Microbiological quality and inhibitory potential of selected Croatian apiary honeys

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Summary

The quality of honey is mainly determined by its sensorial, chemical, physical and microbiological properties. The purpose of this research was to evaluate microbiological properties of 72 honey samples and to determine the number and/or presence of aerobic mesophilic and spore-forming bacteria, moulds, yeasts, sulphite-reducing clostridia, bacteria from the *Enterobacteriaceae* family and *Staphylococcus aureus*. Microbiological quality of tested samples was considered good and pathogenic bacteria were not present. Inhibitory potential of selected honey samples was also investigated. Among tested honey concentrations (0.1%, 5%, 10%, 25%, 50% and 75%), the final concentration of 75% had the highest potential. Honeydew and chestnut honey exhibited the strongest inhibitory effect against tested bacterial species, while the lowest inhibition was exhibited by linden (lime tree) honey. Comparing the samples of the same honey type, considerably different inhibitory activity can be detected. Overall, the most sensitive bacterium to the inhibitory effect of tested honey samples was *S. aureus*, while the most resistant one was *Enterococcus faecalis.*

Keywords: honey, microbiological quality, inhibitory potential, pathogenic bacteria

Introduction

Honey is a natural sweet substance, produced by honeybees from the nectar and secretions of living parts of plants, or excretions of plant-sucking insects. It consists mainly of carbohydrates and water, but also contains small amounts of proteins, enzymes, amino acids, minerals, trace elements, vitamins, aroma compounds and polyphenols (Bogdanov et al., 2008). Honey commonly consists of water (17.2%), fructose (38.19%), glucose (31.28%), sucrose (1.31%), reducing disaccharides (7.31%), high sugars (1.5%), and other compounds (White et al., 1962).

Microflora associated with honey bees and their food (bee bread, pollen) is comprised of Gram-variable pleomorphic bacteria, moulds (*Penicillium* and *Aspergillus* genera), bacteria of *Enterobacteriaceae* family, spore-forming bacterial rods (mostly *Bacillus* spp.), and yeasts (Gilliam, 1997). Microorganisms associated with honey include bacteria, moulds and yeasts of various genera. However, only the spore-forming bacteria (*Clostridium* spp., *Bacillus* spp.) can survive in honey for extended periods of time at higher temperatures (20 °C) (Olaitan et al., 2007). It is known that honey may contain *Clostridium* spores. *Clostridium botulinum* can cause infant botulism, and its presence in honey may represent a potential health hazard for some infants fed with honey (Midura et al., 1979).

Since ancient times, honey has been used for medicinal purposes in many cultures. Lately, honey has been rediscovered as a possible remedy for gastroenteritis, gastric ulcers, wounds, and as a suitable sweetener for diabetic patients (Jeffrey and Echazarreta, 1996). Manuka honey has been reported as being a promising functional food for the treatment of wounds and stomach ulcers because of its antibacterial activity against bacteria such as *Helicobacter pylori* and *S. aureus* (Atrott and Henle, 2009). There have been numerous reports of honey having antimicrobial properties (Mandal and Mandal, 2011; Baltrušaityte et al., 2007; Cooper et al., 1999; Miorin et al., 2003; Maeda et al., 2008; Lusby et al., 2005). The antimicrobial effect has been attributed to osmolarity, acidity, hydrogen peroxide, various plant compounds (Molan, 1992b), and recently, to the production of antimicrobial compounds by bacteria present in honey (Hyungjae et al., 2008).

Natural honey exhibits a large variation in the antimicrobial activity against pathogenic bacteria because the composition of active components in plants depends on various factors, particularly plant cultivar and chemotype, and climatic conditions (Baltrušaityte et al., 2007). This unpredictable antibacterial activity of non-standardized honey may hamper its introduction as an antimicrobial agent (Mandal and Mandal, 2011). Moreover, the antimicrobial activity of honeys that rely mostly on the release of hydrogen peroxide might be reduced *in vivo* by catalase activity in tissues and blood. Hence, honeys which antimicrobial activity stems partly from a phytochemical component (manuka honey, chestnut, honeydew honey) might be more effective in comparative clinical trials (Cooper et al., 1999).

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The objective of this study was to determine the microbial populations of 72 Croatian apiary honey samples, and investigate the antibacterial activity of 20 selected honey samples against six medically important bacteria. Only 10 of the 20 honey samples showed a statistically significant antibacterial activity.

