International Journal of Food Microbiology 336 (2021) 108895

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Improved control of *Listeria monocytogenes* during storage of raw salmon by treatment with the fermentate Verdad N6 and nisin

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ARTICLE INFO

Keywords:
Fermentate
Food safety
Food quality
Microbiota
Sensory analysis

ABSTRACT

Fresh Atlantic salmon (*Salmo salar*) represents a healthy, nutritious food with global distribution and increasing consumption and economic value. Contaminating *Listeria monocytogenes* in fresh salmon represents a health hazard to consumers, is linked to extensive product recalls and is a major challenge for salmon processors. Verdad N6, a commercially available buffered vinegar, was evaluated as a treatment for raw salmon fillets either alone or in combination with the antimicrobial peptide nisin, with regard to anti-listerial effects under processing and storage, and influence on sensory quality and background microbiota. Salmon fillets were surface contaminated with *L. monocytogenes* and immersed in solutions of Verdad N6 or treated with nisin or a combination of these two treatments. Levels of *L. monocytogenes* were determined during vacuum-pack refrigerated storage. The use of Verdad N6 resulted in increased lag times and substantially reduced growth of *L. monocytogenes*. The inhibitory effects were dependent on Verdad N6 levels, immersion time, and storage time and temperature. A 5 s immersion in 10% Verdad N6 solution at 4 °C reduced growth of *L. monocytogenes* from log 2.8 to log 1 after 12 days of storage. Nisin (0.2–1 ppm) had listericidal effects up to 1 log but did not inhibit regrowth when used alone. Appropriate combinations of Verdad N6 and nisin led to *L. monocytogenes* levels no higher after 12 days of storage than the initial levels. The inhibitory effects were markedly lower at 7 °C than at 4 °C. Salmon with Verdad N6 showed reduced levels of total counts during storage indicating a longer shelf-life, and a shift in the dominating bacteria with reduced and increased relative levels of *Enterobacteriaceae* and lactic acid bacteria, respectively. Sensory analyses of raw and cooked Verdad N6 treated a non-treated salmon resulted in small differences. In summary, Verdad N6 is an option for production of high-quality raw salmon with increased shelf-life and enhanced food safety through its *Listeria* inhibiting effects. The application of Verdad N6 in combination with nisin treatment can further reduce the listeria-risks of these products.

1. Introduction

Fresh Atlantic salmon (*Salmo salar*) represents a healthy, nutritious food product with global distribution and increasing economic value. The global production of salmon was in 2019 around 2.6 million tons with Norway as the leading producer of farmed Atlantic salmon (around 1.4 million tons) followed by Chile and Scotland (FAO, 2020). Salmon export from Norway reached NOK 72 billion (Euro 6.5 billion) in 2019, a seven per cent increase from 2018. The world-wide and often long distance export of Atlantic salmon requires a particular focus on high quality processing, handling and storage to reduce potential microbial risks to a minimum while maintaining the microbial and sensory quality of the salmon throughout its storage time. The high-quality requirements are independent of the salmon being consumed fresh, cooked or as ready-to-eat (RTE) products (e.g. smoked or graved salmon). Raw fish and RTE meals were ranked first and second, respectively, among food products with the highest number of reported food safety incidents/recalls in a recent review. For these products presence of *L. monocytogenes* represented a major cause of recalls (Lüth et al., 2019; Soon et al., 2020).

The vast majority of listeriosis infections are foodborne. Although cases are relatively few, the severity of infections and the high proportion of fatal cases (20–30%) have placed *L. monocytogenes* among the top five pathogens responsible for the greatest burden of costs of illness and loss of quality-adjusted life years (Hoffmann et al., 2012; Rigby et al., 2017). *L. monocytogenes* is therefore regarded the biggest microbial challenge for the salmon industry. This is linked to the ubiquitous nature of the bacterium and its ability to survive or multiply at

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https://doi.org/10.1016/j.ijfoodmicro.2020.108895
Received 16 June 2020; Received in revised form 15 September 2020; Accepted 16 September 2020
Available online 08 October 2020

Table 1
Strains of *L. monocytogenes* used in the present work.

Strain no.	Serotype	MLVA/ST[a]	Source[b]	Other designations; reference
MF3860	1/2a	6-10-5-16-6/20	Salmon processing, Plant S4	(Møretrø et al., 2017)
MF3939	1/2a	5-8-15-10-6/14	Salmon processing, Plant S3	(Møretrø et al., 2017)
MF4001	1/2a	5-8-15-10-6/14	Salmon processing, Plant S2	(Møretrø et al., 2017)
MF4077	1/2a	6-9-18-16-6/8	Salmon processing, Plant S1	(Møretrø et al., 2017)
MF4588	1/2a	7-7-10-10-6/7	Salmon processing, Plant S1	(Møretrø et al., 2017)
MF4804	1/2a	6-7-14-10-6/121	Salmon processing, Plant S2	(Møretrø et al., 2017)
MF2184	1/2b	7-8-0-16-0/3	Meat processing, outbreak	2583/92; (Rudi et al., 2006)
MF3009	1/2b	n.d./5	Cattle	FSL J2-064; (Fugett et al., 2006) https://www.ncbi.nlm.nih.gov/nuccore/AARO00000000.2/
MF3039	4b	n.d./6	Human, cerebrospinal fluid, outbreak	FSL N1-227; (Cantinelli et al., 2013) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3889766/)
MF3710	4b	7-7-20-6-10/n.d.	Human, cerebrospinal fluid	CCUG3998; Culture Collection University of Göteborg

[a] MLVA designation according to Møretrø et al. (2017). ST numbers refer to Institute Pasteur MLST database (http://bigsdb.web.pasteur.fr./listeria/listeria.html).
[b] Plant designation according to Møretrø et al. (2017).

low temperatures in raw, fresh salmon, in products of the salmon industry and in processing environments. The presence of *L. monocytogenes* on raw, fresh salmon causes overall increased risks of *L. monocytogenes* in the final products with potential adverse effects. Several outbreaks and product recalls, due to contaminated salmon products, have been reported. For example, human cases of listeriosis have been linked to cold-smoked fish (Jami et al., 2014; Lassen et al., 2016; Ricci et al., 2018).

The lack of efficient *Listeria* control strategies in many fresh, RTE foods along with increasing numbers of documented listeriosis cases caused by such foods has promoted a zero tolerance policy of *L. monocytogenes* meaning a required absence of the bacterium in 25 g samples of risk foods and raw materials in several countries. In the EU, the regulations state that levels of *L. monocytogenes* must not exceed 100 CFU/g at the end of shelf-life. For baby foods and foods for medical purposes EU also has a zero tolerance (Anon, 2005; FDA, 2020).

The prevalence of *L. monocytogenes* in fresh and processed salmon is likely to vary considerably. A European-wide survey in 2010 and 2011 revealed a prevalence of 17.4% in cold-smoked fish samples at retail and with 2% of the total number of samples exceeding the 100 CFU/g limit at the end of shelf life (Anon, 2013). Prevalence and levels on raw, fresh processed or unprocessed salmon are less documented, but raw salmon is likely to be a significant source according to previous reports (Rørvik, 2000; Wieczorek and Osek, 2017). *L. monocytogenes* can grow readily on raw cold-stored salmon and can thus represent a potential risk in sushi, sashimi and similar products and be an important source for *L. monocytogenes* in cold-smoked or graved salmon risk products. Implementation of strategies that inhibit *L. monocytogenes* growth in contaminated salmon along the food chain is of outmost importance.

Another challenge of fresh salmon is the highly perishable nature with growth of spoilage microorganisms and enzymatic activities as primary determinants for shelf life (Gram and Huss, 1996). Low temperature storage near the freezing point is required to limit the growth of spoilage flora and obtain an acceptable shelf life for fresh salmon subjected to long distance travel from catch (slaughter) to further processing or consumption. Still, psychrotrophic bacteria including *Shewanella* spp., *Pseudomonas* spp., *Photobacterium* spp. and *Acinetobacter* spp. will partially be present on the raw material and also contaminate the fish during processing and promote spoilage if allowed to grow (Fogarty et al., 2019; Gram and Huss, 1996; Møretrø et al., 2016). There has been no consensus on which bacteria or bacterial levels to be used to indicate end of shelf life for fresh salmon. Thus, the importance of hygienic processing cannot be emphasized enough for the production of salmon with excellent microbial quality. However, no hygienic actions can guarantee *Listeria*-free products. This has led to enhanced interest in decontamination strategies and especially for non-thermal "clean label" technologies that are able to maintain or even improve the overall quality of perishable raw salmon products. Most studies have focused on strategies for improved control of *L. monocytogenes* in risk cold-smoked salmon, e.g. use of organic acids and their salts (Heir et al., 2019), phages (Soni et al., 2014), nisin (Soni et al., 2014), protective cultures (Ghanbari et al., 2013), UV-light (Holck et al., 2018) and a number of other compounds (Pedrós-Garrido et al., 2020; Tocmo et al., 2014). Fewer studies have tested the effect of such strategies on microbial control of raw salmon. Technologies with some potential application for microbial quality enhancements in raw, fresh salmon include treatments with UV-light (Holck et al., 2018; Ozer and Demirci, 2006; Pedros-Garrido et al., 2018), salts of organic acids (Sallam, 2007b), nisin (Takahashi et al., 2011) and combinatory treatments (Miks-Krajnik et al., 2017).

