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Reduction and inhibition of *Listeria monocytogenes* in cold-smoked salmon by Verdad N6, a buffered vinegar fermentate, and UV-C treatments

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ABSTRACT

Contamination, survival and growth of *Listeria monocytogenes* in cold-smoked salmon represent serious health hazards to consumers and major challenges for salmon processors. Verdad N6, a commercially available buffered vinegar, was evaluated as an ingredient in cold-smoked salmon with regard to anti-listerial effects under processing and storage, sensory quality and consumer preference, effects on background microbiota and yield during production. Cold-smoked salmon with Verdad N6 added in the dry-salting process was produced. Salmon fillets were surface contaminated with a mix of *L. monocytogenes*. Levels of *L. monocytogenes* were determined during vacuum pack refrigerated storage for 29 days. The use of Verdad N6 resulted in increased lag times and reduced growth rates of *L. monocytogenes*. The inhibitory effects were dependent on Verdad N6 levels (0–2%), storage time and temperature (4 or 8 °C), type of contamination (between slices or on non-sliced salmon) and degree of smoking. The presence of dextrose (1%) in the recipe had no significant effects on *L. monocytogenes* levels after storage. On sliced salmon, complete growth inhibition at 4 °C storage could be obtained using 1% Verdad N6 compared to a 3 log increase in *L. monocytogenes* counts in control salmon. At abuse temperatures (8 °C), corresponding *L. monocytogenes* levels increased < 2 log and 5–6 log during 29 days storage. On non-sliced salmon, 1% Verdad N6 provided complete growth reductions at 4 and 8 °C storage while *L. monocytogenes* in control salmon increased 2.3 and 4.6 log, respectively, in the same period. The use of Verdad N6 in combination with bactericidal UV-C treatments (fluence 50 mJ/cm^2) provided an initial 0.8 log reduction and complete *L. monocytogenes* growth inhibition on subsequent storage at 4 and 8 °C. Salmon with Verdad N6 showed reduced levels of total counts during storage and a shift in the dominating bacteria with reduced and increased relative levels of *Photobacterium* and lactic acid bacteria, respectively. A consumer test showed no consistent differences in liking of salmon with and without Verdad N6. In summary, Verdad N6 is an option for the production of high quality cold-smoked salmon with enhanced food safety through its robust listeriostatic effects. The application of Verdad N6 in combination with listericidal UV-C light treatment can further reduce the listeria-risks of this ready-to-eat food product category.

1. Introduction

Strategies for improved control of *L. monocytogenes* in ready-to-eat (RTE) food products are needed to reduce the burden of this serious food-borne pathogen. Although human listeriosis generally has a low incidence, listeriosis can develop to severe infections with common case fatality rates of 20–30% (Lomonaco et al., 2015). Globally, listeriosis was estimated to cause > 23,000 illnesses and almost 5500 deaths in 2010 (de Noordhout et al., 2014). In EU, an increasing trend over recent years in listeriosis incidences has been observed. In 2016, a total of 2536 human listeriosis cases were reported, while 19 member states reported 247 deaths due to listeriosis in 2016 (EFSA and ECDC, 2017). Listeriosis is the disease with the third highest mortality rate among foodborne infection in the US (Scallan et al., 2011). Recent estimates placed L. *monocytogenes* among the top five pathogens responsible for the greatest burden of costs of illness and loss of quality-adjusted life years (QALYs; (Hoffmann et al., 2012)). *L. monocytogenes* is also the most troublesome and costly bacteria for many food manufacturers due to persistent “house strains”, product contaminations and recalls despite extensive *Listeria* control and testing programs. The need for product redesigns or internal product rejections further increase the economic burden of *L. monocytogenes* to the food industry.

Risk products include processed foods with extended shelf life having high *L. monocytogenes* prevalence and contamination rate and

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Table 1
Strains 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 Gothenburg

[a] MLVA designation according to Møretrø et al. (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. (Møretrø et al., 2017).

that support growth of this bacterium under refrigeration storage (Buchanan et al., 2017). Several types of ready-to-eat (RTE) foods of meat, dairy and fish origin do not undergo bactericidal heat treatment before consumption and are potentially high-risk products. The overall prevalence of *L. monocytogenes* in cold-smoked salmon appears to be high. A European-wide baseline survey in 2010 and 2011 revealed that 17.4% of 599 cold-smoked fish samples at retail were contaminated with *L. monocytogenes* at sampling (EFSA, 2013). Generally, the numbers were low, but 2% of the total number of samples (mostly cold-smoked salmon) exceeded levels of 100 cfu/g at the end of shelf-life. Although this and previous reports show that prevalence of *L. monocytogenes* in cold-smoked salmon vary considerably (see review Jami et al., 2014), smoked fish is the food item that most often harbors *L. monocytogenes*, and in levels exceeding the critical limit of 100 cfu/g (EFSA, 2009). Occasionally, cold-smoked salmon with *L. monocytogenes* exceeding levels of 10^5–10^6 cfu/g have been reported (Acciari et al., 2017; EFSA, 2013; Gombas et al., 2003). Factors that underlie differences in prevalence and contamination levels include variations in *L. monocytogenes* incidences in processing environments and differences in cold-smoked salmon recipe and process parameters. The data confirm that cold-smoked salmon is a non-uniform product with regard to product characteristic and *L. monocytogenes* risks, and that strategies to ensure the microbial safety of this product category are required.

Few human cases of listeriosis have been documented to be linked to cold-smoked fish (Ericsson et al., 1997; Jami et al., 2014; Miettinen et al., 1999). However, the increased applications of integrated surveillance and whole genome sequencing have recently shown to be powerful tools and linked previous undetected cases or clusters of cases to outbreaks (Gillesberg Lassen et al., 2016; Jensen et al., 2016; Lassen et al., 2016; Ricci et al., 2018).

Food processing environments represent the primary sources of *L. monocytogenes* contamination. The need for a facility-based approach which includes constant monitoring to control *L. monocytogenes* can therefore not be underestimated (Buchanan et al., 2017; Ferreira et al., 2014; Holch et al., 2013). However, extensive control programs will not eliminate *Listeria* in salmon processing environments nor in products. The zero-tolerance for *Listeria* that is practiced in several countries highlights the need for additional measures that can reduce the incidence and levels of *Listeria* and provide safer products and reduce costly recalls of foods.

Technologies to control *L. monocytogenes* in cold-smoked salmon include methods with bacteriostatic and/or bactericidal effects. Salts of organic acids like potassium lactate and sodium diacetate are widely used in the meat industry and have also significant potential for prevention of *L. monocytogenes* growth in cold-smoked salmon (Kang et al., 2014; Neetoo et al., 2008; Tang et al., 2013; Vogel et al., 2006; Yoon et al., 2004). Further enhanced *Listeria* control by combining bacteriostatic treatments with treatments that kill *L. monocytogenes* in food products has been documented. The effect of chemical, biological or physical strategies (e.g. lauryl arginate, nisin, protective cultures, bacteriophages, high hydrostatic processing, irradiation, UV light) have been evaluated (see reviews (Løvdal, 2015; Tocmo et al., 2014)). Nevertheless, the application of such measures in salmon processing industry is nearly absent which likely is due to limited anti-listerial effects, inadequate validations performed, negative effects on product quality, and/or regulations for use and declaration requirements for these strategies.

