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Improved Microbial Control of CO_2 Packaged Salmon Fillets Compared toWhole, Gutted Salmon

Anlaug Ådland Hansen[1*], Birgitte Moen[1], Pål Georg Storø[2], Marit Kvalvåg Pettersen[1]

[1]Nofima, Osloveien 1, N-1430 Ås, Norway

[2]SalMar ASA, Industriveien 51, N-7266 Kverva, Norway

[*]**Corresponding author:** Anlaug Ådland Hansen, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, PO Box 5003, N - 1432 Ås, Norway. Tel: +4764970104; +4798638084; Email: anlaug.hansen@nofima.no

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Abstract

The microbial load and composition during storage (2˚C) of transport batches of industrial packaged pre-rigor filleted salmon And Head-On-Gutted (HOG) salmon were studied in two different experiments (Experiment 1 and 2). In Experiment 1 HOG salmon stored aerobically were compared to Vacuum- and modified at mosphere (MAP, 20-25% CO_2) packaged fillets. In addition, the effect of processing time was evaluated (early and mid shift). In Experiment 2 (mid shift only) the aim was to further optimize the packaging of fillets by evaluating increased CO_2 levels by use of CO_2 emitter and/or gas flushing. The bacterial load was lower in HOG salmon compared to vacuum, but similar to MAP. Lower bacterial levels for fillets, compared to HOG, were obtained by introducing more CO_2. The microbiota was influenced by packaging and processing time. At early shift, a higher relative value of *Pseudomonas* was observed in HOG salmon compared to the fillets (dominated by *Carnobacterium* and *Enterobacteriaceae*), while *Photobacterium* dominated at mid shift independently of packaging (Experiment 1). Increased levels of CO_2 resulted in higher relative values of *Carnobacterium* and *Photobacterium*, but lower bacterial load, compared to vacuum (more *Pseudomonas* (Experiment 2)). This study show that better bacterial control can be obtained for industrially packaged pre-rigor filleted salmon compared to HOG by applying CO_2 emitter and/or gas flushing.

Keywords: Microbiota; Packaging; Salmon; Spoilage; Storage

Introduction

The total amount of slaughtered and gutted salmon produced in Norway was in 2016 of 1.2 mill tons, including 83 311 tons of fresh/chilled fillets (Norwegian Seafood Council). There is a potential for increasing value and better utilization of the salmon. Improved knowledge on slaughtering processes and fillet quality for pre-rigor filleting compared to post-rigor filleting [1] led to the introduction of filleting prior rigor mortis; pre-rigor filleting, immediately after slaughter. A post-rigor filleting (filleting after rigor mortis) means about two days of intermediate storage after slaughtering (gutting), which is less effective compared to the pre-rigor filleting. Filleting enables total utilization of the entire salmon by exploiting the by-products after removing the filet, which include heads, backs, skins, bones and viscera. However, today only about 5-10% of total amount of fresh fillet is produced as pre-rigor fillets. One of the challenges is to achieve at least as good quality preservation and shelf life of fillets compared to whole, gutted salmon, naturally protected by the skin. Today, the most common bulk packaging methods of salmon, both for the fillets and the gutted salmon is aerobe storage in boxes of Expanded Polystyrene (EPS) added ice (about 20 kg fish per package). However, there have been done improvements in packaging technologies, mainly based on studies performed for retail packages for different products including salmon, and the use of CO_2 emitter [2-8]. Probably these experiences are transferable to bulk transport package sizing, as described for 3 kg units of salmon fillets processed under laboratory conditions [9].

Some industrial tests are performed though in order to study alternative packaging materials for transport packaging [10,11]. However, as the skin of the fish protects the flesh from contamination, it is important to have alternative packaging materials and packaging methods to preserve microbial quality and thereby prolonging shelf life of the fillets at least as good as for the whole fish. Additionally, the packaging concepts needs to be transport efficient with as much edible products per transport package as possi-

ble. Spoilage of fresh fish is a consequence of microbial, chemical and biochemical activities. Microbial metabolism causes development of volatile compounds that give characteristic odors that is well documented [12-14]. Studies have been performed by characterization of the spoilage potential for different bacteria detected on raw fish [15-20]. *Photobacterium phosphoreum* is a spoilage bacteria identified in MAP and anaerobe packaged fish [4,16,21-23], and *Pseudomonas* and *Shewanella* are commonly described in aerobe or anaerobe packages, respectively [24]. Higher levels of *Pseudomonas* spp. and *Shewanella* spp. are found on fillets produced early on the production day compared to later processed fillets, while *Photobacterium* was not detected on fillets, but on whole salmon and from seawater [25]. Spoilage bacteria can to different extent be inhibited by adding CO_2 gas, either as traditional modified atmosphere packaging or by adding a CO_2 emitter [5,23].

Shelf life study reported by [9] for 3-4 kg units, 2-3 layers of half fillets, shows shorter time of shelf life when stored aerobically compared to fillets packaged in MAP added CO_2 emitter (gas to product volume ratio of 1/2). A recommended gas to product volume ratio (g/p ratio) in MAP is reported to be 3/1 for quality preservation [26]. An increased availability and dissolved CO_2 is shown to improve modified atmosphere packaging and bacterial inhibition [27]. Studies performed with both pre-rigor filleted Atlantic salmon, Atlantic cod and chicken breast fillets (retail packages) show that CO_2 emitter can compensate for reduced headspace of gas during storage and achieve prolonged shelf life by improved quality preservation [3-5,9,23]. Prior studies show that CO_2 gas can be added by use of a CO_2 emitter to a vacuum package , or by adding 100% CO_2 by flushing a small gas volume, both resulting in absorption so that the package looks like a vacuum package, as showed by A. Å. Hansen et al. [23] and [28], respectively. Hence, by adjusting capacity of the CO_2 emitter, a CO_2 emitter can develop a headspace of CO_2 in a vacuum package. Bulk packaging (vacuum and MAP) is reported for whole, gutted salmon with better microbial quality compared to traditional aerobe packaging in EPS boxes [29]. Still, transport efficient bulk packaging of fillets by use of CO_2 with low headspace (lower than 1/2), is to our knowledge not studied.

