

Bacterial diversity and changes towards spoilage micro flora of iced Alaska pink salmon

Abstract

Fresh, iced Alaska pink salmon were stored upto 20days with samples of skin, gills and belly cavity analyzed for bacterial flora developments. Bacterial colonies isolated from plates used to estimate aerobic bacterial populations were identified using rapid cellular fatty acid analysis. *Acidovorax* spp. and *Brevundimonas* spp. were identified as part of the fresh fish microflora. The group consisting of *Psychrobacter*, *Moraxella* and *Acinetobacter* were present throughout salmon storage but were found only associated with fish skin and gills. *Pseudomonas fluorescens* and *P. putida* formed a major percentage of the spoilage flora and *Shewanella putrefaciens* comprised the minor portion in sampled tissues. The replacement of using the Sherlock Microbial Identification System for classical taxonomic tests resulted in time, material and labor savings. Also, the database provided a deeper view of the bacterial diversity and changes in bacterial flora that occurred during iced salmon storage.

Keywords: selective transfer, superficial layer, structural analysis, intensity x-rays, width of diffraction lines, crystalline network constant

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Abbreviations: SSO, specific spoilage organisms; MIS, microbial identification system; CFU, colony forming units; PCA, plate count agar

Introduction

The most common method to determine the microbial load of fresh fish is the aerobic plate count and the spoilage threshold is 107 colony-forming units/g.¹ This value can vary during post-harvest storage of seafood and depends on the initial bacterial load, post-harvest handling, storage conditions, temperature, specific tissues sampled and composition of the bacterial flora.²⁻⁴ Although the microflora on fish is diverse, a small group of bacteria designated as “specific spoilage organisms” (SSO), namely *Pseudomonas* spp. and *Shewanella putrefaciens*, are able to cause deterioration and odor in iced fish.⁵ These SSO can interact with other bacteria to affect the dynamics in the fish microflora.⁶

The identification of bacteria involved in changes to the fish microflora can help in modeling the microbial community structure for particular seafood, assessing interactions within the community, predicting and extending shelf life of a product.⁵ The SSO load is estimated by using selective media but these media can support growth of non-target organisms giving an incorrect number of SSO.⁷ Hence, it would be advisable to identify the micro-organisms to the species level.

Identification of these micro-organisms by classical techniques is a time consuming, labor intensive and sometimes expensive process. The Sherlock® Microbial Identification System (MIS) is a fully automated system using direct computerized comparison of fatty acid analysis of test strains against the library database of >100,000 profiles to provide a rapid identification.^{8,9} For seafood bacteria identification, the Sherlock® MIS has been limited to bacteria associated with aquaculture striped bass and biogenic amine-forming bacteria in canned anchovies.¹⁰⁻¹² Recently, we determined that the Sherlock MIS could confirm and update the taxonomic classification of bacteria

from our culture collection in a comparison study with strains from the American Type Culture Collection.¹³

The purpose of this study was to rapidly identify bacteria associated with the fresh salmon microflora found on iced fish skin, gills and belly cavity by using the Sherlock MIS and to follow the spoilage microflora developing during fish storage.

Materials and methods

Fish processing

Fresh seine-caught pink salmon (*Oncorhynchus gorbuscha*) in the Gulf of Alaska and held in chilled seawater for <24h were procured during summer from a seafood processor in Kodiak, AK. Fish without physical damage were iced and brought to the Kodiak Seafood and Marine Science Centre’s pilot plant, de-iced, washed and maintained under iced condition till further processing. Fish were brought on two separate occasions and used for experiments to imitate commercial conditions of processing and handling of fillets.

