Meat Science 117 (2016) 36–40

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Psychrophile spoilers dominate the bacterial microbiome in musculature samples of slaughter pigs

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ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 8 February 2016
Accepted 22 February 2016
Available online 24 February 2016

Keywords:
16S rRNA pyrosequencing
Musculature
Microbiome
Contamination
Bacterial
Slaughter
Microbiota

ABSTRACT

The aim of this study was to disentangle the microbial diversity on porcine musculature. The hypervariable V1–V2 region of the 16S rRNA gene was amplified from DNA samples of clinically healthy slaughter pigs (n = 8). Pyrosequencing yielded 37,000 quality-controlled reads and a diverse microbiome with 54–159 OTUs per sample was detected. Interestingly, 6 out of 8 samples were strongly dominated by 1–2 highly abundant OTUs (best hits of highly abundant OTUs: *Serratia proteamaculans*, *Pseudomonas syringae*, *Aeromonas allosaccharophila*, *Brochothrix thermosphacta*, *Acidiphilium cryptum* and *Escherichia coli*). In 1 g musculature scraping, 3.20E+06 16S rRNA gene copies and 4.45E+01 *Enterobacteriaceae* rRNA gene copies were detected with qPCR. We conclude that i.) next-generation sequencing technologies help encompass the full content of complex, bacterial contamination, ii.) psychrophile spoilers dominated the microbiota and iii.) *E. coli* is an effective marker species for pork contamination, as it was one of very few abundant species being present in all samples.

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1. Introduction

Microbial contamination during pig slaughter and pork processing is often a hidden process which is complex to trace and monitor (Choi et al., 2013; Giovannacci et al., 2001). The microbial load of meat strongly depends on the spread of microorganisms during carcass-processing in slaughterhouses (Sheridan, 1998). Preventive approaches, such as hygiene compliance, and meat inspection carried out strictly according to regulations are the basis of good practice during slaughter (Brunner, Marx, & Stolle, 1997). However, if a contamination occurs, pork provides a manifold niche for microbes because it has a high water content, is rich in nutrients and is often for longer time spans stored until processing. Meat spoilage results in discoloration, off-odors, slime or reduced storage life, all being criteria for consumers' complaint or meat rejection. Aside from product and economical losses, the presence of pathogenic microorganisms (and/ or their toxins) can cause foodborne diseases and should be prevented in raw meat, regardless of further production steps (Sofos, 2005). Pork-specific spoilage organisms (SSO) were determined by culture dependent and independent methods in the last decades (Borch, Kant-Muermans, & Blixt, 1996; Jiang et al., 2010; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008; Schirmer, Heir, & Langsrud, 2009). In considering that most of pathogenic microbes detected on raw meat are regarded to originate from the animal that provides the meat (Gill, 2005), it becomes obvious that plenty microbes originate from the porcine gut microbiota and from the pig's environment prior to slaughter (Koutsourmanis & Sofos, 2004). Psychrotrophic microbes were found on machines and surfaces of slaughter equipment within abattoirs and are known to contaminate pork (Gill, 2005). The consortium of microbes commonly found in meats was previously presented, consisting of 42 genera (Nychas et al., 2008). The proteolytic bacteria *Pseudomonas* spp. and *Serratia* spp., members of the psychrotolerant *Enterobacteriaceae*, were mostly found to be responsible for metabolizing abundant nutrient sources and to produce meat spoilage (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006). Also *Brochothrix* spp. and lactic acid bacteria are known to be abundant members of the aerobic spoilage community (Doulgeraki, Ercolini, Villani, & Nychas, 2012; Holzapfel, 1998; Hultman, Rahkila, Ali, Rousu, & Bjorkroth, 2015). Other genera isolated frequently from fresh pork were *Acinetobacter*, *Aeromonas*, *Enterococcus* and *Moraxella* (Nychas et al., 2008). Despite high efforts with conventional cultivation and cultivation-independent based methods, the full

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http://dx.doi.org/10.1016/j.meatsci.2016.02.034
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bacterial spectrum on fresh pork directly after slaughter was not described until now. However, safety issues associated with microbial populations in food production areas deserve a detailed characterization (Xu, He, & Zhang, 2013).

