Analysis of bacteria associated with honeys of different geographical and botanical origin using two different identification approaches: MALDI-TOF MS and 16S rDNA PCR technique

Pawel Pomastowski1*, Michał Złoch1, Agnieszka Rodzik1,2, Magda Ligór1,2, Markus Kostrzewa3, Bogusław Buszewski1,2

1 Interdisciplinary Center for Modern Technologies, Nicolaus Copernicus University in Torun, Torun, Poland, 2 Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University in Torun, Torun, Poland, 3 Bruker Daltonik GmbH, Bremen, Germany

* p.pomastowski@umk.pl

Abstract

In the presented work identification of microorganisms isolated from various types of honeys was performed. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rDNA sequencing were applied to study environmental bacteria strains. With both approaches, problematic spore-forming Bacillus spp., but also Staphylococcus spp., Lysinibacillus spp., Micrococcus spp. and Brevibacillus spp. were identified. However, application of spectrometric technique allows for an unambiguous distinction between species/species groups e.g. B. subtilis or B. cereus groups. MALDI TOF MS and 16S rDNA sequencing allow for construction of phyloproteomic and phylogenetic trees of identified bacterial species. Furthermore, the correlation between physicochemical properties, geographical and botanical origin and the presence bacterial species in honey samples were investigated.

Introduction

Honey, a supersaturated solution of sugars (mostly glucose and fructose) produced by Apis mellifera, is the first and most reliable sweetener used by human beings [1]. In addition to the high nutritional value that makes it a highly consumed food product around the world, honey is also known for its healing, antioxidant as well as antimicrobial properties [2],[3]. High antibacterial effect of honey is primarily related to the high sugar concentration. This implicates its hyperosmotic nature, high viscosity, and low water content which in consequence prevent the growth or even survival of most vegetative forms of human pathogenic microorganisms by their desiccation as well as limited atmospheric oxygen penetration [4],[5]. Moreover, natural acidity of this product, the ability to produced hydrogen peroxide, and presence of numerous phytochemical factors such as phenols, terpenes or flavonoids (e.g. pinacambrin) cause that honey has a permanent place in the treatment of wound infections and burns [6],[7],[8].
Despite its richness in sugar and inhibins, honey cannot be considered as sterile since many studies proved that it is subject to bacterial and fungal contaminations derived from two kind of sources: primary and secondary [9],[10],[11]. The first ones include pollen, dirt, dust, air, water, flowers, as well as the digestive tracts of honeybees and are considered as natural sources which are difficult to control [12] [13]. The secondary sources are those arising from the honey manipulation by people, thus are closely connected with hygiene of processing, handling, and storage [11]. Such sources includes skin, mouth, and nose of food handlers, equipment, buildings as well as cross-contamination during harvest and processing in honey houses, however, they can be easy control by the application of good manufacturing practices [14],[15]. Microorganisms found in honey must demonstrate the ability to withstand the concentrated sugar, acidity and other severe conditions, thus in most cases they are present in latent forms (dormant) such as spores [4]. Therefore, besides different species of molds and yeasts, the major microbiological contaminants of honey include spore forming bacteria, e.g. *Clostridium* spp. and *Bacillus* spp. [16], [17], [13]. Although studies on microbial contamination of honey are mainly focused on the occurrence of *C. botulinum* [18], *Bacillus* spp. are also microorganisms of concern since some of them (e.g. *B. cereus*) are associated with spoilage of food and foodborne outbreaks [15], [19].

In contrast to the physicochemical properties, microbial contamination of honey has not been thoroughly investigated so far, which is reflected in the lack of proper legislation concerning this issue in the European Union [13]. In the available literature related to honey microbiota investigation, the technique used for microorganisms identification most frequently still is 16S rDNA sequencing, also considered as a gold standard of microorganism identification. However, molecular assays require a high level of expertise and can be quite expensive. Thus, they are not ideally suitable for routine identification which primarily requires rapidity and low cost at the same time [20]. Therefore, since last 10 years a novel identification approach called Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI--TOF MS) steadily is gaining in popularity due to its high accuracy of identification, the robustness as well as rapidity of obtaining results [21]. This technique relies mostly on the detection of microbial protein patterns (proteomic approach), and analysis of such large biomolecules is possible thanks to the so-called soft ionization mechanism [22], [23]. To date, there are no scientific reports on the wider use of MALDI-TOF MS in the investigation of the microbiological composition of different types of honey. Therefore, the main goal of this study was to identify bacterial species present in honey samples using two different diagnostic approaches—genomic (16S rDNA sequencing) and proteomic (MALDI-TOF MS via MALDI Biotyper platform) in order to compare their usefulness in the characterization of the bacterial composition and thus in controlling microbiological purity of honey. Moreover, the influence of the physicochemical features and geographical origin of honey on their bacterial composition was analyzed.

### Results

#### Physicochemical properties of honey

Investigated honey samples significantly differed in both pH, total acidity, color, and electrical conductivity (Table 1). pH ranged from 3.3 to 5.0, however, most of the honeys (70%) had a pH value ≤4.0. The highest pH values were noted for both honeydew honeys and goldenrod nectar—>4.3. Considering acidity, values of TA ranged from 12.8 (RNSK) to 44.0 (BBS) and in most cases (70%) not exceeding 30 mval/kg. Investigated honeys demonstrated high variety in their color—from white (18–34 mm in Pfund scale) to dark amber (>118 mm) and were mostly represented by darker ones—65%. EC ranged from 0.183–0.187 for rape nectar to...
Table 1. List of investigated honeys with physicochemical parameters.

| Country | Name                        | Acronym | pH | Total acidity [mval/kg] | Color [mm] | Electrical conductivity [mS/cm] |
|---------|-----------------------------|---------|----|-------------------------|------------|-------------------------------|
| Poland  | buckwheat Sądecki Bartnik   | BSB     | 3.9| 35.0                    | 107.50     | 0.310                         |
|         | buckwheat Barć Świętokrzyska| BBS     | 3.6| 44.0                    | 184.63     | 0.398                         |
|         | buckwheat Karczowska Górne  | BKG     | 4.0| 29.0                    | 231.05     | 0.467                         |
|         | multflorous Sądecki Bartnik | MSB     | 3.6| 16.0                    | 75.32      | 0.292                         |
|         | multflorous Wilga           | MW      | 4.3| 30.0                    | 59.72      | 0.854                         |
|         | multflorous B Szymanski     | MBS     | 3.7| 16.8                    | 18.25      | 0.249                         |
|         | honeydew Sądecki Bartnik    | HSB     | 4.5| 31.0                    | 151.08     | 1.236                         |
|         | honeydew Sądecki Bartnik Stróże | HSBS  | 4.4| 35.0                    | 108.99     | 1.241                         |
|         | rape Sądecki Bartnik        | RSB     | 3.6| 15.0                    | 114.07     | 0.183                         |
|         | goldenrod Ślesnicza Pasieka Stryków | GSPS | 3.3| 42.7                    | 58.85      | 0.331                         |
|         | goldenrod nectar            | GN      | 5.0| 17.0                    | 243.93     | 0.416                         |
|         | rape nectar Solec Kujawski  | RNSK    | 4.0| 12.8                    | 34.34      | 0.187                         |
|         | sunflower Olekszyn          | SO      | 3.9| 21.0                    | 62.07      | 0.404                         |
| Australia| lime Tomasz Strecker Apiary Lysomice | LTSAL | 3.9| 34.0                    | 281.56     | 0.537                         |
|         | bush Tasmania                | BT      | 4.2| 26.0                    | 129.66     | 0.677                         |
|         | leatherwood Tasmania        | LT      | 4.3| 21.0                    | 88.81      | 0.704                         |
|         | clover Tasmania              | CT      | 3.6| 22.0                    | 20.10      | 0.255                         |
| Italy   | multiflorous Miele di Millefiori | MMDM  | 3.8| 26.0                    | 106.39     | 0.410                         |
| Ukraine | sunflower Black Sea Bartnik | SSB     | 3.7| 25.0                    | 114.44     | 0.374                         |
| Portugal| forest Madeira              | FM      | 4.0| 30.0                    | 150.96     | 0.670                         |

Pfund scale: <9 –water white; 9–17 –extra white; 18–34 –white; 35–50 –extra light amber; 51–85 –light amber; 86–114 –amber; >114 –dark amber

1.236–1.241 for honeydew honeys, however, most of the samples were characterized by lower conductivity (<0.500).