Materials and methods

Honey samples

The study was carried out with 72 unpasteurized locally produced honey samples, which were provided by the Faculty of Food Technology Osijek (Table 1). Tested honey samples were divided into 7 groups depending on their floral source: false indigo (*Amorpha fruiticosa* L.), black locust (*Robinia pseudoacacia* L.), Jerusalem thorn (*Paliurus spina-christi* Mill.), common sage (*Salvia officinalis* L.), chestnut (*Castanea sativa* L.), linden (*Tilia* spp.) and honeydew honey. Additionally, two multifloral honey types were tested of different geographic origin.

Table 1. Characterization of honey samples

Honey type	Honey samples	Number of samples
False indigo	M-24, M-89, M-98, M-121	4
Black locust	M-09, M-23, M-32, M-39, M-44, M-45, M-66, M-94, M-97, M-128	10
Jerusalem thorn	M-05, M-07, M-70	3
Common sage	M-02, M-14, M-16, M-31, M-33, M-35, M-51, M-55, M-68, M-110	10
Chestnut	M-10, M-13, M-18, M-64, M-81, M-100, M-101, M-103, M-113, M-125	10
Linden honey	M-22, M-38, M-40, M-52, M-83, M-120, M-130, M-138	8
Honeydew	M-08, M-15, M-37, M-53, M-76, M-87, M-115	7
Multifloral 1	M-11, M-20, M-54, M-56, M-73, M-88, M-96, M-107, M-108, M-135	10
Multifloral 2	M-03, M-34, M-61, M-63, M-77, M-86, M-95, M-105, M-114, M-116	10

Microbial counts

Ten grams of each sample was mixed with 90 mL of sterile saline solution (0.85 w/v NaCl) in sterile stomacher bags and homogenized in BagMixer® 400 P stomacher (Interscience, France) for 60 seconds. Subsequent dilutions were also made with sterile saline depending on the tested microorganism. Aliquots of the appropriate dilution were then pipetted into sterile Petri dishes and homogenized with the appropriate media. All colonies were counted and expressed as the number of tested microorganism per gram of sample.

Aerobic mesophilic bacteria (AMB) were counted on tryptic glucose yeast agar (TGY agar; Biolife, Italy) after the inoculation and incubation at 28 °C for 7 days.

Moulds and yeasts were counted on standard yeast extract glucose chloramphenicol agar (YEGC agar; Biolife, Italy) after incubation at 25 °C for 7 days.

Samples for aerobic spore-forming bacteria (ASB) count were pasteurized in a water bath at 80 °C for 10 minutes and then counted after inoculation and incubation on TGY agar (Biolife, Italy) at 28 °C for 7 days.

Enumeration and detection of *S. aureus* was performed on mannitol salt agar (MS agar; Biolife, Italy) after incubation at 37 °C for 48 hours.

For the detection and enumeration of sulfite-reducing clostridia homogenized and pasteurized (10 minutes at 80 °C) honey samples were inoculated into test tubes containing sulphite polymyxin sulphadiazine agar (SPS agar; Biolife, Italy). After solidification, additional 1.5 mL of sterile molten agar (50 °C) was poured in the test tubes to ensure better anaerobic conditions. Inoculated media was then incubated at 37 °C for 3-5 days. After incubation, test tubes were checked for growth of characteristic black colonies.

The presence of *Enterobacteriaceae* family was detected by transferring 1 mL of each sample in *Enterobacteriaceae* broth Mossel (Biolife, Italy) and incubating at 37 °C for 24 hours (Stevenson and Segner 2001). For test tubes with positive results for the presence of *Enterobacteriaceae* family, honey samples were mixed with sterile saline and with molten and cooled violet red bile glucose agar (VRBG agar; Biolife, Italy).

After solidification agar was covered with an additional layer of the same agar to improve anaerobic conditions. The same step was done with violet red bile lactose agar (VRBL agar; Biolife, Italy) for the enumeration of coliform bacteria. Inoculated media were then incubated at 37 °C for 24 hours and at 37 °C for 48 hours for the enumeration of *Enterobacteriaceae* and coliform bacteria, respectively.

Bacterial strains

Six foodborne pathogens were used in this study. Of the six bacterial strains three were Gram-negative - *Escherichia coli*, *Salmonella* Enteritidis, *Yersinia enterocolitica*, and three were Gram-positive bacteria - *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus* ATCC 6538. All bacteria used were from the collection of cultures from the Department of Biology and Microbiology, Faculty of Food Technology Osijek. Each of the six pathogenic bacteria were cultured on TGY agar at 37 °C, except for *Yersinia enterocolitica*, which was incubated at 25 °C.

