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Spoilage evaluation of raw Atlantic salmon (*Salmo salar*) stored under modified atmospheres by multivariate statistics and augmented ordinal regression

L. Kuuliala[a,b,*], M. Sader[b], A. Solimeo[a], R. Pérez-Fernández[b], M. Vanderroost[a], B. De Baets[b], B. De Meulenaer[c], P. Ragaert[a], F. Devlieghere[a]

[a] *Research Unit Food Microbiology and Food Preservation (FMFP), Department of Food Technology, Safety and Health, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium*
[b] *Research Unit Knowledge-based Systems (KERMIT), Department of Data Analysis and Mathematical Modelling, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium*
[c] *Research Unit Food Chemistry and Human Nutrition (nutriFOODchem), Department of Food Technology, Safety and Health, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium*

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ABSTRACT

The development of quality monitoring systems for perishable food products like seafood requires extensive data collection under specified packaging and storage conditions, followed by advanced data analysis and interpretation. Even though the benefits of using volatile organic compounds as food quality indices have been recognized, few studies have focused on real-time quantification of the seafood volatilome and subsequent systematic identification of the most important spoilage indicators. In this study, spoilage of Atlantic salmon (*Salmo salar*) stored under modified atmospheres (% $CO_2/O_2/N_2$) and air was characterized by performing multivariate statistical analysis and augmented ordinal regression modelling for data collected by microbiological, chemical and sensory analyses. Out of 25 compounds quantified by selected-ion flow-tube mass spectrometry, ethanol, dimethyl sulfide and hydrogen sulfide were found characteristic under anaerobic conditions (0/0/100 and 60/0/40), whereas spoilage under air was primarily associated with the production of alcohols and ketones. Under high-O_2 MAP (60/40/0), only 3-methylbutanal fulfilled the identification criteria. Overall, this manuscript presents a systematic and widely applicable methodology for the identification of most potential seafood spoilage indicators within the context of intelligent packaging technology development. In particular, parallel application of statistics and modelling was found highly beneficial for the performance of the quality characterization process and for the practical applicability of the obtained results in food quality monitoring.

1. Introduction

The seafood industry is continuously confronted with challenges caused by the increasing complexity of the modern society. The growing interest in food quality and safety, sustainability and convenience is accompanied with the globalization of supply chains and demands towards minimal processing (Mikš-Krajnik et al., 2016). These challenges call for the development of innovative packaging technologies that aim at monitoring and/or improving the quality of food products (Vanderroost et al., 2014). Intelligent packaging technologies can be used for sensing, monitoring and communicating internal and external changes in the product or its environment (Kerry et al., 2006; Restuccia et al., 2010; Yam et al., 2005). Consequently, the importance of these technologies in the food sector has been foreseen to continuously increase in the near future (Fang et al., 2017; Ghaani et al., 2016; Poyatos-Racionero et al., 2018; Realini and Marcos, 2014).

The development of intelligent packaging technologies is particularly beneficial for valuable and highly perishable food products that are commonly packed under modified atmospheres (MAs), such as salmon fillet portions. Spoilage of these products typically manifests itself in the accumulation of volatile organic compounds (VOCs) in the package headspace, eventually leading to the generation of unpleasant

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[*] Corresponding author at: Research Unit Food Microbiology and Food Preservation (FMFP), Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium.
E-mail address: Lotta.Kuuliala@UGent.be (L. Kuuliala).

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off-odors and consumer rejection. Salmon is associated with high consumer demand, production volumes and commercial value and is commonly recognized as one of the most valuable fishery products (Churchill et al., 2016; Møretrø et al., 2016). Even though several studies have discussed the composition and/or growth of salmon microbiota under different conditions (Emborg et al., 2002; He et al., 2014; Macé et al., 2012; Mikš-Krajnik et al., 2017; Milne and Powell, 2014; Møretrø et al., 2016; Powell et al., 2015; Saraiva et al., 2017; Wu and Sun, 2013), studies focusing on the volatilome and its relation to microbiological and/or sensory quality are still limited in number and primarily restricted to aerobic conditions (Mikš-Krajnik et al., 2016; Zaragozá et al., 2014) or inoculated samples (Fall et al., 2012; Jaffrès et al., 2011; Macé et al., 2013).

Monitoring of spoilage metabolites is considered as a promising approach in intelligent packaging and has already led to the development of novel packaging solutions (Fang et al., 2017; Ghaani et al., 2016; Poyatos-Racionero et al., 2018). However, intelligent packaging technology development calls for a comprehensive understanding of spoilage processes under well-defined storage conditions. This requires not only extensive data collection by microbiological, chemical and sensory analyses, but also advanced statistics and modelling. Even though methods such as principal component analysis (PCA) and/or partial least squares regression analysis (PLS) have frequently been applied in food quality analysis (Ioannidis et al., 2018; Kuuliala et al., 2018a; Mataragas et al., 2007; Mikš-Krajnik et al., 2016; Parisi et al., 2002), seafood spoilage modelling has traditionally focused on microbial growth, typically in samples inoculated with certain microbial species. Many previously developed models have been incorporated in freely available software such as the Seafood Spoilage and Safety Predictor (SSSP) (Dalgaard et al., 2002), Fish Shelf Life Prediction Program (FSLP) (Nuin et al., 2008) and Fishmap (Alfaro et al., 2013). In contrast, models based on chemical spoilage of seafood are still scarcely available in the scientific literature.

The aim of the present study was 1) to evaluate the spoilage of Atlantic salmon (*Salmo salar*) packed under different MAs or air by multivariate statistical analysis and mathematical modelling and, consecutively, 2) to develop a systematic methodology for the identification of VOCs that could be used as seafood spoilage indicators in intelligent packaging solutions. Salmon spoilage was examined by microbiological, chemical and sensory spoilage analyses throughout storage time in order to build a multidisciplinary dataset. VOCs were quantified by selected-ion flow-tube mass spectrometry (SIFT-MS) and spoilage-indicating compounds were identified on the basis of PLS and data quality characterization. Finally, the relation between VOC concentrations and sensory quality was examined by developing an augmented ordinal regression model.

2. Materials and methods

2.1. Data collection

2.1.1. Packaging and storage

Norwegian farmed ASC certified Atlantic salmon (gutted weight ca. 4.5 kg) was gutted, filleted pre-rigor and skinned at a commercial seafood processing plant, transported to Belgium by air and delivered in polystyrene boxes under ice within four days after slaughter. Four individual batches were delivered at separate time points between February and June 2017, each weighing 9–12 kg and containing fillets (á 1–1.5 kg, two fillets per fish) from at least three individual fish. Immediately after the delivery, the fillets were manually mixed for 1 min in order to achieve a uniform surface contamination, cut into portions (203 ± 2 g) and packed under gaseous atmospheres (% $CO_2/O_2/N_2$) 0/0/100 (A), air (B), 60/0/40 (C) or 60/40/0 (D) with a gas-product ratio 2:1, using a tray sealer MECA 900 (DecaTechnic, Herentals, Belgium), multilayer trays (PP/EVOH/PP, O_2 transmission rate 0.001 cm^3/tray*24 h at 23 °C and 50% R.H.) and top film (PA/EVOH/PA/PP, O_2 transmission rate 6.57 cm^3/m^2*24h*atm at 23 °C, 50% R.H. and 1 atm). Two atmospheres were tested per batch (batch 1: A + B; batch 2: C + D; batch 3: A + B, batch 4: C + D), meaning that each atmosphere was tested twice using different initial batches. After packing, the samples were stored at (4.0 ± 0.7) °C for up to 11–15 days.

