Tracking bacteriome variation over time in *Listeria monocytogenes*-positive foci in food industry

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Abstract

The variation in microbial composition over time was assessed in biofilms formed *in situ* on selected non-food and food contact surfaces of meat and fish industries, previously identified as *Listeria monocytogenes*-positive foci. First, all samples were analysed for the detection and quantification of *L. monocytogenes* using ISO 11290-1 and ISO 11290-2 norms, respectively. Although the pathogen was initially detected in all samples, direct quantification was not possible. Psychrotrophic bacteria counts were among resident microbiota in meat industry samples ($\text{Mean}_{\text{max}} = 6.14 \log \text{CFU}/\text{cm}^2$) compared to those from fish industry ($\text{Mean}_{\text{max}} = 5.85 \log \text{CFU}/\text{cm}^2$). Visual analysis of the biofilms using epifluorescence microscopy revealed a trend to form microcolonies in which damaged/dead cells would act as anchoring structures. 16S rRNA gene metagenetic analysis demonstrated that, although Proteobacteria (71.37 %) initially dominated the bacterial communities at one meat industry location, there was a dramatic shift in composition as the biofilms matured, where Actinobacteria (79.72 %) became the major phylum present in later samples. This change was largely due to an increase of *Norcardiaceae*, *Micrococcaceae* and *Microbacteriaceae*. Nevertheless, for the other sampling location, the relative abundance of the dominating phylum (Firmicutes) remained consistent over the entire sampling period (Mean = 63.02 %). In fish industry samples, Proteobacteria also initially dominated early on (90.69 %) but subsequent sampling showed a higher diversity in which Bacteroidetes and Proteobacteria were the most abundant phyla accounting for the 48.04 and 37.98 %, respectively by the last sampling period. Regardless of the location, the community profiles of the endpoint samples were similar to those reported previously. This demonstrated that in a given industrial setting there is a trend to establish a determinate biofilm structure due to the

environmental factors and the constant incoming microbiota. This information could be used to improve the existing sanitisation protocols or for the design of novel strategies.

Highlights

- Bacteriome composition variation was assessed in *L. monocytogenes*-positive spots.
- An *in situ* sampling system was used to reproduce the actual bacteriome.
- HTS analysis allowed to assess the dynamics of each bacterial community.
- In meat industry samples, Actinobacteria and Firmicutes were the most dominant phyla.
- In fish industry samples, Proteobacteria and Bacteroidetes dominated instead.

1. Introduction

In Europe, in spite of novel cleaning and disinfection techniques being constantly designed and applied in the food industry, the incidence of *Listeria monocytogenes* among the population still displays a constant increasing trend (EFSA and ECDC, 2018). This Gram-positive bacterium is the causative agent of human listeriosis (Swaminathan and Gerner-Smidt, 2007) with 2480 reported cases (0.48 per 100000 population) and a fatality rate of 13.8 %, the highest amongst those zoonotic agents included in the annual European Food Safety Authority report (EFSA and ECDC, 2018). Relevant to the food industry, *L. monocytogenes* can associate with other bacterial species and form polymicrobial biofilms (Carpentier and Chassaing, 2004; Rodríguez-López et al., 2019, 2015), which enables it to persist for long periods (Carpentier and Cerf, 2011).

Currently, social interactions among bacteria present in a biofilm have become one of the main fields of research in microbiology (Liu et al., 2016; Madsen et al., 2016) with especial relevance to food safety (De Filippis et al., 2018b). Recent advances in culture-independent techniques such as high throughput sequencing (HTS), in addition to the simplification and automatisation of the existing protocols, have turned HTS into a valuable research tool in studies dealing with polymicrobial associations (De Filippis et al., 2018b, 2018a; Parente et al., 2018). A clear example is the development of powerful online databases focused on the elucidation of bacterial interrelationships present in food systems (Parente et al., 2019, 2016).

Among the studies regarding the composition of bacterial communities present in food-related industrial premises, i.e. slaughterhouses, fish smokehouses, etc., those dealing

with biofilms harbouring foodborne pathogens are of a special relevance (Lapointe et al., 2019; Papaioannou et al., 2018; Puga et al., 2014; Rodríguez-López et al., 2015). This is motivated due to the capacity of these structures to host pathogenic species at very low levels (Langsrud et al., 2016), thus somehow masking their presence when classical detection methods, such as agar plating, are used. Furthermore, their increased capability to endure environmental stresses such as desiccation (Burgess et al., 2016; Møretrø et al., 2013), or cleaning and disinfection protocols (Fagerlund et al., 2017; Møretrø et al., 2017; van der Veen and Abee, 2011) make polymicrobial biofilms an issue of serious concern in terms of food safety. Regarding the latter, recent studies involving *L. monocytogenes*-carrying communities, have demonstrated how the differential composition of the community, significantly influences the eventual effectivity of both chemical (Fagerlund et al., 2017) and enzyme-based (Puga et al., 2018; Rodríguez-López et al., 2017a) sanitisation protocols. Gaining further insight into the composition of the different bacterial communities could give us key information in order to develop target-specific cleaning and disinfection strategies. Nevertheless, the vast majority of previous studies on polymicrobial biofilms were based on discontinuous sampling or *in vitro* model biofilms not based in natural associations. Therefore, a partially biased perspective of the actual scenario is obtained since the experimental model does not take into consideration the variation over time of the sessile communities present in *L. monocytogenes* contamination foci.

