Bacterial and fungal communities associated with the surface of hemp seeds and poppy seeds

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Summary
Microbial contamination of oil seeds can cause their deterioration leading to reduction of the quality of the produced oil. Knowledge of the levels and composition of microbial communities may be therefore helpful to set up the effective control measures. Seeds of hemp and poppy (blue and white cultivars) from two farms in Slovakia were analysed by culture-based microbiological methods and by metagenomic high-throughput sequencing of 16S rDNA and 28S rDNA to provide information on surface bacterial and fungal communities. The results of culture-based analysis showed a comparatively high level of contamination by bacteria and by fungi of hemp seeds. High-throughput sequencing revealed that the bacterial community of hemp seeds consisted mainly of families Enterobacteriaceae and Pseudomonadaceae. The fungal community was composed mainly of Pleosporaceae, Davidiellaceae and Tremellaceae. In poppy seeds, the bacterial community was found to be composed mainly of Enterobacteriaceae (genus *Klebsiella*), Pseudomonadaceae and Xanthomonadaceae or Streptomycetaceae. The fungal community was composed mainly of Pleosporaceae and Davidiellaceae (genus *Cladosporium*), while some samples were characterized by increased levels of Trichocomaceae (genus *Aspergillus*). The determined presence of pseudomonads and aspergilli in certain seeds may indicate that more effective cleaning and sanitation will be required as they could affect the produced oil.

Keywords
hemp; poppy; microbiome; metagenome; high-throughput sequencing; DNA

Industrial cultivars of hemp (*Cannabis sativa*) and poppy (*Papaver somniferum*), which are characterized by low levels of psychoactive metabolites, are attractive crops to produce oil. The oils produced from their seeds are established as nutritive and health-promoting food, containing high levels of polyunsaturated fatty acids and other biologically active compounds. Hemp seed oil has an optimum proportion of ω-6 to ω-3 fatty acids (3 : 1). Composition of poppy seed oil is less favourable from the nutritional point of view, as the dominant fatty acid is linoleic acid, but it contains other health-promoting compounds such as phytosterols, tocopherols or phenolics [1–3]. For small farmers in Slovakia, hemp and poppy seed oils represent products with a high added value.

Seeds of various crops can undergo changes in chemical composition, which appear mainly at the harvest or during the post-harvest storage. These are connected to manipulation and contact with contaminated surfaces, soil or dust. Depending on the level of mechanical forces applied, the changes regard the surface of the seeds or may involve the inner tissues. Among the chemical reactions that may take place, oxidation of lipids is the most important for the quality of oil seed crops. By the contact with contaminated surfaces, soil or dust, the seeds may also become contaminated with microorganisms, which can proliferate during the storage and produce enzymes that rapidly degrade the seeds, transforming its components to less valuable or even toxic compounds and imparting

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them off-flavour, such as peroxides, aldehydes or ketones. Some fungi, such as certain aspergilli, can produce toxins. Producers need to apply various measures to control the chemical and microbiological deterioration of oil seeds during their storage prior to processing [4–6].

Microbial contamination of hemp seeds was previously studied in Serbia [7] and of poppy seeds in India [8]. However, those studies were limited by using traditional culture-based techniques that cannot distinguish specific taxonomic groups of microorganisms. In the present study, we obtained more detailed classification of the contaminating microorganisms by making use of the recently developed technique of high-throughput sequencing. Its contemporary effective format involves metagenomic DNA isolation from the sample, polymerase chain reaction-based amplification of a polymorphic DNA marker (16S rDNA for prokaryotes and 18S rDNA, 28S rDNA or internal transcribed spacer for eukaryotes), high-throughput sequencing on Illumina MiSeq platform (Illumina, San Diego, California, USA) and bioinformatic data processing using DNA sequence databases. A typical result of this kind of analysis is a list of taxons, usually families or genera, with their relative abundance [9–11].

By applying this molecular method, we attempted to enrich the previous knowledge on the composition of bacterial and fungal communities associated with hemp seeds and poppy seeds. Seeds from two farms in Slovakia were analysed targeting V3-V4 hypervariable region of the 16S rDNA gene for prokaryotes and D1 domain of the 28S rDNA gene for eukaryotes. Samples were analysed also by culture-based techniques to obtain a quantitative overall picture of microbial contamination. The knowledge gained could be useful for producers to improve the cleaning and sanitation procedures as well as storage conditions of hemp seeds and poppy seeds.

