International Journal of Food Microbiology 223 (2016) 33–40

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Short communication

Bacterial diversity of floor drain biofilms and drain waters in a *Listeria monocytogenes* contaminated food processing environment

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form 3 February 2016
Accepted 5 February 2016
Available online 6 February 2016

Keywords:
Drain water
Drain biofilm
Floor drain
16S rRNA gene pyrosequencing
Bacteria
Listeria monocytogenes

ABSTRACT

Sanitation protocols are applied on a daily basis in food processing facilities to prevent the risk of cross-contamination with spoilage organisms. Floor drain water serves along with product-associated samples (slicer dust, brine or cheese smear) as an important hygiene indicator in monitoring *Listeria monocytogenes* in food processing facilities. Microbial communities of floor drains are representative for each processing area and are influenced to a large degree by food residues, liquid effluents and washing water. The microbial communities of drain water are steadily changing, whereas drain biofilms provide more stable niches. Bacterial communities of four floor drains were characterized using 16S rRNA gene pyrosequencing to better understand the composition and exchange of drain water and drain biofilm communities. Furthermore, the *L. monocytogenes* contamination status of each floor drain was determined by applying cultivation-independent real-time PCR quantification and cultivation-dependent detection according to ISO11290-1. Pyrosequencing of 16S rRNA genes of drain water and drain biofilm bacterial communities yielded 50,611 reads, which were clustered into 641 operational taxonomic units (OTUs), affiliated to 16 phyla dominated by *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. The most abundant OTUs represented either product- (*Lactococcus lactis*) or fermentation- and food spoilage-associated phylotypes (*Pseudomonas mucidolens*, *Pseudomonas fragi*, *Leuconostoc citreum*, and *Acetobacter tropicalis*). The microbial communities in DW and DB samples were distinct in each sample type and throughout the whole processing plant, indicating the presence of indigenous specific microbial communities in each processing compartment. The microbiota of drain biofilms was largely different from the microbiota of the drain water. A sampling approach based on drain water alone may thus only provide reliable information on planktonic bacterial cells but might not allow conclusions on the bacterial composition of the microbiota in biofilms.

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1. Introduction

The food processing environment may harbor a variety of possible spoilage organisms and food-borne pathogens (e.g. *Listeria monocytogenes*) which could easily re-contaminate food products during production (Oliver et al., 2005). Even though European and US legislation have strict regulations concerning material coming into contact with food, epidemic outbreaks caused by zoonotic agents are frequently reported (EFSA and ECDC, 2015; Ferreira et al., 2014; Halberg Larsen et al., 2014).

Contamination of food products could be fostered by biofilms on equipment; their formation is favored by accumulation of food residues, spoilage organisms and food-borne pathogens (Donlan, 2002). Many manufacturing processes may provide niches of low sanitation level, where biofilms can prosper, including stainless steel (e.g. ultrafiltration membrane, valves, air separator), rubber surfaces (e.g. belt, packing machine, air separator, liner, short milking tube) and floors (Faille and Carpentier, 2009; Marchand et al., 2012). Furthermore, bacteria thriving in biofilms can be inherently resistant to antimicrobial agents, facilitating possible persistence of spoilage organisms or food-borne pathogens (Allison et al., 2000; Costerton et al., 1999; Giaouris et al., 2015). In recent years, biofilms have been shown to be important for harborage of various food-borne pathogens (Allison et al., 2000; Costerton et al., 1999; Giaouris et al., 2015; Halberg Larsen et al., 2014; Shi and Zhu, 2009) thereby increasing the risk for food safety violations.

Several studies described floor drain microbial communities (Bokulich and Mills, 2013; Fox et al., 2014; McBain et al., 2003; Schirmer et al., 2013). Sanitation and disinfection procedures can be inappropriate especially when cleaning is not manually supported or when floor drains are poorly accessible (Ayebah et al., 2006; Berrang et al., 2008; Donlan and Costerton, 2002; Folsom and Frank, 2006;

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.02.004
0168-1605/© 2016 Published by Elsevier B.V.

Tompkin, 2002). Floor drains in processing and packaging areas are often placed at inaccessible positions and can be difficult to maintain and have low water loading capacity. Floor drains in storage rooms or building compartments outside the hygiene area are often neglected and not cleaned on a regular basis (Ferreira et al., 2014; Fox et al., 2014; Muhterem-Uyar et al., 2015). To control the occurrence of food-borne pathogens, drain water samples are frequently used as sample matrix for hygiene monitoring (Wagner and Stessl, 2014).

Floor drains may become contamination hotspots for *L. monocytogenes* and other *Listeria* spp. Particularly when sanitation is applied in parallel to processing, food could be re-contaminated by bacteria transmitted via aerosols or spray water (Berrang and Frank, 2012; Berrang et al., 2010; Gudbjornsdottir et al., 2004; Zhao et al., 2006). Unfortunately, no optimal and universally applicable standard for monitoring drain water and drain biofilm in a practical setting is available (Marchand et al., 2012). Moreover, monitoring of floor drains is a challenge, because bacterial populations in drain water are dynamic and change depending on effluents or food residues deriving from the processed food, and may also hide the presence of pathogens. Drain water samples are often taken during routine hygiene monitoring without inclusion of drain biofilm matrix. We hypothesize that including both, drain water and drain biofilm samples in *Listeria* monitoring programs, might yield more reliable insights into the presence of *Listeria* and the composition of microbial communities originating from floor drains (Chmielewski and Frank, 2003). The interdependence of drain water and drain biofilm microbiota has not been studied yet.

