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CONSIDERATIONS ON THE DEGREE OF YEAST AND MOULD CONTAMINATION OF BEE HONEY

CONSIDERAŢII ASUPRA GRADULUI DE CONTAMINARE CU LEVURI ŞI MUCEGAIURI A MIERII DE ALBINE

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ABSTRACT

Honey, unlike other foods, does not represent a favourable substrate for the survival and multiplication of microorganisms; due to its particular composition, honey has the ability to inhibit or even destroy them. However, rare types of microorganisms (such as yeasts and moulds) persist in honey, usually in a latent state, and some species of yeasts, due to the fact that they are pronounced osmophiles, can multiply and produce the fermentation of the product, making it unfit for human consumption. Normally, microorganisms are found in honey in the form of spores, and non-sporogenous ones can only be present in fresh honey. In this sense, we started a series of studies to analyse the risk of fermentation processes in honey depending on the variety. A total of 236 honey samples were collected and processed by complex laboratory methods, referring to 4 assortment categories (with economic importance): acacia honey, linden honey, manna honey, and polyflora honey. The laboratory analyses were focused on determining the total number of yeasts and moulds by using the colony counting technique (CFU/g). Following the statistical processing and interpretation of the obtained results, minimal contamination was found; no sample exceeded the maximum level accepted for the total number of yeasts and moulds, 100 CFU/g. Within the assortment categories, contamination at the lowest level was found for manna honey, acacia honey, and linden honey samples, compared to polyflora honey, where contaminant levels were found that can be considered uniform.

Keywords: yeasts, moulds, honey, lab analysis

REZUMAT

Mierea, spre deosebire de alte alimente, nu reprezintă un substrat favorabil supravieţuirii şi multiplicării microorganismelor; datorită compoziţiei ei particulare, mierea are capacitatea de a le inhiba sau chiar distruge. Totuşi, rare tipuri de microorganisme (cum ar fi levurile şi mucegaiurile) persistă în miere, de regulă în stare latentă, iar unele specii de levuri, datorită faptului că sunt pronunţat osmofile, pot să se multiplice şi să producă fermentarea produsului, făcându-l impropriu pentru consum uman. În mod obişnuit, în miere microorganismele se găsesc sub formă de spori, iar cele nesporogene pot fi prezente numai în mierea proaspătă. În acest sens am demarat o serie de cercetării pentru a analiza riscul proceselor fermentative ale mierii în funcţie de sortiment. Au fost recoltate şi prelucrate prin metode complexe de laborator un număr total de 236 probe miere, prelevările referindu-se la 4 categorii sortimentale (cu importanţă economică), miere de salcâm, miere de tei, miere de mană şi miere polifloră. Analizele de laborator au fost axate pe determinarea numărului total de levuri şi mucegaiuri, în acest scop folosindu-se tehnica numărării coloniilor (CFU/g). În urma prelucrării statistice şi interpretării rezultatelor obţinute a fost constatată o contaminare minimală, nici o probă nu a depăşit nivelul maxim acceptat pentru număr total de levuri şi mucegaiuri, 100 CFU/g. În cadrul categoriilor sortimentale, contaminarea la cel mai scăzut nivel a fost constatată pentru probele de miere de mană, miere de salcâm şi probele de miere de tei, comparativ cu mierea polifloră, în care au fost constatate niveluri contaminante care pot fi considerate uniforme.

Cuvinte cheie: levuri, mucegaiuri, miere, analize de laborator

Honey is the natural product obtained from insect species included in the Order *Hymenoptera*, of which the best-known species is the European honey bee (*Apis mellifera*). In some countries, honey is also obtained from some species of ground bumblebees or semi-domesticated or wild wasps. The bees collect extrafloral floral nectar, pollen, and various vegetable waxes, process them with the help of the oral apparatus and salivary enzymes, turning them into honey, and store the product thus defined in the cells of the honeycombs in the hive to constitute their energy food. Due to its particular composition, bee honey does not represent a favourable substrate for the survival and multiplication of microorganisms, as it possesses the ability to inhibit or destroy them. For this reason, the microbiological examination of honey was not a concern for specialists in the field.

