QUANTIFICATION OF FIRMICUTES, ACTINOBACTERIA, AND GAMMAPROTEOBACTERIA FROM BOHEMIAN HONEY*

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Honey, which has been used as an ancient remedy for infected wounds, has been shown in laboratory studies to have antimicrobial action against a spectrum of bacteria and fungi. Because very little quantitative information exists on the microbiota of honey, the aim of this study was to quantify the Actinobacteria, Firmicutes, and Gammaproteobacteria groups in samples of honeydew honey and blossom honey from six regions in the Czech Republic, using quantitative real-time PCR analysis with specific primers based on the *16S rRNA* gene. Gammaproteobacteria and Firmicutes were clearly the most abundant, predominating Actinobacteria in both types of honey. Most of the Firmicutes were detected in samples from South Bohemia (mean gene copies per 1 g honey: 5.6×10^5) and Ústí nad Labem Region (3.7×10^5), which contained the lowest number of Gammaproteobacteria (15.5×10^3). The Actinobacteria were prevalent in samples from Plzeň (4.3×10^3) and Central Bohemia (5.4×10^3), where conversely the Firmicutes were least abundant. Honey thus contains bacterial species with probiotic activity and oligosaccharides which can act as prebiotics, suggesting that its incorporation into the human diet may potentially impart significant health benefits to consumers compared with 'empty calories' consumed as refined sugar.

Blossom, honeydew, Czech Republic, qPCR, microbiota

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INTRODUCTION

Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, and then deposit, dehydrate, store, and leave in the honey comb to ripen and mature (FAO, 2001). This sweet substance is most commonly consumed in its unpreserved state; that is, liquid, crystallized, or in the comb. In these forms, it is used as medicine, eaten as food, or incorporated as an ingredient into various food recipes. In confectionery production, honey is still included in many traditional products which are consumed locally in considerable quantities and also exported. Honey is appreciated not only for its taste and flavour, but also for its high nutritive value and contribution to human health. There are many published reports describing a wide range of therapeutic effects (e.g. antibacterial, antiviral, antioxidative, and anti-inflammatory properties) of honey which are useful in stimulating the healing of wounds and burns and treating gastric ulcers and gastritis (Radwan et al., 1984; Jeddar et al., 1985; Subrahmanyam, 1991; Schramm et al., 2003; Al-Waili, 2004; Simon et al., 2006; van den Berg et al., 2008; Mandal, Mandal, 2011; Anthimidou, Mossialos,

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2013; Tomblin et al., 2014; Almasaudi et al., 2015, 2017; Boyanova et al., 2015; Tsang et al., 2015). The high osmolality, acidity, and presence of hydrogen peroxide (H_2O_2) in natural honey make the growth of microorganisms in the substance difficult (Wahdan, 1998; Brudzynski, 2006; Kwakman et al., 2010; Mandal et al., 2010a, b; Aurongzeb, Azim, 2011). Non-peroxide factors, such as lysozyme, phenolic acids, polyphenols, and flavonoids, also contribute to honey's antibacterial properties (Molan, 1992; Weston, 2000; Taormina et al., 2001; Brady et al., 2004; Kucuk et al., 2007; Hsieh et al., 2008; Al-Hindi et al., 2011; Al-Waili et al., 2011; Kwakman et al., 2011; Brudzynski et al., 2012; Kwakman, Zaat, 2012). In addition, because honey contains some propolis and bee pollen, part of its antimicrobial activity may be due to the presence of antimicrobial substances present in these components (Viuda-Martos et al., 2008; Redzic et al., 2011). Given these conditions, few microorganisms have the capacity to develop or remain in honey, and the microbes present are likely derived from primary or secondary sources of contamination. The primary sources of microbial contamination include pollen, the digestive tracts of honey bees, dirt, dust, air, and flowers. Secondary sources of microbial contamination in honey are humans, equipment, containers, wind, dust, etc. (Snowdon, Cliver, 1996; Olaitan et al., 2007). There are two recognised types of honey that differ in composition and which therefore should have different properties and bacterial spectra. Blossom honey (BH) is derived from the nectar of plants, whereas honeydew honey (HH) is derived mainly from the excretions of plant-sucking insects (Hemiptera) on the living parts of plants or the secretions of living parts of plants (FAO, 2001). Consumers have different demands for BH and HH; in many countries, nectar honey is valued more highly than HH, but in some countries, including the Czech Republic, HH is preferred (Sanz et al., 2005; Sanova et al., 2017). However, in spite of its usefulness, honey is known to contain certain microbes and is in fact described as a reservoir for microorganisms. The microbes present in honey are those that can withstand its concentrated sugar, acidity, and other antimicrobial components. Conventional microbiology and PCR-based studies have reported several species of cultivable and non-cultivable bacteria, yeasts, and filamentous fungi from honey, the compositions of which are tightly associated with the honey's botanical and geographical origins (Kropf et al., 2009; Castro-Vazquez et al., 2010; Kaskoniene, Venskutonis, 2010; Olivieri et al., 2012; Lazarevic et al., 2013; Sinacori et al., 2014). One of the widely used PCR techniques is quantitative PCR which analyses environmental and clinical microbiological samples. However, this technique has limitations in that it tends to underestimate or overestimate microbial counts, because its main limitation is its inability to discriminate between dead and live cells including DNA of some of them that can be found in the environment (Masters et al., 1994; Wolffs et al., 2005; Sontakke et al., 2009; Maciel et al., 2011; Pathak et al., 2012; Li et al., 2013).