Iced skin-on fillet storage

Within one hour, the first batch of fish (n=3) were headed, gutted, trimmed to remove the fins and washed briefly in tap water. Dressed fish were hand-filletted into boneless, skin-on fillets and rinsed with chilled tap water to remove bloodstains and slime. These fillets were placed individually in plastic bags closed with rubber bands to prevent contact with melting ice water. The bags were placed in a tote having a loose fitting lid on layer of flaked ice, then covered with more ice and kept in a walk-in cold room (4±1°C). The melting ice was replenished every 48h for 14days. The fish fillets had an average temperature maintained at 2±1°C. A random bag was pulled every threedays up to day15.

Gutted fish storage in ice

The second batch of pink salmon (n=4) was immediately degutted and rinsed thoroughly with tap water. Fish were placed belly down to

prevent water accumulation and spaced apart on a layer of flaked ice, then covered with more ice. The totes were covered with lids and kept in the cold room. Melting ice was replenished every 24h for 19days. Initially and at days 4, 6 and 20, one fish was taken for sampling.

Bacterial analysis

On each sampling day, the fish skin and belly cavity were swabbed (area of 10cm²) separately for conducting aerobic plate counts.¹⁴ Gills were aseptically cut and 25g of sample was macerated in 225mL of 0.1% (w/v) peptone water and serial dilutions were made in the diluents.¹⁵ Aliquots (0.1mL) of each serial dilution were spread-plated on duplicate plate count agar (PCA; Difco Lab, Detroit, MI) supplemented with 0.5% w/v NaCl. These plates were incubated at 25°C for 48-72h and then plates with 30-300 colonies were selected for counting.¹⁶ Total number of colonies counted from duplicate plates were averaged and used to calculate log colony-forming units (CFU) per cm² for skin and belly cavity and log CFU/g for gills.

From representative plates at each sampling period, ten colonies were randomly selected and restreaked to purity. These isolates were tested using the KOH reaction for cell wall type,¹⁷ catalase activity¹⁸ and oxidase test.¹⁹ Morphological characteristics (shape and motility) of the selected micro-organisms were observed under microscope (Montagesaez T-UL, 467065-9914, Zeiss, Germany) by using the 40X high-dry and 100X oil-immersion objectives. The pure cultures were collected individually from the PCA and stored in sterile 50% glycerol (Sigma Chemical Co., St. Louis, MO) and 40µL dimethylsulfoxide (Sigma) at -80°C until identification.

Frozen cultures were thawed and resuscitated in brain heart infusion (BBL, Sparks, MD) overnight at 25°C and then streaked on trypticase soy broth agar (30g of Trypticase soy broth (BBL) and 15g granulated agar (BBL) per liter) plates and incubated at

28°C for 24h. Fatty acid methyl esters were extracted and analyzed in duplicates using the procedure of Paisley²⁰ and the GC model 6850 (Agilent Technologies, Wilmington, DE) coupled with a flame ionization detector. The GC conditions were monitored by the Sherlock® MIS software (Microbial ID Inc., Newark, DE). The GC was calibrated using a calibration standard (MIDI Part No. 1300-AA) for the Sherlock® Rapid Method.²⁰ During all the calibration mixture analysis, the peak percent named for the standard was >99% with the root mean square error <0.003. Strains with Similarity Index (SI)>0.5 with a separation of >0.1 between first and second ranks are considered as good library comparisons. If a closely-related genus or species was listed <0.1SI, the identification of that particular isolate was reported as a combination.

Results and discussion

The APC on the skin was <4 log CFU/cm² for the first week then reached 6.6log CFU/cm² at 15days of iced storage (Figure 1). Low initial counts in the belly cavity (Figure 2) indicated that the intestines were intact after capture till the fish were gutted and did not contaminate the belly cavity. Low bacterial counts could be due to the washing effect during on-board chilled seawater storage, evisceration and sample rinsing prior to the start of the experiment.²¹ Additionally, the washing effect of melting ice and its replacement with fresh ice every 24h could have also led to low APC during initial iced storage. Subsequent growth of bacteria on the skin surface, gills and belly cavity was attributed to the psychrotrophic or psychrotolerant nature of bacteria adapted to chill temperatures.²² Although the initial count of belly was approximately similar to the skin, the APC reached approximately 8 log CFU/cm² while gills with a higher initial APC count reached approximately 7log CFU/g, at 20days of storage (Figure 3).

