WALAILAK JOURNAL
http://wjst.wu.ac.th Agricultural Technology and Biological Sciences

Isolation and Identification of Bacteria in Flower Bee Pollen

Dinko DINKOV

Department of Hygiene and Technology of Animal Foodstuffs, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

(Corresponding author's e-mail: dinkodinkov@abv.bg)

Received: 22 July 2016, Revised: 5 July 2017, Accepted: 17 August 2017

Abstract

The main food safety requirements include accurately formulated criteria for the presence of specific microorganisms. The inconsistent literature data about the microbial species diversity in bee pollen during its collection, processing, and storage necessitate integral microbiological examinations. The purpose of this research work was to prove the algorithm for isolation and species level differentiation of bacteria from the *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Bacillaceae*, and *Micrococcaceae* families on fresh and dried flower bee pollen from Bulgaria.

Keywords: Bee pollen, differentiation, microorganisms, species

Introduction

Bee pollen is a valuable food collected by bees after they visit the flowers of plants. They gather it, adding specific enzymes to prepare the so-called pellets- small balls, or conglomerates of pollen balls, 2.5 to 3.5 mm in size [1]. They are brought to and stored in the cells of the beehive. Subsequently, bees process the pollen in the cells mixing it with nectar or honey and packing it with wax caps. Bee families use the pollen gathered in the hive as a main source of protein [2]. The pollen is collected for human consumption in the form of pellets before its storage in cells. When bee pass through the openings of pollen traps on the entrance of hives, the flower pollen stuck to their bodies falls on the ground and, after purification, sieving, dying, and packaging [3], dry (or desiccated) bee pollen is allowed for human consumption [4]. In recent years, consumers have turned their attention to natural products, with increasing numbers of enthusiasts favoring bee pollen collected from pollen traps and dried bee pollen.

In 2008, general criteria for bee pollen quality and safety were proposed [5]. In spite of this, detailed scientific investigation on the reliability of these criteria with respect to product quality and safety is not available. There are no certified specific methods for microbiological analysis of flower bee pollen consumed by humans. The literature data about microbial contamination of this product are also contradictory. Some researchers [6] reported a high extent of microbial contamination of bee pollen with bacteria from the family *Enterobacteriaceae*, and it is acknowledged that some members of this family are human pathogens. It should be emphasized that normative documents stipulating the national requirements for bee honey [1,4] neither write down specific microbiological criteria or requirements for microbiological methods for the examination of bee pollen.

The main food safety requirements include accurately formulated criteria for the presence of specific microorganisms [7]. The inconsistent literature data about microbial species diversity in bee pollen during its collection, processing, and storage necessitate integral microbiological examinations.

The purpose of this research work was to prove the algorithm for isolation and species level differentiation of bacteria on fresh and dried flower bee pollen from different regions of Bulgaria after primary processing, followed by one-year vacuum-packed cold storage at 0 - 4 °C and −18 °C.

Materials and methods

In June 2014, fresh and dried bee pollen samples were collected from 8 regions of Bulgaria (n = 32). The samples originated from beehives in industrial pollution-free areas, 3 km away from farmland with intensive crop production [8].

In order to determine the microbiological parameters after one year of storage, pollen samples were primary processed by sieving (fresh) or drying (dry or desiccated bee pollen), and vacuum-packed into polyethylene bags using a miniVac packaging machine (Vac-Star AG, Switzerland, available: http://www.vac-star.com/en/p1-miniVAC.html). Until the time of microbiological analyses (after one year), samples of dried bee pollen were cold stored (0 - 4 °C) [4], while fresh pollen samples were kept frozen at –18 °C [9].

Preparation of samples for microbiological analysis

Twenty-five g of bee pollen were diluted with 225 ml buffered peptone water (Merck, Darmshtadt, Germany), then homogenized for 10 min at 200 rpm in a Stomacher and left for 30 min at room temperature. From this dilution, serial dilutions were made to 10^{-4} in sterile physiological saline.

Isolation of microorganisms from the Bacillaceae family

By means of automated pipette and sterile pipette tips, aliquots of 100 µl from the initial and serial dilutions were spread onto plates with Plate Count Agar (PCA), (Merck, Darmstadt, Germany). Plates were incubated at 37 °C and, after 72 h, isolates with similar colony and microscopic features were selected from the grown colonies for further species differentiation of *Bacillaceae* family members.