Despite the recent literature on the microbiota of honey, very little and only inconsistent information exists on the quantity of bacteria contained within this sweet substance. The aim of this study was therefore to compare samples of honeydew and blossom honey from six regions in the Czech Republic, using the quantitative real time polymerase chain reaction (qPCR) to quantify the Actinobacteria, Firmicutes, and Gammaproteobacteria groups specifically. Bacteria within these three groups are probiotic species or species with potential probiotic activity, which contaminate honey via the digestive tract of honey bees, nectar of flowers, or honeydew.

MATERIAL AND METHODS

Honey samples

The two honey types (22 samples of BH and 6 of HH) were sampled from six regions (Pardubice, Ústí nad Labem, Hradec Králové, Plzeň, South Bohemia, and Central Bohemia) in the Czech Republic (Table 1, Fig. 1). The samples and bee management represented traditional beekeeping practices in the Czech Republic. The honey samples were collected into disposable tubes and immediately frozen on dry ice, because freshness is related to storage and it is the problem of crystallized honey. Very few bacteria or microorganisms can survive in an environment like that, they

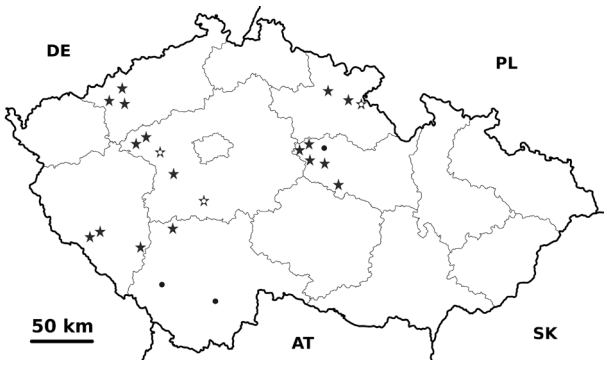

Fig. 1. Origin of the honey samples

Star indicates the place where blossom honey was sampled, a dot indicates the origin of honeydew honey, and a cross with a white dot indicates the place where both blossom honey and honeydew honey were sampled. Městečko u Křivoklátu (Central Bohemia) and Jaroměř (Hradec Králové) show only one cross because the two samples of blossom honey were from different beekeepers

