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Review

Bacterial Contamination of Flower Bee Pollen Production

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Abstract

The literature shows contradictory results regarding the prevalence of microorganisms, as well as the contamination of flower bee pollen. Here is a summary of the species-level differentiation of *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae*, *Micrococcaceae* and *Bacillaceae* isolates obtained from investigations on fresh and dried flower bee pollen from different regions of Bulgaria.
***Key words**:* bee pollen, contamination, microorganisms

Резюме

Проучванията в научната литература за преобладаващите микроорганизми и контаминацията на цветния пчелен прашец показват противоречиви резултати. Представено е обобщение за видовото диференциране на изолати от сем. *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae* и *Bacillaceae* при изследвания на неизсушен и изсушен цветен пчелен прашец от различни региони на България.

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 of size (Central Cooperative Union, 1991). They are brought and stored in the cells of the beehive. Subsequently, bees additionally 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 (Bogdanov, 2014). Under the popular name of 'perga' (from Russian) or bee bread, the pollen stored in cells, mixed with honey or wax, is not regulated for human consumption in the EC countries. Pollen is obtained under the form of pellets before its storage in cells. When bees pass through the openings of the pollen traps at the entrance of hives, the flower pollen stuck to their bodies falls on the ground and after purification, sieving, drying and packaging (Stratev *et al*., 2014) is allowed for human consumption (Ordinance No9, 2005). During the last years, consumers have turned their attention to natural products, with an increasing number of enthusiasts favouring bee pollen collected from pollen traps and dried.

At a global scale, general criteria for bee pollen quality and safety were proposed in 2008 (Campos *et al*., 2008). So far, there has been no detailed scientific investigation on the reliability of these criteria with respect to the quality and safety of this bee product. 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 (Shevtsova *et al*., 2014) reported a high level of microbial contamination of bee pollen with bacteria from the family *Enterobacteriaceae* and it is acknowledged that some members of the family are human pathogens. It should be emphasised that the statutory documents stipulating the national requirements for bee honey (Central Cooperative Union, 1991; Ordinance No9, 2005) do not indicate any specific microbiological criteria and requirements for microbiological methods of examination of bee pollen.

The main food safety requirements comprise accurately formulated criteria for the presence of

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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 present the summarised data about the microbial species from the families *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae* and *Bacillaceae* isolated from dried and fresh bee pollen from 8 regions of Bulgaria, stored either frozen or in refrigerated conditions.

Materials and Methods

In June 2014, fresh and dried bee pollen samples were collected from eight regions of the country: Strandzha, Sliven, Stara Zagora, Shoumen, Lovech, Vratsa, Veliko Tarnovo and Karlovo. The samples originated from bee hives in industrial pollution-free areas, 3 km away from farmland with intensive crop production (Bogdanov, 2006).

In order to determine the microbiological parameters after one-year storage, pollen samples were vacuum-packed into polyethylene bags using a miniVac packaging machine (Vac-Star AG, Switzerland, available at: http://www.vac-star.com/en/p1-miniVAC.html).

Immediately before vacuum-packaging, the water activity of samples (Aw) was determined by automated analyser HygroLab (Rotronic AG, Switzerland). Measurements were run in duplicate and the result was retained after reaching a constant Aw value.

Until the time of microbiological analyses, samples of dried bee pollen were cold-stored (0-4ºC), (Ordinance No9, 2005), while fresh pollen samples were kept frozen at –18 °C (Dominguez-Valhondo *et al*., 2011).

Preparation of samples for microbiological analysis

Twenty-five g of bee pollen were diluted with 225 ml of buffered peptone water (Merck, Darmshtadt, Germany), then homogenised 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 an 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 hours, isolates with similar colony and microscopic features were selected from the grown colonies for further species differentiation of *Bacillaceae* family members.

