JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

BACTERIAL CONTAMINATION OF VACUUM STORED FLOWER BEE POLLEN

Dinko Dinkov

Address(es):
Department of Hygiene and technology of animal foodstuffs, Veterinary legislation and management, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.

*Corresponding author: dinkodinkov@abv.bg

doi: 10.15414/jmbfs.2018.7.4.387-391

ARTICLE INFO

Received 26. 7. 2017
Revised 4. 10. 2017
Accepted 20. 11. 2017
Published 1. 2. 2018

Regular article

OPEN ACCESS

ABSTRACT

Water content, water activity (Aw) and mean microbiological characteristics were determined in fresh and dried flower bee pollen collected from apiary from North east region in Bulgaria. Higher microbial counts were established for fresh than for dried bee pollen after long-time (1 and 2-years) vacuum-packed cold storage (0-4 ºC and - 18 ºC). It was found contamination with *Pantoea* spp., *Prot. mirabilis*, *Citrob. freundii*, *Serratia fonticola*, *Raoultella planticola/ornithinolytica*, *S. hominis* (subspecies *hominis*), *S. sciuri* (subspecies *sciuri*), *Flavimonas orizihabitans*, *Achromobacter denitrificans*, *Arthrobacter globiformis*, *Psychrobacter phenylpyruvicus*, *B. pumilis* and *Enterococcus faecalis* in the dried flower bee pollen intended for human consumption. Fresh bee pollen that is an intermediate product not intended for human consumption besides these contained *Prot.vulgaris*, *E.coli*, *Staph. epidermidis* and *B. subtilis*.

Keywords: microorganisms, vacuum stored, flower bee pollen

INTRODUCTION

It is known that bees and bee pollen are a kind of bioindicators of anthropogenic pollution of the environment (**Bogdanov, 2006**). That is why the certification of bee pollen for human consumption assuring a safe level of contaminants from anthropogenic source and microbiological contaminants from environment, primary collection and processing of flower pollen by the bees and then by humans (**Stratev *et al*., 2014**).
Experience in Switzerland showed that from a microbiological and sensory point of view pollen remains stable until 1.5 years of storage at room temperature. Under these conditions pollen keeps its sensory and microbiological quality for a storage period of 2 years, if stored in a cool, dry and dark place **(Bogdanov *et al*., 2003**). Storage of pollen at 0 to 10 degrees in vacuum has been proposed in order to prevent antioxidant spoilage (**Solomka, 2001**). Pollen aging over 3 years is demonstrated to reduce the free radical scavenging activity by up to 50% (**Campos *et al*., 2003**). I was found that the dehydration process did not interfere in vitamin content (B(1), B(2), PP and B(6)), (**de Arruda *et al*., 2013**).
According to the Bulgarian industry standard introduced in 1991, dried bee pollen should contain up to 13% moisture (**Central Cooperative Union, 1991**). Ordinance from 2005 has stipulated additional requirements to drying and an allowance of up to 12% humidity of end product (**Ordinance №9, 2005**).
Scientific studies indicate that some diseases in plants could be transmitted through the pollen (**Card *et al*., 2007; Flores *et al*., 2005**). The 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 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 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.
In the moment not found studies about bacterial contamination of long time stored fresh and dried bee pollen harvested from the same apiary and processed with sieving, blowing with electric fans and drying. The aims of the present survey was the water content, water activity (Aw) and species level bacterial contamination of fresh and dried bee pollen originating from North east region in Bulgaria, after one- and two-years vacuum-packed cold storage - 0-4 ºC (dried bee pollen) and -18 ºC (fresh bee pollen).