Three randomly selected packages per atmosphere were analyzed every second day of the storage experiment, starting from day 1 (day after packaging). After headspace gas analysis, SIFT-MS, microbiological sampling and physicochemical measurements (Sections 2.1.2–2.1.4), remaining fillet portions were packed under vacuum in high barrier film bags (PA/EVOH/PA/PE, O_2 transmission rate < 2.7 cm^3/m^2*24h*bar at 23 °C and 0% R.H.) and stored at −32 °C for up to 80 days.

In addition to the aforementioned storage experiments, two additional experiments were respectively carried out under atmospheres (% $CO_2/O_2/N_2$) 60/5/35 and 60/21/19. Both atmospheres were tested once using independent fish batches (at least two individual fish per batch) and the collected data was only included in model development (Section 2.3).

2.1.2. Quantification of VOCs by SIFT-MS

Quantification of VOCs was carried out in accordance with Sader et al. (2018). Briefly, preliminary screening was carried out on the basis of a literature survey and qualitative mass spectrometry experiments (data not shown). The 25 selected compounds (Table 1) were directly quantified from the package headspace through a septum inserted on the package lid, using a selected-ion flow-tube mass spectrometer (Voice 200, Syft Technologies™, Christchurch, New Zealand). Each package headspace was sampled for approximately 300 s while simultaneously connected to a 100% N_2 bag (Nalophan™, Scentroid, Whitchurch-Stouffville, ON, Canada) in order to avoid package collapse and external contamination. Respectively, background was determined from a 100% N_2 bag on each sampling day.

All measured concentrations (ppb) were corrected in accordance to the flow rate, measured with Gilibrator-2 (Sensidyne, St. Petersburg, FL, USA) prior to sampling. Averages, standard deviations and relative standard deviations ($SD_{\%}$) were determined for each VOC during an individual SIFT-MS scan. $SD_{\%}$ (%) was calculated according to Eq. (1):

$$SD_{\%} = SD_m / x_m * 100 \tag{1}$$

where x_m is the average and SD_m the standard deviation of a given VOC concentration during a scan (ca. 40 data points). In case of multiple product ions representing a given VOC, one product ion was selected for quantification on the basis of optimizing the following consecutive criteria: 1) $SD_{\%} < 25\%$, 2) high branching ratio, 3) minimum number of *m/z* conflicts, 4) lowest concentration. Next, Limit of Quantification (LOQ) values were determined for each VOC by using Eq. (2):

$$\mathrm{LOQ} = 6 * SD_{background} \tag{2}$$

where $SD_{background}$ is the standard deviation of the background (100% N_2). Individual LOQ values were determined for each VOC on each day of analysis (Supplementary Table 1).

2.1.3. Microbiological and physicochemical analyses

The materials and methods of microbiological analysis have previously been described by Kuuliala et al. (2018b). Briefly, 30 ± 0.1 g of salmon was aseptically diluted ten times in physiological saline peptone solution (PPS; 0.85% m/v NaCl, 0.1% m/v peptone) and homogenized for 1 min. Appropriate decimal dilutions (PPS) were used for pour plates (ca. 50 °C) or spread plates (room temperature). Total psychrotrophic counts (TPC) were enumerated on Marine Agar (MA) by spread plating and on Iron Agar Lyngby supplemented with L-cysteine (IAL) by pour plating, hydrogen sulfide (H_2S) producers on IAL by pour plating, lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe Agar (MRS) by pour plating with an overlay (3–5 ml), pseudomonads on Pseudomonas

Table 1

Volatile organic compounds (VOCs; denoted C1–C25) quantified using SIFT-MS: precursor ions, mass to charge ratios (*m/z*), branching ratios (b), reaction rate coefficients (k) and product ions.

VOC	Code	Precursor	*m/z*	b (%)	k	Product ion
Acids						
Acetic acid	C1	NO^+	90	100	9.0 E−10	$NO^+.CH_3COOH$[a]
		NO^+	108		9.0 E−10	$NO^+.CH_3COOH.H_2O$
3-Methylbutanoic acid	C2	NO+	132	70	2.5 E−09	$C_5H_{10}O_2.NO^{+}$[a]
Alcohols						
2,3-Butanediol	C3	NO^+	89	100	2.3 E−09	$C_4H_9O_2{}^{+}$[a]
		NO^+	107		2.3 E−09	$C_4H_9O_2{}^+.H_2O$
Ethanol	C4	NO^+	45	100	1.2 E−09	$C_2H_5O^{+}$[a]
		NO^+	63			$C_2H_5O^+.H_2O$
		NO^+	81			$C_2H_5O^+.2(H_2O)$
3-Methyl-1-butanol	C5	H_3O^+	71	100	2.8 E−09	$C_5H_{11}{}^{+}$[a]
		NO^+	87	85	2.3 E−09	$C_5H_{11}O^+$
Isobutyl alcohol	C6	NO^+	73	95	2.4 E−09	$C_4H_9O^{+}$[a]
		$O_2{}^+$	33	50	2.5 E−09	CH_5O^+
Aldehydes						
3-Methylbutanal	C7	NO^+	85	100	2.4 E−09	$C_5H_9O^{+}$[a]
Aromatic hydrocarbons						
Ethyl benzene	C8	NO^+	106	100	2.0 E−09	$C_8H_{10}{}^{+}$[a]
Propyl benzene	C9	NO^+	120	100	2.0 E−09	$C_9H_{12}{}^{+}$[a]
Styrene	C10	NO^+	104	100	1.7 E−09	$C_8H_8{}^{+}$[a]
Ketones						
Acetone	C11	NO^+	88	100	1.2 E−09	$NO^+.C_3H_6O$[a]
Acetoin	C12	NO^+	118	100	2.5 E−09	$C_4H_8O_2.NO^{+}$[a]
2,3-Butanedione	C13	NO^+	86	65	1.3 E−09	$C_4H_6O_2{}^{+}$[a]
Butanone	C14	NO^+	102	100	2.8 E−09	$NO^+.C_4H_8O$[a]
Sulfur compounds						
Carbon disulfide	C15	$O_2{}^+$	76	100	7.0 E−10	$CS_2{}^{+}$[a]
Dimethyl sulfide	C16	H_3O^+	63	100	2.2 E−09	$(CH_3)_2S{\cdot}H^{+}$[a]
		NO^+	62	100	2.2 E−09	$(CH_3)_2S^+$
		$O_2{}^+$	47	25	2.2 E−09	CH_3S^+
		$O_2{}^+$	62	60	2.2 E−09	$(CH_3)_2S^+$
Dimethyl disulfide	C17	H_3O^+	95	100	2.6 E−09	$(CH_3)_2S_2{\cdot}H^+$
		NO^+	94	100	2.4 E−09	$(CH_3)_2S_2{}^{+}$[a]
Dimethyl trisulfide	C18	H_3O^+	127	100	2.8 E−09	$C_2H_6S_3H^+$
		H_3O^+	145		2.8 E−09	$C_2H_6S_3H^+.H_2O$
		NO^+	126	100	1.9 E−09	$C_2H_6S_3{}^{+}$[a]
Hydrogen sulfide	C19	H_3O^+	35	100	1.6 E−09	H_3S^{+}[a]
		H_3O^+	53		1.6 E−09	$H_3S^+.H_2O$
Methyl mercaptan	C20	H_3O^+	49	100	1.8 E−09	$CH_4S{\cdot}H^{+}$[a]
		H_3O^+	67		1.8 E−09	$CH_4S{\cdot}H^+.H_2O$
Esters						
Ethyl acetate	C21	H_3O^+	89	100	2.9 E−09	$CH_3COOC_2H_5{\cdot}H^{+}$[a]
		H_3O^+	107		2.9 E−09	$CH3COOC_2H_5{\cdot}H^+.H_2O$
		$O_2{}^+$	31	20	2.4 E−09	CH_3O^+
		O^{2+}	61	40	2.4 E−09	$C_2H_5O_2{}^+$
Amines						
Ammonia	C22	H_3O^+	18	100	2.6 E−09	$NH_4{}^+$
		H_3O^+	36			$NH_4{}^+.H_2O$
		$O_2{}^+$	17	100	2.4 E−09	$NH_3{}^{+}$[a]
Dimethylamine	C23	H_3O^+	46	100	2.1 E−09	$(CH_3)_2NH.H^{+}$[a]
Piperidine	C24	H_3O^+	86	90	3.4 E−09	$C_5H_{12}N^{+}$[a]
Trimethylamine	C25	NO^+	59	100	1.6 E−09	$(CH_3)_3N^{+}$[a]

[a] Product ion was selected for quantifying the respective VOC.