Isolation of bacteria from the family Pseudomonadaceae

By means of automated pipette and sterile pipette tips, aliquots of 100 µl from the initial and serial dilutions were spread onto plates with Violet Red Bile Glucose agar (VRBG agar), (Merck, Darmstadt, Germany). Plates were incubated for 24 h at 37 °C, and then typical bacteria from the family Pseudomonadaceae were isolated from specific colonies grown on VRBD agar (Merck, Darmstadt, Germany).

Isolation of bacteria from the family Enterobacteriaceae

Aliquots of 100 µl from the initial and serial dilutions were inoculated on MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar, (Merck, Darmstadt, Germany), and plates were incubated at 37 °C for 24 h. The remaining amount from the initial dilution (1:10) was left for enrichment at 37 °C for 18 h. Then followed a secondary enrichment in 2 enrichment broths: selenite broth (37 °C, 24 h) and Rappaport-Vassiliadis medium (41 °C, 24 h) (Merck, Darmstadt, Germany). By the 42^{nd} hour, enrichment broths were inoculated on MacConkey agar and XLD agar (Merck, Darmstadt, Germany), and plates were incubated again at 37 °C for 24 h.

If lactose-negative or lactose-positive *Enterobacteriaceae* colonies were detected after the 24 h primary incubation or after the 66 h incubation from enrichment broths on MacConkey and XLD agar plates, isolates were further analyzed by Gram staining of microscopic preparations, followed by the principal protocol for initial laboratory differentiation of *Enterobacteriaceae* on Kligler iron agar, motility test medium, indole, and H_2S (Merck, Darmstadt, Germany), as well as *Salmonella* serums [10].

Isolation of bacteria from the Staphylococcaceae family

For identification of microorganisms from the family *Staphylococcaceae*, a preliminary enrichment of 1 ml of the initial dilution was made in 9 ml Triptic Soy Broth (TSB) supplemented with 7.5 % sodium chloride. The latter was added as it suppresses other bacteria and helps the isolation of staphylococci and especially of enterotoxin-producing *Staphylococcus (S.) aureus*, which is markedly resistant to 7.5 % NaCl [11]. For selective enrichment of staphylococci, a specific Giolotty and Cantoni Broth (GC) (Merck, Darmshtadt, Germany) were also used.

The enrichment broths incubated at 37 °C for 24 h were re-inoculated on Baird Parkar Agar (BPA), (Merck, Darmstadt, Germany) supplemented with 0.0025 % potassium telluride [12]. The plates were incubated at 37 °C and, after 24 - 48 h, typical dark-black staphylococcal colonies were observed. Subsequent investigations of isolates were done by Gram staining, determination of catalase and oxidase activity, and inoculation of BP agar sectors for single colony growth. After re-incubation (24 - 48 h, 37 °C), the obtained pure cultures were examined again by Gram staining, catalase and oxidase activity, presence of pigmentation after inoculation on ordinary agar, hemolytic activity on blood agar, and plasma coagulase activity on rabbit plasma.

The subsequent identification to the species level was done with 9 isolates from each region with similar colony and primary biochemical features, tentatively identified as members of the families *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, and *Bacillaceae*. They were stored until analysis at −18 °C in Eppendorf tubes with TSB (Merck, Darmstadt, Germany) supplemented with 20 % glycerol. Prior to identification of species, isolates were cultured on blood agar for growth of single colonies of pure cultures, followed by 24 h incubation at 37 °C. The species differentiation of obtained pure cultures was done through the identification system BioLog Gen III microplates (BioLog, Hayward, USA).

Identification of isolates through the system BioLog Gen III microplates with 96 wells (Biolog, Hayward, USA)

In brief, separate colonies from the grown isolates were taken with a special swab with a pointed tip, put into tubes containing inoculating fluid IF-A (Catalog No.72401, Biolog, Hayward, USA), and homogenized to obtain microbial suspension for GEN III plates. In each well of the GEN III plate, 100 µl microbial suspension was added, and plates were incubated at 33 °C for 24 h. Results were read visually by the change of color in the wells and compared to positive (10th well) and negative (1st well) controls. Data were interpreted with OmniLog software from the BioLog Gen III microplate system (Biolog, Hayward, USA).