In order to overcome this situation, a recent study carried out by Ripolles-Avila, Hascoët, Martínez-Suárez, Capita, & Rodríguez-Jerez, (2019) first described the development of an *in situ* system aimed to detect *L. monocytogenes* and quantify its associated microbiota on food and non-food contact surfaces in an Iberian pork

processing plant. Their outcomes allowed for the establishment of different zones of potential cross-contamination linking microbial abundancy and environmental factors. The present study goes one-step further in the elucidation of the composition of *L. monocytogenes*-carrying bacterial communities using two different homemade stainless steel (SS) *in situ* sampling devices specifically designed for the purposes of this study. This was intended to a) re-confirm the presence of *L. monocytogenes* on food and non-food surfaces previously detected as *L. monocytogenes*-positive sites in meat and fish processing facilities (Rodríguez-López et al., 2019) and b) ascertain the bacteriome variation over time in biofilms formed when exposed to the same environmental conditions, sanitisation and activity of each particular premise.

2. Material and Methods

2.1. Rationale and selection of sampling sites

Sampling sites, thereafter named as *locations*, were evaluated and identified as *Listeria monocytogenes*-positive with high counts in a direct quantification following ISO 11290-2 norm (ISO, 2004, 1998) in a previous survey performed on food and non-food contact surfaces of fish, meat and dairy industries (Rodríguez-López et al., 2019). Since no *L. monocytogenes* direct quantification was possible in dairy industries, two locations from meat industry and two from fish industry were chosen instead.

In all cases, visits to the industrial settings, sampling device placing and subsequent harvesting was performed by an external company (Deinal Soluciones Agroalimentarias, S.L., Lugo, Spain).

2.2. Locations description

In total, four different *L. monocytogenes*-positive locations were defined and visited between January and August 2018 (Supplementary Table 1). The characteristics of each location are described below.

2.2.1. Meat industry

- Location no. 96 (porcine and bovine abattoir): Non-food contact surface, AISI 304 SS waterway of about 30 cm wide set out horizontally and situated fitted on the floor in front of one of the skinning areas (Fig 1A). This facility is used as a drainage system being in contact with skin parts, hair, blood and other fluids in addition to several liquid residues derived from the normal slaughtering activity of the premise. Of note, this location is constantly exposed to running water carrying several sanitisation products used for either surfaces or operator-hands cleaning and disinfection.

- Location no. 107 (porcine and bovine abattoir): Food contact surface, S235JR or S275JR carbon steel platform. The sampling surface was the vertical part of the platform of approximately 2.5 – 3 cm high and 2 m length (Fig. 1B). This surface was air-exposed and presented visible rusting signs. This location was situated in the area of the abattoir where the elimination of the hoofs and the initiation of the skinning process takes place. Based on the information given by the external company, the slaughtered animal grazes this surface when passing through the trail hanged from metal hooks.

2.2.2. *Fish industry*

- Location no. 168 (fish processing facility): Non-food contact surface, AISI 304 SS 30 x 30 cm squared drainage lid situated on the floor in one of the fresh product chambers (Fig. 1C). In addition to running water and other liquid residues, it is important to highlight that the temperature of the chamber was particularly low (approximately 0 °C) in order to preserve the quality of the product and the relative humidity in the environment was high.

- Location no. 225 (fish market): Food contact surface, AISI 316 table used as support for a scale for fresh fish (Fig. 1D). This location is air-exposed in a moistened environment, although the humidity levels were lower compared to the previous location. Of note, this location was particularly accessible to operators, so constant hand touching is expected to happen. Regarding sanitisation, this surface was subjected to routine cleaning and disinfection procedures with mechanical action (wiping) and running water that was drained out through a near sewage.

2.2. *Coupon-based in situ sampling device*

In order to obtain the maximum number of replicas of the microbial communities present in the different locations, two different sampling devices entirely made of AISI 304, 2B SS (Comevisa, Vigo, Spain) were specifically designed considering the characteristics and the dimensions of each location and the normal activity of the premise.

On one hand, sampling device A consisted of a SS plate (125 x 75 x 1 mm) in which 8 coupons (20 x 20 mm) were present. (Fig. 2A). On the other hand, sampling device B consisted of a SS plate (185 x 25 x 1 mm) in which 4 coupons (10 x 40 mm) were present (Fig. 2B). In both devices, coupons were physically joined to the plate by a tab forming a sole structure but that were easily detached for subsequent processing in the laboratory. In both cases, the coupons of the sampling devices were pretreated as follows: thorough wash with tap water and industrial soap (Sutter Wash; Sutter Ibérica, S.A., Madrid) in order to remove grease residues, further rinsed with distilled water and autoclaved at 121 °C for 20 min.

In locations 96, 168 and 225 sampling device A was used whereas in location 107, sampling device B was preferred due to the dimensions of the surface (Fig. 1). In all cases, devices were firmly fixed with glue (TotalTech Ceys; Ceys S.A., L'Hospitalet de Llobregat, Spain) and exposed to the normal activity of the plant until sampling in order to mimic the colonisation and biofilm formation by resident microbiota in each premise.

2.3. Sample collection from contamination locations

Devices (SS plates) were taken one week after placing and at least 4 hours after the starting of the normal processing activity of the different premises as recommended elsewhere in order to maximise bacterial recovery (Carpentier and Barre, 2012). Subsequent collection was carried out with at least one-week difference between samplings. It is important to remark that sampling times were subjected to the permission of each premise, so in some cases, sampling was not possible to be made with a definite periodicity.