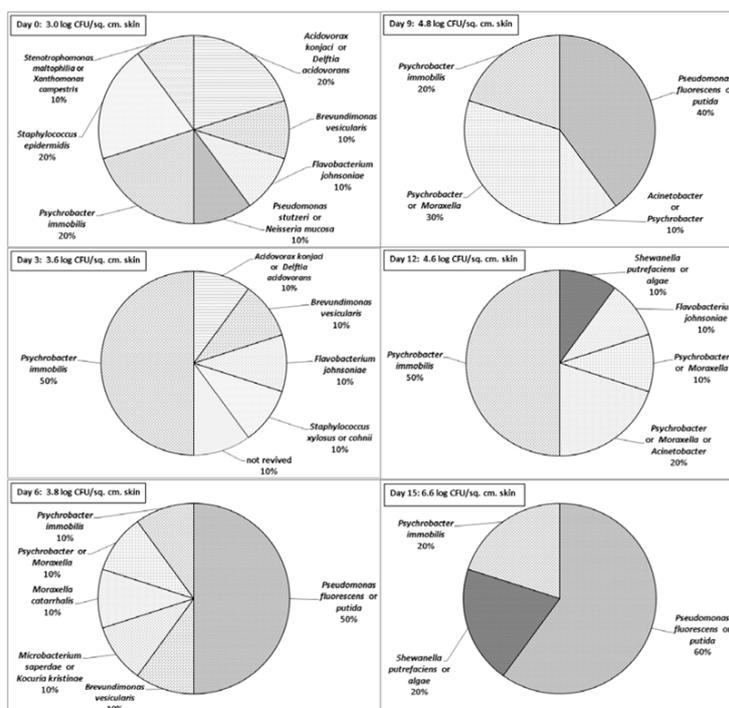


Figure 1 Bacterial flora changes in skin of Alaska pink salmon during 15days in iced storage.

