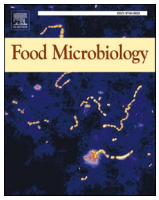
Food Microbiology 109 (2023) 104138

Contents lists available at ScienceDirect

Food Microbiology

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



Bacterial community development and diversity during the first year of production in a new salmon processing plant

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ARTICLE INFO

Keywords:
Bacterial community
Species-level identification
ON-Rep-seq
Food processing environment
Food safety
Food spoilage

ABSTRACT

The bacterial diversity and load on equipment in food processing facilities is constantly influenced by raw material, water, air, and staff. Despite regular cleaning and disinfection, some bacteria may persist and thereby potentially compromise food quality and safety. Little is known about how bacterial communities in a new food processing facility gradually establish themselves.

Here, the development of bacterial communities in a newly opened salmon processing plant was studied from the first day and during the first year of operation. To focus on the persisting bacterial communities, surface sampling was done on strategical sampling points after cleaning and disinfection. To study the diversity dynamics, isolates from selected sampling and time points were classified by Oxford Nanopore Technology-based rep-PCR amplicon sequencing (ON-rep-seq) supplemented by 16S rRNA gene or *rpoD* gene sequencing (for *Pseudomonas*). An overall increase in bacterial numbers was only observed for food-contact surfaces in the slaughter department, but not in filleting department, on non-food contact surfaces or on the fish. Changes in temporal and spatial diversity and community composition were observed and our approach revealed highly point-specific bacterial communities.

1. Introduction

Microorganisms are constantly introduced into food processing facilities via raw material, water, equipment, and staff, and may compromise both the shelf life of the product and the food safety. In a salmon processing facility, the cold marine water environment and the chilled processing facilities favor Gram negative, psychrotrophic bacteria (Guðbjörnsdóttir et al., 2005; Langsrud et al., 2016; Møretrø et al., 2016). *Pseudomonas* spp., *Enterobacteriaceae* and *Acinetobacter* spp. are generally dominant on food processing surfaces in seafood, meat and dairy industry (Møretrø and Langsrud, 2017) whereas *Pseudomonas* spp. and *Shewanella* spp. were the most common bacteria detected in the salmon processing environment (Møretrø et al., 2016). Many microorganisms can attach to surfaces and form biofilm and this ability cause challenges in the food industry (Mizan et al., 2015; Møretrø et al., 2016). The possibility for transmission of bacteria from food contact surfaces to the food product is well documented (Hinton et al., 2004; Midelet and Carpentier, 2002; Møretrø et al., 2016; Sheen, 2008; Truelstrup Hansen and Vogel, 2011), which highlights the importance of preventing the formation of bacterial biofilm in food processing environments.

The food processing facilities routines on cleaning and disinfection (C&D) aim to keep the bacterial load as low as possible. However, environmental bacteria occasionally survive the C&D treatment when exposed to the disinfectant concentrations used in industrial environments (Fagerlund et al., 2017). Additionally, some types of equipment and surfaces used in the food industry might be particularly difficult to clean and cleaning procedures may sometimes be suboptimal. Together, these issues may result in residual bacteria forming a persisting background microbiota (Nivens et al., 2009).

Persisting bacteria can form biofilm structures that can host and protect potentially pathogenic bacteria such as *Escherichia coli* and *Listeria monocytogenes* (Giaouris et al., 2013; Gomes et al., 2017; Langsrud et al., 2016; Schwering et al., 2013). *L. monocytogenes* is of profound concern for the food industry, including the salmon industry in Norway, and there are numerous studies on how *L. monocytogenes* survive and persist in biofilms in food producing facilities, as reviewed by Fagerlund et al. (2021) and Lianou et al. (2020). These reviews summarize that both strain variability in *L. monocytogenes* and interactions with the background microbiota affect the survival and persistence of pathogens. The persistence of some *L. monocytogenes* in food producing

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https://doi.org/10.1016/j.fm.2022.104138
Received 31 March 2022; Received in revised form 26 August 2022; Accepted 6 September 2022
Available online 9 September 2022

environments is suggested to be caused by the match between the specific *L. monocytogenes* strain and the surrounding microbiome, and not solely because of specific intrinsic traits (Fagerlund et al., 2021). This highlights the significance in identifying the background bacterial microflora in food processing premises down to species level.

Microbial analysis in food processing environments, as performed by the industry, has traditionally been limited to conventional spread plating and culture dependent methods. In recent years the food industry has taken interest in sequencing-based methods but so far, sequencing-based methods have mostly been used for detection and identification of pathogens and for source tracking of disease outbreaks (Jagadeesan et al., 2019; Klijn et al., 2020; Nouws et al., 2020; Painset et al., 2019; Sekse et al., 2017). Metagenomic studies have also been frequently performed during the last years (Yap et al., 2022) but mainly as a research tool and not as a "routine analysis" for the food industry. However, as the industry gets more familiar with the possibilities of the technology, and feasibility in terms of costs and ease-of-use improves, the number of applications is likely to increase. We believe that making use of state-of-the-art sequencing technology can help to provide additional knowledge about the background microbiota in food processing facilities and hereby assist in ensuring a microbiologically high quality and long shelf life of food products.

The aim of this study was to analyze the development and dynamics, both quantitatively and qualitatively, of the residing bacterial communities in the food processing environment of a newly opened salmon processing facility. For the identification of bacterial isolates to species-level, we aimed to explore the potential of the ON-rep-seq method, a newly developed method that is based on REP-PCR combined with sequencing of the DNA fragments on an Oxford Nanopore device (Krych et al., 2019).

2. Materials and methods

2.1. Sampling in cleaned and disinfected processing environment

The sampling location was a newly opened salmon processing facility at the coast of Mid-Norway. The facility receives salmon from several marine farming locations in the region, and the fish is pumped into the facility directly from the well boat without the use of waiting pens. The facility produces gutted whole fish packaged in Styrofoam boxes with ice, whole fillets packaged in Styrofoam boxes and frozen, and vacuum-packed portioned fillets with or without skin. Bacterial sampling in the facility was performed thirteen times throughout the first year of production (at day 0, 6, 13, 20, 40, 75, 110, 131, 159, 229, 271, 320, 362) at the same 24 fixed sampling points each time (Fig. 1). A short description of the sampling points is given in Table 1. The

Table 1
Overview of the different sampling points, category (FCS = food contact surface in slaughter department or filleting department, NFCS = non-food contact surface, F = fish), sampling type, water, cloth, swab or fish fillet, and approximate sampling area.

