JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

POLLEN CAN - TESTING OF BEE POLLEN FERMENTATION IN MODEL CONDITIONS

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doi: 10.15414/jmbfs.2018.8.2.805-811

ARTICLE INFO

Received 13. 6. 2018
Revised 6. 9. 2018
Accepted 6. 9. 2018
Published 1. 10. 2018

Regular article

OPEN ACCESS

ABSTRACT

The aim of the study was to simulate bee work with pollen by its fermentation in model conditions and evaluate the final product, which is called - pollen can. Bee pollen grain has thick wall, which is reason of its poor digestibility in organism. Bee pollen after fermentation is more suitable for human organism because of easily-available nutrients. Fresh bee pollen was added into the solution water-honey (with/without yoghurt) and was fermented. We tested 3 types of pollen and 3 types of honey in 3 ways of fermentations, which differed in starter addition, access of oxygen during fermentation and its time. The physico-chemical and microbiological parameters were measured in raw materials and products. Pollen can contained approximately 40% of water, 5% of fat and pH was approximately 4.5. These physico-chemical properties were influenced notably by pollen as raw material. The highest water content was found in rape pollen and consequently rape pollen cans. TPC (total plate count) of pollen can ranged from 4.10 to 7.10 log CFU.g^{-1} after fermentation and depend on type of pollen and fermentation process. We observed decrease of bacteria from *Enterobacteriaceae* family and microscopic filamentous fungi after fermentation in all 3 testing performances, whereas counts of sporulating aerobic microorganisms, yeasts and preliminary LAB (lactic acid bacteria) were stable, in comparison to pollen as raw material. Bacilli and clostridia from group of sporulating bacteria were recorded and identified. Value of pH less than 4.5 is important due to avoid clostridia germination.

Keywords: beekeeping, protein, better digestibility, lactic acid bacteria, microscopic fungi

INTRODUCTION

After a period of fascination with highly processed products, the return to natural foods, whose nutritional value is confirmed by the results of scientific research, is currently observed around the world, mainly in the developed countries. Each bee product is pharmacologically active and may, therefore, be the source of many active substances. Of particular importance the new products are derived from bee products with specified pharmacokinetics and pharmacodynamics, which may be the basis for many new forms of drugs or dietary supplements (**Kieliszek *et* al., 2018**).

Honey is the main source of saccharides for bee colony and pollen is the chief source of protein not only for bees but also for many other solitary insects and insects living in colonies (**Linskens *et* Jorde, 1997**). According to **Campos *et* al. (2010)**, it contains high concentration of reducing sugars, essential aminoacids, unsaturated/saturated fatty acids and mineral substances as Zn, Cu, Fe and high K/Na ratio and significant quantities of several vitamins: provitamín A, vitamin E (tocopherol), niacin, thiamine, folic acid and biotin. Bee pollen is considered as "only perfectly complete food" (**Kostić *et al.*, 2015**). The old Egyptians describe pollen as "a life-giving dust" **(Bogdanov, 2017)**. The amount of nutrition-relevant components is largely dependent on the botanical source of pollen (**Campos *et al.*, 2010**). Pollen is known because of wide range of its biological effects. Pollen increases immunity against harmful physical, chemical and biological agents, which is helpful e. g. for patients with infections of the upper respiratory tract, or pneumonia or leukemia, because pollen increases organism's immunity to infections (**Kieliszek *et* al., 2018**). Immune system of 80% is joined with intestinal tract (**Vidová *et* al., 2013**). Two agents are important for intestinal microbiota – probiotic and prebiotic. It has universal validity – for people and animals. **Kačániová *et* al. (2013)** found increase of lactobacilli and enterococci and decrease of *Enterobacteriaceae* family in intestine of broiler chickens after pollen addition into the feed mixture.

There are three types of pollen: native plant pollen, bee pollen (pollen loads formed by bee foragers) and comb pollen (pollen processed and stored in the hive). Bee pollen is partially processed by the bees in outdoor conditions and collected by the beekeeper. Bees do not consume pollen as collected by foragers – they store pollen pellets in the comb cells and add honey, nectar or glandular secretions to the stored mass and then it is called comb pollen (or perga or bee bread) (**Herbert *et* Shimanuki, 1978**). Comb pollen undergoes lactic acid fermentation and can be thus preserved (**Bogdanov, 2017**).

Some countries such as Brazil, Argentina, Switzerland or France have legally recognized bee pollen as a food supplement presenting identity and quality standards, as well as the limits for each parameter to be analyzed (**de Arruda *et al.*, 2017**). In Slovakia, there are not legislative limits for pollen, there are only general legislative for dietary supplements. According to **Bogdanov *et* al. (2017)**, international proposal of chemical standard for pollen loads has been recently made as follows: water content – not more than 8%, total protein content – not less than 15%, sugar content – not less than 40% and fat – not less than 1.5%.