Evaluations of such microbial mitigation strategies must also consider effects on sensory quality parameters under relevant processing and storage conditions. Such information is still lacking and is highly required for the food and salmon industry to consider implementation of such strategies in their processing. In a recent study, we evaluated and showed the potential of using a commercially available "clean-label" acetate-based fermentate (Verdad N6) for effective *L. monocytogenes* growth inhibition and overall product quality improvements in cold-smoked salmon (Heir et al., 2019). In the present study, the effects of Verdad N6 and the antimicrobial peptide nisin used separately or in combination against *L. monocytogenes* and the innate microbiota on contaminated raw, fresh salmon were evaluated under conditions relevant for practical implementation. The influence of the Verdad N6 treatments on the sensory properties of both raw and heat-treated salmon was also investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains of *L. monocytogenes* used in the study are shown in Table 1. The strains used included strains isolated from salmon and salmon processing facilities (n = 6), strains associated with human listeriosis outbreaks (n = 3) and one strain from cattle. The strains represented three serovars associated with human listeriosis and various MLVA and Multi Locus Sequencing Types (ST). Strains were cultured as previously reported (Heir et al., 2019). In short, strains were cultured individually in Brain Heart Infusion (BHI; Oxoid AS, Oslo, Norway) at 37 °C, 24 h and thereafter mixed to contain equal cell numbers of each of the strains. The 10-strain cell culture mix was stored at 4 °C for 20–24 h before dilutions to working solutions were performed in 0.9% NaCl.

2.2. Antimicrobial compounds for L. monocytogenes growth inhibition and reduction

The Verdad N6 powder (Verdad N6; Corbion, Amsterdam, The Netherlands) is a white distilled vinegar produced by fermentation. The Verdad N6 powder was solubilized in distilled water to provide in-use

solutions in the range 2.5–18% (w/w). The solutions were kept refrigerated (4 °C) and used freshly made. Nisin (Merck Life Science AS, Oslo, Norway) solutions in distilled water were made from the purchased 2.5% stock (equivalent to 25,000 ppm) to obtain in-use nisin solutions in the range 250–1000 ppm nisin. The nisin solutions were freshly made and kept refrigerated until use.

2.3. Preparation and contamination of fresh salmon with L. monocytogenes

Fresh salmon fillets with skin were packed by batch in boxes of extruded polystyrene (XPS) with plastic sheets separating the fillets from the added ice and received from Mowi ASA (Bergen, Norway) one day after slaughter and filleting. At day two after slaughter, the salmon fillets were cut into approximately 3 × 3 × 1 cm^3 pieces maintaining the original fillet surface. The *L. monocytogenes* cocktail (20 µl, 5 × 10^7 CFU/ml) was spread onto the 3 × 3 cm^2 salmon fillet surface by a sterile plastic spreader unless otherwise stated. The contaminated salmon were left for 30 min at 4 °C until further treatments. In parallel experiments, contamination of the skin side of salmon pieces was performed using a similar procedure. To verify anti-listerial effects on larger, retail-size salmon fillets, mid-loin fillets (approximately 300 g) were obtained refrigerated, fresh and vacuum packed 1 day after slaughter and filleting (Salmar, Kverva, Norway). The fillets were unpacked and contaminated on a defined surface area (3 × 3 cm^2) with the *L. monocytogenes* cocktail as described above prior to Verdad N6 treatments.

2.4. Treatment of salmon with Verdad N6 and nisin

Salmon pieces (3 × 3 × 1 cm^3) contaminated with *L. monocytogenes* were immersed in cold (4 °C) Verdad N6 solutions (approximately 50 ml per piece) using different concentrations (2.5, 5, 10 and 18%) and immersion times (5 s and 300 s). Control samples were immersed in sterile water (0% Verdad N6) or left untreated (no immersion). After immersion, excess liquid was dripped off and the samples were vacuum packed or subjected to nisin treatment (for combined Verdad N6 and nisin treatments). Mid-loins were immersed using a procedure like that of the small salmon pieces except for employing approximately 500 ml Verdad N6 solution per mid-loin fillet on a subset of Verdad N6 concentrations only. Nisin treatments were performed by spreading 20 µl of cold (4 °C) nisin solution onto the *L. monocytogenes* contaminated salmon surface by a sterile plastic spreader. Added nisin levels (0.2–1 ppm) were calculated according to the approximate weight of the salmon (ca. 10 g per piece). Nisin exposures were performed both as single treatments and as subsequent treatments of already Verdad N6 treated salmon. Salmon samples without added *L. monocytogenes* were also treated with Verdad N6 and nisin, packed and stored under identical conditions to assess the effects of Verdad N6 and nisin on the indigenous background microbiota of the raw salmon. For nisin treatments, the total surface of the salmon piece was exposed to nisin, otherwise the treatment was as described above. Treated salmon samples were left for 30 min at refrigeration temperature before the samples were put in stomacher bags, vacuum packed and stored at 4 °C or 7 °C. All experiments with *L. monocytogenes* were performed in a Biosafety level 3 pilot processing plant.

2.5. Culture dependent and independent microbial analyses

Bacterial counts of *L. monocytogenes* on salmon stored at 4 °C and 7 °C were recorded at day 0, 1, 5 and 12 after contamination and treatments (unless otherwise indicated). Ninety ml peptone water was added to each sample (10 g) in stomacher bags before stomaching for 1 min and plating appropriate 10-fold dilutions in peptone water on Rapid L'mono agar and incubating at 37 °C for 48 h for *L. monocytogenes* determination. Samples for psychrotrophic bacterial count determinations were added 40 ml peptone water, stomacked and plated on Long and Hammer agar, aerobic incubation at 15 °C for five days. Microbiota profiling using high-throughput paired end sequencing of bacterial 16S rRNA gene amplicons (MiSeq, Illumina) was performed on samples that had been subjected to selected treatments and stored for 12 and 19 days. Sample preparation was as recently described (Heir et al., 2019) with the following minor changes. DNA was extracted from thawed pellets using the DNeasy PowerLyzer PowerSoil kit according to the manufacturers protocol (Qiagen, Hilden, Germany). Five parallel samples were selected for each treatment and storage day (two samples were discarded due to low PCR product concentration). Purified and quantified DNA was diluted to 4 nM, and the MiSeq protocol "Preparing DNA Libraries for Sequencing on The MiSeq" provided by Illumina was then followed using 7 pM sample and 10% PhiX spike control DNA to increase the diversity in the sample pool. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

The sequences were processed in QIIME2 Studio (qiime2-2019.1; Bolyen et al., 2019). Briefly, the data were: demultiplexed using demux, paired ends were joined using vsearch (Rognes et al., 2016), quality filtered based on a q-score above 30, denoised using deblur, and taxonomy was achieved using classify-sklearn with the Greengenes 16S 13_8 database (Amir et al., 2017; Bokulich et al., 2013; McDonald et al., 2012; Pedregosa et al., 2011). The level 6 (genus) table derived from Qiime2 Studio was used for bar chart illustrations. The relative frequency table was exported to a text file and further processed in Excel. The taxonomy- and feature table was used to investigate Amplicon Sequence Variants (ASVs) representing *Enterobacteriaceae* and the most frequent ASVs were submitted to Ribosomal Database Project Seqmatch (RDP) (https://rdp.cme.msu.edu/) to get more information about possible genera.