Results and discussion

Table 1 presents the data about detected *Enterobacteriaceae* and *Pseudomonadaceae* species in bee pollen. The performed analyses showed most coliforms, and some other Enterobacteriaceae members which were not so far reported in this bee product. The interpretation of data should take into consideration the fact that, apart from coliforms, the *Enterobacteriaceae* family includes other bacteria, some of them (*Salmonella*, *E. coli*, etc.) pathogenic, as well as ubiquitous opportunistic or facultatively pathogenic microorganisms, which rarely cause disease in humans [13]. However, there are no data for contemporary evaluation of the risk from this presence, as well as about criteria for the admissibility of opportunistic or facultative pathogenic microorganisms in foods, with respect to consumer safety.

On the other hand, scientific studies indicate that some diseases in plants could be transmitted through pollen [14,15]. Environmental pollution, the activities of bees during pollination of plants, collection and transportation of pollen, and human activities during pollen collection from pollen traps and its primary processing (sieving, drying, packaging) are all important factors, as well as environmental factors- rain, dew, fog, or spray irrigation could also be involved in the contamination of pollen [16]. It is acknowledged that, prior to and during the bringing the pollen to the hive, bees moisture the pollen with nectar and place it in the baskets on their legs, which make the product susceptible to additional microbial contamination.

In all of the studied regions, microorganisms from the *Pantoea* spp. were present in both dried and fresh bee pollen. In the latter, *Pantoea* (*P.*) *agglomerans* was detected in the Vratsa and Sliven regions. *P. agglomerans* bgp 6 was established only in dried and fresh bee pollen samples collected from the Shoumen regions, as well as in fresh product from Strandzha.

It is acknowledged that *P. agglomerans*, which was prevalent in bee pollen according to our studies (**Table 1**), was used in agriculture as a biological antagonist of fungal diseases in plants [17]. Some authors believe that *P. agglomerans* was detected in bees and in bee products in hives after the visit of

bees on plants [18]. *P. agglomerans* has been also isolated from various plants in the Black Sea region [19]. Recently, some researchers have classified *P. agglomerans* as opportunistic pathogens, which are dangerous mainly for immunocompromised subjects. The bacterium was detected in patients with arthritis [20], as well as occasionally as a causative agent of septicemia in newborns [21]. It was found that Pantoea spp. rarely causes disease in healthy people [13]. *P. agglomerans* is not included in the recommendations for European microbiological criteria for bee pollen [5]. Based on our results, we suggest the future examination of bee pollen for contamination with *P. agglomerans* in our geographical regions as well, which could justify the inclusion of this microorganism in microbiological requirements of the product.

The additional investigations of antibiotic sensitivity of 5 strains of *P. agglomerans* and *P. agglomerans* bgp 6, isolated from dried and fresh bee pollen in 4 surveyed regions (Shoumen, Strandzha, Sliven, and Karlovo), with regard to their sensitivity to antibiotics from the main groups of antibacterial drugs used in human medicine, concluded that there was a minor risk for transfer of antibiotic resistance from bee pollen contaminated with *P. agglomerans* [10].

Table 1 Incidence rate* of contamination with microorganisms from Enterobacteriaceae and Pseudomonadaceae families on flower bee pollen from different regions of Bulgaria (n = 32).

Isolated species	Dried bee pollen (n = 19)		Fresh bee pollen (n = 13)	
	No. of samples contaminated	Incidence rate (%)	No. of samples contaminated	Incidence rate (%)
	Pantoea species			
Pantoea agglomerans	13 (All regions except for Shoumen)	68.4	3 (Vratsa and Sliven)	23
Pantoea agglomerans bgp 6	6 (Shoumen)	31.6	10 (Shoumen and Strandzha)	77
	*Citrobacte*r species			
Citrobacter freundii	9 (Lovetch, Shoumen, St. Zagora)	47	-	-
	Proteus species			
Proteus vulgaris	1 (Sliven)	5.3	10 (Shoumen and Strandzha)	77
Proteus mirabilis	6 (Shoumen)	31.6	6 (Shoumen)	46
	Serratia species			
Serratia odorifera	3 (V. Tarnovo)	15.8	-	-
Serratia liquefaciens/grimesii	-	-	4 (Strandzha)	31
	E. coli			
E. coli	-	-	8 (Vratsa and Shoumen)	62
	Salmonella			
Salmonella	-	-	-	-
	Pseudomonas species			
Flavimonas oryzihabitans	-	-	2 (Vratsa)	15

*The incidence rate was calculated as number of positive samples over the total number of samples dried / fresh bee pollen, all multiplied by 100.

