Safety aspects of stingless bee pot-pollen from the Philippines

Sicherheitsaspekte von Cerumen-Pollen stachelloser Bienen von den Philippinen

Ma. Desiree Belina-Aldemita^{1,2}, Vera Fraberger^{1*}, Matthias Schreiner¹, Konrad J. Domig¹, Stefano D'Amico³

¹ Department of Food Science and Technology, BOKU - University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria

² Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna 4031, Philippines

³ Institute for Animal Nutrition and Feed, AGES - Austrian Agency for Health and Food Safety, Spargelfeldstraße 191, 1220 Vienna, Austria

* Corresponding author: vera.fraberger@boku.ac.at

Received: 22 March 2020, received in revised form: 26 May 2020, accepted: 14 June 2020

Summary

Philippines stingless bee (*Tetragonula biori* Friese) pot-pollen are known for their advantageous effects on human health due to a high nutritional value and a broad range of beneficial physiological and pharmacological effects. However, safety aspects regarding this product were rarely examined. Therefore, pollen samples from the Philippines were analyzed for inorganic contaminants and microbiological quality. Obtained values for heavy metals (e.g., arsenic 0.015–0.032 mg/kg, cadmium 0.053–0.153 mg/kg and lead 0.017–0.155 mg/kg) were generally lower compared to previous studies and most were within the safety limits. Further, microbial counts for total aerobic microorganisms, yeasts and molds, Enterobacteriaceae, *E. coli*, *Salmonella*, and *Staphylococcus aureus* were in accordance with the recommended values. Microbial population was diverse and the identified species were mostly endospore-formers such as *Bacillus* spp. and *Clostridium* spp., and molds because of the low water activity and low pH of the pot-pollens. Results indicated that good practices in agriculture, hygiene and manufacturing in every step of the production chain must be implemented in order to enhance the quality and safety of this bee product. Overall, pot-pollens from *T. biori* are safe for human consumption if hygienic principles are applied.

Keywords: Pollen, stingless bee, inorganic contaminants, microbiological quality, safety

Zusammenfassung

Pollen aus Cerumen von stachellosen Bienen (*Tetragonula biori* Friese) sind für ihre positiven Effekte auf die menschliche Gesundheit bekannt, da sie eine hohe ernährungsphysiologischen Wertigkeit und eine Vielzahl an pharmakologischen Effekten ausüben. Bis dato gibt es jedoch kaum Daten zu sicherheitsrelevanten Aspekten. Daher wurden anorganische Kontaminanten und die mikrobiologische Belastung von Cerumen-Pollen in unserer Studie beleuchtet. Generell konnten geringere Konzentrationen an Schwermetallen (z. B. Arsen 0,015–0,032 mg/kg, Cadmium 0,053–0,153 mg/kg und Blei 0,017–0,155 mg/kg) im Vergleich zu bisherigen Studien nachgewiesen werden. Die Keimzahl der Pollenproben für Gesamtkeimzahl, Enterobacteriaceae, *E. coli*, *Salmonella* und *Staphylococcus aureus* lagen unter den Richtwerten. Es wurde eine hohe mikrobielle Diversität ermittelt, wobei vor allem Mikroorganismen, welche bei niedrigen pH-Werten und Wasseraktivitäten persistieren können, nachgewiesen wurden. Dazu zählen Endosporenbildner der Gattungen *Bacillus* und *Clostridium* sowie Schimmelpilze. Die Resultate dieser Studie zeigten, dass Qualitätssicherungssysteme entlang der Produktionskette von großer Bedeutung sind, um die Produktqualität sowie Sicherheit zu gewährleisten. Insgesamt stellen die untersuchten Pollen aus Cerumen der Bienenart *T. biori* Friese ein sicheres Lebensmittel dar.

Schlagworte: Pollen, Stachellose Biene, anorganische Schadstoffe, mikrobiologische Qualität, Sicherheit

1. Introduction

Commercial bee pollen from honey bees (*Apis mellifera*) are collected by beekeepers from forager bees upon their entry to the hive by special pollen load traps while those from stingless bees are obtained from cerumen pots inside the hive. Similar to honey bee pollen, pot-pollen is an agglomerate of pollen grains from different botanical sources in combination with honey and glandular secretions, which are incorporated by the bees. Pot-pollen is analogous to the bee bread of *A. mellifera*, which undergoes lactic acid fermentation and thus is a matured and stabilized pollen product (Menezes et al., 2013). It is a product of great commercial interest due to its high nutritional value and wide range of beneficial physiological and pharmacological activities (Belina-Aldemita et al., 2019). However, apart from the nutritional and bioactive components, bee pollen may also contain undesirable components, such as inorganic contaminants and harmful microorganisms. Contamination of bee pollen can be attributed to various factors and sources, such as the environment, weather, plant materials, insects and animals, humans and agricultural devices, and it can even be derived from bees themselves (Belhadj et al., 2012).

Most inorganic contaminants are widely distributed in nature. At the same time, rapid modernization and anthropogenic activities have caused an increased pollution of our environment. Due to this fact, increased levels of inorganic contaminants along the food chain can be observed. The presence of these substances in food such as bee pollen can promote potential public health concerns considering the frequency of oral exposure (Das et al., 2018). One group of these contaminants are heavy metals, which can cause adverse health effects in numerous organ systems of humans, such as reproductive, endocrine, nervous, and cardiovascular systems, if the tolerable daily intake is exceeded (Rani et al., 2014; Wani et al., 2015; Baker et al., 2018).

Microbial contamination in bee pollen is inevitable due to the various habitats and enormous biodiversity of microorganisms, of which the ubiquitous occurring *Bacillus* spp. has to be pointed out. In addition, storage of pollen in the hive is associated with microorganisms, which promote biochemical changes that alter nutritional quality, enhance digestion and absorption of nutrients, and prevent spoilage and diseases (Menezes et al., 2013). Hence, some microorganisms are inherent in this product. However, microbial population must be monitored to maintain its quality as

a variety of harmful microorganisms may grow if the pot-pollen is not properly handled (De-Melo et al., 2015).

The Codex Alimentarius (2019) recommends maximum levels of contaminants and toxins in food and feed, which includes heavy metals, such as arsenic, cadmium, and lead. Moreover, the general quality criteria for honey bee pollen proposed by scientists of the International Honey Commission (Campos et al., 2008) included limits for heavy metals and pathogenic microorganisms, which were based on national standards from different countries such as Brazil, Bulgaria, Poland, and Switzerland. Hence, this viewpoint on the bee pollen quality is as important as its physicochemical, nutritional, and bioactive properties.

Data regarding safety of the Philippine stingless bee pot-pollen is still scarce. The results of our previous studies already presented the high nutritive value and antioxidative potential of pot-pollen produced by *T. biroi* Friese (Belina-Aldemita et al., 2019; Belina-Aldemita et al., 2020). To examine the quality of these pot-pollen to a greater extent, the present study focused on inorganic contaminants and the microbiological quality. The findings of this work may contribute to the assessment of quality parameters for Philippine stingless bee pot-pollen with respect to its safety features. Further, it may also provide useful information for beekeepers in the management of their apiary or meliponary, such as planning the beehive location and handling of bee products (e.g., bee pollen), in order to ensure consumer safety.

2. Materials and methods

2.1. Samples

Eight polyfloral pot-pollen samples produced by *T. biroi* Friese were gathered from October 2016 to May 2017 from seven apiaries in the Philippines. The detailed data about collection were presented in our previous paper (Belina-Aldemita et al., 2019). Samples were obtained directly from the cerumen pots of three independent hived colonies using a metallic spoon. These were air-dried and further dehumidified (Kolin KDM-18DHS dehumidifier, Philippines) for 12 h at 30°C and 80% relative humidity. The dried samples (about 60 g) were placed in sealed plastic containers and transported to Austria at room temperature. Pollen samples were homogenized and stored at -24°C for further analysis.

2.2. Determination of inorganic contaminants in pot-pollen

Pot-pollen samples (0.5 g) were solved using concentrated HNO_3 (65% w/w) and H_2O_2 (30%, suprapure), before being subjected to acid assisted microwave digestion (Anton Paar Multiwave 3000, Rotor 16MF100, Austria). ICP Multi Element Standard Solution VI (CertiPur, suprapure, Merck KGaA, Germany) were used to prepare the external calibration. An indium ICP standard of 1 ng/g (Inorganic Ventures, Christiansburg, USA) was used for internal normalization with respective dilution of samples, standards and blank solutions.

