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ORIGINAL ARTICLE

Growth and spoilage metabolites production of a mesophilic *Aeromonas salmonicida* strain in Atlantic salmon (*Salmo salar* L.) during cold storage in modified atmosphere

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Keywords

Aeromonas, growth kinetics, modified atmosphere packaging (MAP), nuclear magnetic resonance (NMR), salmon, spoilage metabolites, trimethylamine (TMA).

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Abstract

Aims: The aim of the study was to quantify the growth kinetic parameters and spoilage-associated metabolites of an inoculated strain of *Aeromonas salmonicida* in pre-*rigor* filleted Atlantic salmon (*Salmo salar* L.) stored in vacuum (VP) or modified atmosphere (MAP 60/40% CO_2/N_2) at 4 and 8°C.

Methods and Results: The maximum growth rate of *A. salmonicida* in VP salmon stored at 4°C was 0·56 ± 0·04 day^{-1} with no detectable lag-phase and the concentration of *Aeromonas* reached 8·33 log CFU per g after 10 days. The growth rates and maximum population density of *Aeromonas* in MAP salmon were lower but the applied atmosphere did not inhibit the growth. A selection of metabolites associated with fish spoilage were quantified using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The concentration of trimethylamine (TMA) was significantly affected by storage time and temperature, packaging atmosphere and inoculation with *A. salmonicida* (General Linear Model (GLM), $P < 0{\cdot}001$ for all factors).

Conclusion: The study presents preliminary results on *A. salmonicida* as a potential spoilage organism in vacuum-packaged salmon during cold storage. The combination of refrigeration and a packaging atmosphere consisting of 60/40 % CO_2/N_2 did not completely inhibit the growth but prevented the formation of TMA.

Significance and Impact of the Study: Little information is available on the spoilage potential of *Aeromonas* spp. in minimally processed salmon products under different packaging conditions. The study clearly demonstrates the importance of hurdle technology and provides data to further elucidate the significance of *Aeromonas* spp. as a spoilage organism.

Introduction

Ready-to-eat (RTE) seafood products such as chilled raw salmon (*Salmo salar L.*) packaged in modified atmosphere (MAP) or under vacuum (VP) are popular in Europe because of their convenience and high nutritional value (Odeyemi *et al.* 2018). Raw salmon loins are frequently used in homemade sushi and sashimi (Norwegian Seafood Council (NSC) 2016) or prepared by gentle heating. Fish is a highly perishable product, and global post-harvest fish losses are estimated to be 27% (FAO 2018). Packaging has become one of the most important hurdles to prevent spoilage of cold stored fish caused by bacterial growth, enzymatic and oxidative reactions (Sivertsvik *et al.* 2002). The shelf-life extending effect of MAP and vacuum packing (vacuum packaging can be considered as a specific case of MAP) depends on fish species, fat content, initial microbial population, gas composition (concentration of CO_2 available in the atmosphere and availability of O_2), the ratio of gas volume to product

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volume and most importantly storage temperature (Sivertsvik *et al.* 2002). Microbial growth and metabolism are the major causes of spoilage of most raw and minimally processed seafood (Mikš-Krajnik *et al.* 2016). It is recognized that only a small fraction of the microbial population, the specific spoilage organisms (SSOs), contribute to spoilage by producing the metabolites associated with spoilage of a product resulting from their ability to grow faster than the remaining microbiota under the applied storage conditions (Gram and Dalgaard 2002).

The formation of undesirable spoilage compounds such as amines, sulphides, alcohols, aldehydes, ketones and organic acids with off-flavours depends on the endogenous bacteria present on the product, the seafood species, the post-harvest processing methods and environment, packaging atmosphere (MAP, vacuum or air) and storage temperature (Sivertsvik *et al.* 2002). Most marine fish spoilage bacteria, at oxygen-limiting conditions, reduce trimethylamine oxide (TMAO) to trimethylamine (TMA). This metabolite contributes to the typical ammonia-like and fishy off-odours in spoiled seafood and is often used for determination of fish freshness (Olafsdottir *et al.* 1997). The spoilage of MAP salmon is mainly caused by *Photobacterium phosphoreum* (Gram and Dalgaard 2002; Macé *et al.* 2013), *Brochothrix thermosphacta*, genera of lactic acid bacteria such as *Carnobacterium* spp. but also members of Enterobacteriaceae (Powell and Tamplin 2012) and *Aeromonas* spp. (Joffraud *et al.* 2001; Møretrø *et al.* 2016) are potential spoilage organisms. The fact that *Aeromonas* spp. is frequently isolated from spoiled seafood (Provincial *et al.* 2013), implicates its role as a spoilage organism in these types of food. However, the knowledge about the growth and spoilage potential of *Aeromonas* spp. in refrigerated MAP and vacuum salmon products is very limited.

