Microorganisms in Vacuum Stored Flower Bee Pollen

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Contamination with sanitary microorganisms from *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Micrococcaceae* and *Bacillaceae* families in flower bee pollen from Bulgaria after one-year vacuum-packed cold storage has been found. Dried flower bee pollens intended for human consumption were with high incidence rate of contamination with *Pantoea* sp. (*P. agglomerans* and *P. agglomerans bgp6*) (100%), *Citrobacter freundii* (47%), *Proteus mirabilis* (31.6%), *Serratia odorifera* (15.8%) and *Proteus vulgaris* (5.3%). Bee pollens were also positive for the culture of microorganisms from *Staphylococcaceae*, *Micrococcaceae* and *Bacillaceae* families: *Staphylococcus hominis* subsp hominis, *Staphylococcus epidermidis*, *Arthrobacter globiformis*, *Bacillus pumilis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*. It was concluded that, if consumed directly, the vacuum-packed cold stored dried bee pollen, harvested according hygienic requirements from bee hives in industrial pollution-free areas without intensive crop production, is not problem for healthy human.

Key Words: Bee pollen, *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Micrococcaceae*, *Bacillaceae*

INTRODUCTION

Bee pollen is a valuable food collected by bees after they visit the flowers of plants. They gather it and by adding specific enzymes prepare the pellets - small balls or con-glomerates of pollen balls 2.5 to 3.5 mm of size (1). In beehive, bees process the pollen in the cells by 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). Under the popular name of perga (from Russian) or beebread, the pollen stored in cells mixed with honey or wax is not regulated for human consumption in European Community (EC) countries. People obtain the pollen under the form of pellets before its storage in cells. When bees 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, it is allowed for human consumption (Ordinance No9 of 22 June 2005 on the Ministry of Agriculture and Forest, Issued by the Ministry of Agriculture and Forestry). During the last few years, consumers turned their attention to natural products, with increasing number of enthusiasts favoring the products from pollen traps and dried bee pollen.

In 2008, general criteria for the bee pollen's quality and safety were proposed (3). However, so far, there are no detailed scientific investigations on the reliability of these criteria with respect to the quality and safety of the bee products. There are also no certified specific methods for microbiological analysis of flower bee pollen consumed by people. The literature data about microbiological contamination of the bee pollen presented this product as a potentially hazardous medicinal plant product, as it is exposed for contamination by pathogenic bacteria. Some researchers reported a high extent of microbial contamination of bee pollen with bacteria from the family *Enterobacteriaceae*, and it is acknowledged that some bacteria of the family are human pathogens (4, 5). It should be emphasized that normative documents stipulating the national requirements to bee honey do not indicate specific microbiological criteria or requirements for microbiological methods of examination of bee pollen.

The food safety requirements comprise accurately formulated criteria for the presence of sanitary 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 work was to present the summarized data about the microbial species from the *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Micrococcaceae* and *Bacillaceae* families isolated from vacuum stored flower bee pollens from 8 regions of Bulgaria (country from southeastern Europe in north-eastern part of the Balkan Peninsula).

MATERIALS AND METHODS

Samples and Sample preparation

In June 2014, fresh and dried bee pollen samples were collected according to the hygienic requirements from eight regions of Bulgaria: Strandzha, Sliven, Stara Zagora, Shoumen, Lovech, Vratsa, Veliko Tarnovo and Karlovo. The samples are originated from beehives in industrial pollution-free areas, 3 km away from farmland with intensive crop production (6).

In order to determine the microbiological parameters after one-year storage, pollen samples were vacuum-packed into polyethylene bags using a miniVac packing machine (VacStar AG, Switzerland). Immediately before vacuum-packing, the water activity (a_w) of samples was determined by automated analyzer HygroLab (Rotronic AG, Switzerland). Measurements were run in duplicate, and the result was retained after reaching a stable a_w reading. Data were presented as mean values (Table 1). Until the time of microbiological analyses, samples of dried bee pollen were cold stored (0~4℃), while fresh pollen samples were kept at -18℃ (7).

For microbiological analysis, 25 g of bee pollen was diluted with 225 ml of 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

For the isolation of microorganisms from the family *Bacillaceae*, aliquots of 100 µl from the initial and serial dilutions were spread onto Plate Count Agar (PCA) plates (Merck). Plates were then incubated aerobically at 37℃ for 72 h, and isolates with similar colony and microscopic features were selected from for further species differentiation of *Bacillaceae* family members.