Antibacterial activity assay

Prior to the antibacterial activity test of honey samples against the six pathogenic bacteria they were regenerated three times on TGY agar at 37 °C (except for *Yersinia enterocolitica* at 25 °C). 20 different honey samples were selected from five floral groups – Jerusalem thorn (M07, M70), common sage (M14, M51, M55 and M110), chestnut (M13, M18, M64, M100 and M125), linden (M38, M83, M52 and M130) and honeydew honey (M08, M15, M76, M87 and M115). After regeneration the cultures were grown in tryptic glucose yeast broth at 37 °C (Biolife, Italy) (*Y. enterocolitica* at 25 °C) for 18 hours, and 100 µL was transferred into flasks with 150 mL of sterile molten and cooled TGY agar (50 °C). The growth media was then transferred into Petri dishes, and after solidification, placed in a cooler at 4 °C for 24 hours. 6 wells (8 mm) were made in each Petri dish with a sterile cork borer, and in every well 100 µL of each honey sample was pipetted (0.1%, 5%, 10%, 25%, 50%, 75%; w/v honey/sterile distilled water). Petri dishes were then incubated at 37 °C (*Y. enterocolitica* at 25 °C) for 18 hours and the inhibition zone was measured in two perpendicular directions for each agar well (lurlina and Fritz, 2005).

Statistical analysis

Each honey sample was analyzed in duplicate. Results are shown as mean values. Microbial count results were analyzed using Microsoft® Office Excel 2003 for Windows (Microsoft Corporation, Redmond, USA) and GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, USA). The differences between honey sample inhibitions were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test with $\alpha = 0.05$. This analysis was carried out using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, USA) and XL Stat 2009.3.02 (Addinsoft, Brooklyn New York, USA).

Results and discussion

Microbial counts

Honey is considered a microbiologically very stable product. High sugar content, low water activity level (a_w), low pH value, the presence of hydrogen peroxide and other compounds that have inhibitory properties, all contribute to the antimicrobial effect against different types of microorganisms. In spite of having a measurable inhibitory effect on microorganisms, honey is not considered a sterile product. Certain microorganisms can tolerate the extreme conditions found in honey, like spore-producing bacteria (*Clostridium* spp.), xerophilic moulds and osmophilic yeasts (Snowdon and Cliver, 1996).

The highest number of aerobic mesophilic bacteria was detected in false indigo honey (93 CFU/g) compared to all other samples (Table 2). The honey samples studied here had similar numbers to those reported by Piana et al. (1991) who found count values varying from 1 to 55 CFU/g. According to published data, aerobic bacteria counts for honeys can range from 0 to several thousand per gram (Snowdon and Cliver, 1996). Aerobic mesophilic bacteria are a large group of bacteria which have the potential for causing honey spoilage under the right conditions. Hosney et al. (2009) found that honey contains bacteria of the genera *Lactobacillus*, *Streptococcus*, *Micrococcus* and *Bacillus*. Bacteria do not replicate in honey and, as such, high number of vegetative bacterial cells could indicate recent contamination from a secondary source (Snowdon and Cliver, 1996). Although bacterial growth in properly stored honey is virtually impossible (primarily because of the low a_w level), the obtained results show their presence in tested honey samples. In the same honey type a low mean value of moulds and yeasts was detected (15 CFU/g and 3 CFU/g, respectively). The data suggest that moulds may survive but do not tend to grow in honey. This was also reported by Piana et al. (1991). Among all tested microbial groups only moulds and yeasts can survive at low a_w conditions (down to a_w 0.6). Moulds are associated with the intestinal contents of bees, their hive, and the environment in which the bees forage. Hence, high mould count may be indicative of a recent contamination from a secondary source (Snowdon and Cliver, 1996). The growth of these three groups of microorganisms is not possible in properly stored honey. Moulds and yeasts can grow at lower a_w and pH levels than most bacteria, and their presence in honey can be an indicator of diminished quality. In analyzed false indigo honey samples the number of moulds and yeasts was not above 100 CFU/g. Aerobic spore-forming bacteria, like mesophilic bacteria, represent a common causative agent of spoilage of different foodstuffs when optimal conditions arise, and even though they were present in low numbers in tested samples (22 CFU/g), the underlying problem is their ability to resist the inhibitory effect of honey via their spores. Although the number of these bacteria in honey is not strictly defined, it can be useful to determine the quality and microbiological stability of honey. All honey samples were also tested for the presence of bacteria from *Enterobacteriaceae* family, *S. aureus* and sulphite-reducing clostridia. None of the samples contained the aforementioned bacteria. Our results agree with those obtained by Iurlina and Fritz (2005) which show that faecal coliforms, *Clostridium* sulfite-reducers or *S. aureus* were not found in any of the tested samples. Moreover, Nakano and Sakaguchi (1991) and Tysset et al. (1970b) were not able to detect sulphite-reducers or *S. aureus* in honey as well. Vegetative forms of pathogenic bacteria can, if introduced, survive for extended periods of time at cold temperatures, but their presence in honey has never been detected (Snowdon and Cliver, 1996).