In this case-study different packaging methods for pre-rigor filleted salmon were compared to traditional packaging of whole, gutted salmon (head-on-gutted, HOG), processed at different time of the day. The aim of the study was to study the microbial load and composition of pre-rigor fillets compared to the whole, gutted salmon and to study the effect of processing time (Experiment 1). Furthermore, improved packaging of the fillets was investigated in order to achieve improved microbial control of the fillet product (Experiment 2). The study was performed at an industrial processing site to achieve realistic microbial processing conditions.

Materials and Methods

Raw Material

This study was performed through two experiments. In Experiment 1, whole gutted salmon (head-on-gutted; HOG) packaged in Expanded Polystyrene Boxes (EPS) added wet ice, was compared to pre-rigor filleted fillets (3 kg units) packaged in Modified Atmosphere (MAP) and vacuum packaged fillets. In Experiment 2, based on experience from Experiment 1, only fillets were used, but with increased units (11 kg fillet per package), packaged with different levels of CO_2 compared to vacuum packaging (Table 1).

Packaging method	Experiment 1 (2 fillets per package, 3 kg)	Experiment 2 (7 fillets per package, 11 kg)	Short name
Whole gutted (EPS with ice) (Head-on-gutted)	X		"HOG salmon"
MAP (60% CO_2, 40% N_2)	X		"MAP"*
Vacuum	X	X	"Vacuum 1" - Exp.1 *
			"Vacuum 2" - Exp.2**
Soft vacuum + CO_2 emitter		X	"Vac+CO_2emitter"**
MAP (100% CO_2) + CO_2 emitter		X	"High-CO_2"**
MAP (100% CO_2) + CO_2 emitter + High barrier film		X	"High-CO_2+High- barrier"***
Sampling time	Day 1, 9, 15, 20	Day 1, 9, 20	
Time of processing at day 0	Early shift (at 8 am), Mid shift (at 14 pm)	Mid shift (at 13 pm)	
Packaging materials: *PA/EVOH/PA/PE (140 µm) bags,**PA/PE: 65 µm for top web and 150 µm for bottom web, ***PA/EVOH/PA/PE: 70 µm for top web and 150 µm for bottom web.			

Table 1: Packaging methods used in the experiments.

Atlantic salmon (*Salmo salar* L.) were slaughtered, filleted pre-rigor and packaged at the processing plant Innovamar AS (Salmar, Frøya, Norway). The mean weight of the slaughtered salmon was about 6 kg. Both experiments in this study were performed during wintertime (February and November). The packages of fillets included 3 kg unit (2 fillets per package; packaged side by side, Experiment 1) and 11 kg unit (6-7 fillets per package; sampling from the top fillet, Experiment 2). Only the first experiment contained HOG salmon. Vacuum packaging of fillets was included in both experiments. The fillets were packaged with skin on, and placed flesh against flesh in the packages. In Experiment 1 fillets processed in the morning (one hour after production start, at about 8 am) and at the mid shift (after about seven hours of production, at 14 pm) were used, and were taken from the same holding netpen prior slaughter. Fillets in Experiment 2 originated from only mid shift (Table 1). The fillets were packaged prior onset of rigor.

Packaging

Fillets were packaged by a chamber machines C 400 / C 450 / C 500 (Multivac), both for modified atmosphere packaging (MAP, 60% CO_2 and 40% N_2) and vacuum packaging (evacuation of air without visible space of air) (Experiment 1). The volume of gas in MAP was sufficient to make a "Visible" layer of gas around the fillets, to achieve a transport efficient package. In Experiment 2, a thermoforming machine (R145 Multivac) was used to form and seal the packages. Thereafter 100 % CO_2 was added manually by making two (needle) holes in opposite positions of the sealed plastic bag, flushing CO_2 gas through the package for about 100 sec, putting a septa on one of the wholes and thereafter filling sufficient gas volume to surround the fillets (gas/product volume ratio about 1/2). All packages were added one liquid absorbent pad, or CO_2 emitter pads that develop CO_2 gas inside the package (both delivered by McAirlaid's, Steinfurt, Germany).

In Experiment 1 bags made by PA/EVOH/PA/PE 140 my (Wipack MB 140 HFP, 220 mm x 600 mm, oxygen transmission rate of <2 ml/m^2/24h at 23˚C/50 % RH) were used. In Experiment 2 the top web contained PA/PE (Wipak CO 65 µm, 65 ml/m^2/24h at 23˚C/50 % RH, Wipak, Nastola, Finland), and the bottom web were made by PA/PE (Nordform 215 150 µm, 27 ml/m^2/24h at 23˚C/80 % RH, Wipak). The high barrier packaging material for thermoforming was PA/EVOH/PA/PE (Wipak MB70HFP 70µm, <5 ml/m^2/24h: (23˚C/150 50%RH) and Wipak NFO XX150: 150µm : <3 ml/m^2/24h: (23˚C/ 50%RH)). 6-7 packages of 3 kg units, and 2 packages of 11 kg units were placed in each EPS box added about 3 kg wet ice on top for transport to the Nofima laboratory at Ås (Norway). As a reference, gutted salmon packaged in Expanded Polystyrene Boxes (EPS) added wet ice (about 3 kg) was used. Table 1 gives an overview of the different packaging methods and Sampling.