According to **Roulston *et* Cane (2000)**, there are differences in digestibility among pollen types in connection with pollen wall porosity, thickness and composition, although hummingbirds digest pollen very poorly, most animals, including those that do not regularly consume pollen, can digest 50-100% of

ingested grains. **Kieliszek *et* al. (2018)** stated that pollen must be thoroughly chewed because its nutrients are used by an organism only on a level of 10-15% in raw form. **Linskens *et* Jorde (1997)** published that under the influence of enzymes of gastrointestinal tract digestion of pollen proteins, polysaccharides and lipids take place and the unbound elements, i. e. monosaccharides, amino acids, vitamins and fatty acids are subjected to the normal process of resorption. **Zhang *et* al. (2017)** studied the influence of fermentation on pollen morphology and stated that fermentation is an efficient method for pollen wall breakdown, but its mechanism remains relatively unclear. **Zhou *et* al. (2018)** evaluated the digestion and fermentation *in vitro* of polysaccharides from bee pollen of Goji (Chinese wolfberry – *Lycium chinense*) and found enhancing of short-chain fatty acids production and modulating of gut microbiota composition via increasing the relative abundances of genera *Prevotella*, *Dialister*, *Megamonas*, *Faecalibacterium*, *Alloprevotella* and decreasing the numbers of genera *Bacteroides*, *Clostridium* XIVa, *Parabacteroides*, *Escherichia*/*Shigella*, *Phascolarctobacterium*, *Parasutterella*, *Clostridium* sensu stricto and *Fusobacterium*.

There is a necessity of fast processing of fresh bee collected pollen because of high water content. High humidity of fresh bee pollen (about 20-30 g/100 g) is an ideal culture media for microorganisms like bacteria and yeasts (**Bogdanov, 2017**). The fresh bee pollen can be frozen, dried or lyophilized. Interesting way of pollen processing is its fermentation, which is partial simulation of comb pollen production. **Krell (1996)** described the process of fermentation as pollen can ("home bee bread") production after Dany 1988 with addition of pollen to solution of water and honey and possible addition of lactic acid bacteria. The bee bread has already been processed by the bees for storage with the addition of various enzymes and honey, which subsequently ferments; this type of lactic acid fermentation is similar to that in yoghurts (and other fermented milk products) and renders the end product more digestible and enriched with new nutrients (**Krell, 1996**).

Microorganisms, especially lactic acid bacteria, have an important role in the process of pollen transformation. **Olofsson *et* Vásquez (2008)** detected the indigenous bacterial microbiota, dominated by *Lactobacillus* and *Bifidobacterium* phylotype related to *Lactobacillus kunkeei*, *Bifidobacterium asteroides* and *B. coryneforme*. **Endo *et* Salminen (2013)** analyzed FLAB (fructophilic lactic acid bacteria) in the samples from bee hive, including bee pollen and they found *Lactobacillus kunkeei* as dominant FLAB. **Lamei *et* al. (2017)** used the - term honeybee specific Lactic Acid Bacteria (hbs-LAB) originating from the honey crop (also known as honey stomach or honey sack). These bacteria probably play significant role in pollen fermentation.

Despite the fact that pollen stores are rich supplies of nutrient which makes it fit for consumption it must be recommended only with restraint because of its allergenic potential (**Linskens *et* Jorde, 1997**). Pollen proteins are considered to be the allergens (**Bogdanov, 2017**). Some proteins are breakdown during the fermentation and potentially have lower allergenic effect. However, each allergic person probably can have own answer of organism. The field about allergy of pollen after fermentation could be searched in the future.

The objective of our study was to test the production of pollen can, i. e. the bee pollen fermentation in model conditions, at 3 various ways – with/without access of oxygen in initial fermentation caused only by microorganisms naturally occurred in pollen and without access of oxygen in initial fermentation caused by microorganisms from pollen and yoghurt (added as starter).

MATERIAL AND METHODS

Material

We used water, fresh bee pollen, honey and yoghurt (in one variant) for pollen can development. There were three different types of pollen and honey. Characterization of raw material is in the table 1. Pollen was kept in the freezer until the production testing (at about 1 week).

Table 1 Raw materials for pollen can production

Sample	Origin / Obtaining	Geographical origin	Year
POLLEN			
A	early spring (from plants before *Brassica napus* blooming) / directly from beekeeper	Male Krstenany (Slovakia)	2017
B	rape (*Brassica napus*) / directly from beekeeper	Male Krstenany (Slovakia)	2017
C	late spring (from plants after *Brassica napus* blooming) / directly from beekeeper	Dolny Pajer* - (Slovakia)	2017
HONEY			
1	blossom / directly from beekeeper	Male Krstenany (Slovakia)	2017
2	rape (*Brassica napus*) / directly from beekeeper	Male Krstenany (Slovakia)	2017
3	blended (*Tilia cordata* + honeydew) / directly from beekeeper	Dolny Pajer* - (Slovakia)	2017
YOGHURT	cow whole milk / commercial yoghurt from small producer	eastern part of Slovakia	2018

* unpolluted area surrounded by forest with cottages and only a few inhabitants

Fermentation – production of pollen can

In the table 2, there is a reception of all examined types of pollen can. We proceeded after Dany 1988, as described by **Krell (1996)**: Water was boiled, and then cooled (30 ± 2 °C). Honey was added. Solution was carefully mixed. Yoghurt was added (for 3rd experimental variant) and mixed. Pollen was added and mixed to obtain homogenous material. Then products were bottled. For successful fermentation, air space above the food was set on 20-25% of total volume.