Agar (PA) supplemented with *Pseudomonas* CFC supplement SR 103E by spread plating and *Brochothrix thermosphacta* on Streptomycin Sulfate Thallous Acetate Actidione Agar supplemented with selective supplement SR 151E (STAA) by spread plating. All plates were incubated at 22 °C for 2 (PA and STAA), 3 (IAL and MRS) or 5 days (MA) before enumeration.

Physicochemical analyses (headspace gas composition (% v/v CO_2/O_2), pH and color) were carried out as presented by Kuuliala et al. (2018b). Briefly, headspace gas composition was determined with CheckMate® 9900 CO_2/O_2 (Dansensor A/S, Ringsted, Denmark) prior to SIFT-MS. Within 30 min after opening, pH was determined from three random fillet spots using a pH electrode (Lab® 427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected to a pH meter (SevenEasy, Mettler Toledo GmbH) and color from ten random fillet spots using a spectrophotometer (CM 2500d, Konica Minolta Sensing Inc., New Jersey, USA). Color was determined through a small Petri dish (diameter 230 mm) using the CIELAB color space, a standard 10° observer and Illuminant D65.

2.1.4. Sensory evaluation

Olfactory sensory evaluation was performed in the UGent SensoLab in individual booths under red illumination according to the principles presented by Kuuliala et al. (2018b). For a given test, four frozen fillet portions representing different days of storage (each being a randomly selected daily replicate) within the same storage experiment (same initial batch and atmosphere) were thawed overnight at 2 °C, cut to portions (5.0 ± 0.1 g) in closed plastic cups (odor-free, transparent, diameter 67 mm; AVA, Temse, Belgium), labelled with three-digit random codes and arranged according to Latin Square Design on evaluation trays. The panelists thus received individual trays which contained similar sets of four samples in different order.

During a given evaluation session, the panelists (8–12 persons from FMFP laboratory staff, having experience in sensory evaluation of seafood) carried out two tests. Firstly, an acceptance test was carried out using a five-point ordinal scale with labels "very good" (VG), "good" (G), "satisfactory" (S), "marginal" (M) and "spoiled" (SP) in the order of decreasing degree of freshness (VG > G > S > M > SP). For this test, a fresh reference from the same initial batch was also provided. Secondly, a new tray was provided for a ranking test (most fresh to least fresh). A second evaluation session was in most cases performed for evaluating samples from the critical days determined on the basis of the first session. In total, 18 sessions were arranged.

The results of the acceptance test were used for determining the rejection percentage ($R_{\%}$) of each evaluated sample (Eq. (3)):

$$R_{\%} = \frac{n_M + n_{SP}}{n_{total}} * 100 \tag{3}$$

where n_M and n_{SP} are the numbers of panelists who evaluated the sample as marginal or spoiled, respectively, and n_{total} is the total number of panelists. The sample was considered spoiled when $R_{\%}$ exceeded 50%.

2.2. Statistical analysis

Partial least squares regression analysis (PLS) was used for the identification of most important spoilage indicators in accordance with Kuuliala et al. (2018a). Briefly, data from the two experiments per condition A-D were combined and the VOC concentrations were converted to logarithmic values and z-scores. PLS was carried out with JMP. v. 13 using the NIPALS algorithm and leave-one-out cross validation. Individual packages were treated as samples, VOCs as independent variables and sensory rejection ($R_{\%}$) as the response variable.

Three identification criteria were defined for screening spoilage indicators, consisting of two data quality criteria (Cr-1 and Cr-2) and a PLS criterion (Cr-3). A given VOC fulfilled these criteria if:

- Cr-1: in at least 33% of all tested packages per condition (A-D), $SD_{\%} < 25\%$
- Cr-2: in at least 33% of all tested packages per condition (A-D), concentration > LOQ
- Cr-3: 1) positive correlation with the rejection percentage, 2) Variable Importance in Projection (VIP) coefficient > 1 and 3) positive regression coefficient

Cr-1 and Cr-2 were thus defined by considering all tested packages per condition, irrespective of the storage time of a given package. VOCs that fulfilled all three identification criteria were considered the most important spoilage indicators.

2.3. Augmented ordinal regression model

An augmented ordinal regression model for predicting the evaluation of future samples on an ordinal scale was learned in accordance with a method developed by Sader et al. (2019). Generally, an ordinal regression model is used to model a relationship between ordinal outcomes (i.e., different levels of freshness) and feature representations. In the present study, the feature representation consisted of the measured VOC concentrations of each salmon sample and the outcomes consisted of the frequency distribution of sensory evaluation labels provided by the panelists.

To learn an ordinal regression model, the model parameters, such as weight parameters, were optimized to predict the probability of a sample being assigned each of the possible labels. The weight parameters represent the influence that the features (i.e. VOCs) have on the evaluation of a sample on the given ordinal scale. Due to the limited availability of acceptance data in the present study, the ordinal regression model was enhanced two-fold by integrating: 1) ℓ_1 regularization, which encourages the sum of the absolute values of the weight parameters to be small, and 2) information obtained from the ranking tests. The ranking information consisted of the frequency distribution of all pairs resulting from each ranking provided by the panelists.

To tune a learned ordinal regression model, nested 10-fold cross-validation was used in order to avoid using the same data for determining optimal tuning parameters (Cawley and Talbot, 2010). Firstly, inner cross-validation was performed on nine folds to determine the optimal values of the tuning parameters that provide the best model. From each model that was selected by the inner cross-validation, weight parameters were used to determine whether a VOC was relevant. Subsequently, the frequency of non-relevant features was computed over the ten models. Secondly, outer cross-validation was performed to evaluate the model selected by the inner cross-validation on the remaining fold. The general performance was estimated as the mean performance over all the folds. Since each sample had two distributions – an observed one and a predicted one – a measure of predictive performance was computed on the basis of the Kullback-Leibler (KL) divergence (Kullback and Leibler, 1951):

$$D_{KL}\left(P||Q\right) = \sum_i P(i) \log \frac{P(i)}{Q(i)} \tag{4}$$

where $P(i)$ and and $Q(i)$ are the values of the observed and predicted distributions P and Q for the ith label. A lower KL value corresponds to a higher model performance. To obtain the general performance of the model over the entire dataset, the mean KL divergence was computed over all samples in the dataset.

3. Results

3.1. Headspace composition (% CO_2/O_2), pH and color

The evolution of headspace gas concentrations (CO_2/O_2) is presented in Fig. 1. Throughout storage time, O_2 levels were negligible under anaerobic conditions (A and C) and remained at ca. 50% under high-O_2 MAP (D) after slight initial increase coinciding with CO_2 decrease. Under air (B), decrease of O_2 was observed from day 5 on. Increase in CO_2 levels was observed under low-CO_2 conditions (A and B) from day 5 on.