Sampling point	Sampling point category	Sampling type	Sampling area/ volume
2. Inlet water		Water	100 mL
3. Drain under inlet	NFCS	Cloth	30 × 30 cm
4. Drain under bleeding tank	NFCS	Cloth	30 × 30 cm
5. Conveyor	FCS, slaughter	Cloth	30 × 30 cm
6. Conveyor	FCS, slaughter	Cloth	30 × 30 cm
7. Drain under orientation rig	NFCS	Cloth	30 × 30 cm
8. Slide above conveyor	FCS, slaughter	Cloth	30 × 30 cm
9. Slide above conveyor	FCS, slaughter	Cloth	30 × 30 cm
10. Gutting machine, suction	FCS, slaughter	Swab	10 × 10 cm
11. Gutting machine, holder	FCS, slaughter	Swab	10 × 10 cm
13. Tail cutter	FCS, slaughter	Cloth	90 × 10 cm
14. Head cutter knife	FCS, slaughter	Cloth	2 x Ø25 cm
15. Head cutter, holder	FCS, slaughter	Swab	10 × 10 cm
16. Peg band before fileting	FCS, fillet	Swab	5 × 20 cm
17. Conveyor after fileting	FCS, fillet	Cloth	30 × 30 cm
18. Conveyor before skinning	FCS, fillet	Cloth	30 × 30 cm
19. Skinning machine	FCS, fillet	Cloth	30 × 30 cm
20. Filet turner, slide	FCS, fillet	Cloth	30 × 30 cm
21. Filet turner, arm	FCS, fillet	Cloth	30 × 30 cm
22. Drain under filet turner	NFCS	Cloth	30 × 30 cm
23. Drain under packaging	NFCS	Cloth	30 × 30 cm
24. Waste funnel, backbone	NFCS	Cloth	30 × 30 cm
25. Waste funnel, skin	NFCS	Cloth	30 × 30 cm
26. Drain, personnel sluice	NFCS	Cloth	30 × 30 cm
F. Fish fillet before packaging	Fish	Fish fillet	25 g
S. Skin, gutted whole fish	Fish	Swab	10 × 10 cm
G. Gills, gutted whole fish	Fish	Swab	Gills on both side of fish

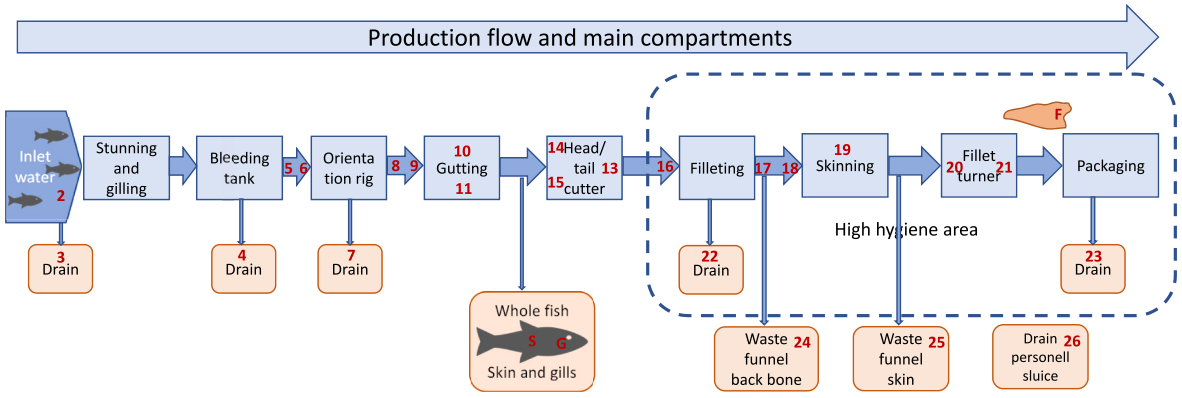

Fig. 1. Schematic diagram of the processing facility. Main equipment and machinery are drawn in light blue squares, conveyors in dark blue arrows, while sampled drains and waste funnels (non-contact surfaces) are drawn in orange. Sampling points are marked with red numbers (2–26). Product samples were taken of filets (F) just before packaging in addition to swab samples of skin (S) and gills (G) of whole fish ready for packaging. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sampling points were chosen in collaboration with the Quality Manager and the Cleaning Manager at the facility. Samples of fish fillet and swab samples of skin and gills of gutted whole fish were collected at four different occasions. All sampling was performed after cleaning, disinfection and air drying in the mornings before startup of normal production. Sampling was performed by swabbing 100 cm^2 with a sterile swab (Promedia ST-25 PBS, r-biopharm, Germany) in 10 mL phosphate buffered saline (PBS) or by swabbing 900 cm^2 (30 × 30 cm) with sterile clothes pre-moistened with 25 mL PBS (Sodibox, France). The choice of swabbing method depended on the type and area of the surface. Clothes and swabs were kept cold in Styrofoam boxes with gel-ice during the transportation (max. 3 h) from the facility to the lab. Spread plating was performed the same day.

2.2. Quantification of general and specific bacteria

Additional PBS was added to the bag with the sampling cloth to a total weight of 50 g more than a clean, unused cloth in its bag, before it was mashed in a Stomacher for 30 s. The cloth was aseptically removed from the bag and ten-fold serial dilutions of the liquid was made before plating on several growth media. Swab samples were also serially diluted 10-fold before plating on growth media. Calculations of CFU/cm^2 were done by multiplying CFU/mL with the volume of the diluent and divided by the swabbed area.

Total aerobic count (AC) and H_2S-producing bacteria were analyzed on Iron Agar (IA) (Oxoid, CM0964) with L-cyctein added to 0.04% final concentration, incubated at 22 °C for 72 h. Aerobic psychrotrophic counts (APC) were analyzed on Long & Hammer agar (LH) (van Spreekens, 1974) with Fe(III)NH_4Citrat added to a final concentration of 0.025%, incubated at 15 °C for 5 days, according to NMKL Method No 184. Analysis for *Pseudomonas* spp. (PsC) was performed on Oxoid™ *Pseudomonas* CFC selective agar (CFC) (CM0559/SR0103, Thermo Fisher Scientific) incubated at 25 °C for 48 h, while *E. coli* and other coliform bacteria was analyzed on Oxoid™ Chromogenic Coliform Agar (Oxoid, CM1205) incubated at 37 °C for 24 h. Detection of *Listeria* spp. was performed according to the Oxoid Listeria Precise™ Method with minor adjustments. Ten mL of the stomacher-liquid or 3 mL of the swab liquid was added to 90 mL or 27 mL respectively of ONE Listeria Enrichment Broth (Oxoid, CM1066B) giving a 10-fold dilution and incubated at 30 °C for 24 h. Positive samples for presumptive *Listeria* spp., seen by a color change in the broth from brown to black, were streaked (10 μL) on to *Brilliance*™ *Listeria* Differential Agar plates (Oxoid, CM1080B with added *Brilliance*™ Listeria Selective Supplement SR0227E and *Brilliance*™ Listeria Differential Supplement SR0228E). Negative samples were left at 30 °C for up to 7 days to also detect damaged, stressed, and slow growing *Listeria* strains.