Table 2 Detailed receipt for tested variants of pollen can

Pollen can	Water	Honey	Yoghurt	Pollen
1st experimental variant				
1	75 ml	**1** – 45 g	-	**A** – 300 g
2	75 ml	**2** – 45 g	-	**B** – 300 g
3	75 ml	**3** – 45 g	-	**C** – 300 g
2nd experimental variant				
4	75 ml	**1** – 45 g	-	**A** – 300 g
5	75 ml	**2** – 45 g	-	**B** – 300 g
6	75 ml	**3** – 45 g	-	**C** – 300 g
3rd experimental variant				
7	75 ml	**1** – 45 g	1 g	**A** – 300 g
8	75 ml	**2** – 45 g	1 g	**B** – 300 g
9	75 ml	**3** – 45 g	1 g	**C** – 300 g

There were tested 3 types of fermentation (I, II, III). Descriptions of tested variants are shown in the table 3. We used for fermentation only microorganisms naturally occurred in the pollen (and partially in honey) for variant I and II. We used the yoghurt as an additional easily-available starter of fermentation for variant III. The fermentation process consisted of initial and next fermentation. The differences were in realising of initial fermentation and time of next fermentation, which was undertaken without access of oxygen in airtight bottles. In variant I and III, initial fermentation was according to **Krell (1996)** - without oxygen - because of LAB stimulating. Variant II was traditional, according **Hajdušková (2006)**, where bottles were covered by linen cloth; consequently oxygen was present during the initial fermentation.

Table 3 Design of fermentation

Experimental variant	Initial fermentation				Next fermentation		Total fermentation (time)
	material with microorganisms	conditions			temperature	time	
		temperature	time	O_2 access			
I	pollen	30 ± 1 °C	2 days	no	RT (23 ± 2 °C)	16 days	18 days
II	pollen	RT (23 ± 2 °C)	5 days	yes	RT (23 ± 2 °C)	13 days	18 days
III	pollen + yoghurt	30 ± 1 °C	2 days	no	RT (23 ± 2 °C)	6 days	8 days

RT – room temperature

Physico-chemical analysis

The raw materials and tested variants of pollen can were analysed from physico-chemical quality point of view. In the honey, we tested water content (by refractometric method), free acidity (by titration to pH 8.3), electrical conductivity (by conductometric method) and hydroxymethylfurfural - HMF (by Reflectoquant®, Merck, Germany). The first three parameters were measured in accordance to **IHC (2009)**. In pollen and pollen can, we found water content (detected by drying at 100 ± 2 °C for 4 h to constant weight), free acidity (by titration to pH 8.3), pH (by pH-meter) and fat content (by fat extractor ANKOM^{XT15} Technology, USA).

Basic microbiological analysis

Raw materials (pollen, honey and yoghurt) as well as examined pollen cans (after total fermentation) were tested from microbiological point of view. We used dilution plating method for quantitative detection of total plate count (TPC), sporulating aerobic microorganisms (SAM), *Enterobacteriaceae* family, preliminary lactic acid bacteria (LAB) and microscopic fungi (MF). Description of individual parts of microbial examination is recorded in the table 4. Preliminary LAB means that they were found by dilution plating method with observation of colony morphology, without next biochemical or molecular-genetic confirmation.

Table 4 Microbial examination of raw materials and products

Microbial group	Medium	Inoculation	Cultivation		
			temperature	time	O_2 requirement
TPC	GTY	pouring	30 °C	2-3 days	aerobic
SAM	NA 2	pouring*	25 °C	3 days	aerobic
Enterobacteriaceae	VRBG	pouring	30 °C	1-2 days	aerobic
preliminary LAB	MRS	pouring**	37 °C	3 days	aerobic**
MF	DG 18	pouring	25 °C	5-7 days	aerobic

TPC – total plate count, cultivated on GTY – agar with glucose, tryptone and yeast extract (HiMedia®, India); SAM – sporulating aerobic microorganisms, inoculated by *pouring – after heat shock (at 80 °C for 10 min), cultivated on NA 2 – nutrient agar no. 2 (HiMedia®, India); *Enterobacteriaceae* family, cultivated on VRBG – violet red bile glucose agar (HiMedia®, India); LAB – lactic acid bacteria, inoculated by **double-pouring (decrease of oxygen in medium), cultivated on MRS – de Man, Rogosa and Sharpe agar (HiMedia®, India); MF – microscopic fungi, cultivated on DG 18 – Dichloran Glycerol agar - with chloramphenicol (HiMedia®, India)

Statistical processing of results

Individual measurements were performed at least 2 times with average expression. Results for experimental variants were processed in MS Excel 2007 as mean ± sd (standard deviation). Data from microbiological analysis were calculated as log CFU.g^{-1}. Significant difference was assessed if it was at least 1.00 log CFU.g^{-1}.