MATERIAL AND METHODS

In June 2014, a total of 6 samples fresh and dried bee pollen were collected from apiary from North east region in Bulgaria (apiary from reg.Shoumen) and processed with sieving, blowing with electric fans and drying (dried bee pollen). In June 2016 a two fresh and dried samples bee pollen were collected and processed from the same apiary (n=4). The samples originated from beehives in industrial pollution-free areas, 3 km away from farmland with intensive crop production (**Bogdanov, 2006**).
The analyses for water content were performed as stated in the national legislation (Central Cooperative Union, 1991). The statistical analysis was done via the Student's t-test (Statmost™ for Windows). All analyses were performed twice and results were presented as means for small samples (n=2), or as means ± standard deviations, minimum and maximum values for larger samples (n=6).
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: http://www.vac-star.com/en/p1-miniVAC.html).
Immediately before vacuum-packaging, the water activity of samples (Aw) was determined by automated analyzer 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 №9, 2005**), while fresh pollen samples were kept frozen at –18 °C (**Dominguez-Valhondo *et al*., 2011**). Fresh bee pollen from 2014 not investigated for quality and microbiological parameters after two years storage because these conditions not required for this intermediate product.

Preparation of samples for microbiological analysis

Twenty-five g of bee pollen were diluted with 225 ml 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 *Micrococcaceae, Moraxellaceae* families and *class Bacilli*

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 hours, isolates with similar colony and microscopic features were selected from the grown colonies for further species differentiation.

Determination of *Enterobacteriaceae* microbial counts and 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 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 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) is 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, serial dilutions were made to 10^{-4} from enrichment broths in sterile physiological saline. Aliquots of 100 µl from the initial and serial dilutions were inoculated on MacConkey agar and XLD agar (Merck, Darmstadt, Germany), and plates were incubated at 37 °C for 24 h.
If lactose-negative or lactose-positive *Enterobacteriaceae* colonies were detected after the 24-hour primary incubation or after the 66-hour 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* serums (Sifin Service GmbH, Berliner Allee 317-321, Berlin, Germany, http://sifin.de/) (**Dinkov, 2016**).

Isolation of bacteria from *Staphylococcaceae* and *Brevibacteriaceae* families

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 markedly resistant to 7.5% NaCl (**Koch, 1942**). For selective enrichment of staphylococci, a specific Giolitti and Cantoni Broth (GC) (Merck, Darmshtadt, Germany) was also used.
The enrichment broths incubated at 37 ºC 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, 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 -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 isolated were taken with a special swab with pointed tip, put into tubes containing special 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 hours. Results were read visually by the change of colour in the wells and compared to positive (10th well) and negative (1st 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 content, water activity and total *Enterobacteriaceae* bacterial counts determination. The highest water content of fresh pollen was detected in a samples from 2014 (25.5 ± 0.77 %), followed by fresh bee pollens from 2016 (24.15%), dried bee pollen from 2015-2016 (15.2 ± 1.85 %) and dried bee pollen from 2016 (12.2 %). There were statistically significant differences (P<0.01), between water content of fresh and dried pollen produced on 2014 (Tab 1).
The highest water activity was established for fresh bee pollen harvested on 2014 (0.725). Dried bee pollen samples had water activity between 0.180 and 0.183, and fresh pollen samples – within 0,684 and 0.725 (Tab 1).
Higher microbial counts were established for fresh bee pollen from 2014 (3.7×10^5 CFU/g) and 2016 (7.1×10^4 CFU/g), than for dried pollen (1.1×10^4– 6.9×10^4 CFU/g). The highest *Enterobacteriaceae* bacterial counts were established in fresh bee pollen from 2014 (1.4×10^4 CFU/g) and 2016 (0.75×10^3 CFU/g). Dried bee pollen samples from 2014 possessed 3.6×10^3 CFU/g and 1×10^3 CFU/g after 2-years storage and also in dried bee pollen from 2016 (Tab 1).

Table 1 Water content, water activity, total count of microorganisms and total *Enterobacteriaceae* bacterial counts determination of vacuum stored fresh and dried flower bee pollen from North east region in Bulgaria ($p<0.05$ [1]).

