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Potential of high-throughput sequencing for broad-range detection of pathogenic bacteria in spices and herbs

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A broad-range culture-independent method was developed and evaluated regarding its sensitivity of detection of pathogenic bacteria in spices and herbs, with focus on paprika powder. The method involved DNA extraction using cetyltrimethylammonium bromide (CTAB), 16S rDNA amplification using universal bacterial polymerase chain reaction, and high-throughput sequencing on Illumina MiSeq platform. The sensitivity of the method was evaluated with series of model samples contaminated at different levels with *Salmonella enterica* and *Escherichia coli* (as representatives of Gram-negative bacteria) and *Staphylococcus aureus* (as a representative of Gram-positive bacteria). For spices (paprika, black pepper), the method had a screening-level sensitivity with limits of detection in the range of 10^4–10^5 CFU/g, and a semi-quantitative response. Low sensitivity (LOD $\geq 10^7$ CFU/g) was observed with herbs (oregano, parsley). The developed method demonstrated a good potential for microbiological screening of spices, with a prospect of further improvement of sensitivity based on progress in high-throughput sequencing technology.

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1. Introduction

Spices and herbs are widely used as additives to foods either at culinary preparation of meals at home or at industrial production of processed foods. These minor components of foods are also known as important vehicles of pathogenic bacteria, thus contributing to the food-borne illness burden. In this regard, *Salmonella enterica* is the most frequent pathogenic bacterium in spices, followed by *Bacillus* spp. and *Clostridium perfringens* (EFSA, 2013; Food and Agriculture Organization of the United Nations (FAO) & World Health Organization (WHO), 2014; Lehmacher, Bockemühl, & Aleksic, 1995; Van Doren et al.,2013; Zweifel & Stephan, 2012).

As certain types of spices and herbs are imported from geographically distant countries, they can be contaminated with microorganisms that are unusual in the place of processing and consumption. The microbial contamination is, in case of this commodity, more likely to reach the consumer as spices and herbs can only to a limited extent be antimicrobially treated prior to packing and distribution, as this would affect their organoleptic properties. Due to the established practice of controlling only selected pathogenic, toxinogenic and indicator microorganisms, "exotic" or unusual microorganisms may escape detection by routine control laboratories. The coverage of the control system could be improved if a broad-range detection method is available. However, such method should be sensitive enough to be compatible with food safety requirements. A candidate for this purpose is the 16S rDNA-based metagenomic approach, which has become recently more attractive as the supporting technology, high-throughput sequencing, has become widely available (Ju & Zhang, 2015; Karlsson et al., 2013; Mayo et al., 2014).

In case of characterization of bacterial consortia, the approach is based on isolation of total DNA from the sample, amplification of a fragment of 16S rDNA, which is universally present in bacteria, high-throughput sequencing and data processing in order to

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identify individual taxons or groups of taxons. Isolation of DNA always needs to be adapted to the matrix, taking into account its physical and chemical properties, and should produce amplifiable DNA. Regarding spices and herbs, a wide evaluation of DNA isolation methods (both based on solid-phase extraction, SPE, and liquid-liquid extraction, LLE) was performed previously and the LLE technique with treatment with cetyltrimethylammonium bromide (CTAB; Jankiewicz, Broll, & Zagon, 1999) was adapted to a range of matrices of this type, such as ground red paprika, black pepper, cinnamon, parsley, oregano and thyme. The adaptation consisted in using different volumes of solutions and different centrifugation speeds (Minarovičová et al., 2017).

This study was aimed at adaptation and evaluation of the metagenomic approach based on high-throughput sequencing for the detection of pathogenic and toxinogenic bacteria in spices and herbs. Because the power of the approach at characterization of diversity in microbial consortia is well established, the main parameter of our interest was the sensitivity of the approach. The evaluation was based on determination of detection limits using series of model samples of paprika (as a representative of spices) and oregano (as a representative of herbs) contaminated at different levels with *Salmonella enterica* or *Escherichia coli* (as representatives of Gram-negative bacteria) and *Staphylococcus aureus* (as a representative of Gram-positive bacteria). Additionally, the potential of *Staph. aureus* detection in black pepper and parsley were also analysed. In order to eliminate possible strain-specific properties, regarding susceptibility of bacterial cells to lysis and subsequent DNA extractability, mixtures of three strains were always used for preparation of model samples.

2. Materials and methods

2.1. Plant material used

Dried fruits of paprika (*Capsicum annuum*) and black pepper (*Piper nigrum*), ground to the particle size of 140 μm–355 μm and <500 μm respectively, microbiologically decontaminated by steam treatment, and untreated dried leaves of oregano (*Oreganum vulgare*) and parsley (*Petroselinum crispum*), ground to the particle size of <355 μm and <300 μm respectively, were obtained from FUCHS Gewürze GmbH, Dissen, Germany. The samples were specifically prepared for research use in frames of the SPICED project.