Identification of aerobic and anaerobic sporulating bacteria

Sample of pollen can (no. 9) was tested at Czech University of Life in Prague in term of sporulating bacteria detection. Firstly, we performed the heat shock (80 °C/10 min) of basic sample solution. We inoculated dilutions from 10^{-1} to 10^{-3} by pouring of TPY agar (Oxoid, UK) with addition of glucose at 1 g.l^{-1}. Plates were cultivated under the aerobic conditions at 30 °C for 1 day and under the anaerobic conditions at 37 °C for 1 day. Then, isolates were transferred into liquid medium for 24 h under the appropriate conditions. After 24 h, bacteria were visualised by microscope with phase contrast and they were identified based on ribosomal protein analysis on an Autoflex speed matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker, Daltonik, Germany) according to **Salmonová *et* al. (2018)**, i. e. extracted by ethanol-formic acid extraction, transferred onto an MTP 384 polished steel BC target (Bruker, Daltonik, Germany) and overlaid with HCCA matrix solution (Bruker, Daltonik, Germany). Measurements of each strain were performed in duplicate. Identification was considered as successful if the score value (degree of similarity to the reference spectrum) was at least 2.0 and was identical at species level. According to **Wieser *et* al. (2012)**, score value provides information about the validity of the identification; the score value above 2.0 is generally considered to be a valid species level identification; and values between 2.0 and 1.7 represent reliable genus level identifications.

RESULTS AND DISCUSSION

Quality of raw materials

Physico-chemical quality of honey samples is recorded in the table 5. All samples met requirements of **Council Directive 2001/110/EC** that has validity in EU countries including Slovakia. Honey had low water content and low HMF content and low free acidity. That indicates ripe unheated honey of high quality from beekeeper of good beekeeping practise without formic acid treatment during the honey production. Electrical conductivity confirms honey origin. At present, measurement of electrical conductivity is the most useful quality parameter for the classification of unifloral honeys, which can be determined by relatively inexpensive instrumentation (**Bogdanov *et* al., 2004**). There is usually a positive correlation among the colour, mineral content and electrical conductivity of honey (**da Silva *et* al., 2016**). Honey, used in tested pollen can, had electrical conductivity to 0.8 mS.cm^{-1}. According to beekeeper, sample 1 and 2 were blossom and sample 3 was blended (honeydew and blossom origin). The determination of electrical conductivity is the fastest method for routine honey control as a criterion of the differentiation between blossom and honeydew honeys (**Bogdanov *et* Martin, 2002**). **Kukurová *et* al. (2008)** stated that electrical conductivity of forest honey in Slovak Republic was 0.55-1.00 mS.cm^{-1} before 2004. Forest honeys from Slovakia are often honeydew or blended in term of their origin, but the strict requirement for electrical conductivity of blended honey is not known nowadays.

Table 5 Physico-chemical quality of honey

Sample	Water content [%]	Free acidity [meq.kg^{-1}]	Electrical conductivity [mS.cm^{-1}]	HMF [mg.kg^{-1}]
Honey 1	16.3	27.15	0.33	10.80
Honey 2	16.5	13.70	0.12	16.50
Honey 3	16.4	27.75	0.37	11.90
mean ± sd (n = 3)	**16.4 ± 0.1**	**22.87 ± 7.94**	**0.27 ± 0.13**	**13.07 ± 3.02**

HMF - hydroxymethylfurfural

Physico-chemical quality of pollen samples is in the table 6. Fresh bee collected pollen contains about 20-30 g water per 100 g (**Campos *et* al., 2010**). Rape pollen (B) had the highest water content (more than 30%) and it was the most acidic, what indicated the lowest pH and the highest free acidity. According to **Bíro *et* al. (2010)**, rape pollen is suitable for animal nutrition because its nutrients are easily and highly digestible; rape pollen is typical by medium content of nitrogen compounds, relatively low content of fiber and high content of nitrogen-free extract, i. e. easy-digestible saccharides. There are some risks joined with pollen consumption, too. Rape bee pollen at concentrations of 0.50% and 0.75% positively affected the body weight gain of female rats; however this higher pollen consumption in feed reduced the triglycerides serum content in female rats and increased the cholesterol serum content in male and female rats (**Gálik *et* al., 2016**). Fat content was variable. The highest value was reached in early spring pollen (A) and the lowest value in late spring pollen (C). There are considerable differences of the fat composition, depending on the botanical origin; there are mainly polar and neutral fats (mono-, di- and triglycerides), as well as small amounts of fatty acids, sterines and hydrocarbons (**Bogdanov, 2017**). Range for fat content in pollen is wide, from 1.1% to 25.0% (**Bíro *et* al., 2010**). Expression of fat and other nutrients as % DM (percentage from dry matter content) is more suitable and precise, because of variable water content in fresh as well as dehydrated pollen. **Taha (2015)** found fat content from 1.82% to 5.38% DM in pollen from Saudi Arabia. Average fat content in rape pollen was 3.92% DM, however typical time for growing and blooming of rape is January in Saudi Arabia (**Taha, 2015**). **Fuenmayor *et* al. (2014)** found fat content from 2.80 to 9.70% DM in Colombian bee pollen. Results of fat in tested pollen are in accordance to the published ranges. According to **Herbert *et* Shimanuki (1978)**, the chemical composition of pollen varies with plant species, environment during pollen development, age of plant when pollen developed, nutrient status of the plant, methods of pollen extraction and storage.