2.6. Sensory analyses by a trained sensory panel

The sensory analyses were performed to determine effects of Verdad N6 treatments of salmon fillet on relevant sensory quality parameters. A highly trained panel of nine assessors (9 women; aged, 37–64 years) at Nofima (Ås, Norway) performed a sensory descriptive analysis (DA) according to the "Generic Descriptive Analysis" as described by (Lawless and Heymann, 2010) and the ISO standard 13299. The assessors are regularly tested and trained according to ISO standard 8586, and the sensory laboratory follows the practice of ISO standards 8589. Separate sensory analyses were performed on raw and heat-treated mid-loin salmon fillets. Sensory analyses on raw fillets were done on fillets stored five days after treatments while fillets for sensory analyses after heat treatment were prior to heat treatment stored frozen at (−40 °C) in the dark for 60 days, before being thawed for 16 h at 4 °C. For raw and heat-treated salmon, 22 sensory attributes were evaluated: sourness odour, marine odour, cloying odour, fish odour, pungent odour, rancid odour, colour hue, colour strength, whiteness, sourness flavour, salt taste, acidic taste, bitter taste, marine flavour, cloying flavour, fish flavour, pungent flavour, rancid flavour, aftertaste, hardness, juiciness and tenderness. In a pretest session, the assessors were calibrated on samples that were considered the most different on the selected attributes typical for the treated salmon samples to be tested. For raw salmon evaluation, each assessor was served two slices of 0.5 cm thickness twice per type of sample. The slices were served at room temperature (19 ± 1 °C) in white plastic beakers covered with a metal lid. For heat-treated salmon evaluation, each assessor was served one slice of 1.5 cm thickness per sample. Heat treatment of salmon were performed in a combi-oven (Electrolux Air-o-steam, Model AOS061EANQ) at 75 °C with 50% steam and 50% heat for 8 min (core temperature of 62 °C). Samples were served in preheated porcelain bowls with a warm metal lid. All attributes were evaluated on an unstructured 15 cm line scale with labelled end points going from "no intensity" (1) to "high intensity" (9). Each assessor evaluated all samples at individual speed on a computer system for direct recording of data (EyeQuestion, Software Logic8 BV, Utrecht, the Netherlands). All

samples were served to the panel coded with a three-digit number in duplicates following a balanced block design. Tap water and unsalted crackers were available for palate cleansing.

2.7. Analyses of physiochemical parameters

Quantitative levels of organic acids in raw salmon were measured using the Acetic acid UV-method for the determination of acetic acid in foodstuffs and other materials (cat. No. 10148261035; R-BIOPHARM AG, Darmstadt, Germany). For sample preparation, up to 10 g of salmon fillets were homogenized and the supernatant were centrifuged, decanted and filtrated. The filtrate was adjusted to pH 10.0 using 2 M KOH and placed on ice for 20 min and filtered. The clear filtrate was used as substrate in the enzymatic reactions performed according to the manufacturer's instructions. The content of the acetic acid in each sample (g/100 g) was calculated after absorbance measurements at 340 nm according to the protocol of the manufacturer.

2.8. Statistical analyses

Three to five parallels of both treated samples and untreated control samples were produced for each experiment on each day, and the experiments were repeated two to four times on different days. Two-sample *t*-tests were used to compare combinations of factor levels at specific time points. Analysis of variance (ANOVA) was used to determine statistically significant effects on the bacterial reduction by the treatments. All analyses were performed in R (R_Core_Team, 2016). A significance level of $\alpha = 0.05$ was used, meaning that samples were considered statistically different for P-values < 0.05.

For sensory performance, ANOVA using a two-way mixed model with the assessor and interaction effects considered random and samples as a fixed effect was performed on the descriptive sensory data to identify the sensory attributes that discriminated between samples. Least significance differences were calculated by Tukey's test ($P < 0.05$). A principal component analysis (PCA) analysis on the average of the sensory descriptive data was performed with mean centered data and no standardization. The statistical software used for the sensory analysis was EyeOpenR (Logic8 BV). For the multivariate data analysis, Unscrambler X Version 10.4.1 was used for the PCA.

3. Results

3.1. The fermentate Verdad N6 provides growth inhibition of L. monocytogenes on fillets and surface skin of fresh salmon

Fresh salmon pieces contaminated with *L. monocytogenes* on the salmon fillet muscle side were immersed in Verdad N6 solutions and stored vacuum packed for 12 days (Fig. 1). The degree of growth inhibition of *L. monocytogenes* was dependent on concentration of Verdad N6, immersion time, storage time and temperature of storage. While *L. monocytogenes* multiplied readily on untreated salmon samples (0% Verdad N6) with a 2.8 log cell count increase, the inhibitory effects of 5 s Verdad N6 treatment of salmon ranged from total inhibition to 2.0 log increase in *L. monocytogenes* counts during 12 days storage at 4 °C depending on the concentration. Increasing the immersion time from 5 s to 300 s showed that substantial growth inhibition during 12 days of storage (≤ 1 log increase in *L. monocytogenes* counts) was obtained at all tested levels (range 2.5% − 18%) of Verdad N6. At 7 °C storage, the untreated control showed 2.2 log increase after only five days storage. Substantial growth inhibitory effects for the Verdad N6 treated samples, with 0.6–1.5 log increases in this time period, were observed. The growth inhibition at 7 °C appeared less efficient after 12 days of storage, but *L. monocytogenes* levels of control samples reaching the stationary phase may somewhat mask the difference between treated and untreated salmon.

The outer skin surface of fresh salmon is likely to be exposed to *L. monocytogenes* and represents risks for contamination of processing surfaces and final products. Therefore, the skin surface of *L. monocytogenes* contaminated salmon samples was also subjected to Verdad N6 treatments, packaging and storage at 4 °C (Fig. 2). The analyses showed that the treatments provided growth inhibition of *L. monocytogenes* on salmon skin during storage with between 0.9 and 2.8 log lower *L. monocytogenes* counts observed in Verdad N6-treated salmon compared to the untreated control after 12 days storage. Again, growth inhibitory effects of *L. monocytogenes* were dependent on Verdad N6 levels, immersion time and storage time. Higher Verdad N6-levels or increased immersion times were needed to obtain the same inhibitory effects on the skin side contaminated salmon compared with fillet side contaminated salmon (Figs. 1 and 2). For both types and at all tested conditions, effective *L. monocytogenes* growth inhibition was obtained the first days of the storage compared to the untreated controls. The increased lag times after treatments were often followed by reduced growth when combinations of Verdad N6 levels and immersion times were sufficient.

The small size salmon samples used in the above immersion experiments may not reflect the conditions for larger, industrially processed fillets. Immersion experiments were therefore conducted on whole, industrially processed salmon loins. The experiments on loins were scaled up, else performed as for the small salmon samples except for using 3-fold lower amounts of Verdad N6 solutions/g salmon. The results showed somewhat reduced inhibitory effects for treated loins (Fig. 3) compared to similarly treated small size salmon fillet pieces (Fig. 1). However, the inhibitory effects between loins and small size samples immersed for 5 s in 5% or 10% Verdad N6 were small with only up to 0.2 log difference in *L. monocytogenes* counts after 12 days storage at 4 °C. Somewhat larger differences in *L. monocytogenes* counts at day 12 were obtained between loins and small size fillet samples immersed for 300 s in 5% and 10% Verdad N6 (0.6 log difference).

3.2. Combined Verdad N6 and nisin treatments provide enhanced L. monocytogenes control in fresh salmon

The Verdad N6 clearly showed *L. monocytogenes* growth inhibitory effects, but no listericidal effects of the treatments were evident. The peptide nisin was therefore selected and evaluated as a listericidal surface treatment strategy both alone and in combination with Verdad N6. Fresh salmon samples were contaminated with *L. monocytogenes* and treated with nisin (range 0.2–1 ppm). Nisin alone provided initial reductions in *L. monocytogenes* levels between 0.6 and 1.0 log (Fig. 4A) which after 12 days storage (vacuum, 4 °C) provided 0.6–1.5 log lower counts in treated samples compared with controls. The reductions compared with controls after nisin treatment at 7 °C were at a similar range after five days, but much smaller after 12 days (Fig. 5A), partially reflecting that *L. monocytogenes* levels in controls approached stationary phase. At the tested nisin levels, *L. monocytogenes* counts increased during the 12 days storage period with 1.3–2.1 log at 4 °C (Fig. 4A) and 3.1–3.5 log at 7 °C (Fig. 5A). The results showed dose-dependent reductions of *L. monocytogenes*. It also revealed that at higher contamination levels surviving *L. monocytogenes* can grow and increase several logs during storage especially at abuse storage temperatures. Successive treatments combining nisin and Verdad N6 to obtain both reductions and growth inhibition of *L. monocytogenes* during storage were therefore evaluated.

The results of the combined treatments showed that nisin provided *L. monocytogenes* reductions also on Verdad N6 treated salmon (Fig. 4B, C, D). At 5% and 10% Verdad N6 a lag phase of at least five days was observed and the additional reduction caused by nisin after 12 days storage at 4 °C were 1.2–1.7 log and 0.6–1.2 for 5% and 10% Verdad N6, respectively. Interestingly, the combined treatments when using 5% or 10% Verdad N6 and 1 ppm nisin gave levels of *L. monocytogenes* not higher at day 12 than the levels on day 0 before any treatments.