Elements were analyzed on an inductively coupled plasma quadrupole mass spectrometer (ICP-MS; NexIon 2000 B, Perkin Elmer, USA) in duplicate with the following settings: nebulizer gas flow rate: 0.96 L/min, auxiliary gas flow rate: 1.25 L/min, plasma gas flow rate: 18 L/min, lens voltage: 14.5 V, ICP radiofrequency power: 1500 W, analog stage voltage: -1825 V, pulse stage voltage: 1600 V according to Belina-Aldemita et al. (2019). Elemental concentrations were calculated according to the routine data evaluation procedures. Standard uncertainties U ($k = 2$) were estimated considering measurement precision (Horsky et al., 2016).

Estimated daily intake (EDI) of the inorganic contaminant was calculated based on the recommended daily consumption for pollen (10 g) and average adult body weight of 60 kg using the formula of the Codex Alimentarius (2014) on evaluation of dietary exposure to food additives.

2.3. Microbiological quality of pot-pollen

Five grams of each pot-pollen sample were aseptically weighed into a Stomacher bag with filter and homogenized using a Stomacher Lab-Blender (Stomacher 400 Circulator, Seward, UK) with 45 mL of sterilized buffered peptone water (BPW, Merck, Germany). Further, successive 10-fold dilutions were prepared using the same diluent (1:10 v/v). All growth media were prepared according to the manufacturer's instructions. Aliquots (0.1 or 1 mL) of the respective dilutions were placed in duplicate on the respective growth media using pour plate or spread plate method.

The following microorganisms were enumerated using the corresponding cultivation media according to the indicated incubation temperature and time. Aerobic mesophilic microorganisms on plate count (PC) agar (Merck, Germany) at 30°C for 72 h (ISO 4833-1), yeasts and molds on

dichloran-glycerol-18 (DG-18) agar (Oxoid, UK) at 25°C for 5 d (ISO 21572-2), coliforms (Enterobacteriaceae) on violet red bile dextrose (VRBD) agar (Merck, Germany) at 37°C for 48 h under anaerobic condition, coagulase-positive *Staphylococcus* on Baird-Parker (BP) agar (Merck, Germany) at 37°C for 48 h and lactic acid bacteria (LAB) on De Man, Rogosa and Sharpe (MRS) agar (Merck, Germany) at 30°C and 37°C for 48 h under anaerobic condition. For the enumeration of *Bacillus* species, sample homogenate, previously pasteurized at 80°C for 10 min, was used, which was further incubated on tryptic soy (TS) agar (Merck, Germany) at 37°C for 48 h. Microbial counts were expressed as the negative decimal logarithm of the colony-forming units per gram of dehydrated pot-pollen ($\log_{10}$ CFU/g).

The occurrence of sulphite-reducing clostridia spores was determined using the most probable number (MPN) method. One mL sample of the first three dilutions was placed into sterilized test tubes containing differential reinforced clostridial broth (DRCM) (Merck, Germany) and paraffin, and further subjected to heating in a water bath at 80°C for 10 min. The paraffin was allowed to solidify on top of the mixture, creating anaerobic conditions. The tubes were incubated at 30°C for 5 d. A positive result was indicated by a color change to black and gas formation. The results were expressed as $\log_{10}$ MPN/g.

The detection for the presence of *Salmonella* was carried out using the Vitek immunodiagnostic assay system (VIDAS UP *Salmonella* [SPT], bioMérieux). The enrichment protocol was used as recommended by the manufacturer, with some modifications. Briefly, five grams of each pot-pollen sample were aseptically weighed into a Stomacher bag with filter and homogenized using a Stomacher Lab-Blender (Stomacher 400 Circulator, Seward, UK) with 45 mL of sterilized BPW (Merck, Germany). *Salmonella* supplement (bioMérieux, France) was added to the mixture, mixed manually and then incubated for 24 h at 41°C. After incubation, 2 mL of enrichment broth were heated at 95°C for 5 min. An aliquot (0.5 mL) of the boiled suspension was placed into the sample well of the VIDAS reagent strip. The assay was performed in a mini-VIDAS (bioMérieux, France).

2.4. Identification of microorganisms through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Colonies with different morphologies obtained from PC agar, VRBD agar, BP agar, and TS agar plates were streaked

on PC agar and incubated at corresponding temperatures and condition for 18 to 24 h. Typical colonies from MRS agar plates were streaked on MRS agar and incubated at corresponding temperatures anaerobically for 48 h. Suspensions from DRCM tubes with positive results were streaked on reinforced clostridial agar (RSA, Merck) and incubated anaerobically at 30°C for 72 h.

Identification of isolates was carried out in their exponential growth phase using the Microflex LT Benchtop MS (Bruker Daltonics, Germany) equipped with FlexControl 3.4 software and MALDI Biotyper Compass database (version 4.1.80). Sample preparation was done according to the manufacturer's recommendation for the extended direct transfer method. A score above 2.00 indicated reliable identification at the species level.

2.5. Molecular identification of microorganisms

For bacterial cultures, single colonies were suspended in nutrient broth (Merck, Germany) and incubated at their respective cultivation condition for 48 h. One milliliter of the cell suspension was centrifuged at 8000 rpm for 6 min at 4°C. Total DNA from bacteria was isolated using the peqGOLD Bacterial DNA Kit (VWR International, Germany) according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) using the 16S rDNA-universal primers bak4 (5'-AGGAGGTGATCCARC-CGCA-3') and bak11w (5'-AGTTTGATCMTGGCT-CAG-3') (Dasen et al., 1998) was carried out in a total reaction volume of 25 µL consisting of 12.5 µL AccuStart II Master Mix (Quanta BioSciences, USA), 9.5 µL sterile water, 1 µL of each primer and 1 µL of DNA. Fragments were amplified in a thermocycler (Eppendorf Mastercycler Nexus SX1, Germany) with the following reaction profile: initial denaturation at 94°C for 3 min; 30 cycles of denaturation (94°C for 30 sec), annealing (56°C for 30 sec), and extension (72°C for 2 min); and a final extension step at 72°C for 7 min. A no template control was performed with each run by replacing the template DNA with sterile water in the PCR mixture.

For fungal cultures, typical colonies from DG-18 agar plates were streaked on PC agar and incubated at 25°C for 72 h. Total DNA was extracted using the Dneasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The 26S rDNA-specific primers NL1 (5'-GCA TAT CAA TAA GCG GAG CAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Waite et al., 2009) were used in PCR with the following

program: initial denaturation at 94°C for 5 min; 28 cycles of denaturation (94°C for 1 min), annealing (52°C for 30 sec), and extension (72°C for 40 sec); and a final extension step at 72°C for 10 min.

The presence of amplicons was verified through agarose gel electrophoresis. A double-tiered (2% w/v) agarose gel (Biozyme Scientific, Germany) was prepared. The gel was run at 80 V for 30 min in the SEA-2000 electrophoresis apparatus (Elchrom Scientific, Switzerland) at 20°C in 0.75X TAE buffer (AppliChem, Germany). Results were visualized using GelRed (Biotium, USA) and GelDoc XR+ Gel Documentation System (Bio-Rad, USA).

The amplicons were purified using the peqGOLD Cycle Pure Kit (S-Line) (VWR International, Belgium) according to the manufacturer's instructions. Purified DNA was sent to Microsynth (Austria) for DNA sequencing service. For identification, sequences were further subjected to the National Center for Biotechnology Information (NCBI) database (GenBank) using the basic local alignment search tool (BLASTn) algorithm.

3. Results and discussion

3.1. Inorganic contaminants of pot-pollen

The contents of inorganic contaminants quantified in pot-pollen samples are summarized in Table 1. Aluminum (Al) was present at the highest levels with sample 8 having an exceptionally high content compared to other samples. The respective sample was obtained from an urbanized area. Thus, its Al levels may reflect the environmental pollution of the city. Obtained values were in the same range as those reported for *Apis mellifera* bee pollens from Serbia (Kostić et al., 2015) and Turkey (Kalaycıoğlu et al., 2017). Some bee pollen samples from Brazil (Morgano et al., 2010) and China (Yang et al., 2013) even contained higher amounts reaching up to 268.0 and 218.2 mg/kg, respectively. Although, only approximately 5% of Al intake is absorbed, exposure to it should be kept at a minimum since its possible effects on human health are still not fully understood (Stahl et al., 2017).