Table 6 Physico-chemical quality of pollen

Sample	Water content [%]	pH	Free acidity [meq.kg^{-1}]	Fat [% DM*]
Pollen A (n = 2)	21.93	5.71	346.00	5.75
Pollen B (n = 2)	31.45	4.83	391.50	4.77
Pollen C (n = 2)	20.16	5.75	334.00	2.42
mean ± sd	**24.51 ± 5.43**	**5.43 ± 0.52**	**357.17 ± 30.33**	**4.55 ± 1.41**

* % DM – percentage in dry matter

In the table 7, the microbiological quality of raw materials is recorded. In the honey, we found low microbial counts, below 2.00 log CFU.g^{-1}. We did not find microscopic fungi. In ripe honey of high quality, we can observe mainly inactive forms of microorganisms, what was confirmed in our study. The difference of TPC and evaluated microbial groups between the honey and pollen was approximately 4 log CFU.g^{-1}. The difference of microbiological quantity between honey and pollen is the same as in last research (**Kňazovická *et* al., 2011**), where TPC as well as TPC of anaerobic microorganisms were at level 5 log CFU.g^{-1} in fresh bee pollen. According to **de Arruda *et* al. (2017)**, who tested 62 samples of Brazilian pollen, microbial counts were at level 1-2 log CFU.g^{-1} for TPC, microscopic fungi, coliform bacteria and staphylococci. The values are lower comparing with pollen samples in our study, because Brazilian bee pollen samples were dehydrated. There is only limited number of studies about quantity of microorganisms in fresh pollen; however there are many studies about identified microorganisms from pollen. **Endo *et* Salminen (2013)** found fructophilic LAB in the pollen. These bacteria have positive influence on health of bees, animals and humans. On the other hand, we found high number of filamentous microscopic fungi. Average value is above 4.00 log CFU.g^{-1}. From hygienic point of view the microbiological safety is the main quality criterion, especially the absence of pathogenic germs and fungi in the pollen (**Bogdanov, 2017**).

Table 7 Microbiological quality of raw materials [log CFU.g^{-1}]

	TPC	*Enterobacteriaceae*	SAM	preliminary LAB	Yeasts	MFF
Honey (n = 3)	1.20 ± 0.17	< 1.00	1.30 ± 0.30	< 1.00	< 1.00	< 1.00
Pollen (n = 6)	5.96 ± 0.38	5.05 ± 0.83	2.48 ± 0.28	5.00 ± 0.88	5.03 ± 0.76	4.70 ± 0.51
Yoghurt (n = 1)	4.95	1.00	2.97	5.06	4.16	< 1.00

TPC – total plate count, SAM – sporulating aerobic microorganisms, LAB – lactic acid bacteria, MFF – microscopic filamentous fungi

Quality of fermented products

In the table 8, there are results of physico-chemical analysis of pollen can. In general, we recorded increase of water content by 60%, increase of free acidity by 40%, decrease of pH by 17% and decrease of fat by 1-2% (as fat in DM) in pollen can comparing to pollen as a raw material. Water content was higher because of its addition. In all experimental variants of fermented pollen, pH decreased. Low pH has protective function. Spoilage of bee bread is avoided by the low pH, caused by lactic and acetic acid fermentation and alcoholic fermentation, and by the presence of natural products with antibiotic properties produced by resident microbiota of stored bee pollen (**Menezes *et* al., 2018**). The order of pollen from the most acidic to less acidic was following: before fermentation B < A < C and after fermentation C < B < A. In pollen C, fermentation was intensive according to pH values. The most acidic products were from 2nd experimental variant. Free acidity measurement was a little bit problematic. The values are of wide range. We recommend automatic titrator with pH detection for next trials. For this trial, we considered the pH values as more precise. Fat values were similar before and after fermentation. Mostly, the values decreased. Exception was sample 1, where the increase was recorded. **Herbert *et* Shimanuki (1978)** found 4.90% fat in bee-collected pollen from USA and 5.40% fat in perga from the same region. There are considerable differences of the fat composition, depending on the botanical origin **Bogdanov (2017)**. The highest values are found in dandelion (*Taraxacum officinale*) and mustard (*Brassica* sp.), and the lowest in birch (*Betula* sp.) brush and buckthorn (*Rhamnus* sp.) (**Herbert *et* Shimanuki, 1978**). The next factor, influencing the fat content and composition, is microbiota during the fermentation. Ether extracts may include fats, fatty acids, some vitamins, pigments, higher alcohols, waxes, sterols and saturated hydrocarbons (Solberg *et* Remedios, 1980 cit. **Roulston *et* Cane, 2000**). **Zhou *et* al. (2018)** observed the degradation of polysaccharides and increasing of short-chain fatty acids during fermentation of pollen grains by gut microbiota. **Kačániová *et* al. (2018)** isolated bacteria from gut of local Slovakian bees and identified them, as follows: *Lactobacillus gasseri*, *L. amylovorus*, *L. kunkeei*, *L. fructivorans* and *Paenibacillus larvae*.