Both pH and color remained constant under all tested conditions. Despite a slight decreasing trend over time, high standard deviations led to pH values remaining at ca. 5.9–6.2 throughout storage time (Fig. 1). Respectively, variables L*, a* and b* yielded 51.0 ± 1.3, 12.7 ± 0.8 and 12.3 ± 0.8 (average ± standard deviation) when averaged over the levels of storage time and condition.

3.2. Microbiological analysis

TPC enumerated on MA are presented in Fig. 2. Three out of four initial fish batches had 3.7–4.2 log CFU g^{-1} on day 1; however, high counts observed in batch 2 (initial average 6.6 ± 0.1 log CFU g^{-1}) led to over 2 log difference between independent storage experiments in case of both conditions C and D and thus prevented averaging over the levels of the initial fish batch. Under 100% N_2 (A) and air (B), TPC exceeded 7 log CFU g^{-1} approximately on day 4 and entered the stationary phase after day 7–9. Under both CO_2-rich MAP conditions (C and D), 7 log TPC was exceeded on day 6 (high initial counts) or 11

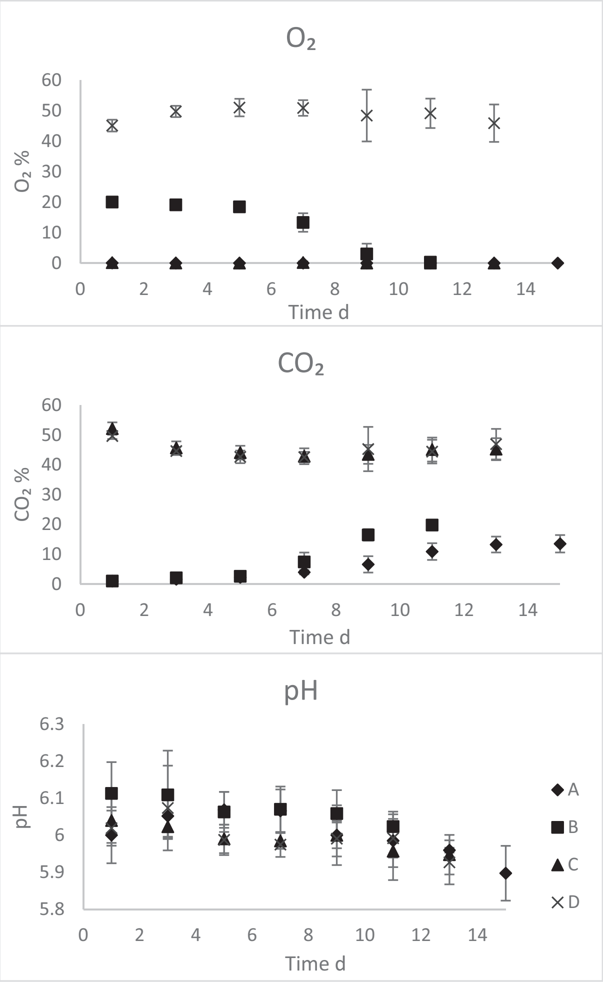

Fig. 1. Headspace gas concentrations (% O_2/CO_2) and pH of Atlantic salmon fillet portions stored under conditions (% $CO_2/O_2/N_2$) A (0/0/100), B (air), C (60/0/40) and D (60/40/0) at 4 °C.

(low initial counts). Enumeration of TPC on IAL produced similar results; generally, < 0.5 log difference between MA and IAL was observed under all tested conditions (results not shown).

The results of the other enumerations are shown in Fig. 3. The initial salmon microbiota (day 1) was primarily comprised of LAB, pseudomonads and *B. thermosphacta*; their relative abundances when compared to each other were subject to some batch-to-batch variation. Growth of LAB showed a highly similar trend under all tested conditions, irrespective of the headspace gas composition and the initial microbial load. LAB counts were in most cases similar or higher than the respective H_2S producer counts that varied even between two initial batches stored under the same conditions. Under 100% N_2 (A), H_2S producers did mostly not exceed 7 log CFU g^{-1} by the end of storage time. Under the other tested conditions, this limit was reached at ca. day 7 in the first experiments (batches 1–2) and not at all in the replicated experiments (batches 3–4). In most cases, day 7 also represented the point of reaching the stationary growth phase.

Pseudomonas spp. growth showed at least 2 log increase by the end of storage time under 100% N_2 (A) and air (B), exceeded 7 log CFU g^{-1} at ca. day 7 and stabilized thereafter. On the other hand, only moderate growth was observed under CO_2-rich MAs (C and D). Finally, the growth of *B. thermosphacta* was promoted by aerobic conditions, starting from low initial levels (ca. 4 log CFU g^{-1}) and reaching similar levels as TPC (Fig. 2).

3.3. Sensory evaluation

The results of the acceptance tests were used for determining the rejection percentage ($R_{\%}$) for each labelled sample. Before TPC reached 7 log CFU g^{-1}, sensory quality was in most cases acceptable (Supplementary Fig. 1). However, 50% rejection was exceeded at this stage of microbial growth under low-O_2 conditions (A and C), while most samples stored under high-O_2 (B and D) were acceptable until 7.6 log CFU g^{-1}. Samples from conditions A and C were usually scored higher than samples from conditions B and D at a given TPC level. After reaching 7.6 log CFU g^{-1}, all samples were rejected irrespective of the applied storage conditions. However, high variation interfered with the determination of the relationship between TPC and rejection percentage under all tested conditions.

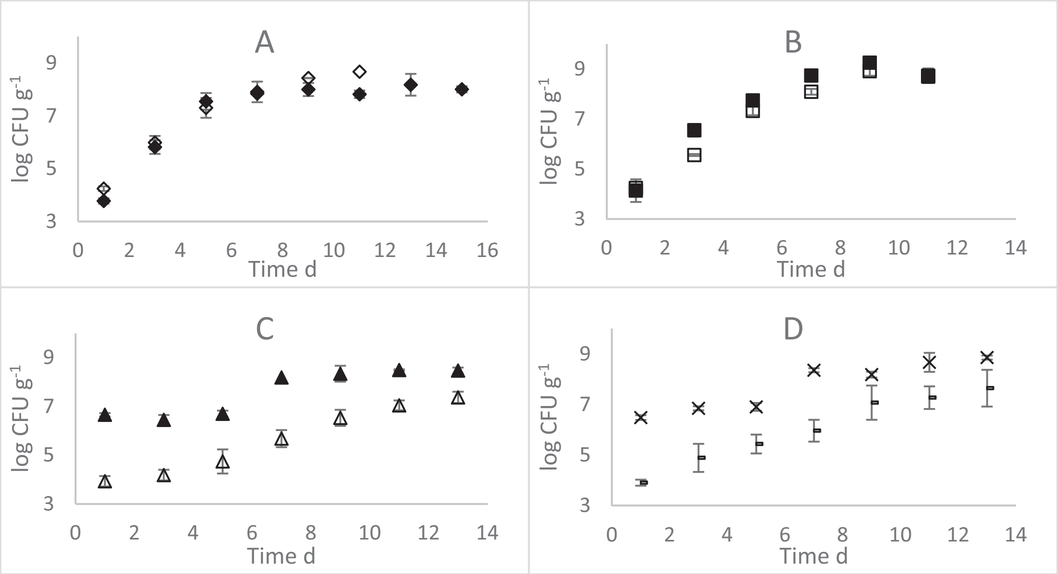

Fig. 2. Counts of total viable psychrotrophic bacteria (TPC) in Atlantic salmon fillet portions stored under conditions (% $CO_2/O_2/N_2$) A (0/0/100), B (air), C (60/0/40) and D (60/40/0) at 4 °C. Respective symbols represent data from two independent storage experiments (filled: batch 1–2, open: batch 3–4).