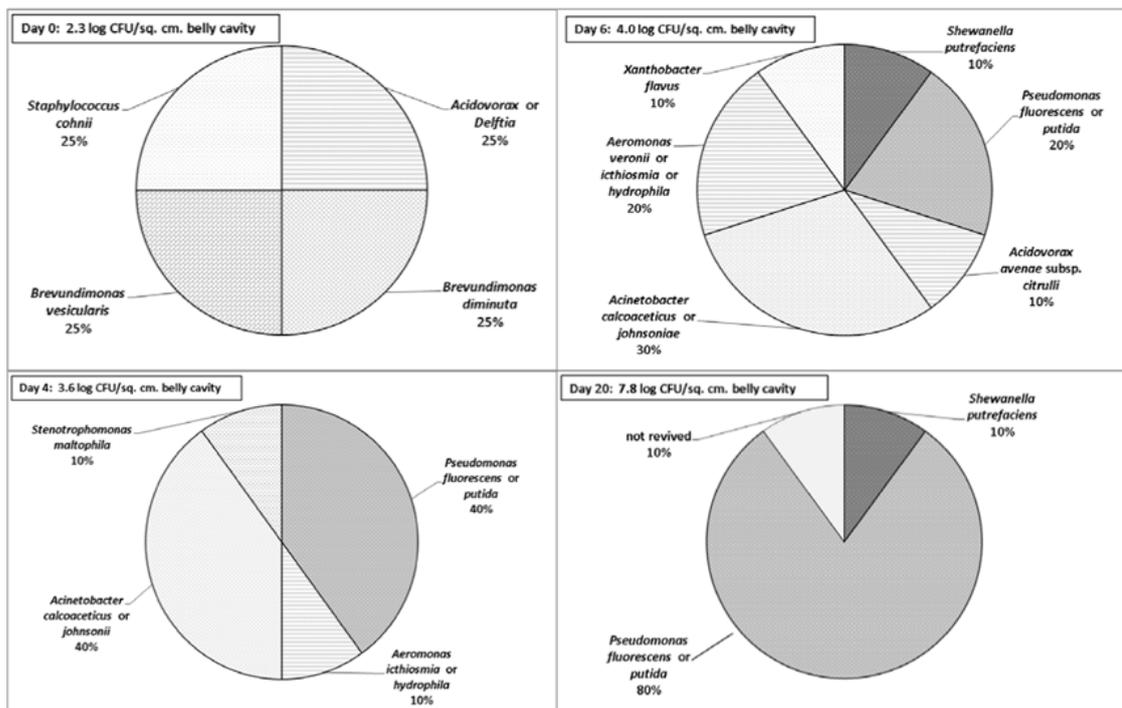


Figure 2 Bacterial flora changes in belly cavity of Alaska pink salmon during 20days in iced storage.

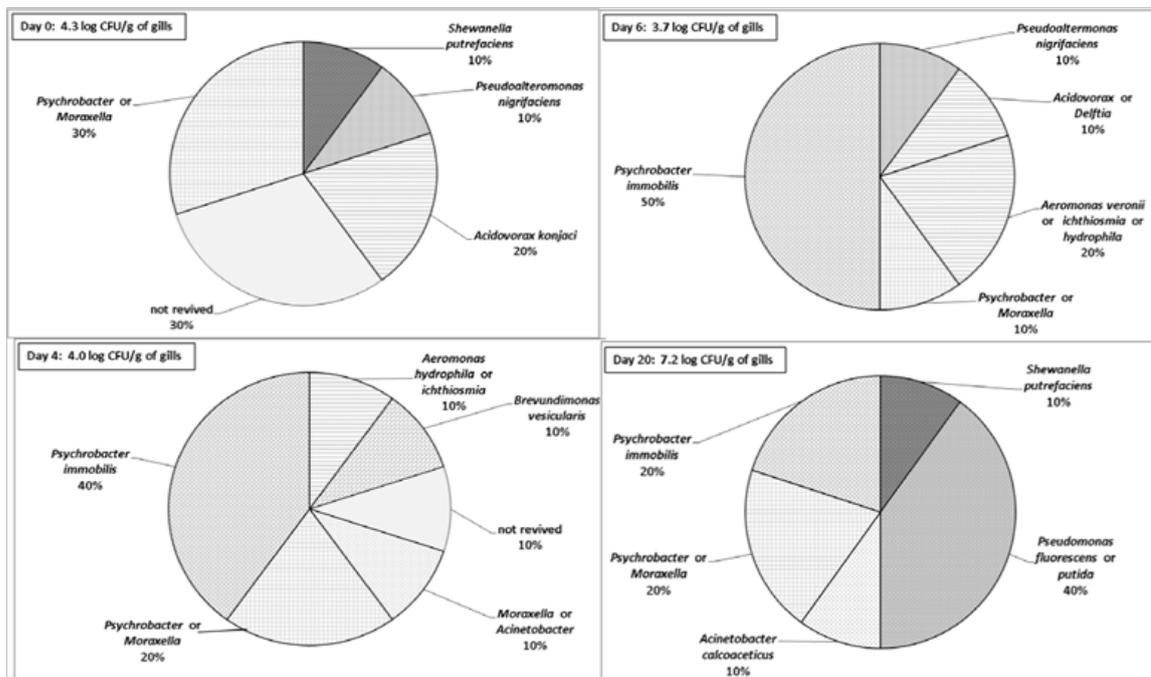


Figure 3 Bacterial flora changes in gills of Alaska pink salmon during 20days in iced storage.