Barium (Ba) levels were high as well, but most obtained values were in agreement to those reported by Morgano et al. (2010), Kostić et al. (2015) and Sattler et al. (2016). The high level of Ba in sample 1 could be attributed to the construction activities in the area, which may have released Ba from welding and cement. Ba toxicity depends on its

Table 1. Inorganic contaminants in pot-pollen samples (mg/kg^a) produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines
 Tabelle 1. Konzentration an anorganischen Kontaminationen von philippinischen Pollenproben (mg/kg^a) produziert von *Tetragonula biroi* Friese

Element		Pollen sample							
		1	2	3	4	5	6	7	8
Aluminum	Al		21.4 ± 1.4	9.5 ± 0.4	30.4 ± 2.8	93.6 ± 3.4	18.6 ± 1.1	14.9 ± 1.2	89.0 ± 7.0
			0.122 ±	0.064 ±	0.098 ±	0.224 ±	0.091 ±	0.083 ±	0.167 ±
Vanadium	V		0.016	0.006	0.005	0.020	0.009	0.007	0.015
			0.683 ±	0.371 ±	0.299 ±	0.643 ±	0.312 ±	0.439 ±	0.888 ±
Chromium	Cr		0.109	0.045	0.031	0.095	0.040	0.055	0.071
			0.155 ±	0.154 ±	0.071 ±	0.058 ±	0.138 ±	0.044 ±	0.108 ±
Cobalt	Co		0.023	0.008	0.005	0.004	0.010	0.004	0.005
			0.874 ±	0.469 ±	0.442 ±	0.481 ±	0.511 ±	0.451 ±	0.994 ±
Nickel	Ni		0.087	0.058	0.041	0.056	0.075	0.055	0.079
			0.061 ±	0.076 ±	0.080 ±	0.075 ±	0.077 ±	0.079 ±	0.082 ±
Gallium	Ga		0.018	0.012	0.010	0.002	0.006	0.011	0.009
			0.018 ±	0.017 ±	0.016 ±	0.028 ±	0.015 ±	0.019 ±	0.019 ±
Arsenic	As		0.004	0.009	0.008	0.006	0.006	0.007	0.009
			0.141 ±	0.096 ±	0.116 ±	0.282 ±	0.275 ±	0.219 ±	0.514 ±
Molybdenum	Mo		0.011	0.010	0.007	0.039	0.024	0.018	0.034
			0.091 ±	0.053 ±	0.153 ±	0.078 ±	0.057 ±	0.085 ±	0.074 ±
Cadmium	Cd		0.014	0.006	0.018	0.013	0.013	0.012	0.012
			23.48 ±	3.13 ±	3.41 ±	2.06 ±	4.71 ±	1.19 ±	7.05 ±
Barium	Ba		1.61	0.26	0.29	0.20	0.42	0.11	0.37
			0.0030 ±	0.0089 ±	0.0031 ±	0.0007 ±	0.0083 ±	0.0040 ±	0.0017 ±
Thallium	Tl		0.0004	0.0011	0.0006	0.0004	0.0019	0.0010	0.0003
			0.017 ±	0.022 ±	0.033 ±	0.108 ±	0.025 ±	0.072 ±	0.052 ±
Lead	Pb		0.003	0.001	0.002	0.009	0.002	0.006	0.006

^a Values of inorganic contaminants are expressed as mean ± standard uncertainty ($k = 2$)

^a Werte anorganischer Verunreinigungen ausgedrückt als Mittelwert ± Standardabweichung ($k = 2$)

solubility and its main target in humans is the cardiovascular system. However, there is only limited data about its neurological and reproductive toxicity (Poddalgoda et al., 2017). Nickel (Ni) and Chromium (Cr) concentrations were partially in accordance to those reported by Morgano et al. (2010), but were generally lower compared to other studies (Yang et al., 2013; Kostić et al., 2015; Sattler et al., 2016; Kalaycioğlu et al., 2017). The trivalent form of Cr [Cr(III)] is an essential trace element and plays an important role in glucose metabolism, while the hexavalent form [Cr(VI)] has toxic effects and is classified as carcinogen (Bhattacharya et al., 2016). Incorporation of both Ni and Cr into the pot-pollen can be attributed to the usage of stainless-steel equipment, coal combustion, and excessive application of chemical fertilizer (Ali et al., 2019).

Cadmium (Cd) levels fell within the range observed by previous studies (Kostić et al., 2015; Morgano et al., 2010; Sattler et al., 2016), but those of lead (Pb) and arsenic (As) were generally lower compared to former reports (Morgano et al., 2010; Sattler et al., 2016; Yang et al., 2013; Kostić et al.,

2015). According to Campos et al. (2008), the concentrations of heavy metals (in mg/kg) should not exceed 0.1 for Cd, 0.5 for Pb, and 0.5 for As. The amount of these metals in all pot-pollen samples were within the safety limit, except for sample 3, which contained 0.153 ± 0.018 mg/kg Cd. However, this value is still lower than the maximum level set by the Codex Alimentarius (2019) for Cd in wheat, which is 0.2 mg/kg. These metals may be incorporated into the pollen samples from various sources. Cd, Pb and As are emitted during coal combustion. Cd and As may even derive from fertilizers and pesticides, while Pb may come from either acid batteries, old plumbing systems or vehicular emissions from combustion Pb-containing gasoline (Ali et al., 2019). These heavy metals are considered as carcinogens and very toxic, causing adverse health effects in numerous organ systems of humans such as the reproductive, endocrine, nervous, and cardiovascular (Rani et al., 2014; Wani et al., 2015; Baker et al., 2018).

This study is the second report on molybdenum (Mo) in bee pollen, which is in accordance with the first report by

Yang et al. (2013) who studied *A. mellifera* pollen from China. Mo can be integrated into the pot-pollen upon exposure to fossil-fuel combustion, welding activities, or usage of stainless-steel materials in the apiary. Mo is considered as an essential trace element as it is a cofactor in certain molybdoproteins but has an antagonistic effect against copper (Cu) (Bhattacharya et al., 2016). Further, it was found to be associated with increased concentrations of uric acid in the blood and urine (Smedley and Kinniburgh, 2017).

Only two studies are known that analyzed vanadium (V) in bee pollen so far. Both revealed greater contents of V in their pollen samples (3.99–4.89 mg/kg and 0.759–3.943 mg/kg) than in this study (Sattler et al., 2016; Kalaycioğlu et al., 2017). Usage of stainless-steel equipment in the apiary may also incorporate V into the pot-pollen. There is little evidence about the toxicity of V in humans but animal studies have demonstrated that V can cause hematological and biochemical changes and neurobehavioral injury (Ghosh et al., 2015).

Values obtained for cobalt (Co) fell within the range reported by Morgano et al. (2010) and Kostić et al. (2015) but were lower compared to those reported by Yang et al. (2013). Contamination of Co in pot-pollen may be caused by coal combustion, lithium-ion batteries and fertilizers (Farjana et al., 2019). The organic form of Co has a significant role in the human metabolism, whereas the inorganic form is toxic (Bhattacharya et al., 2016).

This study determined gallium (Ga) and thallium (Tl) for the first time in bee pollen. Due to missing data, no comparison could be drawn. Ga has relatively low toxicity as it is used in medicine. However, it may cause side effects such as interruption to iron metabolism (Chitambar, 2010). Tl is considered to be very toxic but is not classified as human carcinogen (Staff et al., 2014).

The estimated daily intake (EDI) of the inorganic contaminants (shown in Table 2) was calculated based on the formula of the Codex Alimentarius (2014) on the evaluation of dietary exposure to food additives. Based on EDI and the oral reference dose (RfD) or tolerable daily intake (TDI) as recommended by United States Environmental Protection Agency (US EPA), Risk Assessment Information System (RAIS), Agency of Toxic Substances and Disease Registry (ATSDR), Health Canada (HC), Scientific Committee on Health, Environmental and Emerging Risks (SCHEER), pot-pollens from *T. biroi* are safe for human consumption.