Sampling were performed at early and mid shift after 1, 9, 15 and 20 days of storage in Experiment 1 (only early shift at Day 20), and at only mid shift after 1, 9 and 20 days in Experiment 2 (Table 1). There were five (Experiment 1) and four (Experiment 2) replicates per treatments per sampling time. The temperature of the refrigerating rooms (one room per experiment) were set to 2˚C for both experiments.

Analyses

Gas in Headspace

The levels of CO_2 and O_2 were measured in the packages of MAP and in the packages that included CO_2 gas and CO_2 emitter. The instrument being used was CheckMate 9900 O_2/CO_2 analyzer (PBI Dansensor, Ringsted, Denmark).

Culturable Analyses of Bacterial Growth

Bacterial plate counting were performed at each sampling time. A piece of 10 x 3 cm and 0.5 cm depth (approximately 10 grams) was cut from the top fillet per package of fillets. Analyses of top and bottom fillets were performed in another experiment, but did not show any differences (data not shown). For the HOG salmon, the fish where filleted by hand before sampling. Sampling was done about 10 cm from the tail position, at the dorsal part of the fillet (3x3 cm2 and 1 cm depth). The piece of muscle was diluted by approximately 90 ml peptone water until 1/10 dilution was attained, and thereafter run in a stomacher for 60 s. Appropriate 10-fiold dilutions were made and spread on the different agar plates. Total viable count, and sulphur producing bacteria (counting black colonies) were both enumerated by use of Iron agar (Oxoid, Basingstoke, Hampshire, U.K.), incubated at 15˚C for 5 - 7 days. In Experiment 1 additional Long & Hammer (15˚C for 5 - 7 days) [30] was used for total viable count, but gave similar results as for Iron agar (results not shown). MRS agar (Man-Rogosa-Sharke, Oxoid) was used for lactic acid bacteria, incubated at 25˚C for 48 hours, and CFC agar (Cephaloridine Fucidin Cetrimide) was used for enumeration of *Pseudomonas* (25˚C for 48 hours). *Enterobacteriaceae* was grown on VRBGA (Violet Red Bile Glucose Agar) at 37˚C for 24 hours.

The limits for detection were 20 cfu/gram. All agar plates were aerobically incubated. In Experiment 1 five replicates/samples per packaging treatment at each sampling time (after 9, 15 and 20 days of storage) were measured, and in Experiment 2 three to four replicates/samples per packaging treatment at each sampling time were used (after 9 and 20 days of storage). Experiment 1 and Experiment 2 represent two different batches of salmon.

DNA Extraction

Only samples from day 15 (Experiment 1) and day 20 (Experiment 1 and Experiment 2) were included for DNA extraction and Next Generation Sequencing (NGS) analysis. Nineteen samples from Experiment 1 (day 15 (early shift, n=4; mid shift, n=6) and day 20, n=9) and fifteen samples from Experiment 2 (day 20) were selected for NGS (Next Generation Sequensing). From each of these 34 samples, 45 ml of the stomacher solutions were centrifuged at 100×g for one minute, 40 ml of the supernatant was then

filtered using a 20µm Steriflip filter (Millipore). One (if total cfu > 4 log) or 5 ml (if total cfu < 4 log) of the supernatant was transferred to an Eppendorf tube and centrifuged at 13000×g for 5 min. The pellets were frozen at -20˚C until DNA extraction using the FastDNA-96™ Fecal DNA Kit (MP Biomedical) (with FastDNA-96™ Lysing Matrix E Rack) and following the manufacture's MP-96 Inhibitor Removal Plate protocol.

Bacterial Microbiota (NGS, MiSeq (Illumina))

PCR was performed in triplicates and paired end sequencing (2×150bp) was performed using the protocol presented in [31]. Briefly, the V4 region of the 16S rRNA gene was amplified with region-specific primers that included the Illumina flowcell adapter sequences. The reverse amplification primer also contained a twelve base barcode sequence that supports pooling of different samples. Samples were purified with Ampure (Agencourt Bioscience Corporation) and quantified using the Quant-iT Picogreen ds DNA with picogreen before pooling. The sample pool was purified and quantified as described above, diluted to 4nM and sequenced on a MiSeq (Illumina) following the protocol provided by Illumina. In addition to the experimental samples, the MiSeq run also contained a control library made from phiX Control v3, which, in this run, accounted for 10% of reads. The library quantification and sequencing were performed at Nofima. The MiSeq Control Software (MCS) version used was RTA v1.18.42.

The forward and reverse reads were joined in QIIME version 1.8.0, and the barcodes corresponding to the reads that failed to assemble were removed. The sequences were then demultiplexed in QIIME (version 1.9.1) allowing zero barcode errors and a quality score of 30 (Q30) using the QIIME toolkit [32]. The total number of sequences written was 3,817,922 with a median sequence length of 253 bp. The mean number of sequences per sample was 114,875 sequences (max 304,767; min 23,808). Reads were assigned to their respective bacterial taxonomy using an openref Operational Taxonomic Unit (OTU) picking workflow. Reads that did not match a reference sequence were discarded resulting in 3022 OTUs with n>2, each of these represents a phylotype and may be a representative of a bacterial species. Beta diversity- and taxa plots were generated using the core diversity command in QIIME 1.9.1.The level 6 (genus) table derived from QIIME was used for bar chart illustrations.