Determination of Enterobacteriaceae microbial counts and isolation of bacteria from the family Pseudomonadaceae

By means of an 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 hours at 37 °C and then typical coliform colonies (those of dark-red colour and diameter ≥ 0.5 mm) were counted. The 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 XLD (Xylose Lysine Deoxycholate) agar (Merck, Darmstadt, Germany), and plates were incubated at 37°C for 24 h. The remaining amount of the initial dilution (1:10) was left for enrichment at 37°C for 18 h. Then followed a secondary enrichment in two enrichment broths: selenite broth (37°C, 24 h) and Rappaport-Vassiliadis medium (41°C, 24 h), (Merck, Darmstadt, Germany). By the 42nd 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 analysed 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* (Sifin Service GmbH, Berliner Allee 317-321, Berlin, Germany, http://sifin.de/) (Dinkov, 2016).

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 TSB supplemented with 7.5% sodium chloride. The latter was added as it suppresses other bacteria, helps the isolation of staphylococci and especially of enterotoxin-producing *S. aureus*, which is mark-

edly resistant to 7.5% NaCl (Koch, 1942). For selective enrichment of staphylococci, a specific Giolotty and Cantoni Broth (GC) (Merck, Darmshtadt, Germany) was also used.

The enrichment broths incubated at 37ºC for 24 h were re-inoculated on Baird Parkar Agar (BPA), (Merck, Darmstadt, Germany) supplemented with 0.0025% potassium tellurite (Sawhney, 1986). The plates were incubated at 37°C and after 24-48 h, the typical dark-black staphylococcal colonies were observed. The subsequent investigation of isolates was done by Gram staining, determination of catalase and oxidase activity, 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, haemolytic 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 -18ºC in Eppendorf tubes with TSB (Tryprone Soy Broth), (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-hour 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 (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 IF-A medium and homogenised 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 colour in the wells and compared to positive (10^{th} well) and negative (1^{st} well) controls. Data were interpreted with OmniLog software of BioLog Gen III microplate system (Biolog, Hayward, USA).

Results

Table 1 presents the results from water activity and total *Enterobacteriaceae* bacterial counts determination. The highest water activity was established for dried bee pollen from the Strandzha (0.450/21.9ºC) and Lovech (0.388/23.8ºC) regions, followed by Sliven (0.309/23.2ºC), Veliko Tarnovo (0.298/22.3ºC) and Shoumen (0.183/20.2ºC) regions. Dried bee pollen samples had water activity between 0.183 and 0.450, and fresh pollen samples – within 0.715 and 0.725 (Table 1).

The highest *Enterobacteriaceae* bacterial counts were established 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 established in the

Table 1. Water activity and total count of microorganisms from *Enterobacteriaceae* family in flower bee pollen from different regions of Bulgaria

Regions	Dried bee pollen		Regions	Fresh bee pollen	
	Water activity (Aw / °C)	Enteroba-cteriaceae (CFU/g)		Water activity (Aw / °C)	Enteroba-cteriaceae (CFU/g)
Lovech	0.388/23.8ºC	7.5×10^2	Vratsa	0.715/20.8ºC	1.32×10^4
Strandzha	0.450/21.9ºC	8.5×10^3	Strandzha	0.718/22.2ºC	5×10^4
Shoumen	0.183/20.2 ºC	3.6×10^3	Shoumen	0.725/20.6ºC	1.4×10^4
Sliven	0.309/23.2 ºC	1.5×10^3	Sliven	0.722/23.4 ºC	3.7×10^4
V. Tarnovo	0.298/22.3 ºC	1.4×10^3	-		
St. Zagora	0.234/22.9ºC	not detected *	-		
Karlovo	0.403 /25ºC	not detected *	-		

* not detected - *no microorganisms from the family Enterobacteriaceae were present following direct inoculation of 100 mL of the initial dilution on VRBD agar*

Table 2. Microorganisms from *Enterobacteriaceae and Pseudomonadaceae* families in fresh and dried flower bee pollen from different regions of Bulgaria.