So far, there are no data about reporting the occurrence of *Citrobacter freundii* in flower bee pollen, which was detected in dried bee pollen samples from the regions of Lovech, Shoumen, and Stara Zagora (**Table 1**). It should be noted that bacteria from genera *Citrobacter* and *Pantoea* spp. do not pose a risk for healthy people, and are frequently encountered in the environment. However they are also placed in the opportunistic species group, causing neonatal meningitis and abscesses in humans [22].

The dried bee pollen from the Sliven region was shown to contain *Proteus (Prot.) vulgaris*. This microbial species was also found out in fresh pollen samples from the Strandzha and Shoumen regions. *Prot. mirabilis* was present only in both types of bee pollen from Shoumen (**Table 1**).

Proteus vulgaris is another opportunistic or facultative pathogenic microorganism, causing disease in subjects with immunodeficiency states, kidney fibrosis, or HIV [23]. It has been demonstrated that, when predisposing factors were present, *Prot. vulgaris* could induce urinary tract, skin, and wound infections [24]. There is evidence that *Prot. mirabilis* has been more commonly found in the intestinal content of diarrheic subjects than in healthy humans, which could be attributed to its role as a human intestinal pathogen [25]. The interpretation of our results should take into consideration the not entirely elucidated role of *Prot. mirabilis* as a human pathogen, which could be dangerous after bee pollen consumption, as well as its absence in recommendations for microbiological criteria for bee pollen [5]. Finally, yet importantly, *Prot. mirabilis* was detected only in fresh and dried pollen samples from a single region (Shoumen, **Table 1**).

Bacteria from the genus *Serratia* were demonstrated only in bee pollen from 2 regions. The dried pollen samples from Veliko Tarnovo contained *Serratia odorifera*, and fresh pollen from Strandzha contained *Serratia liquefaciens/grimesii*. The microorganism *Serratia liquefaciens/grimesii* (**Table 1**) is also classified as a potential human pathogen, and is encountered in several plants [24]. The available literature sources provide no data about the involvement of Serratia odorifera, detected in the dried bee pollen from Veliko Tarnovo, in human diseases.

E. coli were detected in fresh bee pollen from the Vratsa and Shoumen regions (**Table 1**). Furthermore, the organism was not detected after drying and one-year storage in vacuum package of bee pollen from Shoumen. It follows that drying as a primary step of the primary processing of floral bee pollen with regard to inhibition of *E. coli* replication is recommended. This microorganism was not found in dried pollen samples, in line with recommendations stipulating its absence in dried pollen intended for human consumption [5].

Bacteria from the *Pseudomonadaceae* family was found only in fresh bee pollen from the Vratsa region (**Table 1**). *Flavimonas oryzihabitans* was initially detected in rice, hence its name [26]. So far, there is no information about the occurrence of this bacterium in bee pollen. *Pseudomonas* spp., which are also from the group of opportunistic bacteria, could cause mainly skin and wound infections [24]. Some authors reported *Flavimonas oryzihabitans* as an agent of postoperative septicemia infections in newborn babies [27] and of peritonitis secondary to peritoneal dialysis [28].

Future research should investigate the possible relationship between skin infections occurring from the collection of fresh bee pollen from pollen traps contaminated with opportunistic bacteria from the family *Enterobacteriaceae* (**Table 1**). There is therefore a need for observation of a higher level of precautions, not only during processing, but also by using disposable gloves when working with pollen traps and during the primary processing of the product.

Table 2 depicts the results from detected *Staphylococcaceae* microbial species. Our examinations did not establish coagulase-positive staphylococci, acknowledged as human pathogens. The dried bee pollen from all of the studied regions, except for Strandzha, contained *Staphylococcus (S.). hominis* (subspecies *hominis*). This organism was present only in fresh pollen samples from the Sliven region.