Bacteria isolation

As a result of the isolation, 38 bacterial colonies were selected for further identification (H1 – H38). From most of the investigated honeys (15) one or two colonies were chosen due to very low bacterial content, while 5 samples—BSB, MW, GSFS, SO, and MMDM were represent by 3 to 5 colonies proportionally to bacterial abundance.

16S rDNA identification

Based on the sequencing of the 16S rDNA region, all isolated bacterial strains were identified and represent group of Gram-positive bacteria (Fig 1). Almost 95% of isolates belonged to the class *Bacilli* (phylum *Firmicutes*) among which 34 strains were able to produce endospores—mostly represent by *Bacillaceae* family (~89%)—while 2 strains were identified as *Staphylococcus epidermidis* were characterized by both nonmotility and nonsporulation. Within *Bacillus* genus, *B. subtilis* group was the most frequently identified species (11 out of 31 strains) followed by *B. cereus* complex (9). However, the reliable species distinction within the mentioned groups was impossible due to obtained small differences in 16S rDNA sequences (< 0.5%) (Table 2). Similar phenomenon was observed for isolates most similar to members of *B. pumilus* group (H18, H21, and H38) and its closely related relatives such as *B. altitudinis* or *B. xiamensis* or *B. aerius*—H8, H15, H20. Moreover, individual cases revealed the presence of *B. megaterium* (2 cases), *B. circulans* as well as *B. nealsionii*. Regarding other genera within
Firmicutes phylum, two Paenibacillaceae strains (P. alvei and Brevibacillus limnophilus) and one Lysinibacillus sp. were detected. Only two isolates belonged to another phylum of bacteria—H24 isolated from rape nectar (RNSK) and H32 from leatherwood honey (LT)—both classified as Micrococcus genus members (phylum Actinobacteria). Obtained phylogenetic tree showed grouping of isolates according to individual species as well as revealed presence of bigger clusters containing closely related species—1. Bacillus subtilis group, 2. Bacillus cereus group, as well as 3. Bacillus pumilus group and its close relatives (Fig 1). Nevertheless, considering level of identification, only for 24% isolates obtained reliable identification to the species while in 76% of cases observed classification at the genus level.

**MALDI-TOF/MS identification**

Used procedure for sample preparation and MALDI-TOF MS analysis allowed to obtain MS spectra for all tested bacterial strains (Fig 2). 36 and 35 bacterial strains were classified in single spectrum and MSP mode using MALDI Biotyper platform, respectively (Table 3). Taking into account raw spectra, 69% isolates were classified to the species level as high-confidence identification (Score value >1.999) from which 42% (29% in total) were identified with a very high log(score) (>2.3), mostly related to B. cereus. Moreover, score values for all isolates identified as B. cereus were > 1.999 (high confidence level), while in case of B. subtilis 36% isolates were identified only at the low confidence level (1.700–1.999). In total, a quarter of the identifications have reached only the low confidence level and in the case of 2 strains—H28 (SO) and H34 (LTSAL)—no reliable identification was obtained. Considering consistency of obtained results, half of the identifications reached species level, 42% genus level, while 8% was defined as neither species nor genus consistency. In MSP mode, percentage of identification at the high confidence level was slightly higher compared to the raw spectra—74%, however, number of not reliable identification raised from 2 to 3 strains—in addition to H28 and H34 isolates also H19 strain failed (GSPS). Similar to the raw spectra mode, all B. cereus strains were identified at high confidence level (Table 3), while 2 strains of B. subtilis were recognized only at low confidence level. Consistency of identification in MSP mode was also higher than in the case of raw spectra analysis—61% samples with species consistency. Omitting not reliable identifications, results of organisms matching for each isolate were similar except for bacteria H17 derived from RSB honey, which was identified as L. boronitolera on the raw spectra analysis while in MSP mode as L. fusiformis—both on high confidence level.

Phyloproteomic relationships between isolates presented on MSP dendrogram (Fig 3) revealed the presence of 8 groups of closely related bacterial species (A–H). Similar to the phylogenetic tree, 3 bigger clusters were distinguished—B. pumilus (C2), B. subtilis (D), and B. cereus (H2) group. Moreover, unidentified isolates H19 and H28 were also placed close to the related strains which were corresponding species according to 16S rDNA identification—B. licheniformis (cluster D1) and B. nealsonii (G), respectively. MALDI-TOF MS analysis did not allow a reliable identification for isolates H19, H28, and H34, nevertheless, only the last two were identified at species level using molecular technique—as B. nealsonii and B. limnophilus, respectively.

**Impact of physicochemical properties of honeys on the bacterial composition**

Grouping of the samples on the first two PCs-plane (76.05% of the explained variance) revealed that pH, EC as well as TA significantly influence the bacterial species composition of honeys.
Table 2. The result of bacteria identification based on 16S rDNA sequencing.