High-throughput sequencing (HTS) overcomes the limitation of biased cultivation and timely DNA cloning and disentangles the complete bacterial diversity in a sample. Meat microbiology has already benefited from the advances of HTS (Ercolini, 2013). Shifts during ripening of fresh pork sausages and changes under treatment with lactate–diacetate were explored and linked to chemical changes in the sausage matrix (Benson et al., 2014) and the bacterial microbiomes of a meat processing plant were identified, comprising mainly of psychrotrophs (Hultman et al., 2015).

The aim of this study was to explore the full spectrum of microbes on pig musculature directly after slaughter. Therefore, eight carcass halves were sampled at a slaughterhouse in Austria and processed with a high-throughput sequencing pipeline. Individual microbiome compositions were found for each sample and the total amount of 425 bacterial phylotypes confirmed the complexity of contamination events during slaughter. Psychrophile spoilers dominated the bacterial flora.

2. Methods

2.1. Sampling of musculature tissues, DNA extraction and pyrosequencing

Porcine musculature was sampled at an IFS (International Food Standard) certified slaughterhouse in Austria, where meat inspection was carried out by local veterinarians. Musculature scrapings cranial to the first rib (area scraped = 10 cm^2) were taken from eight slaughter pigs which originated from three different farms. Genomic DNA was extracted from 250 mg of musculature scraping material using the PowerSoil™ DNA Isolation kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. The DNA concentration was determined by a Qubit® Fluorometer (Invitrogen, Carlsbad, USA) and adjusted to 25 ng/μl in DEPC-treated water (Thermo Fisher Scientific, Vienna, Austria).The V1/V2 region of the 16S rRNA gene was amplified by using the primer panel F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg, Barns, Pelletier, & Lane, 1991) and R357 (5′-CTGCTGCCTYCCGTA-3′) (Dorsch & Stackebrandt, 1992) PCR reactions and conditions used were previously reported (Mann, Dzieciol, Metzler-Zebeli, Wagner, & Schmitz-Esser, 2014). Amplicons were purified (Transgenomic Inc., Omaha, USA) and eluted and DNA concentrations were determined with a PicoGreen® dsDNA Assay Kit (Life Technologies, Carlsbad, USA). PCRs of pooled samples were performed with the GS Titanium MV emulsion PCR Kit (Roche 454 Life Science, Branford, CT) and sequencing was performed with the GS-FLX Titanium Sequencing Kit "XLR70" (Roche 454 Life Science) at the Center for Medical Research, Core Facility Molecular Biology, Medical University Graz, Austria.

2.2. Bioinformatics

All sequences were processed with the software mothur, version 1.34.0 (Schloss et al., 2009), based on a published workflow (Schloss & Westcott, 2011). Sequences with low quality (a minimum average quality score of 35 in a window size of 50 bp) and short sequences with a minimum sequence length of under 162 bp were filtered out. Primers and barcodes were trimmed and a filter for maximal homopolymer length of 8 was applied. Chimeric sequences and sequencing errors were removed with "pre.cluster" and "chimera.uchime" commands, which are implemented in mothur. Uncorrected pairwise distances were used for the assignment of OTUs (operational taxonomic units). OTUs were calculated based on a distance limit of 0.03 (97% similarity) and OTUs containing less than five sequences were removed. The RDP naïve Bayesian rRNA Classifier and the SILVA SSU reference database v102 (Pruesse et al., 2007; Wang, Garrity, Tiedje, & Cole, 2007) were used for taxonomic classification of sequences and a taxonomy was assigned to all OTUs. Diversity indices were calculated with bootstrapped sequencing data in Explicet (Robertson et al., 2013). Explicet was also used for the Bray–Curtis analysis and for the visualization of all OTUs detected. The 30 most abundant OTUs were also classified against type strains using the online Greengenes database http://greengenes.lbl.gov (DeSantis et al., 2006). The heatmap was created using JColorGrid (Joachimiak, Weisman, & May, 2006).