A total of 8 coupons (1 plate for device A, 2 plates for device B) were collected in each sampling: 2 were used for microscopy assays, 4 for quantification of adhered viable count (AVC) of psychrotrophic bacteria and for detection and enumeration of *L. monocytogenes* and the last 2 for metagenetic assays.

In all cases, sampling devices were collected aseptically by the external company, placed fixed in a closed clean container to minimise dislodging of the adhered biomass, and transported to the laboratory at room temperature within the next 24 h to be processed for the different assays, as described in subsequent sections.

2.4. Assessment of AVC bacteria

In all cases, each coupon's upper surface containing the adhered biomass was thoroughly rubbed with two sterile cotton swabs moistened in sterile buffered peptone water (BPW; Cultimed, Barcelona, Spain) and the cotton tips were pooled in a sterile tube containing 2 ml of sterile Fraser Broth Base (Oxoid, Hampshire, England). Next, the tube was vigorously vortexed for at least 1 min in order to dislodge cells from swabs. Assessment of recovery of the adhered biomass in rubbed coupons was carried out staining one coupon from each location with a 1 mg/ml DAPI solution (Invitrogen) and visualised under a Leica 6000DM epifluorescence microscope (Leica, Wetzlar, Germany), confirming that no cells remained (data not shown).

2.4.1. AVC psychrotrophic bacteria quantification

From each suspension made in section 2.4., decimal dilutions (up to 1:10⁷) were made in BPW and 100 µl of each dilution was plated on TSA plates and incubated for 72 h at 17 °C. Results were expressed in log CFU/cm².

2.4.2. Sampling for *L. monocytogenes*

2.4.2.1. *Listeria monocytogenes* direct enumeration

Listeria monocytogenes enumeration was performed following a modified protocol of that specified in the ISO 11290-2 norm (ISO, 2004, 1998). Firstly, the suspensions (see section 2.4.) were incubated at 20 °C for 1 h to favour the reactivation of injured cells. Next, from each tube, 500 µl of the suspension was used to inoculate an ALOA agar plate (Scharlab, Barcelona, Spain). The aliquot was allowed to partially dry for 30 min in the biological safety cabinet (BSC), in order to facilitate the subsequent spreading. Plates were then incubated at 37 °C for 24 h. Following this, ALOA plates were visually analysed for typical *L. monocytogenes* morphology (blue colony presenting a clear halo). In case of colony absence or the size of the colony was too small to be picked, plates were further incubated for 24 h. In positive plates, five presumptive colonies were picked and streaked onto Tryptone Soy Yeast Extract Agar (TSAYE; Tryptone Soy Agar (TSA; Cultimed; Barcelona, Spain) supplemented with 0.6 % Yeast Extract (Cultimed)) and incubated at 37 °C for 24 h. Finally, isolates were subcultured twice on TSAYE to ensure purity, each time incubating plates at 37 °C for 24 h, for strain confirmation as described later. Results were expressed in log CFU/cm².

2.4.2.2. *Listeria monocytogenes* detection

The rest of the suspension (see section 2.4.) was used for *L. monocytogenes* detection following a modification of the ISO 11290-1 norm (ISO, 1996). For primary enrichment, the corresponding amount (22.4 µl) of Half-Fraser Selective Supplement (Oxoid) was added to the remaining aliquot (1.4 ml) and was incubated at 30 °C for 24 h. Next, 100 µl was transferred (in triplicate) to a new tube containing 10 ml of sterile Fraser Broth (Fraser Broth Base with Fraser Selective Supplement; Oxoid) and incubated at 37 °C for 48 h to carry out secondary enrichment. Eventually, a loop from primary and secondary enrichments, regardless of medium colour shift, was streaked onto an ALOA plate and incubated at 37 °C for 24 h. In case of presumptive colonies, these were picked and subcultured on TSAYE for species confirmation.

In all samples, this procedure was stopped if a positive in the enumeration phase took place.

2.4.2.3. Confirmation of presumptive *L. monocytogenes* isolates

In order to confirm the presence of *L. monocytogenes*, a 16S rRNA gene amplification, sequencing and analysis was carried out. Firstly, genomic DNA was extracted following the protocol described by Vázquez-Sánchez, López-Cabo, Saá-Ibusquiza, & Rodríguez-Herrera, (2012) and amplification was performed in a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA) using primer pair 27FYM and 1492R' as described elsewhere (Weisburg et al., 1991). PCR product was resolved in a 1.5 % agarose gel stained with Red Sefe Nucleic Acid Staining Solution (iNtRON Biotechnology, Sangdaewon-don, South Korea) to check amplicon size, using Hyperladder 50 bp (Bioline, Singapore) as a

molecular weight marker, and visualised in a GelDoc 2000 Apparatus equipped with Quantity One Software (Bio-Rad). Next, product was purified using a GeneJet gel extraction kit (Thermo Scientific, Vilnius, Lithuania) and subsequent sequencing was carried out using an AB 3130 Genetic Analyser (Applied Biosystems, Foster City, CA) at Centro de Apoyo Científico y Tecnológico a la Investigación (CACTI; Vigo, Spain). Electropherograms were processed and double analysed with Nucleotide-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Ribosome Database Project (RDP; <http://rdp.cme.msu.edu/>) algorithms.

2.4.2.4. *Listeria monocytogenes* isolates maintenance

Stock cultures were made in Brain-Heart Infusion Broth (BHI; Scharlab, Barcelona) containing 50 % glycerol 1:1 (v/v) mixed (final glycerol concentration = 25 %) and flash frozen at – 80 °C.