Isolated species	Dried bee pollen	Fresh bee pollen
	Regions	Regions
Pantoea species		
Pantoea agglomerans	All regions without Shoumen	Vratsa and Sliven
Pantoea agglomerans bgp 6	Shoumen	Shoumen and Strandzha
Citrobacter species		
Citrobacter freundii	Lovech, Shoumen, St. Zagora	-
Proteus species		
Proteus vulgaris	Sliven	Shoumen and Strandzha
Proteus mirabilis	Shoumen	Shoumen
Serratia species		
Serratia odorifera	V. Tarnovo	-
Serratia liquefaciens/ grimesii	-	Strandzha
E. coli species		
E.coli	-	Vratsa and Shoumen
Pseudomonas species		
Flavimonas oryzihabitans	-	Vratsa

samples from Stara Zagora and Karlovo regions. The highest total microbial counts for *Enterobacteriaceae* in fresh pollen samples were found again in the samples from Strandzha (5×10^4 CFU/g), 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 data about *Enterobacteriaceae* and *Pseudomonadaceae* species detected in bee pollen. The analyses have shown mostly coliforms and some other *Enterobacteriaceae* members, which have not been so far reported in this bee product.

In all studied regions, microorganisms from the *Pantoea* spp. were present in both dried and fresh bee pollen. In the latter, *Pantoea agglomerans* was detected in Vratsa and Sliven regions. *Pantoea agglomerans bgp 6* was established only in dried and fresh bee pollen samples collected form the Shoumen regions, as well as in fresh product from Strandzha. *Citrobacter freundii* was found in dried pollen samples from Lovech, Shoumen and Stara Zagora regions (Table 2).

Dried bee pollen from the Sliven region was shown to contain *P. vulgaris*. This microbial species was also found in fresh pollen samples from Strandzha and Shoumen regions. *P. mirabilis* was present only in both types of bee pollen from Shoumen.

Bacteria from the genus *Serratia* were demonstrated only in bee pollen from two of the regions. Dried pollen samples from Veliko Tarnovo contained *Serratia odorifera*, and fresh pollen from Strandzha – *Serratia liquefaciens/grimesii*.

The fresh pollen samples from two of the surveyed regions (Vratsa and Shoumen) were positive for *E.coli*. Bacteria of the *Pseudomonadaceae* family were only found in fresh bee pollen from the Vratsa region - contamination with *Flavimonas oryzihabitans* was detected (Table 2).

Table 3 gives the results about detected *Staphylococcaceae* microbial species. Our examinations did not establish coagulase-positive staphylococci, acknowledged as human pathogens. Dried bee pollen from all studied regions except for Strandzha contained *S. hominis ss hominis*. This organism was present only in fresh pollen samples from the Sliven region.

Dried and fresh pollen samples were most

Table 3. Microorganisms from *Staphylococcaceae* family in fresh and dried flower bee pollen from different regions of Bulgaria

Dried bee pollen		Fresh bee pollen	
Staphylococcaceae			
Regions	Microorganisms	Regions	Microorganisms
Shoumen	*S. hominis ss hominis*	Shoumen	*S. epidermidis*
Strandzha	*S. epidermidis*	Strandzha	*S. epidermidis*
Sliven	*S. hominis ss hominis*	Sliven	*S. hominis ss hominis*
Stara Zagora	*S. hominis ss hominis*	Vratsa	*S. epidermidis*
Karlovo	*S. hominis ss hominis*	-	
V.Tarnovo	*S. hominis ss hominis*	-	
Lovech	*S. hominis ss hominis*	-	

Table 4. Microorganisms from *Bacillaceae* and *Micrococcaceae* families in fresh and dried flower bee pollen from different regions of Bulgaria

Dried bee pollen		Fresh bee pollen	
Fam. Bacillaceae			
Regions	Microorganisms	Regions	Microorganisms
Shoumen	*B. pumilis* *Arthrobacter globiformis*	Shoumen	*B.subtilis* *B. pumilis* *A. globiformis*
Strandzha	*B. pumilis*	Strandzha	*B. pumilis*
Sliven	*B. pumilis*	Sliven	*B. pumilis*
Stara Zagora	*B. amyloliquefaciens* *B. pumilis*	Vratsa	*B.subtilis* *A. globiformis*
Karlovo	*B. amyloliquefaciens* *B. pumilis*	-	
V.Tarnovo	*B. pumilis*	-	
Lovech	*B. pumilis* *B. amyloliquefaciens*	-	

commonly contaminated with *B. pumilis* (Table 4). While *B. subtilis* was not found in dried pollen samples, it was detected in fresh product from two of the studied regions – Shoumen and Vratsa.