| Strain                  | Related species from NCBI  | Identity [%] | Given accession number |
|-------------------------|----------------------------|--------------|------------------------|
| **H1.**                 | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045834               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.9         |                        |
|                         | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7        |                        |
| **H2.**                 | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045824               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.9         |                        |
|                         | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7        |                        |
| **H3.**                 | *Bacillus megaterium* NBRC15308 [NR_112636] | 100         | MH045836               |
|                         | *Bacillus flexus* NBRC15715 [NR_113800]  | 98.9        |                        |
| **H4.**                 | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045835               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.9         |                        |
|                         | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7        |                        |
| **H5.**                 | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045838               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.8         |                        |
|                         | *Bacillus vallismortis* NBRC101236 [NR_104919] | 99.6    |                        |
| **H6.**                 | *Paenibacillus alvei* NBRC3343 [NR_113577] | 99.7         | MH046040               |
|                         | *Paenibacillus apiarius* DSM5581 [NR_040890] | 96.9              |                        |
| **H7.**                 | *Bacillus subtilis* JCM1465 [NR_113265] | 100          | MH045840               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 100          |                        |
|                         | *Bacillus flexus* NBRC15715 [NR_113800]  | 99.7        |                        |
| **H8.**                 | *Bacillus altitudinis* 41KF2b [NR_042337] | 100          | MH046864               |
|                         | *Bacillus aerius* 24K [NR_118439]      | 100          |                        |
|                         | *Bacillus xiamenensis* MCCC1A000008 [NR_148244] | 99.9  |                        |
| **H9.**                 | *Bacillus wiedmannii* strain FSL W8-0169 [NR_152692] | 100          | MH046863               |
|                         | *Bacillus subtilis* JCM1465 [NR_113265] | 100          |                        |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 100          |                        |
|                         | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 99.9  |                        |
|                         | *Bacillus cereus* ATCC14579 [NR_074540] | 99.9        |                        |
| **H10.**                | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045847               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.9         |                        |
|                         | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7        |                        |
| **H11.**                | *Staphylococcus epidermidis* Fussel [NR_036094] | 99.9         | MH045848               |
|                         | *Staphylococcus caprae* ATCC35538 [NR_024665] | 99.4              |                        |
| **H12.**                | *Bacillus cereus* ATCC14579 [NR_074540] | 100          | MH046867               |
|                         | *Bacillus tropicus* MCCC1A01406 [NR_157736] | 99.9        |                        |
|                         | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 99.9  |                        |
| **H13.**                | *Bacillus subtilis* JCM1465 [NR_113265] | 99.9         | MH045851               |
|                         | *Bacillus tequilensis* 10b [NR_104919]  | 99.9         |                        |
|                         | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7        |                        |
| **H14.**                | *Bacillus circulans* NBRC13626 [NR_112632] | 99.7         | MH045850               |
|                         | *Bacillus nealonii* DSM15077 [NR_044546]  | 98.1        |                        |
| **H15.**                | *Bacillus altitudinis* 41KF2b [NR_042337] | 99.9         | MH045854               |
|                         | *Bacillus aerius* 24K [NR_118439]      | 99.9         |                        |
|                         | *Bacillus xiamenensis* MCCC1A000008 [NR_148244] | 99.8  |                        |
| **H16.**                | *Bacillus onubensis* 0911MAR22V3 [NR_149252] | 98.8         | MH045852               |
|                         | *Bacillus humi* LMG22167 [NR_025626]    | 98.7        |                        |
| **H17.**                | *Lysinibacillus macroides* LMG18474 [NR_114920] | 99.7         | MH045853               |
|                         | *Lysinibacillus boronitolerans* NBRC103108 [NR_114207] | 99.4  |                        |
|                         | *Lysinibacillus pakistanensis* NCCP-54 [NR_113166] | 99.3          |                        |
| **H18.**                | *Bacillus pumilus* ATCC7061 [NR_043242]  | 99.9         | MH045855               |
|                         | *Bacillus zhangzhouensis* MCCC1A08372 [NR_148786] | 99.9    |                        |
|                         | *Bacillus safensis* NBRC100820 [NR_113945] | 99.8      |                        |
| **H19.**                | *Bacillus haynesii* NNRL B-41327 [NR_1157609] | 99.7        | MH045856               |
|                         | *Bacillus licheniformis* DSM13 [NR_118996]  | 99.6        |                        |
|                         | *Bacillus sonorensis* NBRC101234 [NR_113993] | 99.5     |                        |
| **H20.**                | *Bacillus altitudinis* 41KF2b [NR_042337] | 100          | MH046869               |
|                         | *Bacillus aerius* 24K [NR_118439]      | 100          |                        |
|                         | *Bacillus xiamenensis* MCCC1A000008 [NR_148244] | 99.9  |                        |

(Continued)
investigated honeys (correlation with the respective factors ≥ ±0.75) (Fig 4). Members of *B. cereus* group were most frequently present in the honeys with higher pH values (more alkaline), lower acids content as well as higher electrical conductivity. Contrary, isolates classified to the *B. pumilus* group preferred lower pH and were not affected by high acid content. The

| Strain | Related species from NCBI [Accession number] | Identity [%] | Given accession number |
|--------|--------------------------------------------|--------------|-----------------------|
| H21.   | *Bacillus pumilus* ATCC7061 [NR_043242]   | 99.9         | MH045860              |
|        | *Bacillus safensis* NBRC100820 [NR_113945]| 99.8         |                       |
| H22.   | *Staphylococcus epidermidis* Fussel [NR_036904] | 100         | MH045861              |
|        | *Staphylococcus epidermidis* NBRC100911 [NR_113957] | 99.9       |                       |
|        | *Staphylococcus caprae* ATCC35538 [NR_024665] | 99.5       |                       |
| H23.   | *Bacillus subtilis* ICM1465 [NR_113265]   | 100         | MH046866              |
|        | *Bacillus subtilis* IAM12181 [NR_112116]   | 99.9         |                       |
|        | *Bacillus tequilensis* 10b [NR_104919]   | 99.9         |                       |
| H24.   | *Micrococcus luteus* NCTC28665 [NR_075062] | 99.6         | MH045862              |
|        | *Micrococcus flavus* LW4 [NR_043881]    | 98.3         |                       |
| H25.   | *Bacillus tropicus* MCCC1A01406 [NR_157736] | 99.9         | MH045979              |
|        | *Bacillus cereus* ATCC14579 [NR_074540]   | 99.9         |                       |
|        | *Bacillus wiedmannii* strain FSL W-8-0169 [NR_152692] | 99.9      |                       |
| H26.   | *Bacillus megaterium* NBRC15308 [NR_112636] | 100         | MH045943              |
|        | *Bacillus flexus* NBRC15715 [NR_113800]   | 98.9         |                       |
| H27.   | *Bacillus tequilensis* 10b [NR_104919]   | 99.9         | MH045978              |
|        | *Bacillus subtilis* 168 [NR_102783]   | 99.9         |                       |
|        | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.7       |                       |
| H28.   | *Bacillus nealsomii* DSM13077 [NR_044546] | 99.4         | MH045980              |
|        | *Bacillus circulans* NBRC13626 [NR_112632] | 98.9       |                       |
| H29.   | *Bacillus thuringiensis* ATCC10792 [NR_114581] | 100        | MH045982              |
|        | *Bacillus toyonensis* BCT-7112 [NR_121761] | 100         |                       |
|        | *Bacillus pacificus* MCCC1A06182 [NR_157733] | 99.9      |                       |
| H30.   | *Bacillus cereus* ATCC14579 [NR_074540]   | 100         | MH045984              |
|        | *Bacillus tropicus* MCCC1A01406 [NR_157736] | 99.9         |                       |
|        | *Bacillus wiedmannii* strain FSL W-8-0169 [NR_152692] | 99.9    |                       |
| H31.   | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 99.9  | MH045985              |
|        | *Bacillus cereus* ATCC14579 [NR_074540]   | 99.9         |                       |
| H32.   | *Micrococcus aloeverae* AE-6 [NR_134088] | 99.9         | MH045983              |
|        | *Micrococcus yunnanensis* YIM5004 [NR_116578] | 99.8       |                       |
|        | *Micrococcus luteus* NCTC2665 [NR_075062] | 99.5       |                       |
| H33.   | *Bacillus tequilensis* 10b [NR_104919]   | 99.9         | MH045986              |
|        | *Bacillus subtilis* ICM1465 [NR_113265]   | 99.9         |                       |
|        | *Bacillus mojavensis* NBRC15718 [NR_112725] | 99.9     |                       |
| H34.   | *Brevibacillus limnophilus* DSM6472 [NR_024822] | 99.2       | MH045990              |
|        | *Brevibacillus brevis* NBRC15304 [NR_041524] | 98.6      |                       |
| H35.   | *Bacillus cereus* ATCC14579 [NR_074540]   | 100         | MH046870              |
|        | *Bacillus tropicus* MCCC1A01406 [NR_157736] | 99.9         |                       |
|        | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 99.9 |                       |
| H36.   | *Bacillus wiedmannii* strain FSL W-8-0169 [NR_152692] | 100        | MH046865              |
|        | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 100      |                       |
|        | *Bacillus cereus* ATCC14579 [NR_074540]   | 99.9         |                       |
| H37.   | *Bacillus wiedmannii* strain FSL W-8-0169 [NR_152692] | 100        | MH046868              |
|        | *Bacillus proteolyticus* strain MCCC1A00365 [NR_157735] | 100      |                       |
|        | *Bacillus cereus* ATCC14579 [NR_074540]   | 99.9         |                       |
| H38.   | *Bacillus pumilus* ATCC7061 [NR_043242]   | 99.9         | MH045994              |
|        | *Bacillus zhangzhouensis* MCCC1A08372 [NR_148786] | 99.8     |                       |
|        | *Bacillus safensis* FO-36b [NR_041794]   | 99.7         |                       |

https://doi.org/10.1371/journal.pone.0217078.1002

Identification of honey bacteria

PLOS ONE | https://doi.org/10.1371/journal.pone.0217078 | May 23, 2019 | 7 / 20
most ubiquitous type of bacteria among the analysed samples was \textit{B. subtilis} group, for which occurrence was not significantly affected by the investigated physicochemical parameters.

