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Identification of biofilm hotspots in a meat processing environment: Detection of spoilage bacteria in multi-species biofilms

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ABSTRACT

Biofilms are comprised of microorganisms embedded in a self-produced matrix that normally adhere to a surface. In the food processing environment they are suggested to be a source of contamination leading to food spoilage or the transmission of food-borne pathogens. To date, research has mainly focused on the presence of (biofilm-forming) bacteria within food processing environments, without measuring the associated biofilm matrix components.

Here, we assessed the presence of biofilms within a meat processing environment, processing pork, poultry and beef, by the detection of microorganisms and at least two biofilm matrix components. Sampling included 47 food contact surfaces and 61 non-food contact surfaces from eleven rooms within an Austrian meat processing plant, either during operation or after cleaning and disinfection. The 108 samples were analysed for the presence of microorganisms by cultivation and targeted quantitative real-time PCR based on 16S rRNA. Furthermore, the presence of the major matrix components carbohydrates, extracellular DNA and proteins was evaluated.

Overall, we identified ten biofilm hotspots, among them seven of which were sampled during operation and three after cleaning and disinfection. Five biofilms were detected on food contact surfaces (cutters and associated equipment and a screw conveyor) and five on non-food contact surfaces (drains and water hoses) resulting in 9.3 % of the sites being classified as biofilm positive. From these biofilm positive samples, we cultivated bacteria of 29 different genera. The most prevalent bacteria belonged to the genera *Brochothrix* (present in 80 % of biofilms), *Pseudomonas* and *Psychrobacter* (isolated from 70 % biofilms). From each biofilm we isolated bacteria from four to twelve different genera, indicating the presence of multi-species biofilms.

This work ultimately determined the presence of multi-species biofilms within the meat processing environment, thereby identifying various sources of potential contamination. Especially the identification of biofilms in water hoses and associated parts highlights the need of a frequent monitoring at these sites. The knowledge gained about the presence and composition of biofilms (i.e. chemical and microbiological) will help to prevent and reduce biofilm formation within food processing environments.

1. Introduction

Biofilms are microorganisms embedded in a self-produced matrix most often adhering to a surface (Vert et al., 2012). Biofilm development is described by different steps (Carpentier and Cerf, 1993; Chmielewski and Frank, 2003). The first step of biofilm formation is adhesion, which is reversible and is followed by irreversible adhesion, also termed attachment (Vert et al., 2012). After attachment the cells start to proliferate and produce extracellular polymeric substances (EPS), characteristic for the biofilm matrix. The major EPS components are carbohydrates, proteins and extracellular DNA (eDNA) (Flemming and Wingender, 2010) and the resulting biofilm is highly diverse, dependent on the involved microorganisms (Flemming et al., 2016). Through further matrix production and division of cells the biofilm matures. Within the biofilm certain niches are formed (e.g. by oxygen and nutrient supply) and the heterogeneous biofilm arises, hosting multiple specialised inhabitants (Flemming et al., 2016). The last step of biofilm formation is dispersal and/or detachment, in which single

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cells or clusters of cells detach actively or passively. These cells can adhere to another surface and start to build a new biofilm.

Microorganisms living in a biofilm also demonstrate distinct gene expression profiles and phenotypic variations (Flemming et al., 2016), potentially resulting in dormancy states, i.e. viable-but-non-culturable (VBNC) cells or persister cells (Flemming et al., 2016). Due to the protective nature of the matrix and these phenotypic variations, microorganisms in biofilms show higher tolerance to antimicrobial disinfectants and toxic metals, as well as other environmental stressors, such as desiccation or UV-radiation (Flemming et al., 2016; Srey et al., 2013).

In the food producing and processing environment various bacteria, including spoilage and pathogenic bacteria, have been detected (Giaouris et al., 2014). Many of them have been shown to be able to attach to surfaces and form biofilms in an experimental setting, including the foodborne pathogens *Salmonella* spp., *Listeria monocytogenes*, enterohemorrhagic *E. coli*, *Campylobacter jejuni* and *Staphylococcus aureus* (Giaouris and Simões, 2018). This has led to the assumption that biofilms have an important role in the contamination of food products in the food processing environment (Marouani-Gadri et al., 2009), as food processing plants harbour numerous sites prone for biofilm development. Indeed, spoilage organisms and/or pathogenic bacteria can be transferred to the food product by i) direct contact to the food product, if the biofilm has developed on a food contact surface (FCS) (Myszka and Czaczyk, 2011) or ii) if parts of biofilms detach and are transferred from non-food contact surfaces (NFCS) to FCS during operation or cleaning and disinfection and subsequently contaminate the food product (Kumar and Anand, 1998).

Food processing environments also expose microorganisms to a variety of stresses, such as alternations in moisture content, times of nutrient-richness and starvation, different temperatures and disinfection procedures. While biofilms can help microorganisms to survive these harsh conditions (Flemming et al., 2016), data on the presence of biofilms in the food producing environment are very limited. To date, only two studies, both performed by Maes et al., analysed the presence of bacteria and matrix components (carbohydrates, proteins, uronic acids), one in a food producing environment (Maes et al., 2017) and one in primary meat production facilities, namely broiler houses (Maes et al., 2019b). Within the study of food processing plants, they investigated eight different Belgian food companies (including two meat processing plants). They showed that 17 % of the analysed sites harboured a biofilm, being defined by the presence of bacteria and at least one of the analysed matrix components (Maes et al., 2017).

The aim of our study was to identify biofilm hotspots in a meat processing environment by analysing 108 samples, including FCS and NFCS. To do so, we determined the presence of bacteria, by cultivation and targeted quantitative real-time PCR, and three matrix components (carbohydrates, eDNA and proteins). This work allowed us to identify ten biofilm positive sites in a meat processing environment and provided first insight into their multi-species community including known spoilage bacteria.