The main objective of this study was to validate the anti-listerial effect of Verdad N6, a commercially available blend of “label-friendly” buffered vinegar when used as ingredient in the salting process of cold-smoked salmon. The study aimed to determine how Verdad N6 in cold-smoked salmon affected 1) growth of *L. monocytogenes* when varying recipe, process and storage parameters 2) the sensory quality and consumer preference of the salmon 3) other quality characteristics including growth and composition of background microbiota and yield during production. The effects of combining the use of Verdad N6 and bactericidal UV-C treatments of cold-smoked salmon were also evaluated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes strains used in the experiments are shown in Table 1. The 10 strains used included six strains isolated from salmon and salmon processing facilities, three strains associated with human listeriosis outbreaks and one strain from cattle. The strains represented three serovars commonly associated with human listeriosis and various MLVA and Multi Locus Sequencing Types (ST). The strains were maintained at −80 °C in Brain Heart Infusion (BHI) broth with 15% glycerol. For each experiment, strains were cultured on BHI agar at 37 °C, 24 h and single colonies were picked to inoculate 2 ml BHI broth before incubation at 37 °C for 24 h. This pre-culture was used for inoculation (1%) of each strain in individual tubes of 2 ml BHI broth. After incubation at 37 °C for 24 h, the bacterial cultures were 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 for cold adaptation. Dilutions to working solutions were performed in 0.9% NaCl.

2.2. Antimicrobial compound for L. monocytogenes growth inhibition

The Verdad N6 powder (Verdad N6) was obtained from Corbion (Amsterdam, The Netherlands). It is a white distilled vinegar produced by fermentation. The compound was added in the dry-salting procedure to fresh salmon fillets prior to cold-smoking as described below.

2.3. Production and preparation of cold-smoked salmon

Fresh salmon fillets with skin were packed on ice and received from Marine Harvest ASA (Bergen, Norway) one day after slaughter and filleting. At day two after filleting, the salmon fillets were added NaCl, Verdad N6 and dextrose (only some fillets) and prepared for cold-smoking according to the following procedure: The tail part of fresh salmon fillets were discarded and each of the remaining fillets were divided in two parts and individually weighed. For salmon added Verdad N6, dry salt mixtures of NaCl (3% w/w), Verdad N6 (concentrations range 0.1–2% w/w), and dextrose (1% w/w; in a subset of the fillets) were prepared according to the weight of each salmon fillet. The salmon fillets were dry salted in separate plastic bags to obtain controlled levels of NaCl, Verdad N6 and dextrose and to avoid spill. Control samples were similarly prepared fillets but without Verdad N6. The plastic bags with salmon fillets and salts were sealed under mild vacuum and stored at 4 °C for 64–68 h to obtain controlled and even distribution of salts and sugar in the fillets prior to smoking. After salt distribution, the salmon fillets were unpacked and weight yields after salting were determined. The salmon fillets were cold-smoked in a programmable smoking cabinet (DOLESCHAL, process control unit SC2000; Inject Star Maschinenbau GmbH, Hagenbrunn bei Wien, Austria) using smoke generated from beech chipwood (Räuchergold KL 2/16; J. Rettenmaier & Söhne GmbH, Rosenberg, Germany). The fillets were placed horizontally on stainless steel meshes and exposed to one of two smoking processes; mild or extended smoking. Mild smoking was performed at 25 °C and included an initial drying step of 30 min with air circulation, followed by five cycles of smoking/smoke circulation for a total of approximately 3.5 h. The extended smoking was identical to the mild smoking process, but included three additional cycles of smoking with total time approximately 5.5 h. Smoking was followed by weight yield determination, vacuum packing and storage of each fillet at 0 °C for approximately 64 h to allow time for diffusion of smoke compounds in the salmon fillets prior to the *L. monocytogenes* contamination experiments.

2.4. Contamination of salmon with L. monocytogenes

Smoked salmon fillets were contaminated with *L. monocytogenes* on day three after smoking. Two contamination scenarios, reflecting contamination of either sliced or non-sliced salmon, were tested. For sliced salmon, slices of approximately 5 g were each added 20 µl of the 10 strain *L. monocytogenes* cocktail (5×10^4 cfu/ml) on the surface before non-inoculated 5 g slices of salmon were placed on the *L. monocytogenes* contaminated salmon surface to obtain 10 g samples. For non-sliced salmon, pieces of approximately $3 \times 3 \times 0.5$ cm^3 were cut, and 20 µl of the *L. monocytogenes* cocktail (5×10^5 cfu/g) were spread on the undamaged muscle surface by a sterile plastic spreader. The contaminated salmon samples were put in separate stomacher bags, and thereafter vacuum-packed and stored at 4 °C or 8 °C. Control samples without added *L. monocytogenes* were packed and stored under identical conditions to assess the indigenous background microbiota of the smoked salmon. All experiments with *L. monocytogenes* were performed in a Biosafety level 3 pilot processing plant.

2.5. Bactericidal treatment of salmon with UV-C light

In the UV-C light experiments, samples were treated in a custom-made aluminium chamber ($1.0 \times 0.5 \times 0.6$) m^3 equipped with two UV-C lamps (UV-C Kompaktleuchte, 2×95 W, BÄRO GmbH, Leichlingen, Germany) in the ceiling. The UV-C light emitted was essentially at 253.7 nm, measured using a UVX Radiometer (Ultra-Violet Products Ltd., Cambridge, UK) equipped with a UV-C sensor (model UVX-25, Ultra-Violet Products Ltd., Cambridge, UK). Samples of non-sliced salmon were placed in empty petri dishes at 6 cm distance from the lamps at 10 mW/cm^2, which is close to a maximum when using commercial lamps. An exposure time for 5 s gave a fluence of 0.05 J/cm^2. The parameters of distance and exposure times selected were considered relevant for application in salmon production lines. After UV-C treatment, samples were put in stomacher bags, vacuum-packed and stored at 4 °C or 8 °C.