3.2. Microbiological quality of pot-pollen

3.2.1. Microbial ecology in pot-pollen samples

Since bee pollen is exposed to various possible sources of contamination, they are more susceptible to rapid degradation due to the growth of spoilage microorganisms when pollen is not properly handled. Analysis of its microbiological quality is essential in order to assess the condition during production, storage, and distribution (De-Melo et al., 2015). Campos et al. (2008) recommended the following limits of the microbiological content in bee pollen: $< 5 \log_{10}$ CFU/g for total aerobic microorganisms, $< 4.70 \log_{10}$ CFU/g for yeasts and molds, maximum of $2 \log_{10}$ CFU/g for Enterobacteriaceae, absence in 1 g for *Escherichia coli* and *Staphylococcus aureus*, and absence in 10 g for *Salmonella*. Table 3 summarizes the microbial population recorded for the pot-pollen samples. The aerobic mesophilic counts, yeasts and molds, and Enterobacteriaceae showed levels below the recommended limits by Campos et al. (2008). Aerobic mesophilic counts were lower than reported for pollen samples originating from *A. mellifera* (Feás et al., 2012; Belhadj et al., 2012; Nogueira et al., 2012; De-Melo et al., 2015) and *Melipona scutellaris* (Oliveira Alves et al., 2018). Yeasts and molds were in the same range as previously reported by other authors (Feás et al., 2012; Belhadj et al., 2012; Nogueira et al., 2012; De-Melo et al., 2015), whereas the study conducted by Oliveira Alves et al. (2018) showed no detectable growth of yeasts and molds. Based on the identification using either MALDI-TOF MS or DNA analysis (section 3.2.2), *E. coli* was absent in all samples. *E. coli* was absent even in the examined samples of Feás et al. (2012) and Nogueira et al. (2012), whereas reported values for total coliforms of other studies were in the range of < 1.00 to $4.00 \log_{10}$ CFU/g (Belhadj et al., 2012; De-Melo et al., 2015; Arruda et al., 2017). All samples exhibited values $< 1.0 \log_{10}$ CFU/g for coagulase-positive *Staphylococcus*. Based on the identification part (section 3.2.2), *Staphylococcus aureus* was absent in all samples. Further, this species was not detected in any of the examined samples of Feás et al. (2012) and Nogueira et al. (2012) but present in the samples of other researchers with values in the range of < 1 to $4 \log_{10}$ CFU/g (Belhadj et al., 2012; De-Melo et al., 2015; Arruda et al., 2017).

Using the MPN method, only two (samples 2 and 6) out of the eight pot-pollen samples had no detectable sulphite-reducing clostridia spores. However, other studies reported

Table 2. Reference dose for chronic oral exposure as recommended by agencies and average estimated daily intake of the determined inorganic contaminants in pot-pollen samples produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines

Tabelle 2. Referenzwerte zur chronisch oralen Aufnahme und geschätzten durchschnittlichen Tagesdosis der analysierten anorganischen Kontaminanten durch philippinische Pollenproben produziert von *Tetragonula biroi* Friese

Element		Oral RfD or TDI (mg/kg-day) ^a	Average EDI (mg/kg-day) ^b
Aluminum	Al	0.3 (SCHEER)	8.54×10^{-3}
Vanadium	V	V (+5): 0.009 (US EPA)	2.28×10^{-5}
Chromium	Cr	Cr (+3): 1.5, Cr (+6): 0.003 (US EPA)	8.58×10^{-5}
Cobalt	Co	0.0003 (RAIS), 0.01 (ATSDR)	1.66×10^{-5}
Nickel	Ni	0.02 (US EPA)	1.04×10^{-4}
Gallium	Ga	–	1.28×10^{-5}
Arsenic	As	0.0003 (US EPA)	3.39×10^{-6}
Molybdenum	Mo	0.023 (HC), 0.005 (US EPA)	4.45×10^{-5}
Cadmium	Cd	0.001 (HC), 0.0005 (US EPA)	1.37×10^{-5}
Barium	Ba	0.2 (US EPA)	9.81×10^{-4}
Thallium	Tl	–	6.91×10^{-7}
Lead	Pb	0.0036 (HC)	1.01×10^{-5}

^a Oral reference dose (Oral RfD) or Tolerable daily intake (TDI) as recommended by agencies and committees such as United States Environmental Protection Agency (US EPA), Risk Assessment Information System (RAIS), Agency of Toxic Substances and Disease Registry (ATSDR), Health Canada (HC), and Scientific Committee on Health, Environmental and Emerging Risks (SCHEER); ^b Estimated daily intake (EDI) of inorganic contaminants calculated based on recommended daily consumption for pollen (10 g) and average adult body weight (60 kg) using the formula of the Codex Alimentarius (2014) on evaluation of dietary exposure to food additives

^a empfohlene orale Referenzdosis (Oral RfD) oder tolerierbare tägliche Aufnahme (TDI) nach US EPA (United States Environmental Protection Agency), RAIS (Risk Assessment Information System), ATSDR (Agency of Toxic Substances and Disease Registry), HC (Health Canada) und SCHEER (Scientific Committee on Health, Environment and Emerging Risks); ^b geschätzte tägliche Aufnahme (EDI) anorganischer Kontaminanten, berechnet auf Basis des empfohlenen täglichen Verzehr von Pollen (10 g) und des durchschnittlichen Körpergewichts von Erwachsenen (60 kg) nach Codex Alimentarius (2014) zur Bewertung der ernährungsbedingten Exposition gegenüber Lebensmittelzusatzstoffen

the absence of clostridia in all the samples analyzed previously (Arruda et al., 2017; De-Melo et al., 2015; Feás et al., 2012; Nogueira et al., 2012). The concentrations of anaerobic spores of the examined samples within our study were in a range of 0.56 to 2.18 log₁₀/g. No recommended values for these microorganisms are given for bee pollen legislation. However, compared to the recommended value (3 log₁₀ CFU/g) for *Clostridium perfringens* in herbs and spices (Deutsche Gesellschaft für Hygiene und Mikrobiologie), results did not exceed the limits.

Salmonella could not be detected in any sample, which is in accordance with previous studies (De-Melo et al., 2015; Feás et al., 2012; Belhadj et al., 2012; Nogueira et al., 2012). *Salmonella* is an important group of intestinal pathogens that causes gastroenteritis and typhoid fever. Species are responsible for many food-borne diseases and causes significant morbidity and mortality worldwide (Ahmer and Gunn, 2011).

LAB and *Bacillus* spp. in pot-pollen were investigated as these microorganisms were previously reported to be responsible for the fermentation of bee pollen. Furthermore, *Bacillus*

species are even considered to be vital for the survival of stingless bees. Although the role of these microorganisms related to bees is still undefined, they seem to be important in food digestion and disease defense of bees through secretion of enzymes for pre-digestion of the pollen and secretion of antibiotic substances, respectively (Menezes et al., 2013). LAB counts were < 3 log₁₀ CFU/g, while counts of *Bacillus* spp. ranged from < 2 to 4.11 log₁₀ CFU/g. As there are no existing recommended limits or previously reported counts for these microorganisms in bee pollen, no direct comparison can be drawn. However, compared to the recommended values for presumptive *B. cereus* in herbs and spices, counts within the pollen sample (3 log₁₀ CFU/g) did not exceed the safety level. Thus, the product can be considered as safe for human consumption (Deutsche Gesellschaft für Hygiene und Mikrobiologie).