For the determination of *Enterobacteriacea*e microbial counts and isolation of bacteria from the family *Pseudomonadaceae*, aliquots of 100 µl from the initial and serial dilutions were spread onto Crystal-violet neutral-red bile D(+)Glucose (VRBD) agar (Merck). Plates were incubated for 24 h at 37℃ and then, typical coliform colonies (those of dark-red color and diameter ≥ 0.5 mm) were counted. Experiments were performed twice, and data were presented as mean values (Table 1).

For the isolation of bacteria from the family *Enterobacteriaceae*, aliquots of 100 µl from the initial and serial dilutions were inoculated onto MacConkey agar and Xylose Lysine Deoxy cholate (XLD) agar plates (Merck), and plates were incubated at 37℃ for 24 h. The remaining amount from the initial dilution (10^{-1}) was left for enrichment at 37℃ for 18 h, followed by a secondary enrichment in two

enrichment broths: selenite broth (37℃, 24 h) and Rappaport-Vassiliadis medium (41℃, 24 h) (Merck). By the 42nd hour, enrichment broths were inoculated onto MacConkey agar and XLD agar (Merck), and plates were incubated again at 37℃ for 24 h. Both lactose-negative and lactose-positive *Enterobacteriaceae* colonies were further analyzed by Gram stain followed by the principal protocol for initial laboratory differentiation of *Enterobacteriaceae* on Kligler iron agar, motility test medium, indole and H_2S production (Merck), as well as with group and monospecific test reagents Anti-Salmonella (Sifin Service GmbH, Berlin, Germany) (8).

For identification of microorganisms from the family *Staphylococcaceae*, a preliminary enrichment of 1 ml of the initial dilution was made in 9 ml Tryptic Soy Broth (TSB) supplemented with 7.5% sodium chloride (NaCl). NaCl was added as it suppresses other bacteria and helps the isolation of staphylococci, especially of enterotoxin-producing *Staphylococcus aureus* (*S. aureus*), which is markedly resistant to 7.5% NaCl (9). For selective enrichment of staphylococci, a specific Giolotty and Cantoni Broth (GC) (Merck) was also used. The enrichment broths incubated at 37℃ for 24 h were re-inoculated on Baird Parkar Agar (BPA) plates (Merck) supplemented with 0.0025% potassium telluride (10). The plates were then incubated at 37℃ for 24~48 h, and typical dark-black staphylococcal colonies were observed. After re-incubation on BPA (24~48 h, 37℃), obtained pure cultures were examined by Gram stain, catalase and oxidase activity, presence of pigmentation after inoculation on ordinary agar, hemolytic activity on blood agar, and plasma coagulase activity on rabbit plasma.

Identification of the isolates to the species level

The subsequent identification to the species level was done with 9 isolates from each region with similar colony appearance and primary biochemical features, which are tentatively identified as the members of families *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae* and *Bacillaceae*. Isolates were stored at -18℃ in TSB (Merck) supplemented with 20% glycerol until analysis. Prior to identification, isolates were cultured on blood agar at 37℃ for 24 h to obtain single colonies of pure cultures.

Species identification was done through BioLog Gen III

Table 1. Water activity (a_w) and total count of microorganisms from the family *Enterobacteriaceae* on flower bee pollen from different regions of Bulgaria (n=32)

Regions	Dried bee pollen		Regions	Fresh bee pollen	
	Water activity (a_w/℃)	*Enterobacteriaceae* (CFU/g)		Water activity (a_w/℃)	*Enterobacteriaceae* (CFU/g)
Lovetch (n=1)	0.388/23.8℃	7.5×10^2	Vratsa (n=2)	0.715/20.8℃	1.32×10^4
Strandzha (n=4)	0.450/21.9℃	8.5×10^3	Strandzha (n=4)	0.718/22.2℃	5×10^4
Shoumen (n=6)	0.183/20.2℃	3.6×10^3	Shoumen (n=6)	0.725/20.6℃	1.4×10^4
Sliven (n=1)	0.309/23.2℃	1.5×10^3	Sliven (n=1)	0.722/23.4℃	3.7×10^4
V. Tarnovo (n=3)	0.298/22.3℃	1.4×10^3		–	
St. Zagora (n=2)	0.234/22.9℃	Not detected[*]		–	
Karlovo (n=2)	0.403/25℃	Not detected[*]		–	

[*]Not detected: no microorganisms from the family Enterobacteriaceae were present following direct inoculation of 100 µl of the initial dilution on VRBD agar

microplate identification system (BioLog, Hayward, USA). In brief, single colony was taken with a special swab with pointed tip, put into tubes containing IF-A medium and homogenized to obtain microbial suspension for GEN III plates. In each well of the GEN III plate, 100 μl of microbial suspension was added and plates were then incubated at 33℃ 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 Omni-Log software of BioLog Gen III microplate system.