S. hominis (subspecies *hominis*) is a member of the resident microflora of human skin, occasionally causing infections in immunocompromised people [29]. Gram-positive cocci, and especially *S. epidermidis*, are encountered in bees and bee pollen [30]. *S. epidermidis* as a part of normal skin microflora rarely causes disease, except in immunosuppressed patients [31].

The wide spread of *S. hominis* (subspecies *hominis*) in dried bee pollen after primary processing, proved in our studies, suggests a possible secondary contamination with this bacterium during sieving and drying. The opposite relationship was found in *S. epidermidis*. It was detected in most of the surveyed

regions, but was present in dried pollen samples only from the Strandzha region (**Table 2**). The absence of *S. epidermidis* could be attributed to the mechanical removal of the agent with the secondary contaminants of pollen during sieving.

Table 2 Incidence rate of contamination with microorganisms from Staphylococcaceae family on flower bee pollen from different regions of Bulgaria (n = 32).

Dried bee pollen (n = 19)			**Fresh bee pollen (n = 13)**		
Regions / Isolated species	**No. of samples contaminated**	**Incidence rate (%)**	**Regions / Isolated species**	**No. of samples contaminated**	**Incidence rate (%)**
Shoumen / *S. hominis* (subspecies *hominis*)	6	32	Shoumen / *S. epidermidis*	6	46
Strandzha / *S. epidermidis*	4	21	Strandzha / *S. epidermidis*	4	31
Sliven / *S. hominis* (subspecies *hominis*)	1	5	Sliven / *S. hominis* (subspecies *hominis*)	1	8
Stara Zagora / *S. hominis* (subspecies *hominis*)	2	10.5	Vratsa / *S. epidermidis*	2	15
Karlovo / *S. hominis* (subspecies *hominis*)	2	10.5	-	-	-
V.Tarnovo / *S. hominis* (subspecies *hominis*)	3	16	-	-	-
Lovech / *S. hominis* (subspecies *hominis*)	1	5	-	-	-
Total *S. hominis* (subspecies *hominis*)	15	79	Total *S. hominis* (subspecies *hominis*)	1	8
Total *S. epidermidis*	4	21	Total *S. epidermidis*	12	92

The dried and fresh pollen samples were most commonly contaminated with *Bacillus pumilus* (**Table 3**). While *B. subtilis* was not found in dried pollen samples, it was detected in fresh product from 2 of the studied regions, Shoumen and Vratsa.

The second most prevalent species in fresh bee pollen after *B. pumilus* was *B. subtilis*, which was not found in any of the dried samples. In our studies, the microbial species *B. amyloliquefaciens* in dried bee pollen samples (from the Stara Zagora, Karlovo, and Lovech regions), and *Arthrobacter globiformis* in the fresh pollen from Vratsa and Shoumen, were demonstrated for the first time (**Table 3**).

Table 3 Incidence rate of contamination with microorganisms from *Bacillaceae* and *Micrococcaceae* families on flower bee pollen from different regions of Bulgaria (n = 32).

Dried bee pollen (n = 19)			**Fresh bee pollen (n = 13)**		
Regions/ Isolated species	**No. of samples contaminated**	**Incidence rate (%)**	**Regions/ Isolated species**	**No. of samples contaminated**	**Incidence rate (%)**
Shoumen / *B. pumilus* *Arthrobacter globiformis*	6	32	Shoumen / *B. pumilus* *B. subtilis* *Arthrobacter globiformis*	6	46
Strandzha / *B. pumilus*	4	21	Strandzha / *B. pumilus*	4	31
Sliven / *B. pumilus*	1	5	Sliven / *B. pumilus*	1	8
Stara Zagora / *B. pumilus* *B. amyloliquefaciens*	2	10.5	Vratsa / *Arthrobacter globiformis* *B. subtilis*	2	15
Karlovo/ *B. pumilus* *B. amyloliquefaciens*	2	10.5	-	-	-
V. Tarnovo / *B. pumilus*	3	16	-	-	-
Lovech / *B. pumilus* *B. amyloliquefaciens*	1	5	-	-	-
Total					
B. pumilus	19	100	*B. pumilus*	11	85
B. amyloliquefaciens	3	16	*B. subtilis*	8	62
Arthrobacter globiformis	6	32	*Arthrobacter globiformis*	8	62

Bacillus spp. were isolated from 59 % of samples stored in cells of honeycombs (bee bread) and from only 18 % of samples collected from bees outside the cells. *B. megaterium* was the most commonly encountered species. *B. circulans* and *B. alvei* were detected only in pollen from honeycomb cells, but not in stored food [32].