2.5. *Epifluorescence microscopy assays*

As described in section 2.4., two coupons from each sample set were used for microscopy analysis. First, organic matter and solid residues were removed from the surface of the coupon with running distilled water. Following this, coupons were stained for 30 min in dark with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies, Eugene, OR, USA) according to manufacturer's instructions. Staining solution was removed by gentle washing with running distilled water and coupons were allowed to air dry in the BSC, avoiding direct light. Samples were then placed on a

glass slide and visualised under a Leica 6000DM epifluorescence microscope equipped with a DFC 365 FX camera using 10x ocular and a 40x objective. Representative images of each coupon were digitally acquired using Metamorph MMAF software (Molecular Devices, Sunnyvale, CA).

2.6. 16S rRNA gene metagenetic analysis

For these assays, each coupon was double swabbed as described in section 2.4. All four swabs belonging to a particular location were pooled in 2 ml of Fraser Broth Base and mixed with 2 ml of 50% glycerol 1:1 (v/v) kept at – 80 °C if the DNA extraction was not performed immediately after swabbing. Once needed, the aliquot was recovered and thawed for its processing.

2.6.1. Library construction and quantification

DNA was extracted from the suspension using a PureLink™ Microbiome DNA Purification Kit (Life Technologies) following the manufacturer's instructions. Then, the 16S rRNA gene was amplified with 16S Ion Metagenomics Kit (Life Technologies) which is designed for rapid analyses of polymicrobial samples using Ion Torrent sequencing technology. Specifically, the kit utilises two primer pools that selectively amplify seven hypervariable regions (V2, V4, V8 and V3, V6-7, V9) of the bacterial 16S rRNA gene, using 2 ng of template DNA per reaction. Following this, all amplicons were equally combined and quantified using the Agilent 2100 Bioanalyser. Construction of libraries was undertaken using the Ion Plus Library Kit for AB Library Builder

System (ThermoFisher Scientific) following the library preparation protocol for short amplicons. Amplified DNA, between 150 and 400 ng, was used for library preparation and each sample was barcoded using the P1 adapter and the barcoded A adapters provided in the Ion Xpress Barcode Adapter Kit (Life technologies). Quantification of the resulting DNA libraries was carried out by qPCR using the Ion Library Quantitation Kit (ThermoFisher Scientific) following manufacturer's instructions, to calculate the dilution factor of each library for a final concentration of 10 pmol. Finally, barcoded libraries were pooled in equimolar amounts (10 µl each) in order to obtain equal representation of each sample in the sequencing process.

2.6.2. Template set up and sequencing

Emulsion PCR was carried out using the Ion PGM Hi-Q view OT2 kit adequate for reads up to 400 bp according to the manufacturer's standard protocol using OneTouch 2 and Ion One Touch ES instruments (ThermoFisher Scientific) as indicated by manufacturers' instructions. Finally, sequencing was performed using the Ion Hi-Q Sequencing View Kit (400 bp) and 316 V2 BC chips in an Ion Personal Genome Machine (PGM; ThermoFisher Scientific).

2.6.3. Data analysis

Base calling and run demultiplexing were undertaken by Torrent Suite Software v. 5.2.2 (ThermoFisher Scientific) using default parameters. Individual BAM files were then generated using FileExporter version 5.2.0.0 (ThermoFisher Scientific). In all cases, mean read length ranged between 236 bp to 241 bp. Next, BAM files were analysed using the 16S Metagenomics 16S workflow v 1.1 of the Ion Reporter Software enabling rapid identification of bacteria present in complex polymicrobial samples using both curated Greengenes (DeSantis et al., 2006) and premium curated MicroSEQ® ID 16S rRNA (Life Technologies) databases.

3. Results and discussion

3.1. Relevance of in situ sampling

Over recent years, food microbiologists have been facing various challenges in the study of biofilms such as the monitoring of these structures in order to determine their composition and fitness linked to the environmental conditions (Alvarez-Ordóñez et al., 2019). Following this line, in this work the sampling procedure was specifically designed to minimise the variations that occurred during collection and subsequent processing of the sample by placing SS coupons directly in sites previously identified as *L. monocytogenes*-positive locations (Rodríguez-López et al., 2019). Recovery of the cells was performed using double swabbing mixed with vigorous vortexing in order to dislodge the cells from the swab, which has proven to be an effective technique for direct evaluation of microbial loads (bacteria and moulds) and downstream HTS studies (Moen et al., 2016).

In situ sampling has been previously used for microbial load direct evaluation (bacteria and moulds) and also to determine the presence of pathogenic bacteria in drinking water systems (Douterelo et al., 2016, 2014), giving information about both the microbial diversity and the potential consequences for public health. Similarly, but in a different context, Moen et al., (2016) used an *in situ* approach to evaluate the biofilm formation and composition by placing SS coupons into domestic SS sinks. Their outcomes revealed that not only the type of material was relevant for the establishment and maturation of the biofilm, but also the overall condition of the surface. Therefore, it was demonstrated that this type of sampling approach is suitable for evaluating the impact of surface ageing on bacterial adhesion. In food industry, Ripolles-Avila, Hascoët, Martínez-Suárez, Capita, & Rodríguez-Jerez, (2019) used a SS-based sensor on different food and non-food contact surfaces in a pork processing plant during two years of sampling to evaluate the level of pathogen contamination linked with the load and diversity of the resident microbiota. Despite the abovementioned studies, to date, no study has been published based on *in situ* sampling over time followed by 16S rRNA gene metagenetic analysis, to the best of the authors' knowledge.