Table 8 Physico-chemical quality of pollen can

	Water content [%]	Dry matter [%]	Free acidity [meq.kg^{-1}]	pH	Fat [% DM]
1st EV (- O_2)					
1	37.07	62.93	415.00	5.08	6.98
2	47.32	52.68	658.00	4.43	3.14
3	33.46	66.54	762.00	3.81	0.85
mean ± sd (n = 3)	**39.28 ± 7.19**	**60.72 ± 7.19**	**611.67 ± 178.08**	**4.44 ± 0.64**	**3.66 ± 3.10**
2nd EV (+ O_2)					
4	41.23	58.77	466.50	4.51	4.55
5	41.48	58.52	563.00	4.50	3.01
6	34.87	65.13	550.00	3.94	0.57
mean ± sd (n = 3)	**39.19 ± 3.75**	**60.81 ± 3.75**	**526.50 ± 52.37**	**4.32 ± 0.33**	**2.71 ± 2.01**
3rd EV (with starter, - O_2)					
7	37.31	62.69	325.50	5.06	5.31
8	44.34	55.66	382.00	4.64	2.79
9	36.92	63.08	315.50	4.60	0.69
mean ± sd (n = 3)	**39.52 ± 4.18**	**60.48 ± 4.18**	**341.00 ± 35.86**	**4.77 ± 0.25**	**2.93 ± 2.31**

EV – experimental variant, DM – dry matter

Zhang *et* al. (2017) observed the fermentation by *Ganoderma lucidum* and *Saccharomyces cerevisiae* on rape pollen grain and suggested some hypothesis for explaining the mechanism of the pollen wall rupture, as follows:
(1) In fermented infancy (days 0-1), the fermentation species could secrete protease and other enzymes to remove the coating of pollen and to expose the germinal apertures and the porous exine;
(2) In the earlier stage of fermentation (days 2-5), cellulose, pectinase, and other enzymes from the species could easily permeate through the inner pollen to destroy the intine. At this time, some bond structures form at the pollen interior, and these structures could completely immobilize the pollen content.
(3) In the middle of the fermentation process (day 6-7), cellulose and pectinase could abscise the bonds, leading to the separation of the exuviae. The content then overflowed at the exine hole;
(4) At the late stage of fermentation (day 8), all of the bonds were abscised, absolutely releasing the entire contents. The content and fragments of the pollen wall remained scattered in the fermentation broth.
Microbial counts in pollen can after fermentation are recorded in the table 9. Botanical origin of pollen seems to be the considerable factor in experimental variants I and II in term of microbial counts, because we found TPC and count of preliminary LAB at level 6.00-7.00 log CFU.g^{-1} in the sample 2 and 5 (from rape pollen - B) as well as absent bacteria from *Enterobacteriaceae* family and absent microscopic filamentous fungi or their presence near the detection limit. In rape pollen after fermentation (sample 2 and 5), increase of preliminary LAB (with counts over 6.00 log CFU.g^{-1}) could inhibit the growth of bacteria from *Enterobacteriaceae* family (counts equal or below 1.00 log CFU.g^{-1}). LAB were frequently isolated from gut of bee, nowadays, they are confirmed from pollen, too (**Olofsson *et* Vásquez, 2008; Lamei *et* al., 2017**). According to **Krell (1996)**

and **Bogdanov (2017)** fermentation at 30 °C for first days without oxygen helps to better development of lactic acid fermentation. The hypothesis was not confirmed by our study. We did not find differences in LAB counts in term of fermentation performance. Specific group of LAB - FLAB prefer aerobic rather than anaerobic conditions for growth and cannot grow on glucose under anaerobic conditions (**Endo *et* Salminen, 2013**). Other samples from experimental variant I and II (1, 3, 4, 6) have lower and similar values of mentioned microbial counts. The counts of SAM as well as yeasts had similar values in experimental variants I and II. In experimental variant III (with addition of yoghurt), we found less differences in term of pollen of botanical origin. In sample 8 (from rape pollen B), the count of microscopic filamentous fungi was lower comparing with sample 7 and 9. The presence of bacteria from *Enterobacteriaceae* family was detected at 3.00 log CFU.g^{-1}. In Slovakia, there are not the legislative regulations for pollen and the research studies could make help for set the limits in the future. For example, **de Arruda *et* al. (2017)** published the standard established by the Argentinean Food Code is 1.5×10^5 CFU.g^{-1} (i. e. 5.18 log CFU.g^{-1}) for aerobic mesophiles (equivalent to TPC) in dehydrated bee pollen. According the results, similar or higher value could be the limit for TPC in fermented pollen from Slovakia and related countries, because pollen is food with relatively high counts of microorganisms, which occur naturally and probably most of them are positive for human health, however it is necessary to eliminate the activities of pathogenic bacteria and microscopic filamentous fungi.