2.3. Preparation of isolates and DNA extraction

Colonies were picked from LH agar plates for isolation and further analysis. For randomized picking of colonies, plates with 10–100 colonies were selected aiming at 20 colonies per sampling point. Plates containing more than 40 colonies were divided in equal sectors and all colonies in one sector were picked (1/2, ¼, 1/8). The isolates were repropagated minimum twice before they were frozen in TSB w/20% glycerol at −80 °C.

Isolates from sampling time 0 (before startup), 1 (one week after startup), 8 (5 months after startup) and 12 (12 months after startup) and selected sampling points (2, 8, 10, 11, 13, 14, 15, 17, 18, 20, 22) (Table 1) were selected for further analysis and thereby thawed and plated on LH agar again, incubated at 15 °C for 5 days and repropagated twice. DNA extraction from the isolates was done with Micro AX Bacteria Gravity-kit (A&A Biotechnology, Poland) following the producer's procedure. DNA quality and integrity was checked by running 10 μL DNA on 1% agarose gel containing GelRed (Biotium, USA) and visualization under UV-light in a G:box (Syngene, USA). DNA concentration was measured spectrophotometrically by a PowerWaveXS (Biotek®, USA) and Take3 plate with software Gen5 2.0. For isolates resulting in low DNA concentration by this procedure, DNA extraction was performed again with Micro AX Bacteria + Gravity-kit (A&A Biotechnology, Poland) which include mutanolysin treatment for lysing Gram-positive bacteria.

2.4. Classification of isolates by ON-rep-seq method

DNA was normalized (1 ng/μL) and subject to ON-rep-seq analysis at University of Copenhagen. Library preparation and amplifications was performed as described by Krych et al. (2019). In brief: A Rep-PCR with REP primers (GTG)5 was performed to amplify fragments of the DNA before the dual step barcoding Rep-PCR was done to incorporate the Oxford Nanopore Technology (ONT) compatible adapters. The samples were pooled, and the library purified before final DNA quantification measurement and end preparation according to 1D amplicon by ligation protocol (ADE_9003_v108_ revT_18Oct2016), and finally loading of the library on a R9.4.1 flow cell.

Data were collected using Oxford Nanopore software: GridION 19.12.2 (https://nanoporetech.com). Guppy 4.4.0 toolkit was used to base call raw fast5 to fastq and demultiplex based on custom adapters. Further, the ON-rep-seq data analysis toolbox (https://github.com/lauramilena3/On-rep-seq), was used to classify the isolates (Krych et al., 2019). From the sequenced amplicons a read length count profiles (LCp) were generated for each sample and a corrected consensus read for all the reads in each peak were generated. Kraken2 (Wood and Salzberg, 2014) metagenomic classifier was used for classification of corrected reads based on NCBI database. For visualization of D_KLsym distance on bacterial LCp, heatmaps was generated based on Ward.D clustering method and modified heatmap3 from R library as previously described in Krych et al. (2019). Details can be found on https://on-rep-seq.readthedocs.io/en/latest/index.html.

2.5. Sequencing of 16S rRNA gene or rpoD gene

Isolates that were not sufficiently classified by ON-rep-seq method were subjected to 16S rDNA sequencing. The universal 16S primers 338f (Huse et al., 2008) and 1492r (Turner et al., 1999) were used, resulting in an amplicon of ~1154 bp, covering V3–V9 variable regions. PCR reactions were performed with 25 μL reactions containing 1x PCR buffer, 200 μM of each nucleotide, total concentration of $MgCl_2$ at 650 μM, 0.4 μM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Initial denaturation at 95 °C for 15 min, 25 cycles of denaturation at 95 °C for 60 s, annealing for 30 s at 58 °C, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min.

Isolates identified as *Pseudomonas* genus by ON-rep-seq, without a clear species classification were subjected to sequencing of the *rpoD* housekeeping gene with primers PsEG30F/PsEG790R, resulting in a 760 bp product (Mulet et al., 2009). The PCR reactions were performed with 25 μL reactions containing 1x PCR buffer, 200 μM of each nucleotide, total concentration of $MgCl_2$ at 650 μM, 0.5 of μM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. PCR products were enzymatically purified by ExoSAP-IT™ (Thermo Fischer Scientific, USA) procedure which entail incubation at 37 °C for 15 min to degrade remaining primers and nucleotides followed by inactivation at 80 °C for 15 min. A quality control of the purified PCR products was performed, and the PCR products were prepared for sequencing according to Eurofins LightRun sequencing acquirements.

Classification of the isolates was done by comparison to sequences currently available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST) using BLASTN search.

2.6. Statistical data analyses

Statistical analyses were performed using IBM SPSS Statistics 27. To analyze the difference in bacterial load for sampling points of different categories (non-food contact surfaces (NFCS), food contact surface (FCS) slaughter dep., FCS filet dep., and fish), a One-way ANOVA with post hoc Tukey test was done. Correlation between the bacterial parameters Aerobic count, aerobe psychrotrophic count, *Pseudomonas* spp. count, H_2S-producing bacteria and coliform count was calculated by a bivariate correlation analysis for Pearson's coefficient.

3. Results

3.1. High variability in bacterial cell counts between the sampling points

A total of 312 different samples were collected after cleaning and disinfection, from 24 different sampling points throughout a newly opened salmon processing facility (Fig. 1). Of these samples, 26 (8%) were negative for all parameters checked.

