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Influence of sampling and DNA extraction on 16S rRNA gene amplicon sequencing - Comparison of the bacterial community between two food processing plants

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ABSTRACT

16S rRNA amplicon sequencing is frequently used to investigate and compare microbiomes. This study focuses on the potential, applicability and limits of 16S rRNA amplicon sequencing for comparing the microbiomes of two dairy production plants, one of which experienced recurrent contamination with *Listeria monocytogenes*. During preceding *in vitro* studies, DNA extraction of *Listeria monocytogenes* and *Salmonella enterica* ser. Typhimurium with two extraction kits (*PowerSoil* and *NucleoSpin*) revealed differences between these exemplary bacteria and both extraction methods. When equivalent samples of the companies were processed independently with both methods, sequencing results demonstrated also bacterium specific variances. These variances do not reflect a direct correlation between Gram-positive or -negative bacteria and the performance of both kits. Despite these differences, the analysis with a non-metric multi-dimensional scaling plot showed grouping of respective samples independent of the DNA extraction method.

Some operational taxonomic units (OTUs) occurred predominantly in one of the companies, but none of them generated a clear hypothesis why one of the plants had recurrent contamination with *L. monocytogenes*. This work provides insights into the microbiomes of the two dairy companies but advises cautions in over-interpreting sequencing results as selective DNA extraction and sampling only reflect components of prevalent bacterial communities.

1. Introduction

Milk products are among those regularly involved in food related outbreaks caused by bacterial hazards (Choi, Lee, Lee, Kim, & Yoon, 2016; FAO, 2013; Oliver, Jayarao, & Almeida, 2005; Verraes et al., 2015). Contamination with pathogens, such as *Listeria monocytogenes,* can occur at various steps of milk processing. One main contamination source is the raw milk, but contamination can also occur via the food processing environment as *L. monocytogenes* readily survives outside the product (Kousta, Mataragas, Skandamis, & Drosinos, 2010). This necessitates regular disinfection hygiene programs to prevent long-term survival of bacteria (Carpentier & Cerf, 2011; Kousta et al., 2010). Further, regular monitoring is essential to verify the efficacy of hygiene measures and for early detection of contamination (Marriott & Gravani, 2006).

Conventional microbiological culture methods are frequently used for monitoring work areas in the food industry, but molecular methods are more and more applied. PCR (polymerase chain reaction) and qPCR (quantitative PCR) are now routinely used for monitoring (Mester et al., 2017). However, 16S rRNA gene amplicon sequencing has also been applied in dairy production, for instance for investigating microbiomes in drain water (Dzieciol et al., 2016).16S Illumina sequencing pertains to the second-generation sequencing methods and uses adapters on primers so they can be densely coated to a solid substrate (Shendure & Ji, 2008). Like other new generation sequencing technologies such as 454 sequencing, SOLiD or HeliScope, this method is associated with

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higher error rates than traditional Sanger sequencing, but it is suitable for high throughput applications (Kircher & Kelso, 2010).

For most efficient and reproducible sequencing, straightforward, streamlined sampling and DNA extraction are indispensable. However, the influence of these steps is often neglected although the assortment of methods for DNA extraction is quite extensive. Several research groups investigated nonetheless the influence of DNA extraction on sequencing results: For instance, Kennedy et al. (2014) demonstrated significant differences in sequencing results when DNA of fecal samples was extracted with two different kits. Further studies with different research backgrounds also revealed the impact of the DNA extraction protocol on sequencing results (e.g. Vishnivetskaya et al., 2014; Willner et al., 2012; Yuan, Cohen, Ravel, Abdo, & Forney, 2012). Such studies investigating various DNA extraction methods point to the impression that for each question, environment and sample type different DNA extraction methods were appropriate. However, other steps in the sequencing pipeline can also bias the results. For example, the selection of primers is a topic often discussed. Since primers are never completely universal, here are considerable biases for 16S rRNA gene amplicon sequencing (e.g. Baker, Smith, & Cowan, 2003; Klindworth et al., 2013; Tremblay et al., 2015).

The aim of this study was to analyze the impact of the two DNA extraction kits *PowerSoil* and *NucleoSpin* for comparing them in respect of sequencing results from samples derived from two dairy production companies. These methods were chosen because the *PowerSoil* kit is optimized for environmental samples often used for next generation sequencing. Since L. *monocytogenes* is a threat to dairy products (Farber & Peterkin, 1991) and DNA extraction of the Gram-positive *Listeria* is more challenging than extraction from Gram-negative bacteria, the *NucleoSpin* Kit that is more suitable for this purpose and subsequent qPCR analysis was chosen (Rossmanith, Krassnig, Wagner, & Hein, 2006). Furthermore, aim of this study was the determination whether recurrent contamination with *L. monocytogenes* in one facility might be associated with a distinct constellation of flora compared with the other facility and whether the sequencing methodology had applicability in addressing this question.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes EGDe (1/2a, internal number 2964), Δ*prfA L. monocytogenes* EGDe (1/2a) and *Salmonella enterica* ssp. *enterica* serovar Typhimurium belong to of the collection of the Institute of Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna, Austria. All bacterial strains were grown overnight in tryptone soya broth with 0.6% yeast extract (TSB-Y; Oxoid, Hampshire, UK) at 37 °C.