Table 1. Places of origin and types of sampled honeys

Region	Type of honey	Region	Type of honey
Central Bohemia		**South Bohemia**	
Hrabří	honeydew	Prachatice	honeydew
Hrabří	blossom	Dobrá Voda	honeydew
Městečko u Křivoklátu	blossom	Čimelice U Písku	blossom
Městečko u Křivoklátu	blossom	**Hradec Králové**	
Městečko u Křivoklátu	honeydew	Dvůr Králové	blossom
Řeřichy	blossom	Jaroměř	blossom
Senomaty	blossom	Jaroměř	blossom
Tmaň u Berouna	blossom	Přibyslav	blossom
Ústí nad Labem		Přibyslav	honeydew
Kadaň	blossom	**Pardubice**	
Žatec	blossom	Břehy u Přelouče	blossom
Jirkov	blossom	Hlinsko	blossom
Plzeň		Chvaletice	blossom
Klatovy	blossom	Pardubice	honeydew
Spůle	blossom	Rašovy u Přelouče	blossom
Horažďovice	blossom	Slatiňany	blossom

just die, but qPCR detects also dead and live cell of bacteria. This means that freshness of honey does not influence the qPCR results. Every sample was weighed. Approximately 100–150 mg of the honey was used for individual isolation of the total bacterial DNA, using the ZR Faecal DNA MiniPrep Kit (Zymo Research, Irvine, USA).

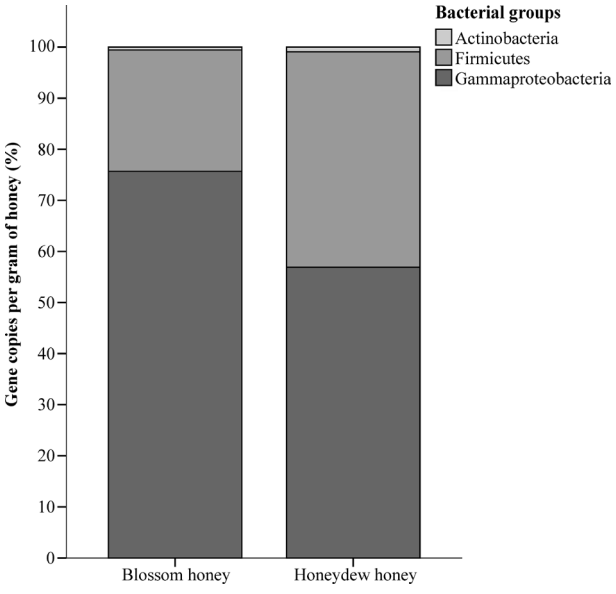

Figure 2. Quantification of bacterial DNA of Actinobacteria, Firmicutes, and Gammaproteobacteria

The number of copies of the *16S rRNA* gene per 1 g honey scaled to 100% is shown on the Y-axis

Quantitative real-time PCR analysis

Bacterial DNA was quantified using a MX3005P thermocycler (Stratagene, La Jolla, USA), based on the *16S rRNA* gene copy numbers with specific primers for Gammaproteobacteria (1080γF, γ1202R), Firmicutes (928F-Firm, 1040FirmR), and Actinobacteria (Act920F3, Act1200R) (De Gregoris et al., 2011) (Table 2). IBM SPSS Statistics Version 20 (IBM, Armonk, USA) was used for the descriptive data analysis and for visualisation of the qPCR data.

RESULTS

The aim of this study was to quantify the probiotic species-containing Actinobacteria and Firmicutes as well as Gammaproteobacteria, as bacteria with potential probiotic activity against honey bees. However, the composition of honey changes depending on its botanical origin (HH or BH) and geographical region, because soil and climate characteristics determine the melliferous flora (Anklam, 1998; Rashed, Soltan, 2004; Castro-Vazquez et al., 2010). Therefore, we compared samples of HH and BH from six regions in the Czech Republic, using qPCR to quantify the three selected bacterial groups. Our results indicated that Gammaproteobacteria predominated in all the samples of honey (56.9% in HH vs 75.7% in

Table 2. Taxon-specific primer pairs used (De Gregoris et al., 2011)

Target group	Primer	Sequence
Gammaproteobacteria	1080γFγ1202R	TCGTCAGCTCGTGTYGTGACGTAAGGGCCATGATG
Firmicutes	928F-Firm1040FirmR	TGAAACTYAAAGGAATTGACGACCATGCACCACCTGTC
Actinobacteria	Act920F3Act1200R	TACGGCCGCAAGGCTATCRTCCCCACCTTCCTCCG

BH), followed by Firmicutes (42.2% in HH vs 23.8% in BH) and Actinobacteria (0.9% in HH vs 0.6% in BH) in order of decreasing abundance (Fig. 2).