At abuse storage temperature (7 °C), *L. monocytogenes* showed

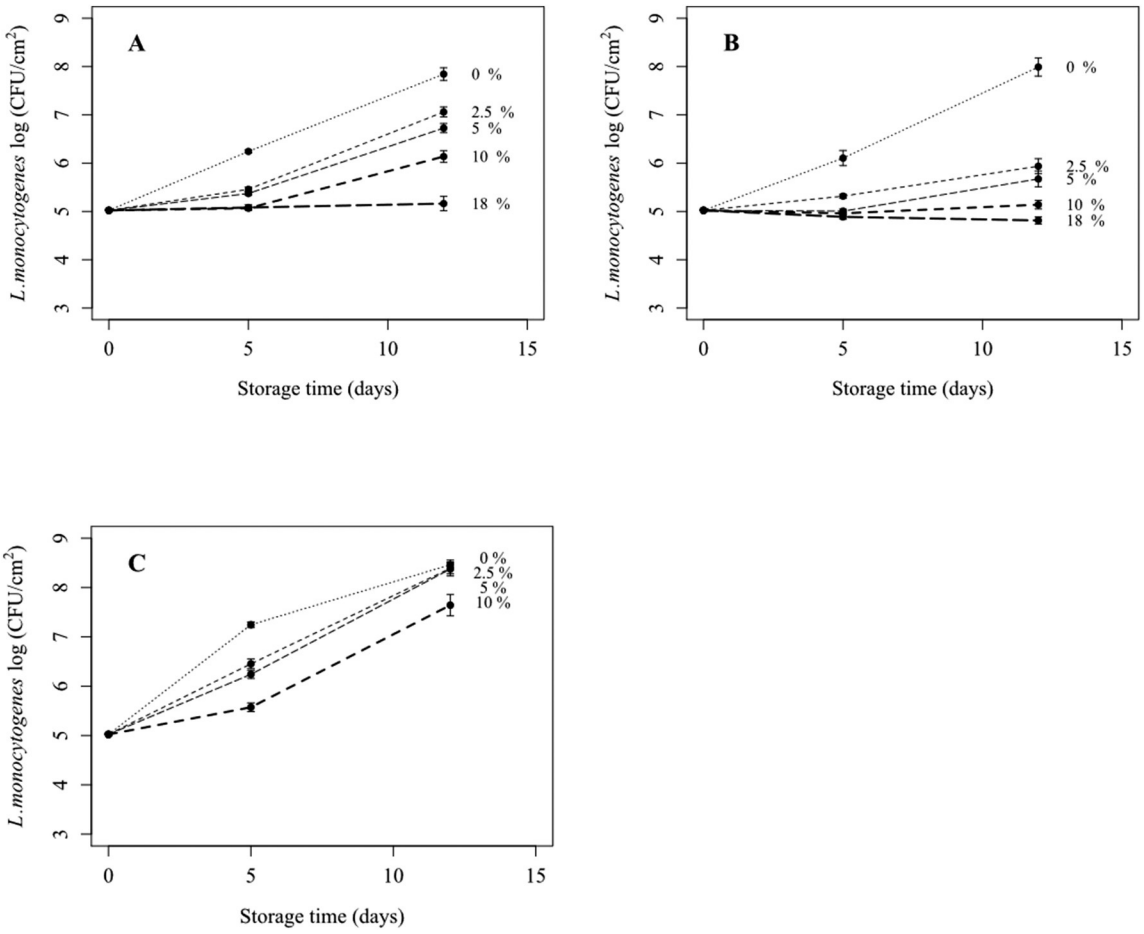

Fig. 1. Inhibition by Verdad N6 of *L. monocytogenes* growth on vacuum-packed fresh salmon (meat side) during storage for 12 days at 4 °C (A, B) and 7 °C (C). Samples of approximately 10 g were immersed in Verdad N6 (0–18 (or 10) %) for 5 s (A, C) or 300 s (B). Standard error of the mean is shown.

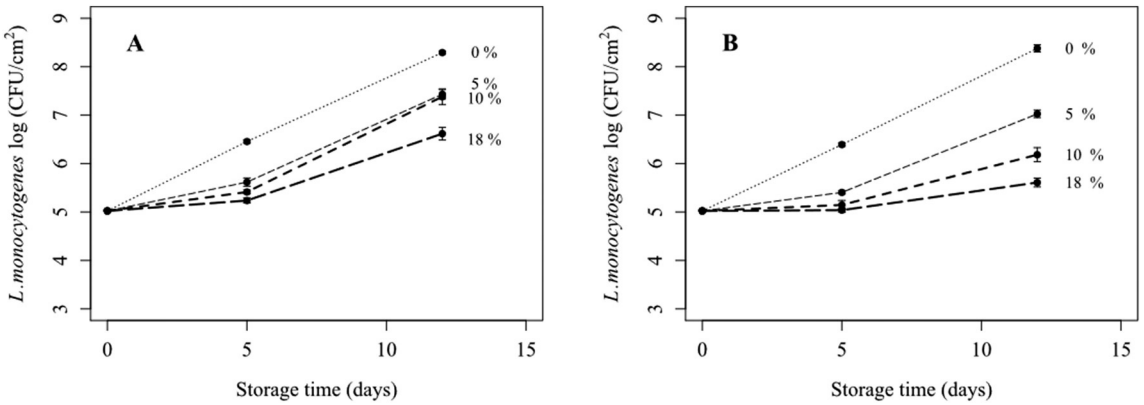

Fig. 2. Inhibition by Verdad N6 of *L. monocytogenes* growth on vacuum-packed fresh salmon (skin side) during storage for 12 days at 4 °C. Samples of approximately 10 g were immersed in Verdad N6 (0–18%) for 5 s (A) or 300 s (B). Standard error of the mean is shown.

significant growth at all tested conditions although using combinations of appropriate levels of nisin and Verdad N6 gave some reduced growth. Using Verdad N6 and nisin at highest tested levels (10% and 1 ppm, respectively), *L. monocytogenes* counts increased 1.5 log compared to 2.6 log using 10% Verdad only and 3.1 log using 1 ppm nisin only after 12 days vacuum storage at 7 °C.

To determine whether the *L. monocytogenes* contamination levels influenced the efficacy of nisin, salmon samples were contaminated with 10^2 and 10^5 CFU/cm^2 before nisin treatment and storage (Supplemental Fig. S1). No differences in reduction or growth were detected.

3.3. Effects of Verdad N6 and nisin treatments on the indigenous microbiota of raw salmon during storage

The analyses of levels and composition of endogenous bacteria in stored salmon (4 °C, vacuum) identified significant and different effects between untreated control salmon and salmon treated with Verdad N6, nisin and combinations of Verdad N6 and nisin (Fig. 6). In controls, mean bacterial total counts were 2.1 log CFU/g at day 0 which

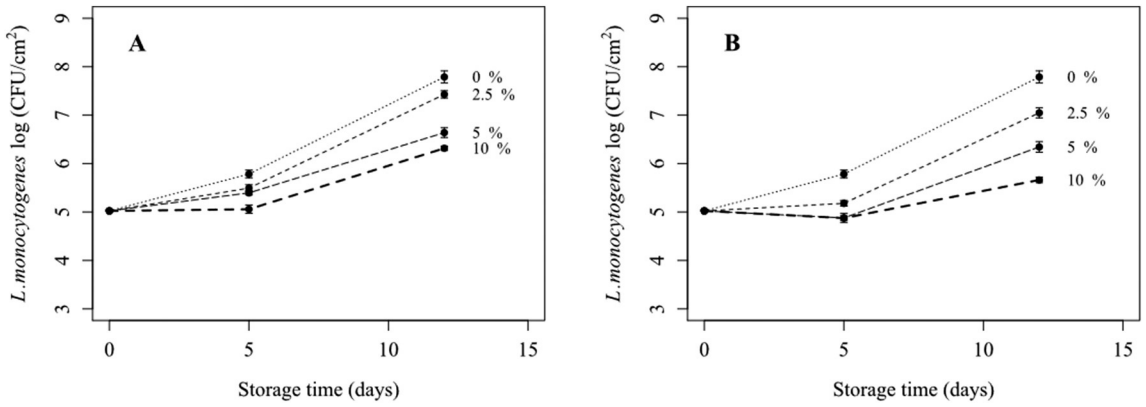

Fig. 3. Inhibition by Verdad N6 of *L. monocytogenes* growth on vacuum-packed fresh salmon loins (meat side) during storage for 12 days at 4 °C. Loins of approximately 300 g were immersed in Verdad N6 (0–10%) for 5 s (A) or 300 s (B). Standard error of the mean is shown.

increased to 7.5 log CFU/g after 12 days of storage. Nisin at 0.5 ppm had no significant effects on bacterial levels compared to untreated control after 12 days of storage with bacterial counts of 7.4 log CFU/g. Treatments with Verdad (5% and 10%) and combinations of Verdad 5% and nisin showed total counts (mean values) of only 5.7, 4.2 and 4.5 log CFU/g, respectively, at day 12. In line with the bacterial counts, nisin treatments had also relatively little effects on the bacterial composition compared with untreated control salmon, both were dominated by *Enterobacteriaceae* and *Pseudomonas* with *Aliivibrio* also being a dominant genus in control samples. Among the *Enterobacteriaceae*, two dominating and mainly equally distributed groups (ASV1 and ASV2) were identified in the RDP Seqmatch analyses. The ASV1 group included the genera *Enterobacter*, *Klebsiella*, *Buttiaxella* and *Pantoea* while the ASV2 group contained *Serratia*, *Rahnella* and *Yersinia*.