The bacterial counts ranged from zero to 5.9 log CFU/cm^2, 5.8 log CFU/cm^2 and 5.3 log CFU/cm^2 for AC, APC and PsC respectively (Fig. 2). The NFCSs had the highest bacterial count among the environmental samples, with an average of 1.9 log CFU/cm^2, 2.2 log CFU/cm^2 and 1.9 log CFU/cm^2 for AC, APC and PsC respectively. Average bacterial count for these three parameters on FCS in the slaughter department were 0.4 log CFU/cm^2, 1.0 log CFU/cm^2 and 0.8 log CFU/cm^2, and on FCSs in the filet department 0.2 log CFU/cm^2, 0.3 log CFU/cm^2 and 0.2 log CFU/cm^2 for AC, APC and PsC respectively. The gutting machine suction stands out with the highest variability in bacterial cell counts for both AC, APC and PsC ranging from zero to 5.8 log CFU/cm^2 (Fig. 2). The inlet water had an average AC of 2.3 log CFU/mL, APC of 3.4 log CFU/mL and PsC of 2.1 CFU/mL. Moreover, fish filet, skin and gills of round fish were sampled at four occasions during the time period. The bacterial counts varied between the samplings and no clear trend was observed (Fig. 2).

A significant difference (ANOVA, posthoc Tukey $p < 0.001$) in bacterial load could be noticed between the three groups of surface sampling points: NFCS, FCS in slaughter department and FCS in filet department for three of the parameters, AC, APC and PsC.

There was a high correlation between AC, APC and PsC for the different sampling points, with Pearson correlation coefficient at 0.863, 0.844 and 0.859 for AC vs. APC, APC vs. PsC and PsC vs. AC respectively ($p < 0.01$).

Coliforms and H_2S-producing bacteria were only sporadically detected and mostly at low numbers. The head cutter knife was the only FCS where coliforms was detected more than once. H_2S-producing bacteria was overall more frequently detected, mostly on the NFCSs. Yet, the correlation between H_2S-producing bacteria and coliforms towards all the above-mentioned bacterial parameters was significant at 0.01 level.

During the time frame of the sampling, no *L. monocytogenes* was detected on the cleaned and disinfected surfaces. *L. innocua* was detected twice; one time on the head cutter knife and one time in the drain below filleting machine.

3.2. The total bacterial load in the facility increased during the first year of processing

To assess the development of the general bacterial load on the FCSs over time, the average bacteria count for all contact sampling points was calculated (Fig. 3). On FCS in slaughter department (Fig. 3A) an increase in the bacterial load was seen for both AC, APC and PsC, but the increase was most obvious for AC. For FCSs in the filleting department (Fig. 3B) a similar trend was observed but the increase was not as distinct as in the slaughter department. For the NFCS, a slight increase in bacterial load over time was observed. The bacterial load on the fish fillets did not increase over time.

3.3. Microbial profiling by ON-rep-seq and 16S rRNA gene amplicon sequencing

A total of 520 isolates were identified by ON-rep-seq resulting in the detection of 75 unique taxa belonging to 27 different genera. Of all these isolates 78% were identified to species level, additional 8% were identified to genus level and 14% remained unclassified. All isolates that were not classified by ON-rep-seq and the isolates that only reached a genus-level classification were subjected to sequencing of the 16S rRNA gene. When combining the identification from ON-rep-seq analysis and 16S rDNA sequencing 84 unique taxa were detected, and these belonged to 34 different genera. From this combined identification 85% of the isolates were identified to species level, 12% were identified to genus level and 4% still remained unclassified.

Members of the genus *Pseudomonas* were the most abundant in this combined classification and accounted for 26% (n = 22) of the taxa and 46% of all isolates (Fig. S1). Other frequently detected species were *Acinetobacter* spp. (14%), *Serratia* spp. (6%), *Chryseobacterium* spp. (5%) and *Aliivibrio* spp. (3%).

Based on the current database only 8% of *Pseudomonas* isolates obtained a clear species classification by ON-rep-seq. Additional 42% were assigned to different unclassified strains of *Pseudomonas*. The remaining 50% of *Pseudomonas* isolates could not be classified by this method. Isolates of *Pseudomonas* genus that did not obtain a clear species classification by ON-rep-seq were subjected to sequencing of the *rpoD* gene (n = 101). Of these 101 *Pseudomonas*, 62 isolates (61%) obtained a clear species classification, *P. fluorescens* being the most abundant (43%) (Fig. S2). Additionally, eight isolates (8%) were assigned to different strains of unclassified *Pseudomons* spp. while the remaining 31 isolates had a high similarity to several different species and could not be classified with certainty or could not be assigned to any known species. Fifteen of these 31 isolates had a similarity to other *rpoD* sequences of less than 98% and could not be assigned to any known species (Girard et al., 2020) while the remaining isolates had high similarity to several *Pseudomonas* spp. belonging to different groups and subgroups.

3.4. Spatial and temporal dynamics of the bacterial communities

Species of *Pseudomonas* were more abundant on the equipment and environment surfaces than in inlet water and on the salmon fillet (Fig. 4). When comparing all the analyzed sampling points, *Pseudomonas* was present in 94% of them. *Photobacterium* was only detected in the inlet water and on the salmon fillet, while *Vibrio* was only detected in the inlet water. *Aliivibrio* and *Psychrobacter* was only detected in inlet water and on conveyor belt in the slaughter department and do not seem to be carried further into the facility. The genera *Janthinobacterium*, *Leucobacter*, *Comamonas*, *Morganella*, *Pseudochrobactrum*, *Arthrobacter*, *Pedobacter*, *Sphingobacterium* and *Galactobacter* were only detected as singletons.

3.5. Species/strain-level resolution reveals time and point-specific communities

ON-rep-seq analysis assigned most of the isolates to species level, but some isolates only to genus level (Fig. 4, Supplement Table S1). Using dynamic classification gives a high resolution and reveals that only a few species are detected at several sampling points or time (Supplemental Table S1). Of all the detected taxa, 61% (n = 51) were detected in only one sample. Only 10% (n = 8) of the taxa were detected in five samples or more. Five of these taxa belonged to different groups, species, or strains of *Pseudomonas* genus while the rest belonged to *Microbacterium* sp., *Serratia liquefaciens* and the remaining group of unclassified isolates. In addition, only 12% (n = 10) of the taxa were detected both in the equipment and on the salmon fillet. Of these taxa, seven belonged to