RESULTS

Table 1 presents the mean values of a_w and bacterial counts of total *Enterobacteriaceae*. Dried bee pollen samples had aw between 0.183 and 0.450, and the highest a_w was

Table 2. 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 reg. Shoumen)	68.4	3 (Vratsa and Sliven)	23.0
Pantoea agglomerans bgp 6	6 (Shoumen)	31.6	10 (Shoumen and Strandzha)	76.9
Citrobacter species				
Citrobacter freundii	9 (Lovetch, Shoumen, St. Zagora)	47.3	–	–
Proteus species				
Proteus vulgaris	1 (Sliven)	5.3	10 (Shoumen and Strandzha)	76.9
Proteus mirabilis	6 (Shoumen)	31.6	6 (Shoumen)	46.1
Serratia species				
Serratia odorifera	3 (V. Tarnovo)	15.8	–	–
Serratia liquefaciens/grimesii	–	–	4 (Strandzha)	30.7
Escherichia species				
Escherichia coli	–	–	8 (Vratsa and Shoumen)	61.5
Pseudomonas species				
Flavimonas oryzihabitans	–	–	2 (Vratsa)	15.3

*The incidence rate was calculated as % rate of number of positive samples over the total number of samples.

established from dried bee pollen from the Strandzha region (0.450/21.9℃) followed by Lovech (0.388/23.8℃), Sliven (0.309/23.2℃), Veliko Tarnovo (0.298/22.3℃), and Shoumen (0.183/20.2℃) regions. The a_w values of fresh pollen samples were 0.715 to 0.725.

Fresh pollens showed higher microbial counts (1.32 × 10^4 ~ 5 × 10^4 CFU/g) than dried pollen samples (7.5 × 10^2 ~ 8.5 × 10^3 CFU/g). The highest *Enterobacteriaceae* bacterial counts were detected in dried bee pollen from Strandzha (8.5 × 10^3 CFU/g), followed by Shoumen (3.6 × 10^3 CFU/g), Sliven (1.5 × 10^3 CFU/g), Veliko Tarnovo (1.4 × 10^3 CFU/g) and Lovech (7.5×10^2 CFU/g) regions. Microorganisms from the family *Enterobacteriaceae* were not detected in samples from Stara Zagora and Karlovo regions. Fresh pollen samples from Strandzha region also showed highest count of *Enterobacteriaceae* bacteria (5 × 10^4 CFU/g), followed by Sliven (3.7 × 10^4 CFU/g), Shoumen (1.4 × 10^4 CFU/g), and Vratsa (1.32 × 10^4 CFU /g) regions.

Table 2 presents the isolated species and incidence rate of *Enterobacteriaceae* and *Pseudomonadaceae* species in bee pollen. Most of isolated species in these families were not so far reported in this bee product. In all studied regions, microorganisms from the *Pantoea* (*P.*) sp. were present in both dried and fresh bee pollens. In fresh bee pollens, *P. agglomerans* was detected in Vratsa and Sliven regions. *P. agglomerans bgp 6* was found only in dried and fresh bee pollen samples collected form the Shoumen region, as well as in fresh product from Strandzha region. *Citrobacter freundii* was found in dried pollen samples from Lovech, Shoumen and Stara Zagora regions.