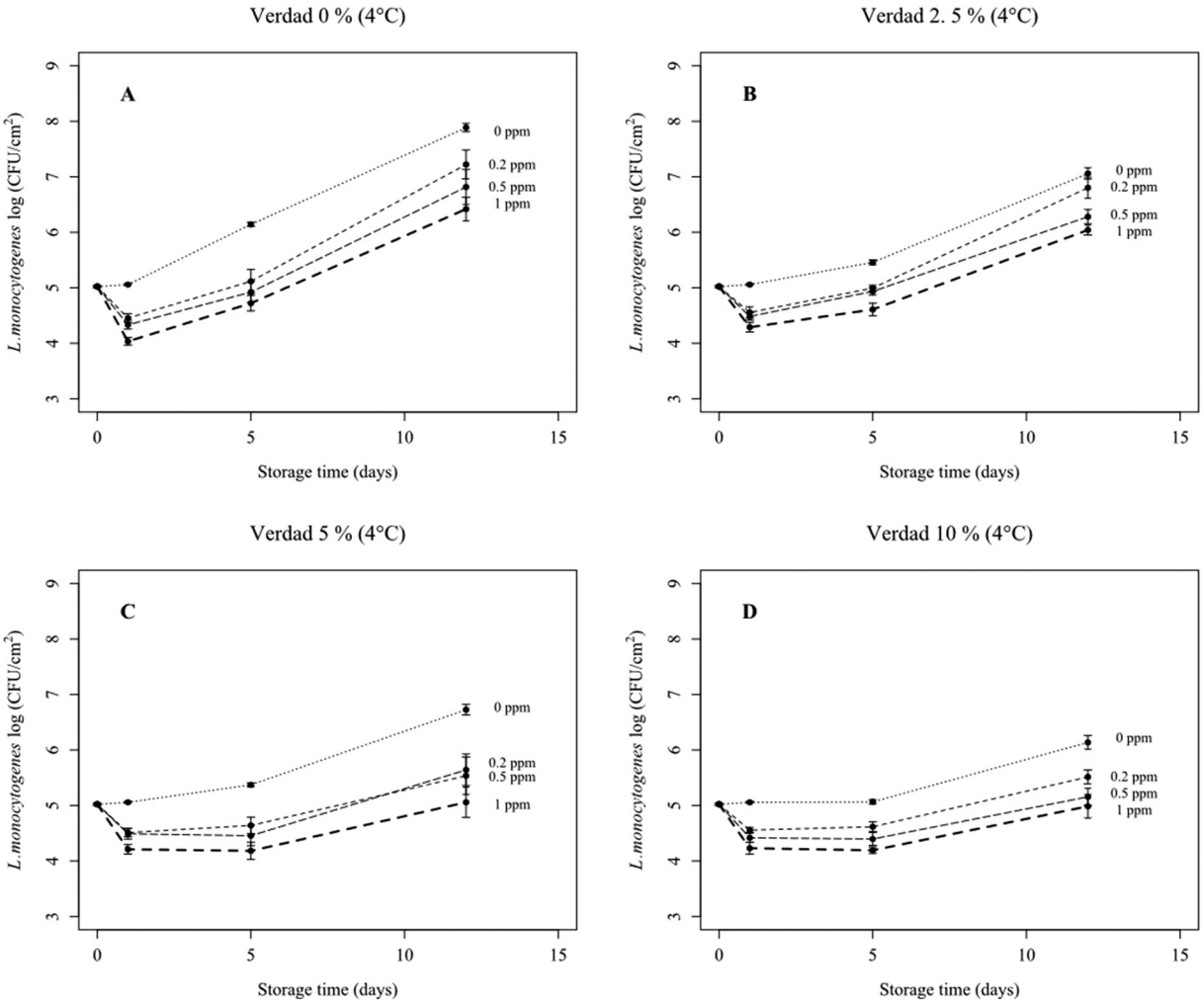

Fig. 4. Inhibition and survival by Verdad N6 and nisin of *L. monocytogenes* on vacuum-packed fresh salmon (meat side) during storage for 12 days at 4 °C. Samples of approximately 10 g were immersed in Verdad N6 at 0% (A), 2.5% (B), 5% (C), 10% (D) for 5 s and subsequently treated with nisin at the indicated concentrations (0–1 ppm). Standard error of the mean is shown.

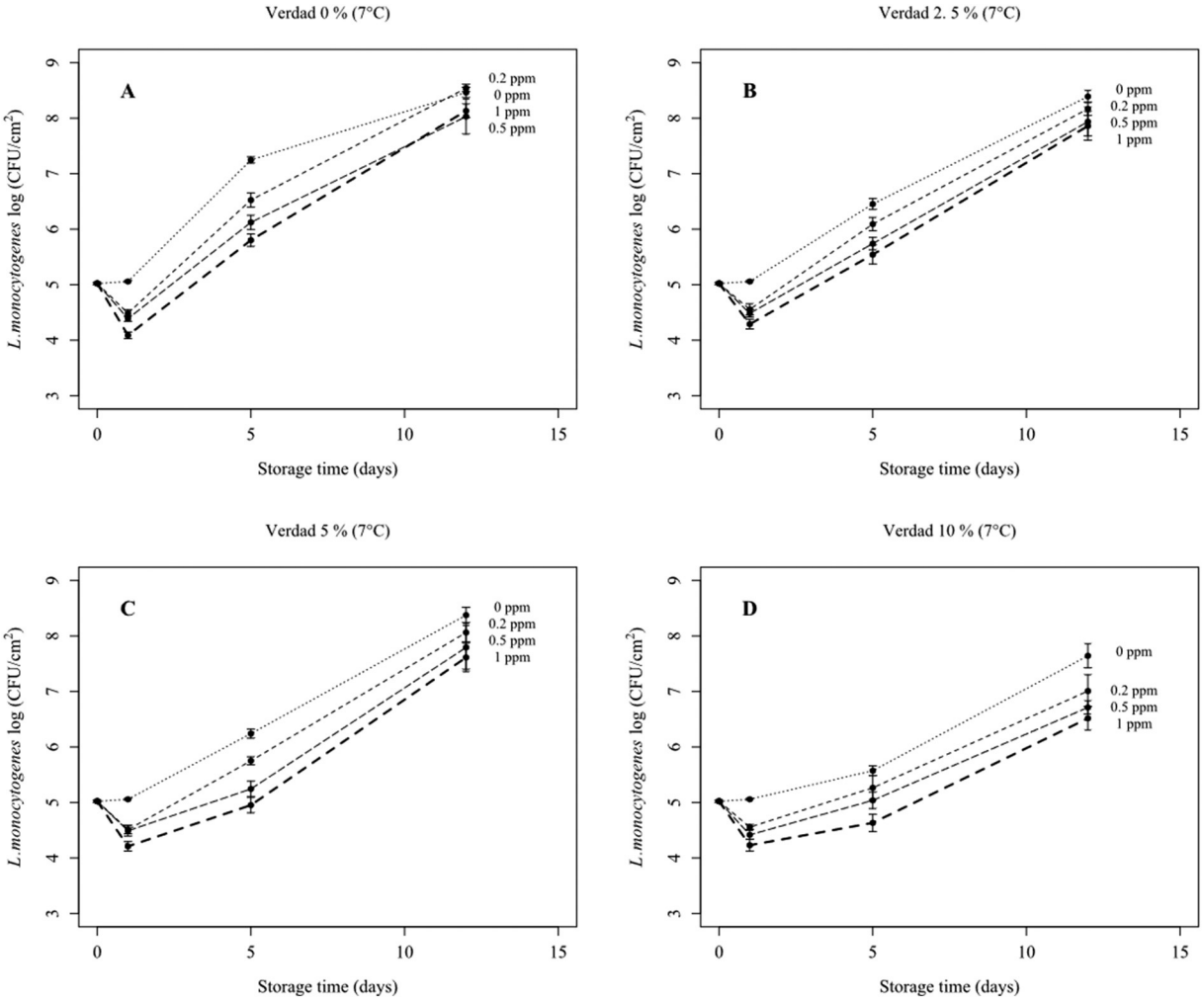

Fig. 5. Inhibition and survival by Verdad N6 and nisin of *L. monocytogenes* on vacuum-packed fresh salmon (meat side) during storage for 12 days at 7 °C. Samples of approximately 10 g were immersed in Verdad N6 at 0% (A), 2.5% (B), 5% (C), 10% (D) for 5 s and subsequently treated with nisin at the indicated concentrations (0–1 ppm). Standard error of the mean is shown.