2. Materials & methods

2.1. Biofilm sampling

From an Austrian meat processing plant 108 samples were taken including 47 FCS and 61 NFCS (Supplementary Table 1). The meat facility processes meat from pork, poultry and beef but has no own slaughter unit. The sampling was aimed to investigate as many different sites possibly relevant for product contamination. In total eleven distinct rooms were sampled, whereas the focus in each room was to sample sites possibly critical for biofilm formation and product contamination. In each investigated room handling with unprocessed or processed meat occurs daily. The sampling was performed on two different dates (in November 2018) since only a limited number of samples could be processed at once. To investigate as many different sites at different conditions the first sampling was performed during operation and the second after cleaning and disinfection. The first sampling was performed in six distinct rooms (filling room, cutting room, weighing room, smokehouse, slicing area, and packaging area) during the daily operation resulting in 61 samples (#1 – #61). On the second sampling date 47 samples (#62 – #108) were taken from five different rooms (tumbling room, curing room, delivery, ham room and cooking room) before operation. These rooms underwent a cleaning and disinfection procedure before sampling was conducted. The temperature in all sampled rooms ranged from 2 to 12 °C. Samples were taken using a scraper-flocked swab method, described by Maes et al. (2017) of an area from 2 – to 10 cm^2. The head of the scraper (Cell Scraper (length: 225 mm, blade width: 20 mm), Carl Roth) and the flocked swab (552C, FLOQSwabs, COPAN) were immediately put into 10 ml 0.25 x Ringer's solution (B. Braun Austria GesmbH). The samples were cooled to 4 °C until further processing, which was done within 24 h.

2.2. Sample processing and EPS extraction

To each sample, 2 g of hydrated (washed twice for 15 min using 0.1× PBS) cation exchange resin (CER, Amberlite® HPR110, 20 – 50 mesh, Sigma-Aldrich) were added. The samples were shaken for 20 min at 500 rpm. An aliquot of 50 µl was then taken for determination of colony forming units (CFU). Subsequently, the samples were centrifuged for 20 min at 3220×*g* at 20 °C, the supernatant was filter sterilised using a 0.22 µm filter (Filtropur S0.2 Sarstedt AG & Co KG) and stored at −20 °C until EPS analysis. The residual pellet containing the cation exchange resin and the biomass was stored at −20 °C until DNA extraction.

2.3. Biofilm characterisation

2.3.1. Determination of CFU

The CFU of the biofilm suspension was determined by serial dilution. Appropriate dilutions were plated on tryptic soy agar supplemented with yeast extract (TSA-Y) in triplicate and incubated at room temperature for 72 h. The colonies (not distinguishing between fungal and bacterial colonies) were enumerated and the CFU/cm^2 was calculated. Additionally, one plate per dilution was incubated at 10 °C for cultivation of psychrotrophic microorganisms.

2.3.2. Enumeration of bacteria by qPCR

The pellet containing CER and DNA was washed using 8 ml Dulbecco's Phosphate Buffered Saline (PBS; Thermo Fisher Scientific). After thoroughly mixing, the sample was incubated for 5 min at RT to allow the separation of the CER and the supernatant. The supernatant was collected, transferred into a new tube and centrifuged for 5 min at 3220×*g*. The supernatant was removed and the DNA of the pellet was extracted using the DNeasy®PowerSoil®ProKit (QIAGEN) according to manufacturer's instructions. Elution was carried out using two times 50 µl 70 °C DEPC-H_2O, leading to a total volume of 100 µl. The DNA concentration was determined using 1 µl DNA and the Spectrophotometer/Fluorometer DS-11 FX+ (DeNovix). The total bacterial cell equivalents (BCE) were determined in duplicate by performing quantitative real-time PCR (qPCR) targeting the 16S rRNA gene as previously described (Dixon et al., 2019) using the primer set F (5′-CCT ACG GGA GGC AGC AG-3′) and R(5′-ATT ACC GCG GCT GCT GG-3′) (standard curve 2.8 log to 6.8 log BCE, primer efficiency 90.1 to 94.9 %). In each run a negative control was included. Additionally, for each DNA extraction, one background control of the kit was included to determine the bacterial contamination of the reagents. The evaluated copy number of these background controls was subtracted from all samples before extrapolation of the total BCE. The total BCE was calculated considering an average of four 16S rRNA gene copies as

estimated using rrnDB (Stoddard et al., 2015; Větrovský and Baldrian, 2013).

2.3.3. Identification of cultivated bacteria

After 72 h of incubation colonies with different morphologies were picked from plates incubated at RT and 10 °C and cultivated on TSA-plates. Genus/species identification was performed from 111 isolates of identified biofilm hotspots using matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics GmbH, Bremen, Germany). For direct transfer method fresh colony material was smeared, in duplicate, on a polished steel MSP 96 target plate (Bruker Daltonik) using a toothpick, overlain with 1 µl of matrix solution (alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) and air dried at room temperature. If the identification log score value was below 2 the protein extraction procedure was performed as previously described (Alispahic et al., 2010). Briefly, 1 µl loopful of bacterial material was suspended in 300 µl of distilled water and 900 µl ethanol was added. The cell suspension was centrifuged at 13000 × *g* for 2 min, and the supernatant was discarded. The centrifugation was repeated, and the residual ethanol was discarded. The pellet was air dried and thoroughly suspended in 30 µl 70% formic acid, and finally an equal volume of acetonitrile was added. After centrifugation at 13000 × *g* for 2 min, 1 µl of the supernatant was transferred to the MALDI target plate and allowed to dry at room temperature before being overlain with 1 µl of matrix solution. Measurement and analysis of the data was done as previously described (Alispahic et al., 2014). If the score value was below 1.9, species identification was done by sequencing and analysing the 16S rRNA gene. Therefore, the genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's instructions. The universal 16S rRNA bacterial primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′- GGY TAC CTT GTT ACG ACT T-3′) were used to amplify the 16S rRNA gene, resulting in a 1522 bp product. Each PCR reaction (50 µl) contained 1 × buffer, 2 mM $MgCl_2$, 250 mM dNTPs, 0.625 U Taq Polymerase (Thermo Fisher Scientific), 200 nM each primer and DEPC-H_2O. A standard thermocycler was used for amplification for 5 min at 95 °C of denaturation, followed by 35 cycles at 94 °C for 40 s, at 52 °C for 40 s, at 72 °C for 60 s and final extension at 72 °C for 7 min. For quality control the PCR-product was checked using gel electrophoresis (1.5%). The samples were sequenced by Sanger sequencing (LGC Genomics) using the 1492R primer and the quality of the obtained sequences was analysed using FinchTV (Version 1.4.0). The species identity of the obtained sequences was determined using the RDP seqmatch tool (Cole et al., 2014). Additionally, the sequence was blasted using the NCBI Nucleotide BLAST (NCBI [cited 2020 Jan 14]). The hit with the highest S_ab score from RDP was retrieved after blasting using NCBI Nucleotide BLAST, with limits to sequences "from type material". The percentage identity as well as the appropriate accession-number were selected from NCBI.