The second most prevalent species in fresh bee pollen after *B. pumilis* 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 (Stara Zagora, Karlovo, Lovech regions) and *A. globiformis* in the fresh pollen from Vratsa and Shoumen were demonstrated for the first time (Table 4).

Discussion

It is acknowledged that higher water activity values are beneficial for the development of microorganisms. This is an important factor guaranteeing food safety during production and subsequent storage (Rahman, 2010). The high A_w is proved to induce the development of microorganisms (Mathlouthi, 2001), including pathogenic bacteria which replicate extensively at water activity values > 0.85, while molds could develop at water activity values >0.6 (Rahman, 2010).

The water activity values substantially lower than 0.85 detected in dried bee pollen in our studies suggest that when drying was properly done, there should be no preconditions for development of microbial pathogens in this product (Table 1). This is confirmed by the established highest levels of water activity (0.450/21.9ºC), corresponding to the highest total microbial *Enterobacteriaceae* counts in dried pollen samples from Strandzha (8.5×10^3CFU/g). Higher *Enterobacteriaceae* microbial counts proportionally to higher water activity levels (0.718/22.2ºC) were found again in fresh pollen samples from the Strandzha region (5×10^4 CFU/g) (Table 1).

At the same time it should be noted that the water activity in fresh pollen from all regions was >0.6 (Table 1), which according to Rahman (2010) is a precondiion for development of some microorganisms in stored bee pollen. The relatively low water activity in some samples, in which detected *Enterobacteriaceae* counts were higher (Table 1), 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 (Gilliam, 1979; Serra and Escola, 1997).

Coliforms are Gram-negative, oxidase-negative, non-spore forming, aerobe and facultative anaerobe rods. Although not taxonomically distinct, the group is functionally defined as lactose-fermenting, gas and acid producing bacteria at 35ºC. The group includes, apart from the genus *Escherichia* (*E. coli* in particular*),* the genera *Citrobacter, Enterobacter* and *Klebsiella*. Some authors place in this group *Serratia* and *Hafnia*. Coliforms are normal residents of the intestinal tract of animals and men, and are also encountered in the environment, soil and water. About 1% of coliforms, mainly *E. coli*, are detected in animal and human gastrointestinal tract. 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 (Tortorello, 2003).

The data about the lack of bacteria from the family *Enterobacteriaceae* in dried bee pollen indicate the essential role of production hygiene and timely drying as the main factors impeding the development of enterobacteria (Table 1).

According to reported projects for international standards for bee pollen, no more than 100 *Enterobacteriaceae* CFU/g are recommended in this product (Campos *et al*., 2008). Compared to these recommendations, samples of vacuum packed dried bee pollen cold-stored for 1 year exhibited values from 7.5×10^2 – 8.5×10^3 CFU/g (Table 1). The interpretation of these data should take into consideration the fact that apart from coliforms, the *Enterobacteriaceae* family also 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 (Sanders and Sanders, 1997). Yet, there are no data for contemporary evaluation of the risk from the presence as well as about criteria for the admissibility of opportunistic or facultatively 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 (Card *et al.,* 2007; Flores *et al.,* 2005). Envionrmental pollution, the activities of bees during pollinaiton of plants, collection and transportation of pollen, human activities during pollen collection from pollen traps and its primary processing (sieving, drying, packaging) are all important factors for contamination of pollen, as well as the air at the site where plants grow (Gilliam, 1979; Serra and Escola, 1997). Other environmental factors – rain, dew, fog, spray irrigation, could be also involved in the contamination of pollen (Lacey and West, 2007). It is acknowledged that prior to and during bringing the pollen into the hive, bees moisten the pollen with nectar and place it in the baskets on their legs, which makes the product susceptible to additional microbial contamination.

Our results for increased counts of *Enterobacteriaceae* in fresh bee pollen (1.32×10^4 - 5×10^4 CFU/g) point at the primary role of secondary contamination with these bacteria, which could occur following their presence in the environment, on plants, bees, and during harvesting of pollen from pollen traps or during its sieving or drying. The data indicating lower enterobacterial counts in dried pollen ($7.5\text{x}10^2$ – $8.5\text{x}10^3$ CFU/g) confirm the importance of proper drying of the product with respect to the inhibition of microbial development and maintenance of acceptable level of safety for consumers.