2.6. Culture dependent and independent microbial analyses

Bacterial counts of *L. monocytogenes* in salmon stored at 4 °C and 8 °C were recorded at day 0, 7, 12, 19 and 29 after contamination (if not otherwise indicated). To each sample in stomacher bags, 40 ml peptone water was added. The samples were stomached for 1 min and appropriate 10-fold dilutions in peptone water were plated on Rapid L'mono agar and incubated at 37 °C for 24 h. Total counts were determined by plating on blood agar plates and aerobic incubation at 15 °C for five days. Microbiota profiling using high-throughput sequencing of bacterial 16S rRNA gene amplicons (MiSeq, Illumina) was performed on selected samples of cold-smoked salmon stored for 29 days. For sample preparation, 12 ml of stomacher solutions were centrifuged at $100 \times g$ for 1 min before 10 ml of the supernatants were centrifuged at $13000 \times g$ for 5 min and the pellets stored at −20 °C. DNA was extracted from thawed pellets using the MoBio PowerLyzer PowerSoil kit according to the manufacturers protocol (Qiagen, Hilden, Germany) and cell lysis in FastPrep-24 homogenizer (MP Biomedicals, Solon, OH) at 6 m/s for 2×40 s. Extracted DNA was eluted in 100 µl Solution C6. PCR was performed in triplicates with amplification of the V4-V5 region of the 16SrRNA gene using region specific primers according to Caporaso et al. (2011, 2012) with redesign performed by Parada et al. (2016) and Apprill et al. (2015). The forward primer was redesigned with a 12-base barcode sequence that supported pooling of different samples (Walters et al., 2016). The triplicate samples were pooled and purified with AMPure XP (Agencourt Bioscience Corporation, Beverly, MA, USA) and quantified by the Quant-iT Picogreen dsDNA Assay (Invitrogen, Life Technologies, Dynal AS, Oslo, Norway). 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 6.3 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 1.18.42. Paired end sequencing was performed using sequencing primers Read1_seq.primer, Read2_seq.primer and Index_seq.primer according to the protocol of Walters et al. (2016). The forward and reverse reads were joined in QIIME (Quantitative Insights Into Microbial Ecology, version 1.9.1), and the barcodes corresponding to the reads that failed to assemble were removed. The sequences were then demultiplexed in QIIME allowing zero barcode errors and a quality score of 30 (Q30) using the QIIME toolkit (Caporaso et al., 2010). 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. The analysis revealed that some of the sequences originated from non-target DNA (Unassigned in the openref analysis) identified as Salmon DNA in BLAST nt search. These non-target DNAs were removed from the data by filtration. The level 6 (genus) table derived from QIIME was used for bar chart illustrations.

2.7. Analyses of physiochemical parameters

Quantitative levels of organic acids in cold-smoked salmon were measured by reversed-phase HPLC. Samples (up to 10 g) were blended and extracted with deionized water and then vortexed followed by sonication. Supernatants were filtered through a 0.45 µm cellulose acetate membrane (Whatman, Merck, Darmstadt, Germany) followed by injection of 10 µl into the HPLC (Shimadzu LC 20 Prominence, SPD-M20A Detector; Shimadzu Europe, Duisburg, Germany). The acids were separated by reversed phase chromatography using a silica-based column TSKgel ODS-100V, particle size 5 µm (Tosoh Bioscience GmbH,

Griesheim, Germany). The mobile phase was 0.1% phosphoric acid with flow rate of 1.0 ml/min at 40 °C. Detection and quantification of the organic acids were obtained by UV–VIS carried out at 210 nm. Levels of NaCl were determined by titration with 0.1 M $AgNO_3$ according to AOAC Method 937.09. The pH of the cold-smoked salmon was measured in the stomacher homogenized solutions using a sensION + pH 31 pH meter (Hach Company, Loveland, CO, USA). Water activity (a_w) of the cold-smoked salmon was measured at 25 °C (AquaLab, series 3TE, Decagon Devices Inc., Washington, USA). At least three replicate samples were used in the analyses. The effect of the salting process on weight yields of the salmon prior to smoking and after smoking were determined by weighing the fillets before salting, after salting and after smoking. The percentage weight of the salmon fillets relative to the raw fillet was calculated.

2.8. Consumer test

Three types of mildly smoked salmon (Verdad N6 1% and 2%, and control) without sugar in the recipe were served in a consumer test. The cold-smoked salmon was stored vacuum-packed for 28 days at 2–4 °C prior to the consumer test. Fifty randomly chosen consumers from Nofima (31 women and 19 men) were first asked" How much do you like the salmon-sample?" using a nine-point hedonic category scale ranging from "I don't like it at all" (1) to "I like it very much" (9). Second and last, the consumers were asked to assess the sensory quality of the salmon using check-all-that-apply, CATA. The consumers were presented with a list of attributes and asked to indicate which words appropriately described their experience with the salmon-sample being evaluated. The parameters used in the CATA were: moderate smoked flavour, sweet, bitter, moderate salt taste, mild, rancid, too much salt taste, too little salt taste, too little smoked flavour, too much smoked flavour, sour, fresh, not fresh, cloying flavour, sharp flavour, doughy texture, faded colour, red colour, firm, yellowish, other. Each consumer was served a slice of smoked salmon from each of the three types in one serving. The slices were served on white plastic plates coded by three-digit numbers. The samples were served in randomized order to each consumer.

2.9. Statistical analyses

Three to five parallels of both treated samples and untreated control samples were produced for each experiment, and the experiments were repeated two to four times on different days. Two-sample *t*-tests with Welch's estimation of degrees of freedom 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. The consumer liking test was modeled in a two-way ANOVA with product and consumers as fixed factors. For the CATA consumer test Cochrans Q test was used in a context for statistical inference of product differences by attribute. The statistical software used in consumer analysis was EyeQuestion and EyeOpenR® (Logic8 BV, Utrecht, The Netherlands).

3. Results

3.1. Inhibitory activity of Verdad N6 on L. monocytogenes in cold-smoked, sliced salmon

Addition of Verdad N6 in the salting process in cold-smoked, sliced, vacuum-packed salmon resulted in reduced growth rates of contaminating *L. monocytogenes* (Fig. 1A, B). The inhibition was dependent on the Verdad N6 concentration, storage time and temperature. At recommended maximum storage temperature (4 °C), the addition of 2% Verdad N6 provided complete inhibition of *L. monocytogenes* during the tested 29 days storage period. Verdad N6 at all levels (0.15–2.0%) increased lag times for *L. monocytogenes* with no growth observed during the first seven days of storage. During the 29 days of storage, a > 3 log increase in *L. monocytogenes* was observed in control samples without Verdad N6 at 4 °C. For storage at abuse temperature (8 °C), *L. monocytogenes* levels increased < 2 log in salmon with 2% Verdad compared with 5–6 log in control samples. Significantly reduced levels of *L. monocytogenes* after 29 days storage (4 or 8 °C) were only obtained using levels at or above 0.5% Verdad N6. The growth inhibiting effects of Verdad N6 were apparently due both to an increased lag phase and to a reduced growth rate once growth commenced. No listericidal effects were observed in these inhibition experiments.

3.2. Influence of recipe and process parameters on Verdad N6 inhibition of L. monocytogenes in cold-smoked, sliced salmon

Differences in cold-smoke salmon processing and recipes may affect *L. monocytogenes* growth in the products. The influence of the degree of smoking and the addition of 1% dextrose in the recipe on the Verdad N6 (at 1% and 2%) inhibiting effect of *L. monocytogenes* was investigated in sliced salmon (Fig. 1C–F). Complete *L. monocytogenes* growth inhibition on sliced salmon could be obtained at both 1 and 2% Verdad N6 when combined with extended smoking for salmon stored at 4 °C. At 8 °C storage, only limited growth of 2.3 log and 0.8 log was observed after 29 days storage for sliced salmon containing 1% and 2% Verdad N6, respectively. The degree of smoking had no significant effects on growth rate of *L. monocytogenes* on sliced control salmon not containing Verdad N6 (Fig. 1A–D). When testing for synergy effects between smoking and added Verdad N6 on *L. monocytogenes* growth, a p-value of 0.052 was obtained for salmon with 1% Verdad and stored at 8 °C (Fig. 1D).