Based on the results obtained in this study, extensively discussed below, it is thus demonstrated that this approach not only allows to characterise the microbial diversity in *L. monocytogenes*-positive locations, but also to assess the effectiveness of the cleaning and disinfection routines performed in a given scenario. This fact is highly relevant since it gives a broader perspective than classical techniques (e.g. RODAC plates, etc.) of the past and current resident microbiota present in a given location, revealing not only the presence of pathogenic bacteria but also the potential favouring

of the recalcitrance of some taxa over time. This characterisation is essential to develop *ad hoc* efficient antimicrobial strategies.

3.2. *Listeria monocytogenes* detection and quantification from samples previously identified as contamination locations.

For a complete list of results regarding detection and quantification, the reader is referred to Supplementary Table 1. As performed previously (Rodríguez-López et al., 2019), detection of *L. monocytogenes* was performed using ISO 11290-1 method (ISO, 1996). In samples coming from meat industry, detection after enrichment and subsequent ALOA plating was possible in samples of 7 days in location no. 107 (sample 107C3) and in samples of 7 and 28 days in location no. 96 (samples 96C14 and 96C11, respectively) in which colonies presented the typical clear halo in the medium. In contrast, in fish industry samples typical colonies on ALOA plates were only present in 98 days samples in both sampling locations (location 168: samples 168C9, 168C10 and 168C11; location 225: samples 225C10 and 225C11), fact that could be derived from the low temperature of the sampling location making the development of *L. monocytogenes* and, thus, its subsequent abundancy to be below the limit of detection for ISO 11290-1. These outcomes were further confirmed as *L. monocytogenes* by 16S rRNA gene sequencing, yielding no false positives (Supplementary Table 1).

These results confirmed those obtained previously in which *L. monocytogenes* was detected in the same sampling locations (Rodríguez-López et al., 2019). The origins of such contamination are of diverse nature and a topic of controversy among the scientific community. Whereas some authors claim that the presence of *L. monocytogenes* can be

related to contact with raw materials (Pagadala et al., 2012), there is evidence demonstrating that this occurrence and direct contact with raw matter are independent facts (Sala et al., 2016). In line with this, locations 96 and 225 should be especially taken into account since they are constantly manipulated by plant operators. This poses a high risk of direct (location 96) and indirect (location 225) cross-contamination due to human factors (Todd et al., 2009) should not be overlooked.

As observed, there was not a continuity in the presence of the pathogen (location 96), meaning that in spite of the cleaning and disinfection routines performed in the different processing plants, there is a recurrent recontamination with the subsequent hazard of transmission to the different food products. Additionally, *L. monocytogenes* surrounding microbiota could have impaired the growth as previously observed *in vitro* in mixed biofilms, up to undetectable levels (Langsrud et al., 2016). This was further confirmed since no quantification was possible in any of the samples collected and processed using the ISO 11290-2 method (ISO, 2004, 1998) (Supplementary Table 1).

3.2. AVC psychrotrophic microbiota dynamics in contamination locations

As depicted in Fig. 4A, high psychrotrophic counts in meat samples were detected. These had a general increasing tendency during the sampling period with maximum counts of 6.55 ± 0.30 and 6.84 ± 0.35 CFU/cm² in location 96 (second and first sampling round, respectively) and 6.91 ± 0.85 CFU/cm² in location 107 (second sampling round). Despite this fact, in the first sampling round of location 107 it seemed that the psychrotrophic load stabilised according to the observed counts (mean value of 4.25 ± 0.03 log CFU/cm²). These values were generally lower than those obtained

previously in the same locations (Rodríguez-López et al., 2019). Nevertheless, a new increase or decrease due to the normal activity of the plant cannot be discarded.

For coupons coming from the fish processing environment, location 168 presented a significantly higher microbial load than location 225 at all sample times. Maximum counts in location 168 were obtained at 98 days (8.12 ± 0.13 log CFU/cm²) whereas in location 225 the highest value was obtained at 42 days followed by a decrease at 98 days (4.08 ± 0.57 and 3.05 ± 0.64 log CFU/cm², respectively). These remarkable differences are attributable to the nature of the sample site. More specifically, location 168 is a non-food contact surface located in a cold, highly moistened environment constantly contaminated with fish waste, such as skin and guts, which would be expected to harbour high microbial loads and, consequently, acting as materials for recurrent contamination of products (Pagadala et al., 2012; Parente et al., 2018). In contrast, location 225 is a food contact surface situated in an area of warmer temperature and lower moisture levels than location 168. Moreover, this particular location is constantly decontaminated by routine sanitisation procedures, which corroborates the lower amount of microbiota found.

3.3. Microscopy assays

Epifluorescence microscopy has proven to be an effective approach for studying biofilms, as it can provide straightforward and meaningful information regarding the development and maturation of the structure over time (Beyenal et al., 2004; Jackson et al., 2001). Additionally, this technique together with LIVE/DEAD® staining, has also been used to assess the effectiveness of food-grade disinfectants in mixed-species

biofilms (Rodríguez-López et al., 2017b). This aspect is particularly relevant to the present study since the samples collected were subjected to routine sanitisation regimes in the processing plants surveyed. Hence, *de visu* analysis of the micrographs gives not only an idea of the amount of bacteria present, but also the level of viability of the whole structure based on the ratio of live and damaged/dead cells (green and red fluorescence, respectively).