The dried bee pollen from Sliven region was shown to contain *Proteus* (*P.*) *vulgaris*. This microbial species was also found in fresh pollen samples from Strandzha and Shoumen regions. *P. mirabilis* was present only in both dried and fresh bee pollens from Shoumen region. Bacteria from

Table 3. 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* subsp hominis	6	31.5	Shoumen / *S. epidermidis*	6	46.1
Strandzha / *S. epidermidis*	4	21.0	Strandzha / *S. epidermidis*	4	30.7
Sliven / *S. hominis* subsp hominis	1	5.2	Sliven / *S. hominis* subsp hominis	1	7.6
Stara Zagora / *S. hominis* subsp hominis	2	10.5	Vratsa / *S. epidermidis*	2	15.3
Karlovo / *S. hominis* subsp hominis	2	10.5	–	–	–
V.Tarnovo / *S. Hominis* subsp hominis	3	15.7	–	–	–
Lovech / *S. hominis* subsp hominis	1	5.2	–	–	–
Total *S. hominis* subsp hominis	15	78.9	Total *S. hominis* subsp hominis	1	7.6
Total *S. epidermidis*	4	21.0	Total *S. epidermidis*	12	92.3

the genus *Serratia* were isolated only in bee pollens from Veliko Tarnovo and Strandzha regions. Dried pollen samples from Veliko Tarnovo contained *Serratia* (*S.*) *odorifera*, and fresh pollens from Strandzha contained *S. liquefaciens/grimesii*.

Escherichia coli (*E. coli*) were found in the fresh pollen samples from two regions, Vratsa and Shoumen. Only fresh bee pollens from the Vratsa region were positive for the culture of bacteria in the *Pseudomonadaceae* family, and *Flavimonas oryzihabitans* was detected.

Table 3 depicts the results from the identification of bacteria in the family *Staphylococcaceae*. Coagulase-positive staphylococcus, acknowledged as human pathogen, has not been detected in this study. The dried bee pollens from all studied regions except Strandzha contained *S. hominis* subsp hominis. Fresh pollen sample from only Sliven region was positive for the culture of *S. hominis* subsp hominis.

The dried and fresh pollen samples were most commonly contaminated with *Bacillus* (*B.*) *pumilis* (Table 4). While *B. subtilis* was not found in any dried pollen samples, it was detected in fresh product from two of studied regions, Shoumen and Vratsa. In this study, the presence of microbial species *B. amyloliquefaciens* in dried bee pollen samples from Stara Zagora, Karlovo, Lovech regions, and *Arthrobacter globiformis* in fresh pollen samples from Vratsa and Shoumen regions was demonstrated for the first time.

DISCUSSION

It is acknowledged that higher value of a_w is beneficial for the growth of microorganisms. This is an important factor guaranteeing food safety during their production and

Table 4. 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. pumilis*; *Arthrobacter globiformis*	6	31.5	Shoumen / *B. pumilis* *B. subtilis*, *Arthrobacter globiformis*	6	46.1
Strandzha / *B. pumilis*	4	21.0	Strandzha / *B. pumilis*	4	30.7
Sliven / *B. pumilis*	1	5.2	Sliven / *B. pumilis*	1	7.6
Stara Zagora / *B. pumilis* *B. amyloliquefaciens*	2	10.5	Vratsa / *Arthrobacter globiformis* *B. subtilis*	2	15.3
Karlovo / *B. pumilis* *B. amyloliquefaciens*	2	10.5	–	–	–
V. tarnovo / *B. pumilis*	3	15.7	–	–	–
Lovech / *B. pumilis* *B. amyloliquefaciens*	1	5.2	–	–	–
Total					
B. pumilis	19	100	*B. pumilis*	11	84.6
B. amyloliquefaciens	3	15.7	*B. subtilis*	8	61.5
Arthrobacter lobiformis	6	31.5	*Arthrobacter globiformis*	8	61.5

subsequent storage (11). The high value of a_w is proved to induce the growth of microorganisms (12), including pathogenic bacteria extensively replicate at a_w values > 0.85, while molds could develop at a_w values > 0.6 (11). In this study, a_w values substantially lower than 0.85 were detected in dried bee pollen, which suggests that when drying is properly done, there should be no preconditions for growth of microbial pathogens in this product (Table 1). This finding is further confirmed by the highest total microbial *Enterobacteriaceae* counts in dried pollen samples from Strandzha with highest values of a_w (0.450/21.9℃) (Table 1).

The high value of a_w is a precondition for the development of some microorganisms in stored bee pollen (12). It has been reported that the relatively low a_w in some samples with higher number of *Enterobacteriaceae* count could be attributed to the secondary contamination of pollen between the moment of its harvesting from bees, its transportation to the hive and the subsequent primary processing (13, 14).