Table 2. Microbial populations of tested honey types

Honey type	Microorganism count (CFU/g)*						
	AMB	P	K	ASB	*Clostridium* spp.	*Enterobacteriaceae*	*S. aureus*
False indigo	93 ± 46	15 ± 6	< 10	21 ± 5	< 10	< 10	< 10
Black locust	28 ± 6	< 10	< 10	10 ± 4	< 10	< 10	< 10
Jerusalem thorn	57 ± 22	37 ± 3	23 ± 12	22 ± 4	< 10	< 10	< 10
Common sage	24 ± 6	60 ± 22	42 ± 29	< 10	< 10	< 10	< 10
Chestnut	42 ± 7	14 ± 5	10 ± 7	15 ± 3	< 10	< 10	< 10
Linden honey	60 ± 16	11 ± 5	< 10	16 ± 7	< 10	< 10	< 10
Honeydew	53 ± 17	34 ± 13	< 10	31 ± 10	< 10	< 10	< 10
Multifloral 1	95 ± 26	25 ± 15	25 ± 17	11 ± 4	< 10	< 10	< 10
Multifloral 2	72 ± 33	10 ± 4	38 ± 32	11 ± 4	< 10	< 10	< 10

*Values are mean ± SEM of duplicates; AMB – aerobic mesophilic bacteria; P – moulds; K – yeasts; ASB – aerobic sporogenic bacteria

Black locust honey samples contained, overall, the lowest number of each tested microbial group. Yeasts were not detected in any of the samples. The low mean value of aerobic mesophilic bacteria count (28 CFU/g) and low counts for moulds and aerobic spore-forming bacteria (6 CFU/g and 10 CFU/g, respectively) make this honey microbiologically very stable and of very good quality.

Multifloral honey is derived from different floral sources and higher variability between microbial count results is to be expected. The aerobic mesophilic bacteria count was relatively high for multifloral 1 honey samples (95 CFU/g), and so was the number of yeasts and moulds compared to other honey samples (both mean values were 26 CFU/g). This fact makes this type of honey more susceptible to spoilage should the water content rise. Osmophilic or sugar tolerant yeasts represent a problem in the honey industry because of their ability to grow under acidic conditions, and are not inhibited by sucrose. They can grow even at limited water levels available in ripe honey, and as a result, they can readily ferment honey (Snowdon and Cliver, 1996).

In the case of Jerusalem thorn honey, the number of yeasts and moulds was rather high compared to other samples (26 CFU/g and 37 CFU/g, respectively), except for common sage. Moreover, the aerobic spore-forming bacteria count was one of the highest of all samples with a mean value of 22 CFU/g, ranging from 15 to 30 CFU/g.

Common sage honey samples had a very low mean value for aerobic mesophilic bacteria (24 CFU/g). The number of yeasts (60/g) and moulds (42/g) was averagely the highest, which makes this type of honey microbiologically unsatisfactory. It should be emphasized, however, that two of the samples with a higher number of yeasts and moulds (250 CFU/g) contributed significantly to the overall result for this type of honey.

On the other hand, microbial counts of chestnut honey were low for each group of microorganisms, as were the counts of linden honey samples. As indicated by Küçük et al. (2007) the high antimicrobial activity of chestnut honey could be the result of high polyphenolic content and higher antioxidant potential characteristic to this type of honey.

In honeydew honey samples, the average microbial load for aerobic mesophilic bacteria was 53 CFU/g, while the average mould and spore-forming bacteria counts were somewhat lower (35 CFU/g and 32 CFU/g, respectively). Yeasts were not detected in any of the honeydew honey samples. Antimicrobial properties discourage the growth or persistence of many microorganisms, and as such, honey can be expected to contain low numbers and limited variety of microbes (Snowdon and Cliver, 1996).