Statistics

The effect of the experimental factor per experiment were "Packaging Method" with the "HOG salmon", "Vacuum 1" and "MAP" used for the Experiment 1, and the"Vacuum 2", "Vacuum+CO_2emitter", "High CO_2" and "High CO_2+High Barrier" for the Experiment 2. They were analyzed separately for each day during storage using analysis of variance (ANOVA, GLM, One-Way, Minitab 17.0) with Tukey's multiple comparisons test where applicable ($p<0.05$). The effect of packaging (Experiment 2 only) for the relative values (MiSeq) × CFU/g was analyzed using analysis of variance (ANOVA, GLM, Minitab 18.0) with Tukey's pairwise comparisons test ($p<0.05$).

Results

Headspace Gas (Experiment 1 and 2)

In Experiment 1, the CO_2 levels of the MAP samples were measured to be 20 ± 3 % and 25 ± 8 for the early and mid-shift samples Day 1, respectively, and thereafter it was relatively stable during further storage at 13 - 16 % CO_2. There were no differences between early and mid-processed samples related to CO_2 or O_2 levels. The residual O_2 levels dropped from 1-2 % at day 1 until 0 % after 20 days of storage.

In Experiment 2, the "Vac + CO_2 emitter" had CO_2-levels of 39 ± 1 % and 27 ± 2 % after 9 and 20 days of storage, respectively. The CO_2 levels were quite more variable for the samples with manual gas flushing: the "High CO_2" had CO_2-levels of 54 ± 12 % and 48 ± 24 % after 9 and 20 days, respectively. The "High CO_2+High-barrier" had 63 ± 26 % and 50 ± 19 % CO_2 after 9 and 20 days, respectively. The residual O_2 levels were about 0.6 ± 0.4 %, 6.3 ± 6.4 %, and 5.4 ± 4.8 % for the "Vac+CO_2emitter", "High-CO_2" and "High-CO_2+High-barrier", respectively, after 20 days of storages.

Bacterial Counts

Experiment 1

The fillet samples of "Vacuum 1" and "MAP" processed at mid shift had similar bacterial counts as the mid shift "HOG salmon" after 9 and 15 days of storage (Figure 1). The "HOG salmon" processed at early shift had lower total bacterial counts (1.7 ± 0.4 log cfu/g) compared to the early shift fillets packaged by vacuum (3.2 ± 0.4 log cfu/g) and by modified atmosphere ("MAP", 2.8 ± 0.1 log cfu/g) after 9 days of storage. During further storage (day 15) of early shift samples, the "HOG salmon" had significantly lower bacterial counts compared to the "Vacuum 1" fillets, but not significantly different to the "MAP" fillets. After 20 days of storage, no differences in total bacterial counts were detected between any of the packaging methods. The counts of *Enterobacteriace* and lactic acid bacteria were higher for the fillet samples ("MAP" and "Vacuum 1") compared to the "HOG salmon". The sulfide producing bacteria were highest for the "Vacuum 1" and lowest for the HOG, showing some effect of the CO_2 for the MAP fillets. On the other hand, the *Pseudomonas* was highest for the HOG salmon (15 and 20 days).

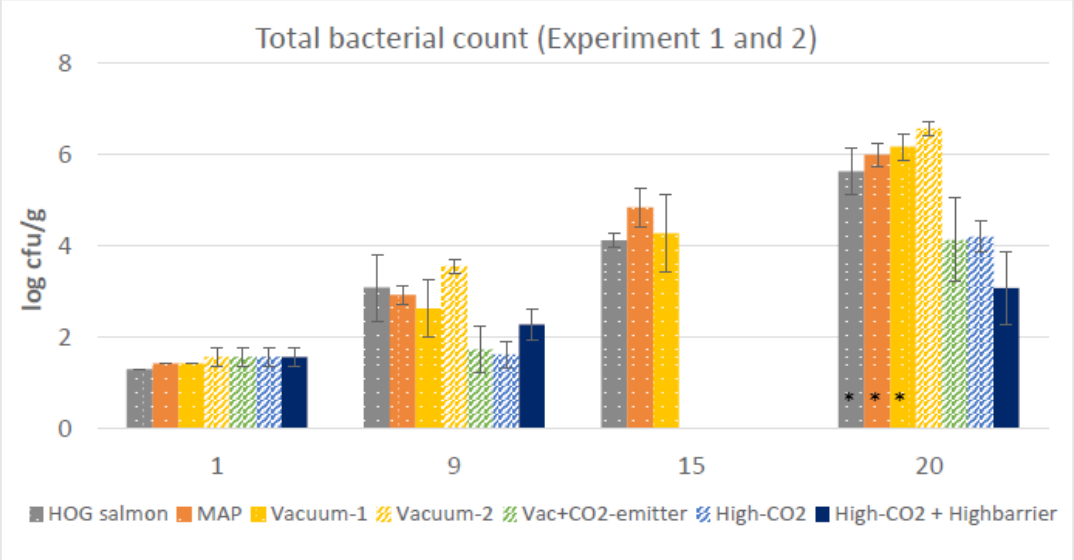

Figure 1: Total bacterial counts of salmon from Experiment 1 and 2, processed at mid shift, of pre-rigor fillets and HOG (head-on-gutted) salmon. Limit for detection was 1.3 log cfu/gram (Iron agar). (*Only early shift for the Experiment 1.).

After 9 days of storage the early shift "HOG salmon" samples had lower total bacterial counts (1.7 ± 0.4 log cfu/g) compared to the mid shift (3.1 ± 0.7 log cfu/g). Such difference between time of processing was not detected for the packaged pre-rigor fillets for any of the growth media used.