3.2.2. Identification of microorganisms isolated from pot-pollen samples

In recent years, MALDI-TOF MS has revolutionized routine identification of microorganisms, being a fast and

Table 3. Average microbial counts of the analyzed pot-pollen samples produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines
Tabelle 3. Durchschnittliche ermittelte Keimzahl der untersuchten philippinischen Pollenproben produziert von *Tetragonula biroi* Friese

Microorganism	Lowest Limit of Detection (LLOD)	Pollen sample							
		1	2	3	4	5	6	7	8
aerobic mesophilic organisms ^a	3.00	3.27 ± 0.04	3.02 ± 0.02	ND	4.54 ± 0.02	3.16 ± 0.11	3.41 ± 0.01	4.28 ± 0.02	4.73 ± 0.08
yeasts and molds ^a	2.00	< 3.00	ND	ND	2.57 ± 0.03	ND	ND	ND	ND
lactic acid bacteria ^a	2.00	< 3.00	ND	ND	ND	ND	ND	ND	ND
Enterobacteriaceae ^a	1.00	ND	ND	ND	< 2.00	ND	ND	ND	ND
coagulase-positive <i>Staphylococcus</i> ^a	1.00	ND	ND	ND	ND	ND	ND	ND	ND
<i>Bacillus</i> spp. ^a	2.00	3.15 ± 0.03	3.06 ± 0.02	ND	3.95 ± 0.01	2.23 ± 0.13	2.29 ± 0.03	3.77 ± 0.13	4.10 ± 0.01
sulphite-reducing clostridia spores ^b	0.48	1.63	ND	0.56	1.97	1.36	ND	1.88	2.18
<i>Salmonella</i>	ND in 5 g	ND	ND	ND	ND	ND	ND	ND	ND

^a Values are expressed as log₁₀ CFU/g ± standard deviation; ^b Values are expressed as log₁₀ MPN/g; ND – not detected or lower than the LLOD of corresponding microorganism

^a Keimzahlen werden angegeben als log₁₀ KBE/g ± Standardabweichung; ^b Werte werden angegeben als log₁₀ MPN/g; ND – nicht detektierbar oder unter der LLOD des zu nachweisenden Mikroorganismus

cost-effective technique (Singhal et al., 2015). MALDI-TOF MS uses proteomics for bacterial identification. This technique has been shown to exceed conventional phenotypical methods as well as bacterial gene amplification, which remains the gold-standard for microorganism identification (Shafer et al., 2017). Although a higher number of species can be identified with 16S rDNA gene sequencing, MALDI-TOF MS constitutes a resource saving first-line identification system (Biswas and Rolain, 2013).

Table 4 summarizes the identified bacterial and fungal species in the pot-pollen samples. The majority of bacterial species was identified through MALDI-TOF MS. Some bacterial colonies that were identified only up to the genus level (a score of 1.70–1.99) as well as fungal isolates were identified through DNA analysis. It was quite expected that endospore-forming bacteria were isolated from the samples as these can survive under extreme environmental conditions. *Bacillus* spp. were identified in all the pot-pollen samples as these microorganisms are ubiquitous in nature. The genus *Bacillus* is one of the most common genera of bacteria that was identified in stingless bee colonies. It was hypothesized that species within this genus play an important role in secreting extracellular enzymes that cause fermentation and conversion of pollen constituents for preservation and protection of stored pollen against spoilage microorganisms (Menezes et al., 2013). Though some of the identified *Bacillus* spp. were reported to be human pathogens (Ehling-Schulz et al., 2019; Celandroni et al., 2016; Desai and Varadaraj, 2013), their counts were low and can therefore be considered to be at a safe level. Bacteria of the *B. pumilus* clade were not identified up to the

species level using the MALDI-TOF MS analysis and partial 16S rDNA sequencing. However, for the scope of this study, differentiation of species within the groups was not of importance. For an effective discrimination, sequencing of DNA gyrase β-subunit and/or RNA polymerase β-subunit genes has been proven to be more reliable in identifying these closely related species (Liu et al., 2013). Most of these *Bacillus* spp. are associated with plants and were reported to have potential application in biomedicine and biocontrol (Liu et al., 2019; Lu et al., 2017; Jouzani et al., 2017; Prazdnova et al., 2019; Wang et al., 2017) as well as potential probiotics (Elshaghabee et al., 2017) and biofertilizer (Pramanik et al., 2019).

Lysinibacillus and *Paenibacillus* are widespread bacteria usually considered as environmental contaminants and rarely connected to human diseases (Wenzler et al., 2015; Sannino and Angert, 2017). Some of these species were also reported to have biosorption capabilities (Mathivanan et al., 2016; Zhang et al., 2019) and insecticidal activities (Xu et al., 2015).

Most clostridia species are non-pathogenic (Patakova et al., 2019) but some virulent, toxin-producing strains may cause fatal infections especially *Clostridium perfringens* (Stelma, 2018). However, low counts of sulphite-reducing clostridia spores were recorded in the pot-pollen and considered to be at a safe level.

LAB are generally recognized as safe (GRAS) for humans and animals. *Lactobacillus* spp. were previously isolated from the digestive tract of honey bee *A. mellifera*, pollen, and bee bread, and were reported to dominate the microbiota (Asama et al., 2015). *Lactobacillus coryniformis* has

Table 4. Bacteria and fungi identified in pot-pollen samples produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines by partial 16S rDNA and 26S rDNA sequencing and/or MALDI-TOF MSTabelle 4. Identifizierung von Mikroorganismen isoliert aus Pollenproben von stachelloser Bienen (*Tetragonula biroi* Friese) von den Philippinen mittels 16S rDNA/26S rDNA Sequenzierung und/oder MALDI-TOF MS

Microorganism	Pollen sample ^a							
	1	2	3	4	5	6	7	8
<i>Bacteria</i>								
<i>Bacillus cereus</i> group ^b	13	3	2	3	3	4	3	3
<i>Bacillus megaterium</i> ^b	1		2	2	1	2	1	
<i>Bacillus subtilis</i> ^b	3		5	1	4	1	2	4
<i>Bacillus amyloliquefacie</i> ^b	1			3	1	2	1	1
<i>Bacillus coagulans</i> ^c						1		
<i>Bacillus pumilus</i> clade ^{b,c}	2	2		1	2	1	2	1
<i>Bacillus flexus</i> ^b	1			2				
<i>Fictibacillus halophilus</i> ^c				1				
<i>Lysinibacillus fusiformis</i> ^{b,c}	1	1	1				1	2
<i>Lysinibacillus macroides</i> ^c					1			
<i>Lysinibacillus sphaericus</i> ^c		2				1		
<i>Paenibacillus spp.</i> ^c			1					
<i>Paenibacillus pabuli</i> ^c	1							
<i>Paenibacillus thiaminolyticus</i> ^b							1	
<i>Virgibacillus halophilus</i> ^c						1		
<i>Lactobacillus coryniformis</i> ^c								1
<i>Pediococcus pentosaceus</i> ^b				1				
<i>Clostridium beijerinckii</i> ^b	3				1		3	
<i>Clostridium perfringens</i> ^b				4				
<i>Clostridium sordellii</i> ^b			1					2
<i>Enterobacter hormaechei</i> ^b				2				
<i>Fungi</i>								
<i>Alternaria sp.</i> ^c					1			
<i>Aspergillus spp.</i> ^c					1	1		
<i>Monascus ruber</i> ^c	1		1				1	1
<i>Rhizopus spp.</i> ^c				1				

^a Scores refer to the number of colonies identified for each microorganism in each sample; ^b Microorganism was identified through MALDI-TOF MS analysis;^c Microorganism was identified through DNA analysis^a Werte beziehen sich auf die Anzahl der identifizierten Isolate pro Mikroorganismus und Probe; ^b identifiziert mittels MALDI-TOF MS; ^c identifiziert mittels DNA Analyse

shown to decrease symptoms linked to respiratory infections in elderly people (Fonollá et al., 2019), whereas *Pediococcus pentosaceus* has been reported to exhibit potential probiotic properties and cholesterol-lowering activity (Damodharan et al., 2015).

Fungal species of the genera *Alternaria*, *Aspergillus*, *Rhizopus*, and *Monascus* were previously isolated from commercial bee pollens (Deveza et al., 2015; Belhadj et al., 2014; González et al., 2005; Villalobos et al., 2010). These

species play important ecological roles and have been used industrially for enzyme, organic acid, and traditional food production (Cong et al., 2017; Londoño-Hernández et al., 2017; Meussen et al., 2012). The presence of *Monascus ruber* in 4 out of 8 pot-pollen samples may be due to its lactic acid tolerance (Weusthuis et al., 2017). Since these fungal species are commonly found in bee hive, they were derived mainly from primary sources (Kiš et al., 2018). Secondary sources of contamination may also contribute,

which include post-harvest treatment by humans or equipment (Pajor et al., 2018). Contrary to previous studies, yeasts were not isolated from any of the pot-pollen samples (De-Melo et al., 2015; Nogueira et al., 2012).