As observed in Fig. 3, there was a clear predominance of live (green-emitting) cells at all sampling times, however, structural differences were observed depending on the location. Specifically for location 107 on day 7, cells were generally uniformly distributed across the surface even though some cloud-shaped structures were present indicating some degree of clusterisation. After this point in time, adhered cells present on the surface formed a more uniform structure with cells sparsely distributed (Fig. 3). Contrarily, location 96 on day 7 of sampling presented a uniform morphology that was gradually modified into a more unequal architecture in which cellular clusters predominated. This clusterisation was particularly evident in day 28 of sampling where large aggregates with red-emitting nuclei surrounded by green cells were present (Fig. 3). This architectural evolution can be explained by the hydrodynamic nature of location 96, being in constant contact with running water, disinfectant residues and organic matter favouring microcolony-based growth (Chmielewski and Frank, 2003). This distribution was previously observed in mixed-species biofilms (Rodríguez-López et al., 2017b) and could have an important role in the development of resistance structures in which dead/injured cells act as a scaffold for the live surrounding cells. This could represent a stabilisation mechanism for the existing biofilm and further strategy for incoming microbiota to adhere (Bayles, 2007). This would be especially relevant for

cleaning and disinfection systems in which organic/biological matter is not effectively removed from the surface.

3.4. Assessment of microbial community dynamics in selected samples via 16S rRNA gene metagenetic analysis.

Among the samples obtained, three different samples from locations 96, 107 (meat industry) and 168 (fish industry), respectively were processed as described in section 2.6. and subjected to subsequent metagenetic characterisation. The criterion applied was 1) the presence of *L. monocytogenes* in the detection assays and 2) the high psychrotrophic counts observed relative to other samples.

3.4.1. Analysed sequences, alpha indexes and rarefaction curves.

Samples were processed using Ion Reporter Software v. 5.10.5.0 (ThermoFisher Scientific) using Metagenomics 16S w 1.1 workflow. Broadly, valid reads (i.e. with a number of valid copies > 10) ranged between 384624 and 663376. The minimum percentage of mapped reads using the curated MicroSEQ® reference library v. 2013.1 and the curated Greengenes database v. 13.5 (<http://greengenes.lbl.gov>) was of a 70.84 % in samples from location 96, 67.63 % for location 107 and 42.24 % for location 168. For more detailed data about the metagenetic analysis, the reader is referred to Supplementary Table 2.

Alpha diversity indexes in samples from meat industry (i.e. locations 96 and 107) had similar values indicating that in both locations there is a high microbial diversity yet

with slightly different dynamics (Table 1). Whereas in location 96 both Simpson's and Shannon-Wiener indexes increase with time meaning an increasing diversity in this particular community directly related with the number of OTUs observed, in location 107 the situation is slightly different (Table 1). As observed for both family and genus, there was an increase in the number of OTUs in the second sample (107_Mar; Fig. 5) and a sharp decrease in the third (107_Apr; Fig. 5) whilst the both diversity indices decreases constantly. This could be an indicator that there is a dominance of some of the members of the biofilm making the community richer but less diverse in the second sampling and less rich and less diverse in the third. Inter-bacterial interactions are intrinsic in polymicrobial biofilms and, combined with environmental factors, can play in favour of a particular family or genus (Giaouris et al., 2015). In particular, location 96 is a permanently humid contamination focus whereas location 107 was a surface mostly dry and seldom in contact with running water. This fact would explain the higher diversity in the first contamination site, since water availability is a key element for bacterial survival in food industry (Esbelin et al., 2018). Of note, the latter is especially relevant if one of these favoured bacteria is of a pathogenic nature since the biofilm can act as a reservoir.

For samples from the fish processing facility (i.e. location 168), the number of OTUs increased nearly 2-fold from the initial to the final times (Table 1, Figure 5). When examining the trends for the Shannon-Wiener and Simpson indices, it can be seen that both experience a slight rise between the first and second sampling times before subsequently reverting on the final day (Table 1). Altogether, these results indicate that, in spite of the greater number of OTUs observed at both taxonomy levels (i.e. family and genus), there was a shift in the relative abundance of the bacterial community in

favour of one (or a few) members. A plausible explanation for this observation could be related to environmental factors such as temperature and salinity, which could apply selective pressure on the resident microbiota in favour of the better adapted organisms (Doyle et al., 2013; Møretrø and Langsrud, 2017; Rodríguez-López et al., 2019), or due to ecological niche competitiveness between bacteria undertaking different survival strategies to the detriment of the less adapted ones (Rendueles and Ghigo, 2012) stabilising, in the last term, the interrelationships amongst bacteria present in the microbiome.

Independently of richness and diversity of the different samples, rarefaction curves constructed based on the mapped sequences that were further classified into the different OTUs (identity for families: > 95 %; identity for genera: > 97 %) clearly indicated that the number of valid sequences increased with the biofilm age (Fig. 5). Since the number of sequences is directly related with the abundance, it is obvious to claim that the older the biofilm, the greater the number of bacteria adhered to the surface. Nevertheless, it is important to keep in mind that HTS analyses are not able to discriminate between live and dead cells, thus unable to determine the biological activity of the biofilm. So, despite the valuable information given, culture and/or metabolic activity-based methods cannot be fully discarded, hence a combination of both is required to give biological meaning to the assay.