Experiment 2

In Experiment 2: The "Vac + CO_2emitter" and "High CO_2" had lower bacterial counts during storage (day 9 and day 20) compared to the "Vacuum 2", and they had also lower total bacterial count as the "HOG salmon" (both early and mid shift samples) in Experiment 1 (Figure 1). The samples containing CO^2 in Experiment 2 did additionally have lower total bacterial counts compared to the "MAP" in Experiment 1. The "High CO_2 + High Barrier" showed indication of even lower total bacterial count after 20 days of storage (3.1 ± 0.8 log cfu/g) compared to the other packaging methods, but it was not significantly different to the other fillet samples. Increasing CO_2 levels also showed increased inhibition and lower bacterial counts of sulfide producing bacteria, *Pseudomonas* and lactic acid bacteria. The vacuum packaged fillets from the two experiments ("Vacuum 1" and "Vacuum 2") had similar bacterial counts during storage.

Bacterial Microbiota

Experiment 1

The NGS results showed that the dominating microbiota was mainly affected by origin (HOG vs fillets) and time of processing. Figure 2-A (weighted beta diversity bi-plot Experiment 1) shows that the HOG samples had a different microbiota compared to the fillets, explained by higher levels of especially *Pseudomonas* and *Acinetobacter* for the HOG salmon compared to the fillets (early shift samples, day 15 and 20). This plot also shows that samples processed during the mid shift (day 15 samples only) had a similar microbiota independent of origin (illustrated by the dotted circle), dominated by *Photobacterium* (>90%). Figure 3 shows a more detailed view of the relative amounts of the dominating taxa from Experiment 1 and Experiment 2.

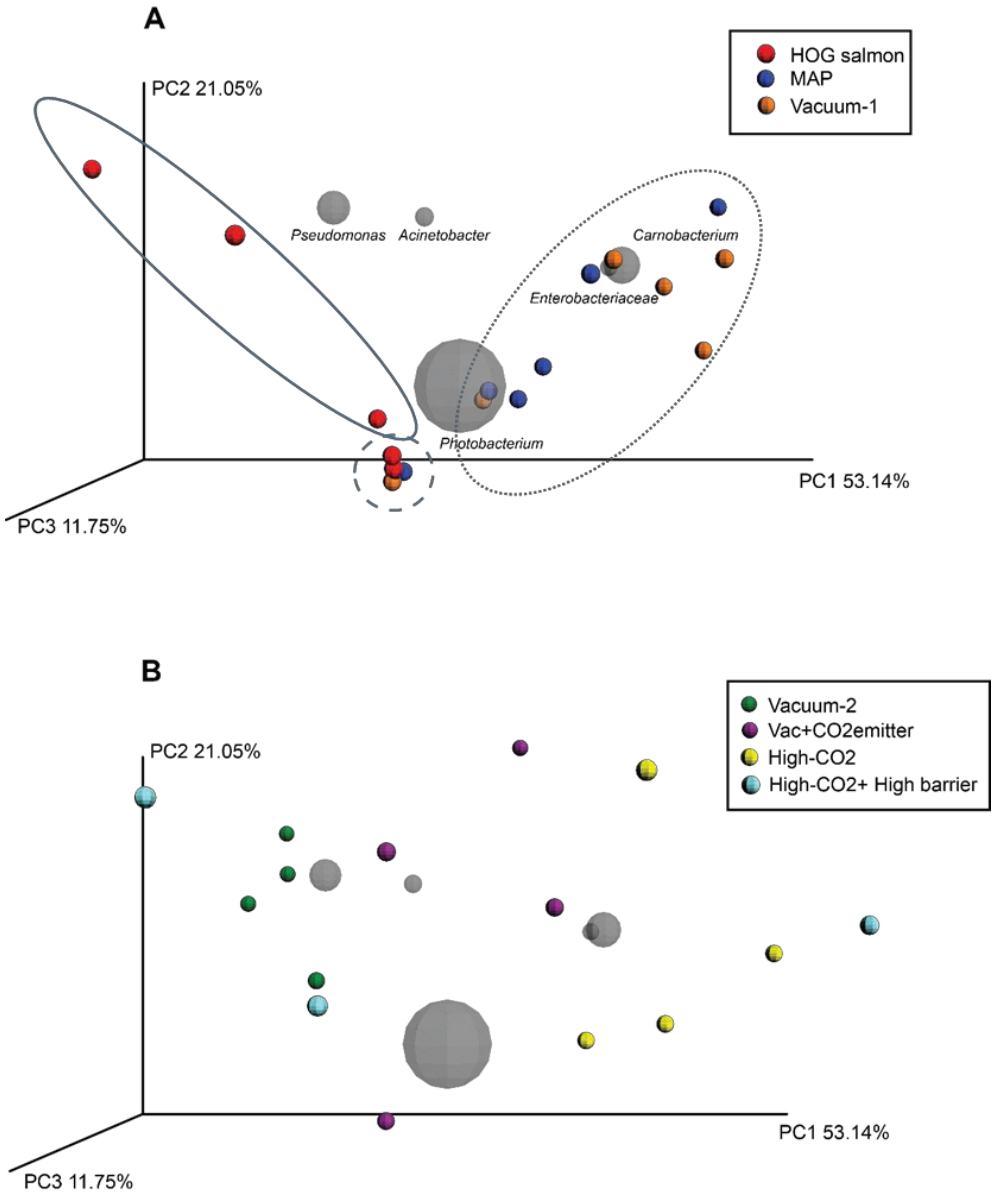

Figure 2: Overall bacteria composition (weighted beta diversity bi-plot) in Experiment 1 (early and mid shift) (A) and Experiment 2 (mid shift) (B). The different samples are colored according to packaging method. The grey spheres represents the five most influential taxa (the taxa are indicated by name in plot A). The striped circle (- - -) in plot A represents the samples from the mid shift (day 15), the dotted circle (……) and the solid lined circle represent samples from the early shift (the Vacuum-1 and MAP, and the HOG samples, respectively, both day 15 and 20).