Antibacterial activity assay

The antibacterial activity assay was conducted with six different concentrations of honey, however, only the values from the highest concentration (75%) are presented in the results. High incidence of results with no inhibition of selected bacteria for lower percentages of honey concentration showed that only samples with the highest concentration have a statistically significant antibacterial activity. Furthermore, only the results of complete inhibition are shown (completely clear zone around the agar well void of bacterial cells), and not the results of partial inhibition (zone around the agar well with reduced number of bacterial cells).

Most bacteria and other microbes cannot grow or reproduce in honey i.e. they are dormant and this is due to antibacterial activity of honey (Olaitan et al., 2007). Honey has been shown to be bactericidal to many different bacteria including: *S. aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Shigella flexneri*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* ser. *typhimurium*, *Shigella sonei*, *Bacillus cereus* and others (Baltrušaityte et al., 2007; Mandal and Mandal, 2011; Hyungjae et al., 2008; Mundo et al., 2004; Taormina et al., 2001).

Among 20 tested honey samples 10 samples with the highest inhibition are shown in the results. The results of the antibacterial activity assay of selected honey samples showed that only honeydew honey has an inhibitory potential against the bacterium *E. faecalis* (Fig. 1). Two honeydew samples, M15 and M87, had an inhibitory potential of 8 mm and 14 mm, respectively, which shows that even samples from the same honey type can have a considerably different inhibitory effect. Mundo et al. (2004) showed, likewise, that the ability of honey to inhibit the growth of microorganisms varies widely, and could not be attributed to a specific floral source or demographic region.

As shown in Fig. 1, the greatest inhibition zones for *Listeria monocytogenes* bacterium were caused by honeydew honey sample M87 (12 mm), followed by linden honey sample M83 (11.5 mm). Inhibition value for other honey samples ranged from 9.5 mm (Jerusalem thorn – M07) to 11 mm (common sage – M51; honeydew honey – M15; chestnut – M64).