The family *Enterobacteriaceae* comprises about 20 genera including coliforms, as well as some other foodborne microorganisms proven to be pathogenic for example the members of *Salmonella*, *Shigella* and *Yersinia* genera (15). According to reported projects for international standards for bee pollen, no more than 100 *Enterobacteriaceae* CFU/g is recommended in this product (3). The interpretation of these data should take into consideration of the fact that apart from coliforms, the *Enterobacteriaceae* family includes also other bacteria, some of them (*Salmonella*, or *E. coli,* etc.) are pathogenic, as well as ubiquitous opportunistic microorganisms, which rarely cause disease in humans (16). Yet, there are no data for contemporary evaluation of the risk from the presence as well as about criteria for the admissibility of opportunistic 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 the pollen (17, 18). Other environmental factors - rain, dew, fog, spray irrigation, could be also involved in the contamination of pollen (19). It is acknowledged that prior to and during bringing the pollen to the hive, bees moisture the pollen with nectar and place it in the baskets on their legs, which makes the product susceptible to additional microbial contamination.

It is acknowledged that *P. agglomerans* which was prevalent in bee pollen according to our studies (Table 2), was used in agriculture as a biological antagonist of fungal diseases in plants (20). Some authors believe that *P. agglomerans* was detected in bees and in bee products in hives after the visit of bees on plants (21). *P. agglomerans* has been also isolated from various plants in the Black Sea region (22). Recently, some researchers classify *P. agglomerans* to opportunistic pathogens, which are dangerous mainly for immunocompromised subjects. The bacterium was detected in patients with arthritis (23), as well as occasionally as a causative agent of septicaemia in newborns (24). It was found out that *Pantoea* sp. rarely causes disease in healthy people (16). *P. agglomerans* is not included in the recommendations for European microbiological criteria to bee pollen (3).

The additional investigations of antibiotic sensitivity of five strains *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: β-lactams (amoxicillin + clavulanic acid: 20/10 μg), aminoglycosides (gentamycin), amphenicols (chloramphenicol), tetracyclines (doxycycline), quinolones (enrofloxacin) and cephalosporins (cephalotin) suggests that there was a minor risk for transfer of antibiotic resistance through *P. agglomerans* in bee pollen (8).

On the basis of our results, we suggest the future examination of bee pollen for contamination with *P. agglomerans* in our geographical regions, which could justify the inclusion of this microorganism in microbiological requirements to the product.

So far, there is no data about reporting the occurrence of *Citrobacter freundii* in bee pollen, which was detected in dried bee pollen samples from the region of Lovech, Shoumen and Stara Zagora (Table 2). It should be noted that bacteria from genera *Citrobacter* do not pose a risk for healthy people and are frequently encountered in the environments. They are also placed in the opportunistic species group, causing neonatal meningitis and abscesses in men

(25).

P. vulgaris is another opportunistic microorganism, causing disease in peoples with immunodeficiency states. It is demonstrated that when predisposing factors are present, *P. vulgaris* could induce urinary tract, skin and wound infections (26). There is evidence that *P. mirabilis* has been more common in the intestinal content of diarrheic subjects than in healthy men, which could be attributed to its role as a human intestinal pathogen (27). The interpretation of our results should take consideration of the not entirely elucidated role of *P. mirabilis* as a human pathogen, which could be dangerous after bee pollen consumption, as well as its absence in recommendations for microbiological criteria to bee pollen (3). Last but not least, it should be outlined that *Proteus mirabilis* was detected only in fresh and dried pollen samples from a single region (Shoumen, Table 2).

The microorganism *Serratia liquefaciens/grimesii* (Table 2) is also classified as a potential human pathogen and is encountered on several plants (26). The available literature sources provide no data about the involvement of *Serratia odorifera*, detected in the dried bee pollen in human diseases (Veliko Tarnovo, Table 2).

E. coli was detected in fresh bee pollen from the Vratsa and Shoumen regions (Table 2). Furthermore, the organism was not detected after drying and one-year storage in vacuum package of bee pollen from Shoumen. It allows recommending the drying as a primary step of the primary processing of floral bee pollen with regard to inhibition of *E. coli* replication. This microorganism was not found in dried pollen samples, in line with recommendations stipulating its absence in dried pollen intended for human consumption (3).

Flavimonas oryzihabitans, found out in fresh bee pollen from the Vratsa region (Table 2) was initially detected in rice, hence its name (28). So far, there is no information about the occurrence of this bacterium in bee pollen. *Pseudomonas* sp., which is also from the group of opportunistic bacteria, could cause mainly skin and wound infections (26). Some authors reported *Flavimonas oryzihabitans* as an agent of postoperative septicemic infections in newborn babies (29) and of peritonitis secondary to peritoneal dialysis (30).