2.3. Quantitative real-time PCR

Total copy numbers of 16S rRNA genes were determined using DNA of musculature samples. Each amplification reaction was pipetted in duplicate with Brilliant III SYBR Green qPCR low ROX master mix (Agilent Technologies, Vienna, Austria), using the forward primer 341F (5′-CCTACGGGAGGCAGCAG-3′) and the reverse primer 518R (5′-ATTACCGCGGCTGCTGG-3′) (400 nmol; Microsynth, Balgach, Switzerland) (Muyzer, de Waal, & Uitterlinden, 1993), and 1 μl DNA/cDNA. All reactions were performed with an initial denaturation step at 95 °C (3 min), followed by 40 cycles of 95 °C for 5 s and 61 °C for 20 s with a fluorescence measurement at the last step of each cycle. Copy numbers of *Enterobacteriaceae* were determined with Brilliant III SYBR Green qPCR low ROX master mix (Agilent Technologies, Vienna, Austria), using the forward primer Eco1457F (5′-CATTGACGTTACCCGCAGAAGAAGC-3′) and the reverse primer Eco1652R (5′-CTCTACGAGACTCAAGCTTGC-3′) (400 nmol; Microsynth, Balgach, Switzerland) (Bartosch, Fite, Macfarlane, & McMurdo, 2004). *Enterobacteriaceae*-specific 16S rRNA gene primers covered the genera *Citrobacter*, *Cronobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Pantoea*, *Pectobacterium* and *Shigella*. The temperature profile was the same as for total copy number real-time PCR, with the exception of the annealing temperature (63 °C). A melting curve ranging from 70 °C to 90 °C, with fluorescence measurements at 1 °C intervals, was done after all real-time PCRs to determine the specificity of the reaction. Negative controls were pipetted in duplicates. For the template DNA used in standard curves, a purified PCR product (universal primer set 27F-1492R) of a mixed DNA sample consisting of 1 μl DNA from 3 musculature samples was used (Metzler-Zebeli et al., 2013). Standard curves were included in each qPCR assay. Quantitative PCRs were performed using a Stratagene Mx3000P real-time PCR System (Agilent technologies, Santa Clara, USA).

3. Results

Over 37,000 pyrotags were generated with pyrosequencing (between 3300 and 5300 pyrotags per sample). Sequences were compared to the reference database SILVA SSU v102 and a quantitative estimation on OTU abundances in each musculature sample was given. In total, 425 OTUs were assigned and between 54 and 159 OTUs (median = 74 OTUs) were found on each musculature sample. Species richness and diversity indices are listed in the Supplementary Table 1. Heterogeneity measures and OTU richness calculations were in accordance with the actual number of OTUs observed. Rarefaction curves were calculated for all samples to ensure that sequencing depth was sufficient: OTUs identified were plotted as a function of sequences obtained per sample and high diversity coverage was achieved with all curves reaching asymptotes (Supplementary Fig. 1A). Interestingly, in musculature samples 2, 3, 4, 6, and 7 a high proportion of low abundant OTUs was dominated by a few highly abundant OTUs. In musculature samples 1 and 5 the microbiome consisted of rare and moderately abundant OTUs, but no clear dominance of one OTU was observed. This is depicted in the rank abundance curves (Supplementary Fig. 1B). These two samples also harbored a more complex community compared to other samples (156 and 159 OTUs observed). In a next step, the microbiomes of samples were compared to each other. The dissimilarity on community level among musculature samples was demonstrated by using the Bray–Curtis analysis (Fig. 1A). A high dissimilarity >70% was found for

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