3.4.2. Taxonomic diversity over time in contamination locations

Results of the 16S rRNA gene metagenetic analysis of samples from meat processing facilities (i.e. locations 96 and 107) showed population dynamics in which a particular

phylum dominated over the others provoking a phylum shift (Fig. 6). Specifically, in samples from location 96 there is a clear dominance of Actinobacteria with an increase from 1.72 % to 79.72 % to the detriment of Proteobacteria that experienced an abundance reduction from 71.37 % to 13.69 %. The predominance of phyla found at this location coincides with results obtained in our previous survey carried out at the same sampling location (Rodríguez-López et al., 2019). This highlights the reproducibility of the *in situ* sampling approach used in this study being in line with the outcomes obtained previously by other authors using similar methods (Ripolles-Avila et al., 2019).

Taking a closer look at this shift in diversity, families that represented the Actinobacteria predomination within the community were *Nocardiaceae* (mainly the genus *Rhodococcus*), *Micrococcaceae* (mainly *Rothia*) and *Microbacteriaceae* (mainly *Pseudoclavibacter*) with relative abundance increases of 11.49, 24.02 and 37.84 %, respectively comparing the first and last sampling times considered in this study (Fig. 7A, 8A). On the other hand, Proteobacteria were diminished, mainly due to a sharp decrease of a 53.44 % in the relative abundance of the *Moraxellaceae* (Fig. 7A), represented mainly by the genera *Acinetobacter* and *Psychrobacter*. These results coincided with those obtained in our previous study (Rodríguez-López et al., 2019) and also were in line with results published by other authors stating that the above mentioned families and genera are among those mostly present in food industry premises (Møretrø and Langsrud, 2017). Of note, the sampling location was a non-food contact surface (drain) constantly in-touch with rinsing water containing biological matter and disinfectant residues.

In contrast, samples from location 107 were mainly dominated by Firmicutes (58.02, 64.73 and 66.31% in samples from February, March and April, respectively) whereas Proteobacteria and Actinobacteria abundances remained almost constant in all three samplings (Fig. 6). This steadiness of the above mentioned phyla could be explained in part due to the characteristics of the location in which few environmental and mechanical variations take place. Of note, there was a sharp decrease in the abundance of Bacterioidetes that ended as a residual phylum in the last sampling (107_Feb: 6.15 %; 107_Mar: 0.87 %; 107_Apr: 0.08 %), which could have been due to environmental factors rather than the fitness of the members of the dominant phylum (Kampmann et al., 2012). More specifically, as depicted in Fig. 8B, in final samples the most representative members among the phylum Firmicutes were mainly the genera *Facklamia* (Aerococcaceae; 16.76%), *Jeotgalicoccus* (Staphylococcaceae; 15.55 %) and in a lesser extent the genus *Clostridium* (Clostridiaceae; 1.57 %). Representatives of all these genera have been previously isolated from environmental sources linked to food processing (Glaeser et al., 2016; Rodríguez-López et al., 2019). Particularly significant is the genus *Clostridium* since it can produce environmentally resistant endospores (Onyenwoke et al., 2004; Talukdar et al., 2017) that can not only endure low moistened environments, but also those in which disinfectants are routinely deployed (Charlebois et al., 2017; Davidson and Harrison, 2002). On the other hand, the major genus present within the Actinobacteria was *Corynebacterium* (Corynebacteriaceae; 20.74%), which has been implicated in the contamination of meat foodstuffs and illnesses in humans (Alibi et al., 2016).

As expected, sample from location 168 (fish industry) showed a completely different picture in terms of taxonomic distribution (Fig. 6). Phyla distribution showed a clear

predominance of Proteobacteria at the first sampling time accounting for a 90.69 % of the total microbiota characterised in the sample. This result was in line with the HTS analysis of biofilm from the same location reported in an earlier study (Rodríguez-López et al., 2019). More specifically, *Aeromonadaceae* (21.07 %), *Moraxellaceae* (26.21 %) and *Pseudoalteromonadaceae* (37.26 %) (Fig. 7C) were the most representative families of the taxon including genera typically associated with marine and coastal environments and forming part of the fish skin commensal microbiota such as *Oceanisphaera* (9.13 %), *Pseudoalteromonas* (27.82 %) and *Psychrobacter* (16.14 %) (Bowman, 2006; Bowman and McMeekin, 2015; Choi et al., 2011; Lokesh and Kiron, 2016; Teixeira and Merquior, 2014) (Fig. 8C). Nevertheless, this abundance decreased in subsequent samplings (168_Jun: 70.71 %; 168_Aug: 37.98 %) giving way to a predominance of the Bacteroidetes (48.04 %) in the last time of sampling (Fig. 6). As depicted in Fig. 7C, this increase of relative abundance was due to the continuous increase of *Flavobacteriaceae*, in particular, the proliferation of the genus *Flavobacterium* (168_May: 2.32 %; 168_Jun: 12.45 %; 168_Aug: 29.58 %) (Fig. 8C). Although generally not pathogenic to humans, members of this genus are often part of the natural microbiota of fresh and salt water environments (Bernardet and Bowman, 2006), and should be considered as potentially hazardous since some species produce cytotoxic metabolites (Kul et al., 2018) and are known pathogens of aquatic animals. In fact, *F. columnare* has been implicated in tremendous economic losses for commercial aquaculture operations (Verma and Rathore, 2015).