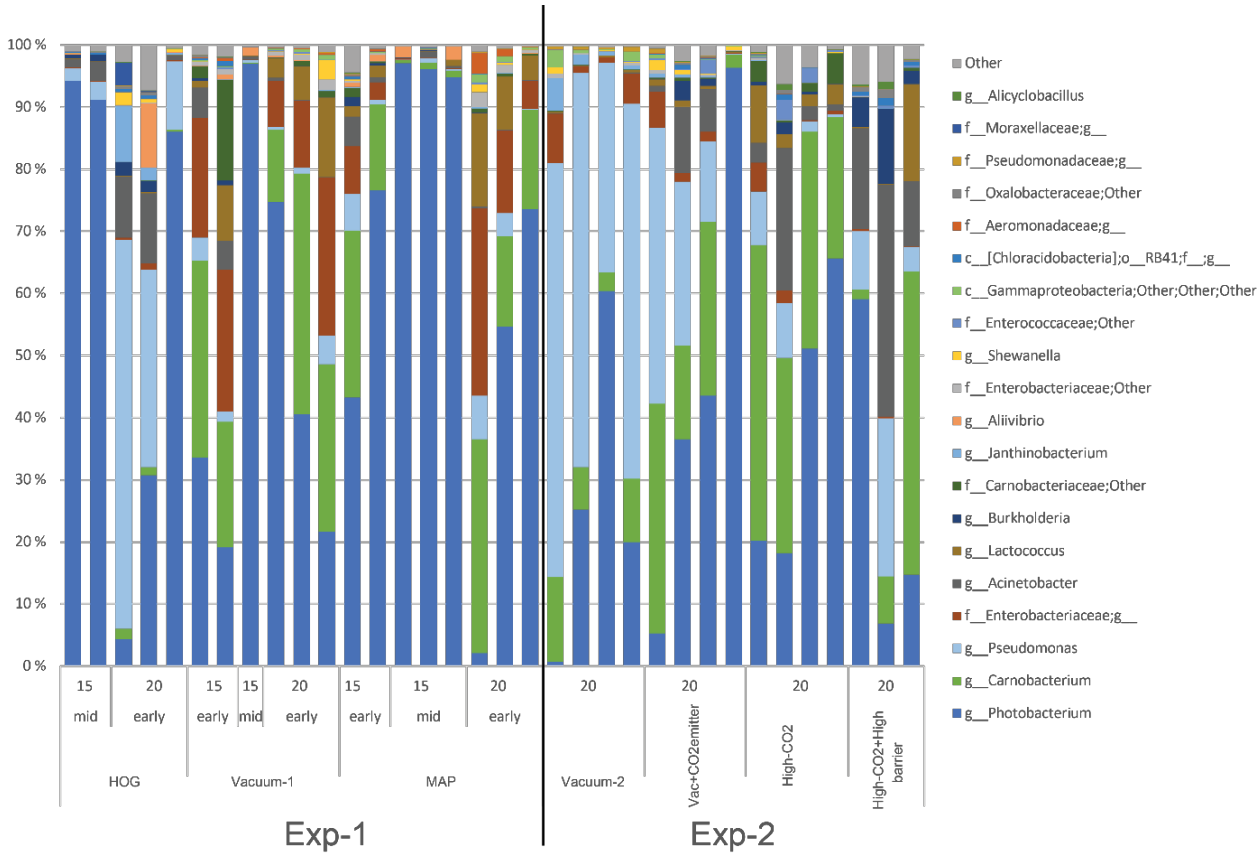

Figure 3: Dominating microbiota (all taxa above 0.1% across all samples) in Experiment 1 (early and mid shifts) and Experiment 2 (mid shift). The dominating taxa in all samples are represented as percent abundance. Taxa with average below 0.1 % across all samples are represented together as "Other". C= class; o= order; f= family and g= genus.

Experiment 2

In Experiment 2 the effect of the amount of CO_2 was apparent. The beta diversity analysis showed an effect of the CO_2 gradient along PC1 (weighted beta diversity bi- plot) (Figure 2-B), with the exception of two of the three "High-CO_2+High barrier" samples. This exception can be explained by the high residual O_2 levels in these samples (see Headspace gas Section). The relative amounts of *Pseudomonas* was higher for the samples with no use of CO_2 ("Vacuum 2") compared to packages with CO_2. These "Vacuum 2" samples were dominated by *Pseudomonas*, *Photobacterium* and *Carnobacterium* (Figure 2-B and 3). Samples with the highest amounts of CO_2 were dominated by *Photobacterium*, *Carnobacterium* and *Acinetobacter*. Figure 4 shows the relative values from the NGS multiplied with the total bacterial numbers. These estimates show that the levels of *Photobacterium*, *Pseudomonas* and Enterobacteriacea were significantly lower for the samples with the highest amounts of CO_2 compared to the "Vacuum 2" samples, and the levels of *Carnobacterium* were significantly lower for the "High-CO_2" and "High-CO_2+High-barrier" samples compared to the "Vacuum 2" and "Vac+CO_2 emitter" samples.