Table 9 Microbiological quality of pollen can after fermentation [log CFU.g^{-1}]

	TPC	SAM	*Enterobacteriaceae*	Preliminary LAB	Yeasts	MFF
1st EV (- O_2)						
1	4.34	2.53	2.81	< 1.00	4.48	2.67
2	7.10	2.37	< 1.00	6.58	4.96	< 1.00
3	4.91	2.78	3.06	3.61	4.83	1.99
mean ± sd (n = 3)	**5.45 ± 1.46**	**2.56 ± 0.21**	**2.94 ± 0.18**	**5.10 ± 2.10**	**4.76 ± 0.25**	**2.33 ± 0.48**
2nd EV (+ O_2)						
4	4.10	2.57	1.95	2.62	4.93	1.49
5	6.88	2.31	1.00	6.44	4.58	1.15
6	4.77	2.92	2.79	4.42	5.90	2.67
mean ± sd (n = 3)	**5.25 ± 1.45**	**2.60 ± 0.31**	**1.91 ± 0.90**	**4.49 ± 1.91**	**5.14 ± 0.68**	**1.77 ± 0.80**
3rd EV (with starter, - O_2)						
7	5.15	1.60	3.92	2.94	4.85	3.70
8	4.76	2.19	3.48	4.84	4.48	1.60
9	4.59	3.39	2.85	4.72	4.41	3.48
mean ± sd (n = 3)	**4.83 ± 0.29**	**2.39 ± 0.91**	**3.42 ± 0.54**	**4.17 ± 1.06**	**4.58 ± 0.24**	**2.93 ± 1.15**

EV – experimental variant, TPC – total plate count, SAM – sporulating aerobic microorganisms, LAB – lactic acid bacteria, MFF – microscopic filamentous fungi

Identification of sporulating bacteria cultivated from pollen can

Sporulating bacteria, isolated from pollen can, were identified (table 10). The most frequent sporulating aerobic bacteria was *Bacillus licheniformis* (4 isolates), then *B. megaterium* (2 isolates) and 1 isolate was identified as *B. sonorensis* (figure 1). *Bacillus licheniformis* and *B. megaterium* are frequently present all over the world and their strains are used in biotechnology (**de Clerck *et* de Vos, 2004**; **Eppinger *et* al., 2011**). *Bacillus sonorensis* is named after the Sonoran Desert (USA), where the organism was collected (**Palmisano *et* al., 2001**).
From sporulating anaerobic bacteria, we found 2 species *Clostridium perfringens* (2 isolates) and *C. baratii* (3 isolates, figure 2). **De Arruda *et* al. (2017)** did not find sulphite-reducing clostridia in 62 samples of Brazilian bee pollen. Spores of bacilli and clostridia are normal part of nature. They are found e. g. in the soil, dust, air. However, vegetative clostridia of some species are pathogenic and can produce the toxins. *Clostridium perfringens* spores are spread in the soil and intestinal tract of animals and humans, in vegetative form and under specific conditions, the bacteria can produce more than 15 toxins (**Lindström *et* al., 2011**). *Clostridium baratii* can produce botulotoxin in rare cases and result in infant botulism (**Khouri *et* al., 2018**). On the other hand, **Stefka *et* al. (2014)** demonstrated that the allergy-protective capacity is conferred by a clostridia-containing microbiota, where clostridia regulate innate lymphoid cell function and intestinal epithelial permeability to protect against allergen sensitization.

Table 10 Identification of sporulating bacteria cultivated from pollen can

isolate no.	species identification	score-value*
AEROBIC		
1	*Bacillus sonorensis*	2.219
2	*Bacillus licheniformis*	2.313
3	*Bacillus licheniformis*	2.261
4	*Bacillus licheniformis*	2.299
5	*Bacillus licheniformis*	2.152
6	*Bacillus megaterium*	2.094
7	*Bacillus megaterium*	1.909
ANAEROBIC		
8	*Clostridium perfringens*	2.271
9	*Clostridium perfringens*	2.408
10	*Clostridium baratii*	2.205
11	*Clostridium baratii*	2.306
12	*Clostridium baratii*	2.205

*Identification results with score values above 2.0 are considered to be correct for determination of the respective species (**Wieser *et* al., 2012**).

According to **Grieger *et* Vařejka (1990)**, food contains clostridia at dose less than 2.00 log CFU.g^{-1} cannot cause the illness, because the counts are reduced during the transport by gastrointestinal tract, but risk groups are children, older people and people with weakened immunity; and food with pH below 4.5 is considered as safe food, because clostridia (especially *Clostridium botulinum*) are not able to multiply and produce botulotoxin under the pH 4.5.