Fresh / Dried bee pollen (n)	Samples from 2014 2015 Fresh (n=6)	Samples from 2014 2015 Dried (n=6)	Samples from 2014 2016 Dried (n= 6)	Samples from 2016 2016 Fresh (n= 2)	Samples from 2016 2016 Dried (n= 2)
Water activity (Aw / °C)	0.725 / 20.6		0.183 / 20.2	0,684 / 24ºC	0,180/20.3
Total count of microorganisms (CFU/g)	3.7×10^5	6.5×10^4	6.9×10^4	7.1×10^4	1.1×10^4
Total count of *Enterobacteriaceae* (CFU/g)	1.4×10^4	3.6×10^3	1×10^3	0.75×10^3	1×10^3

The fresh and dried bee pollen from 2014 after 1-year storage was shown to contain *Prot. mirabilis*. This microbial species was also found out in fresh pollen samples from 2016. *Prot. vulgaris* was present only in fresh bee pollen from 2014 (tab 1).
Table 2 depicts the results from detected species in bee pollen. The performed analyses have shown most coliforms and some other microorganisms which were not so far reported in this bee product. Microorganisms from the *Pantoea* spp. were present in both dried and fresh bee pollen. *Citrobacter freundii* was found out in dried pollen samples from 2014 after 1-year storage (Tab 2). It is important to mention that *P. agglomerans* under stress conditions (e.g. vacuum and long-term cold storage) may enter into the viable and culturable state in different selective mediums (MacConkey agar and XLD agar).
Bacteria from the genus *Serratia* were demonstrated only in bee pollen from two of regions. The fresh and dried pollen samples from 2016 contained *Serratia fonticola*. The fresh pollen samples from 2014 were positive for *E.coli*. Only in fresh and dried bee pollen from 2016 were found bacteria from the *Pseudomonadaceae* family - contamination with *Flavimonas oryzihabitans* and *Achromobacter denitrificans* were detected (Tab 2).
Our examinations did not establish coagulase-positive staphylococci, acknowledged as human pathogens. The dried bee pollen from 2014 contained *Staphylococcus* (*S.*) *hominis (*subspecies *hominis). S. sciuri (*subspecies *sciuri)* was present in fresh and dried bee pollen samples from 2016. Also in samples from 2016 was found *Brevibacterium epidermidis* (Tab 2).

Table 2 Species level differentiation of isolates in long-term stored in vacuum conditions flower bee pollen.

Fresh / Dried bee pollen (n)	Samples from 2014			Samples from 2016	
	2015		2016	2016	
	Fresh (n=6)	Dried (n=6)	Dried (n= 6)	Fresh (n= 2)	Dried (n= 2)
Enterobacteriaceae	*Pantoea agglomerans* *Prot.mirabilis* *Prot.vulgaris* *E.coli*	*Pantoea agglomerans* *Prot. mirabilis* *Citrob. freundii*	*Pantoea agglomerans*	*Pantoea agglomerans* *Pantoea dispersa* *Serratia fonticola* *Prot. mirabilis* *Raoultella planticola/ ornithinolytica*	*Pantoea agglomerans* *Pantoea dispersa* *Serratia fonticola* *Raoultella planticola/ ornithinolytica*
Pseudomonadaceae	-	-	-	*Flavimonas orizihabitans* *Achromobacter denitrificans*	*Flavimonas orizihabitans* *Achromobacter denitrificans*
Staphylococcaceae	*S. epidermidis*	*S. hominis* (subspecies *hominis)*	-	*S. sciuri (*subspecies *sciuri)*	*S. sciuri (*subspecies *sciuri)*
Brevibacteriaceae	-	-	-	*Brevibacterium epidermidis*	*Brevibacterium epidermidis*
Micrococcaceae	*Arthrobacter globiformis*	*Arthrobacter globiformis*	*Arthrobacter globiformis*	-	-
Moraxellaceae	-	-	-	*Psychrobacter phenylpyruvicus*	*Psychrobacter phenylpyruvicus*
Class Bacilli	*B. pumilis* *B. subtilis* *Enterococcus faecalis*	*B. pumilis* *Enterococcus faecalis*	*Enterococcus faecalis*	*B. pumilis*	*B. pumilis*

The dried and fresh pollen samples were most commonly contaminated with *Bacillus* (*B.*) *pumilis.* While *B. subtilis* was not found in dried pollen samples, it was detected in fresh product from 2014. The second most prevalent species in fresh bee pollen after *B. pumilis* was *B. subtilis*, which was not fount in any of dried samples. In our studies, the microbial species *Arthrobacter globiformis* and *Psychrobacter phenylpyruvicus* in the fresh and dried bee pollen samples were demonstrated for the first time. *Enterococcus faecalis* was detected after one and two-years vacuum storage of samples from 2014 (Tab 2).