In Verdad N6 treated samples, a higher prevalence of Gram-positive lactic acid bacteria (*Lactococcus*, *Vagococcus* and some *Carnobacterium*) were present at day 12. After 19 days storage, the difference in the bacterial composition between untreated and Verdad N6 treated salmon was even more pronounced with *Enterobacteriaceae* dominating in samples not treated with Verdad N6 (control and nisin) while Gram positives (*Lactococcus*, *Vagococcus*, *Carnobacterium*) dominated in Verdad N6 treated samples. Of note, in both Day 12 and Day 19 samples, we observed more variations in the bacterial genera composition within the parallels treated with 5% Verdad N6 compared to the parallels treated with 10% Verdad N6.

3.4. Physical and chemical quality parameters

A main component of Verdad N6 is acetate. Low levels of acetate (range 0.045–0.066% (w/w)) were present in both Verdad N6 treated and non-treated salmon fillets although with the highest levels in fillets treated with the highest Verdad N6 concentration examined (10%) (Table 2). The pH of Verdad N6 treated and non-treated salmon showed only small variations at the day of treatment (Table 2 Day 0; pH range 6.29–6.42) and also after 12 days storage (pH range 6.19–6.30).

3.5. Sensory analyses of raw and heat-treated salmon

Sensory analyses on both raw and heat-treated salmon exposed to six different Verdad N6 treatments and a non-treated control were performed by a trained sensory panel. Comparing the Verdad N6 treated raw salmon with untreated control, gave five out of 22 sensory attributes which were statistically different ($P < 0.05$) (Fig. 7, Supplemental Table S1). These were the odour attributes sourness, marine, pungent, and the flavour attributes cloying and pungent. High level (10%) Verdad N6 and long exposure time (300 s) were associated with both pungent odour and pungent flavour. At Verdad N6 levels ≤ 10% and short exposure time (5 s), no significant differences compared to the untreated control were detected. The small differences obtained on the visual attributes colour strength, whiteness and tenderness of different Verdad N6 treated salmon were likely due to natural variations in these attributes among the salmon fillets used in the sensory analyses (Supplemental Table S1).

For heat-treated salmon, statistically significant differences between the six treatments and the control were obtain for seven of the 22 sensory attributes included (Fig. 7, Supplemental Table S2). All these differences (e.g. sourness odour and flavour, fish odour, cloying flavour, pungent odour and flavour) were associated with high Verdad N6 levels (10%) and/or long exposure time (300 s). The sensory attributes sourness odour and flavour were reduced at 10% Verdad N6 treatments and long exposure time, while the attributes pungent odour and flavour and cloying odour and flavour increased at the same treatment conditions. For salmon treated with Verdad 2.5% or 5% for 5 s, no significant differences between these and untreated control were obtained for any

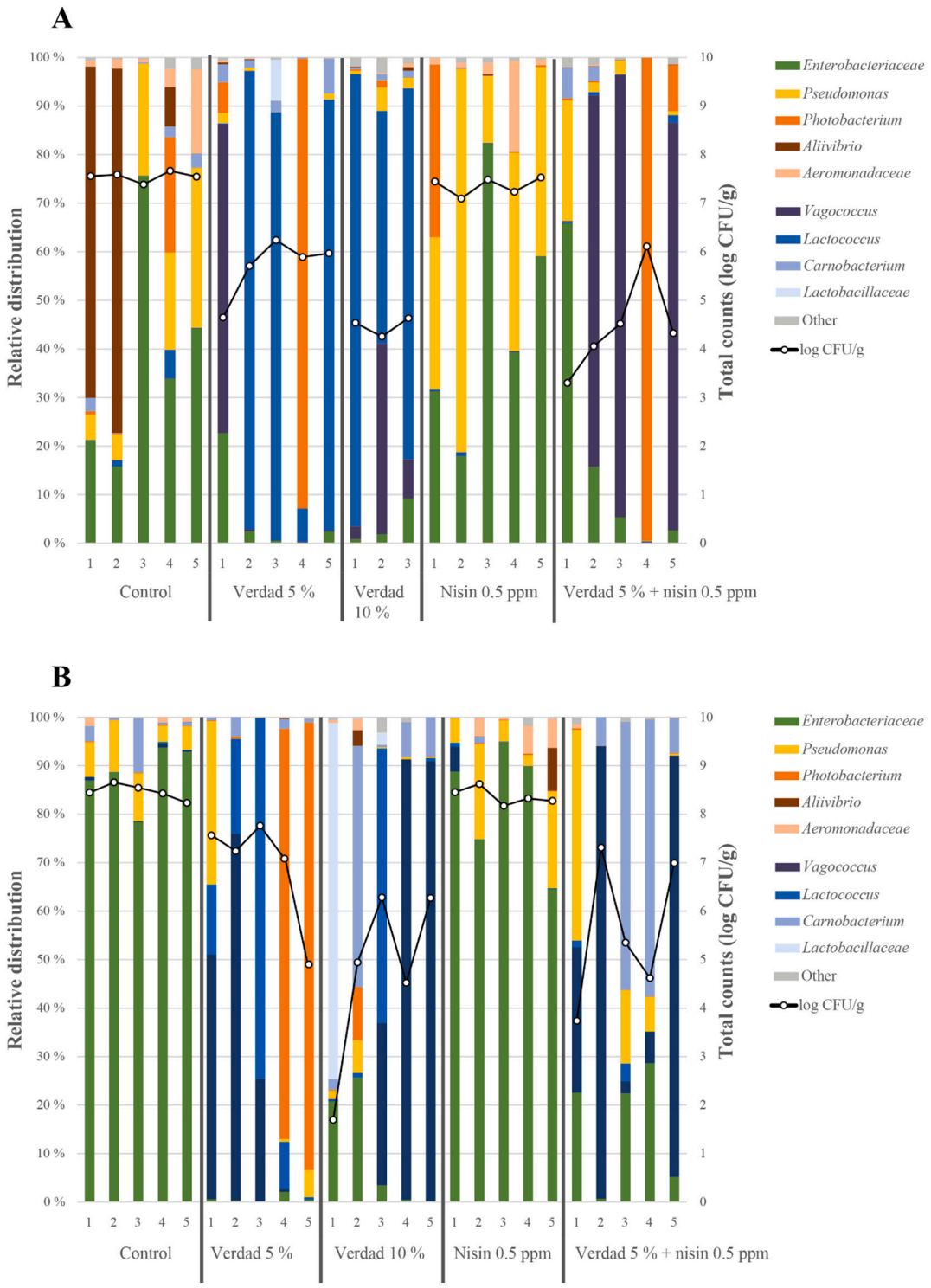

Fig. 6. Total counts and microbiota after storage of vacuum-packed salmon at 4 °C for 12 days (A) and 19 days (B). Only taxa with average over all samples above 0.2% or max value above 2% are represented. The remaining taxa are represented as "Other". The taxa are colored according to family or genus affiliation, and lactic acid bacteria are represented as different shades of blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the 22 attributes.

The principal component analysis bi-plots (scores and loadings; Supplemental Figs. S2 and S3) of raw and heat-treated salmon also illustrated the similarities and differences between the Verdad N6-treated samples and control. For both raw and heat-treated salmon, the first principal component (PC-1) explained 88% which means this PC dominates the interpretation of the two plots.

4. Discussion

Contamination of salmon with pathogenic *L. monocytogenes* at harvest, before slaughtering or during the process from slaughter to final product is a big challenge in the salmon processing value chain. Intervention strategies to control *L. monocytogenes* on salmon have mainly focused on smoked products (Heir et al., 2019; Holck et al.,

Table 2
Acetate and pH levels in untreated and Verdad N6 treated salmon fillets.