An overall view of the results revealed that most of the identified species belonged to endospore-forming bacterial genera such as *Bacillus* spp. and *Clostridium* spp., or molds. This was attributed to the low water activity (a_w) of the dried pot-pollen. Based on our previous study (Belina-Aldemita et al., 2019), a_w values of these samples were about 0.6. Bacterial growth related to food spoilage is mostly inhibited at a_w values below 0.9 and foods with levels below 0.6 are considered stable towards microbial growth. Moreover, pot-pollen samples have an acidic pH. The decrease in the pH value of stored pollen can be attributed to the activity of LAB and *Bacillus* spp., which are introduced into the pollen by bees (Menezes et al., 2013). The fermentation process carried out by these microorganisms resulted in the accumulation of various metabolites, decreasing the pH of the pollen and simultaneously reducing the number of pathogenic microorganisms (Vásquez and Olofsson, 2009). Anderson et al. (2014) stated that the stored pollen is not a suitable growth medium for microorganisms. Results of the mentioned study revealed that the bacterial groups abundant in the stored pollen were acid resistant and osmotolerant. As known, endospores are more resistant than vegetative cells to the action of chemical and physical factors. Thus, endospore-forming microorganisms have a higher possibility to survive under harsh conditions and persist for long periods in the environment. Furthermore, extracts of pollen and bee bread have shown to inhibit Gram-positive bacteria (e.g., *Staph. aureus*, *B. cereus*, *C. perfringens*), Gram-negative bacteria (e.g., *E. coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*), yeasts (e.g., *Zygosaccharomyces bailii*, *Candida magnoliae*), and molds (e.g., *Aspergillus flavus*, *Penicillium expansum*) (Abouda et al., 2011; Hudz et al., 2019; Morais et al., 2011; Kacániová et al., 2012; Joo et al., 2015).

There is a tendency for the pot-pollens to exhibit greater microbial proliferation and diversity due to the tropical conditions being characterized by high humidity and temperature. Additionally, the storage of pollen in the hive involves inoculation of microorganisms by the bees. Furthermore, considering the nutrient content of pollen, it is a reliable food source for a variety of microorganisms. Hence, it is obligatory that beekeepers adopt hygienic standards and proper handling of pot-pollen in every single stage of production. Otherwise, it may result in products that could be

harmful to the consumers. Harvest of pot-pollens should be done with clean devices and containers to avoid cross-contamination. Drying should be carried out immediately after harvest and in a facility with controlled temperature, instead of natural drying (González et al., 2005; De-Melo et al., 2015). As recommended by the Codex Alimentarius (2019), in order to reduce the risk of food-borne illness and spoilage, good practices in agriculture, hygiene and manufacturing must be implemented.

4. Conclusion

This is the first in depth report on heavy metal contaminants and microbiological populations in Philippine stingless bee pot-pollen. Obtained values for heavy metals were generally lower compared to the previous studies and most were within the safety limits. The estimated daily intake of these elements was much lower compared to the tolerable daily intake recommended by authorities. Microbial counts were less than the recommended values. The pot-pollens had a generally low but varying microbial population, most of which were endospore formers such as the *Bacillus* spp. and related genera, and *Clostridium* spp., or molds. The low pH and a_w values of pot-pollens as well as refrigeration temperature effectively inhibited the germination of these microorganisms, thus low counts were recorded. Overall, pot-pollens from *T. biroi* can be considered as safe for human consumption as long as hygienic principles are applied. More samples should be analyzed to get a deeper insight about the influence of varying factors such as geographical and botanical origin, drying and storage conditions, and beekeeper activities on the contents of inorganic contaminants and microbiological populations, as well as the presence of pesticide residues and toxins in stingless bee pot-pollen.

Acknowledgments

We acknowledge the Austrian Agency for International Cooperation in Education and Research (OeAD-GmbH) and the Austrian Federal Ministry of Science, Research and Economy (BMWF) for the Ernst-Mach ASEAN-European Academic University Network (ASEA-UNINET) PhD-scholarship granted to Ms. Belina-Aldemita. Thanks to the UPLB Bee Program and beekeepers for the pot-pollen samples. Thanks to Ms. Christine Oppen of VIRIS

Laboratory, Department of Chemistry, BOKU Tulln for her technical expertise. The authors thank the EQ BOKU VIBT GmbH-Center for Preservation and Aseptic Processing for providing the MALDI-TOF MS Bruker Biotyper for identification of microorganisms.

References

- Abouda, Z., Zerdani, I., Kalalou, I., Faid, M. and M. T. Ahami (2011): The antibacterial activity of Moroccan bee bread and bee-pollen (fresh and dried) against pathogenic bacteria. *Research Journal of Microbiology* 6, 376–384.
- Ahmer, B. M. M. and J. S. Gunn (2011): Interaction of *Salmonella* spp. with the intestinal microbiota. *Frontiers in Microbiology* 2, 1–9.
- Ali, H., Khan, E. and I. Ilahi (2019): Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry* 2019, 6730305.
- Anderson, K. E., Carroll, M. J., Sheehan, T., Lanan, M. C., Mott, B. M., Maes, P. and V. Corby-Harris (2014): Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. *Molecular Ecology* 23, 5904–5917.
- Arruda, V. A. S., Santos, A. V., Sampaio, D. F., Araújo, E. S., Peixoto, A. L. C., Estevinho, M. L. F. and L. B. Almeida-Muradian (2017): Microbiological quality and physicochemical characterization of Brazilian bee pollen. *Journal of Apicultural Research* 56, 231–238.
- Asama, T., Arima, T.-H., Gomi, T., Keishi, T., Tani, H., Kimura, Y., Tatefuji, T. and K. Hashimoto (2015): *Lactobacillus kunkeei* YB38 from honeybee products enhances IgA production in healthy adults. *Journal of Applied Microbiology* 119, 818–826.
- ATSDR. Agency of Toxic Substances and Disease Registry. Retrieved October 2019 from: <https://www.atsdr.cdc.gov>.
- Baker, B. A., Cassano, V. A. and C. Murray (2018): Arsenic exposure, assessment, toxicity, diagnosis, and management: guidance for occupational and environmental physicians. *Journal of Occupational and Environmental Medicine* 60, e634–e639.
- Belhadj, H., Bouamra, D., Dahamna, S., Harzallah, D., Ghabbane, M. and S. Khenouf (2012): Microbiological sanitary aspects of pollen. *Advances in Environmental Biology* 6, 1415–1420.
- Belhadj, H., Harzallah, D., Dahamna, S. and S. Khenouf (2014): Microbiological quality control of marketed pollen. *Der Pharmacia Lettre* 6, 37–42.
- Belina-Aldemita, M. D., Oppen, C., Schreiner, M. and S. D'Amico (2019): Nutritional composition of pot-pollen produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines. *Journal of Food Composition and Analysis* 82, 103215.
- Belina-Aldemita, M. D., Schreiner, M. and S. D'Amico (2020): Characterization of phenolic compounds and antioxidative potential of pot-pollen produced by stingless bees (*Tetragonula biroi* Friese) from the Philippines. *Journal of Food Biochemistry* 44, e13102.
- Bhattacharya, P. T., Misra, S. R., and M. Hussain (2016): Nutritional aspects of essential trace elements in oral health and disease: an extensive review. *Scientifica* 2016, 5464373.
- Biswas, S., and J.-M. Rolain (2013): Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *Journal of Microbiological Methods* 92, 14–24.
- Campos, M. G. R., Bogdanov, S., Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Frigerio, C. and F. Ferreira (2008): Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research and Bee World* 47, 156–163.
- Celandroni, F., Salvetti, S., Gueye, S. A., Mazzantini, D., Lupetti, A., Senesi, S. and E. Ghelardi (2016): Identification and pathogenic potential of clinical *Bacillus* and *Paenibacillus* isolates. *PloS One* 11, e0152831.
- Chitambar, C. R. (2010): Medical applications and toxicities of gallium compounds. *International Journal of Environmental Research and Public Health* 7, 2337–2361.
- Codex Alimentarius (2014): Guidelines for the simple evaluation of dietary exposure to food additives from: <http://www.fao.org/fao-who-codexalimentarius/codex-texts/guidelines/pt/>.
- Codex Alimentarius (2019): General standard for contaminants and toxins in food and feed from: <http://www.fao.org/fao-who-codexalimentarius>.
- Cong, B., Wang, N., Liu, S., Liu, F., Yin, X. and J. Shen (2017): Isolation, characterization and transcriptome analysis of a novel Antarctic *Aspergillus sydowii* strain MS-19 as a potential lignocellulosic enzyme source. *BMC Microbiology* 17, 129.
- Damodharan, K., Lee, Y. S., Palaniyandi, S. A., Yang, S. H. and J.-W. Suh (2015): Preliminary probiotic and