https://doi.org/10.1371/journal.pone.0217078.g002

Fig 2. Exemplary MS spectra obtained for bacterial strains identified as different species. A–H1 (\textit{B. subtilis}); B–H9 (\textit{B. cereus}); C–H8 (\textit{B. pumilus}); D–H20 (\textit{B. altitudinis}); E–H3 (\textit{B. megaterium}); F–H14 (\textit{B. circulans}); G–H19 (\textit{B. licheniformis}); H–H6 (\textit{P. alvei}); I–H17 (\textit{L. boronitolerans}); J–H11 (\textit{S. epidermidis}); K–H24 (\textit{M. luteus}).
Regarding other types of bacteria, the residual representatives of Bacilliaceae family were more often present in the honeys with lower EC and pH values while *M. luteus* preferred low total acids content.
Considering botanical origin, the highest species diversity was observed in sunflower honeys—5 different *Bacillus* species: *subtilis*, *pumilus*, *cereus*, *nealsonii*, and *megaterium*. The same number of species was demonstrated by samples of multiflorous and goldenrod honeys, however, representing less number of subgroups—4 and 3, respectively. Moreover, in the case of multiflorous samples, *B. cereus* was most dominant species—5 out of 9 identified isolates. The lowest biodiversity among samples with multiple isolates was indicated for buckwheat honeys—3 species, mostly *B. subtilis* (5 out of 7). Regarding geographical origin, most of the bacterial strains isolated from honeys derived from outside Poland belong to the *B. cereus* group—5 compared to the 3 from polish ones, which constituted 56% and 10% of all isolates for each variant, respectively.

**Discussion**

Results of our studies are in agreement with the generally accepted statement that Gram (+) bacteria are most often expected to be the highly dominant group [24] since due to high
osmotic pressure honey is considered a harsh environment for the growth of microorganisms. Lack of the Gram (-) bacteria presence in investigated samples may indicate the application of good manufacturing practices during honey handling since their occurrence is most often associated with secondary sources of contamination such as people or equipment [10]. Moreover, 89% of the identified strains were able to produce endospores which indicate that their were rather present in a dormant form than in vegetative ones. Olaitan et colleagues [4] claimed that most of the microbial species cannot grow and reproduce in honey, thus, only spore-forming microorganisms can survive such conditions. Nevertheless, in 4 honeys presence of non sporulating *Staphylococcus epidermidis* and *Micrococcus luteus* was noted (Fig 1). As they are a part of the natural microflora of the human skin, they occurrence may be associated with handling and storage of honey without appropriate care on good manufacturing practices [12], [14]. On the other hand, *Micrococcus* spp. are typical colonizers of the hive and both *Micrococcus luteus* and *Staphylococcus* spp. have been recorded for gastrointestinal tracts of honeybees, from where they can be easily transferred to the honey [25]. For this reason, it is difficult to definitely assess whether the source of these strains of bacteria is primary or secondary. Nevertheless, it is emphasized that improved procedures of honey harvesting and handling is needed to reduce the introduction of such microbes as *Staphylococcus* species [24].
The vast majority of the isolated strains belonged to the genus *Bacillus* which should be expected since their symbiotic relationship with honeybees had been previously reported [26], [27]. Among them, two species (species groups)—*B. subtilis* and *B. cereus*—were the most frequently isolated strains (over half of all identified bacteria). Both species are ubiquitous in the environment and are regularly found in the honey [28], [29]. According to the literature, the presence of both mentioned species is associated with the potential spoilage of honey [19], however, *B. cereus* is the microorganism of particular concern since it is classified as medically important human pathogen, emerging infectious agent, and principal foodborne pathogen at the same time [30]. Therefore, *B. cereus* presence in the honey should be under strict control. It does not change the fact that this species is regularly found in honey samples [27]. Iurlina and Fritz [8] reported occurrence of *B. cereus* in 23% among 70 Argentinean honeys which demonstrated presence of bacterial growth. Similar percentage of *B. cereus* in Argentinean honeys reported Alippi [29]—20% and in works Monetto et al. [31] percentage of *B. cereus* in Argentinean commercial honeys reached 78%. In turn, studies on honeys derived from Turkey and Portugal revealed lower results—4% and 14%, respectively [16], [3]. This findings may indicate the dependence of *B. cereus* presence on geographical origin of honey which is in agreement with results of our studies—the percentage of *B. cereus* among Poland honey samples was more than 4 times lower compare to the samples derived from abroad. Moreover, regardless of country of origin, most of the identified *B. cereus* were found in the multifloral honeys (5 out of 9). Considering the fact, that multifloral honey is the most common honey available in large quantities on the market [10], our and cited works may suggest to take special care of such kind of honey in terms of *B. cereus* presence as a potential vehicle of infection and the route of foodborne outbreaks.

According to the main purpose of the study, we used two different bacteria identification methods. While using 16S rDNA sequencing for honey bacteria identification is common, currently MALDI-TOF MS analyzer is getting more popular. Despite the increasing use of MALDI in the identification of clinical bacteria, the identification of environmental species is still limited [32]. This is due to the fact that commercial databases used for the MALDI approach contain less environmental reference spectra, in comparison with BLAST type repositories used in identifying microorganisms with 16S rDNA sequence [33]. Therefore in our study, the *Brevibacillus limnophilus* strain was identified only using 16S rDNA approach (Table 2). Its MALDI identification has not been possible due to the lack of reference spectra in the MALDI Biotyper database. Since it is believed that microorganisms originated from environmental samples are more diverse which implicate difficulties in their identification [20], Kopcakova et colleagues [34] emphasized the need to expand the current reference spectra database to improve the identification power of MALDI-TOF MS techniques in terms of environmental bacteria. One of the approaches to increase the identification power of the MALDI-TOF MS on genus and species level in case of environmental bacterial strains technique is the construction of home (local) databases with the parallel use of molecular techniques (eg 16S rDNA) [35]. Despite the fact that MALDI reference spectrum databases are more limited compared to molecular ones, percentage of the correct species identification via MALDI Biotyper platform was almost 3 times higher compared to the 16S rDNA sequencing. Such findings resulted from dominance of the 2 big bacteria clusters—*B. subtilis* and *B. cereus* group. The first one comprises of such species as *B. subtilis* subsp. *subtilis*, *B. amyoliquefaciens*, *B. licheniformis*, *B. atrophaeus*, *B. mojavensis*, *B. Vallismortis*, *B. subtilis* subsp. *spizizenii*, and *B. soronensis* [36], while *B. cereus* is a parent species of group containing *B. thuringensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. anthracis* [37], [29]. Distinguishing species within this two groups is difficult due to their high genetic similarities [38] which causes analysis of 16S rDNA sequences insufficient in distinguishing individual species [39].
Indeed, despite the very high 16S rDNA sequence similarity (>99.5%), all individuals were classified only as *B. subtilis* group or *B. cereus* complex since distance scores to the next closest species were <0.5% and according to recommended guidelines are insufficient to reliable species identification, so additional housekeeping gene sequencing is required [40]. In turn, application of MALDI Biotyper enabled reliable species identification within *B. subtilis* and *B. cereus* group in most cases except for 4 strains most similar to *B. subtilis* as well as strain H19 which was most similar to the *B. licheniformis* and *B. soronensis* species (Table 2). However, Similar findings were noted in works Lasch et al. [41], [42] or Fernandez-No et al. [43] in which obtained accurate classification of *B. cereus* and *B. subtilis* group. In contrast, in the distinguishing of *B. licheniformis* and *B. soronensis* MALDI approach is considered as not very useful since their share more phenotypic traits with each other than with any other taxon [44]. In another study Dieckmann et al. [45] noted that 16S rDNA sequencing failed to resolve problems with intra- and inter-species classification of *Pseudoalteromonas* sp. isolates derived from marine sponges while application of MALDI-TOF MS technique enabled discrimination of very closely related species with high confidence. Similar findings revealed Angolini et al. [46] investigating petroleum microorganisms. It suggests that use of proteomic-based approaches such as MALDI-TOF MS is a good solution for distinguishing some species sharing very similar 16S rDNA sequences as in the case of honey bacteria, without the need to analyze additional genes. MALDI approach seems to be more efficient for *Bacillus sp.* identification e.g. distinction between *B. cereus* and *B. subtilis*, in comparison with 16S rDNA sequencing. However, MALDI has also some restrictions in *Bacillus cereus* group differentiation. As an example, proper identification between *B. cereus* and *B. anthracis* is still challenge [47].