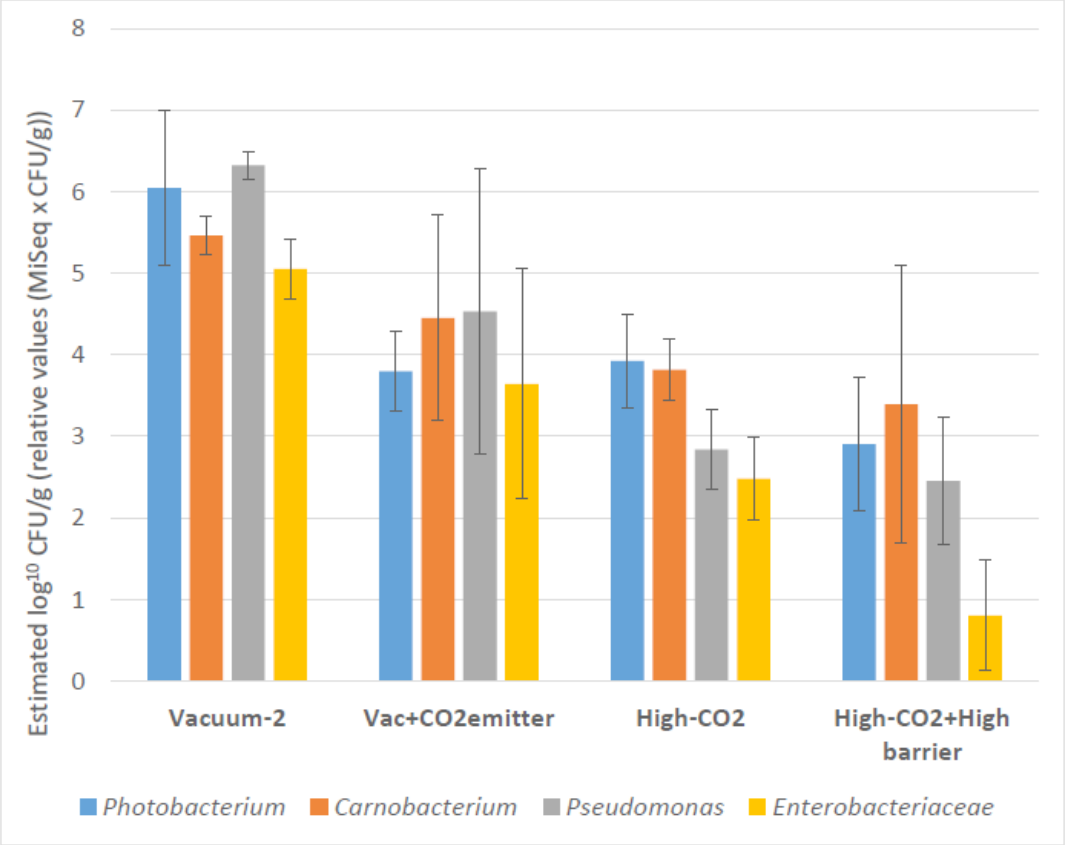

Figure 4: The relative abundance in relation to the total bacterial load (log CFU/g) of the fou rmost important taxa (*Photobacterium*, *Carnobacterium*, *Pseudomonas* and *Enterobacteriaceae*) for the Experiment 2 (day 20, mid shift). The values are based on the relative amounts (%) from the HTS results and the total number of bacteria in the samples (relative values × CFU/g) after 20 days of storage (the mid shift of the processing day).

Discussions

Bacterial Counts

Our experiments showed that one can achieve similar or lower bacterial counts with packaging of fillets compared to "HOG salmon". By increasing the CO_2 levels, despite the variable CO_2 levels according to the packaging methods being used in Experiment 2, the microbial quality can even be improved for the fillet samples. The effect of time of processing was only significant for the "HOG salmon" after 9 days of storage for the total bacterial count, and was not detected for the fillet samples. Based on this, the time of processing for the fillets was not included in the Experiment 2. However, possibly difference in bacterial counts due to time of processing is important to be aware of when planning sanitation of processing environment of salmon fillets, as up to 90 % reduction in initial levels of bacteria can be achieved by strict hygiene conditions [25]. Prior studies on salmon fillets (laboratory scale with strict hygiene) show that low initial bacterial counts (about 4 log cfu/g) can be kept low during about 14 days of 0˚C storage when packaged in modified atmosphere [3,9].

Similar low initial counts were measured in the presented experiments, but growth inhibition during storage require high availability of CO_2 and in the presented study these levels were lower compared to what used by [9] (about 65 - 80% CO_2 with use of CO_2 emitter) . [29] showed that gutted salmon stored at 3˚C in traditional boxes of expanded polystyrene (in air, with ice) reached total bacterial count of about 6 log cfu/g after 13 days of storage (initial level of 2 log cfu/g) [29]. These studies indicate the importance of storage temperature and packaging conditions on the bacterial level and thereby microbial quality and shelf life. Based on the experience from the Experiment 1, it was hypothesized that improved bacterial inhibition was possible by adding more CO_2 gas to the package of fillets, even for packages of more than two fillets in each. In the presented study, Experiment 1 and 2 showed that bacterial levels developed differently depending on the packaging atmosphere. Increased CO_2 levels resulted in better bacterial inhibition, despite 11 kg fillet per packaging units. The "Vac+CO_2emitter" achieved lower bacterial count compared to the "MAP", however, not different to the samples with even higher CO_2-levels, probably because of the variations of CO_2 in

headspace due to the manual flushing. It is shown in several studies that increased CO_2 levels, or partial pressure of CO_2, results in increased inhibition of bacterial growth [23,27,33,34]. The "HOG salmon" preserved microbial quality as good as the samples added 20 - 25% CO_2 gas ("MAP"), but still, not as good as the packages with the highest CO_2 levels ("Vac+CO_2emitter" and "High-CO_2"). This also show that CO_2 emitter can compensate for gas flushing/ modified atmosphere packaging, and that vacuum packaging can be sufficient with high CO_2 capacity of the CO_2 emitter.