Sugar is commonly used by certain processors as an ingredient in cold-smoked salmon. The inhibitory effects of Verdad N6 at 1% and 2% were not influenced by addition of 1% dextrose during the salmon production compared with corresponding salmon without dextrose when tested after 29 days storage at either 4 (Fig. 1A, E) or 8 °C (Fig. 1B, F). In salmon without Verdad, 1% dextrose provided lower *L. monocytogenes* levels at both 4 °C ($p = 0.02$) and 8 °C ($p = 0.03$) storage for 29 days compared to salmon without dextrose.

3.3. Influence of recipe and process parameters on Verdad N6 inhibition of L. monocytogenes on cold-smoked, non-sliced salmon

Cold-smoked salmon is often stored and sold non-sliced. The muscle surface of cold-smoked salmon is likely to exhibit other conditions for *L. monocytogenes* growth and survival than between the slices of sliced products. The growth of *L. monocytogenes* on the fillet surface of non-sliced cold-smoked salmon produced without added sugar was determined (Fig. 2A–D). *L. monocytogenes* growth was completely prevented on mildly smoked salmon produced with 1% Verdad N6 and stored at 4 °C (Fig. 2A) or 8 °C for 29 days (Fig. 2B). Significant growth of *L. monocytogenes* was observed on the surface of non-sliced, mildly smoked salmon fillet controls without Verdad N6, with levels increasing 2.3 log and 4.6 log after 29 days storage at 4 °C and 8 °C, respectively. By extended smoking, complete growth inhibition was obtained for salmon stored at 4 °C, independent of the presence of 1% Verdad N6 or not (Fig. 2C; $p = 0.095$). At 8 °C storage for 29 days, *L. monocytogenes* levels where lower (0.8 log; $p = 0.047$) in salmon with 1% Verdad N6 than in salmon without (Fig. 2D).

3.4. Combinations of Verdad N6 and UV-C treatments for L. monocytogenes control on non-sliced cold-smoked salmon

Optimal *Listeria* control in cold-smoked salmon would include both killing and growth inhibitory strategies. We therefore determined the

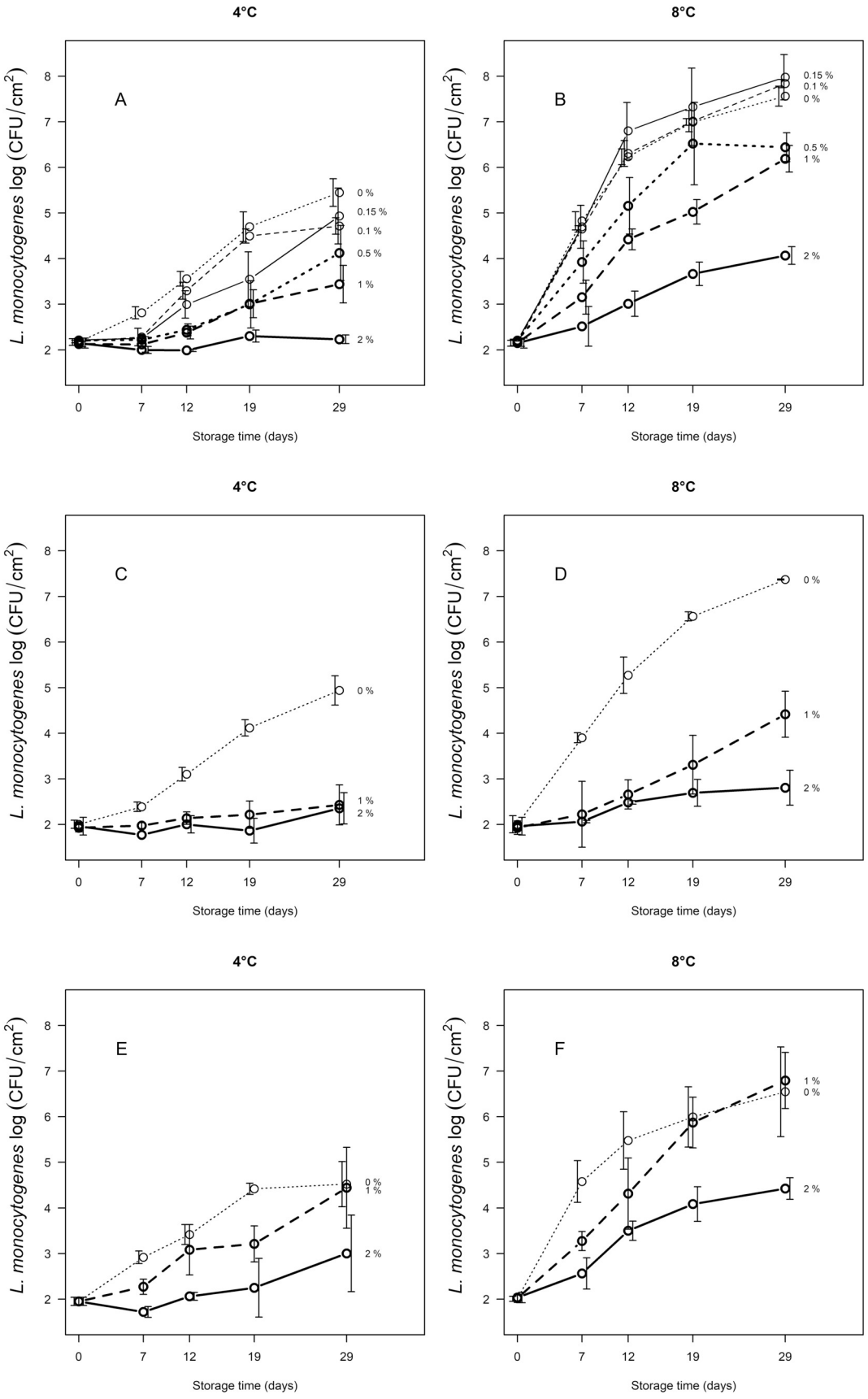

Fig. 1. Inhibition by Verdad N6 on *L. monocytogenes* growth in sliced, vacuum-packed cold-smoked salmon during storage for 29 days at 4 °C (A, C, E) and 8 °C (B, D, F). Levels of added Verdad N6 (0–2%) in the salmon are indicated. Effects on mildly smoked salmon without dextrose (A, B), extensively smoked salmon without dextrose (C, D) and on mildly smoked salmon added 1% dextrose in the recipe (E, F) are shown. Mean values of two to four experiments and standard error of the mean are shown.