- technological characterization of *Pediococcus pentosaceus* strain KID7 and *in vivo* assessment of its cholesterol-lowering activity. *Frontiers in Microbiology* 6, 768.
- Das, K. K., Reddy, R. C., Bagoji, I. B., Das, S., Bagali, S., Mullur, L., Khodnapur, J. P. and M. S. Biradar (2018): Primary concept of nickel toxicity - an overview. *Journal of Basic and Clinical Physiology and Pharmacology* 30, 141–152.
- Dasen, G., Smutny, J., Teuber, M. and L. Meile (1998): Classification and identification of propionibacteria based on ribosomal RNA genes and PCR. *Systematic and Applied Microbiology* 21, 251–259.
- De-Melo, A. A. M., Estevinho, M. L. F. and L. B. Almeida-Muradian (2015): A diagnosis of the microbiological quality of dehydrated bee-pollen produced in Brazil. *Letters in Applied Microbiology* 61, 477–483.
- Desai, S. V. and M. C. Varadaraj (2013): Behavior of vegetative cells and spores of *Bacillus cereus* in two food systems. *Journal of Food Safety* 33, 291–301.
- Deutsche Gesellschaft für Hygiene und Mikrobiologie. Retrieved 10/24/2019 from: <https://www.dghm.org/>.
- Deveza, M. V., Keller, K. M., Lorenzon, M. C. A., Nunes, L. M. T., Sales, É. O. and O. M. Barth (2015): Mycotoxicological and palynological profiles of commercial brands of dried bee pollen. *Brazilian Journal of Microbiology* 46, 1171–1176.
- Ehling-Schulz, M., Lereclus, D. and T. M. Koehler (2019): The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. *Microbiology Spectrum* 7, GPP3-0032-2018.
- Elshagabee, F. M. F., Rokana, N., Gulhane, R. D., Sharma, C. and H. Panwar (2017): *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Frontiers in Microbiology* 8, 1490.
- Farjana, S. H., Huda, N. and M. P. Mahmud (2019): Life cycle assessment of cobalt extraction process. *Journal of Sustainable Mining* 18, 150–161.
- Feás, X., Vázquez-Tato, M. P., Estevinho, L., Seijas, J. A. and A. Iglesias (2012): Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules* 17, 8359–8377.
- Fonollá, J., Gracián, C., Maldonado-Lobón, J. A., Romero, C., Bédmar, A., Carrillo, J. C., Martín-Castro, C., Cabrera, A. L., García-Curiel, J. M., Rodríguez, C., Sanbonmatsu, S., Pérez-Ruiz, M., Navarro, J. M. and M. Olivares (2019): Effects of *Lactobacillus coryniformis* K8 CECT5711 on the immune response to influenza vaccination and the assessment of common respiratory symptoms in elderly subjects: a randomized controlled trial. *European Journal of Nutrition* 58, 83–90.
- Ghosh, S. K., Saha, R. and B. Saha (2015): Toxicity of inorganic vanadium compounds. *Research on Chemical Intermediates* 41, 4873–4897.
- González, G., Hinojo, M. J., Mateo, R., Medina, A. and M. Jiménez (2005): Occurrence of mycotoxin producing fungi in bee pollen. *International Journal of Food Microbiology* 105, 1–9.
- Health Canada (2010): Federal contaminated site risk assessment in Canada, Part II: Health Canada toxicological reference values (TRVs) and chemical-specific factors, version 2.0 from: www.healthcanada.gc.ca.
- Horsky, M., Irrgeher, J. and T. Prohaska (2016): Evaluation strategies and uncertainty calculation of isotope amount ratios measured by MC ICP-MS on the example of Sr. *Analytical and Bioanalytical Chemistry* 408, 351–367.
- Hudz, N., Yezerska, O., Grygorieva, O., Brindza, J., Felsőciová, S., Kačániová, M. and P. Wiczorek (2019): Analytical procedure elaboration of total flavonoid content determination and antimicrobial activity of bee bread extracts. *Acta Poloniae Pharmaceutica - Drug Research* 76, 439–452.
- Joo, M.-J., Jung, H., Kim, S.-h. and W. B. Yoon (2015): The antibacterial effect of flower pollen on *Escherichia coli* O157:H7 in ground pork. *Journal of Food Safety* 35, 355–361.
- Jouzani, G. S., Valijanian, E. and R. Sharafi (2017): *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Applied Microbiology and Biotechnology* 101, 2691–2711.
- Kačániová, M., Vukovic, N., Chlebo, R., Hascík, P., Rovná, K., Cubon, J., Dzugan, M. and A. Pasternakiewicz (2012): The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. *Archives of Biological Sciences* 64, 927–934.
- Kalaycıoğlu, Z., Kaygusuz, H., Döker, S., Kolaylı, S. and F.B. Erim (2017): Characterization of Turkish honeybee pollens by principal component analysis based on their individual organic acids, sugars, minerals, and antioxidant activities. *LWT* 84, 402–408.
- Kiš, M., Furmeg, S., Jaki Tkalec, V., Zadravec, M., Denžić Lugomer, M., Končurat, A., BeniĆ, M. and D. Pavliček (2018): Characterisation of Croatian honey by physicochemical and microbiological parameters with mold identification. *Journal of Food Safety* 38, e12492.