MATERIALS AND METHODS

Samples

Hemp seed samples were collected from a farm in Slovakia. They were collected after harvesting and after five-months storage. Poppy seed samples were obtained from two farms in Slovakia, where they were cultivated, hand-harvested and then also processed. From the first farm, seeds of the white type of poppy (cultivar Albín) were obtained. From the second farm, seeds of the same cultivar Albín of the white type of poppy were obtained, together with seeds of the blue type of poppy (cultivars Major and Zeno). The seeds were dried to moisture content of 70 $g \cdot kg^{-1}$ and stored at 10–20 °C in dry conditions so that their moisture content did not exceed 80 $g \cdot kg^{-1}$.

Culture-based microbiological analysis

Seed samples of 10 g were homogenized in 90 ml of 10 $g \cdot kg^{-1}$ bacteriological peptone (Lab M, Heywood, United Kingdom) with 85 $g \cdot kg^{-1}$ NaCl (Merck, Darmstadt, Germany) in a 400 ml Bag-Filter P bag with a hydrophilic filter of a pore size of 250 μm (Interscience, Woburn, Massachusetts, USA) using a homogenizer Stomacher 400 (Seward, Basingstoke, United Kingdom). The filtrate was used for downstream culture-based microbiological analyses. Total mesophilic aerobic bacteria were quantified according to ISO 4833-1:2013 [12]. Coliforms were determined according to ISO 4832:2006 modified by the use of Chromocult Coliform Agar (Merck) [13]. Yeasts and moulds were determined according to ISO 21527-2:2008 [14]. Coagulase-positive staphylococci were determined according to ISO 6888-2:1999 [15]. *Bacillus cereus* counts were determined according to ISO 7932:2004 [16]. Presence of *Listeria monocytogenes* was analysed according to ISO 11290-1:2017 [17]. Presence of *Salmonella* spp. was analysed according to ISO 6579-1 [18].

DNA isolation

Amounts of 5 g of seeds were washed with 10 ml sterile distilled water and the wash was centrifuged at 1 900 ×*g* for 25 min. A volume of 1 ml of the supernatant was removed and centrifuged at 10 000 ×*g* for 5 min. The sediment was subjected to DNA isolation by chaotropic solid-phase extraction using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, protocol for G^+ bacteria.

Polymerase chain reaction, library preparation and sequencing

Fragments of prokaryotic 16S rDNA covering V3 and V4 regions (341–805) were amplified by polymerase chain reaction (PCR) with primers 16S-Amplicon-F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and 16S-Amplicon-R (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'). Fragments of eukaryotic 28S rDNA covering D1 domain were amplified by PCR with primers LS2NL-Fwd (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAG TCG AGT TGT TTG GGA AT-3') and N4L-Rev (5'-GTC TCG TGG GCT CGG AGA TGT GTA

TAA GAG ACA GGG TCC GTG TTT CAA GAC GG-3'). Oligonucleotides were synthesized by Eurofins Genomics, (Ebersberg, Germany). PCR was performed in 25 μl volumes in a Veriti thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using Kapa Taq HotStart PCR kit (Kapa Biosystems, Wilmington, Massachusetts, USA), 0.12 μmol·l^{-1} primers, 0.5 μmol·l^{-1} dNTP mixture (Thermo Fisher Scientific) and 3 μl of DNA solution. The temperature programme involved initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 54 °C and 60 °C for 16S and 28S primers, respectively, and polymerization at 72 °C for 60 s. The reaction was ended by final polymerization for 7 min at 72 °C. PCR products were purified using AMPure XP magnetic beads (Beckman Coulter Genomics, Brea, California, USA) according to the manufacturer's instructions and indexed using Nextera XT Index Kit v2 Set B (Illumina) according to the manufacturer's instructions. Prepared libraries were purified by AMPure XP magnetic beads, DNA concentration was checked by fluorimetry using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and fragment integrity was checked by electrophoresis in Bioanalyzer 2100 (Agilent, Santa Clara, California, USA). Concentration of libraries was normalized to 4 nmol·l^{-1}, they were pooled, denatured by adding 0.2 mol·l^{-1} NaOH (1 : 1) and loaded to MiSeq cartridge (Ilumina). Samples were sequenced in a 300-cycle paired-end mode in MiSeq system (Ilumina). Typical read counts for individual samples were greater than 100 000.