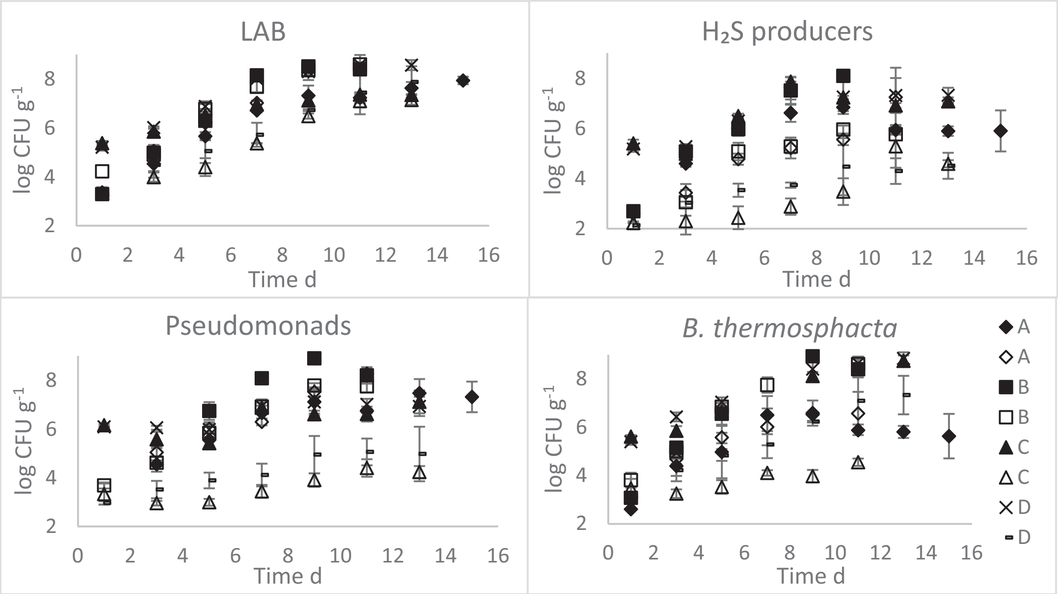

Fig. 3. Counts of lactic acid bacteria (LAB), H_2S producers, pseudomonads and *B. thermosphacta* in Atlantic salmon fillet portions stored under conditions (% CO_2/O_2/N_2) A (0/0/100), B (air), C (60/0/40) and D (60/40/0) at 4 °C. Respective symbols represent data from two independent storage experiments (filled: batch 1–2, open: batch 3–4).

3.4. Identification of spoilage indicators

The SIFT-MS data quality was characterized by determining $SD_{\%}$ and LOQ values for the 25 quantified VOCs. Since most of the VOCs had low initial concentrations ($<$ 100 ppb), small deviations during a SIFT-MS scan often led to relatively high initial $SD_{\%}$ values that decreased as a function of time. Because of the low initial concentrations, LOQs (Supplementary Table 1) were also often not exceeded during the early days of storage. In order to avoid excluding useful spoilage indicators on the basis of average $SD_{\%}$ and/or average LOQ, all VOCs were included in the statistical analyses and the data quality criteria Cr-1 and Cr-2 were used in addition to the PLS criterion Cr-3 for screening most important spoilage indicators.

Independent PLS models were built for each of the studied conditions. Under conditions A-C, the number of factors that minimized the root mean predicted residual sum of squares (PRESS) and was thus used for building the models was 2, 2 and 4, respectively. The first two retained factors covered 85.4, 89.7 and 87.7% of the variance observed in $R_{\%}$. Furthermore, the proportion of VOCs having > 75% of their variance explained by the first two factors was 52, 24 and 20%. Under condition D, root mean PRESS was minimized by one factor; however, other models were significantly different from the one-factor model and did thus not fulfill the van der Voet test criterion (van der Voet, 1994). For this reason, two factors were selected for illustrating the performance of the PLS analysis.

Correlation loading plots and VIP vs. regression coefficient plots (Supplementary Fig. 2) were used for identifying the VOCs that fulfilled the PLS criterion (Cr-3). The results of applying the three identification criteria (Cr-1, Cr-2, Cr-3) are summarized in Table 2. Three main types of VOCs could be identified (Table 2): 1) VOCs that fulfilled all three criteria, 2) VOCs that fulfilled 1–2 criteria, and 3) VOCs that did not fulfill any criteria. Compounds belonging to the first group were considered to be the most important spoilage indicators; Fig. 4 presents their evolution as a function of TPC. Typically, initial concentration levels were below 200 ppb and the onset of exponential increase was observed at ca. 7–7.5 log CFU g^{-1}.

The identification criteria highlighted several alcohols, aldehydes and ketones (Fig. 4). Ethanol was identified under conditions A-C and reached the highest levels among all quantified VOCs. Approximately 6-fold concentration levels were detected by the end of storage under low-O_2 conditions (A and C) when compared to aerobic conditions (B and D). Most of the other compounds in this group were characteristic to air (B); furthermore, 3-methylbutanal was the only compound that fulfilled all three criteria under high-O_2 MAP (D). Apart from ethanol, none of the alcohols, aldehydes and ketones reached the ppm range by the end of storage under any of the tested conditions. The production of the only studied ester, ethyl acetate, was greatly favored by high-O_2 conditions (B and D).

Sulfuric compounds were produced especially under low-O_2 conditions (A and C; Fig. 5). Dimethyl sulfide was initially present at trace concentrations and increased exponentially over 200 ppb under low-CO_2 conditions (A-B) and over 600 ppb under high-CO_2 conditions (C-D). Methyl mercaptan was recognized as a spoilage indicator under 100% N_2 (A) and increased exponentially up to the ppm range. H_2S was highly characteristic to both anaerobic conditions (A and C), reaching 5–20 ppm on the late days of storage.

3.5. Augmented ordinal regression model

On the basis of the frequency of non-relevance for the augmented ordinal regression model (Table 2), the studied VOCs could be classified into three main groups. It was observed that 3-methyl-1-butanol, dimethyl amine, ethyl acetate, methyl mercaptan, 3-methylbutanal, acetone, butanone, ammonia and carbon disulfide were considered non-relevant 0% of the time. Therefore, these VOCs were recognized as strong contributors for the model, regardless of the applied atmosphere. Moreover, it was observed that H_2S, 3-methylbutanoic acid, dimethyl disulfide, ethanol and isobutyl alcohol were considered non-relevant > 50% of the time and therefore were considered to have limited relevance for the model. Finally, the remaining nine VOCs that were considered non-relevant 10–50% of the time were considered to have intermediate relevance for the model.