After 20 days of storage bacterial level reached approximately 6 log cfu/g (Figure 1) for the "HOG salmon", "Vacuum 1", "Vacuum 2" and "MAP", which probably is within acceptable limit of microbial quality and shelf life [35]. The "Vac + CO_2 emitter", "High CO_2" and "High-CO_2-High barrier" only reached about 3 - 4 log cfu/g, which probably not represent any microbial spoilage of the fillets. However, it is the fraction of spoilage bacteria being present on the product that decide the level of quality degradation [24]. As reported by [9] a total bacterial count of 4-6 log cfu/g can be a critical range for sensory degradation, with detection of *Photobacterium* sp. as dominating bacteria. It is also shown that sensory attributes that are interpreted to be negative associated, increase during storage with the same patterns as for bacterial growth. However, a lag in the odor intensity is often observed compare to the bacterial growth [3,4]. Unpublished data from our lab, of artificially contamination of salmon fillets with *Photobacterium* show ended shelf life of aerobic ice stored salmon fillets at bacterial count of 7 log cfu/g, characterized with fermented, cloying and ammonia odor. Consequently, there might be different bacterial limits for spoilage and end of shelf life depending on the dominating microbial composition and packaging conditions. Changes in texture will also influence shelf-life, but according to Hansen et al. [9] there were no significant change in texture during storage until 14 days of storage for chilled MAP fillets stored at 0˚C, and only slightly reduction after 21 days of storage.

Bacterial Microbiota

The dominant bacteria during storage are likely to be involved in the spoilage of the product. In Experiment 1, the presented study detected a dominance of *Photobacterium* at mid-shift (day 15) and a more diverse microbiota at early shift, independent of packaging method and raw material (HOG salmon or vacuum and MAP fillets). A prevalence of *Pseudomonas* and *Acinetobacter* for the HOG salmon compared to the fillet samples at the end of storage (20 days) were found, however, one HOG sample was dominated by *Photobacterium* (Figure 2-B). Different contamination during a day is therefore reasonable to suggest. Several studies have been devoted to spoilage bacteria on salmon [3,15,16,25,28,36-38], but the effect of the time of processing during a day has not been described after storage with different packaging. However, studies have shown that during storage there can be a shift in microbiota, demonstrated by *Pseudomonas* sp. at the beginning of storage, followed by *Photobacterium* and Lactococcus piscium after 7 and 10 days of storage (50% CO_2, 50% N_2) [37].

In the presented study *Pseudomonas* was an initial dominating bacteria based on colonies identification (data not shown), for both fillets and HOG salmon, hence for the fillets the dominating bacteria changed during 20 days of storage to be dominated by the more CO_2 tolerant *Photobacterium*, *Carnobacterium* and *Acinetobacter.* However, despite their dominance, the total bacterial count (including *Photobacterium*) is lower for the CO_2- samples compared to the vacuum packaged samples, as increased CO_2 levels in Experiment 2 led to both changes in microbiota and the total bacterial counts, showing improved bacterial control by improved packaging. Inhibition of *Photobacterium* is shown for cod samples; a lower total bacterial count dominated by *Photobacterium*, with increasing CO_2 levels [23]. Furthermore, *Acinetobacter* is detected in prior study of thawed modified atmosphere packaged cod loins, showing that this group of bacteria may affect quality negatively in high numbers [10]. In the presented study *Acinetobacter* was detected in some HOG salmon samples and fillets samples, but probably not in numbers affecting quality negatively. *Carnobacterium* has been detected in several studies as one of the dominating bacteria during storage of MAP products. Some studies report that *Carnobacterium* sp. are not spoilage despite relatively high bacterial counts [39,40]. By others, *C. divergens* and *C.maltaromaticum* are confirmed to cause fish spoilage in raw seafood products both stored aerobically, in vacuum and in MAP, and that some strains can be food preservative [41].

Møretrø et al. [25] report higher levels of bacteria (*Pseudomonas* and *Shewanella*) on fillets produced early at a production day compared to fillets produced later the same day. They did not detect *Photobacterium* on the ice stored fillets. Similarly, *Pseudomonas* and *Shewanella* were detected at processing equipment after sanitation, while *Photobacterium* was not detected after sanitation, but on the whole salmon and seawater. Bagge-Ravn et al. [42] studied the microbial diversity on equipment in four fish processing plants and detected dominance by *Pseudomonas* and yeasts, followed by *Acinetobacter* and *Neisseriaceae* after cleaning and disinfection. A better cleaning throughout the processing day might therefore be relevant in order to achieve better microbial control and prolonged shelf life, especially related to the presence of *Photobacterium*, as also reported in the presented study, with a 75-98% dominance of *Photobacterium* at the mid shift. Related to the dominance of this bacterium and lower level of *Photobacterium* with increasing CO_2 levels, it is reasonable to suggest that microbial quality and load of big batches (here; 3 and11 kg units) of salmon fillets can be better controlled by improved packaging as also detected in previous studies [3,43].

Conclusions

The experiments presented in this work show that packages of fillets can achieve better microbial quality and control by use of increased levels of CO_2, compared to the HOG salmon, either by

gas flushing or by use of CO_2 emitter pad. Similar bacterial counts were found between times of processing during a day, except for the HOG salmon, which had higher bacterial load at mid shift compared to early shift, detected after 9 days of storage. A higher prevalence of *Photobacterium* on fillets processed at mid shift during a processing day compared to early shift was detected despite similar count numbers. Improved microbial control during a production day is therefore reasonable to recommend as such difference might lead to different quality and shelf life. Still, we have shown that the total bacterial load (including *Photobacterium*) can be kept low by use of CO_2 in the packages. The results present effect of packaging methods based on realistic pictures of the total microbial load and microbiota during storage of pre-rigor fillets processed under industrial conditions. Further studies should emphasize sensorial changes during similar processing and storage conditions.

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