Data processing and analysis

Sequencing data were processed using Geneious software (Biomatters, Auckland, New Zealand). Reads shorter than 50 bp and non-confident sequences ($q \geq 30$) were discarded. The forward and reverse reads were matched resulting in fragments with an average length of 450 bp covering the entire V3 and V4 region. Obtained sequences from 16S rDNA analysis were classified by Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, Maryland, USA) using 16S rDNA Biodiversity Tool with RDP database [19]. Analysis of 28S rDNA sequences was performed by MG-RAST version 4.0.3 with SILVA database [20]. Limit of sequence confidence was set to 80 %.

RESULTS AND DISCUSSION

Culture-based analysis of the rinses from the surface of hemp seeds and poppy seeds revealed a comparatively high level of microbial contamination (Tab. 1). Levels of total mesophilic aerobic bacteria for hemp seeds (10^5–10^6 CFU·g^{-1}) were similar to those previously reported in the literature [7], while those of poppy seeds (10^3–10^5 CFU·g^{-1}) were higher than those previously reported in the literature [8]. Levels of fungi (10^5 CFU·g^{-1}) for hemp seeds were higher than those previously reported in the literature [7], while those of poppy seeds (10^3–10^5 CFU·g^{-1}) were similar to those previously reported in the literature [8]. We found a significantly lower content of coliform bacteria (< 50 CFU·g^{-1}) in both poppy seeds samples of the Albín cultivar, compared to the blue poppy seeds of cultivars Major and Zeno (10^3–10^5 CFU·g^{-1}).

Levels of yeasts were not high in any sample with the exception of hemp seeds at harvest, which decreased during storage. Coagulase-positive staphylococci were not detected in any sample with the exception of hemp seeds after storage, which indicated post-harvest contamination, but their levels did not exceed the threshold for toxin production. *Bacillus cereus* was detected in hemp

Tab. 1. Results of microbiological analysis.

Seed sample	Contents [CFU·g^{-1}]					
	Total mesophilic aerobes	Coliforms	Fungi	Yeasts	Coagulase-positive staphylococci	*Bacillus cereus*
Hemp at harvest	6.6×10^5	2.3×10^4	1.1×10^5	< 1000	< 50	$< 5 \times 10^3$
Hemp after storage	4.9×10^6	4.2×10^5	1.1×10^5	< 10	3.6×10^3	5.9×10^3
Poppy Major	2.6×10^3	1.0×10^3	1.8×10^2	< 10	< 50	< 10
Poppy Zeno	7.7×10^5	2.5×10^4	4.5×10^3	< 100	< 50	< 50
Poppy Albín 1	1.2×10^3	< 50	2.7×10^1	< 10	< 50	< 50
Poppy Albín 2	3.6×10^4	< 50	1.8×10^2	< 100	< 50	< 50

Tab. 2. Composition of bacterial communities of hemp seeds.

Family	Relative abundance [%]	
	Hemp seeds after harvest	Hemp seeds after 5-months storage
Enterobacteriaceae	25.1	25.2
Pseudomonadaceae	17.5	17.7
Rhizobiaceae	8.5	7.0
Sphingomonadaceae	8.3	5.8
Xanthomonadaceae	6.2	2.6
Comamonadaceae	2.6	2.4
Flavobacteriaceae	2.3	3.5
Moraxellaceae	2.0	3.7
Oxalobacteraceae	1.6	2.7
Lactobacillaceae	1.2	1.7
Methylobacteriaceae	1.1	1.6
Microbacteriaceae	1.0	1.7
Enterococcaceae	1.1	1.0
Sphingobacteriaceae	0.0	2.3
Rhodobacteraceae	0.0	1.6
Aurantimonadaceae	0.0	1.5
Sanguibacteraceae	0.1	0.6
Others (including chloroplasts)	21.4	17.4

Average results of the analysis of three samples are presented.

Tab. 3. Composition of fungal communities of hemp seeds.