Revealed differences in identification quality between *B. cereus* and *B. subtilis* group may results from influence of endospores production. It is known that proteins expression in endospores differ from vegetative cells of *Bacillus* species mainly due to high amounts of small acid soluble proteins which play crucial role in endospore formation [48]. It was observed, that with increasing time of incubation from 12-48h the numbers of spores increase, while the number of signals on the MS spectrum decreases. This causes a reduction of the identification power of MALDI approach. Shu and Yang [49] claimed that fresh cultures (<12h) are ideal samples for classification and identification of *Bacillus* species since inconsistencies of their identification are mostly because of endospores production which is dramatically influenced by incubation time. Based on UK standards for Microbiology Investigations Identification of Bacillus species, *Bacillus cereus* and *Bacillus subtilis* belongs to two different endospores groups with different time of sporulation which may influence quality of MS spectra and thus further bacteria identification. As a solution for this problem Lasch et al. [42] proposed the protein enrichment protocol where application of combined TFA treatment promoted the protein isolation from spores. However, this analytical solution does not prevent the sample from isolation of protein from mixed bacterial culture (different time and stage of sporulation), on the one hand, leading to an increase of proteins signals in MALDI spectra, but on the other hand causing misidentification [50]. However, there are some reports which suggest that in the case of *Bacillus* strains, species identification could be also performed based only on the protein profiling of their spores using both top-down and bottom-up approaches which was proved for such species as *B. cereus*, *B. globigii* or *B. atrophaeus* [51], [52]. Nevertheless, considering vegetative cells, our results suggest using different MALDI sample pre-treatment protocols for individual group of honey bacteria contradicts the statement of Santos et al. [20] that there is the need of a universal sample pre-treatment protocol to overcome misidentification problems.

Results of our studies also revealed that both physicochemical parameters, as well as the origin of the honey, have a significant influence on the bacterial composition since they are very often correlated with each other. However, this effect strongly depended on the bacterial
species. Occurrence of the B. cereus strains was considerably influenced by the TA, pH as well as EC values, while, in the case of B. subtilis no significant differences were observed (Fig 4). B. cereus strains mostly preferred more alkaline honey, thus with lower acid content. The impact of EC, which in honey is associated with ash content, was lower, however, still statistically significant. It was found that among the most important variables associated to the levels of bacteria in honey were ash (related to the solids and mineral content) and acidity [33]. It is also believed that in undiluted honey the acidity is a significant antibacterial factor [4] since the optimum pH for most bacteria is between 7.2 and 7.4 while natural acidity of the honey ranges between pH 3.2 and 4.5 [54]. It is believed that among Bacillus spp. B. cereus generally reflects higher tolerance to antimicrobial properties of the honey[19]. The results of our research are contradictory to this statement because B. cereus showed the least tolerance among all identified Bacillus species. Nevertheless, impact of the botanical origin of honey must be also taken into account since is associated with some physicochemical properties such as color and thus mineral and organic content including acids (pH) [4], [55]. Sinacori et colleagues [5] investigating various types of honeys have shown that the microbiota of multifloral honeys showed the highest values for genotype richness and diversity indexes. Moreover, the authors revealed that some species were detected in almost all honeys, which may indicate their strong adaptation to such kind of matrix, while some species were detected only occasionally, and thus their presence cannot be correlated with the honey origin. Similar to this findings, investigated samples of multiflorous honeys found themselves in a group of honeys with the highest species diversity of bacteria with dominance of B. cereus strains, while strains belonged to the B. subtilis group proved to be the most ubiquitous.

Conclusions

Our studies revealed that regardless geographical and botanical origin, honeys are by far dominated by spore-forming Bacillus spp. According to literature and our own observations, the most frequently isolated honey bacteria belong to the B. subtilis or B. cereus group, which raises big problems with identification to the species level via 16S rDNA technique. Solution for this problem in some cases can be using MALDI-TOF MS and suitable software, such as MALDI Biotyper platform. Application of such an approach can significantly improve quality of the identification analysis—in our studies nearly three times—and avoids the need for sequencing of additional housekeeping genes so lets keep short laboratory workflow. It is particularly important in the case of B. cereus, which is a microorganism of special concern in terms of honey spoilage and foodborne illness outbreaks. However, the influence of endospores formation should be taken into account since it significantly affects MS profiles and thereby microbial identification. As a solution for this obstacles, shorter incubation times (less than 12 hours) are recommended. Nevertheless, in the case of B. cereus group even the use of a standard 24-hour incubation protocol seemed to be sufficient for reliable species indentification. Considering that both the geographical and botanical origin as well as the physicochemical parameters of honeys, affecting the composition of bacteria and the fact that these parameters are very often related, this indicates a great need for further studies on a larger scale using a greater number of different types of honey. For this purpose use of the MALDI-TOF MS technique seems to be the most promising approach, as indicated by our study. Such studies can significantly expand knowledge about microbial composition and occurrence of the specific species in the different type of honey which are relevant in view of safe food handling and processing. This might give the opportunity to consider honey as a source of microorganisms which are able to survive in suboptimal conditions (e.g. high sugar concentrations) or as good material for microbial inocula preparation.
Materials & methods

Honey samples

During the study, 20 different honeys belonging to the collection of the Department of Environmental Chemistry and Bioanalytics of the Nicolaus Copernicus University in Toruń were tested. Most of them – 14 – derived from Poland while the rest originated from different countries around the world – Australia (3), Italy (1), Ukraine (1), and Portugal (1). Moreover, honeys varied in botanical origin: multiflorous – 4; buckwheat – 3; honeydew, rape, sunflower, and goldenrod – 2; clover, leatherwood, bush, forest as well as lime – 1. Full names of the honeys with given acronyms are presented in Table 1.

Colour of honey samples

Collected honey samples (4 g of each, dissolved in water 8 mL) were heated up to 50 °C to dissolve sugar crystals. The spectrophotometric measurements were performed by use of UV-Vis spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA USA). The colour of sample was determined by measurement of the absorbance of a 50% honey solution (w/v) at wave length $\lambda = 635$ nm. The honeys were classified according to the Pfund’s scale after conversion of the absorbance (Abs) values:

$$mm\ Pfund = -38.70 + 371.39 \times Abs$$

where mm Pfund is the intensity of honey colour in the Pfund’s scale; Abs is the absorption of honey solution. Two replicates were performed for each honey sample.

pH and acidity of honey

10 g of honey was dissolved in water 75 mL. Next pH of the solution was measure by use of pH-meter CPC-501 (Elmetron, Chorzów, Poland). A glass electrode was used. Measurements have been made in triplicate.