Aeromonas species are widespread in marine environments and frequently isolated from various water sources (Ørmen and Østensvik 2001; Martínez-Murcia *et al.* 2016) and seafood (Hoel *et al.* 2015; Yano *et al.* 2015; De Silva *et al.* 2019). The genus *Aeromonas* consists of Gram negative, facultative anaerobic, rod-shaped bacteria (Martin-Carnahan and Joseph 2005). To date, there are 36 recognized species (Navarro and Martinez-Murcia 2018), and a subset of species (*A. hydrophila*, *A. caviae*, *A. dhakensis* and *A. veronii*) are more implicated in human disease (Tomás 2012; Figueras and Beaz-Hidalgo 2015). Other *Aeromonas* species, for example, *Aeromonas salmonicida* and *Aeromonas trota* are reported as potential food spoilage organisms, especially in MAP seafood from tropical or warmer waters where they have been found in high concentrations and are likely to be the SSO. Strains of *A. salmonicida* can grow relatively uninhibited in seafood during cold storage in normal atmosphere (Provincial *et al.* 2013; Hoel *et al.* 2018) and are implicated in spoilage of ice-stored sea bream (*Sparus aurata*; Parlapani *et al.* 2013) and common carp (*Cyprinus carpio*; Beaz-Hidalgo *et al.* 2015) and in MAP (50/50% CO_2/N_2) shrimps (*Penaeus vannamei*; Macé *et al.* 2014). In the case of *A. hydrophila*, a high CO_2 atmosphere (80%) in combination with storage at 0°C was required to inhibit growth in sea bream (Provincial *et al.* 2013), while others have shown that a 60/40% CO_2/O_2 is sufficient to reduce growth of a mixed *Aeromonas* population in pearlspot (*Etroplus suratensis* Bloch) (Ravi Sankar *et al.* 2008).

The increased interest in seafood products that are easy to prepare, minimally processed or intended for raw consumption poses new microbiological challenges. Understanding the dynamics of microbial growth throughout the food chain is fundamental to correctly determine the shelf-life of a product, to control microbial spoilage as well as inhibit potential pathogenic bacteria. The aim of the present study was to quantify the growth kinetics parameters of an environmental mesophilic strain of *A. salmonicida* in salmon fillet as affected by packing atmosphere (vacuum and MAP; CO_2/N_2; 60/40%) at optimal storage temperature (4°C) and at minor temperature abuse (8°C). Moreover, to evaluate the spoilage potential of *A. salmonicida* in salmon using ^{1}H nuclear magnetic resonance (NMR) spectroscopy to detect and quantify potential spoilage metabolites during storage.

Materials and methods

Experimental design

The experimental design was set up to study the effect of packaging atmosphere (vacuum and MAP; 60/40% CO_2/N_2) and storage temperature (4 and 8°C) on the growth kinetic parameters and spoilage metabolites of *A. salmonicida* strain SU2, a strain previously isolated from sushi (Hoel *et al.* 2015). The design resulted in eight groups; un-inoculated control samples and inoculated samples packaged in vacuum stored at 4°C (hereby designated VPC4 and VPI4, respectively), and at 8°C (VPC8 and VPI8), un-inoculated control samples and inoculated samples packaged in 60/40% CO_2/N_2 stored at 4°C (MAPC4 and MAPI4), and at 8°C (MAPC8 and MAPI8). All samples were stored at 4 and 8°C for 16 days (VP) and 21 days (MAP). Samples for microbiological analysis were taken at day 0, 2, 4, 6, 8, 10, 12, 14 and 16 (VP) and at day 0, 3, 6, 9, 12, 15, 18 and 21 (MAP) after packaging ($n = 3$ for each group at each sample point, except for day 0 where $n = 4$). The only exception was sampling of VPI8 that was terminated after 14 days due to evident spoilage. Headspace gas analysis of the packaging

atmosphere was carried out on all packages prior to sampling. Samples for metabolite analysis were taken at day 0, 6 and 14 (VP) and day 0, 6 and 15 (MAP) after packaging and kept at −80°C until extraction and further analysis.