Fig. 5 presents the KL divergence as a function of the number of integrated ranking tests. Increasing the amount of ranking information led to a decrease in both mean KL divergence value and its variation (shaded), thus improving the performance of the model. The highest decrease was observed between 0 and 5 added ranking tests, whereas only moderate changes in the mean KL divergence or its variance were

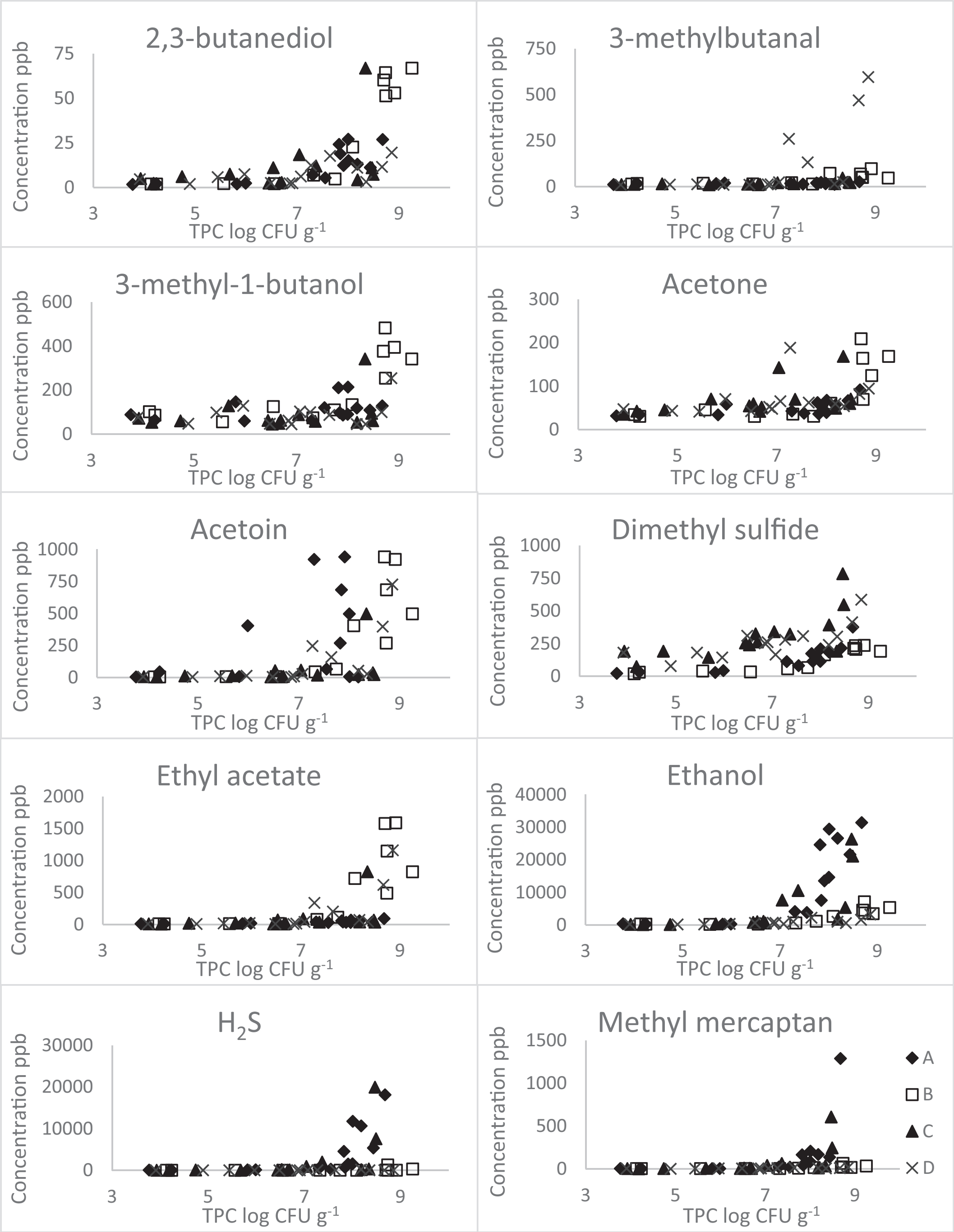

Fig. 4. Concentrations (ppb) of most important spoilage indicators (Table 2) as a function of total viable psychrotrophic counts (TPC) in Atlantic salmon fillet portions stored under conditions (% $CO_2/O_2/N_2$) A (0/0/100), B (air), C (60/0/40) and D (60/40/0) at 4 °C. Isobutyl alcohol remained below 100 ppb throughout storage time under all tested conditions and was omitted from the figure.

observed after integrating seven or more ranking tests.

4. Discussion

The composition and growth of the seafood microbiota is dependent on the initial contamination, handling practices and storage conditions. The initial microbiological quality of salmon used in the present study was slightly lower than observed by de la Hoz et al. (2000) and higher than observed by Mikš-Krajnik et al. (2016) or Zaragozá et al. (2014), possibly due to the impact of delivery times. Furthermore, highly similar counts were enumerated on MA and IAL under all tested conditions. This is in contrast with the results of Broekaert et al. (2011) and Kuuliala et al. (2018b) who suggested that pour plating techniques can reduce the TPC due to the elimination of thermally sensitive psychrotrophic bacteria, such as *Photobacterium phosphoreum*. The results of the present study thus suggest low relative abundance of highly heat-sensitive bacteria in the tested salmon samples. In fact, the role of *Photobacterium* spp. in salmon spoilage is still not completely understood. Although its absence in Australian-produced salmon has been demonstrated (Milne and Powell, 2014; Powell and Tamplin, 2012), the prevalence of this genus (often specified as *P. phosphoreum*) has been well shown in naturally contaminated salmon in the northern hemisphere (Emborg et al., 2002; Hansen et al., 2009; Jääskeläinen et al., 2019; Macé et al., 2012; Møretrø et al., 2016). Most recently, Jääskeläinen

Table 2

Most important spoilage indicators of Atlantic salmon stored under atmospheres (% $CO_2/O_2/N_2$) A (0/0/100), B (air), C (60/0/40) and D (60/40/0) according to the identification criteria Cr-1, Cr-2 and Cr-3 (1–3) and their frequency of non-relevance for the augmented ordinal regression model. Compounds denoted with bold font fulfilled the three criteria at least under one of the tested conditions (see also Fig. 4).

VOC	Code	A			B			C			D			Frequency of non-relevance
		1	2	3	1	2	3	1	2	3	1	2	3	
Acetic acid	C1			x			x			x			x	0.1
3-Methylbutanoic acid	C2													0.6
2,3-Butanediol	C3	x	x	x	x	x	x							0.1
Ethanol	C4	x	x	x	x	x	x	x	x	x	x	x		0.9
3-Methyl-1-butanol	C5	x	x		x	x	x	x	x		x	x		0
Isobutyl alcohol	C6		x	x	x	x	x						x	0.5
3-Methylbutanal	C7	x	x		x	x	x	x	x	x	x	x	x	0
Ethyl benzene	C8			x		x	x							0.4
Propyl benzene	C9													0.3
Styrene	C10													0.4
Acetone	C11				x	x	x					x	x	0
Acetoin	C12	x	x	x	x	x	x			x			x	0.1
2,3-Butanedione	C13		x		x	x			x			x	x	0.3
Butanone	C14		x						x			x		0
Carbon disulfide	C15	x	x		x	x		x	x		x	x		0
Dimethyl sulfide	C16	x	x	x	x	x	x	x	x	x	x	x		0.3
Dimethyl disulfide	C17			x										1
Dimethyl trisulfide	C18													0.3
Hydrogen sulfide	C19	x	x	x	x	x		x	x	x				1
Methyl mercaptan	C20	x	x	x			x			x			x	0
Ethyl acetate	C21			x	x	x	x			x			x	0
Ammonia	C22								x		x	x		0
Dimethylamine	C23			x			x			x				0
Piperidine	C24													0.2
Trimethylamine	C25								x			x		0.4

Cr-1: average relative standard deviation < 25% in ≥33% of the tested packages per condition.

Cr-2: VOC concentration > LOQ in ≥33% of the tested packages per condition.