DISCUSSION

According to literature data, the water content of fresh bee pollen varies between 20 and 30% (**Bogdanov, 2014**). The higher values established for pollen originating from the Sliven (31.25%) and Strandzha (30.82±0.02) regions could be attributed to frequent precipitation during the pollen collection period in 2014 (**Dinkov *et al*., 2014**).

It is acknowledged that higher water activity values are beneficial for the development of microorganisms. This is an important factor guaranteeing food safety during their 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 (**Rahman, 2010**). The water activity values substantially lower than 0.85 detected in dried bee pollen in our studies suggested that when drying was properly done, there should be not preconditions for development of microbial pathogens in this product (Tab 1).

At the same time it should be noted that the water activity if fresh pollen from all regions was >0.6 (Tab 1), which in the view of Rahman (2010) is a precondition for development of some microorganisms in stored bee pollen. The relatively low water activity in some samples in which detected *Enterobacteriaceae* counts were higher (Tab 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 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 low quantity 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 (Tab 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 $1 \times 10^3 - 3.6 \times 10^3$ CFU/g (Tab 1). The interpretation of these data should took into consideration the fact that apart coliforms, the *Enterobacteriaceae* family includes also 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.

Our results for increased counts of *Enterobacteriaceae* in fresh bee pollen (0.75×10^3 – 1.4×10^4 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 ($1 \times 10^3 - 3.6 \times 10^3$ CFU/g) confirm the important 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), was 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 insolated from various plants in the Black Sea region (**Mudryk, 2012**). Recently, some researchers classify *P. agglomerans* to 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**). 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 to the product.

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, 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 concluding that there was a minor risk for transfer of antibiotic resistance through *P. agglomerans* in bee pollen (**Dinkov, 2016**).

P.agglomerans forms aggregate structures called `symplasmata'. A symplasmatum is a multicellular aggregate structure in which several (at least two) to hundreds of individual cells tightly bind together (**Feng *et al*., 2003**). These structures may be connected with long time persistence of *P. agglomerans* in vacuum-stored bee pollen.

Members of the genus *Citrobacter* (*Citrobacter diversus*) have been encountered in bee pollen (**Belhadj *et al*. 2014**). So far, there are no data about reporting the occurrence of *Citrobacter freundii*, which was detected in dried bee pollen samples from Shoumen (Tab 2). It should be noted that bacteria from genera *Citrobacter* and *Pantoea* spp. do no pose a risk for healthy people and frequently encountered in the environments. They are also placed in the opportunistic