Family	Relative abundance [%]	
	Hemp seeds after harvest	Hemp seeds after 5-months storage
Pleosporaceae	14.2	14.1
Davidiellaceae	17.3	15.2
Tremellaceae	13.2	17.5
Dipodascaceae	1.6	6.1
Unclassified (derived from Hypocreales)	0.9	8.2
Unclassified (derived from Dothideomycetes)	3.6	2.3
Unclassified (derived from Filobasidiales)	1.7	0.4
Phaeosphaeriaceae	0.7	1.0
Phyllachoraceae	0.6	1.1
Nectriaceae	1.4	1.4
Leptosphaeriaceae	1.2	0.5
Sporormiaceae	0.0	0.4
Others (including plants)	43.6	31.8

Average results of the analysis of three samples are presented.

seeds after storage, which again indicated post-harvest contamination and might be significant if the seeds would be used for food purposes without proper heat treatment. *Listeria monocytogenes* and *Salmonella* spp. were not detected in any sample (data not shown).

High-throughput sequencing of amplified 16S rDNA for hemp seeds after harvest and after 5-months storage showed that the bacterial community consisted mainly of families Enterobacteriaceae and Pseudomonadaceae (Tab. 2). The sequencing data were analysed also on the genus level but the results were apparently skewed due to low confidence at most of the sequences (data not shown). The dominant bacterial families remained stable during 5-months storage, while some increase in relative abundance was detected for Oxalobacteraceae, Sphingobacteriaceae, Comamonadaceae, Microbacteriaceae, Moraxellaceae, Lactobacillaceae, Enterococcaceae and Sanguibacteraceae. The increase in Moraxellaceae may be important regarding safety of employees handling the seeds, as some species cause respiratory tract infections [21]. The increase in lactic acid bacteria may be important because it is accompanied by the production of acids with a decrease in pH, which is favourable regarding enzymatic lipid oxidation [22].

High-throughput sequencing of amplified 28S rDNA for hemp seeds after harvest and after 5-months storage showed that the fungal community consisted mainly of families Pleosporaceae, Davidiellaceae and Tremellaceae (Tab. 3). Analysis of the sequencing data on the genus level suggested that the main genera of Pleosporaceae were Alternaria and Lewia, while the main genus of Davidiellaceae was Cladosporium. However, the values of relative abundance were apparently skewed due to low confidence at most of the sequences (data not shown). The dominant fungal families remained stable during 5-months storage, while a remarkable relative increase was detected for Dipodascaceae and unclassified fungi derived from Hypocreales. The increase in the latter fungal families should not pose a technological or safety threat.

Results of high-throughput sequencing of amplified 16S rDNA for poppy seed samples are presented in Tab. 4. The results showed comparatively high levels of Enterobacteriaceae, Pseudomonadaceae and Xanthomonadaceae (the latter apparently replaced by Streptomycetaceae in the bacterial community of Zeno cultivar seeds). The analysis of the sequencing data on genus level suggested that the main genus of Enterobacteriaceae was *Klebsiella* but further information was

difficult to deduce as the results were apparently skewed due to low confidence at most of the sequences (data not shown). The results regarding the samples of Albín cultivar from the second farm showed a markedly lower level of Enterobacteriaceae and presence of several bacterial families absent from the first farm, which probably reflects the better hygienic conditions associated with the lower volume of production, together with the higher altitude and cooler climate of the fields.

Results on fungal communities of poppy seed samples as provided by high-throughput sequencing of amplified 28S rDNA are presented in Tab. 5. The results showed that the fungal community consisted mainly of families Davidiellaceae, Pleosporaceae, Trichocomaceae and a group related to *Trichothecium*. Analysis of the sequencing data on the genus level suggested that the main genera of Pleosporaceae were *Alternaria* and *Lewia*, the main genus of Davidiellaceae was *Cladosporium* and the main genus of Trichocomaceae was *Aspergillus*.

The study demonstrated the power of amplicon-based high-throughput sequencing, which gained comprehensive knowledge of the bacterial and fungal communities of the seeds. Relevant levels of pseudomonads in hemp seeds and in poppy seeds were identified, which is an important information as these bacteria are persistent in the environment, proliferate at temperatures close to zero and are equipped with a range of degradative enzymes, in particular oxygenases, that can degrade or transform fatty acids that will reduce the quality of oil produced from the seeds [23, 24]. Presence of *Aspergillus* might be a concern if toxinogenic species were involved [25, 26].