2.3.4. Determination of EPS components

2.3.4.1. Determination of carbohydrates. The carbohydrate concentration was increased by evaporation for 1 h at 95 °C on a Thermoblock. Afterwards, the carbohydrate concentration was determined using a phenol-sulphuric acid method described by Masuko et al. (2005). In short, to 50 µl of sample, 150 µl concentrated sulphuric acid and 30 µl 5% phenol were added. After a heating step at 95 °C for 5 min the absorbance at 490 nm was measured using a plate reader (TECAN). The amount of glucose equivalents was calculated using a standard curve of glucose (270 – 5400 ng/50 µl). The limit of quantification was 6.28 mg/l glucose equivalents.

2.3.4.2. Presence of eDNA. eDNA was precipitated overnight using ethanol precipitation according to Zetzmann et al. (2015). Briefly, 2.5 × ethanol (100%), 0.1 × Na-acetate (3 M) and 0.1 × $MgCl_2$ (0.1 M) were added to the sample. After incubation for 24 h at −20 °C the DNA was recovered by centrifugation. After one washing step with 70% ethanol, the pellet was resuspended in water. The presence of eDNA was confirmed by measurement (two times, in duplicate) on a NanoDrop Spectrophotometer 2000c (Thermo Scientific). The detection limit for eDNA was 2 ng/cm^2.

2.3.4.3. Presence of proteins. Proteins were precipitated with cold trichloroacetic acid/acetone (final concentration 10%) supplemented with sodium deoxycholate (final concentration 0.2%) at 4 °C for 16 h (according to Rychli et al., 2016), in duplicate. Collection of precipitated proteins was done by centrifugation (30 min, 20817 × g, 4 °C), the pellet was air dried and suspended in 0.05 M Tris-HCl. The proteins were analysed using SDS-PAGE (15%) followed by silver-staining (Supplementary Table 2). If at least one clear band was visible, the sample was considered to contain proteins.

2.4. Controls

As a positive control we used a *Pseudomonas* (*P.*) *simiae* strain grown in a static biofilm model for 28 days at 10 °C. The *P. simiae* strain was isolated in a previous study from a conveyor belt. The species was confirmed by sequencing and analysing of the *gyrB* gene as described by Agaras and Valverde (2018), resulting in 99.73 % sequence identity to *P. simiae*. The biofilm was grown as follows: one colony of *P. simiae* was inoculated in 1:2 tryptic soy broth supplemented with yeast (TSB-Y) and grown over night at 20 °C with shaking. Afterwards the *P. simiae* overnight culture was adjusted to an optical density of 0.1 in 20 ml 1:10 TSB-Y in a 50 ml tube. A sterilised glass slide (7.6 × 2.6 × 0.1 cm, Thermo Scientific) was inserted and incubated at 10 °C with shaking. After the first 24 h the medium was changed. Additionally, every third day the medium was changed. The biofilm was harvested after 28 days by vortexing for 2 min in a tube with 10 ml of 0.25 × Ringer's solution and 15 g of sterile glass beads (Ø 4 ± 0.3 mm, Carl Roth). The fluid was further processed as the samples from the environment. As negative control (NC) a sterile scraper and swab was used and processed like the environmental samples (n = 2).

In all four laboratory grown biofilms used as positive controls a bacterial load above 8.4 log CFU/cm^2 and all evaluated matrix components could be detected. All negative controls were negative for bacteria and for any matrix components (Supplementary Table 3).

2.5. Statistics

Statistical analysis was performed using SPSS.20 software (SPSS Inc., Chicago USA). Brown Forsythe and Welch test were applied to confirm variance homogeneity. *t*-Test with independent variables (variance homogeneity) was used to compare the bacterial load (CFU/cm^2, BCE/cm^2), the amount of carbohydrates (µg/cm^2 glucose equivalent) and eDNA (µg/cm^2) of samples during operation and after cleaning and disinfection and of FCS and NFCS. To determine significant differences between the bacterial load, the amount of carbohydrates and eDNA of samples of the different rooms a posthoc test (Tukey-HSD in the case of variance homogeneity) was used. *p*-Values < 0.05 were considered to be statistically significant.

3. Results

3.1. Bacterial load on surfaces in an Austrian meat processing company

In total, 108 different samples were taken from 11 different rooms in a meat processing company (filling room, cutting room, weighing room, smokehouse, slicing area, packaging area, tumbling room, curing room, delivery, ham room, cooking room). We detected bacteria in 93 samples (86 %) either by cultivation and/or by quantification of the bacterial DNA.