species group, causing neonatal meningitis and abscesses in men (**Joaquin *et al.*, 1991**).
Prot. vulgaris is another opportunistic or facultatively pathogenic microorganism, causing disease in subjects with immunodeficiency states, kidney fibrosis or HIV (**Steinkamp *et al*., 2005**). It is demonstrated that when predisposing factors were present, *Prot. vulgaris* could induce urinary tract, skin and wound infections (**Berg *et al*., 2005**). There is evidence that *Prot. mirabilis* has been more commonly found out in the intestinal content of diarrheic subjects than in healthy men, which could be attributed to its role as a human intestinal pathogen (**Müller, 1986**). 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 (**Campos *et al*., 2008**). Last but not least, it should be outlined that *Prot. mirabilis* was detected in fresh and dried pollen samples from 2014 and fresh samples from 2016 (Tab 2).
The microorganism *Serratia sp.* (Tab 2) is also classified as a potential human pathogen and is encountered on several plants (**Berg *et al*., 2005**). The available literature sources provide no data about the involvement of *Serratia fonticola*, detected in the dried bee pollen in human diseases.
E. coli were detected in fresh bee pollen from the Shoumen region (Tab 2). Furthermore, the organism was not detected after drying and one-year storage in vacuum package of bee pollen. 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 (**Campos *et al*., 2008**).
Flavimonas oryzihabitans, found out in fresh bee pollen (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 are 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**).
*S. hominis (*subspecies *hominis)* is a member of the resident microbiota 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**). *S. epidermidis* was detected as a part of normal skin microbiota rarely causes disease, except for immunosuppressed patients (**Levinson, 2010**). The wide spread of *S. hominis (*subspecies *hominis)* in dried bee pollen proved in our studies after its being primarily processed suggest a possible secondary contamination with this bacterium during sieving and drying (Tab 2). The opposite relationship was found out in *S. epidermidis*. It has been detected in fresh bee pollen samples from 2014. The absence of *Staph. epidermidis* could be attributed to the mechanical removal of the agent with the secondary contaminants of pollen during the sieving. In available references not found data for detection of *Staph. sciuri* subsp. *sciuri* detected in bee pollen samples from 2016 (Tab 2).
Some authors consider the microorganisms from *class Bacilli* as an alternative for plant disease control (**Borriss *et al*., 2011**). *Bacillus* sp. were isolated from 59% of samples stored in cells of honeycombs (beebread) 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 of isolates of class *Bacilli* detected during our studies were identified as *B. subtilis* (Table 2), 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 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 (**Regulation 1441, 2007**). This *Bacillaceae* member was detected in none of regions surveyed during our study (Tab 2).
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 (Tab 2). 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. Based on our results from the absence of *B. subtilis* in dried pollen samples (Tab 2), we could hypothesise that sieving, proposed as an important element of the primary processing of pollen (**Stratev *et al*., 2014**) has minimised the chance for contamination. The predominant member of family *Bacillaceae* in our studies was *B. pumilis* (Tab 2). 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. pumilis* in fresh pollen could be attributed to its storage in a frozen state (-18 ºC).
It is shown that *B. pumilis* and *B. subtilis* are the main representatives of the family *Bacillaceae*, encountered in spices (**Muhamad *et al*., 1986**). *B. pumilis* 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 risk for people is still unclear. With this regard it should be noted that *Bacillus* spp. do not replicated 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 0.180 and 0.183, whereas fresh pollen samples: from 0.684 and 0.725 (Tab 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 out in bees and wax moths (**Gilliam and Lorenz, 1983**). Some authors use *Arthrobacter globiformis* for testing the antibacterial peptides in the haemolymph of bees for evaluation of their immunity level (**Sadd and Schmid-Hempel, 2009**). In our studies, *Arthrobacter globiformis* was detected in pollen samples from 2014 after one- and two years vacuum storage (Tab 2).A 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 (Tab 2).

CONCLUSION

The species-level differentiation of *Enterobacteriacea* isolates did not establish microorganisms that could cause enteric diseases in humans (Regulation 1441, 2007). *P. agglomerans* was found after one- and two-years vacuum cold storage (0-4 ºC) in dried flower bee pollen. Existing after one-year storage *Prot. mirabilis* not detected after two-years vacuum cold storage (0-4 ºC) in dried bee pollen. 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.

Acknowledgments: The author wishes to express his most sincere gratitude to beekeepers from North east region in Bulgaria (apiary from reg.Shoumen) for sending bee pollen samples.