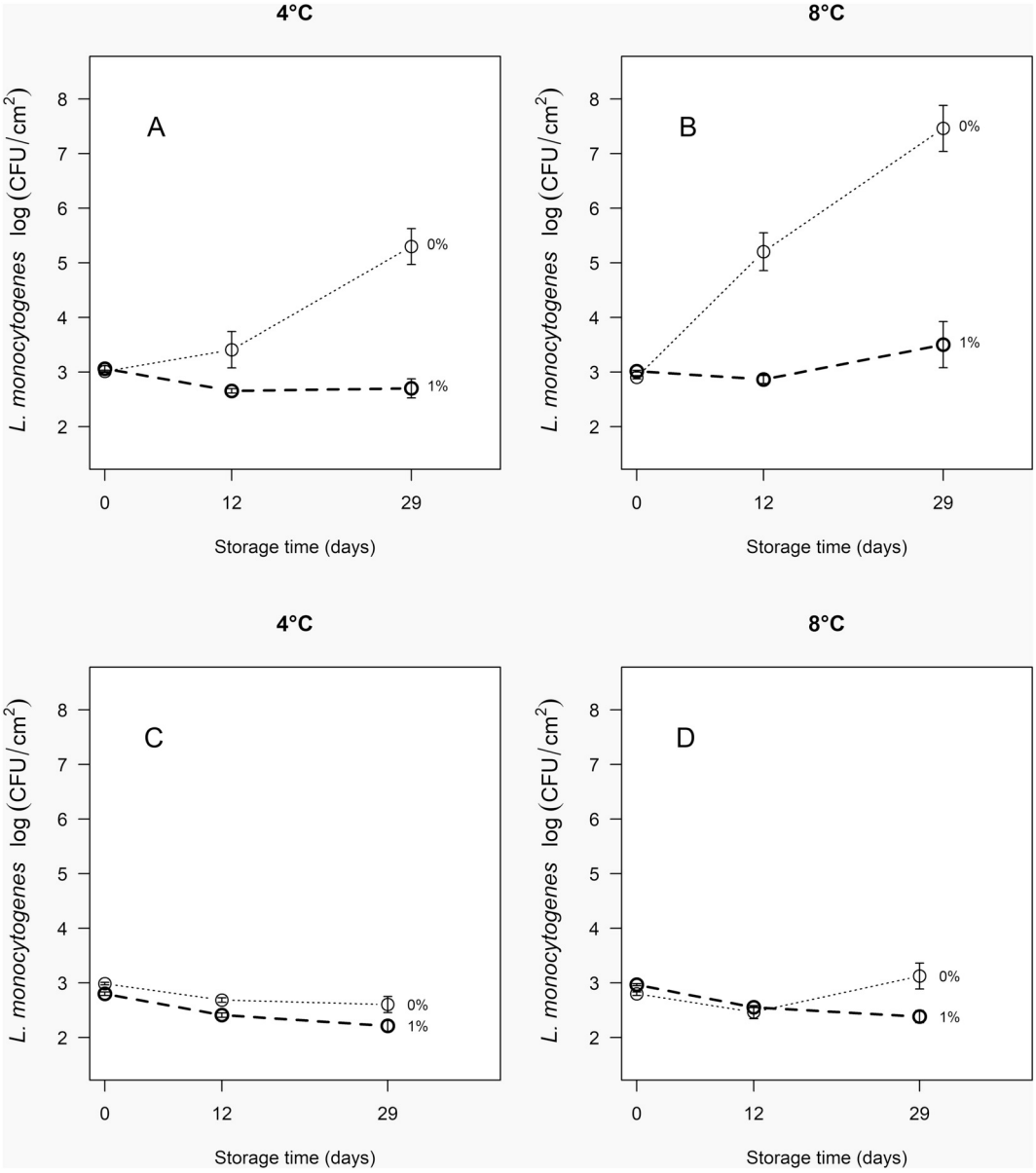

Fig. 2. Inhibition by Verdad N6 on *L. monocytogenes* growth on non-sliced, cold-smoked, vacuum-packed salmon during storage for 29 days at 4 °C (A, C) and 8 °C (B, D). The levels of added Verdad N6 (0, 1%) in the salmon are indicated. Prior to vacuum-packing and storage, the salmon were smoked using mild smoking process (A, B), or extended smoking process (C, D). Mean values of two experiments and standard error of the mean are shown.

effect of a bactericidal treatment using UV light on *L. monocytogenes* contaminated cold-smoked salmon produced with Verdad N6. Exposure of mildly smoked, non-sliced spiked salmon to UV-C light (50 mJ/cm^2) resulted in an immediate 0.8 log reduction in *L. monocytogenes* on the fillets irrespective of the presence of Verdad N6 or not. This reduction was followed by complete inhibition of *L. monocytogenes* growth at both storage temperatures in salmon with 1% Verdad N6 (Fig. 3A, B). In corresponding UV-C treated control salmon without Verdad N6, *L. monocytogenes* counts reached 4.0 log and 6.7 log at Day 29 at 4 and 8 °C, respectively. These values were still about 1 log lower than for comparable non-UV-C treated salmon (Fig. 2A, B), implying similar growth rates of *L. monocytogenes* on UV-C treated and non-UV-C treated samples.

3.5. Effect of Verdad N6 on the indigenous microbiota of cold-smoked salmon

Samples of sliced, mildly smoked salmon manufactured without dextrose were examined for growth of total indigenous microbiota during storage at 4 °C (Fig. 4). Cold-smoked salmon with 2% Verdad N6 showed significantly reduced levels in total counts during the 29 days storage at 4 °C compared with salmon with no or lower levels of Verdad N6. Lower levels of Verdad N6 gave variable results on inhibition of the background microbiota (not shown). Extended smoking without presence of Verdad N6 had minor effects on total counts in sliced salmon (not shown). Some samples stored for 29 days were subjected to culture independent microbiota analyses. Analyses indicated that Verdad N6 contributed to a shift in dominating bacteria on the salmon during storage (Supplementary Fig. S1). Salmon without Verdad N6 showed an overall dominance of *Photobacterium* spp. while increasing relative levels of *Carnobacterium* spp. and other lactic acid bacteria were obtained in salmon with 1% and 2% Verdad N6.

3.6. Physical and chemical quality parameters

Analyses of organic acid content of salmon with and without Verdad

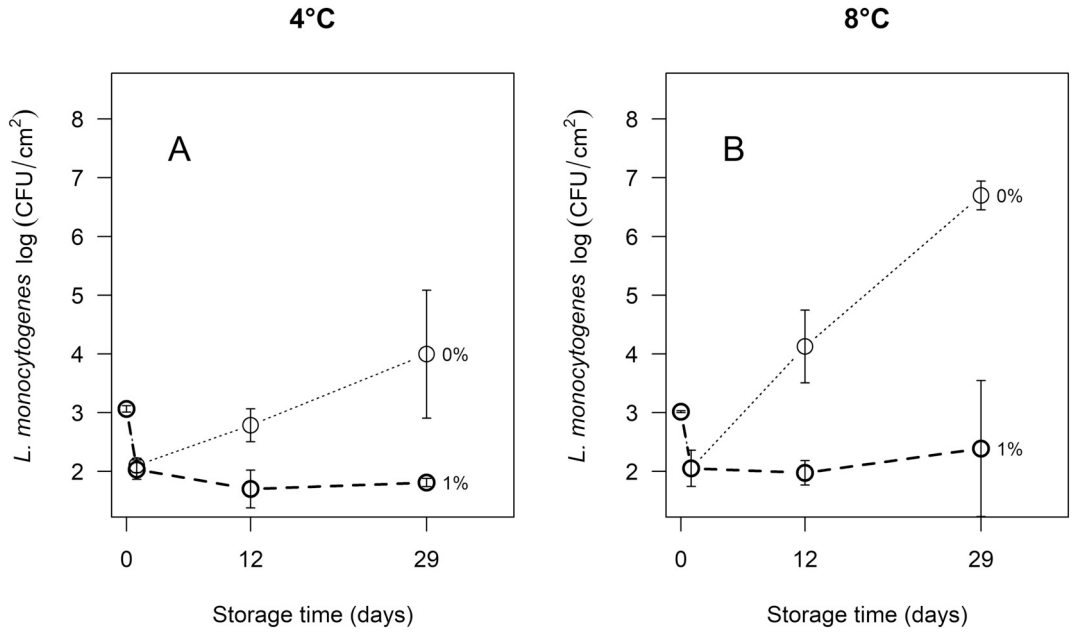

Fig. 3. Reduction and subsequent growth of *L. monocytogenes* on UV-C treated (0.050 J/cm^2), non-sliced, mildly smoked, cold-smoked, vacuum-packed salmon with 0% or 1% Verdad N6 during storage for 29 days storage at 4 °C (A) and 8 °C (B). Mean values and standard error of the mean of three to five parallels are shown.