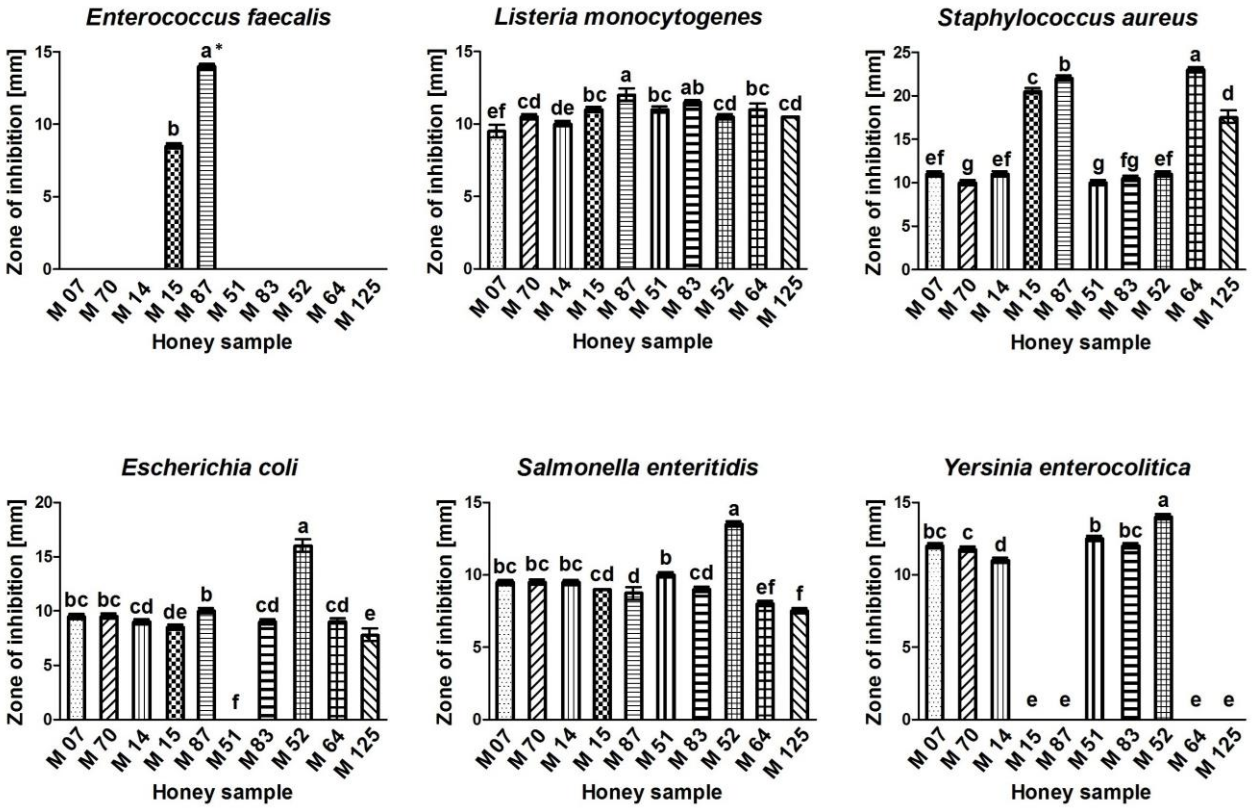

Fig. 1. Inhibition zone diameter of tested pathogenic bacteria

Colums with the same letter represent values that are not statistically different ($p < 0.05$)
*Values are expressed as mean ± SEM of duplicates

Although the tested bacteria discussed before were all Gram-positive, variation in inhibition potential can be seen for different honey samples and honey types. *S. aureus* showed the highest sensitivity toward honey inhibition compared to other bacteria. Largest inhibition zones were caused by chestnut honey M64 and honeydew honey M87 (24 mm and 22 mm, respectively). Linden honey sample (M52) and Jerusalem thorn (M07) showed the weakest inhibitory potential. Cooper et al. (1999) found that honeys completely inhibit *S. aureus* at greater dilutions, which may indicate that the inhibition mechanism is not achieved only through their osmolarity. Similarly, Selcuk and Nevin (2002) found that *S. aureus* failed to grow at a concentration of 40% or above in apiary honeys from Turkey. The inhibitory effect against the bacterium is probably partly due to enzymatic formation of hydrogen peroxide in honeys as suggested by Baltrušaityte et al. (2007), Mundo et al. (2004) and Taormina et al. (2001). Proteinaceous substances found in some honeys are also responsible for some of the inhibitory activity against *S. aureus* (Hyungjae et al., 2008).

The inhibitory results of different honey samples against *E. coli* showed only slight differences in inhibition for all samples (8 mm to 11 mm) except for linden honey sample M52 (16 mm), and common sage sample M51, which showed no inhibition. In this case, it could be concluded that the primary inhibition mechanism is achieved through high sugar content or osmolarity (except for samples M52 and M51). According to Nakano and Sakaguchi (1991) *E. coli* is not usually present in honey. Moreover, it cannot survive for very long (< 10 days at 20 °C) if introduced into honey (Tysset and Durand, 1973).

As can be seen in the results for *E. coli*, the results for bacterium *Salmonella* Enteritidis show, overall, the same slight differences in inhibitory potential between tested honey samples. Linden honey sample M52 had the highest inhibitory effect (13.5 mm), while the chestnut honey sample M125 had the lowest effect (7.5 mm). Other honey samples had their inhibition zones in the range from 8 mm to 10 mm.

The common sage honey samples (M14 and M51), linden honey samples (M83 and M52) and Jerusalem thorn honey samples (M07 and M70) showed a relatively high inhibitory effect against *Y. enterocolitica*. The results ranged from 11 mm (M14) to 14 mm (M52). On the other hand, chestnut honey samples (M64 and M125) and honeydew honey samples (M15 and M87) showed no inhibitory effect against the bacterium.

Conclusions

Among different honey types a limited variety and low number of microorganisms can be found which indicates a relatively high antimicrobial and inhibitory potential against pathogenic bacteria, spore-forming bacteria, aerobic mesophilic bacteria, moulds and yeasts. The number of these microorganisms depends on the floral source and geographic origin of honey, even though a high variability among the same type of honey can be detected. At sufficiently high concentrations honey inhibits pathogenic bacteria where, overall, the most sensitive bacterium was *S. aureus*, and the most resistant one was *E. faecalis*. In addition, honeydew and chestnut honey exhibited the strongest inhibitory effect against tested bacterial species, while the lowest inhibition was exhibited by linden honey. The study demonstrated the relevance of honey as a healthy alimentary product, and as a possible source of biologically active ingredients which could have important clinical applications.

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