It is acknowledged that *P. agglomerans* which was prevalent in bee pollen according to our studies (Table 2), is used in agriculture as a biological antagonist of fungal diseases in plants (Nunes *et al*., 2001). Some authors believe that *P. agglomerans* was detected in bees and in bee products in hives after the visit of bees on plants (Loncaric *et al*., 2009). *P. agglomerans* has been also isolated from various plants in the Black Sea region (Mudryk, 2012). 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 (Kratz *et al*., 2003), as well as occasionally as a causative agent of septicaemia in newborns (Bergman *et al*., 2007). It was found out that *Pantoea* spp. rarely causes disease in healthy people (Sanders and Sanders, 1997). *P. agglomerans* is not included in the recommendations for European microbiological criteria to bee pollen (Campos *et al*. 2008). On the basis of our results, we suggest

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 the microbiological requirements to the product.

The additional investigations of antibiotic sensitivity of five strains of *P. agglomerans* and *P.agglomerans bgp 6* isolated from dried and fresh bee pollen in 4 of the surveyed regions (Shoumen, Strandzha, Sliven, Karlovo) with regard to their sensitivity to antibiotics from the main groups of antibacterial drugs used in human medicine: ß-lactams (amoxicillin + clavulanic acid: 20/10 μg), aminoglycosides (gentamicin), amphenicols (chloramphenicol), tetracyclines (doxycycline), quinolones (enrofloxacin) and cephalosporins (cephalotin) allowed to conclude that there was a minor risk for transfer of antibiotic resistance through *P. agglomerans* in bee pollen (Dinkov, 2016).

Members of the genus *Citrobacter* (*Citrobacter diversus*) have been encountered in bee pollen (Belhadj *et al*., 2014). So far, there are no data about the occurrence of *Citrobacter freundii*, 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* and *Pantoea* spp. do not pose a risk to healthy people and are frequently encountered in the environments. They are also placed in the opportunistic species group, causing neonatal meningitis and abscesses in humans (Joaquin *et al.,* 1991).

Proteus vulgaris is another opportunistic or facultatively pathogenic microorganism, causing disease in subjects with immunodeficiency disorders, kidney fibrosis or HIV (Steinkamp *et al*., 2005). It is demonstrated that when predisposing factors are present, *Prot. vulgaris* could induce urinary tract, skin and wound infections (Berg *et al.,* 2005). There is evidence that *P. mirabilis* has been more commonly found in the intestinal content of diarrhoeic subjects than in healthy persons, which could be attributed to its role as a human intestinal pathogen (Müller, 1986). The interpretation of our results should take into consideration the fact that the role of *P. mirabilis* as a human pathogen is not entirely elucidated, which could be dangerous after consumption of bee pollen, as well as its absence in recommendations for microbiological criteria to bee pollen (Campos *et al*., 2008). Last but not least, it should be outlined that *Prot. 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 in several plants (Berg *et al*., 2005). 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 2). Furthermore, the organism was not detected in bee pollen from Shoumen after drying and one-year storage in vacuum packages. Drying is recommended as the first 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 (Campos *et al*., 2008).

Flavimonas oryzihabitans, found in fresh bee pollen from the Vratsa region (Table 2) was initially detected in rice, hence its name (Kodama *et al*., 1985). So far, there is no information about the occurrence of this bacterium in bee pollen. *Pseudomonas* spp., which is also from the group of opportunistic bacteria, could cause mainly skin and wound infections (Berg *et al*., 2005). Some authors reported *Flavimonas oryzihabitans* as an agent of postoperative septicaemic infections in newborn babies (Freney *et al*., 1988) and of peritonitis secondary to peritoneal dialysis (Bending *et al*., 1989).

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 ss hominis is a member of the resident microflora of human skin, occasionally causing infections in immunocompromised people (Palazzo *et al*., 2008). Gram-positive cocci and especially *S. epidermidis* are encountered in bees and bee pollen (Gilliam and Lorenz, 1983). It has also been evidenced that *S. epidermidis* as a part of normal skin microflora rarely causes disease, except for immunosuppressed patients (Levinson, 2010).