Treatment, exposure time	Acetate (g/100 g)[a]	pH[b]	
		Day 0	Day 12
None (Control)	0.045 ± 0.002	6.33 ± 0.02	6.19 ± 0.02
Verdad N6 5%,5 s	0.046 ± 0.003	6.42 ± 0.02	6.27 ± 0.02
Verdad N6, 5%, 300 s	nd	6.33 ± 0.01	6.27 ± 0.03
Verdad N6, 10%, 5 s	0.066 ± 0.003	6.31 ± 0.01	6.30 ± 0.01
Verdad N6, 10%, 300 s	nd	6.29 ± 0.04	6.22 ± 0.03

[a] nd: not determined.
[b] pH was measures at the day of treatment (Day 0) and after 12 days of storage (vacuum, 4 °C). Mean values from three parallels and standard deviations are shown.

2018), while studies on application on fresh, raw salmon are few. The present study aimed to evaluate the suitability of Verdad N6, a label-friendly, buffered vinegar fermentate used alone or in combination with the bactericidal bacteriocin nisin to enhance the microbial safety and quality of raw, fresh salmon.

The acetate-rich Verdad N6 fermentate provided a dose-dependent growth inhibition of *L. monocytogenes* with significant growth reductions on fresh salmon fillets. The inhibitory effect is caused by undissociated acetic acid being able to penetrate the bacterial membrane and acidify the interior of the cell. No significant listericidal effects were observed. Other studies on raw salmon and rainbow trout fillets reported both initial reductions (0.5–1.4 log) and growth inhibition of bacteria (Kilinc et al., 2009; Sallam, 2007a), but no studies on *Listeria* were performed.

L. monocytogenes strains may differ in growth characteristics in foods and susceptibility to antimicrobial compounds (Bucur et al., 2018; Heir et al., 2018; Møretrø et al., 2017). In the experiments, we therefore applied a mix of 10 *L. monocytogenes* strains of which six strains were from salmon processing with different sequence type (ST) to obtain results for *L. monocytogenes* diversity in salmon products. Control experiments showed that contaminating *L. monocytogenes* adhered to salmon fillet and skin surfaces and was not washed off during subsequent immersion in solutions. Since contamination of salmon with *L. monocytogenes* can be recent or have resided on the fish for longer periods, we contaminated salmon both 30 min and 24 h before treatments and obtained similar results (not shown).

Presence of *L. monocytogenes* on both salmon fillet and skin surface represents risks for contamination of processing equipment, environments and further cross contamination of raw and processed fresh and RTE salmon products. The distribution and processing of different types of raw, fresh salmon (head on gutted, fillets with and without skin) makes testing the effects of control strategies on both fillets and skin side appropriate. Also, on the skin side we observed dose dependent growth inhibition of *L. monocytogenes* using Verdad N6, although significantly lower effects compared with that on the fillet side. The reason for this is, however, uncertain. We are not aware of other studies reporting *L. monocytogenes* growth and survival on skin of fresh salmon in conjunction with organic acid salt treatments.

Combinations of appropriate Verdad N6 levels and immersion times seemed essential to ensure large enough local concentration of acetate at the treated salmon surface to obtain a lag phase of at least five days. During extended storage, the local surface concentration of the acetate containing Verdad N6 were likely lowered due to diffusion of the compound throughout the salmon meat and the surface-bound bacterial growth resumed. Measured acetate levels in the treated salmon samples were low (Table 2) but reflect the likely diffusion of acetate during storage and not the local surface concentration present after treatment. In our recent study on cold-smoked salmon, Verdad N6 was used as an ingredient in the dry-salting process prior to cold smoking (Heir et al., 2019). This provided higher levels of acetate within the whole salmon samples and effective *Listeria* inhibition also in sliced cold-smoked salmon. Application of Verdad N6 by dry salting is not relevant for raw, fresh salmon. We therefore solved Verdad N6 in water and submerged the fish samples into solutions of Verdad N6 of appropriate levels to ensure that all parts of the surface were treated. We also used a short (5 s) and a long (300 s) immersion period, although both being shorter than the 10 min dipping time used in the previous study of Sallam (2007a). In industrial, high throughput processing, showering or spraying of the fish may be a preferred alternative above a short immersion. The longer immersion period gave us an indication of the maximum possible inhibition using this strategy. However, for cost-effectiveness one would desire using combinations of as low concentrations as required, short immersion times and consider reuse of the solutions. It is practical to work with small size samples in the laboratory. The small samples had a higher surface to volume ratio which could lead to higher final concentrations of Verdad N6 after diffusion of the acetate during storage. It was therefore important to also examine the effect on larger samples. Significant inhibition of growth of *L. monocytogenes* was observed on salmon loin fillets of 300 g, although somewhat lower than for the smaller samples. This can be explained, at least partly by recognizing that in the experiments we used a higher fish/immersion fluid ratio for the loins than for the smaller pieces. The results, nevertheless, showed that the method with short immersion times (5 s) may be implemented on large fillets in industrial production.

The contamination level of *L. monocytogenes* on salmon is usually low, less than 10 CFU/g (Skjerdal et al., 2014). We used high inoculum levels (10^5 CFU/cm^2) in our experiments (certain exceptions, see below) to be able to determine the degree of reduction when using nisin in the experiments and to obtain good counting statistics. These high numbers may in some cases mask the differences in growth between controls and Verdad N6 treated samples where controls reach stationary phase e.g. at 7 °C (Fig. 1C).

Strategies that provide both reduction and growth inhibition of *L. monocytogenes* on contaminated salmon during transport and storage are required to meet the needs for the salmon industry to approach the stringent zero tolerance requirements in increasing number of markets. The antibacterial effect of nisin, a polycyclic antibacterial peptide produced by *Lactococcus lactis* that is used as a food preservative in cheese, processed meats, beverages and cold-smoked salmon (Gharsallaoui et al., 2016; Kang et al., 2014; Soni et al., 2014; Tang et al., 2013), was therefore evaluated. Nisin has GRAS (Generally Recognized As Safe) status and is the only purified bacteriocin that is widely approved as food additive (see Elsser-Gravesen and Elsser-Gravesen, 2014 and references therein). Nisin is active in the parts-per-million range and is effective against many Gram-positive bacteria including *L. monocytogenes*. In this study, nisin was applied both alone and in combination with Verdad N6. Bacteriocins are interacting with the membrane of target organisms, and at low inoculum levels there is more bacteriocin available per target cell, so potentially, given possibilities for diffusion, the reduction by nisin could be higher at low inoculum levels. However, the reduction was the same for both high and low inocula (Supplemental Fig. S1). The results confirm that reductions will also occur for the low contamination levels observed in commercial production.

Several previous studies have investigated the use of nisin on cold-smoked salmon usually claiming 1–3 log initial reduction of *L. monocytogenes* (Kang et al., 2014; Katla et al., 2001; Nilsson et al., 1997; Tang et al., 2013). Dipping fresh salmon in 0.2% nisin in combination with 5–10% Microgard (a fermentate containing another bacteriocin), provided a 1.5 log initial reduction and inhibited further growth of *L. monocytogenes*, and increased its shelf-life with 3–5 days at 6 °C (Zuckerman and Ben Avraham, 2002). When we tested 24 *L. monocytogenes* isolates, of which 13 represented common MLST sequence types (ST) from salmon and salmon processing against nisin (10 ppm) in a simple liquid assay, all strains were shown to be sensitive to the bacteriocin (1.7–2.6 log reductions; data not shown). The lack of