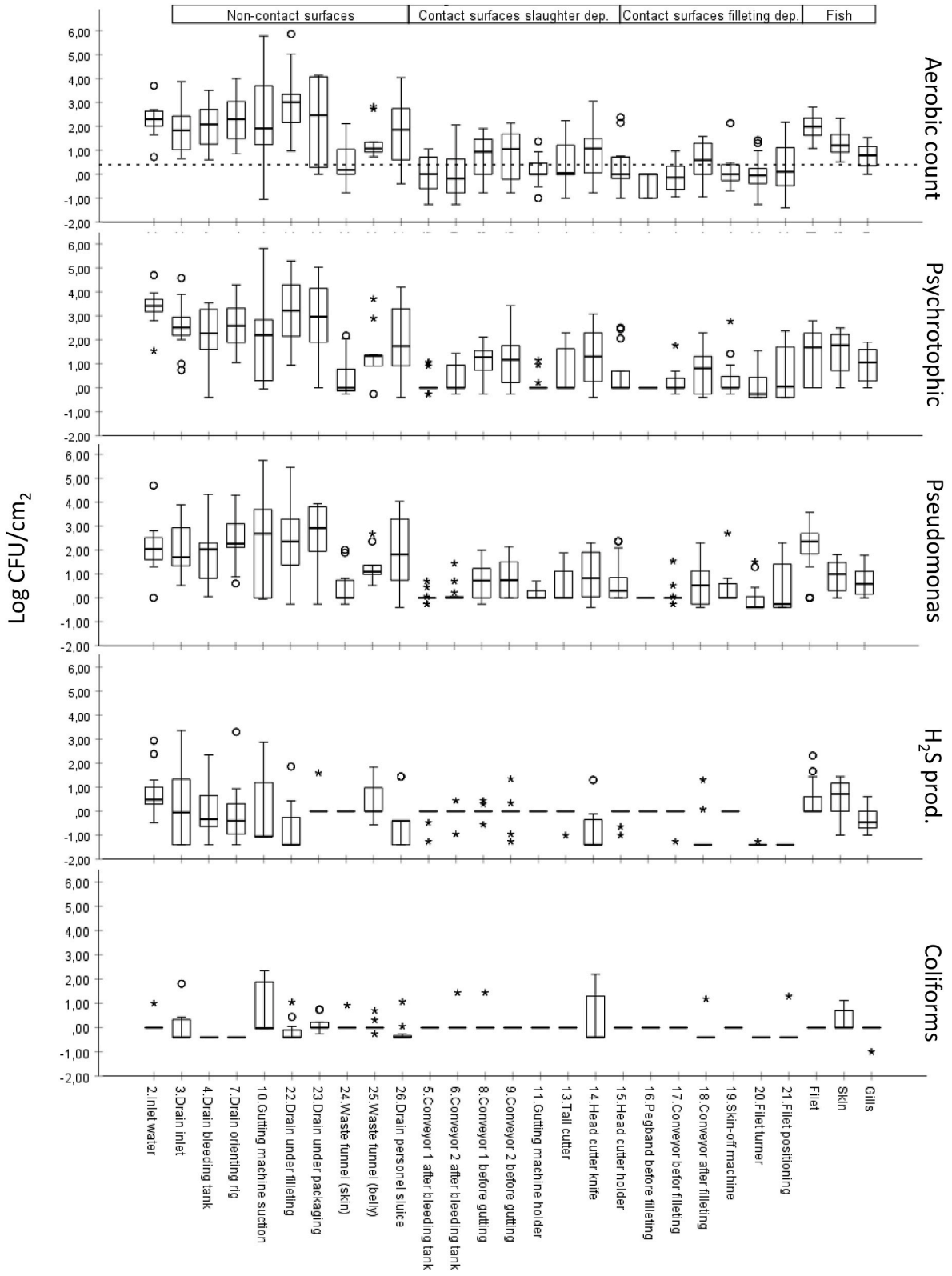

Fig. 2. Spatial variation in bacterial load. Aerobic cell count, Psychrotrofic cell count, *Pseudomonas* spp., H_2S-producing bacteria and coliforms from each sampling point.The analysis for aerobic count and H_2S-producing bacteria has a lower detection limit then the analysis for Psychrotrofic count, *Pseudomonas* and coliforms and negative log values means that the cfu/cm^2 was between 0 and 10. The boxes indicate the interquartile range of the data, the black line inside each box is the median and the whiskers extend to the most extreme values within 1,5 x interquartile range. Outliers are market with * or °. The sampling points on the x-axis is divided into four different sample categories, non-contac surfaces, contact surfaces slaghter department, contact surfaces fillet department and fish, as indcated on top of the figure. The dotted line at log 2,5 cfu/cm^2 for aerobic count indicate the acceptance level for aerobic cell count on cleaned and disinfected surfaces s stated by Griffith (2016). Note that sampling point 2: inlet water is included in this figure to show the bacterial level in the water (in log CFU/ml), but since it is not included in the surface category, it was omited from further statistical analyses. The log CFU value for "Filet" is in cfu/g, while the rest are in log cfu/cm^2 due to the nature of the samples.

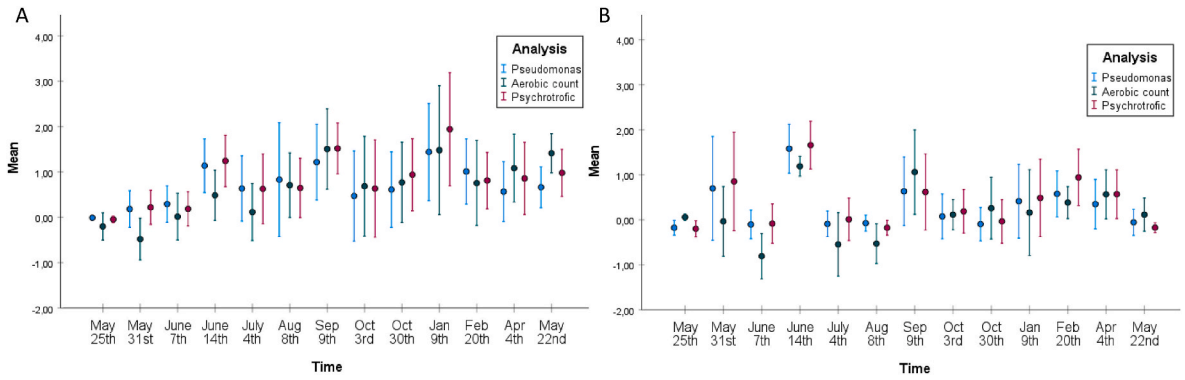

Fig. 3. Trend in bacterial load over time. The diagrams show the average log CFU/cm^2 for, **A**: the food-contact surfaces in the slaughter departmend and **B**: the food-contact surfaces in the filleting department, and how it developed over time from the first sampling in May (before start-up of regular production in the facility) to the last sampling in May one year after. All samples were taken in the morning after cleaning and disinfection, before production startup. For contact surfaces in the slaughter department (A), an increase in bacterial count for both aerobic, aerobic psychrotrophs and for *Pseudomonas* was observed. For the sampling points in the filleting department (B) no general increase was observed, but rather a high variation in bacterial counts between different sampling points.