N6 showed that a main component of Verdad N6 was acetate. Approximate 20-fold higher acetate levels were evident in salmon added 2% Verdad N6 than in control salmon (Table 2). Levels of lactate were not significantly different in salmon with and without Verdad N6. The pH of cold-smoked salmon with and without Verdad N6 showed insignificant differences, and the pH remained essentially unchanged at pH 6.0 during the 29 days storage at 4 °C (Supplementary Table S1). Levels of NaCl were in the range 2.60–3.13%. Higher drip/water loss was observed with increasing levels of added Verdad N6 (Table 2). The yields of salmon with Verdad N6 were 1.7 to 3.2% lower than for control salmon after salting, but with yield differences reduced to 1.3 to 2.3% after extended smoking for salmon with 1% and 2% Verdad N6, respectively. Despite this, only minor and non-significant difference in the water activities (range 0.965–0.981) were observed.

3.7. Consumer test on cold-smoked salmon with Verdad N6

The eating quality of mildly smoked, sliced cold-smoked salmon manufactured without dextrose in the production process, containing 0 (control), 1 and 2% Verdad N6 was assessed in a consumer test with 50 respondents. The salmon was stored vacuum-packed at 2–4 °C for 28 days in the dark prior to the consumer test. The respondents were served one slice of each salmon type and were first asked, "How much do you like the salmon-sample?" Averaged answers were 6.24 (control), 6.08 (1% Verdad N6) and 5.70 (2% Verdad N6) on a scale ranging from 1 to 9 corresponding to "I don't like it at all" to "I like it very much", respectively, and with no statistically significant differences between the salmon types obtained ($p = 0.273$). The respondents were also asked to describe the samples according to 21 predefined attributes in a CATA test. Only two attributes, faded colour and red colour, were statistically different between salmon with Verdad N6 and the control (Fig. 5). Salmon with 1 and 2% Verdad N6 was considered less pale and with higher intensity of red colour compared with control. In conclusion, no consistent differences in consumer liking in cold-smoked salmon with and without Verdad N6 were evident.

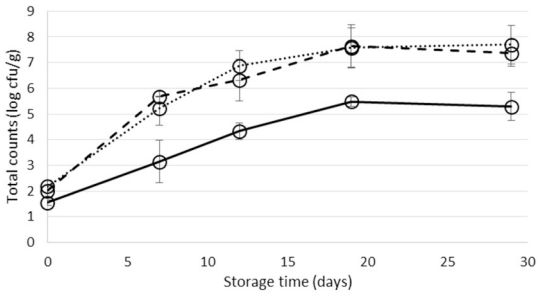

Fig. 4. Influence of three levels of Verdad N6 (0% dotted line; 1% hatched line; 2% continuous line) on total counts on sliced, mildly cold-smoked, vacuum-packed salmon during storage for 29 days at 4 °C. Mean values and standard error of the mean of three replicates are shown.

4. Discussion

L. monocytogenes levels and prevalence in cold-smoked salmon exceed those of other RTE risk products of meat and cheese (EFSA, 2013). Despite high prevalence, application of specific intervention strategies to control *L. monocytogenes* on cold-smoked salmon are not common. This study reports that Verdad N6, a label-friendly ingredient option, can substantially retard the growth of *L. monocytogenes* and provide significant risk reduction even when cold-smoked salmon are stored at abuse temperatures. The present study aimed to evaluate the suitability of this intervention used alone or in combination with bactericidal UV-C treatment in cold-smoked salmon products and production processes. Cold-smoked salmon is sold sliced or non-sliced with higher probabilities of *L. monocytogenes* contamination reported for the former category (Ricci et al., 2018). These products can provide different microenvironments for bacterial survival and growth. Both product categories were therefore included. The present study also highlights the enhanced food safety risks of keeping cold-smoked salmon above recommended storage temperatures.

The acetate-rich Verdad N6 fermentate provided a dose-dependent growth inhibition of *L. monocytogenes* with significant growth reductions obtained in cold-smoked products. The inhibitory effect is caused by undissociated acetic acid being able to penetrate the bacterial membrane and acidify the interior of the pathogen. The effects of various organic acid salts and especially sodium and potassium salts of lactate and diacetate on growth inhibition of *L. monocytogenes* have been documented in cold-smoked salmon (Neetoo et al., 2008; Tang

Table 2
Levels of acetate, lactate and NaCl in cold-smoked salmon and calculated yields of cold-smoked salmon without (control) and with added Verdad N6.

Treatment (% Verdad N6 added)	Acetate (%)[a]	Lactate (%)[a]	NaCl (%)[a]	Yield (%)[b]		
				After salting	After mild smoking	After extended smoking
Control (0)	0.051 ± 0.010	0.575 ± 0.090	2.60 ± 0.17	98.6 ± 0.5	95.0 ± 1.2	91.6 ± 1.2
Verdad (1)	0.440 ± 0.077	0.648 ± 0.044	3.02 ± 0.40	96.9 ± 0.3	93.4 ± 0.9	90.3 ± 0.9
Verdad (2)	1.059 ± 0.138	0.615 ± 0.059	3.13 ± 0.47	95.4 ± 0.6	92.1 ± 0. 8	89.3 ± 1.6

[a] All percentages are (w/w). Mean values of four to six parallels from two replicate experiments and standard deviations between the samples are shown.
[b] The yield was calculated at two processing steps; after salting and after smoking (mild and extended). The yields were determined as the weight of the salted or smoked salmon in percentage of the weight of the raw salmon fillet prior to salting or smoking. Mean values of at least four parallel samples from each of two to four replicate experiments and standard deviations between the samples are shown.

et al., 2013; Vogel et al., 2006; Yoon et al., 2004). Acetates have been applied in combination with lactate in most studies. Kin et al. reported listeriostatic effect of brines with potassium acetate and potassium lactate in smoked catfish and with no negative effect on the quality and sensory parameters (Kin et al., 2012). Vogel et al. showed lower inhibitory effects of lactate/acetate than lactate/diacetate combinations on injected cold-smoked salmon (Vogel et al., 2006). A study including naturally contaminated cold-smoked salmon indicated the addition of acetic and/or lactic acid through injection to be an appropriate mitigation strategy to prevent high and potentially critical levels of *L. monocytogenes* in final products (Mejlholm et al., 2015). Introductory to the current study, comparisons of blends of lactate and acetate, added by injection to the salmon, and Verdad N6 distributed by dry salting of the salmon indicated the latter to provide more effective *L. monocytogenes* growth inhibition in the produced cold-smoked salmon (data not shown). Based on these results as well as its "label friendly" nature, Verdad N6 was selected for further studies. Due to differences in experimental conditions, direct comparisons between studies are difficult, but the ability to increase the lag phase, reduce the growth rate and provide reduced levels of *L. monocytogenes* during storage seem to be comparable between dry-salted Verdad N6 (this study) and lactate/diacetate surface treated cold-smoked salmon.