- Kostić, A. Ž., Pešić, M. B., Mosić, M. D., Dojčinović, B. P., Natić, M. M. and J. Đ. Trifković (2015). Mineral content of bee pollen from Serbia. *Arhiv za Higijenu Rada i Toksikologiju* 66, 251–258.
- Liu, D., Li, K., Hu, J., Wang, W., Liu, X. and Z. Gao (2019): Biocontrol and action mechanism of *Bacillus amyloliquefaciens* and *Bacillus subtilis* in soybean phytophthora blight. *International Journal of Molecular Sciences* 20, 2908.
- Liu, Y., Lai, Q., Dong, C., Sun, F., Wang, L., Li, G. and Z. Shao (2013): Phylogenetic diversity of the *Bacillus pumilus* group and the marine ecotype revealed by multilocus sequence analysis. *PloS One* 8, e80097.
- Londoño-Hernández, L., Ramírez-Toro, C., Ruiz, H. A., Ascacio-Valdés, J. A., Aguilar-Gonzalez, M. A., Rodríguez-Herrera, R. and C. N. Aguilar (2017): *Rhizopus oryzae* - Ancient microbial resource with importance in modern food industry. *International Journal of Food Microbiology* 257, 110–127.
- Lu, X., Zhou, D., Chen, X., Zhang, J., Huang, H. and L. Wei (2017): Isolation and characterization of *Bacillus altitudinis* JSCX-1 as a new potential biocontrol agent against *Phytophthora sojae* in soybean [*Glycine max* (L.) Merr.]. *Plant and Soil* 416, 53–66.
- Mathivanan, K., Rajaram, R. and V. Balasubramanian (2016): Biosorption of Cd(II) and Cu(II) ions using *Lysinibacillus fusiformis* KMNTT-10: equilibrium and kinetic studies. *Desalination and Water Treatment* 57, 22429–22440.
- Menezes, C., Vollet-Neto, A., Contrera, F. A. F. L., Venturieri, G. C. and V. L. Imperatriz-Fonseca (2013): The role of useful microorganisms to stingless bees and stingless beekeeping. In Vit, P., Pedro, S. R. M. and D. Roubik (Eds.): *Pot-honey: A legacy of stingless bees*. Springer, New York, NY, USA, pp. 153–171.
- Meussen, B. J., Graaff, L. H. de, Sanders, J. P. M. and R. A. Weusthuis (2012): Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals. *Applied Microbiology and Biotechnology* 94, 875–886.
- Morais, M., Moreira, L., Feás, X. and L. M. Estevinho (2011): Honeybee-collected pollen from five Portuguese natural parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology* 49, 1096–1101.
- Morgano, M. A., Teixeira Martins, M. C., Rabonato, L. C., Milani, R. F., Yotsuyanagi, K. and D. B. Rodriguez-Amaya (2010): Inorganic contaminants in bee pollen from Southeastern Brazil. *Journal of Agricultural and Food Chemistry* 58, 6876–6883.
- Nogueira, C., Iglesias, A., Feás, X. and L. M. Estevinho (2012): Commercial bee pollen with different geographical origins: a comprehensive approach. *International Journal of Molecular Sciences* 13, 11173–11187.
- Oliveira Alves, R. M., Sodré, G. S. and C. A. L. Carvalho (2018): Chemical, microbiological, and palynological composition of the “samburá” *Melipona scutellaris* pot-pollen. In Vit, P., Pedro, S. R. M. and D. W. Roubik (Eds.): *Pot-pollen in stingless bee melittology*. Springer International Publishing AG Cham, Switzerland, pp. 349–360.
- Pajor, M., Worobo, R. W., Milewski, S. and P. Szveda (2018): The antimicrobial potential of bacteria isolated from honey samples produced in the apiaries located in Pomeranian Voivodeship in Northern Poland. *International Journal of Environmental Research and Public Health*, 15.
- Patakova, P., Branska, B., Sedlar, K., Vasylykivska, M., Jureckova, K., Kolek, J., Koscova, P. and I. Provaznik (2019): Acidogenesis, solventogenesis, metabolic stress response and life cycle changes in *Clostridium beijerinckii* NRRL B-598 at the transcriptomic level. *Scientific Reports*, 9, 1371.
- Poddalgoda, D., Macey, K., Assad, H. and K. Krishnan (2017): Development of biomonitoring equivalents for barium in urine and plasma for interpreting human biomonitoring data. *Regulatory Toxicology and Pharmacology* 86, 303–311.
- Pramanik, P., Goswami, A. J., Ghosh, S. and C. Kalita (2019): An indigenous strain of potassium-solubilizing bacteria *Bacillus pseudomycoides* enhanced potassium uptake in tea plants by increasing potassium availability in the mica waste-treated soil of North-east India. *Journal of Applied Microbiology* 126, 215–222.
- Prazdnova, E. V., Mazanko, M. S., Chistyakov, V. A., Denisenko, Y. V., Makarenko, M. S., Usatov, A. V., Bren, A. B., Tutelyan, A. V., Komarova, Z. B., Gorlov, I. F., Weeks, R. and M. L. Chikindas (2019): Effect of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 on the productivity, reproductive aging, and physiological characteristics of hens and roosters. *Beneficial Microbes* 10, 395–412.
- RAIS (Risk Assessment Information System). Retrieved October 2019 from: <https://rais.ornl.gov/>.
- Rani, A., Kumar, A., Lal, A. and M. Pant (2014): Cellular mechanisms of cadmium-induced toxicity: a

- review. *International Journal of Environmental Health Research* 24, 378–399.
- Sannino, D., and E. R. Angert (2017): Genomic insights into the thiamin metabolism of *Paenibacillus thiaminolyticus* NRRL B-4156 and *P. apiarius* NRRL B-23460. *Standards in Genomic Sciences* 12, 59.
- Sattler, J. A. G., De-Melo, A. A. M., Nascimento, K. S. d., Melo, I. L. P., Mancini-Filho, J., Sattler, A. and L. B. Almeida-Muradian (2016): Essential minerals and inorganic contaminants (barium, cadmium, lithium, lead and vanadium) in dried bee pollen produced in Rio Grande do Sul State, Brazil. *Food Science and Technology* 36, 505–509.
- SCHEER (2017): SCHEER final opinion on tolerable intake of aluminium with regards to adapting the migration limits for aluminium in toys from: https://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scheer_consultation_04_en.
- Shafer, D., Liu, H., Dong, J., Liu, W., Loft, J., Phelps, T., and Y. Zhan (2017): Comparison of direct smear and chemical extraction methods for MALDI-TOF mass spectrometry identification of clinical relevant anaerobic bacteria. *Frontiers in Laboratory Medicine* 1, 27–30.
- Singhal, N., Kumar, M., Kanaujia, P. K. and J. S. Vird (2015): MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology*, 6, 791.
- Smedley, P. L. and D. G. Kinniburgh (2017): Molybdenum in natural waters: A review of occurrence, distributions and controls. *Applied Geochemistry* 84, 387–432.
- Staff, J. F., Cotton, R. J., Warren, N. D., and J. Morto (2014): Comparison of urinary thallium levels in non-occupationally exposed people and workers. *International Archives of Occupational and Environmental Health* 87, 275–284.
- Stahl, T., Falk, S., Rohrbeck, A., Georgii, S., Herzog, C., Wiegand, A., Hotz, S., Boschek, B., Zorn, H. and H. Brunn (2017): Migration of aluminum from food contact materials to food—a health risk for consumers? Part I of III: exposure to aluminum, release of aluminum, tolerable weekly intake (TWI), toxicological effects of aluminum, study design, and methods. *Environmental sciences Europe* 29, 19.
- Stelma, G. N. (2018): Use of bacterial spores in monitoring water quality and treatment. *Journal of Water and Health* 16, 491–500.
- US EPA. Integrated Risk Information System. Retrieved September 2019 from: <http://www.epa.gov/iris>.
- Vásquez, A. and T. C. Olofsson (2009): The lactic acid bacteria involved in the production of bee pollen and bee bread. *Journal of Apicultural Research* 48, 189–195.
- Villalobos, C. M. B., Preciado, G. L., Jaime, O. A. M. and J. J. T. Morales (2010). Mycoflora asociated to bee pollen collected by domesticated bees (*Apis mellifera* L.). *Revista Electrónica Nova Scientia* 2, 93–103.
- Waite, J. G., Jones, J. M. and A. E. Yousef (2009): Isolation and identification of spoilage microorganisms using food-based media combined with rDNA sequencing: ranch dressing as a model food. *Food Microbiology* 26, 235–239.
- Wang, T.-T., Ding, P., Chen, P., Xing, K., Bai, J.-L., Wan, W., Jiang, J.-H. and S. Qin (2017): Complete genome sequence of endophyte *Bacillus flexus* KLBMP 4941 reveals its plant growth promotion mechanism and genetic basis for salt tolerance. *Journal of Biotechnology* 260, 38–41.
- Wani, A. L., Ara, A., and Usmani, J. A. (2015). Lead toxicity: a review. *Interdisciplinary Toxicology* 8, 55–64.
- Wenzler, E., Kamboj, K. and J.-M. Balada-Llasat (2015): Severe sepsis secondary to persistent *Lysinibacillus sphaericus*, *Lysinibacillus fusiformis* and *Paenibacillus amylolyticus* bacteremia. *International Journal of Infectious Diseases* 35, 93–95.
- Weusthuis, R. A., Mars, A. E., Springer, J., Wolbert, E. J., van der Wal, H., Vrije, T. G. de, Levisson, M., Lepince, A., Houweling-Tan, G. B., Pha Moers, A., Hendriks, S. N., Mendes, O., Griekspoor, Y., Werten, M. W., Schaap, P. J., van der Oost, J. and G. Eggink (2017): *Monascus ruber* as cell factory for lactic acid production at low pH. *Metabolic Engineering* 42, 66–73.
- Xu, K., Yuan, Z., Rayner, S. and X. Hu (2015). Genome comparison provides molecular insights into the phylogeny of the reassigned new genus *Lysinibacillus*. *BMC Genomics* 16, 140.
- Yang, K., Wu, D., Ye, X., Liu, D., Chen, J. and P. Sun (2013): Characterization of chemical composition of bee pollen in China. *Journal of Agricultural and Food Chemistry* 61, 708–718.
- Zhang, J., Wang, Y., Shao, Z., Li, J., Zan, S., Zhou, S. and R. Yang (2019): Two selenium tolerant *Lysinibacillus* sp. strains are capable of reducing selenite to elemental Se efficiently under aerobic conditions. *Journal of Environmental Sciences (China)* 77, 238–249.