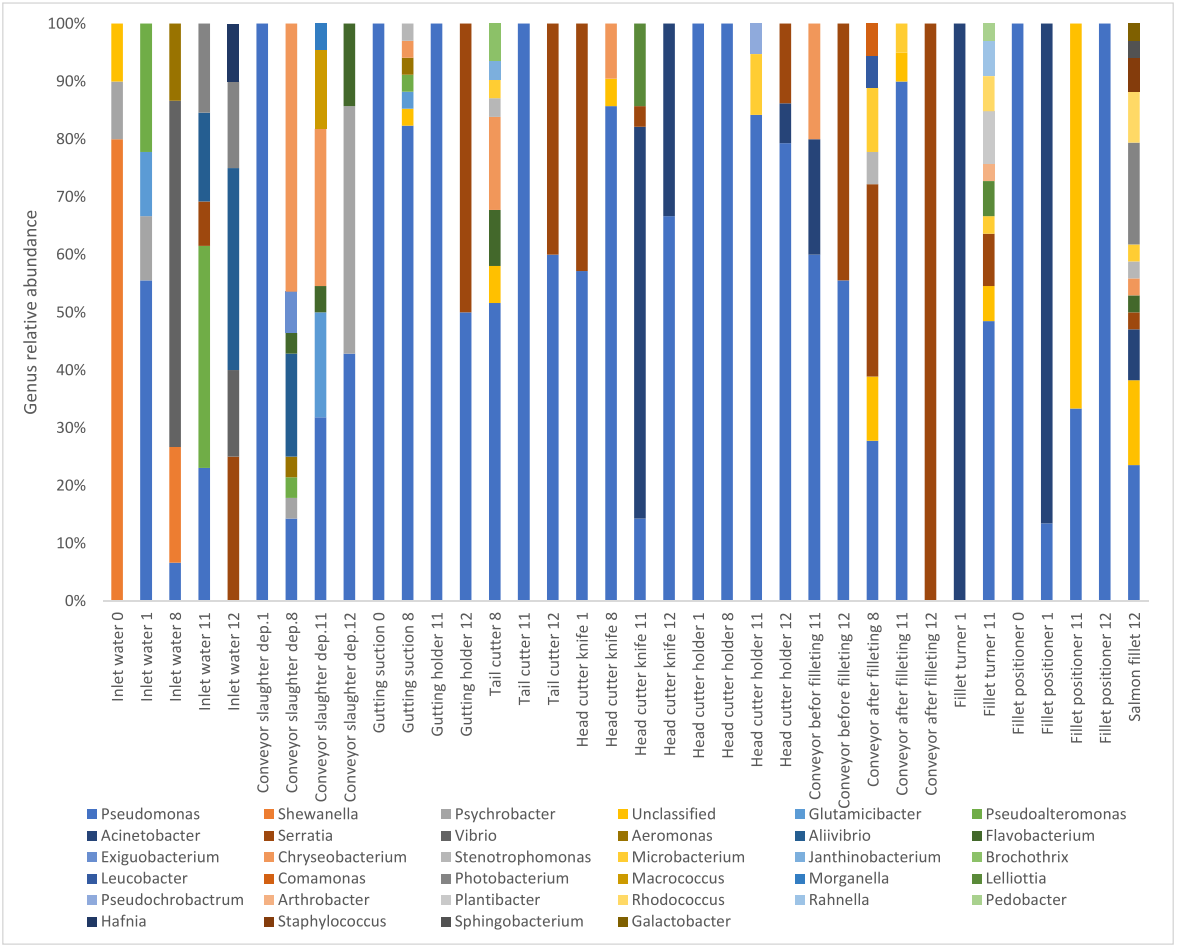

Fig. 4. Genus relative abundance. Barchart demonstrating bacterial abundance for each sampling point. The x-axis is sorted by sampling point and sampling time (0, 1, 8, 11, 12). Missing points means that there are no isolates collected from that point either because of overgrown plates (Conveyor slaughter dep. time 0) or there were no growth (the rest). The identification of bacterial community has been conducted using ON-rep-seq and 16S rRNA gene amplicon sequencing. A list of detectes species within each genus can be found in Table S1.

Pseudomonas genus while the rest belonged to *Microbacterium* sp., *Serratia liquefaciens* and the remaining group of unclassified isolates. The overall diversity among the isolates is visualized in heatmaps generated by ON-rep-seq method (Supplemental Figs. S3–S8).

4. Discussion

In this study we aimed to classify members of the bacterial communities in the equipment and the food processing environment of a newly opened salmon processing plant by sampling the same sampling

points 13 times through a period of one year after start-up. We identified most of the isolates to species-level by using a 3rd generation sequencing based method, ON-rep-seq, and by this documented the development and dynamics of the bacterial communities during this period.

Initially quantification of different bacterial groups from cleaned and disinfected surfaces in the salmon processing environment was performed with standard cultivation methods and as expected, the bacterial counts were generally low. However, the microbial diversity was relatively high. Presently, there are no general acceptance limits for hygiene samples, however, NSW Food Authority states in its guidelines for environmental swabbing that a total viable count of >10 CFU/cm^2 (average over time) is unacceptable on cleaned and disinfected FCSs in meat and poultry abattoirs (NSW Food Authority, 2012). It has also been indicated that a contamination level below 2,5 CFU/cm^2 after cleaning and disinfection should be achievable (Griffith, 2016).

In this study we observed that 40% of the surface samples had a contamination level below 2,5 CFU/cm^2. Additionally, 27% of the surface samples had a contamination level above 10 CFU/cm^2. This is in concordance with the findings from Møretrø et al, 2016 where they reported AC on Iron agar to be > 3 log CFU/cm^2 for the most contaminated FCSs.

As the processing plant was entirely new and the equipment had only been used for test runs prior to our first sampling, a general increase of bacterial load on the surfaces over time was expected. This was observed on the FCSs in the slaughter department and slightly on the FCSs in the filleting department. Through this first year of production, the facility was not always run at maximum capacity, hence, all the production lines in the filleting department were not used every day. This affected the cleaning and disinfection (C&D) routines in the facility. C&D of the production lines in the filleting department was performed if the line had been used or were to be used the next day. Based on information from the facility about when the production line of our focus had been used, there was no correlation between recent C&D and bacterial cell counts.