The ability of honey to inhibit or even destroy mi-

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croorganisms is due to a complex of factors, the most important of which are: the high sugar content and very low free water content, which causes dehydration, shrivelling and death of microbial cells; acidity (pH ≤ 4.5); the glucose oxidation system in honey, which determines the formation of hydrogen peroxide, a toxic product for microorganisms, known long before its identification as "*inhibin*"; the very low protein content and high C/N ratio of honey; lack of oxygen in honey, atmospheric oxygen not being able to penetrate honey due to its viscosity (most contaminating microorganisms need oxygen to develop); the presence in honey of some chemical substances and enzymes unfavourable to the development of microorganisms (e.g., pinocembrin, lysozyme, phenolic acids, terpenes, benzyl alcohols, and various volatile substances); the unfavourable electrical charge created by the reducing sugars in honey inhibits the multiplication of moulds and aerobic bacteria (2, 3, 4, 21, 22).

However, rare types of microorganisms persist in honey, usually in a latent state, and some yeast species can multiply and modify the product, making it unfit for human consumption. This has recently led producers and specialists to pay more and more attention to the microbiological quality of honey and to require its microbiological examination before marketing. Sometimes honey is used as the main ingredient in the preparation of other products that are not thermally treated (fermented salami, various syrups, etc.) or in some confectionery products. In these cases, the antimicrobial properties of honey are reduced or disappear by diluting it, and the contained microorganisms can multiply. Likewise, fraudulently diluted honey obviously loses its antimicrobial properties. Contamination of honey with microorganisms occurs at the level of the hive by bees during the collection and deposition of nectar, as well as after the collection and primary processing of honey. Contamination during the harvesting and primary processing of honey has the most important hygienic and sanitary significance, because it often includes pathogenic or potentially pathogenic microorganisms from the people who carry out these operations. It is determined by the unhygienic working conditions and the inadequate microbiological quality of the water used. Moreover, the inadequate microbiological quality of the water used by bees can also contribute to contamination during the collection and deposition of nectar in the combs. According to current data,primary and industrial processing can increase or decrease total number of microorganisms in honey.

Data from the literature regarding the types and number of microorganisms that can be found in honey are quite limited. These microorganisms are represented by yeasts, moulds and bacteria. Under natural conditions, honey from the hive has a limited variety and a reduced number of microorganisms.

Normally, microorganisms are found in honey in the form of spores, and non-sporogenous ones can only be present in fresh honey. The main property of the yeasts that produce the fermentation of honey is that they are pronounced osmophilic, so they can develop in concentrated sugar conditions. But they also have a threshold below which development is no longer possible (2, 3, 21, 22). This threshold is at the level of 20% water content. However, the threshold has a somewhat relative character because, in the case of massive contamination, yeasts can develop even if the water content of the honey is somewhat lower than 20%.

Honey moulds come from the intestinal contents of the bees, from the hive, or from the environment where the bees work or where the honey is processed. They can multiply on honeycombs kept in moist spaces. Contamination of honey with moulds is relatively frequent, but with a small number of spores (tens or hundreds), which indicates that they can survive in the product but cannot multiply. Some osmotolerant species survive for a long time in honey, multiply, and form colonies visible to the naked eye. Specialised literature indicates the presence of black mould in manna honey. The most common species belong to the genera *Aspergillus*, *Atrichia*, *Bettsia alvei*, *Cephalosporium*, *Chetonium*, *Coniothecium*, *Hormiscium*, *Penicillium*, *Triposporium*, *Uredinaceae*, *Ustilaginaceae*, etc.

The dominant yeasts found in fermented honey are species included in the genera *Debaryomyces* and *Saccharomyces*. When they are in large numbers and find conditions of high humidity and moderate temperature, they multiply, their number being able to reach values ≥1,000,000 UFC/g, triggering the phenomenon of spoilage through fermentation. Fermentation causes the transformation of sucrose into alcohol, CO_2, organic acids, and various compounds with undesirable odours and flavours. Alcohol can turn into acetic acid, and carbon dioxide gives honey a foamy and cloudy appearance. Other yeast species found in honey belong to the genera *Ascosphaera*, *Hansenula*, *Lipomyces*, *Nematospora*, *Oosporium*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, *Schwuniomyces*, *Trichosporon*, *Torula*, *Torulopsis*, *Zygosaccharomyces*, etc. Since the usual temperature for keeping honey (15–20 °C) is the favourable temperature for the development of yeasts, it follows that the decisive factor for the initiation of the fermentation process is the water content, to which the level of contamination of the honey has a secondary contribution (21, 22).