Some of the isolates of the family *Bacillaceae* detected during our studies were identified as *B. subtilis* (**Table 3**), determined by other researchers as a common species in both collected pollen and pollen stored in comb cells. Other representatives of this family, isolated from bee pollen, were *B. megaterium*, *B. licheniformis*, *B. pumilus*, and *B. circulans* [33].

It has been demonstrated that some *B. cereus* and *B. pumilus* strains could produce enterotoxins and, therefore, could be considered dangerous in cold stored foods due to their psychrotrophic nature and their potential for growth at temperatures ≤ 6 °C [34]. It should be also noted that, from the bacilli acknowledged as human pathogens, some references determine *B. cereus* as surely pathogenic. Allowances of up to 50 CFU/g of this bacterium in powdered milk intended for children until 6 months of age are already regulated [7]. This *Bacillaceae* member was detected in none of the regions surveyed during our study (**Table 3**).

The predominant member of the family *Bacillaceae* in our studies was *B. pumilus* (**Table 3**). This bacterium is psychrotrophic, able to replicate at low temperatures, at which the product was usually stored in our experiments. The less frequent detection of *B. pumilus* in fresh pollen could be attributed to its storage in a frozen state (−18 °C).

B. subtilis is used for plant disease control [35]. In our studies, the share of *B. subtilis* among *Bacillaceae* isolates from fresh bee pollen was considerable (**Table 3**). It should be emphasized that *B. subtilis* was not encountered in dried bee pollen. This could be due to sieving, which removes the

particles carrying additional *B. subtilis* contamination from the environment. Based on our results from the absence of *B. subtilis* in dried pollen samples (**Table 3**), we can hypothesize that sieving, proposed as an important element of the primary processing of pollen [3], has minimized the chance of contamination.

B. amyloliquefaciens (**Table 3**) is also associated with its occurrence on plants. It stimulates plant growth, and is used for the control of bacterial and fungal plant diseases. This is why some authors consider the microorganism as an alternative for plant disease control [36].

It has been shown that *B. pumilus* and *B. subtilis* are the main representatives of the family *Bacillaceae*, encountered in spices [37]. *B. pumilus* was also encountered in cold-stored flours [38]. The pathogenic potential of this bacterium, and the possibility for production and accumulation of endotoxins posing risk to people, is still unclear. In this regard, it should be noted that *Bacillus* spp. does not replicate at Aw < 0.92 [39]. To prevent the development of *B. cereus*, the storage of foods at < 4 °C is recommended, as at these temperatures the spores of *B. cereus* cannot develop into vegetative forms and, hence, accumulate toxins [39].

Soil microorganisms from *Arthrobacter* spp. are found in bees and wax moths [30]. Some authors have used *Arthrobacter globiformis* for testing the antibacterial peptides in the hemolymphs of bees for evaluation of their immunity levels [40,41]. In our studies, *Arthrobacter globiformis* was detected in pollen samples from the Vratsa and Shoumen regions (**Table 3**).

Conclusions

In the study, methods for differentiation of bacterial microflora in bee pollen from the *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Bacillaceae*, and *Micrococcaceae* families were presented, followed by identification of isolates through the system BioLog Gen III microplates (Biolog, Hayward, USA). Microbial quality in dried flower bee pollen from different regions of Bulgaria after one-year vacuum-packed cold storage (0 - 4 °C) was in compliance with the European microbiological quality guidelines for other foods [7].

Acknowledgements

The author wishes to express his most sincere gratitude to beekeepers from Bulgaria for sending bee pollen samples.