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 2). There is therefore a need for observation of a higher level of precautions not only during processing, but also using disposable gloves when working with pollen traps and during the primary processing of the product.

S. hominis subsp hominis is a member of the resident microflora of human skin, occasionally causing infections in immunocompromised people (31). Gram-positive cocci and especially *S. epidermidis* are encountered in bees and bee pollen (32). It was found that *S. epidermidis* as a part of normal skin microflora rarely causes disease, except for immunosuppressed patients (33).

The wide spread of *S. hominis ss* hominis in dried bee pollen proved in our studies after its being primarily processed (Table 3), suggest a possible secondary contamination with this bacterium during sieving and drying. The opposite relationship was found out in *S. epidermidis*. It has been detected in most of surveyed regions, but was present in dried pollen samples only from the Strandzha region (Table 3). The absence of *S. epidermidis* could be attributed to the mechanical removal of the agent with the secondary contaminants of pollen during the sieving.

Bacillus sp. was 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* is the most commonly encountered species. *B. circulans* and *B. alvei* were detected only in pollen from honeycomb cells, but not in stored food (34).

Some of isolates of family *Bacillaceae* detected during our studies were identified as *B. subtilis* (Table 4), 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, are *B. megaterium*, *B. licheniformis*, *B. pumilus* and *B. circulans* (13).

It is demonstrated that some strains of *B. cereus* and *B. pumilis* are enterotoxin producers and therefore could be considered dangerous in cold stored foods due to their psychotropic nature and potential of growth at temperatures

≤ 6℃ (35). 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 (36). This *Bacillaceae* member was detected in none of regions surveyed during our study (Table 4).

B. subtilis is used for plant disease control (37). In our studies, the share of *B. subtilis* among *Bacillaceae* isolates from fresh bee pollen was considerable (Table 4). 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. On the basis of results from the absence of *B. subtilis* in dried pollen samples (Table 4), we could hypothesize that sieving, proposed as an important element of the primary processing of pollen has minimized the chance for contamination.

The predominant member of family *Bacillaceae* in our studies was *B. pumilis* (Table 4). This bacterium is psychotropic, able to replicate at low temperatures at which the product was usually stored in our experiments. The less frequent detection of *B. pumilis* in fresh pollen could be attributed to its storage in a frozen state (-18℃).

B. amyloliquefaciens (Table 4) was also associated with its occurrence on plants. Some authors consider the microorganism as an alternative for plant disease control (38).

It is shown that *B. pumilis* and *B. subtilis* are the main representatives of the family *Bacillaceae*, encountered in spices (39). *B. pumilis* was also encountered in cold stored flours (40). The pathogenic potential of this bacterium and the possibility for production and accumulation of endotoxin posing risk for people is still unclear. With this regard it should be noted that *Bacillus* sp. do not replicated at $a_w <$ 0.92 (41).

It should be outlined that dried pollen samples in our study exhibited aw between 0.183 and 0.450, whereas fresh pollen samples: from 0.715 to 0.725 (Table 1). Therefore, the one-year cold storage of dried or frozen storage of fresh pollen did not create prerequisites for *Bacillus* sp. replication.

Soil microorganisms from *Arthrobacter* sp. are found out in bees and wax moths (32). Some authors use *Arthrobacter globiformis* for testing the antibacterial peptides in the hemolymph of bees for evaluation of their immunity level (42, 43). In our studies, *Arthrobacter globiformis* was detected in pollen samples from Vratsa and Shoumen regions (Table 4).

One aspect that could be improved in future legislation for bee pollen is some more details on how the pollen is consumed (3). If, for example, it is incorporated into a food at temperatures that will support the growth of some of the microflora, then there could be a potential health problem.

The data on vacuum-packed dried flower bee pollen from eight different Bulgarian's regions did not establish microorganisms that could cause enteric diseases in humans (36). To prevent the development of *B. cereus*, the storage of dried bee pollen at < 4℃ is recommended as at these temperatures the spores of *B. cereus* could not develop into vegetative forms and hence, accumulate toxin (41). In the future it is recommended the future investigations for pathogenic potential of *B. pumilis* in stored bee pollen.

From our results for dry bee pollen we could conclude that, if consumed directly, the low numbers of non-enteric microorganisms in this product may not be problem for healthy human.

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