In beekeeping practise, especially during large harvests, some beekeepers extract honey from the combs before the majority of the cells are closed, so before the bees finish processing. The water content of this honey is around the 20% threshold or even exceeds it, so there is an imminent danger of fermentation. Other times, the honey is extracted from the combs, packed,

and stored in poor hygienic conditions, so it has a high level of contamination with fermentation germs and can ferment even if it has a water content of slightly less than 20%. Honey has a pronounced hygroscopic character, so if it is kept in humid spaces and in improperly closed containers, it can reach the risk zone for the establishment of fermentation.

MATERIALS AND METHODS

A total of 236 honey samples from 4 types of honey were collected and analysed: acacia honey, manna honey, linden honey, and polyflora honey, aiming to collect an approximately equal number of samples. The samples were collected from commercial lots (after processing and packaging) and come from producers in different areas of the country. The collection was done respecting the sampling conditions and the identification and transport conditions, and the samples were placed in sterile vials and provided with tight closure systems.

The determination of the number of yeasts and moulds was carried out in accordance with the specifications of reference SR ISO 7954/2001 by the colony counting technique at 25 °C. Working technique: 10 g of honey is dissolved in 90 ml of physiological serum, respecting sterility during the work, after which successive decimal dilutions are made. 1 ml of each dilution to be analysed is placed in two sterile Petri dishes. Pour about 15 ml of DDCA medium (yeast –dextrose – chloramphenicol – agar), previously melted and maintained at 45 °C ± 1 °C in a water bath, into each Petri dish. Carefully mix the inoculum with the medium, then let it solidify, placing the boxes on a cold horizontal surface. In parallel, a control box is prepared to verify sterility. Incubate the seeded Petri dishes at 25 °C ± 1 °C, placing them in the incubator with the lid down. After 3-5 days of incubation, count the colonies in each Petri dish. After 5 days, those boxes containing less than 150 colonies are retained. If there are portions of mould with invasion growth in the boxes or if it is difficult to count the well-isolated colonies, their number is recorded after three or four days of incubation (24, 25). If necessary, a microscopic examination is performed to differentiate, according to their morphology, the colonies of yeasts and moulds from those of bacteria. Boxes containing no more than 300 colonies at the level of two successive dilutions are retained. It is necessary that one of these boxes contain at least 15 colonies. The number of microorganisms per gram of product is then calculated using a calculation formula, with the results being rounded to two significant figures. As a consequence, the number of microbes per gramme of product is recorded, which is reported as a value between 1.0 and 9.9 multiplied by 10x, where x is the power equivalent to ten (23-25).

RESULTS AND DISCUSSIONS

Microorganisms in honey can have several origins, and these sources of contamination can be systematised into primary and secondary sources. The primary sources depend on the period of harvesting and laying of honey in the combs, and they includes contaminants on the surface of flowers, dust, air, pollen, and the digestive tract of bees. The types of microorganisms associated with bees are similar, but not identical, to those found in honey. This means that honey can be contaminated by sources other than bees. Nectar contains very few microorganisms, while pollen and its substitutes can be important sources, especially of yeasts. Sweets used to feed bees can also be an important source of contamination for honey. The secondary sources of contamination for honey are the

Table 1

The results of the determinations of yeasts and moulds from honey samples

No. UFC/g	Acacia honey	Manna honey	Linden honey	Polyflora honey	Total samples
0-10	1	10	5	0	**16**
11-20	2	12	4	2	**20**
21-30	3	8	5	2	**18**
31-40	12	6	7	5	**30**
41-50	14	4	8	12	**38**
51-60	10	5	9	14	**38**
61-70	7	1	11	13	**32**
71-80	4	1	3	8	**16**
81-90	4	0	4	6	**14**
91-100	5	0	2	7	**14**
> 100	0	0	0	0	**0**
Total samples	**62**	**47**	**58**	**69**	**236**

same as for food (e.g., equipment, utensils, surfaces, air, and people).