2.2. Microorganisms

Salmonella Infantis Lj 9 was a food isolate from University of Ljubljana, Slovenia; *S.* Typhimurium CCM 4419 was a collection strain from Czech Collection of Microorganisms, Brno, Czech Republic; *S.* Enteritidis SVU 26 was a food isolate from Veterinary and Food Institute in Bratislava, Slovakia. *Escherichia coli* CCM 2024 and *E. coli* CCM 3988 were collection strains from Czech Collection of Microorganisms, Brno, Czech Republic, and *E. coli* SZU 106 was a food isolate from National Institute of Public Health, Center for Health, Nutrition and Food, Brno, Czech Republic. *Staphylococcus aureus* NCTC 10656 was a collection strain obtained from National Collection of Type Cultures, Salisbury, United Kingdom, *Staph. aureus* VUP 622 was isolated from food and identified in Food Research Institute NAFC, Bratislava, Slovakia, and *Staph. aureus* HPL 468/1 was a clinical isolate from HPL Laboratories of Clinical Microbiology, Bratislava, Slovakia. Pure cultures of each strain were grown for 16–18 h in Brain Heart Broth (Merck, Darmstadt, Germany) at 37 °C with shaking of 2 Hz.

2.3. Preparation of model samples

Pure bacterial suspensions of three strains of each species were mixed at a ratio of 1:1:1; (v/v/v). The obtained mixture was decimally diluted (10^1–10^7-fold) with 9 g/L NaCl. Densities of diluted bacterial suspensions were checked by plating on Baird-Parker agar (Merck) for *Staph. aureus,* on Xylose lysine deoxycholate agar (Merck) for *S. enterica*, and on Chromocult coliform agar (Merck) for *E. coli.* The plates were incubated at 37 °C and colonies were counted after 24 h and 48 h. An amount of 1 g of dried mild paprika powder was contaminated with 100 μL of a strain mixture of a specified density, to obtain contamination levels ranging from 10^1 to 10^7 CFU per gram of matrix.

2.4. DNA extraction

DNA was extracted from samples, including uncontaminated matrices, by a chaotropic liquid-liquid extraction (LLE) method using cetyltrimethylammonium bromide (CTAB; Official Collection of Test Methods, 1998; Jankiewicz et al., 1999) modified by Minarovičová et al. (2017). The success of DNA extraction from samples with different contamination levels was checked by real-time polymerase chain reaction (PCR) according to Minarovičová et al. (2017). DNA from bacterial strains was extracted by chaotropic solid-phase extraction using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

2.5. DNA amplification

A fragment of 16S rDNA was amplified by PCR using a temperature programme composed of initial denaturation step (95 °C for 2 min), followed by 35 cycles (94 °C for 1 min; 54 °C for 1 min; 72 °C for 2 min) and final polymerization step (72 °C for 10 min). Each sample of extracted DNA was amplified in 6 replicates to increase the amount of the product. The reaction volume (25 μL) contained 2.5 μL Cheetah Taq Dilution Buffer (Biotium, Hayward, CA, USA), 1.5 mM $MgCl_2$, 250 μM of each dNTP (Applied Biosystems, Foster City, CA, USA), 500 nM of primer 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′; Lane, 1991), 500 nM of primer 1062R (5′-ACA GCC ATG CAG CAC CT-3′; Youssef et al., 2009; both oligonucleotides synthesized by Sigma-Aldrich), 1.5 U Cheetah Hotstart Taq DNA polymerase (Biotium) and 3 μL of the template DNA solution. PCR was carried out in a Veriti thermocycler (Applied Biosystems). After PCR, the products were analysed by 1.5% agarose gel electrophoresis using 100 bp DNA Ladder (Biolabs, Ipswich, MA, USA) to check the amplicon homogenity, replicates were pooled and purified by QIAquick PCR Purification Kit (Qiagen).

2.6. High-throughput sequencing

PCR product clean-up was carried out by Zymo DNA Clean and Concentrator-5 (Zymo Research, Irvine, CA, USA) according to the standard protocol. Sample quantification was carried out by Qubit High sensitivity assay (Life Technologies, Carlsbad, CA, USA), which enabled precise concentration determination and further sample dilution to 0.2 ng/μl. An amount of 0.5 ng of sample in a volume of 2.5 μL was used for transposome-based shot-gun library preparation using Nextera XT Library Preparation Kit (Illumina, San Diego, CA, USA) by a protocol optimized to the initial amount of sample. All amplicons (also shorter than 500 bp) generated by sample tagmentation and further PCR amplification were recovered by use of 1.8× Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). The molarity of the final DNA library was assessed and calculated from library fragment size (bp) determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and library

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