Of 60 isolates from skin-on salmon fillets collected over 15days of iced storage, 93% were Gram-negative and the remainder was Gram-positive. The micro flora changed from very diverse on day 0 to comprising three genera by day 15 (Figure 1). From the 104 bacterial isolates collected from the belly cavity and gills, 97% were Gram-negative and the remainder was Gram-positive. These trends were similar to the ones observed on salmon skin fillets indicating that the majority of spoilage microflora on pink salmon belongs to Gram-negative group of bacteria.²¹

Identification of bacterial isolates indicated a presence of some under reported seafood bacterial species such as *Acidovorax*, *Delftia* and *Brevundimonas* during the early part of iced salmon storage. Nedoluha and Westhoff^{10,23,24} indicated the presence of these genera identified using Sherlock® MIS in aqua cultured striped bas.^{10,23,24} The formation of *Acidovorax*²⁵ and *Delftia*²⁶ and their low presence during the first fewdays of iced fish storage may indicate their non-importance in fish spoilage as compared to *Pseudomonas* spp. and *S. putrefaciens*. *Brevundimonas vesicularis* and *B. diminuta* are

delineated from *Pseudomonas*.²⁷ Although *Brevundimonas* spp. are mainly associated with clinical specimens,²⁸ *B. vesicularis* and *B. diminuta* have been isolated from seafood.^{5,29,30}

Bacteria belonging to genus *Flavobacterium* were identified in the spoilage microflora of pink salmon along with *Chryseobacterium*³¹ and *Elizabethkingia*.³² These bacteria have been reported only as *Flavobacterium* spp. which provides no information about speciation. Isolates from pink salmon identified using Sherlock® MIS as *F. johnsoniae*, *C. indologenes* and *C. meningosepticum* have been reported by other researchers.^{10,23,24,33}

The seafood bacteria grouping of *Psychrobacter*, *Moraxella* and *Acinetobacter* were isolated from iced pink salmon and were confirmed using the Sherlock® MIS and comprised 20-50% of the skin microflora. Species-level overlap was due to a very low SI and <0.1 SI difference between first ranks of identifications. Presence of these bacteria throughout storage irrespective of sampling regimen could indicate their commensal role in pink salmon spoilage development. As the ice storage progressed, the percentage of the microflora was maintained, then decreased possibly due to competitive exclusion by motile *Pseudomonas* spp. during the later stage of iced storage, similar to observations by McMeekin TA.³⁴

Primarily *P. fluorescens* - *P. putida* (40-80%) and *S. putrefaciens* (10%) were identified at the three sampling locations of pink salmon on the final day of iced storage (Figures 1–3). The occurrence of *P. fluorescens* and *P. putida* increased with a corresponding decrease in the variety of bacterial species present during storage. The predominance of these organisms can be attributed to the ability of *Pseudomonas* to grow rapidly at chill temperature³⁵ and suppressed growth of other bacteria.^{36,37} The importance of *Pseudomonas* spp. as SSO can be attributed to the ability of psychrotrophic *Pseudomonas* strains that exhibit lipolytic and proteolytic activity resulting in fish spoilage.^{38,39} *Pseudomonas putida* is recognized as a less important fish spoilage bacterium due to a lack of proteolytic activity⁴⁰ but isolates from Mediterranean hake produced putrescine.⁴¹

The bacterial flora observed on gills and skin for day 0 of pink salmon was typically a diverse microflora and expected of fresh fish harvested from Alaskan waters. As storage progressed, there was a change in the microflora, as observed in a previous report.⁴² The presence of *P. fluorescens* or *P. putida* at 80% of the total microflora on final day of ice storage in all the tissues reinforces the importance of *Pseudomonas* spp. in the spoilage process⁴³ of pink salmon stored in ice. Presence of *P. immobilis* and *M. catarrhalis* was observed throughout the storage period and their commensal contribution to the spoilage process needs further research.

In conclusion, the Sherlock® MIS was useful in identifying psychrotrophic seafood bacteria to the species level and can be used as a rapid method for assessing seafood quality and estimating remaining shelf life.

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Conflict of interest

Author declares that there is no conflict of interest.

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