During operation 61 samples were taken in six rooms. Within these

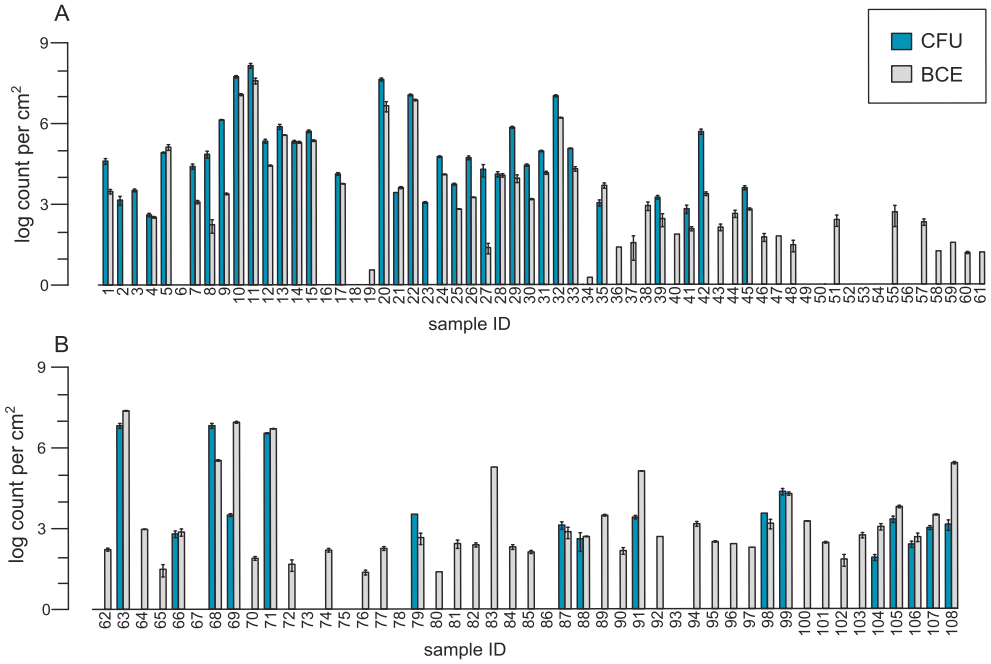

Fig. 1. Bacterial load of the samples taken during operation (A) and after cleaning and disinfection (B) determined by cultivation (log CFU/cm^2, blue bars) and by qPCR (log BCE/cm^2, grey bars). Mean values of CFU measurements determined in triplicate ± standard deviation and BCE-measurements determined in duplicate ± standard deviation are given. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples, the presence of bacteria was detectable in 52 samples either by cultivation (34 samples) and/or by quantification of bacterial DNA (48 samples). In 18 samples we detected bacteria only by qPCR. The total microbial count Fig. 1 ranged from 2.6 log CFU/cm^2 (#4, control panel – keyboard) to 8.1 log CFU/cm^2 (#11, drain), whereas the BCE ranged from 0.3 log BCE/cm^2 (#34, trolley) to 7.6 log BCE/cm^2 (#11, drain).

After cleaning and disinfection, we could confirm the presence of bacteria in 41 of 47 samples (taken in five rooms) either by cultivation (16 samples) and/or by quantification of bacterial DNA (41 samples). In 25 of these samples, we detected bacteria only by qPCR. Here the total bacterial cell count Fig. 1 ranged from 1.9 log CFU/cm^2 (#104, shovel) to 6.8 log CFU/cm^2 (#63, screw conveyor) and the BCE from 1.4 log BCE/cm^2 (#80, plate) to 7.4 log BCE/cm^2 (#63, screw conveyor).

We could cultivate less bacteria from sites after cleaning and disinfection (34 %) than from sites sampled during operation (56 %). However, there were no statistically significant differences in the quantification of bacteria during operation and after cleaning and disinfection. Overall, the lowest levels of microbial presence were detectable in the slicing area (#41 – #52) and in the packaging area (#53 – #61), with mean BCE-values of 2.5 log BCE/cm^2 and 1.9 log BCE/cm^2, respectively. Of the total 47 FCS and 61 NFCS that were sampled, we confirmed the presence of bacteria in 38 FCS (81 %) and 53 NFCS (87 %).

3.2. Presence of matrix components

Carbohydrates could be detected in 45 samples (26 during work/19 after cleaning and disinfection), leading to 41.7 % carbohydrate positive sites Figs. 2 and 4. The carbohydrate content ranged from 1.09 (#40, rack in slicing room) to 197.88 µg/cm^2 glucose equivalents (#63, screw conveyor) Fig. 2.

In six samples (5.56 %), eDNA could be detected Figs. 3 and 4. The minimum eDNA-level was 2.03 µg/cm^2 (#69, drain) and the maximum eDNA-level was 61.50 µg/cm^2 (#10, inside the nozzle of a water hose) Fig. 3.

Using SDS-PAGE and subsequent silver-staining, proteins could be detected in nine samples (seven during work and two after cleaning and disinfection), resulting in 8.3 % protein-positive samples Fig. 4.

3.3. Identified biofilm hotspots

Since biofilms consist of microorganisms and matrix components, we defined a biofilm hotspot as a site contaminated with bacteria and the presence of at least two matrix components.

Under such criteria, we identified ten biofilm hotspots corresponding to 9.3 % of 108 sites Fig. 4. In four of the samples (#10, #11, #63 and #69) all three matrix components (carbohydrates, eDNA and proteins) as well as the presence of bacteria could be detected. Additionally, six samples (#5, #12, #13, #14, #20, #71) were positive for two of the three matrix components and bacteria.

In the filling room we identified three biofilm sites on a cutter waggon (#5), the inside of a nozzle of a water hose (#10) and the drain (#11). In the cutting room, biofilms at three different cutters (#12, #13, micro-cutter (#14)) and in the nozzle of a water hose (#20) were present. Lastly, the screw conveyor (#63) and the drain (#69) in the tumbling room, in addition to the water hose (#71) in the curing room, also harboured a biofilm. All together, we detected a biofilm at five FCS (10.6 % positive) and five NFCS (8.2 % positive).