Cr-3: PLS selection criteria (VIP > 1, regression coefficient > 0, positive correlation with rejection %) were met.

et al. (2019) observed the dominance of *Photobacterium* spp. under air or vacuum at 3 °C, whereas Fogarty et al. (2019) found that *Photobacterium* spp., H_2S producers, LAB, *Pseudomonas* spp. and *B. thermosphacta* showed highly similar growth rates under air at 2 °C. In terms of spoilage potential, *P. phosphoreum* has been associated with high TMA production in seafood (Dalgaard, 1995; Kuuliala et al., 2018b), whereas it has not become known as a major H_2S producer and its capacity to produce volatile sulfides has indeed suggested to be limited (Gram and

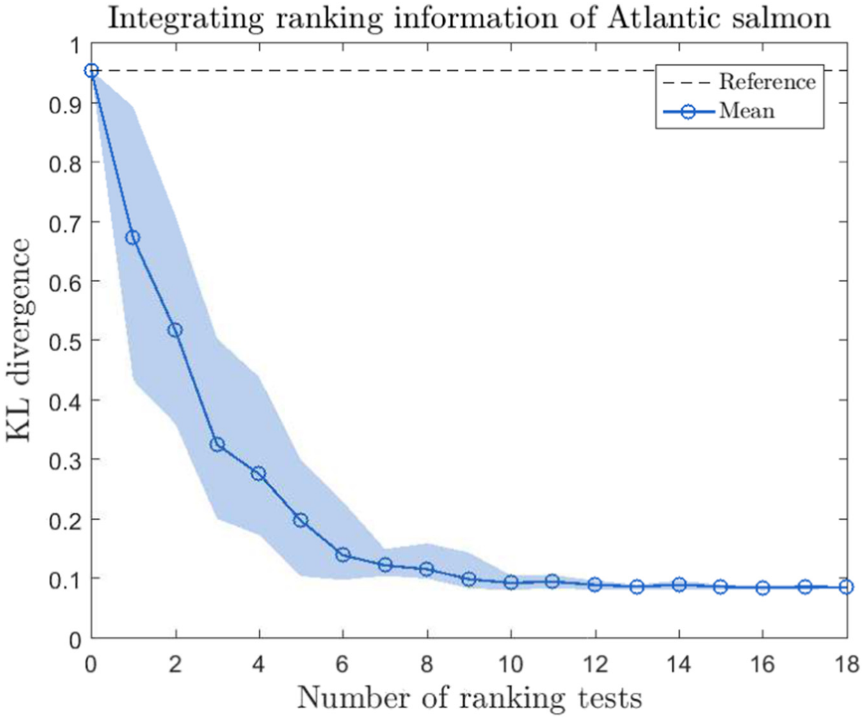

Fig. 5. Kullback-Leibler (KL) divergence as a function of incorporated ranking tests in the augmented ordinal regression model of Atlantic salmon stored under different gaseous atmospheres at 4 °C.

Huss, 1996). In contrast to these findings, negligible TMA levels were observed under all tested conditions throughout storage time in the present study; furthermore, very high H_2S levels (> 5000 ppb) were reached under anaerobic conditions (A and C), which could be due to *Shewanella* spp. (López-Caballero et al., 2001). Hence, when taking into account the general prevalence of *Photobacterium* spp. in Norwegian salmon, the present results suggest that this genus may have expressed unexpected patterns in growth and/or metabolism under the tested conditions. Further studies regarding their growth and spoilage potential in naturally contaminated salmon would thus be highly relevant for the scientific community.

The observed impact of different atmospheres on microbial growth is well in line with previous results. Generally, *Pseudomonas* and/or *Shewanella* spp. have been recognized as specific spoilage organisms (SSOs) of marine fish stored under air, whereas *P. phosphoreum*, LAB and/or *B. thermosphacta* have often been found dominant under CO_2-rich MAP conditions (Drosinos and Nychas, 1996; Drosinos et al., 1997; Gram and Dalgaard, 2002; Møretrø et al., 2016). Under air, pseudomonads, LAB, *B. thermosphacta* and H_2S producers indeed formed a major part of the microbiota throughout storage time, as in several previous studies (Dabadé et al., 2015; Kuuliala et al., 2018b; Mikš-Krajnik et al., 2016; Parlapani et al., 2014; Parlapani et al., 2015). Under conditions C and D, pseudomonads' growth was inhibited, most likely due to the effect of elevated CO_2 levels (Gram and Huss, 1996). In contrast, growth of H_2S producers and LAB was observed under all tested conditions, although H_2S producers were less prominent under MAP (A, C-D). Respective results have been observed in Atlantic cod (Kuuliala et al., 2018b) where the combination of CO_2 and O_2 was suggested as a possible reason for the inhibitive effect of MAP on H_2S producers in accordance with López-Caballero et al. (2001) and Noseda et al. (2012a). No change in pH was recorded under any of the tested conditions even though a considerable increase in LAB counts (eventually exceeding 7–8 log CFU g^{-1}) was observed, most likely because pH was measured from the fillet interior rather than from its surface. Counts of H_2S producers were determined as black colonies on IAL, likely belonging to the *Shewanella* or *Aeromonas* genera (Emborg et al., 2002; Møretrø et al., 2016). In conclusion, it is important to note that since spoilage is not necessarily due to the same micro-organisms under different conditions, the selectivity of microbial enumeration can be expected to affect the applicability of the resulting datasets in VOC modelling. Respectively, SSO levels have been suggested to correlate with quality deterioration better than TPC (Fogarty et al., 2019). However, incorporating plate count data in volatilome models may not only require SSO identification, but also strategies for overcoming the challenges associated with the stationary growth phase (Kuuliala et al., 2018a) and for ensuring that the model matches the reality inside the food package even under unexpected scenarios. For these reasons, the obtained plate count data were excluded from model development and instead used for evaluating the microbiological relevance of the modelling output as well as for comparing the deterioration of microbiological quality under different storage conditions.

The interest towards advanced data-driven methods in food quality research has been constantly increasing during the past decades and led to numerous outcomes, as summarized in comprehensive reviews (Ghasemi-Varnamkhasti et al., 2018; Maione et al., 2019; Ropodi et al., 2016) and automated tools (Estelles-Lopez et al., 2017). In this study, the previous work of Kuuliala et al. (2018a) was used as a basis for determining the three criteria (Cr-1, Cr-2, Cr-3) that were used in screening potential spoilage indicators. Extending the screening to VOCs that fulfill 1–2 criteria should be considered with caution. Firstly, VOCs that do not fulfill the PLS criterion (Cr-3) can be considered to have limited indicative potential. Secondly, although excluding VOCs that do not fulfill the data quality criteria (Cr-1 and Cr-2) could allow additional VOCs to exceed the VIP/regression coefficient thresholds, this would decrease the dataset size and could highlight only a few most influential VOCs. Finally, the cut-off limit of sufficient sample coverage in the data quality criteria should be selected in accordance with the product shelf life and the duration of the storage experiment. In particular, it should be considered that VOC concentration levels associated with freshness are typically relatively low– even below LOQ – and are thus characterized by high initial $SD_{\%}$ values during the early days of storage. Since these VOCs can still be useful spoilage indicators, the evolution of $SD_{\%}$ and exceeding the LOQ should be studied throughout storage time instead of simply relying on averages. In the present study, a cut-off limit of 33% was thus chosen in order to allow some initial variation before the onset of exponential increase.