Of note, the relative abundance of the phylum Firmicutes also increased as the bacterial community matured, achieving a final value of 12.34 % in the final sampling time (Fig. 6). According to the mapped sequences, *Listeriaceae* was detected in the first two

sampling times despite its relative abundance being < 1 % of the bacteriome in both cases (results not shown). Moreover, an increasing proportion of lactic acid bacteria (LAB), especially the *Carnobacteriaceae* (*Carnobacterium* sp.), was observed in the three samples analysed (Fig. 7). Since LAB are known antagonists of the *Listeriaceae*, this could in part explain the low numbers of members of this family detected in the samples (Rodríguez-López et al., 2015). Despite this protective role against *Listeriaceae* members, LAB such as *Carnobacterium* sp. are described as fish spoilers (Laursen et al., 2005; Laursen, Leisner, & Dalgaard, 2006; Leisner, Laursen, Prévost, Drider, & Dalgaard, 2007) or coexist with other spoilage microbiota (Stellato et al., 2015), a fact that cannot be neglected as since it represents a potential issue of concern in food industry.

4. Conclusions

Since classical, broad-spectrum sanitisation procedures are known to have several deficiencies, modern food safety systems nowadays require extensive knowledge of the microbiota present in a given setting. In this study, *in situ* sampling coupled with the use of 16S rRNA metagenetic profiling provided a broad perspective of the evolution of the resident bacterial communities present in the biofilms over time, while linking them to the environmental factors associated with specific locations within the processing facilities examined in this study. The acquisition of such transversal ecological knowledge of food industry biofilms is essential for the development of novel target-directed cleaning and disinfection regimes. Additionally, outcomes of sequential time sampling compared with those obtained previously, demonstrate that the environmental

factors favour a specific bacterial community in every particular location; data that could be used in predictive systems in food-related industrial settings, in order to improve safety of the final product.

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918 **Fig. 1.** Images of the sampling locations located in the different food-related premises
 919 previously identified as *Listeria monocytogenes*-positive (Rodríguez-López et al.,
 920 2019). Yellow arrows indicate the places where the sampling devices were fixed.

Fig. 2. Scheme diagrams and actual images of SS coupon-based sampling devices used for sampling in this study. Values are the dimensions in mm of the coupons representing the actual sample to be processed in the different assays.

Fig. 3. Biofilm formation on *in situ* coupons placed on contamination locations 96 and 107, both located in meat industry premises. Micrographs correspond to 40x field epifluorescence images obtained after LIVE/DEAD staining in which green cells represent undamaged (live) cells and red cells represent those either damaged or dead. White arrows point out cellular clusters present in the sample. Scale bar = 50 μ m.

Fig 4. Mean adhered viable counting (AVC) values (n =3) of plate count assays of the dynamics of psychrotrophic microbiota from coupons collected *in situ* in *L.*

monocytogenes-associated contamination locations in meat (A) and fish (B) industries.

Error bars = SD.

Fig 5. 16S rRNA gene metagenetic analyses rarefaction curves depicting the number of operational taxonomic units (OTUs) for families and genera obtained in the different sampling locations at different sampling times. In case of a lack of extension of the plot all the way of the x-axis, is an indicator of a lower amount of mapped sequences. Curve plateaus indicate an approximation to completeness.

Fig. 6. Phyla distribution obtained in 16S rRNA gene metagenetic analysis from *in situ* communities in meat and fish food industry samples. In all cases, in the “others” sections, the phyla with an abundance < 1% were represented.

A.

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B.

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Fig. 7. Family relative abundances obtained in the 16S rRNA gene metagenetic assays (mapped sequences) from samples collected *in situ* in the contamination locations no. 96 (A) and 107 (B), both belonging to meat industry and location 168 (C) from fish industry. In the “other” box, those families with a relative abundance < 1% were included.

Fig. 8. Genera relative abundances obtained in the 16S rRNA gene metagenetic assays (mapped sequences) from samples collected *in situ* in the contamination locations no. 96 (A) and 107 (B), both belonging to meat industry and location 168 (C) from fish industry. In the “other” box, those genera with a relative abundance < 1% were included.

Table 1. Alpha diversity indexes and observed taxonomic units (OTUs) obtained in the metagenetic analysis of samples of biofilms formed *in situ* in industrial premises.

Sector	Sample ID	Family			Genus		
		Simpson	Shannon-Wiener	OTUs	Simpson	Shannon-Wiener	OTUs
Meat	96_Feb	0,684	2,314	50	0,680	2,459	69
	96_Mar	0,723	2,704	70	0,726	2,885	100
	96_Apr	0,779	2,961	75	0,830	3,288	114
	107_Feb	0,907	4,040	67	0,893	4,077	109
	107_Mar	0,886	3,842	81	0,876	3,957	128
	107_Apr	0,884	3,229	59	0,846	3,414	86
Fish	168_May	0.745	2.420	22	0.754	2.655	27
	168_June	0.757	2.618	30	0.781	2.781	33
	168_Aug	0.709	2.496	43	0.732	2.625	52

Supplementary info

Supplementary Table 1. Complete list of samples and results regarding *Listeria monocytogenes* detection and direct quantification based on the protocols described in ISO 1290-1 and ISO 11290-2 norms (ISO, 2004, 1998, 1996) with subsequent confirmation based on 16S rRNA gene amplification and analysis (Weisburg et al., 1991).

Supplementary Table 2. Technical data obtained during 16S rRNA gene metagenetic analyses from *in situ* samples collected from *L. monocytogenes*-positive locations 96, 107 (meat industry) and 168 (fish industry).