3.4. High abundance of Brochothrix, Pseudomonas and Psychrobacter in biofilms

From the ten samples classified as biofilm positive we isolated and cultivated bacteria of 29 different genera (16 Gram-negative and 13 Gram-positive) Fig. 5. The genera of the isolated bacteria were distributed among the bacterial phyla as follows: 14 Proteobacteria, eight Actinobacteria, five Firmicutes and two Bacteroidetes. The most prevalent bacteria belonged to the genera *Brochothrix,* isolated from eight biofilms (80 %), *Pseudomonas* and *Psychrobacter,* of which both have been isolated from seven biofilms (70 %). From each biofilm we isolated bacteria from at least four different genera: a minimum of four from the screw conveyor (#63) and water hose (#71), and a maximum of 12 from the drain (#12).

We detected *Brochothrix* spp., *Pseudomonas* spp. and *Psychrobacter* spp. in all four cutter associated biofilms. Further *Carnobacterium* spp. was isolated at all cutter-associated sites except the micro-cutter. All three water hose biofilms harboured *Rhodococcus* spp. and from two of them we additionally isolated *Flavobacterium* spp., *Microbacterium* spp. and *Stenotrophomonas* spp.. From both drain biofilms the genera

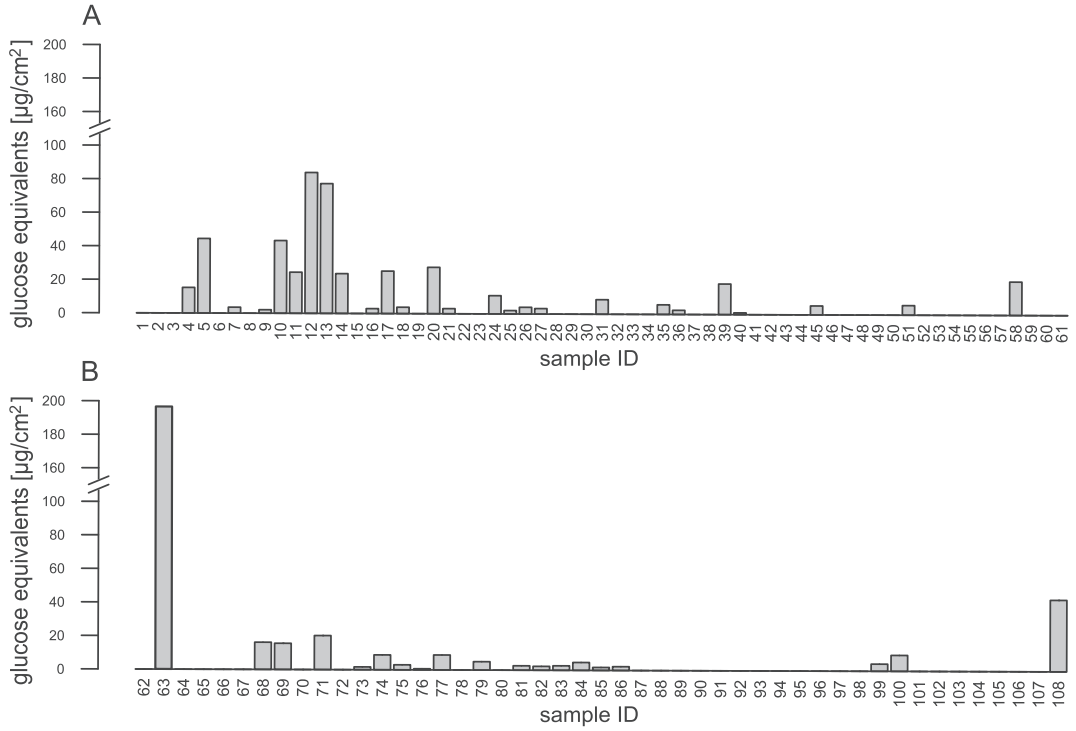

Fig. 2. Carbohydrate content, given as glucose equivalents per cm^2, of samples obtained during operation (A) and after cleaning and disinfection (B). Values represent the mean ± the standard deviation of at least six measurements. Due to data representation the standard deviation is not seen (standard deviation values from 0.001–0.01).

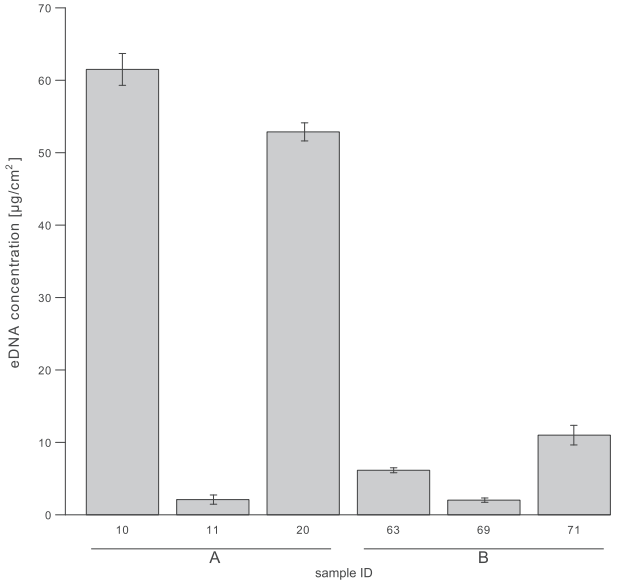

Fig. 3. eDNA concentration (µg/cm^2) of eDNA positive samples obtained during operation (A) and after cleaning and disinfection (B). Values represent the mean value ± the standard deviation of at least two measurements.

Brochothrix, Pseudomonas and *Psychrobacter* were isolated; all other genera were not shared between the drain biofilms.

At a lower incubation temperature (10 °C) we were able to cultivate bacteria of three additional genera: *Chryseobacterium, Paeniglutamicibacter* and *Xanthomonas*, present in drain and water hose biofilms (Supplementary Fig. 1).