The aim of this study was to analyze the microbial communities of drain water and drain biofilm samples from floor drains in a food processing environment harboring *L. monocytogenes* (Ruckerl et al., 2014). We used pyrosequencing, quantitative real-time PCR (qPCR) and cultivation approaches to obtain insights into the composition and dynamics of the microbial communities of floor drains. A better knowledge on the microbial communities in floor drains should improve our understanding of (re)contamination processes in food production facilities.

2. Materials and methods

2.1. Facility description and floor drain sampling

Four sampling sites were defined and visited in February 2013 in a cheese processing facility producing composite cheese products (vegetables stuffed with pasteurized curd cheese) and cow, sheep and goat milk cheeses. General plant information and a floor plan are provided in Ruckerl et al. (2014).

The floor drain sampling sites ($n = 4$) included the cooling chamber, and cutting, washing and processing areas of the plant. In the cooling chamber, where packaged raw material was stored, the accumulation of food soil was low. In the cutting and washing area, pickled vegetables were cut and washed. Finally, vegetables were stuffed with pasteurized curd cheese in the processing area. All samples were taken during food production. From each floor drain sampling site, 1000 ml drain water were transferred to sterile polypropylene bottles and drain biofilms were taken from an approx. 20×20 cm area by using sterile dry sponge sticks (3 M). 500 ml of each drain water sample and one drain biofilm sponge sample were used for both the cultivation-dependent and -independent approaches. Samples were cooled on ice during transport (one hour) to the laboratory and then processed immediately.

2.2. Cultivation-dependent L. monocytogenes detection

500 ml drain water was centrifuged at 10 °C for 30 min with $11{,}000 \times g$. The sediment was transferred to 500 ml half-strength Fraser broth (Solabia Biokar Diagnostics). The sponge samples containing the drain biofilm were enriched in 100 ml half-strength Fraser broth. The cultivation was performed according to the ISO-11290-1 (ISO, 1996). Half- and full-strength Fraser broths were streaked on Palcam agar (Solabia Biokar Diagnostics) and Agar Listeria according to Ottaviani and Agosti (ALOA, Merck). *Listeria* positive colonies were subjected to multiplex PCRs targeting: i) the invasion-associated protein (*iap*) gene (Bubert et al., 1999) and ii) 16S rRNA gene specific for the genus *Listeria* and iii) the *hly* gene specific for *L. monocytogenes* (Border et al., 1990) after short Chelex® 100-Resin (BioRad) DNA extraction (Walsh et al., 1991).

2.3. Sample preparation for cultivation-independent approaches

Drain water samples were centrifuged at $11{,}000 \times g$ for 20 min at 4 °C. Drain biofilm samples were diluted in 100 ml sterile $1 \times$ PBS buffer and homogenized for 7 min in a lab blender (Stomacher 3500 Seward, UK). Subsequently, the biofilm slurry was pelleted at $11{,}000 \times g$ for 20 min at 4 °C. DNA isolation was performed from 250 mg of the remaining pellet using the PowerSoil™ DNA Isolation kit (MoBio Laboratories) according to the manufacturer's instructions.

2.4. PCR amplification of bacterial 16S rRNA genes and pyrosequencing

PCR amplification of V1–V2 regions of bacterial 16S rRNA genes, library preparation and pyrosequencing were performed at the Center for Medical Research, Core Facility Molecular Biology, Medical University of Graz, Austria, as described recently (Hund et al., 2015).

2.5. Sequence processing and analysis

All pyrosequencing reads were analyzed using the software package mothur, version 1.30.2 (Schloss et al., 2009). For details see Hund et al. (2015). Reads were clustered into operational taxonomic units (OTUs) using a distance limit of 0.03 (97% similarity). For calculation of species richness and diversity estimators, data were randomly normalized to the same number of sequences per sample ($n = 2312$ sequences). Venn diagrams were plotted using mothur. The 50 most abundant OTUs were analyzed for taxonomic affiliation to their closest related type strains in the Ribosomal Database Project (RDP) website (Cole et al., 2014).

2.6. Quantitative real-time PCR assays

Quantitative *L. monocytogenes* real-time PCR (qPCR) was performed targeting a 274 bp fragment of the *prfA* gene (Rossmanith et al., 2006) and primers targeting the 16S rRNA gene of the genus *Listeria* (Muller et al., 2013). The 16S rRNA gene PCR quantification of bacterial communities in floor drain samples followed protocols described in Metzler-Zebeli et al. (2013). Details on primer sequences and probes are listed in Supplementary Table 1. All PCRs were performed in an Mx3000p thermocycler (Stratagene). The 25 μl mastermix contained 5 μl DNA template. qPCR results were expressed as bacterial cell equivalents (BCE). All samples were pipetted manually in quadruplicates and analyzed using the MxPro Mx3000p qPCR software v.4.10. Negative controls without template DNA were included in duplicates for each 96-well plate. DNA standard curves for qPCRs were generated by plotting the quantification cycle (Cq) values at 1000 (*prfA*, 16S RNA *Listeria* spp.) and 200 (16S rRNA domain bacteria) fluorescence units against the log initial quantities of the tenfold dilution series of purified genomic DNA from *L. monocytogenes* EGD-e. Based on a molecular size of 2.94 Megabases (Mb) of *L. monocytogenes* EGD-e genome (GenBank accession number: NC_003210), 3.17×10^5 genomes ng^{-1} DNA were calculated. The copy number of the target genes (*prfA* — single copy; 16S rRNA five to six copies) was taken into account when extrapolating *L. monocytogenes* and *Listeria* spp. BCE from qPCR.

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