No general increase over time was observed on the NFCSs but rather a considerable variability in cell counts from one sampling to the next. The high variability (0–5,9 log CFU/cm^2) in the NFCS can probably be explained by the cleaning and disinfection routines in the facility where they, in addition to daily cleaning and disinfection, practiced an extraordinary disinfection of the drains and waste funnels rotational in different parts of the facility.

One NFCS of special interest is the gutting machine suction. We observed a considerable increase in bacterial count for both total AC, APC and PsC in the suction of the gutting machine during the first 9 months (Supplemental Fig. S9). The sampled unit is a special steel pipe that sucks out the viscera of the fish by using vacuum. The end of the pipe can be in contact with the abdominal cavity of the fish and, with prolonged use parts may be worn out and the possibility of flush back is present (personal communication, QC manager at facility). This exact equipment is known to be a high risk area for bacterial contamination and also for *Listeria monocytogenes* colonization due to low accessibility for cleaning (Løvdal et al., 2017). During our study the facility experienced an increasing problem with frequent detection of *L. monocytogenes* in this specific equipment. Because of this, an extraordinary disassembly and cleaning measures were effectuated by the facility personnel between our samplings 10 and 11. This resulted in bacterial counts at this point dropping to below the detection limit for all bacterial parameters except AC in Iron agar (IA). In our study, this sampling point is characterized by the highest variability in bacterial cell counts.

The bacterial communities associated with salmon, production equipment and salmon fillet is dominated by Gram-negative, psychrotrophic bacteria and, according to Broekaert et al. (2011), LH agar is the best suited medium to analyze for these communities. The bacteria detected on IA and on LH agar will in many cases be partly overlapping depending on the environmental conditions, therefore the high correlation between the total AC on IA and APC on LH was expected. Broekaert et al. (2011) also showed that most species of psychrotrophic *Pseudomonas* grew well on LH agar, and due to the high prevalence of *Pseudomonas* spp. in food processing environments (Cobo-Díaz et al., 2021; Maes et al., 2019; Møretrø et al., 2016), a correlation between *Pseudomonas*, APC and AC was expected.

It is increasingly common to analyze bacterial communities by culture independent methods as metagenomic sequencing or sequence based microbial profiling (McHugh et al., 2021; Solden et al., 2016; Zwirzitz et al., 2020). As this study was part of a larger project where characterization of isolates was a goal, it was of our interest to get bacterial isolates from the samples. For this reason, a culture-based approach was chosen and subsequently the sequencing-based method, ON-rep-seq was used to analyze and differentiate the isolates down to species or strain level. We have previously used this method for differentiating between *L. monocytogenes* isolates from a specific industry case (Thomassen et al., 2021), and here we explore it at a much larger and more diverse set of isolates.

The ON-rep-seq method classified 78% of the isolates to species or strain level, 8% to genus level and 14% remained unclassified. Many of the isolates that were only identified to genus level belonged either to the genus *Pseudomonas* or to a less described species in the respective genus. ON-rep-seq classification resolution relies on the database completeness and quality. Hence, its performance is affected by the number and diversity of the reference genomes currently in the database. For this reason, our results were supplemented with 16S rRNA gene amplicon sequencing for isolates unclassified by ON-rep-seq, as curated databases for 16S rRNA genes could provide additional information despite lower resolution. The most prevalent genera among these isolates were *Chryseobacterium* (n = 15), *Photobacterium* (n = 11), *Shewanella* (n = 8) and *Glutamicibacter/Arthrobacter* (n = 6). Isolates classified as for example *Chryseobacterium* spp. or *Shewanella* spp. by 16S sequencing had a few consensus reads from ON-rep-seq that was annotated to different species of *Chryseobacterium* or *Shewanella* respectively. This indicates that the reason for classification failure by ON-rep-seq was the lack of matching sequences in the database. For isolates classified as *Photobacterium* spp. many consensus reads were assigned as unclassified or classified at various taxonomic levels indicating no matching regions for this bacterium in the database.

Nearly half of the isolates (46%, n = 237) belonged to the genus *Pseudomonas*. This was expected as *Pseudomonas* spp. has been found to be the most common bacteria in several different food premises regardless of sampling method or choice of analyzing method (Cobo--Díaz et al., 2021; Gram and Huss, 2000; Maes et al., 2019; Møretrø et al., 2016; Parlapani and Boziaris, 2016). The species classification obtained by ON-rep-seq for isolates belonging to *Pseudomonas* genus was relatively low as 50% of the isolates could not be classified. For some *Pseudomonas* isolates the classification was ambiguous as the different consensus reads were assigned to different species. *Pseudomonas* isolates where a species classification could not be called with confidence were subject to sequencing of the *rpoD* gene (n = 101). Sequencing of this housekeeping gene has been suggested as an effective and accurate tool for identification and classification of *Pseudomonas* isolates by Girard et al. (2020). In our case it resolved a species classification for 61% (n = 62) of the *Pseudomonas* isolates subjected to this analysis. The most abundant species according to the *rpoD* sequencing was *P. fluorescens* or uncertain species of *P. fluorescens* group. Several of the isolates that were most similar to the unclassified *Pseudomonas* strains Myb193, FDAARGOS_380, LG1D9 or NC02, had the highest similarity to *P. fluorescens* by *rpoD* analysis with similarities between 98.58 and 99.72%, 98.15–100%, 98.72–99.57% and 99.72–99.86% respectively. If counting these in addition to the strains already classified as *P. fluorescens,* makes *P. fluorescens* by far the most abundant species in this material accounting for 23% of all the isolates.

It was clear that the species level identification resolution of ON-rep-seq for *Pseudomonas* was significantly reduced compared to other genera. By using two different methods in the attempt to classify the

Pseudomonas isolates we obtained a higher number of reliable species classifications but also some partly contradictive taxonomy assignment between ON-rep-seq and *rpoD* sequencing were registered. ON-rep-seq assign many *Pseudomonas* isolates to *P. koreensis* species because this was the species where most of the consensus reads had the best match. But with many of the other consensus reads matching several different *P. fluorescens* strains, this species assignment is questionable. However, Gomila et al. (2015) revealed that several *Pseudomonas* strains previously assigned as *P. fluorescens* clustered intertwined with *P. koreensis* subgroup and close to *P. koreensis* type strains in phylogenetic analysis based on four concatenated housekeeping genes (16S rRNA, *rpoB*, *rpoD* and *gyrB*). The same study also reported that about 30% of sequenced genomes of non-type strains were not correctly assigned at the species level and 20% were not identified at all. The genus *Pseudomonas* is one of the largest bacterial genera with almost 200 recognized species and over 500 full genomes available in Genbank (Koehorst et al., 2016; Nikolaidis et al., 2020). This makes species classification of *Pseudomonas* very complicated (Gomila et al., 2015; Lalucat et al., 2020; Özen and Ussery, 2012), particularly for methods that relay on shotgun sequencing or extragenic regions sequencing. It is therefore clear that poor performance of ON-rep-seq on classification of *Pseudomonas* spp. is related to the meagre quality of the databases.