4. Discussion

In this study, we identified ten biofilm positive samples in a meat processing environment by analysing 108 sites. Our definition of a biofilm positive sample, which combined cultivation and cultivation-independent methods along with the detection of a minimum of two matrix components (carbohydrates, eDNA and/or proteins), is more robust than the definitions used before to monitor biofilms.

To our knowledge, there is only one research group, that included the analysis of matrix components (Maes et al., 2017, 2019b) in the identification of biofilm hotspots in the food processing environment. All other studies to date have focused on attached bacteria (among others Dzieciol et al., 2016; Røder et al., 2015). In one of the Maes et al. studies, they investigated eight different Belgian food companies after cleaning and disinfection and determined that 17 % of the sites harboured biofilms (Maes et al., 2017). The biofilm definition in their study was based on the following criteria: the presence of cultivable bacteria (> 1 CFU/cm^2) and at least one matrix component (carbohydrates, proteins or uronic acids). Uronic acid, a component of bacterial alginate, is a highly abundant carbohydrate in the biofilm matrix, mainly in biofilms of alginate producing *Pseudomonas* spp. (Flemming and Wingender, 2010). When applying the biofilm criteria from Maes et al., 43 % of the sites in our study would be categorised as biofilm positive sites, including 34 % of samples after cleaning and disinfection. However, since there is a high probability to detect residues of raw materials or processed food in the food producing environment, the detection of a single matrix component, such as carbohydrates could overestimate the number of biofilm hotspots. In fact, we detected carbohydrates in 45 samples of the 108 samples (41.7%), including 28 samples, in which we could also isolate bacteria (62 % from carbohydrate positive sites). In seven other samples, the presence of bacteria was only detectable by qPCR targeting the bacterial 16S rRNA gene.

This study included 18 samples during operation and 25 after cleaning and disinfection, in which the presence of bacteria could only be detected by qPCR and not by cultivation. Besides targeting bacteria

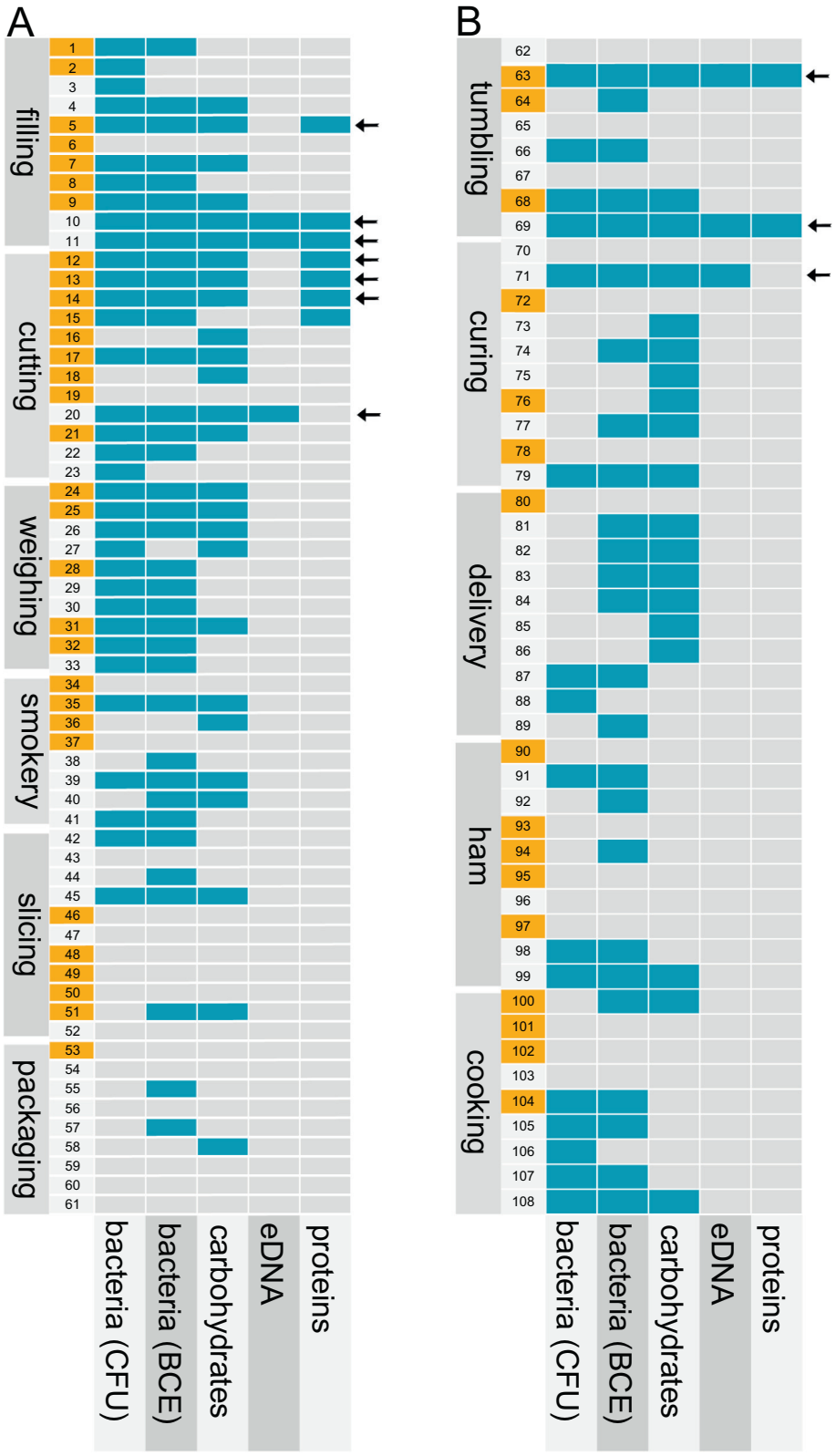

Fig. 4. Identification of biofilm hotspots. Blue fields indicate the detection of bacteria (by CFU determination and qPCR) and the detection of EPS components (carbohydrates, eDNA and proteins) in samples obtained during operation (A) and after cleaning and disinfection (B). Arrows indicate identified biofilms. Sampling sites highlighted in orange indicate food contact surfaces (FCS). The type of work or abbreviations of the room names are given. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

difficult to cultivate or in the VBNC state, qPCR targets also DNA of dead cells, possibly leading to an overestimated number of bacteria positive samples. The higher number of samples positively confirmed only by qPCR after cleaning and disinfection (53.2 %) than during operation (29.5 %) demonstrates the effect of cleaning and disinfection. However, the VBNC state can also be promoted by cleaning and