Sample and inoculum preparation

Fresh vacuum-packed pre-*rigor* filleted back loins from farmed Atlantic salmon (*Salmo salar* L.) were purchased from a nearby fish processing plant and brought to the laboratory 1 day post-harvest. The loins were cut in 90 ± 2g pieces and stored at 2°C for approximately 16 h before inoculation and packaging (at 2 days post-harvest). For preparation of inoculum, 1 ml of a frozen stock culture of *A. salmonicida* strain SU2 was thawed and transferred to a 250 ml baffled conical flask containing 100 ml tryptone soy broth (TSB) (Oxoid, Oslo, Norway). The culture was grown over night at 37°C, and 1 ml of the overnight culture was transferred to a new conical flask containing 100 ml TSB. The culture was grown overnight at 8°C to adapt to cold storage, and then 1 ml was transferred to a new conical flask and grown overnight under the same conditions. Thereafter, the overnight culture was diluted to an appropriate optical density (OD) (600 nm, Shimadzu UV spectrophotometer) corresponding to a bacterial concentration of approximately log 4 CFU per ml. The relatively high concentration of inoculum was applied to ensure detection of the produced metabolites in a naturally contaminated system. For inoculation, 0·9 ml of the culture was added on the top of each piece of salmon and spread evenly on the surface using a sterile spreader. The inoculated fish were covered and allowed to dry for 30 min before packaging.

Packaging and storage

The VP samples were packaged in 20-μm polyamide (PA)/70-μm polyethylene (PE) bags (120 × 80 mm, Star-Pack Productive, Boissy-l'Aillerie, France) with a Webomatic Supermax-C vacuum machine (Webomatic, Bochum, Germany). Air was evacuated to an end pressure of 10 mbar before sealing. The MAP samples were placed in 230 ml semi-rigid crystalline polyethylene terephthalate trays (Faerch Plast, Holstebro, Denmark) with an absorbent underneath using a semi-automatic tray sealing packaging machine (TL250, Webomatic) to obtain a gas/product ratio of 2·6. The air was evacuated and then filled with the gas mixture prior to heat sealing of the top film (40-μm PE, ethylene vinyl alcohol (EVOH), PA and PET) (Topaz B-440 AF, Plastopil, Almere, The Netherlands). CO_2 and N_2 (food grade quality) were mixed using a MAP Mix 9000 gas mixer (Dansensor, Ringsted, Denmark) to obtain a packaging atmosphere of 60% CO_2 and 40% N_2. To ensure correct gas mixture, the gas composition was measured in 10 dummies (sealed packages without products) with an O_2 and CO_2 analyzer (Checkmate 9900 analyzer, Dansensor).

Headspace gas analysis

The headspace gas composition was measured at each sampling point to confirm that the sealed trays contained the desired atmosphere using an O_2 and CO_2 analyzer (Checkmate 9900 analyzer, Dansensor). Before inserting the syringe for headspace gas collection, a rubber septum (Nordic Supply, Skodje, Norway) was placed on the sealing foil to avoid introduction of surrounding atmosphere.

Microbiological analysis

A 10-g piece of salmon was aseptically transferred to a sterile stomacher bag and diluted 1 : 10 with sterile peptone water (0·85% NaCl (Merck) and 0·1% neutralized bacteriological peptone (Oxoid)) and homogenized for 60s using a Stomacher 400 lab blender (IUL Masticator, Barcelona, Spain). Appropriate serial dilutions were made in peptone water. *Aeromonas* spp. were quantified on Starch Ampicillin Agar (SAA) supplemented with 10 mg l^{-1} Ampicillin (Sigma-Aldrich) as described in NMKL (Nordic Committee on Food Analysis 2004 method no. 150). The plates were incubated at 37°C for 24 and 48 h. Total aerobic plate count (APC) including H_2S-producing bacteria (black colonies) were quantified on Lyngby's iron agar (Oxoid) supplemented with 0·04% L-cysteine (Sigma-Aldrich). The plates were incubated at 22°C for 72 ± 6 h.

Identification of presumptive *Aeromonas* in un-inoculated samples

A few colonies growing on SAA from the MAPC8 samples were transferred to TSB and grown overnight at 37°C. Total genomic DNA was extracted from 1 ml culture using the protocol for Gram-negative bacteria in the DNeasy Blood & Tissue kit (Qiagen, Oslo, Norway). An approximately 1100 bp fragment of the bacterial 16S ribosomal RNA (rRNA) was amplified by PCR using primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) (Muyzer *et al.* 1993) and 1492R (5′-ACGGYTACCTTGTTAC GACT-3′) (Turner *et al.* 1999) (0·4 μmol l^{-1} each primer, Sigma-Aldrich). The PCR reactions were performed with 50 μl reactions containing 1 × PCR buffer (1·5 mmol l^{-1} $MgCl_2$), 200 μmol l^{-1} of each nucleotide, 2·5 U Taq Polymerase (Qiagen) and template DNA. The PCR amplification cycles were as follows: initial

denaturation and hot start of enzyme at 95°C for 15 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30 s and polymerization at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were confirmed by electrophoresis in a 1·5% agarose gel (SeaKem, Basel, Switzerland) in 1 × TAE buffer and stained with Gel Red Nucleic Acid Gel Stain (Biotium, CA) (20 000× diluted). The PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific, Oslo, Norway) and Eurofins Genomics (Germany) performed the sequencing. The obtained DNA sequences were compared to available sequences in the NCBI GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov) for identification on genus level.