The HH samples contained more Firmicutes (mean gene copies per 1 g honey: 2.6×10^5) than the BH samples (1.2×10^5) (Fig. 3A). We also quantified the species of this bacterial phylum in samples from different Bohemian regions (Fig. 3B), with most of them being detected in samples from South Bohemia (5.6×10^5) and Ústí nad Labem (3.7×10^5). The least was in the honey from the region of Plzeň (2.1×10^4). As mentioned above, the most abundant group of bacteria was Gammaproteobacteria, likely contaminating the honey through the process of honey production, and it was higher in the BH samples (mean gene copies per 1 g honey: 3.9×10^5 vs 3.5×10^5 in HH samples) (Fig. 4A). However, this difference was not significant because the main source of Gammaproteobacteria is the digestive tract of honey bees. Samples of honey from the region of Ústí nad Labem contained only 15.5×10^3 gene copies of Gammaproteobacteria (Fig. 4B). The differences in Gammaproteobacteria among the other five regions were not so considerable in comparison with those seen for Firmicutes and Actinobacteria. The least abundant group was Actinobacteria, which was more prevalent in HH (mean gene copies per 1 g honey: 5.8×10^3) than in BH (3.0×10^3) (Fig. 5A). The Actinobacteria were prevalent in samples from the regions of Plzeň (4.3×10^3) and Central Bohemia (5.4×10^3), where conversely the Firmicutes were the least abundant. Counts of Actinobacteria (5.6×10^2) were the lowest in South Bohemia (Fig. 5B).

DISCUSSION

Phylum Firmicutes, which includes two clusters (Firm4 and Firm5) that are largely restricted to the bee