After measurements of pH, in a beaker with a honey solution a magnetic stirrer was placed and the titration continued by use of standard solution NaOH (0.1 M) to obtain pH 8.3.

$$Total\ acidity\ (TA)\ \text{[mval/kg]} = V_{\text{NaOH}} \times 10$$

$V_{\text{NaOH}}$ – volume of NaOH 0.1 M solution used during the titration of honey solution

Conductivity of honey

The electrical conductivity (EC, mS × cm$^{-1}$) of the honey has been measured for 1 mL of water solution (20%) of the honey, on a dry matter basis, at the temperature 20°C. At first 2 g of honey was dissolved in water 8 mL. The sample was transferred to the conductivity cell and the conductivity of the solution was measured by use of equipment CPC-501 (Elmetron, Chorzów, Poland).

Measurements were performed for each sample in the triplicate repetition. Data are presented as mean values. The level of statistical significance required to measure differences between the means for all analyses was $P = 0.05$.

Bacteria isolation

For isolation of aerobic bacteria, the serial dilution method was used. 10 g of honey was added into the 90 ml of sterile physiological saline solution (0.87% NaCl), mixed well, and then 100 µl of obtaining suspension ($10^{-1}$) were plated on TSA medium (Triptic Soy Agar, Sigma-Aldrich,
Germany) and incubated for 24h at 37˚C. After incubation, single colonies were transferred onto new TSA plates in order to obtain pure bacterial cultures using reductive culture method. Pure cultures were stored on TSA slants at 4˚C. Microorganisms from the same passage were used for both identification methods.

16S rDNA identification of bacterial isolates

Total bacterial DNA was extracted from overnight cultures (TSA, 37˚C) using the Bacterial Genomic Extraction GPB Mini Kit and GPB Lysozyme (GenoPlast Biochemicals, Poland). Regions of 16S rDNA were amplified using universal primers for bacteria: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3'), thermostable Taq DNA polymerase (Qiagen, Hilden, Germany), Mastercycler pro S thermocycler (Eppendorf AG, Hamburg, Germany), and following PCR program: 1. 95˚C, 1 min. (initialization); 2. 95˚C, 15 s (denaturation); 3. 55˚C, 15 s (annealing); 4. 72˚C, 90 s (elongation); 2.– 4. repeated 30x; 5. 72˚C, 7 min (final elongation); 6. 10˚C (cooling for final hold). PCR products were then purified using Extractme Genomic DNA kit (Blirt S.A., Poland) followed by direct DNA sequencing via sanger dideoxy method using the same 27F and 1492R primers. Quality of obtained chromatograms of 16S rDNA sequences were checked using Chromas ver. 2.6.2 software (Technelysium Pty Ltd, Australia). Contigs were assembled via BioEdit Sequences Alignment Editor ver. 7.2.5 (Tom Hall, USA), and finally, consensus sequences were compared with known 16S rDNA genes present in the The National Center for Biotechnology Information (NCBI) BLAST database [56]. Obtained sequences were submitted to GenBank and received accession numbers. Evolutionary relationships of investigated bacterial strains were presented on a phylogenetic tree created using the Neighbor-Joining method [57] with computing the evolutionary distances by the Maximum Composite Likelihood method (bootstrap test—500 replicates, values lower than 70% were hidden) [58] via MEGA7 ver. 7.0.21 software [59].

MALDI-TOF/MS identification of bacterial isolates

α-Cyano-4-hydroxycinnamic acid (HCCA) solution in standard solvent (acetonitrile (ACN) 50%, water 47.5% and trifluoroacetic acid 2.5%) at final concentration: 10 mg/ml was used as matrix. For sample preparation, ethanol/formic acid (EtOH/FA) extraction procedure was performed according to Bruker’s guideline with small modifications. A single colony of bacteria was transferred into an Eppendorf tube containing 300 μl of deionized water and mixed thoroughly. Subsequently, 900 μl of absolute EtOH were added and thoroughly vortexed, then centrifuged at max speed for 20 min. The supernatant was discarded, and the remaining cell pellet was dried by evaporation of EtOH residue at 37˚C for 5–10 minutes to increase the extraction efficiency. Then, 1–5 μl of 70% FA was added to the dried cell pellet proportionally to the amount of biological material and mixed by pipetting. Next, an equal volume of can was added, mixed carefully, centrifuged at max speed for 3 min., and 1 μl of supernatant was transferred onto a MALDI MTP 384 ground steel target sample spot (Bruker Daltonik GmbH, Germany). Finally, the dried sample spot (after ~15 min.) was overlaid with 1 μl of HCCA matrix solution and air dried. Target with samples was analyzed in an ultrafleXtreme MALDI–TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Germany) equipped with the smartbeam-II laser–positive mode. The cut–off for applied liner MBT Standard methods (Bruker Daltonik GmbH, Germany) was at m/z range: 2000–20 000, acceleration voltage at 25 kV, global attenuator offset at 20% and attenuator offset at 34% and its range at 34%. Applied laser power at 40% and smartbeam parameter set at MBT focus were applied. The one single spectra above 8000 unit intensity was collated manually by 500 shots in-one-single spectra to frequency 2500. For calibration Bruker Bacterial Test Standard (BTS), algorithm Reference Mass
Assignment and interactive calibration with of quadratic mode. Before calibration, each spectra of bacteria were subjected to smooth and baseline operations. Validated mass spectra were processed with the use of software provided by the manufacturer—flexControl and flexAnalysis, and subsequently used for bacterial identification via MALDI Biotyper Compass platform (Bruker Daltonik GmbH, Germany) based on the both raw spectra (RAW) and Main Spectra (MSP) according to manufacturer protocol [60]. The spectra of 3 microbial replicates was collected in triplicate.

Statistical analysis
Effect of investigated physicochemical properties of honeys on their bacterial composition was analysed by principal component analysis (PCA) using STATISTICA version 12 software (StatSoft, Poland). During the analysis, the results of 16S rDNA identification and covariance as a measure of the linear relationship between variables were used.

Acknowledgments
This study was supported by PLANTARUM project No. BIOSTRATEG2/298205/9/NCBR/2016 from National Centre for Research and Development, Poland.

Author Contributions
Conceptualization: Paweł Pomastowski.
Data curation: Michał Złoch.
Formal analysis: Agnieszka Rodzik.
Methodology: Michał Złoch.
Project administration: Bogusław Buszewski.
Resources: Magda Ligor, Bogusław Buszewski.
Supervision: Markus Kostrzewa.
Visualization: Agnieszka Rodzik.
Writing – original draft: Paweł Pomastowski.
Writing – review & editing: Markus Kostrzewa.