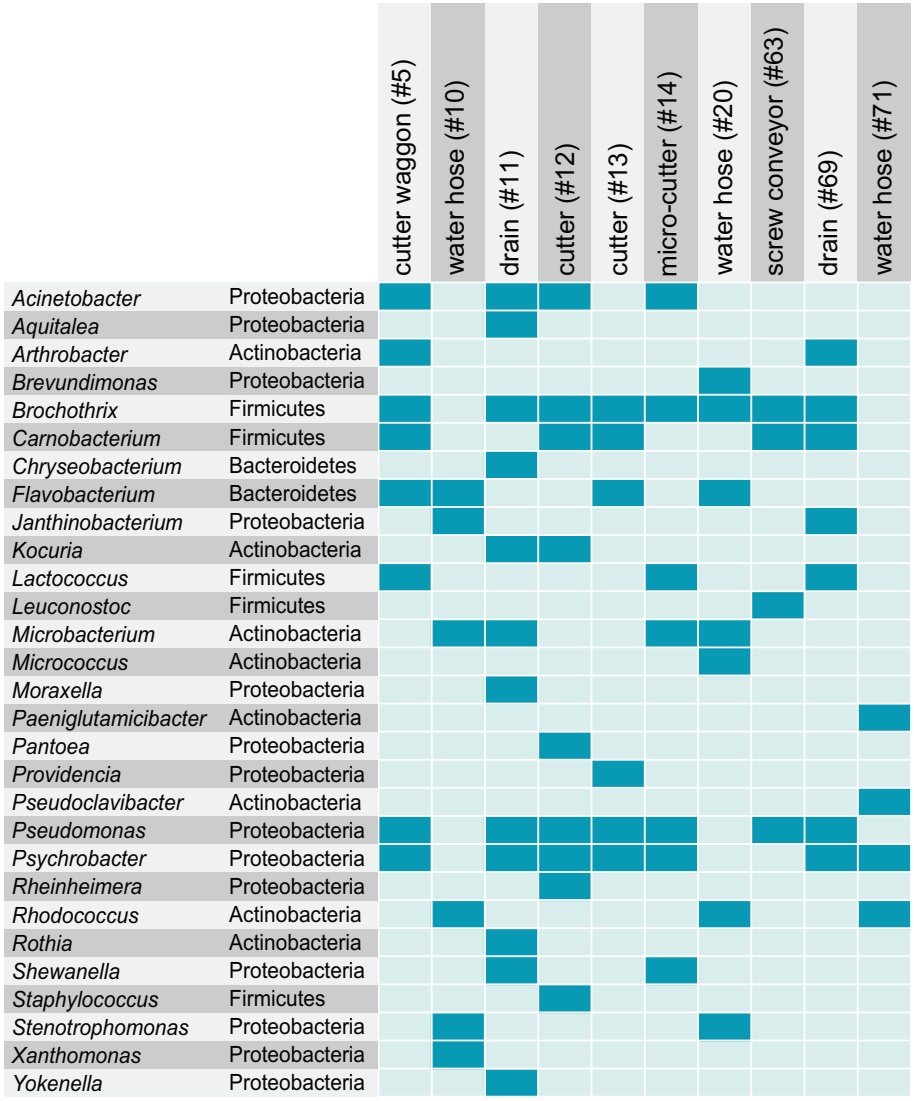

Fig. 5. Identified genera and phyla of bacteria isolated from biofilms. Dark blue indicates isolation of at least one bacterial colony of the indicated genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

disinfection strategies (Ferro et al., 2018; Robben et al., 2018). From three sites microorganisms could only be cultivated, which indicates the presence of fungi. We focused however only on bacteria and neglected the presence of fungi and their potential role in biofilms. It is worth noting that in the identified biofilms, the presence of bacteria was confirmed by both methods, by cultivation and qPCR, respectively. Consistent with biofilms having high cell densities (Flemming et al., 2016), we detected 3.5 to 8.4 log CFU/cm^2 and 4.4 to 7.5 log BCE/cm^2, respectively.

Species identification of bacteria isolated and cultivated from the biofilms confirmed that biofilms in the food producing environment are multi-species. We isolated bacteria from at least four different genera from each biofilm and cultivated up to twelve genera from a biofilm found in a drain by using a single growth media and two incubation temperatures. The diversity of these biofilms was further highlighted by the different matrix components detected.

No pathogens were detected in this work, however, the detection of food borne pathogens often requires specific enrichment methods or selective agars (e.g. ISO 11290-1:2017 for L. *monocytogenes* or ISO 6579-1:2017 for *Salmonella*). It is known that common foodborne pathogens like *L. monocytogenes* can establish within multi-species biofilms with microorganisms isolated from the meat processing environment. Moreover, *L. monocytogenes* has been isolated from drains (Rückerl et al., 2014), which are known to harbour a complex microbial consortium and biofilms (Dzieciol et al., 2016).

The bacteria most frequently isolated from biofilms belonged to *Brochothrix* spp., *Pseudomonas* spp. and *Psychrobacter* spp.. *Brochothrix* (most frequent *B. thermosphacta*) was isolated from all biofilm sites, except two water hoses. This Gram-positive bacterium is known as a spoiler of raw and packaged meat and was previously described to be present on surfaces often in contact with unprocessed meat (Maes et al., 2019a; Møretrø and Langsrud, 2017; Quijada et al., 2018; Røder et al., 2015). Contamination of meat products with *B. thermosphacta*, which is able to survive high-salt and low pH conditions and to grow at refrigeration temperatures, could lead to spoilage (Stanborough et al., 2017). Whether *Brochothrix* spp. can form biofilms is still unknown.