REFERENCES

Belhadj, H., Harzallah, D., Dahamna, S. & Khennouf S. (2014). Microbiological quality control of marketed pollen, *Der Pharmacia Lettre*, 6 (2), 37-42.
Bending, J.W.A., Mayes, P.J., Eyers, D.E., Holmes, B. & Chin, T.T.L. (1989). *Flavimonas oryzihabitans* (*Pseudomonas oryzihabitans*, CDC Group Ve-2): an emerging pathogen in peritonitis related to continuous ambulatory peritoneal dialysis. *J Clin Microbiol* 27:217-8. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC267271/pdf/jcm00061-0241.pdf.
Berg, G., Eberl, L., & Hartmann, A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol* 7 (11): 1673–1685. http://dx.doi.org/10.1111/j.1462-2920.2005.00891.x
Bergman, K.A., Arends, J.P., and Schölvinck, E.H. (2007). Pantoea agglomerans septicemia in three newborn infants. *Pediatr Infect Dis J* 26(5), 453–454. http://dx.doi.org/10.1097/01.inf.0000261200.83869.92
Bogdanov, S. (2014). Pollen: Production, Nutrition and Health: A Review. http://www.bee-hexagon.net/files/file/fileE/Health/PollenBook2Review.pdf. Accessed on 16.06.2016.
Bogdanov, S. 2006. Contaminants of bee products. Review article, *Apidologie* 37, 1–18. http://dx.doi.org/10.1051/apido:2005043
Bogdanov, S., Bieri K., Gremaud G., Iff D, Känzig A., Seiler K.,… & Zürcher K. (2003). Bienenprodukte; 23 B Pollen. *Swiss Food Manual (Schweiz.Lebensmittelbuch)*, 1-6..
Borriss, R., Chen, X.H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B.... & Klenk, H.P. (2011). 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* 61(8), 1786–1801. http://dx.doi.org/10.1099/ijs.0.023267-0
Campos, M.G., Webby, R.F., Markham, K.R., Mitchell, K.A. & Da Cunha, A.P. (2003). Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *J Agric Food Chem* 51(3):742-745. http://dx.doi.org/10.1021/jf0206466
Campos, M.G.R., Bogdanov, S., Almeida-Muradian, L.B., Szczesna, T., Mancebo,... & Ferreira, F. (2008). Pollen composition and standardization of analytical methods. *J Agric Res and Bee World* 47(2),156-163. http://www.bee-hexagon.net/files/file/fileE/IHCPapers/CamposJAR_2008.pdf.
Card, S., Pearson, M. & Clover, G.R. 2007. Plant pathogens transmitted by pollen. *Australas Plant Path* 36(5), 455-461. http://dx.doi.org/10.1071/ap07050