References

[1] Central Cooperative Union. Industry Standard (IS) 2567111-91. Bee Pollen. 1991, p. 1-7.
[2] S Bogdanov. Pollen: Production, Nutrition and Health: A Review. Available at: http://www.bee-hexagon.net/files/file/fileE/Health/PollenBook2Review.pdf, accessed July 2015.
[3] D Stratev, R Balkanska, DD St Mateev and D Dinkov. Processing, storage, labeling and microbiological hazards of organic bee pollen production. *Sci. Tech*. 2014; **4**, 21-7.
[4] Ordinance №9 of 22 June 2005 on the Ministry of Agriculture and Forest, Issued by the Ministry of Agriculture and Forestry, Official Gazette 54/1.07.2005 (BG). Available at: http://econ.bg/l.l_i.129712_at.5.html, accessed July 2016.
[5] MGR Campos, S Bogdanov, LB Almeida-Muradian, T Szczesna, Y Mancebo, C Frigerio and F Ferreira. Pollen composition and standardization of analytical methods. *J. Agr. Res. Bee World* 2008; **47**, 156-63.
[6] T Shevtsova, M Kacaniova, K Garkava, J Brindza and J Petrova. Contamination of *Betula verrucosa Ehrh*. pollen by microorganisms, mycotoxins and heavy metals. *J. Microbiol. Biotech. Food Sci*. 2014; **3**, 509-13.
[7] Regulation 1441. Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Offic. J. Eur. Union* 2007; **322**, 12-29.
[8] S Bogdanov. Contaminants of bee products: Review article. *Apidologie* 2006; **37**, 1-18.

[9] D Dominguez-Valhondo, DB Gil, MT Hernandez and D Gonzalez-Gomez. Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. *Int. J. Food. Sci. Tech*. 2011; **46**, 2204-11.
[10] D Dinkov. Differentiation and antibiotic susceptibility of *Pantoea agglomerans* isolated from flower bee pollen. *East. Acad. J.* 2016; **1**, 99-108.
[11] GFE Koch. Electivnahrboden fur Staphylokokken. *Zentr. Bakteriol. Parasitenk*. *Abt. I Orig*. 1942; **149**, 122-4.
[12] D Sawhney. The toxicity of potassium tellurite to *Staphylococcus aureus* in rabbit plasma fibrinogen agar. *J. Appl. Bacteriol*. 1986; **61**, 149-55.
[13] WEJ Sanders and CC Sanders. *Enterobacter* spp.: Pathogens poised to flourish at the turn of the Century. *Clin. Microbiol. Rev*. 1997; **10**, 220-41.
[14] S Card, M Pearson and GR Clover. Plant pathogens transmitted by pollen. *Aust. Plant Path* 2007; **36**, 455-61.
[15] J Flores, I Gutiérrez and R Espejo. The role of pollen in chalkbrood disease in Apis mellifera: transmission and predisposing conditions. *Mycologia* 2005; **97**, 1171-6.
[16] M Lacey and J West. A Manual for Catching and Identifying Airborne Biological Particles. Available at: http://www.dbbe.fcen.uba.ar/contenido/objetos/aerobiologia.pdf, accessed July 2016.
[17] C Nunes, J Usall, N Teixidó and I Viñas. Biological control of postharvest pear diseases using a bacterium *Pantoea agglomerans* (CPA-2). *Int. J. Food Microbiol*. 2001; **70**, 53-61.
[18] I Loncaric, H Heigl, E Licek, R Moosbeckhofer, HJ Busse and R Rosengarten. Typing of *Pantoea agglomerans* isolated from colonies of honey bees (Apis mellifera) and culturability of selected strains from honey. *Apidologie* 2009; **40**, 40-54.
[19] M Mudryk. Plant-isolated *Pantoea agglomerans*-new look into potential pathogenicity. *Mikrobiol. Z*. 2012; **74**, 53-7.
[20] A Kratz, D Greenberg, Y Barki, E Cohen and M Lifshitz. *Pantoea agglomerans* as a cause of septic arthritis after palm tree thorn injury: Case report and literature review. *Arch. Dis. Child*. 2003; **88**, 542-4.
[21] KA Bergman, JP Arends and EH Schölvinck. *Pantoea agglomerans* septicemia in three newborn infants. *Pediatr. Infect. Dis. J*. 2007, **26**, 453-4.
[22] A Joaquin, S Khan, N Russel and N Fayez. Neonatal meningitis and bilateral cerebellar abscesses due to *Citrobacter freundii*. *Pediatr. Neurosurg*. 1991; **17**, 23-4.
[23] G Steinkamp, B Wiedemann, E Rietschel, A Krahl, J Giehlen, H Barmeier and F Ratjen. Prospective evaluation of emerging bacteria in cystic fibrosis. *J. Cyst. Fibros*. 2005; **4**, 41-8.
[24] G Berg, L Eberl and A Hartmann. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ. Microbiol*. 2005; **7**, 1673-85.
[25] HE Müller. Occurrence and pathogenic role of Morganella-Proteus-Providencia group bacteria in human feces. *J. Clin. Microbiol*. 1986; **23**, 404-5.
[26] K Kodama, K Kimura and K Komagata. Two new species of *Pseudomonas*: *P. oryzihabitans* isolated from a rice paddy, and *P. luteola* isolated from clinical specimens. *Int. J. Syst. Bacteriol*. 1985; **35**, 467-74.
[27] J Freney, W Hansen, J Etienne, F Vandenesch and J Fleurette. Postoperative infant septicemia caused by *Pseudomonas luteola* (CDC Group Ve-l) and *Pseudomonas oryzihabitans* (CDC Group Ve-2). *J. Clin. Microbiol.* 1988; 2**6**, 1241-3.
[28] JWA Bending, PJ Mayes, DE Eyers, B Holmes and TTL Chin. Flavimonas oryzihabitans (Pseudomonas oryzihabitans, CDC Group Ve-2): An emerging pathogen in peritonitis related to continuous ambulatory peritoneal dialysis. *J. Clin. Microbiol*. 1989; **27**, 217-8.
[29] ICV Palazzo, PA Azevedo, C Secchi and ACC Pignatari. *Staphylococcus hominis* stubs. *novobiosepticus* strains causing nosocomial bloodstream infection in Brazil. *J. Antimicrob. Chemoth.* 2008; **62**, 1222-6.
[30] M Gilliam and BJ Lorenz. Gram-positive cocci from apiarian sources. *J. Invertebr. Pathol*. 1983; **42**, 187-95.