Pseudomonas species, isolated from seven biofilms in this study, are among the best studied biofilm formers. Pseudomonads, known to be potential spoilers of refrigerated ready-to-eat products, have been isolated from various food processing environments and are suggested to belong to the residual microbial community (Dzieciol et al., 2016; Maes et al., 2019a; Møretrø and Langsrud, 2017; Røder et al., 2015; Rodríguez-López et al., 2015; Zwirzitz et al., 2019). The formation, matrix composition and control of *Pseudomonas* biofilms is widely

studied (Masák et al., 2014) and it has been shown that *P. fluorescens* protects *L. monocytogenes* against biocides in dual-species biofilms (Puga et al., 2016).

Psychrobacter spp. and *Acinetobacter* spp., isolated from seven and four biofilms respectively, have been previously detected in various meat processing environments, including the Belgian meat companies investigated by Maes et al., 2019a. The genera *Psychrobacter* and *Acinetobacter* have also been isolated from the meat receiving, filling and packaging areas of a meat processing plant (Hultman et al., 2015), from a small slaughterhouse in Denmark (Røder et al., 2015), during manufacturing of traditionally produced sausages (Quijada et al., 2018) and from the air and different food processing sites in a meat producing factory (Fagerlund et al., 2017). Additionally, *Psychrobacter* and *Acinetobacter* are psychrotrophic bacteria and have been associated with food spoilage (Møretrø and Langsrud, 2017).

We detected biofilms on five FCS, one screw conveyor in the tumbling room, a cutter waggon in the filling room, two cutters and a micro-cutter in the cutting room, which could lead to direct contamination of the food product. A biofilm on the screw conveyor was even sampled after cleaning and disinfection. Adjusting the cleaning processes e.g. including mechanical cleaning may have helped to remove this biofilm (Jessen and Lammert, 2003). Specifically, we isolated bacteria of only four different genera from this biofilm, suggesting an effect of cleaning and disinfection on the biofilm composition and diversity. All other biofilms found on FCS, from which we were able to cultivate bacteria from six to nine different genera, were isolated during operation. Further investigations, including microbiome studies are needed to get insights in the bacterial community of biofilms and the potential effect of cleaning and disinfection.

Regardless, most biofilms on the FCS harboured the genera *Brochothrix*, *Pseudomonas, Carnobacterium,* and *Psychrobacter*, which are all typical psychrotrophic bacteria associated with meat spoilage. Therefore, the presence of these bacteria within a biofilm poses an elevated contamination risk for the processed meat.

We further found biofilms in two of nine sampled drains, yet, bacteria could be detected in all drains at high levels (minimum log CFU/cm^2 of 3.23). Drains, which are known to be a niche for foodborne pathogens, such as *L. monocytogenes* (Stessl et al., 2019; Zhao et al., 2004), are a potential contamination source during the cleaning process. If water jets are applied to the floor and drain, the microorganisms could be distributed throughout the room. To keep the bacterial load and the biofilm burden in drains low, these sites should be cleaned regularly. This issue was scientifically proofed by Gagnière et al. (2006), who showed that aerosols produced by high-pressure water cleaning can contaminate water pipes, promoting further transmission of contamination. Moreover, our results are in line with pyrosequencing data from drains, confirming the presence of *Chryseobacterium*, *Lactococcus*, *Microbacterium*, *Pseudomonas*, *Psychrobacter* in drains and drain waters of a *L. monocytogenes* contaminated cheese producing environment (Dzieciol et al., 2016). The drain biofilms sampled in our study additionally harboured meat associated genera like *Brochothrix*, *Carnobacterium* and *Kocuria*.

Notably, we identified water hoses as biofilm hotspots in the food processing environment. Indeed, we isolated biofilms from three out of six water hoses and associated parts. Water hoses are frequently used to wash away residuals of disinfecting agents after disinfection. If microorganisms from a hose attached biofilm disperse into the water, a surface considered to be clean and safe would then be contaminated. This could lead to immediate transmission of microorganisms onto food products during the entire production time. Interestingly, the presence of *Rhodococcus* spp. was confirmed in all water hose biofilms. This genus, isolated previously in a shower head (Lee, 2013), is known to catabolise a wide range of compounds, potentially enabling the growth in a nutrient poor environment like the water hose. Furthermore, *Flavobacterium* spp., *Microbacterium* spp. and *Stenotrophomonas* spp. were isolated from the two biofilms present at the water hose nozzles. In the cutting room we detected, additional to the water hose biofilm, three biofilms on FCS. Three out of the seven taxa were shared between the water hose and at least one of the FCS biofilms. This could be a first indication that bacteria from water hoses biofilms can be transmitted to food contact surfaces. There is limited information about biofilm development in water hoses (Proctor et al., 2017; Soto-Giron et al., 2016; Thomas et al., 2014) and to our knowledge, this is the first study to assess biofilms in water hoses within the food producing environment. The study of Maes et al., which investigated the occurrence of biofilms in drinking water systems of broiler houses focused on the primary production of meat (Maes et al., 2019b). The identification of biofilms in water hoses and associated parts highlights the need of a frequent monitoring at these sites to prevent recontamination.

In conclusion, this study confirmed the frequent presence of multispecies biofilms in the meat processing environment during operation and after cleaning and disinfection. Within the biofilms, typical meat spoilage organisms were isolated. However, additional comparable studies are needed to determine the biofilm-load of the food producing environment in general. Each biofilm represents a potential contamination source and niche for pathogens. Therefore, the development of biofilms should be generally prevented. Understanding development and composition of biofilms in the food processing environment will help us to prevent contamination by inhibiting the formation of biofilms and removing biofilms.

Declaration of competing interest

None.

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Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108668.

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