The investigations carried out on honey samples from Romania revealed a low degree of contamination with yeasts and moulds. The distribution and statistical analysis of the results is shown in Table 1.

Honey has a set of intrinsic properties that ensure the destruction or inhibition of microorganisms. External factors, such as storage temperature, relative air humidity, and the presence and concentration of environmental gases, influence the lifespan of microorganisms in honey. The key to post-harvest control of microorganisms in honey is good processing practises within a well-prepared and fulfilled HACCP plan. The control of microorganisms in the atmosphere in which the processing takes place, as well as on the equipment used, is of particular importance for obtaining honey with appropriate microbiological qualities, but it is difficult to achieve. Experts recommend monitoring the good hygiene practises in the hive and the facilities used to process the honey. Among the recommendations made, we mention: practises used to ripen honey must avoid high moisture content; strict hygiene during extraction; limiting the exposure of honey to air; removing honey residues from the equipment after each use and drying them completely after washing; using containers without microorganisms (sterilised). To sanitise the equipment, chemical disinfectants based on copper, iodine, quaternary ammonium salts, or sodium hypochlorite can be used, according to the instructions (21, 23, 25).

The honey harvested, processed, packaged, and stored in good conditions (hygienic and technological) has a water content in the range of 17–19% and a low level of yeast contamination, so it has a good shelf life. To prevent fermentation and dissolve sugar crystals, it is currently recommended to heat treat honey to inactivate yeast. For this purpose, the honey is heated to 63°C for 30 minutes, which ensures the killing of yeasts. Heating in open vessels also causes dehydration of the honey, which increases its resistance to fermentation. Different time-temperature combinations can be used to inactivate other types of non-sporogenic microorganisms, but these combinations must not exceed the level of pasteurisation. They must not reach sterilisation levels because honey is sensitive to these sterilisation temperatures and degrades. It therefore follows that in order to obtain honey with superior microbiological qualities,the emphasis must be placed on avoiding its contamination and not on removing the microorganisms already present in the honey (23, 24).

Honey stored in combs is an excellent model for how to prevent microbial growth after harvest, although dormant spores may remain in the honey. Unfortunately, the honey has to be removed from the combs and put into different containers in order to be sold. Containers with honey must be packed and stored in conditions that exclude contact with air and that prevent cycles of evaporation of water from the honey and its subsequent condensation on the surface of the product, diluting it and creating favourable conditions for microbial development. The prevention of honey fermentation is achieved by storing it in rooms with temperatures below 10 °C and relative atmospheric humidity below 50% or by treating it thermally. Yeasts that tolerate sugar do not develop, as a rule, below 11 °C or above 38 °C. It should also be noted, as shown above, that honey containing more than 17% water is prone to fermentation, and that honey containing more than 19% water is markedly prone to fermentation (23, 24).

The obtained results demonstrate that the most susceptible to fermentation processes is polyflora honey, which in many cases also has a higher moisture percentage compared to other varieties. Manna honey is kept longer in the combs and harvested later, which gives it a ripening (with implicit loss of moisture) and a decrease in the load of yeasts and moulds.

CONCLUSIONS

Honey has a particular chemical composition, which favours the growth and multiplication of microorganisms or even has a microbicidal effect. From the total of 236 samples, the majority presented a total number of yeasts and moulds at a minimum and medium level, with the exception of several polyflora honey samples that presented a medium to high load. All samples analysed for the total number of yeasts and moulds fell within the legally regulated parameters, a maximum of 100 CFU/g of product, with only a few samples being at the maximum allowed level. The lowest contaminant level was found in the samples collected from manna honey, followed by acacia honey and linden honey (no yeasts or moulds were detected in some samples), compared to polyflora honey samples (in all samples from this category, yeasts and moulds were detected).

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