[31] W Levinson. Review of Medical Microbiology and Immunology. 11th ed. McGraw-Hill, New York, 2010, p. 94-9.
[32] M Gilliam, DW Roubik and BJ Lorenz. Microorganisms associated with pollen, honey and brood provisions in the nest of a stingless bee, *Melipona fasciata*. *Apidologie* 1990; **21**, 89-97.
[33] M Gilliam. Microbiology of pollen and bee bread: The genus *Bacillus*. *Apidoligie* 1979; **10**, 269-74.
[34] B Ray and A Bhunia. Fundamental Food Microbiology, Opportunistic Bacterial Pathogens, Molds and Mycotoxins, Viruses, Parasites and Fish and Shellfish Toxins. 5th ed. CRC Press Taylor & Francis, 2014, p. 1-390.
[35] EE Idris, H Bochow, H Ross and R Borriss. Nutzung von *Bacillus subtilis* als Mittel für den biologischen Pflanzenschutz. VI. Phytohormonartige Wirkung von Kulturfiltraten von pflanzenwachstumsfördernden Bacillus amyloliquefaciens FZB24, FZB42, FZB45 und *Bacillus subtilis* FZB37. *Z. Pflanzenk. Pflanzen*. 2004; **111**, 583-97.
[36] R Borriss, XH Chen, C Rueckert, J Blom, A Becker, B Baumgarth, B Fan, R Pukall, P Schumann, C Sproer, H Junge, J Vater, A Pühler and HP Klenk. Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7T and FZB42T: A proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. *Int. J. Syst. Evol. Microbiol.* 2011; **61**, 1786-801.
[37] LJ Muhamad, H Ito, H Watanabe and N Tamura. Distribution of Microorganisms in Spices and Their Decontamination by Gamma-irradiation. *Agr. Biol. Chem*. 1986; **50**, 347-55.
[38] RF Rogers. *Bacillus* isolates from refrigerated doughs, wheat flour, and wheat. *Cereal. Chem*. 1978; **55**, 671-4.
[39] European Food Safety Authority (EFSA). Opinion of the Scientific Panel on biological hazards (BIOHAZ) on Bacillus cereus and other *Bacillus* spp. in foodstuffs. *Eur. Food Saf. Authority J*. 2005; **3**, 175.
[40] P Korner and P Schmid-Hempel. *In vivo* dynamics of an immune response in the bumble bee *Bombus terrestris*. *J. Invertebr. Pathol*. 2004; **87**, 59-66.
[41] BM Sadd and P Schmid-Hempel. Principles of ecological immunology. *Evol. Appl*. 2009; **2**, 113-21.