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Contamination of salmon fillets and processing plants with spoilage bacteria

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ABSTRACT

The processing environment of salmon processing plants represents a potential major source of bacteria causing spoilage of fresh salmon. In this study, we have identified major contamination routes of important spoilage associated species within the genera *Pseudomonas*, *Shewanella* and *Photobacterium* in pre-rigor processing of salmon. Bacterial counts and culture-independent 16S rRNA gene analysis on salmon fillet from seven processing plants showed higher levels of *Pseudomonas* spp. and *Shewanella* spp. in industrially processed fillets compared to salmon processed under strict hygienic conditions. Higher levels of *Pseudomonas* spp. and *Shewanella* spp. were found on fillets produced early on the production day compared to later processed fillets. The levels of *Photobacterium* spp. were not dependent on the processing method or time of processing. In follow-up studies of two plants, bacterial isolates ($n = 2101$) from the in-plant processing environments (sanitized equipment/machines and seawater) and from salmon collected at different sites in the production were identified by partial 16S rRNA gene sequencing. *Pseudomonas* spp. dominated in equipment/machines after sanitation with 72 and 91% of samples from the two plants being *Pseudomonas*-positive. The phylogenetic analyses, based on partial 16S rRNA gene sequencing, showed 48 unique sequence profiles of *Pseudomonas* of which two were dominant. Only six profiles were found on both machines and in fillets in both plants. *Shewanella* spp. were found on machines after sanitation in the slaughter department while *Photobacterium* spp. were not detected after sanitation in any parts of the plants. *Shewanella* spp. and *Photobacterium* spp. were found on salmon in the slaughter departments. *Shewanella* was frequently present in seawater tanks used for bleeding/short term storage.
In conclusion, this study provides new knowledge on the processing environment as a source of contamination of salmon fillets with *Pseudomonas* spp. and *Shewanella* spp., while *Photobacterium* spp. most likely originate from the live fish and seawater. The study show that strict hygiene during processing is a prerequisite for optimal shelf life of salmon fillets and that about 90% reductions in the initial levels of bacteria on salmon fillets can be obtained using optimal hygienic conditions.

1. Introduction

There is an increasing consumer demand for fresh, chilled fish. This is a result of increased consumption of fish eaten raw (e.g. sushi, sashimi) and a general increased consumer demand of fresh food (CBI Market Intelligence, 2016; Quested et al., 2010). Fresh fish has significant added value compared to frozen fish, but also requires increased attention to the sensory and microbial quality (Gram and Huss, 1996). Farmed salmon is a high volume product in the fresh, chilled fish product category. In Norway, the production of farmed Atlantic salmon in 2014 was about one million metric ton with an export value of about five thousand million Euro (Norwegian Seafood Council, 2015).

Microbial control during processing and storage is a key factor that determines the quality and shelf life of fresh fish. Bacteria on the product can originate from the raw materials or be introduced during processing by e.g. cross contamination from equipment or by food handlers. The microbial quality of the product is depending on the spoilage potential of the microorganisms present and the storage conditions that affect growth and formation of spoilage metabolites (Gram and Huss, 1996).

The most commonly reported spoilage bacteria for aerobically stored chilled fish including salmon are species within the genera *Pseudomonas* (*P.*) and *Shewanella* (*S.*), while the CO_2-resistant *Photobacterium (Ph.) phosphoreum* dominates on fish packed under modified atmosphere (Chaillou et al., 2015; Dalgaard et al., 1993; Emborg et al., 2002; Gram and Huss, 1996; Parlapani and Boziaris, 2016; Tryfinopoulou et al., 2002). *Ph. phosphoreum* is a producer of trimethylamine (TMA), a major spoilage product in fish (Dalgaard, 1995). The most important spoilage products of *Shewanella* spp. are volatile sulfides, but TMA may

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also be produced (Dalgaard, 1995; Joffraud et al., 2001). *Pseudomonas* spp. does not produce TMA but has been associated with quality changes and development of sweet, fruity off-odors in various species of chilled fish (Olafsdottir et al., 2006; Parlapani et al., 2015).

Live salmon can harbour *Pseudomonas* spp., *Shewanella* spp. and *Photobacterium* spp. and can thus be considered an important primary source for these spoilage organisms on processed salmon (Cantas et al., 2011; Gram and Huss, 2000; Hovda et al., 2012; Navarrete et al., 2009). Although good hygienic practices are considered essential in all production of food, little is known about the importance of bacterial cross contamination from product contact surfaces to the fish during processing. Bagge-Ravn et al. (2003) studied the bacterial microbiota on equipment in four fish processing plants, including two smokehouses producing cold smoked salmon and found that *Pseudomonas* spp. and yeasts, followed by *Acinetobacter* and *Neisseriaceae* dominated after cleaning and disinfection. *Photobacterium* spp. was found at low prevalence in one of the two smokehouses after cleaning and disinfection. *Pseudomonas* is frequently isolated after cleaning and disinfection in other types of food industry, e.g. meat and dairy processing plants (Brightwell et al., 2006; Hultman et al., 2015; Mettler and Carpentier, 1998; Møretrø et al., 2013; Stellato et al., 2015). To our knowledge the prevalence of *Shewanella* spp. in fish processing plants has not been reported.

The aim of this study was to identify the main sources of spoilage bacteria in salmon fillets. The microbiota of industrially processed salmon fillets and fillets processed by manual filleting under strict hygienic conditions from seven different processing plants were compared. Furthermore, the prevalence of spoilage bacteria along the processing line in two salmon processing plants was determined to detect high-risk sites for contamination from machines/equipment to products.