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 in *S. epidermidis*. It has been detected in most of the 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 sieving.

Bacillus sp. 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* is the most commonly encountered species. *B. circulans* and *B. alvei* were detected only in pollen from honeycomb cells, but not in stored food (Gilliam *et al*., 1990).

Some 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* (Gilliam, 1979).

It is demonstrated that some *B. cereus* and *B. pumilis* strains could produce enterotoxins and therefore could be considered dangerous in cold stored foods due to their psychrotrophic nature and potential of growth at temperatures ≤ 6ºC (Ray and Bhunia, 2014). It should be also noted that of 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 (Regulation 1441, 2007). This *Bacillaceae* member was detected in none of the regions surveyed during our study (Table 4).

B. subtilis is used for plant disease control (Idris *et al*., 2004). In our studies, the share of *B. subtilis* among *Bacillaceae* isolates from fresh bee pollen was considerable (Table 4). It should be emphasised 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 about absence of *B. subtilis* in dried pollen samples (Table 4), we could hypothesise that sieving, proposed as an important element of the primary processing of pollen (Stratev *et al*., 2014) has minimised the chance of contamination.

The predominant member of family *Bacillaceae* in our studies was *B. pumilis* (Table 4). This bacterium is psychrotrophic, able to replicate at the 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 ºC).

Bacillus amyloliquefaciens (Table 4) was also associated with its occurrence in plants. It stimulates plant growth and is used for control of bacterial and fungal plant diseases. That is why some authors consider the microorganism as an alternative for plant disease control (Borriss *et al*., 2011).

It has been shown that *B. pumilus* and *B. subtilis* are the main representatives of the family *Bacillaceae*, encountered in spices (Muhamad *et al*., 1986). *B. pumilus* was also encountered in cold-stored flours (Rogers, 1978). The pathogenic potential of this bacterium and the possibility for production and accumulation of endotoxin posing a risk to people is still unclear. With this regard it should be noted that *Bacillus* spp. do not replicate at $A_w < 0.92$ (EFSA, 2005). To prevent the development of *B. cereus,* the storage of foods at < 4ºC is recommended as at these temperatures the spores of *B. cereus* could not develop into vegetative forms and hence, accumulate toxin (EFSA, 2005).

It should be outlined that dried pollen samples in our study exhibited water activity 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* spp. replication.

Soil microorganisms from *Arthrobacter* spp. are found in bees and wax moths (Gilliam and Lorenz, 1983). Some authors use *A. globiformis* for testing the antibacterial peptides in the haemolymph of bees for evaluation of their immunity level (Korner and Schmid-Hempel, 2004; Sadd and Schmid-Hempel, 2009). In our studies, *A. globiformis* was detected in pollen samples from Vratsa and Shoumen regions (Table 4).

Having investigated the antibacterial activity of bee pollen (bee bread) obtained from bee-processed product stored in honeycomb cells, Baltrušaytite *et al*. (2007) concluded that it was due to flavonoids and phenolic acids contained in pollen, also reported in other research on this product (Carpes *et al*., 2009).

The occurrence of *Staphylococcus* spp. and *Bacillus* spp. in pollen (Tables 3 and 4) showed that the observed antibacterial effect of pollen against them could be exhibited only by high-concentration ethanol solutions (40-90%) (Carpes *et al*., 2007), but not by consumption of floral bee pollen not processed in ethanol. In support of this, no antibacte-

rial activity of ethanol extracts (0.02%-2.5% v/v) of *Laurus nobilis* L. pollen against *S. aureus*, *B. cereus* and *B. subtilis* was found out (Erkmen and Özcan, 2008).

Gamma irradiation at a decontamination dose of 1.0 Mrad is recommended after processing a drying laboratory evidence of contamination of bee pollen with bacteria from the family *Bacillaceae*, which was successfully applied for reduction of the content of spore-forming bacteria up to 10^3 CFU/g, required by Japanese Hygienic Standard for spice (Muhamad *et al*., 1986).

Conclusion

In dried flower bee pollen from 8 regions of Bulgaria after one-year vacuum-packed cold storage (0-4ºC) microorganisms that could cause enteric diseases in humans were not established (Regulation 1441, 2007).

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