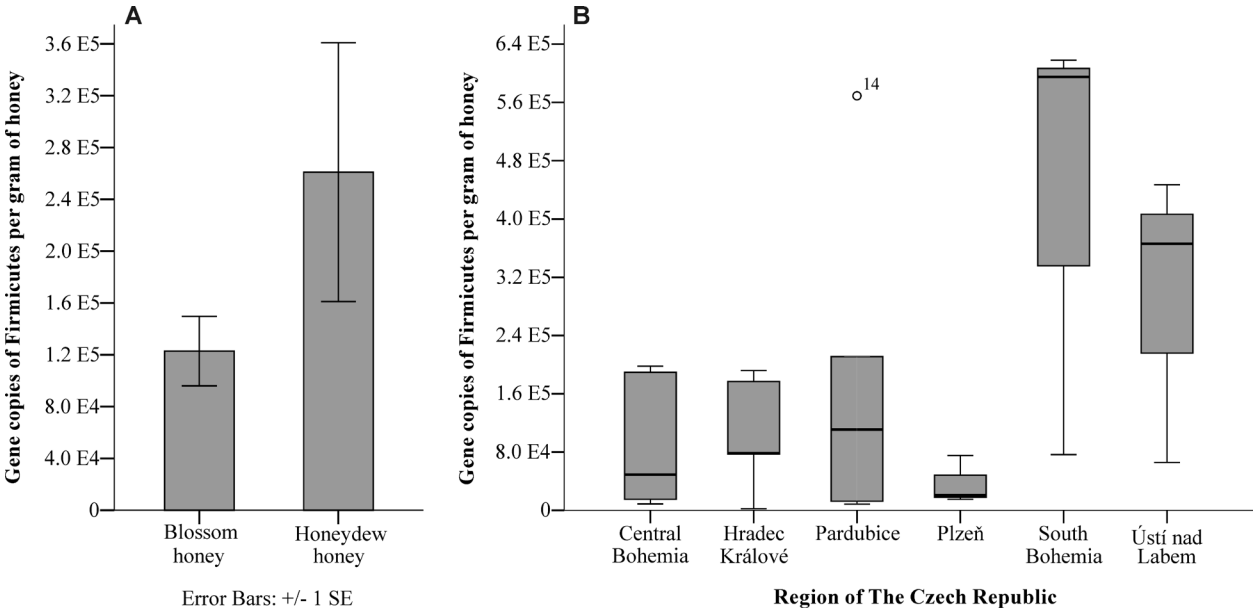

Figure 3. Quantification of bacterial DNA of Firmicutes

(**A**) Quantitative determination (copies of the *16S rRNA* gene per 1 g honey) of Firmicutes in blossom honey and honeydew honey; values are means ± SE. (**B**) Boxplot of quantitative real-time PCR (qPCR) data of the Firmicutes abundance in honey samples from six selected regions of the Czech Republic. The Y-axis shows copies of the *16S rRNA* gene per 1 g honey. Boxes show pooled data from honey samples from each region. The code of outlier 14 refers to the sample of honeydew honey from Pardubice, Region Pardubice

gut (Moran et al., 2012), was more prevalent in the samples of HH, which is generally characterised by higher values of electric conductivity, pH, and acidity (Terrab et al., 2003; Diez et al., 2004; Marini et al., 2004; Conti et al., 2007; Ouchemoukh et al., 2007; Manzanares et al., 2011). These conditions can create a suitable environment for lactobacilli. Moreover, the higher ash and oligosaccharide contents indicate potential prebiotic activity, increasing the populations of bifidobacteria and lactobacilli (Sanz