The antimicrobial effects of the smoking process are due to smoke components including phenols, organic acids, aldehydes and alcohols, and by drying of the salmon surface (Doe, 1998; Porsby et al., 2008; Sunen et al., 2001; Sunen et al., 2003). Levels of salt and type of salting process (brine injection vs. dry-salting) also affect the sensitivity of *L. monocytogenes* to cold smoking (Montero et al., 2007) with the higher reduction in fish subjected to dry-salting (Niedziela et al., 1998; Porsby et al., 2008). *L. monocytogenes* strains may differ in susceptibility to phenols and other antimicrobial smoking compounds (Cornu et al., 2006; Thurette et al., 1998). We applied a mix of 10 *L. monocytogenes* strains of which six strains where salmon industry isolates with different sequence type (ST) to obtain results representative for *L. monocytogenes* diversity. No effect of the smoking process on *L. monocytogenes* inhibition was apparent on sliced control salmon. However, in sliced salmon with Verdad N6, extended smoking provided more effective growth inhibition than mildly smoked salmon. In non-sliced cold-smoked salmon the growth inhibiting effects of extended smoking were further enhanced. Complete inhibition of *L. monocytogenes* even

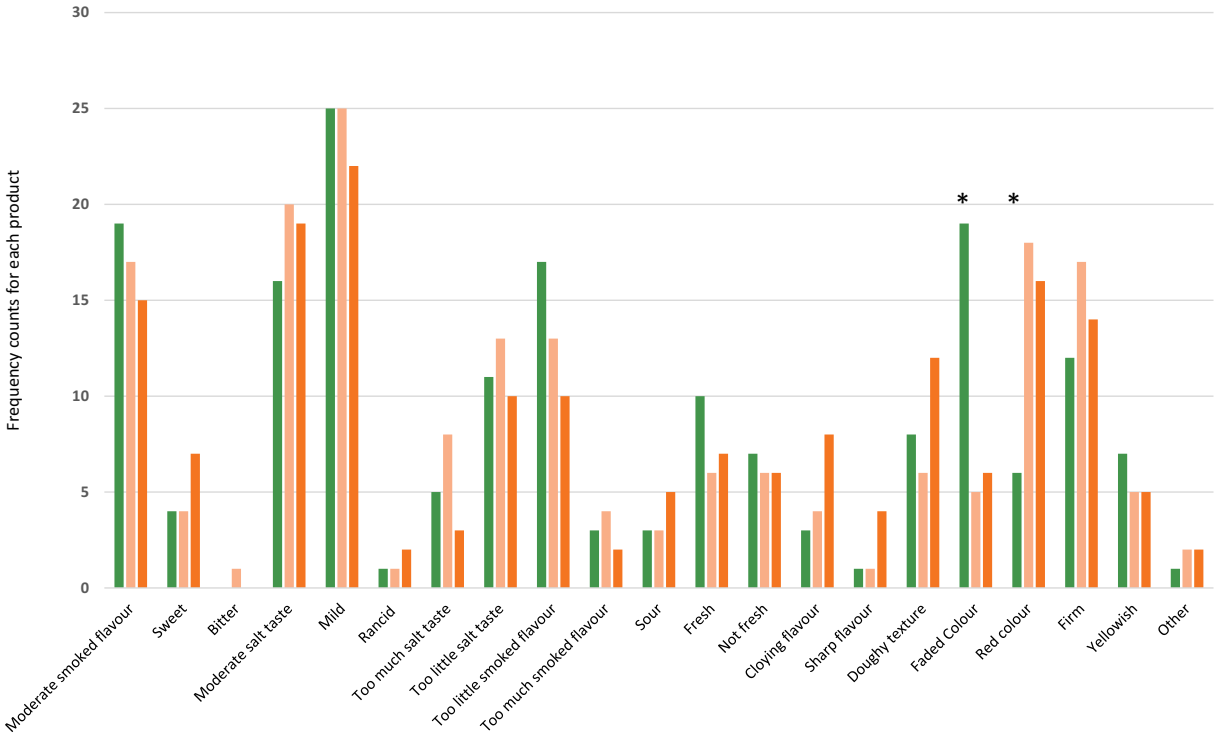

Fig. 5. "Check-All-That-Apply" (CATA) consumer test with 50 respondents on cold-smoked salmon without (green) and with 1% Verdad N6 (light brown) and 2% Verdad N6 (dark brown). The number of times respondents described the products in the consumer test according to the pre-defined characteristics (x-axis). Characteristics with significant differences ($p < 0.05$) between control salmon and salmon with Verdad 1% and 2% according to the consumer responses are indicated by *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

without added Verdad N6 was obtained compared to an approximate 2 log *L. monocytogenes* increase in the mildly smoked control salmon. The increased anti-listerial effect of extended smoking on non-sliced products compared to sliced products is likely due to higher levels of anti-listerial smoking compounds on the surface of the former and that the extended smoking process generates a drier surface that further limits *L. monocytogenes* growth. Since the smoking procedure affects *L. monocytogenes* growth, it has been suggested as a possible critical control point. The anti-listerial effect of Verdad N6 in combination with smoking should be validated in each case as different smoking processes generate different types and levels of antimicrobial compounds (Cornu et al., 2006; Porsby et al., 2008). However, smoking alone seems not to be a trusted strategy to inhibit *L. monocytogenes* growth during storage, especially in products contaminated during slicing (Tocmo et al., 2014).

Predictive models for *L. monocytogenes* growth in foods can be valuable for risk assessment and risk management. Additionally, they can be of practical importance to facilitate optimization of recipe and processing combinations in compliance with e.g. EU regulations on *L. monocytogenes* in RTE foods. A requirement is that the models provide sufficiently accurate predictions so that the models can be used with confidence. In the present study, results on *L. monocytogenes* growth in cold-smoked salmon were compared with predictions obtained from the Food Safety and Spoilage Predictor (FSSP), a software originally developed for processed and RTE seafood (http://fssp.food.dtu.dk/). The FSSP underestimated the *L. monocytogenes* levels at the end of storage (Day 29, 4 °C) for sliced salmon with approximately 1–2.5 log higher levels obtained in the storage trials (mean values) for salmon without and with 1% Verdad N6, respectively, compared to the FSSP estimates. Corresponding data on unsliced cold-smoked salmon showed good correlations between the FSSP predictions and the storage trial data. Similar differences between predicted and storage trial data were evident at 8 °C storage. Processing and environmental factors that are not congruent between conditions supported by the model and conditions applied in the storage trials in this study may explain the observed underestimation *L. monocytogenes* by the FSSP model. Relevant here is e.g. the artificial contamination of salmon with *L. monocytogenes* and no freezing of the smoked salmon prior to storage (this study) vs. naturally contaminated salmon and freezing of the salmon prior to storage. This could provide reduced lag times and higher *L. monocytogenes* levels in the former case.

