

Almasaudi, S.B., Al-Nahari, A.A.M., Abd El-Ghany, E.S.M., Barbour, E., Al Muhayawi, S.M., Al-Jaouni, S., Azhar, E., Qari, M., Qari, Y.A. and Harakeh, S., 2017. Antimicrobial effect of different types of honey on *Staphylococcus aureus.* Saudi Journal of Biological Sciences 24(6): 1255-1261.

Bilandžić, N., Đokić, M., Sedak, M., Solomun Kolanović, B., Varenina, I., Končurat, A. and Rudan, N., 2011. Determination of trace elements in Croatian floral honey originating from different regions. Food Chemistry 128(4): 1160-1164.

Bogdanov, S. and Martin, P., 2002. Honey authenticity: a review. Swiss Bee Research Centre, Liebefeld-Bern, Switzerland, pp. 1-20.

Croatian Regulation, 2011. Croatian national guidelines on microbiological criteria for foodstuffs, 3rd edition. Ministry of Agriculture, Zagreb, Croatia. Available at: http://www.veterinarstvo.hr/UserDocsImages/Vodic%20o%20mikrobioloskim%20kriterijima.doc.

Erkan, M.E., Vural, A., Guran, H.S. and Durmusoglu, H., 2015. Microbiological investigation of honey collected from Şırnak province of Turkey. Journal of the Hellenic Veterinary Medical Society, 66(1): 22-26. https://doi.org/10.12681/jhvms.15579

Felšociova, S., Kačaniova, M., Hleba, L., Petrova, J., Pavlekova, A., Dzugan, M. and Grabek-Lejko, D., 2012. Microscopic fungi isolated from Polish honey. Journal of Microbiology, Biotechnology and Food Sciences 2(3): 1040-1049.

Gilliam, M., 1997. Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiology Letters 155(1): 1-10.

Gonzalez, G., Hinojo, M.J., Mateo, R., Medina, A. and Jimenez, M., 2005. Occurrence of mycotoxin producing fungi in bee pollen. International Journal of Food Microbiology 105(1): 1-9. https://doi.org/10.1016/j.ijfoodmicro.2005.05.001

Grabowski, N.T. and Klein, G., 2017. Microbiology and food-borne pathogens in honey. Critical Reviews in Food Science and Nutrition 57(9): 1852-1862. https://doi.org/10.1080/10408398.2015.1029041

Gradvol, V., Atlaban, N., Lenart, L. and Pavlović, H., 2015. Microbiological quality and inhibitory potential of selected Croatian apiary honeys. Croatian Journal of Food Science and Technology 7(2): 40-46. https://doi.org/10.17508/CJFST.2015.7.2.07

International Organization for Standardization (ISO), 2008. ISO/FDIS 21527-2. Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of yeasts and moulds. Part 2 – colony count technique in products with water activity less than or equal to 0.95. Available at: https://www.iso.org/standard/38276.html

Kačániová, M., Kňazovická, V., Felšöciová, S. and Rovná, K., 2012. Microscopic fungi recovered from honey and their toxinogenety. Journal of Environmental Science and Health, Part A 47(11): 1659-1664. https://doi.org/10.1080/10934529.2012.687242

Kačániová, M., Pavličová, S., Haščík, P., Kociubinski, G., Kńazovická, V., Sudzina, M., Sudzinova, J. and Fikselová, M., 2009. Microbial communities in bees, pollen and honey from Slovakia. Acta Microbiologica et Immunologica Hungarica 56(3): 285-295. https://doi.org/10.1556/AMicr.56.2009.3.7

Kiš, M., Furmeg, S., Jaki Tkalec, V., Zadravec, M., Denžić Lugomer, M., Končurat, A., Benić, M. and Pavliček, D., 2018. Characterization of Croatian honey by physicochemical and microbiological parameters with mold identification. Journal of Food Safety 38(5): e12492. https://doi.org/10.1111/jfs.12492

Kostić, A.Ž., Milinčić, D.D., Petrović, T.S., Krnjaja, V.S., Stanojević, A.P., Barać, M.B., Tešić, Ž.Lj. and Pešić, M.B., 2019. Mycotoxins and mycotoxin producing fungi in pollen: review. Toxins 11: 64.

Kostić, A.Ž., Petrović, T.S., Krnjaja, V.S., Nedić, N.M., Tešić, Ž.L., Milojković-Opsenica, D.M., Barać, M.B., Stanojević, S.P. and Pešić, M.B., 2017. Mold/aflatoxin contamination of honey bee collected from different Serbian regions. Journal of Apicultural Research 56(1): 13-18. https://doi.org/10.1080/00218839.2016.1259897

Li, D.W. and Yang, C.S., 2005. Taxonomic history and current status of *Stachybotrys chartarum* and related species. Indoor Air 15: 5-10. https://doi.org/10.1111/j.1600-0668.2005.00339.x

Petrović, T., Nedić, N., Paunović, D., Rajić, J., Matović, K., Radulović, Z. and Krnjaja, V., 2014. Natural mycrobiota and aflatoxin B1 presence in bee pollen collected in Serbia. Biotechnology in Animal Husbandry 30(4): 731-741. https://doi.org/10.2298/BAH1404731P

Pitt, J. and Hocking, A.D., 2009. Fungi and food spoilage, 4th edition. Springer, New York, NY, USA.

Rozanska, H., 2011. Microbiological quality of Polish honey. Bulletin of the Veterinary Institute in Pulawy 55: 443-445.

Salgado Silva, M., Rabadzhiev, Y., Renon Eller, M., Iliev, I., Ivanova, I. and Santana, W.C., 2017. Microorganisms in honey. In: Arnaut de Toledo, V. (ed.) Honey analysis. InTech, New York, NY, USA, pp. 233-245.

Sinacori, M., Francesca, N., Alfonzo, A., Cruciata, M., Sannino, C., Settanni, L. and Moschetti, G., 2014. Cultivable microorganisms associated with honeys of different geographical and botanical origin. Food Microbiology 38: 284-294.

Snowdon, J.A. and Cliver, D.O., 1996. Microorganisms in honey. International Journal of Food Microbiology 31(1-3): 1-26.

White, J.W., 1975. Composition of honey. In: Crane, E. (ed.) Honey, a comprehensive survey. Heinemann, London, UK, pp. 157-206.