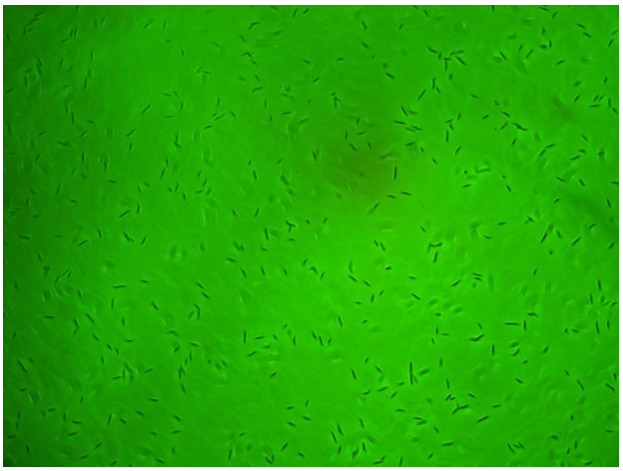

Figure 1 Isolate 1, cultivated from pollen can – *Bacillus sonorensis* (40×10×1.5 by phase contrast)

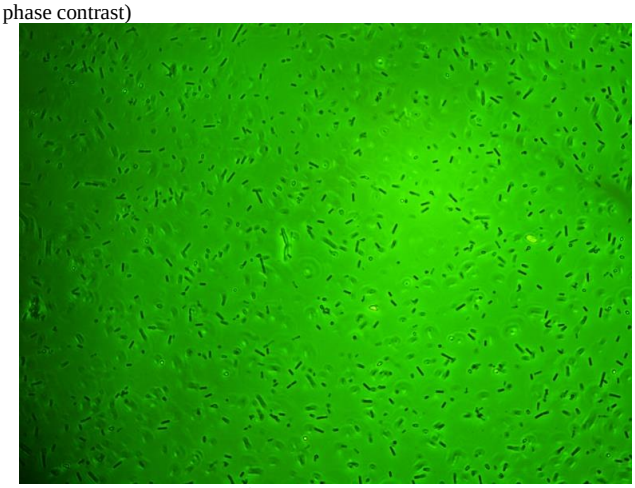

Figure 2 Isolate 11, cultivated from pollen can – *Clostridium baratii* (40×10×1.5 by phase contrast)

De Arruda *et* al. (2017) suggested that the main physico-chemical and nutritional composition of bee pollen *in natura* combined with the presence of microorganisms naturally available in this product are conditions that have to be monitored for the good practices of bee pollen collection, transport, packaging and processing. LAB produce antibacterial compounds as organic acids, hydrogen peroxide, diacetyl, benzoate and bacteriocins (de Vuyst *et* Vandamme, 1994 cit. **Olofsson *et* Vásquez, 2008**).

CONCLUSION

Bees collect the pollen and ferment it in their hives. Bee pollen is better digestible for bees and humans after processing. Fermentation is one of the possible ways for better availability of high quality nutrients for the body. In the study, we tested the production of pollen can by 3 variants of fermentation with 3 different types of honey and pollen. The main factor influenced the pollen can was pollen as raw material, which differs in term of water, fat and pH. Tested variants of fermentation process seem to be suitable in term of physico-chemical quality. Pollen naturally contains microorganisms in relatively high numbers. Fermentation resulted in quantity decrease of bacteria from *Enterobacteriaceae* family and microscopic filamentous fungi, comparing of pollen and pollen can in all 3 tested variants. Counts of SAM, yeasts as well as TPC were kept like in pollen before fermentation. Count of preliminary LAB was also kept on average, but it depended on pollen type. Risk microbial groups are sporulating bacteria and microscopic filamentous fungi, mainly for children, older people and people, who have problems with immune system. Sporulating bacteria from the genus *Bacillus* and *Clostridium* were isolated and identified from pollen can after heat shock. They occurred only in inactive form of spores. Bacilli and clostridia are commonly present in honeys or other foodstuffs or natural materials. The risk is germination of certain clostridia species during the storage. Generally, spores of clostridia can begin to germinate at pH more than 4.5. Therefore, pH seems to be essential for considering the safety of the product. In practise, we suggest to modify the fermentation process considering the pH. In next research, we plan to observe the pollen can quality during the shelf life in term of basic physico-chemical and microbiological analyses and continue with the pollen research to expected result in simple recommendation about production and shelf life of pollen can for beekeepers and bee products processors.

Acknowledgments: This work was supported by the Grant 05-GA SPU 17 "Testing of pollen can production and shelf life" and by AgroBioTech Research Centre built in accordance with the project Building "AgroBioTech" Research Centre ITMS 26220220180.We are thankful to beekeeper Jozef Švercel for material of high quality and really great discussion and cooperation at field of bee products processing. Thanks belong to Regina Bányiová and Natália Karapinová for assistance with laboratory analyses and to Erasmus+ programme for opportunity to mobility and cooperation.

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