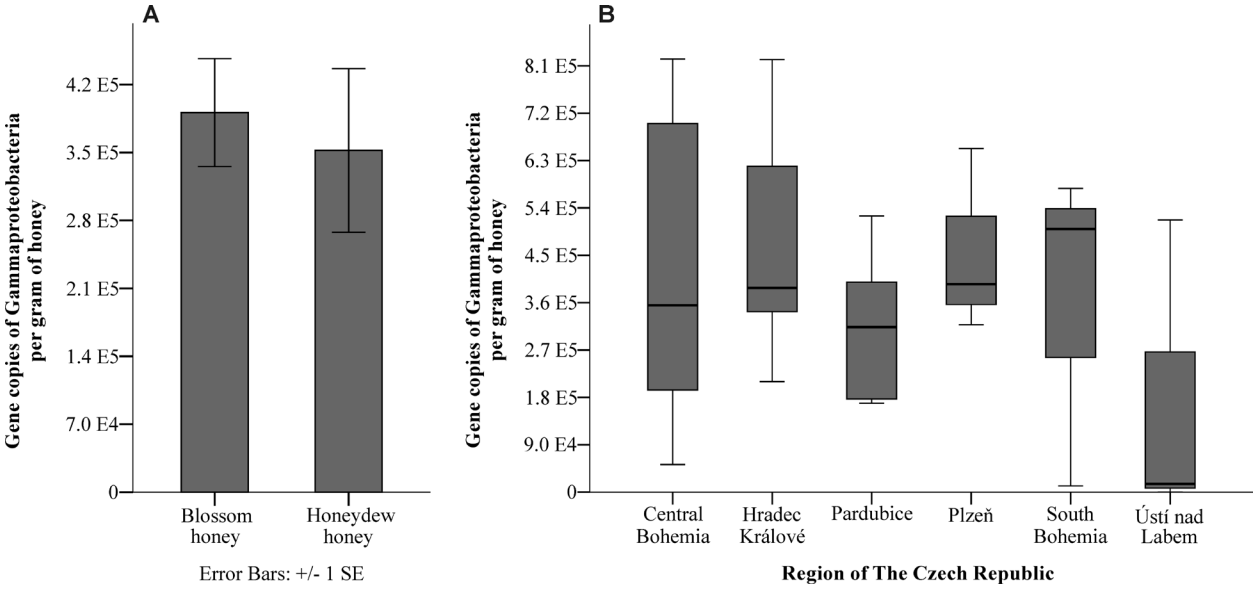

Figure 4. Quantification of bacterial DNA of Gammaproteobacteria

(**A**) Quantitative determination (copies of the 16S rRNA gene per 1 g honey) of Gammaproteobacteria in blossom honey and honeydew honey; values are means ± SE. (**B**) Boxplot of quantitative real-time PCR (qPCR) data of the Gammaproteobacteria abundance in honey samples from six selected regions of the Czech Republic. The Y-axis shows copies of the 16S rRNA gene per 1 g honey. Boxes show pooled data from samples of honey from each region

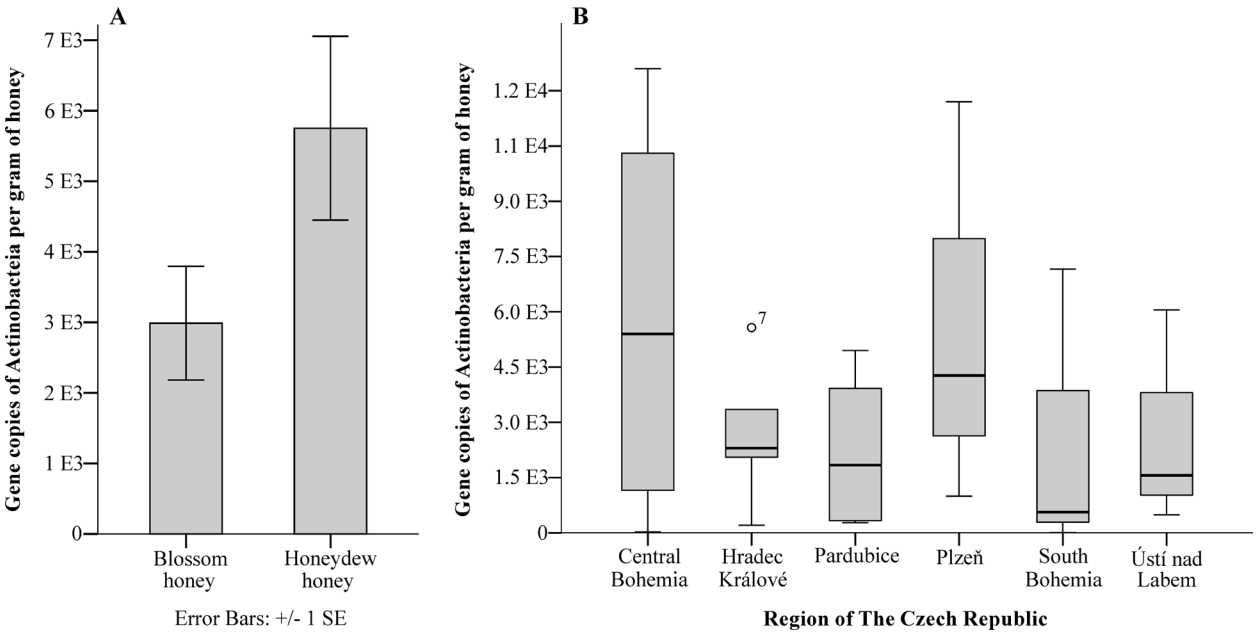

Figure 5. Quantification of bacterial DNA of Actinobacteria

(**A**) Quantitative determination (copies of the 16S rRNA gene per 1 g honey) of Actinobacteria in blossom honey and honeydew honey; values are means ± SE. (**B**) Boxplot of quantitative real-time PCR (qPCR) data of the Actinobacteria abundance in samples of honey from six selected regions of the Czech Republic. The Y-axis shows copies of the 16S rRNA gene per 1 g honey. Boxes show pooled data from samples of honey from each region. The code of outlier 7 refers to the sample of blossom honey from Přibyslav, Region Hradec Králové

et al., 2005). These can be beneficial not only as probiotics in honey, but also for the human gut microbiota, imparting nourishment benefits, such as fermentation ability and the break-down of nutrients to facilitate absorption of short-chain fatty acids, ions, amino acids, and vitamins; protective effects, preventing the invasion of pathogenic microorganisms; and trophic effects in the gut epithelium and digestive system (Anadon et al., 2016).