Furthermore, the LCp profile comparison with heatmaps generated by the ON-rep-seq method indicates rather high strain diversity in the analyzed samples (Supplemental Figs. S3–S8). Fig. S5 contains all the *Pseudomonas* isolates from this study and shows the complexity of this genus. The intertwined clustering of *P. koreensis* and *P. fluorescens*, as reported by (Gomila et al., 2015), can also be seen in Fig. S5, but here also other species are intertwined. Additionally, it is apparent that isolates classified as the same species/strain do not consistently cluster together. In Fig. S6 all the *Acinetobacter* isolates are compared, and the clustering for this genus is much more consistent with the species classification than it is for *Pseudomonas*. It has earlier been reported that rep-PCR, is a well-suited method to differentiate between strains within the genus of *Acinetobacter* (Pasanen et al., 2014; Snelling et al., 1996). And, as ON-rep-seq relays on rep-PCR, a good differentiation of *Acinetobacter* spp. was expected. In our study there is a difference in the diversity between the *Pseudomonas* set of isolates and the *Acinetobacter* set. But the picture we see here (Supplemental Fig. S5) also indicates that the genetic diversity within *Pseudomonas* genus, in addition to previous mentioned database issues, makes the species classification by this method difficult.

The strain level diversity between the *Pseudomonas* group and the *Acinetobacter* was clearly different. The high genetical diversity (big difference in the LCPs) of the *Pseudomonas* genus (Supplemental Fig. S5) in addition to previous mentioned database issues, makes the species classification by this method difficult.

In this study the bacterial communities remaining after C&D seem to vary both through time and space, and many of the detected species were only detected once and at one point, though at a high number. This phenomena with a high day-to-day diversity have also been observed in other studies (Cobo-Díaz et al., 2021; Johnson et al., 2021). In our case we suspect that the reason for this was that the sampled surfaces were daily object to a thorough C&D procedure but, with minor variations from day to day due to manual labor, resulting in variable number of remaining bacteria every day. As very few of the detected bacterial strains were detected at several sampling points and time points, it is not apposite to speak about persistent bacteria based on these results. However, isolates with highest similarity to species/strains *Pseudomonas* sp. MYb193, *Pseudomonas fluorescens*, *Pseudomonas koreensis*, *Pseudomonas* FDAARGOS_380 and to *Serratia liquefaciens*, reoccurred several times, but rarely at the same sampling point. Of all the detected taxa, 61% (n = 51) were detected in only one sample. One reason for this is the low-throughput method by picking colonies for isolation. Possibly a metagenomic approach could have detected a higher diversity of taxa, however a metagenomic approach could have resulted in a lower taxonomic resolution.

Of all the bacterial species detected in this study several of them are known spoilage organisms. *Pseudomonas* spp. has been reported to be the main spoilage organism in iced freshwater fish (Gram and Dalgaard, 2002), in tropical brackish water shrimp stored at 0 °C (Dabadé et al., 2015), and in gutted sea bream (Parlapani et al., 2015) among others.

Both *Photobacterium phosphoreum* and various *Shewanella* spp. are well-known spoilage bacteria in fish (Dalgaard et al., 1997; Gram and Dalgaard, 2002; Gram and Huss, 2000). Of the eleven isolates that belonged to the genus *Photobacterium*, five were most similar to *Photobacterium phosphoreum*, according to 16S rRNA gene sequencing. All the isolates classified as *Shewanella* by 16S rRNA gene sequencing had highest similarity to *S. algidipiscicola*. This species is reported to both reduce TMAO and to produce H_2S (Satomi et al., 2007), thus it must be considered as a spoilage bacterium. In addition, *Microbacterium* sp., *Acinetobacter* sp., *Stenotrophomonas* sp. and several other of those detected in low numbers, have been shown to have spoilage potential (Maes et al., 2019). Based on this we must assume that the detected bacterial flora poses a significant risk for spoilage of the salmon filets produced.

5. Conclusion

In this study of bacterial communities in a salmon processing plant we have documented a generally low contamination level on food contact surfaces but with a few questionable spots. We saw a general increase in contamination level on food contact surfaces, especially in the slaughter department through the first year of production. Bacterial load on salmon filet at the end of the production line does not increase.

A diverse psychrotrophic bacterial community, highly dominated by *Pseudomonas* spp. was detected, and most of the detected species have been reported to have a spoilage potential in seafood.

By classification of bacterial isolates to species-level and differentiating between strains we revealed point-specific bacterial communities, which indicates limited number of persistent bacteria. The detailed knowledge of the bacterial communities on species level can be significant for improving cleaning and disinfection routines and, it can be helpful in evaluating the shelf life and the food safety of the product.

The ON-rep-seq method has a potential in species-level identification for many bacteria in these complex bacterial communities but as also reported for other methods, it has difficulties in clear species classification within the highly divergent *Pseudomonas* genus. Additionally, novel bacteria (not present in databases), non-complete draft genomes, or misclassified genomes, will reduce the resolution of taxonomic classification by this method.

Funding information

This work was funded by Norwegian University of Science and Technology (NTNU). Gunn Merethe B. Thomassen was supported by a Ph.D. grant from NTNU, as part of the OPTiMAT project.

Author contribution

Gunn Merethe Bjørge Thomassen: Conceptualization (equal); Writing – original draft (lead); Formal analysis (equal); Writing – review & editing (equal); Lukasz Krych: Conceptualization (equal); Formal analysis (equal); Writing – review & editing (equal); Susanne Knøchel: Conceptualization (equal); Writing – review & editing (equal); Lisbeth Mehli: Conceptualization (equal); Writing – original draft (supporting); Writing – review & editing (equal).

Ethics statement

None required.

Data availability statement

The ON-rep-seq data analysis toolbox is available from github repository https://github.com/lauramilena3/On-rep-seq.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Data was generated through accessing research infrastructure at University of Copenhagen, including FOODHAY (Food and Health Open Innovation Laboratory, Danish Roadmap for Research Infrastructure). The authors wish to thank the salmon processing plant for constructive cooperation. Thanks to Kirill Mukhatov for valuable help with the statistical data analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104138.

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