2.2. DNA extraction

2.2.1. NucleoSpinTissue kit

DNA was isolated using the *NucleoSpin* Tissue kit (Macherey Nagel, Düren, Germany) following protocol instructions for Gram-positive bacteria. The DNA was eluted twice with 50 µl ddH_2O (70 °C).

2.2.2. PowerSoil DNA isolation kit

DNA was isolated using the *PowerSoil* DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, USA), according to the manufacturer's instructions. The DNA was eluted twice with 50 µl ddH_2O (70 °C).

2.3. Sampling

Surfaces were swabbed using 3M™ Sponge-Sticks soaked with Buffered Peptone Water Broth, type SSL 10BPW (3M, St. Paul, Minneapolis, USA) as recommended by the manufacturer two times swabbing zigzagways. In the second round the swab was turned over and swabbing direction was changed 90°.

To determine swab uptake, 1 ml of three different concentrations of *L. monocytogenes* Δ*prfA* (10^2 cfu/ml, 10^4 cfu/ml, 10^7 cfu/ml) were distributed on the sterilized worktop surfaces (30 cm × 30 cm) and allowed to dry. After swabbing, the swabs were transferred to Stomacher bags containing 100 ml TSB-Y and homogenized for 2 min with a Stomacher device. The solutions were plated directly onto TSA-Y (tryptone soya agar with 0.6% yeast extract) after centrifugation or dilutions. Enumeration of bacterial suspensions was performed using the plate count method. For swab release, 1 ml of *L. monocytogenes* Δ*prfA* was directly added to a swab. As control, 1 ml *L. monocytogenes* Δ*prfA* was directly transferred into Stomacher bags.

2.4. Sample processing

Samples were collected in two different European dairy production plants producing different types of cheeses at equivalent sites (smear waters, salt baths (≈20% NaCl), gullies, smear robots, pallets, cheese boards, floors, the area before the hygiene sluice and air). Liquids (smear waters, salt baths and gully water) were collected in bottles while surfaces (smear robots, pallets, cheese boards, floors, the area before the hygiene sluice) were swabbed using Sponge-Sticks as described in 2.3. The “air” samples were swabs exposed to the air for 2 h. Sampling has taken place for each company within 4 h on two different days in the same calendar week.

Respective swab samples were pooled (when more than one swab was used for one sample type) in plastic bottles containing 250 ml of 1 × PBS (Phosphate Buffered Saline) including 0.05% Tween 80 (PBST). After 30 min agitation in a water bath (200 rpm), samples were centrifuged (all centrifugation steps: 30 min, 3200 g) and the gained pellets stored in the fridge. Afterwards, swabs were transferred to 100 ml of PBST and homogenized with a Stomacher device (2 min). Samples were then centrifuged, merged with the first pellet and resuspended in 2 ml Ringer solution. Bacteria samples were split and 1 ml each used for *NucleoSpin* and *PowerSoil* DNA extraction.

One liter samples of salt baths and gullies were centrifuged and pellets split for DNA extraction as described above. 0.5 L smear water samples were centrifuged, and since the pellets obtained reached a critical size for further processing, they were resuspended with Matrix-Lysis-buffer IV (including $MgCl_2$) for removal of fat, carbohydrates and proteins (Mester, Schoder, Wagner, & Rossmanith, 2014), again centrifuged and the resulting pellet used for DNA extraction as before.

DNA concentration was measured using the Qubit ds Broad Range Kit (Fisher Scientific, Vienna, Austria).

2.5. Sequencing of the V3-V5 region of bacterial 16S rRNA genes

16S rRNA PCRs, amplicon sequencing and library preparation were performed with the Illumina MiSeq sequencing platform using pair-end sequencing protocol (Microsynth AG, Balgach, Switzerland). The V3-5 hypervariable region of bacterial 16S rRNA genes was amplified using primers 341F (5′-CCTACGGGRSGCAGCAG-3′) and 909R (5′-TTTCAGYCTTGCGRCCGTAC-3′) as described before by Metzler-Zebeli et al. (2015). In total, 800,000 reads were obtained.

2.6. Sequencing analysis using mothur

Sequence processing was determined using the *mothur* software, version v1.34.4 according to MiSeq SOP (standard operating procedure) written by the software's authors (Schloss et al., 2009) (www.mothur.org): The two overlapping paired-end reads were combined to contigs. The minimum sequence length was defined as 500 base pairs with an average quality score of more than 30. The maximal number of ambiguous bases was zero; the maximal number of homopolymers was