Dextrose is often added by producers to obtain a mild rounded flavour. We hypothesized that the addition of dextrose during production of cold-smoked salmon could potentially stimulate the growth of *L. monocytogenes* and reduce the inhibitory effect of Verdad N6. However, we observed no significant differences in *L. monocytogenes* counts in salmon with or without added dextrose (1%) after 29 days storage at 4 or 8 °C. Likewise, the *L. monocytogenes* counts showed no significant differences in salmon with 2% Verdad N6, irrespective of the presence of added dextrose or not. In samples with 1% Verdad N6, more variable results were obtained. The overall results showed the potent inhibitory effect of Verdad N6 also in cold-smoked salmon with added dextrose. When using low-levels of Verdad N6, small differences in recipe, process, background microbiota and product parameters can apparently influence *L. monocytogenes* growth and be a possible explanation for the variations observed. A previous study (Peterson et al., 1993) reported no effects of added brown sugar on *L. monocytogenes* growth in brined salmon when no smoking was performed.

Optimal control of *L. monocytogenes* in cold-smoked salmon would include strategies encompassing both bactericidal and bacteriostatic effects. The bactericidal effects of a number of chemical and biological compounds (e.g. lauryl arginate, ozone, bacteriocins, chitosan, epsilon poly lysine), and technologies (e.g. high hydrostatic processing, irradiation) for *L. monocytogenes* elimination in RTE products of meat, dairy, fresh produce and fish origin have been tested (Kang et al., 2012; Kang et al., 2014; Tang et al., 2013; Tocmo et al., 2014). The benefits of many listericidal treatments in cold-smoke salmon processing appear limited due to e.g. negligible listericidal effects (e.g. lauryl arginate, chitosan, and epsilon polylysine (Kang et al., 2014); ozone (Vaz-Velho et al., 2006)) or undesirable changes in product quality (e.g. HHP (de Oliveira et al., 2017; Gudbjornsdottir et al., 2010; Montiel et al., 2014)). In this context, UV-C light for bactericidal treatments of foods have gained increasing interests. UV-C treatments provided immediate reductions (0.8 log) of *L. monocytogenes* with no subsequent growth in non-sliced salmon with 1% Verdad N6. Thus, the combined killing by UV-C light and growth inhibiting effects of Verdad N6 offers a strategy that can provide *L. monocytogenes* reduction in contaminated products and restrict any growth of possible survivors in the products during storage. The listericidal effect of UV-C requires that the *Listeria* in the contaminated product are exposed to the UV-C light and are not shielded in e. g. crevices in the products protecting the bacteria form UV-light exposure, since the UV light does not penetrate well into organic matter. The limited reductions obtained by UV-C treatments may question the cost-benefit of such treatments. High *L. monocytogenes* prevalence have been reported (17.4–22%) in retail cold-smoked salmon at end of shelf life, but with only a minor part (2.0–4.4%) exceeding the EU legal limit of 100 cfu/g (Acciari et al., 2017; EFSA, 2013). Moreover, > 90% of listeriosis cases are caused by ingestion of foods with > 2000 cfu/g (Ricci et al., 2018). This shows the potentially large risk-reducing benefit of using listericidal technologies, although with limited effects, combined with treatments that inhibit *L. monocytogenes* growth.

Increasing concentrations of Verdad N6 (up to 2%) were associated with lower total counts, lower relative levels of *Photobacterium* spp., and increased relative levels of *Carnobacterium* spp. and other lactic acid bacteria at the end of storage (Day 29). *Photobacterium* spp. are known as potential spoilage organisms of fresh salmon and are likely to have a similar role in cold-smoked salmon (Gimenez and Dalgaard, 2004; Stohr et al., 2001). The dominance of *Photobacterium* and lactic acid bacteria in vacuum-packed cold-smoked salmon is in part in agreement with previous reports (Gram and Dalgaard, 2002; Hansen et al., 1998; Leroi et al., 1998; Olofsson et al., 2007). As there is no obvious correlation between bacterial levels or species present and shelf-life of cold-smoked salmon (Hansen et al., 1995; Hansen et al., 1998), further studies are needed to conclude whether the antimicrobial effects of Verdad N6 could extend the overall quality and shelf-life of these products.

A key economic factor for the cold-smoked salmon industry is product yield. During dry-salting, osmosis reduced fillet weight. The yield after salting is dependent on the salting process and lipid content of the fillet. We observed a 1.7–3.2% higher weight loss in fillets added Verdad N6 in the dry-salting process compared with control fillets, but obtained weight losses were still comparable to recent studies (Lerfall et al., 2016; Lerfall and Rotabakk, 2016). The yield differences between salmon with and without Verdad N6 were reduced by more extensive smoking. Our yields of 92–95% and 89–92% for mild and extended smoking, respectively, compare well with yields of dry-salted salmon after smoking, which are reported in the range 82–91% (Birkeland and Bjerkeng, 2005; Cardinal et al., 2001; Rora et al., 2004; Sigurgisladottir et al., 2000). Adaptations of the drying/smoking process can apparently partly counteract the yield loss experienced during dry-salting with Verdad N6.

The consumer test did not identify differences in preference for mildly smoked salmon with or without Verdad N6, despite the 10–20 fold higher levels of acetate in salmon with Verdad N6. In previous studies, although not directly comparable, no adverse sensory effects were attained on smoked fish containing acetate (Kin et al., 2012; Vogel et al., 2006). However, one study on sliced fresh salmon reported reduced oxidation of lipids, but also reduced sensory score on fish containing sodium acetate (Sallam, 2007). In the CATA consumer test only two of 21 predefined attributes showed different scores between the salmon with Verdad N6 and the control. Interestingly, salmon fillet with Verdad N6 were regarded more red and less pale than the control

salmon. The colour of the salmon fillet during smoking is generated through a series of chemical reactions such as protein and lipid oxidation and Maillard reactions (Martins et al., 2000; Zamora and Hidalgo, 2005). The salting process also affects colour development in cold-smoked products (Birkeland and Bjerkeng, 2005). Our data indicated that Verdad N6 containing compounds could affect colour development. The higher red and lower pale score of Verdad N6 containing cold-smoked salmon are important quality parameters that adds value to the use of Verdad N6 as an ingredient.

In conclusion, the commercially available fermentate Verdad N6, with acetate as a main ingredient, provided effective growth inhibition of *L. monocytogenes* when applied as an ingredient in the dry-salting process of cold-smoked salmon. Robust *L. monocytogenes* growth inhibition on cold-smoked salmon was obtained under variable, but relevant conditions for industrial processing and consumer storage, even at abuse temperatures. Verdad N6 and key process parameters can be combined to produce high quality and microbiologically safe cold-smoked salmon with negligible differences in consumer preference and lower levels of potential spoilage organisms. The application of Verdad N6 in combination with UV-C treatments of the smoked salmon offers the advantage of both a *Listeria* growth inhibiting ingredient and a killing step in the production process resulting in a higher safety level. This approach could be an attractive choice with potentially significant positive effects for the whole industry and for individual processors, which are responsible for providing safe products to the consumers.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.10.026.

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Conflict of interest

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

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