The applied atmosphere has a central impact on the microbiological and chemical changes that lead to VOC accumulation. In the present study, the highest number of spoilage indicators was identified under air (B), including ethyl acetate, acetone, isobutyl alcohol and 3-methyl-1-butanol that did not fulfill the identification criteria under other tested conditions. Ethyl acetate could have originated from monoamino monocarboxylic acids or esterification between alcohols and carboxylic acids (Noseda et al., 2012b), likely due to pseudomonads growth (Olafsdottir et al., 2005; Parlapani et al., 2014). Despite being a fermentation product (Noseda et al., 2012b), acetone has rather rarely been associated with seafood quality deterioration. On the contrary, 3-methyl-1-butanol is one of the most frequently detected spoilage-related compounds in different aerobically stored fish species (Alasalvar et al., 2005; Duflos et al., 2006; Edirisinghe et al., 2007; Mikš-Krajnik et al., 2016) and can be produced by several common seafood spoilage bacteria (Parlapani et al., 2014). In contrast, the application of MAs affects the growth and composition of the microbiota when compared to air and may also cause changes in microbial metabolism. For instance, Pin et al. (2002) examined the glucose metabolism of *B. thermosphacta* under different atmospheres at 5 °C and observed a shift from anaerobic to aerobic metabolism when $O_2 > 10.17 + 0.6717{*}CO_2$. Ethanol and lactic acid were produced under anaerobic conditions, whereas the acetoin-diacetyl pathway was dominant under aerobic conditions. In the present study, respectively, acetoin production was favored under aerobic and/or low-CO_2 conditions.

The selection of appropriate storage conditions for a given food product depends on its composition, properties and initial microbiota. O_2-free MAs are often applied for fatty seafood in order to limit the growth of aerobic microbiota and lipid oxidation, while elevated O_2 levels have been found useful for products that benefit from suppressed TMA production (Boskou and Debevere, 1997), redness preservation (Lopez-Galvez et al., 1995) or prolonged survival (Bernárdez and Pastoriza, 2011; Gonçalves et al., 2009). In the present study, the availability of O_2 in the package headspace was found critical in determining whether the emerging off-odors could be characterized as sulfuric (A and C) or rancid (B and C). Sulfurous off-odors most likely originated from few VOCs that were also highlighted by the identification criteria – such as H_2S, dimethyl sulfide and methyl mercaptan – whereas rancidity could be associated with more complex interactions of several VOCs. Previously, one or several sulfuric compounds have been associated with seafood quality deterioration in several studies (Chinivasagam et al., 1998; Noseda et al., 2012a; Noseda et al., 2012b; Parlapani et al., 2018). Furthermore, on the basis of sensory evaluation (Section 3.3), anaerobic off-odor profiles were usually considered more offensive than aerobic profiles at a certain TPC level. This could be due to the characteristically low olfactory thresholds (OTs) of sulfuric compounds, allowing small concentration changes to potentially have a notable sensory impact (Noseda et al., 2012a). Hence, the panelists were likely able to detect emerging off-odors and thus reject the samples close to the onset of exponential VOC increase (ca. 7 log CFU g^{-1}). Overall, storage under anaerobic conditions (A and C) did not improve the shelf life of salmon when compared to aerobic conditions (B and D); moreover, TPC development was dependent on the availability of CO_2. Storage under CO_2-rich MAs could thus be considered universally beneficial for salmon. Furthermore, it should be noted that the evolution of the seafood volatilome is also dependent on storage temperature and its possible fluctuations during storage time. Consequently, the development of novel packaging technologies requires the examination of all relevant and/or possible storage conditions. Using the present methodology under different temperature regimes can thus be expected to have great relevance for the development of food quality monitoring.

Modelling broadens the applicability of the collected datasets and is thus of high importance for the development of food quality monitoring. However, it should be noted that the contribution of a given variable in a regression model may differ from its relevance determined by statistical analysis. For example, even though H_2S and ethanol were often identified as important spoilage indicators by PLS, these VOCs were considered non-relevant for the ordinal regression model. This was most likely due to the fact that the model was produced from the collected data as a whole; these VOCs showed different patterns under different storage conditions, meaning that a given concentration level did not necessarily correspond with a fixed panel response. In contrast, several VOCs were considered highly influential for the model (frequency of non-relevance equal to 0%) despite having limited relevance as spoilage indicators according to the identification criteria; in fact, many of these compounds did not fulfill the quality criteria (Cr-1 and Cr-2). Since modelling utilizes and represents the data as it is, preceding and/or simultaneous statistical analysis can be considered highly beneficial for interpreting and evaluating the obtained output. Secondly, it should be emphasized that the performance of modelling depends on the availability of data, which often conflicts with the realistic limits of experimental data collection. Even though building separate models to represent specific storage conditions could improve the correspondence between the results of statistical analysis and modelling, this approach requires increasing the number of replicated storage experiments. Finally, it should be noted that the incorporation of ranking information improved the performance of the regression model. Generally, ranking can be considered a simpler task than acceptancy testing (Meilgaard et al., 2006) and thus a more suitable alternative for inexperienced panelists. Since the availability of experienced panelists may be limited, ranking could provide a valuable source of additional information for quality assessment and/or modelling (Sader et al., 2018).

The development of intelligent packaging technologies calls for the identification of spoilage-related metabolites that could be used for food quality monitoring. However, because of the impact of several intrinsic and extrinsic factors on the VOC profile evolution, few compounds could be considered as universal spoilage indicators. As previously highlighted (Ioannidis et al., 2018; Kuuliala et al., 2018b), several VOCs simultaneously contribute to the perceived off-odor, which is why food quality monitoring would benefit from the capacity to detect multiple compounds. Furthermore, even though certain VOCs might not directly contribute to the perceived quality because of their high OTs, monitoring of these compounds may still be highly relevant (Ioannidis et al., 2018). For example, ethanol could be linked with several metabolic pathways and microbiota (Casaburi et al., 2015) and could thus be considered as one of the most universal spoilage indicators, despite its high OT (> 29 ppm, according to Devos et al., 1990). Finally, it should be noted that even though the screening of spoilage indicators increases the knowledge regarding food spoilage processes, it does not guarantee that the identified compounds can be conveniently applied in quality monitoring. The relevance of a VOC in packaging technology development is limited if its concentration levels fall below the application's quantification limits. For example, 2,3-butanediol and isobutyl alcohol were recognized as spoilage indicators under certain conditions in the present study, even though these compounds were produced in very low quantities (< 100 ppb). Separating acceptable and unacceptable concentrations would thus require highly sensitive devices. However, given that most of the quantified VOCs did not surpass 1 ppm by the end of storage time, the obtained results highlight the importance of ppb-range sensitivity in food quality monitoring.

5. Conclusions

The development of food quality monitoring is a multidisciplinary process that builds upon extensive data collection and advanced statistical and modelling techniques. The identification criteria applied in the present study allowed for the comprehensive distinction of most important spoilage indicators among the studied VOCs, whereas the performance of the regression model was improved by incorporating ranking information. Given that statistical analysis and modelling have different aims – the former aims at identifying spoilage indicators, the latter at predicting the quality label on the basis of the VOC profile – a versatile view into food spoilage can be expected when using these approaches in parallel. Overall, this study presented a systematic seafood spoilage characterization methodology which directly corresponds to the needs of intelligent packaging technology development.

The three identification criteria were fulfilled by 6, 9, 4 and 1 out of 25 compounds under conditions A-D, respectively. Under anaerobic conditions (A and C), ethanol, dimethyl sulfide, and H_2S were found characteristic irrespective of the applied CO_2 levels. Under air (B), most of the identified VOCs were alcohols and ketones, including four unique identifications (3-methyl-butanol, isobutyl alcohol, acetone and ethyl acetate). Fewer compounds were identified under high-CO_2 MAP (C and D) when compared to low-CO_2 conditions (A and B), 3-methylbutanal being the single identified VOC under aerobic MAP (D). In conclusion, ethanol, dimethyl sulfide, H_2S, methyl mercaptan and acetoin were found abundant under several conditions and often identified as spoilage indicators, suggesting that monitoring these VOCs in the package headspace could lead to significant benefits in the seafood industry. However, because of the notable impact of different intrinsic and extrinsic factors on food quality deterioration, the requirements of the packaging technologies for volatile spoilage indicators should also be carefully considered.

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