Central Cooperative Union. (1991). Industry standard (IS) 2567111-91 Bee Pollen, 1-7 (BG).
de Arruda, VA, Santos Pereira, A.A., Estevinho, L.M. & de Almeida-Muradian, L.B. (2013). Presence and stability of B complex vitamins in bee pollen using different storage conditions. *Food Chem Toxicol* 51:143-148. http://dx.doi.org/10.1016/j.fct.2012.09.019
Dinkov, D. 2016. Differentiation and antibiotic susceptibility of *Pantoea agglomerans* isolated from flower bee pollen, *Eastern Academic Journal* 1, 99-108. ISSN: 2367-7384. http://www.e-acadjournal.org/pdf/article_160424.pdf.
Dinkov, D., Balkanska, R., Stratev, D. & Mateev St. (2014). Water and mineral content of Bulgarian bee pollen, *BJVM* 17(1), 99-100
Dominguez-Valhondo, D., Gil, D.B., Hernandez, M.T., & Gonzalez-Gomez, D. (2011). Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. *Int J Food Sci Tech* 46 (10), 2204-2211. http://dx.doi.org/10.1111/j.1365-2621.2011.02738.x
European Food Safety Authority (EFSA). (2005). Opinion of the Scientific Panel on biological hazards (BIOHAZ) on Bacillus cereus and other Bacillus spp in foodstuffs. *EFSA Journal* 3(4), 175. http://dx.doi.org/10.2903/j.efsa.2005.175
Feng, Y., Shen, D., Dong, X. & Song, W. (2003). *In vitro* symplasmata formation in the rice diazotrophic endophyte *Pantoea agglomerans* YS19, *Plant and Soil* 255(2), 435-444.
Flores, J., Gutiérrez, I. & Espejo, R. (2005). The role of pollen in chalkbrood disease in Apis mellifera: transmission and predisposing conditions. *Mycologia* 97(6), 1171-1176. http://dx.doi.org/10.3852/mycologia.97.6.1171
Freney, J., Hansen, W., Etienne, J., Vandenesch, F. & Fleurette, J. (1988). Postoperative infant septicemia caused by Pseudomonas luteola (CDC Group Ve-l) and Pseudomonas oryzihabitans (CDC Group Ve-2). *J Clin Microbiol* 26(6), 1241-1243. http://jcm.asm.org/content/26/6/1241.full.pdf.
Gilliam, M. & Lorenz, B.J. (1983). Gram-positive cocci from apiarian sources. *J Invertebr Pathol* 42(2), 187-195. http://dx.doi.org/10.1016/0022-2011(83)90061-7.
Gilliam, M. (1979). Microbiology of pollen and bee bread. The genus Bacillus. *Apidoligie* 10(3), 269-274. http://dx.doi.org/10.1051/apido:19790304
Gilliam, M., Roubik, D.W. & Lorenz, B.J. (1990). Microorganisms associated with pollen, honey and brood provisions in the nest of a stingless bee, Melipona fasciata. *Apidologie* 21(2), 89-97. http://dx.doi.org/10.1051/apido:19900201
Idris, E.E., Bochow, H., Ross, H. & Borriss, R. (2004). 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* 111 (6), 583–597. http://www.bashanfoundation.org/borriss/borrissbiovontrol.pdf.
Joaquin, A., Khan, S., Russel, N. & al Fayez, N. (1991). Neonatal meningitis and bilateral cerebellar abscesses due to Citrobacter freundii. *Pediatr Neurosurg* 17(1), 23–24. http://dx.doi.org/10.1159/000120561
Kodama, K., Kimura, K. & Komagata, K. (1985). Two new species of Pseudomonas: P. oryzihabitans isolated from a rice paddy, and P. luteola isolated from clinical specimens. *Int J Syst Bacteriol* 35(4), 467 - 474. http://dx.doi.org/10.1099/00207713-35-4-467
Koch, G.F.E. (1942). Electivnahrboden fur Staphylokokken. *Zentr Bakteriol Parasitenk Abt I Orig* 149, 122-124. http://eurekamag.com/research/024/594/024594596.php#close.
Kratz, A., Greenberg, D., Barki, Y., Cohen, E. & Lifshitz, M. (2003). Pantoea agglomerans as a cause of septic arthritis after palm tree thorn injury; case report and literature review. *Arch Dis Child* 88, 542–544. http://dx.doi.org/10.1136/adc.88.6.542
Lacey, M., and West, J. (2007). A manual for catching and identifying airborne biological particles (pp. 15-34). Springer. http://www.dbbe.fcen.uba.ar/contenido/objetos/aerobiologia.pdf.
Levinson, W. (2010). Review of Medical Microbiology and Immunology (11th ed.), 94–99. https://www.amazon.com/Medical-Microbiology-Immunology-Eleventh-Science/dp/0071700285#reader_0071700285.
Loncaric, I., Heigl, H., Licek, E., Moosbeckhofer, R., Busse, H.J. & Rosengarten, R. (2009). Typing of Pantoea agglomerans isolated from colonies of honey bees (Apis mellifera) and culturability of selected strains from honey. *Apidologie* 40(1), 40–54. http://dx.doi.org/10.1051/apido/2008062