CONCLUSION

The study demonstrated a comparatively high level of contamination of hemp seeds by bacteria and fungi, involving contamination by *Bacillus cereus* at 10^3 CFU·g^{-1}, after 5-months storage. High-throughput sequencing revealed that the bacterial community of hemp seeds consisted mainly of families Enterobacteriaceae and Pseudomonadaceae, while the fungal community was composed mainly of Pleosporaceae (genera *Alternaria* and *Lewia*), Davidiellaceae (genus *Cladosporium*) and Tremellaceae. In poppy seeds, the bacterial community was found to be composed mainly of Enterobacteriaceae (genus *Klebsiella*), Pseudomonadaceae and Xanthomonadaceae or Streptomycetaceae. The fungal community was composed mainly of Pleosporaceae

Tab. 4. Composition of bacterial communities of poppy seeds.

Family	Relative abundance [%]			
	Major	Zeno	Albín 1	Albín 2
Enterobacteriaceae	37.5	72.3	55.7	17.6
Xanthomonadaceae	10.9	0.5	9.5	9.2
Pseudomonadaceae	9.8	4.2	6.4	14.9
Streptomycetaceae	0.0	11.8	0.3	1.2
Rhizobiaceae	18.1	2.5	7.1	2.5
Microbacteriaceae	4.6	0.8	4.2	8.6
Moraxellaceae	3.0	0.5	2.5	1.4
Nocardiaceae	3.4	0.8	4.0	0.8
Micrococcaceae	5.4	0.3	0.4	0.2
Sphingobacteriaceae	0.7	0.1	0.4	2.9
Sphingomonadaceae	1.2	0.0	1.6	0.6
Methylobacteriaceae	1.0	0.5	1.3	0.5
Phyllobacteriaceae	0.0	0.3	1.4	0.9
Enterococcaceae	0.1	0.2	1.8	0.6
Sanguibacteraceae	0.2	0.0	0.2	1.7
Pseudonocardiaceae	0.0	1.9	0.1	1.1
Promicromonosporaceae	0.0	1.4	0.1	0.7
Comamonadaceae	0.3	0.0	0.1	4.8
Oxalobacteraceae	0.3	0.0	0.0	1.0
Others	3.7	2.2	2.6	23.4*

Average results of the analysis of three samples are presented.
* – others including Alcaligenaceae (3.9 %), Brucellaceae (6.4 %), Rhodobacteraceae (2.0 %), Nocardiopsaceae (1.8 %), Caulobacteraceae (1.8 %), Hyphomicrobiaceae (1.7 %) and Chromatiaceae (1.0 %).

Tab. 5. Composition of fungal communities of poppy seeds.

Family	Relative abundance [%]			
	Major	Zeno	Albín 1	Albín 2
Pleosporaceae	23.7	39.2	18.3	34.4
Davidiellaceae	32.1	38.7	48.0	29.5
Tremellaceae	5.3	0.2	6.0	0.6
Filobasidiaceae	6.3	0.0	6.1	1.1
Phyllachoraceae	1.8	4.1	2.9	2.9
Unclassified (derived from Erythrobasidiales)	1.2	0.0	1.4	0.1
Unclassified (derived from Hypocreales)	1.0	3.8	1.3	1.9
Sclerotiniaceae	0.7	1.6	1.0	0.4
Unclassified (derived from Wallemiales)	10.7	0.0	0.0	0.0
Trichocomaceae	8.4	0.1	0.0	1.6
Trichothecium group	0.0	0.0	0.0	14.3
Nectriaceae	0.0	0.8	0.2	2.6
Taphrinaceae	0.0	0.0	0.0	5.1
Others (including plants)	8.8	11.5	14.8	5.5

Average results of the analysis of three samples are presented.

(genera *Alternaria* and *Lewia*) and Davidiellaceae (genus *Cladosporium*), while some samples were characterized by increased levels of Trichocomaceae (genus *Aspergillus*). The levels and composition of microbial contamination does not pose a threat from technological and safety point of view. However, the detected presence of pseudomonads and *Aspergillus* in certain samples may indicate that more efficient cleaning and sanitation will be required, as they could affect the products of processing of the seeds.

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