References
1. Estevinho M, Vázquez-Tato M, Seijas J, Feás X. Palynological, physicochemical, and microbiological attributes of organic lavender (Lavandula stoechas) honey from Portugal. Acta Aliment. Academia Kiado; 2013; 42: 36–44. https://doi.org/10.1556/AAlim.42.2013.1.4
2. Gomes S, Dias LG, Moreira LL, Rodrigues P, Estevinho L. Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. Food Chem Toxicol. 2010; 48: 544–548. https://doi.org/10.1016/j.fct.2009.11.029 PMID: 19909782
3. Erkan ME, Vural A, Guran HS, Durmusoglu H. Microbiological investigation of honey collected from Şımak province of Turkey. J Hell Vet Med Soc. 2017; 66: 22. https://doi.org/10.12681/jhvms.15579
4. Olaitan PB, Adeleke OE, Ola IO. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr Health Sci. Makerere University Medical School; 2007; 7: 159–65. https://doi.org/10.5555/afhs.2007.7.3.159 PMID: 18052870
5. Sinacori M, Francesca N, Alfonzo A, Crucia M, Sannino C, Settanni L, et al. Cultivable microorganisms associated with honeys of different geographical and botanical origin. Food Microbiol. 2014; 38: 284–294. https://doi.org/10.1016/j.fm.2013.07.013 PMID: 24290653
6. Molan PC. Potential of Honey in the Treatment of Wounds and Burns. Am J Clin Dermatol. 2001; 2: 13–19. https://doi.org/10.2165/00128071-200102010-00003 PMID: 11702616
7. Mahendran S, Kumarasamy D. Antimicrobial activity of some honey samples against pathogenic bacteria. Int Lett Nat Sci. 2015; 34: 15–20. https://doi.org/10.18052/www.scipress.com/ILNS.34.15
8. Iurlina MO, Fritz R. Characterization of microorganisms in Argentinian honeys from different sources. Int J Food Microbiol. 2005; 105: 297–304. https://doi.org/10.1016/j.ijfoodmicro.2005.03.017 PMID: 16166924
9. Torres-González A, López-Rivera P, Duarte-Listci G, López-Ramírez Á, Correa-Benítez A, Rivero-Cruz JF. Analysis of volatile components from Melipona beecheei geopropolis from Southeast Mexico by headspace solid-phase microextraction. Nat Prod Res. 2016; 30: 237–240. https://doi.org/10.1080/14786419.2015.1043631 PMID: 26118891
10. Madras-Mawebska B, Rosiak E, Jaworska D, Kulesza K, Wasiak-Zys G, Teper D. Comparison of selected quality characteristics of domestic and Thailand multifloral honeys. Med Weter. 2016; 72: 620–626. https://doi.org/10.21521/mw.5573
11. Laredj H, Waffa R. Microbiological and Physicochemical Characterization of Honeys from the Tiaret Region of Algeria. Asian J Pharm Res Heal Care. 2017; 9: 85. https://doi.org/10.18311/ajprhc/2017/15698
12. Rożanska H. Microbiological quality of Polish honey | Request PDF. Bull Vet Inst Pulawy. 2011; 55: 443–445. Available: https://www.researchgate.net/publication/295654564_Microbiological_quality_of_Polish_honey
13. Vet TJ, Sci A, Dümen E, Akkaya H, Merve Öz G, Sezgin FH. Microbiological and parasitological quality of honey produced in Istanbul. Turkish J Vet Anim Sci. 2013; 37: 602–607. https://doi.org/10.3906/vet-1301-46
14. Adapi P, Obeng AK. Assessment of bacterial quality of honey produced in Tamale metropolis (Ghana). J Food Drug Anal. Elsevier; 2017; 25: 369–373. https://doi.org/10.1016/j.jfda.2016.07.005 PMID: 28911679
15. Fernández LA, Ghilardi C, Hoffmann B, Busso C, Gallez LM. Microbiological quality of honey from the Pampas Region (Argentina) throughout the extraction process. Rev Argent Microbiol. 2017; 49: 55–61. https://doi.org/10.1016/j.ram.2016.05.010 PMID: 27989610
16. Martins H, Martins L, Bernardo F. Bacillaceae spores, fungi and aflatoxins determination in honey Esporos de Bacillaceae, fungos e aflatoxinas em mel. RPCV. 2003; 98: 85–88. Available: http://www.fmv.ulisboa.pt/spcv/PDF/pdf6_2003/546_85_88.pdf
17. Omafuvbe BO, Akanbi OO. Microbiological and physico-chemical properties of some commercial Nigerian honey. African J Microbiol Res. 2009; 3: 891–896. Available: http://www.academicjournals.org/ajmr
18. Fach P, Micheau P, Mazuet C, Perelle S, Popoff M. Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing Clostridium botulinum, Clostridium baratii and Clostridium butyricum. J Appl Microbiol. 2009; 107: 465–473. https://doi.org/10.1111/j.1365-2672.2009.04215.x PMID: 19291235
19. Iurlina MO, Saiz AI, Fuselli SR, Fritz R. Prevalence of Bacillus spp. in different food products collected in Argentina. LWT—Food Sci Technol. Academic Press; 2006; 39: 105–110. https://doi.org/10.1016/J.LWT.2005.01.006
20. Santos IC, Hildenbrand ZL, Schug KA. Applications of MALDI-TOF MS in environmental microbiology. Analyst. 2016; 141: 2827–37. https://doi.org/10.1039/c6an00131a PMID: 27072574
21. Doern CD, Butler-Wu SM. Emerging and Future Applications of Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry in the Clinical Microbiology Laboratory. J Mol Diagnostics. 2016; 18: 789–802. https://doi.org/10.1016/j.jmoldx.2016.07.007 PMID: 27770851
22. Branquinho R, Sousa C, Lopes J, Pintado ME, Peixe L V., Osoário H. Differentiation of Bacillus pumilus and Bacillus safensis Using MALDI-TOF-MS. Desvaux M, editor. PLoS One. Public Library of Science; 2014; 9: e110127. https://doi.org/10.1371/journal.pone.0110127 PMID: 25314655
23. Schubert S, Kostrewa M. MALDI-TOF Mass Spectrometry in the Clinical Microbiology Laboratory; Beyond Identification. Methods Microbiol. Academic Press; 2015; 42: 501–524. https://doi.org/10.1016/BSM.MI.2015.04.004
24. Snowden JA, Cliver DO. Microorganisms in honey. Int J Food Microbiol. 1996; 31: 1–26. Available: http://www.ncbi.nlm.nih.gov/pubmed/8880294 PMID: 8880294
25. Grabowski NT, Klein G. Microbiology and Food-borne Pathogens in Honey. Crit Rev Food Sci Nutr. 2015; 57: 00–00. https://doi.org/10.1080/10408398.2015.1029041 PMID: 26176586
26. Nicholson WL. Roles of Bacillus endospores in the environment. Cell Mol Life Sci. Birkhäuser Verlag; 2002; 59: 410–416. https://doi.org/10.1007/s00018-002-8433-7 PMID: 11964119
Identification of honey bacteria

27. Pucciarelli AB, Schapovaloff ME, Kummeritz S, Seňuk IA, Brumovsky LA, Dallagnol AM. Microbiological and physicochemical analysis of yai (Tetragonisca angustula) honey for assessing quality standards and commercialization. Rev Argent Microbiol. 2014; 46: 325–332. https://doi.org/10.1016/S0325-7541(14)70091-4 PMID: 25576417

28. Finola MS, Lasagno MC, Marioli JM. Microbiological and chemical characterization of honeys from central Argentina. Food Chem. Elsevier; 2007; 100: 1649–1653. https://doi.org/10.1016/J.FOODCHEM.2005.12.046

29. López AC, Alippi AM. Phenotypic and genotypic diversity of Bacillus cereus isolates recovered from honey. Int J Food Microbiol. 2007; 117: 175–184. https://doi.org/10.1016/j.ijfoodmicro.2007.03.007 PMID: 17466403

30. Ecker DJ, Sampath R, Willett P, Wyatt JR, Samant V, Massire C, et al. The Microbial Rosetta Stone Database: a compilation of global and emerging infectious microorganisms and bioterrorist threat agents. BMC Microbiol. 2015; 5: 19. https://doi.org/10.1186/1471-2180-5-19 PMID: 15850481

31. Monetto A. M., Francescavilla A., Rondini A., Manca L., Siravegna M., Fernandez R. A study of botulinum spores in honey. Anaerobe. Academic Press; 1999; 5: 185–186. Available: http://www.academia.edu/7224677/A_Study_of_Botulinum_Spores_in_Honey

32. Keys CJ, Dare DJ, Sutton H, Wells G, Lunt M, McKenna T, et al. Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases. Infect Genet Evol. 2004; 4: 221–242. https://doi.org/10.1016/j.meegid.2004.02.004 PMID: 15450202

33. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry: a Fundamental Shift in the Routine Practice of Clinical Microbiology. Clin Microbiol Rev. 2013; 26: 547–603. https://doi.org/10.1128/CMR.00072-12 PMID: 23824373

34. Kopcakova A, Stramova Z, Kvasnov a S, Godany A, Perhacova Z, Pristas P. Need for database extension for reliable identification of bacteria from extreme environments using MALDI TOF mass spectrometry. Chem Pap. Veristica; 2014; 68: 1435–1442. https://doi.org/10.2478/s11696-014-0612-0

35. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, et al. Re-evaluating prokaryotic species. Nat Rev Microbiol. 2005; 3: 733–739. https://doi.org/10.1038/nrmicro1236 PMID: 16138101

36. Starostin K V., Demidov EA, Bryanskaya A V., Efimov VM, Rozanov AS, Peltek SE. Identification of Bacillus strains by MALDI TOF MS using geometric approach. Sci Rep. Nature Publishing Group; 2015; 5: 16899. https://doi.org/10.1038/srep16899 PMID: 26592761

37. Helgason E, Toursee NJ, Meisal R, Caugant DA, Kolsta A-B. Multilocus sequence typing scheme for bacteria of the Bacillus cereus group. Appl Environ Microbiol. 2004; 70: 191–201. Available: http://www.ncbi.nlm.nih.gov/pubmed/14711642 https://doi.org/10.1128/AEM.70.1.191-201.2004 PMID: 14711642

38. RASKO D, ALTHERR M, HAN C, RAVEL J. Genomics of the group of organisms. FEMS Microbiol Rev. 2005; 29: 303–329. https://doi.org/10.1016/j.femsre.2004.12.005 PMID: 15808746

39. Maughan H, Van der Auwera G. Bacillus taxonomy in the genomic era finds phenotypes to be essential though often misleading. Infect Genet Evol. 2011; 11: 789–797. https://doi.org/10.1016/j.meegid.2011.02.001 PMID: 21334463

40. Janda JM, Abbott SL. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J Clin Microbiol. 2007; 45: 2761–2764. https://doi.org/10.1128/JCM.01228-07 PMID: 17626177

41. Lasch P, Beyer W, Nattermann H, Stammier M, Siegbrech E, Grunow R, et al. Identification of Bacillus anthracis by Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry and Artificial Neural Networks. Appl Environ Microbiol. 2009; 75: 7229–7242. https://doi.org/10.1128/AEM.01971-08 PMID: 19767470

42. Lasch P, Nattermann H, Erhard M, Stämmier M, Grunow R, Bannert N, et al. MALDI-TOF Mass Spectrometry Compatible Inactivation Method for Highly Pathogenic Microbial Cells and Spores. Anal Chem. 2008; 80: 2026–2034. https://doi.org/10.1021/ac0710122 PMID: 18290666

43. Fernández-No IC, Böhme K, Díaz-Bao M, Cepeda A, Barros-Velázquez J, Calo-Mata P. Characterisation and profiling of Bacillus subtilis, Bacillus cereus and Bacillus licheniformis by MALDI-TOF mass fingerprinting. Food Microbiol. 2013; 33: 235–242. https://doi.org/10.1016/j.fm.2012.09.022 PMID: 23200657

44. Rooney AP, Price NPJ, Ehrhardt C, Swezey JL, Bannan JD. Phylogeny and molecular taxonomy of the Bacillus subtilis species complex and description of Bacillus subtilis subsp. inaquosorum subsp. nov. Int J Syst Evol Microbiol. 2008; 58: 2429–2436. https://doi.org/10.1099/ijs.0.009126-0 PMID: 19622642

45. Dieckmann R, Graeber I, Kaesler I, Szewzyk U, von Döhren H. Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by Intact-Cell-MALDI-TOF mass spectrometry (ICM-MS). Appl Microbiol Biotechnol. 2005; 67: 539–548. https://doi.org/10.1007/s00253-004-1812-2 PMID: 15614563
46. Angolini CFF, Pilau EJ, Lopes-Oliveira PF, Garcia INS, Gozzo FC, De Oliveira VM, et al. Classification and Identification of Petroleum Microorganisms by MALDI-TOF Mass Spectrometry. Artic J Braz Chem Soc. 2015; 26: 513–520. https://doi.org/10.5935/0103-5053.20150004

47. Pauker VI, Thoma BR, Grass G, Bleichert P, Hanczarak M, Zöller L, et al. Improved Discrimination of Bacillus anthracis from Closely Related Species in the Bacillus cereus Sensu Lato Group Based on Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. J Clin Microbiol. American Society for Microbiology Journals; 2018; 56: e01900-17. https://doi.org/10.1128/JCM.01900-17 PMID: 29514939

48. Hathout Y, Demirev PA, Ho YP, Bundy JL, Ryzhov V, Sapp L, et al. Identification of Bacillus spores by matrix-assisted laser desorption ionization-mass spectrometry. Appl Environ Microbiol. 1999; 65: 4313–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/10508053 PMID: 10508053

49. Shu L-J, Yang Y-L. Bacillus Classification Based on Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry—Effects of Culture Conditions. Sci Rep. Nature Publishing Group; 2017; 7: 15546. https://doi.org/10.1038/s41598-017-15808-5 PMID: 29138467

50. Chambers T, Culak R, Gharbia SE, Shah HN. Minor Differences in the Proteome of Bacillus subtilis and Bacillus mojavensis Based upon High Abundance/Conserved Protein Mass Spectra; Implications for Rapid, Improved Identification of Two Pathogen Genetically Closely Related. J Proteomics Enzymol. SciTechnol; 2015;04. https://doi.org/10.4172/2470-1289.1000119

51. English RD, Bettina W, Fenselau C, Cotter RJ. Bacillus Spore Identification via Proteolytic Peptide Mapping with a Miniaturized MALDI TOF Mass Spectrometer. American Chemical Society; 2003; https://doi.org/10.1021/ac034624 PMID: 14670049

52. Demirev PA, Feldman AB, Kowalski P, Lin JS. Top-Down Proteomics for Rapid Identification of Intact Microorganisms. Anal Chem. 2005; 77: 4313–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/10508053 PMID: 10508053

53. Estevinho LM, Feias S, Seijas JA, Pilar Vázquez-Tato M. Organic honey from Trás-Os-Montes region (Portugal): Chemical, palynological, microbiological and bioactive compounds characterization. Food Chem. 2014; 146: 548–557. https://doi.org/10.1016/j.foodchem.2013.09.105 PMID: 24176380

54. Karabagias IK, Badeka A, Kontakos S, Karabourioti S, Kontominas MG. Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical parameters and chemometrics. Food Chem. 2014; 146: 548–557. https://doi.org/10.1016/j.foodchem.2013.09.105 PMID: 24176380

55. da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. Honey: Chemical composition, stability and authenticity. Food Chem. Elsevier; 2016; 196: 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051 PMID: 26593496

56. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25: 3389–402. Available: http://www.ncbi.nlm.nih.gov/pubmed/9254694 https://doi.org/10.1093/nar/25.17.3389 PMID: 9254694

57. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4: 406–25. https://doi.org/10.1093/molbev/25.17.3389

58. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci. 2004; 101: 11030–11035. https://doi.org/10.1073/pnas.040206101 PMID: 15258291

59. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

60. Rogowska A, Pomastowski P, Złoch M, Railean-Plugar A, Rafińska K, et al. The influence of different pH on the electrophoretic behaviour of Saccharomyces cerevisiae modified by calcium ions. Sci Rep. 2018; 8. https://doi.org/10.1038/s41598-017-18329-3