Mathlouthi, M. (2001). Water content, water activity, water structure and the stability of foodstuffs. *Food Control* 12(7), 409-417. http://dx.doi.org/10.1016/s0956-7135(01)00032-9
Mudryk, M. (2012). Plant-isolated Pantoea agglomerans-new look into potential pathogenicity, *Mikrobiol Z* 74(6), 53-57, ISSN 0201-8462. http://www. irbis-nbuv.gov.ua/.../cgiirbis_64.exe.
Muhamad, L.J., Ito, H., Watanabe, H. & Tamura, N. (1986). Distribution of Microorganisms in Spices and Their Decontamination by Gamma-irradiation. *Agric Bioi Chem* 50 (2), 347- 355. http://dx.doi.org/10.1080/00021369.1986.10867397
Müller, H.E. (1986). Occurrence and pathogenic role of Morganella-Proteus-Providencia group bacteria in human feces. *J Clin Microbiol* 23, 404–405. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC268658/pdf/jcm00104-0236.pdf.
Nunes, C., Usall, J., Teixidó, N. & Viñas, I. (2001). Biological control of postharvest pear diseases using a bacterium Pantoea agglomerans (CPA-2). *Int J Food Microbiology* 70(1-2), 53–61. http://dx.doi.org/10.1016/s0168-1605(01)00523-2
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). http://econ.bg/l.l_i.129712_at.5.html.
Palazzo, I.C.V., d'Azevedo, P.A., Secchi, C. & Pignatari, A.C.C. (2008). Staphylococcus hominis stubs. novobiosepticus strains causing nosocomial bloodstream infection in Brazil. *J Antimicrob Chemoth* 62(6), 1222-1226. http://dx.doi.org/10.1093/jac/dkn375
Rahman, M.S. 2010. Food stability determination by macro–micro region concept in the state diagram and by defining a critical temperature. *J Food Eng* 99(4), 402-416. http://dx.doi.org/10.1016/j.jfoodeng.2009.07.011
Ray, B. & Bhunia, A. (2014). Fundamental Food Microbiology, Fifth Edition, Opportunistic Bacterial Pathogens, Molds and Mycotoxins, Viruses, Parasites and Fish and Shellfish Toxins, part 28, page 390. CRC Press Taylor& Francis Group. International Standard Book Number-13:978-1-4665-6444-2(e-Book-PDF). https://www.amazon.com/Fundamental-Food-Microbiology-Fifth-Bibek/dp/1466564431/ref.
Regulation 1441. (2007). COMMISSION REGULATION (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, *Official Journal of the European Union*, L 322, 12-29. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:322:0012:0029:EN:PDF.
Rogers, R.F. (1978). Bacillus isolates from refrigerated doughs, wheat flour, and wheat. *Cereal Chem* 55(5), 671-674. http://www.aaccnet.org/publications/cc/backissues/1978/Documents/chem55_671.pdf.
Sadd, B.M. & Schmid-Hempel, P. (2009). Principles of ecological immunology. *Evol Appl* 2, 113–121. http://dx.doi.org/10.1111/j.1752-4571.2008.00057.x
Sanders, W.E. Jr. & Sanders, C.C. (1997). Enterobacter spp.: Pathogens poised to flourish at the turn of the Century. *Clin Microbiol Rev* 10(2), 220-241. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC172917/pdf/100220.pdf. Accessed on 16.06.2016.
Sawhney, D. (1986). The toxicity of potassium tellurite to Staphylococcus aureus in rabbit plasma fibrinogen agar. *J Appl Bacteriol* 61(2), 149-55. http://dx.doi.org/10.1111/j.1365-2672.1986.tb04269.x
Serra, B.J. & Escola, J.R. (1997). Nutrient Composition and Microbiological Quality of Honeybee-Collected Pollen in Spain. *J Agr Food Chem* 45(3), 725-732. http://dx.doi.org/10.1021/jf960265q
Solomka, V. (2001). On bees pollen storage technologies. *Pasika* (3), 22-23.
Steinkamp, G., Wiedemann, B., Rietschel, E., Krahl, A., Giehlen, J., Barmeier, H. & Ratjen, F. (2005). Prospective evaluation of emerging bacteria in cystis fibrosis. *J Cyst Fibros* 4(1), 41–48. http://dx.doi.org/10.1016/j.jcf.2004.10.002
Stratev, D., Balkanska, R., Mateev, St. & Dinkov, D. (2014). Processing, storage, labeling and microbiological hazards of organic bee pollen production. *Science & Technologies* IV(5), 21-27.http://journal.sustz.com/VolumeIV/Number5/Papers/DeyanStratev1.pdf. Accessed on 16.06.2016.
Tortorello, M. (2003). Indicator Orgnanisms for Safety and Quality – Uses and Methods for Detection: Minireview. *J AOAC Int* 86(6), 1208-1217. http://lib3.dss.go.th/fulltext/Journal/J.AOAC%201999-2003/J.AOAC2003/v86n6(nov-dec)/v86n6p1208.pdf. Accessed on 16.06.2016