Although the BH samples contained more Gammaproteobacteria (Fig. 4A), this difference is not significant since the main source of these bacteria is the digestive tract of honey bees, and the honey is therefore contaminated through the process of honey production, when the honey bees ingest nectar and convert it with the help of enzymes. Besides these enzymes, some symbiotic microorganisms associated with the unique bee gut microbiota can also be incorporated into the honey. These consist mainly of eight bacterial phylotypes: two from the Alphaproteobacteria, two from the Gammaproteobacteria, two from *Lactobacillus*, one from *Bifidobacterium*, and one from the Betaproteobacteria (Martinson et al., 2011; Moran et al., 2012). Adult honey bees producing honey were shown to contain 5.1×10^7 gene copies of Gammaproteobacteria per 1 g of total digestive tract content (Hroncova et al., 2015). *Gilliamella apicola* and *Frischella perrara* are the most commonly occurring Gammaproteobacteria in the digestive tract of honey bees. Moreover, aphids producing honeydew were also shown to contain some symbiotic species of Gammaproteobacteria; namely, pea aphid secondary symbiont (PASS), pea aphid U-type symbiont (PAUS), pea aphid T-type symbiont (PABS) and *Buchnera* sp. (Unterman et al., 1989; Chen et al., 1996, 2000; Chen, Purcell, 1997; Fukatsu et al., 2000; Darby et al., 2001; Sandstrom et al., 2001; Tsuchida et al., 2002). However, our BH samples contained an excess of ~3.9×10^4 gene copies of Gammaproteobacteria per 1 g of honey over that of the HH samples.

The least abundant bacterial group was Actinobacteria within the bifidobacteria cluster closely related to the honey bee gut (Rada et al., 1997; Jeyaprakash et al., 2003; Olofsson, Vasquez, 2008; Vasquez, Olofsson, 2009; Martinson et al., 2011; Moran et al., 2012). In general, these bacteria grow on rich media, consistent with their host-associated lifestyle, and require anaerobic or microaerophilic conditions, which is consistent with the likely lowered oxygen availability within the honey bee gut lumen compared with the conditions of the flower nectar. This caused the predominance of Actinobacteria in HH rather than in BH. However, HH has higher antioxidative and antibacterial properties (Prodolliet, Hischenhuber, 1998) which could be caused by the action of probiotic bacteria (bifidobacteria and lactobacilli), including the production of H_2O_2, organic acids, bacteriocins, and strain-specific metabolites (Servin, 2004).

HH has an appreciably higher oligosaccharide content (Doner, 1977; Prodolliet, Hischenhuber, 1998), implying its potential prebiotic activity for increasing the populations of probiotic microbiota in the human gut (Sanz et al., 2005). Thus, daily intake of these new symbionts would be necessary to be able to populate the human body and maintain their benefits (Anadon et al., 2016).

CONCLUSION

Our results showed that HH contains more Firmicutes and Actinobacteria (both groups that contain beneficial bacteria) than BH which is rich in Gammaproteobacteria. The Actinobacteria and Gammaproteobacteria were the most abundant microbes in samples from the region of Central Bohemia. Conversely, the Firmicutes prevailed in honey from the region of South Bohemia. Although honey contains fewer microorganisms than other neutral foods, honeybee products nevertheless contain several lactic acid bacteria and bifidobacteria that act as beneficial probiotics when ingested, suggesting that incorporation of honey into the human diet or as a food ingredient may potentially impart significant health benefits to consumers. Therefore, our recommendation is to serve honey as a substitution for some of the 'empty calories' being consumed as refined sugar.

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