2. Materials and methods

2.1. Salmon fillets from Norwegian salmon processing plants

Fillets were collected in June–September 2012 from seven Norwegian plants with pre-rigor processing of farmed Atlantic salmon. This was done to determine bacterial levels in pre-rigor processed salmon. Similar procedures for collection of the fillet samples were performed in each plant: Twice during a production day, four to six fillets were collected at the end of the processing lines. The first collection was performed early at the production day (within 1 h after start) and the second after mid shift (5–6 h after production start). In addition, from each salmon processor three salmon were collected prior to processing. These salmon were never in contact with processing plant surfaces. They were manually gutted under high hygienic conditions using clean knifes and cutting boards to avoid microbial cross-contamination. These salmon were used as controls representing salmon with optimal hygienic status. Fillets and gutted salmon (controls) were packed on ice in separate boxes (expanded polystyrene; EPS) and sent express to the laboratory. Within 24 h after reception in the lab, the gutted salmon were filleted and skinned under hygienic conditions to avoid cross-contamination. The in-plant produced fillets were skinned likewise. All fillets were stored on ice during the experiment (temperature < 1 °C, confirmed by temperature logging).

2.1.1. Culture-dependent bacterial analyses of salmon fillets

Microbial sampling of salmon fillets (early, mid shift and control samples) from the seven processing plants was performed at Day 1–2 and Day 10. From each fillet, a sample of 3 × 3 cm × 0.5 cm depth (approximately 10 g) was diluted with peptone water (saline with 0.1% Bacto Peptone (Oxoid)) to obtain a 1/10 dilution. Bacterial quantification on homogenized suspensions after stomaching for 60 s was determined by cultivation on Long & Hammer agar (van Spreekens, 1974) incubated at 15 °C for 5–6 days. A total of 186 colonies, representing different plants, processing conditions and storage times were picked and identified by partial 16S rRNA gene analysis. The picked colonies were resuspended in 50 μl of Tris-EDTA buffer in a microtiter plate well, followed by heat treatment at 99 °C for 10 min. After centrifugation at 4500 ×*g* for 3 min, 30 μl supernatant was transferred to a new microtiter plate, which was frozen at −20 °C until further analysis. For amplification of 16S rRNA gene, the supernatant was thawed and 1 μl used as template in a PCR reaction. Briefly, universal primers (Nadkarni et al., 2002) were used for PCR and sequencing. Amplification was performed using 0.25 μM of each primer, 10 μl Qiagen multipleks PCR kit (2×) (Qiagen) to a total volume of 20 μl. The cycling conditions, PCR purification and sequencing were performed as described (Omer et al., 2015). Genus was determined by search of approximately 400 bp in Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.1.2. Culture-independent bacterial analyses of salmon fillets

Culture-independent bacterial analyses of salmon fillet samples were done using Next generation sequencing (NGS) of the variable region 4 of the 16S rRNA gene. Twenty one samples from Day 10 fillets (early, mid shift and controls from seven plants) were prepared. For each sample, 8–12 ml of the salmon-peptone water stomaching suspensions from each of the four to six parallel fillets were combined. For control samples, 16 ml of three parallels were combined. Samples from Day 1 were not prepared due to low total counts. The suspensions were filtrated (20 μm Steriflip (Merck Millipore, Darmstadt, Germany) to remove salmon debris, aliquots of 4.5 ml were centrifuged (13,000 ×g, 5 min), and pellets stored at −20 °C until DNA purification. The pellets were resuspended in 500 μl 2× Tris-EDTA (20 mM Tris-Cl, pH 8.0/2 mM EDTA)/1.2% Triton X-100 (Sigma Aldrich, St. Louis, USA), transferred to a FastPrep tube (Matrix B, MP Biomedicals, Solon, USA) and lysed in a FastPrep bead beater (MP Biomedicals) for 40 s at 6 m/s. The Fastprep tube was centrifuged for 5 min at 14,000 ×g, 360 μl of the supernatant was added 50 μl Proteinase K and 400 μl lysis buffer AL (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA), mixed and incubated for 30 min at 56 °C. 400 μl EtOH was added, mixed and transferred in two steps to a Qiagen column (DNeasy Blood and Tissue Kit, Qiagen). The manufacturer's protocol was followed from here. DNA was used as template for the NGS (MiSeq, Illumina Inc., San Diego, USA) analysis as previously described (Moen et al., 2015). Briefly, a portion of the 16S rRNA gene spanning the variable region 4 (V4) was amplified using the barcoded, universal primer set (515F/806R) (Caporaso et al., 2012). Of the 21 samples, two samples (F control and G control) were excluded due to low PCR product concentration. The library quantification and sequencing were performed at the Norwegian Sequencing Centre (https://www.sequencing.uio.no/). The pre-processing of the data was performed in QIIME (Quantitative Insights Into Microbial Ecology (version 1.6.0)) (Caporaso et al., 2010). The sequences were then demultiplexed in QIIME allowing zero barcode errors and a quality score of 20 (Q20). To remove short sequences (identified as *Salmo salar* mitochondrion), the minimum number of consecutive high quality base calls to include a read as a fraction of the input read length was increased from 0.75 to 0.9. Reads were assigned to their respective bacterial id using de novo operational taxonomic unit (OTU) picking workflow in QIIMEReads that did not match a reference sequence were discarded. In total 15,272 OTUs were written (5277 OTUs when not including singletons), each of these represents a phylotype and may be a representative of a bacterial species. The level 6 (genus level) table was used in further analysis. Only the dominating genera were represented (the other genera were represented as "other").

2.2. Sampling of spoilage bacteria in two salmon processing plants

The prevalence and contamination sources of common spoilage associated bacteria of fresh salmon were further investigated in two salmon processing plants (B and H). The two plants, both processing pre-rigor salmon, were visited in March (plant B) and November (plant H