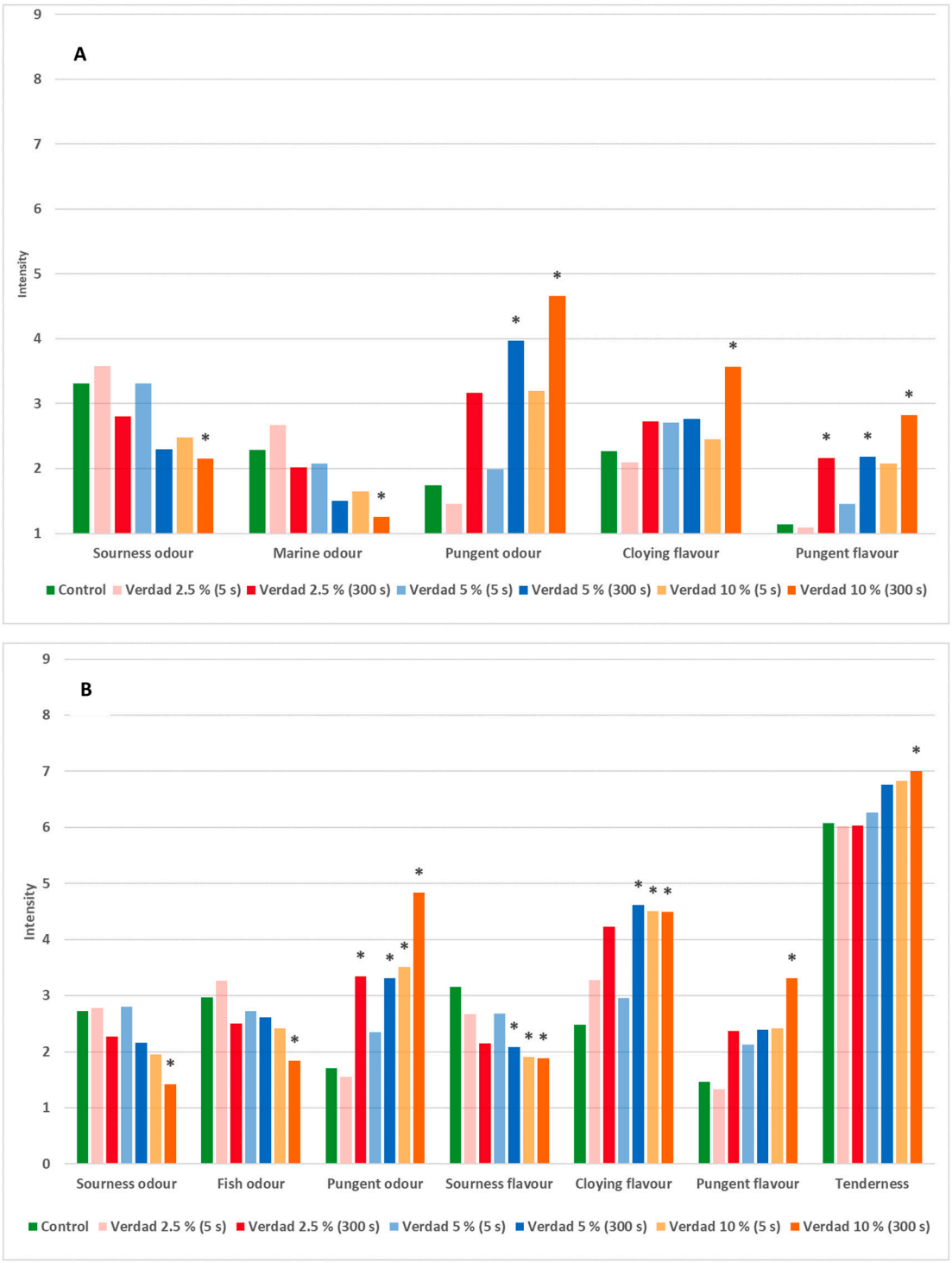

Fig. 7. Sensory analyses of raw (A) and heat-treated (B) salmon subjected to Verdad N6 treatments. Comparisons of mean values of sensory attributes of salmon samples. Samples different ($P < 0.05$; Tukey's test) from control are marked with *.

resistance among salmon related strains increases the usefulness of this strategy. However, one should be aware that inducible nisin resistance in *L. monocytogenes* can be influenced by food-related stresses as well as the genetic composition of the strains (Bergholz et al., 2013; Malekmohammadi et al., 2017), so care should be exercised to minimize development and persistence of such resistance.

Control of temperature was of paramount importance to limit the growth of *L. monocytogenes*. In untreated salmon, the pathogen increased more than 2 log within 5 days when stored at 7 °C (Fig. 5A). Also, when Verdad N6 was added, the inhibition was markedly lower at 7 °C compared with those at 4 °C at similar treatments (Figs. 4 and 5). This growth may reflect both that diffusion is faster at higher temperatures so that the surface concentration of acetate will decline more rapidly, and that the bacteria intrinsically grow faster at higher temperatures. The results underscore the importance of strict control of the cold chain from production throughout transport and storage at retail to consumers.

Previous studies have documented that bacterial growth and

composition of fresh fish during storage is highly dependent on packaging atmosphere and temperature during storage (Churchill et al., 2016; Fogarty et al., 2019; Gram and Huss, 1996; Mace et al., 2013). Verdad N6 (up to 10%) provided lower bacterial counts, lower relative levels of *Enterobacteriaceae* and *Pseudomonas*, and increased relative levels of *Lactococcus*, *Vagococcus* and *Carnobacterium* after storage. In untreated and nisin-treated salmon, psychrotrophic *Enterobacteriaceae* were relatively dominant where analyses revealed genera prevalent in seawater, salmon or salmon processing environments (e.g. *Klebsiella*, *Yersinia* and *Serratia*) to be common (Mace et al., 2012; Møretrø et al., 2016).

The dominance of Gram-positive lactic acid bacteria in Verdad N6 treated salmon indicates how the acetate rich Verdad N6 and the vacuum conditions shape the bacterial community during storage. The selective factor of increasing levels of Verdad N6 was also indicated with a more variable microbial composition in parallel samples treated with 5% Verdad N6 compared to 10% Verdad N6 after both 12- and 19-days storage. Although lactic acid bacteria dominated in most of these samples, high relative levels of *Photobacterium* were detected in a few. In our previous study on cold-smoked salmon, increasing levels of Verdad N6 appeared to reduce the relative levels of *Photobacterium* in the final product. These are known potential spoilage organisms of fresh salmon (Gram and Dalgaard, 2002). Further studies are needed to determine effects of Verdad N6 and other fermentates on specific food spoilage bacteria.

More important than the presence of possible spoilage organisms is the notion that the total number of bacteria in the Verdad N6 treated samples were 2–3 log lower than in the untreated samples. Also, after storage for 19 days, the total counts were on the average around log 6 for salmon treated with 0.5 ppm nisin and 5% Verdad N6. Of interest, *Vagococcus* spp. were dominant in several of the Verdad N6 and nisin treated samples after 12- and 19-days storage. In a recent study, *Vagococcus fluvialis* was the only bacterial species to cause reduced levels of off-odours and thus extend the sensory quality, even beyond 25 days, in vacuum packed salmon gravlax (Wiernasz et al., 2020). Although no obvious correlation between bacterial levels or species present and shelf-life of fresh salmon has been reported (Fogarty et al., 2019), the lower total counts and observed changes in microbiota in Verdad N6 treated versus non-treated salmon indicate that Verdad N6 has a general bacterial growth inhibiting effect being more pronounced on Gram negatives than Gram positive lactic acid bacteria. This would indicate possibilities for a more stable microbial quality, significant extension of the shelf-life, and improved flexibility of the salmon industry to apply fresh, high quality salmon for further processing. However, microbial interactions, characteristics and spoilage developments in salmon during storage are complex. Further studies are needed to conclude whether fermentates (like Verdad N6) and nisin could direct the microbiota development in fresh salmon towards a composition having fewer negative effects on the sensory quality of salmon.

Immersion of salmon in Verdad N6 using short immersion time (5 s) and at levels $\leq$5% did not lead to significant changes in acetate levels or pH, and accordingly most of the sensory parameter values did not change significantly after these treatments. For salmon treated using extended immersion times (300 s) and/or high Verdad N6 levels (10%) more differences in sensory perception were observed although the changes were generally small. Previous studies did not identify differences in preference for mildly smoked salmon with or without Verdad N6 (Heir et al., 2019) or adverse sensory effects on smoked catfish containing acetate (Kin et al., 2012). Reduced lipid oxidation, but also reduced sensory acceptance, of heat treated product was reported for sodium acetate treated fresh salmon (Sallam, 2007a, 2007b). Considering the low acetate concentrations in immersed salmon, it is highly unlikely that this would influence the sensory profile if the fillets were later used in cold smoked fish production.

In conclusion, the commercially available fermentate Verdad N6, with acetate as the main ingredient, provided effective growth inhibition of *L. monocytogenes* when applied to raw salmon fillets by surface treatments. In combination with nisin, enhanced control could be obtained. The inhibition was dependent on the temperature and control of the cold chain was important to achieve good growth reduction. The use of Verdad N6 also lowered the levels of potential spoilage bacteria. There were no or only small sensory changes when using Verdad N6 at short treatment times and at Verdad N6 levels $\leq$ 5%.The approach shows the potential to be used to produce high quality and microbiologically safe fresh salmon. For implementation in the salmon processing industry, further development and optimization for large scale, cost-effective use will be needed. Verification of effects on *L. monocytogenes* applying specific industry relevant conditions using valid challenge tests are further warranted.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108895.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding publication of this paper.

Acknowledgements

We thank Signe Marie Drømtorp and Tom Chr. Johannessen for excellent technical assistance. The Verdad N6 was provided by Corbion, Amsterdam, The Netherlands. This study was made possible by a research grant from The Norwegian Seafood Research Fund – FHF (grant no. 901166) and by grants on research infrastructure (grant no. 208674/F50). The work was also partly financed by the Norwegian Research Council, project 221663. Mowi ASA and Salmar ASA are appreciated for providing fresh salmon to the study. We thank the advisory board of the FHF project with representatives from the Norwegian salmon processing industry for valuable discussions, advice, and input to the work.

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