NMR spectra acquisition and processing

A subset of water-soluble metabolites (TMA: trimethylamine, TMAO: trimethylamine oxide, IMP: inosine monophosphate, H × R: Inosine, Hx: hypoxanthine, 2,3-butanediol, ethanol, glucose, putrescine, tyramine and cadaverine) were analysed as described by Shumilina *et al.* (2015). Metabolites were extracted from the fish muscle using 7·5% trichloroacetic acid (TCA) (Sigma-Aldrich) in water. The samples for NMR analysis were prepared by mixing 55 μl of 10 mmol l^{-1} 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom% D) (Armar Chemicals, Döttingen, Switzerland) and 495 μl of the TCA extract followed by centrifugation at 20 000 × ***g*** (Kubota 3500, Japan) for 5 min (at 8°C) to remove the precipitate. 530 μl of the supernatant was transferred to a standard 5 mm NMR tube.

1D ^{1}H-NMR spectra were acquired at 300 K using a Bruker Avance 600-MHz spectrometer equipped with a 5 mm z-gradient TXI (H/C/N) cryoprobe. All NMR analysis were done at the NMR centre of the faculty of Natural Sciences at NTNU, Trondheim. The ^{1}H-NMR experiments were acquired using the Bruker pulse sequences noesygppr1d with the following settings: 48 number of scans, RG = 144, spectral with 20 ppm. The signal of the external standard TSP was used for spectral calibration (0 ppm). Phase and baseline corrections of the spectra were performed with TopSpin 3.5 pl7 (Bruker, Karlsruhe, Germany) software. An average value of three integrations of the same resonance signal was used for quantification of metabolite concentrations.

Statistical analysis and growth prediction

Statistical analysis on microbial growth were done at log-transformed data. The bacterial counts are presented as means ± standard error (SE), and other mean values are presented as ±1 SD. The log-transformed average bacterial counts were fitted to the primary model of Baranyi and Roberts (1994) (available at www.combase.cc) to estimate the maximum growth rates (μ_{max}) and duration of the lag phases. For analysis of main effects in the metabolite data set, a general linear model (GLM) with packaging atmosphere (MAP or vacuum), inoculation (control or inoculated), and storage temperature (4 or 8°C) as fixed factors was applied. One-way analysis of variance (ANOVA) with Duncan's post hoc test was used for comparison between experimental groups. Effect size in ANOVA (η^2, defined as the proportion of variance accounted for by each of the effects; Tabachnick and Fidell 2014) was calculated as: $\eta^2 = SS_{effect}/SS_{total}$, where SS_{effect} is the sum of squares for the effect of interest, and SS_{total} is the sums of squares for all effects, interactions and errors in the ANOVA. Linear regression was used to assess correlation. The alpha level was set to 5% ($P < 0{\cdot}05$). The statistical analysis was done in IBM SPSS statistical software (version 25, IBM, Armonk, NY).

Results

Headspace gas composition

Analysis of the headspace gas composition was made at each sampling day for the MAP salmon stored at 4°C and 8°C. The initial gas composition was measured to be 60·0 ± 0·4% CO_2 and 40·0 ± 0·5% N_2 and was significantly altered between day 0 and day 3 for all MAP groups ($P < 0{\cdot}001$). The CO_2 concentrations at day 3 were as follows: 42·6 ± 0·9% (MAPC4), 44·4 ± 0·4% (MAPC8), 43·4 ± 0·4% (MAPI4) and 44·5 ± 0·1% (MAPI8). Equilibrium, observed as a stable CO_2 concentration throughout storage, was obtained from day 3 for all groups except MAP8I. The CO_2 level in the latter group increased to 51·5 ± 0·5% at day 21. Overall, the concentration of CO_2 was significantly higher in samples stored at 8°C ($P < 0{\cdot}001$) compared to 4°C, and in inoculated samples versus control samples ($P < 0{\cdot}01$).

Effects of packing atmosphere and storage temperature on the growth of *A. salmonicida* in salmon

Pre-rigor filleted back loins of salmon were inoculated with *A. salmonicida* strain SU2, packed in either vacuum (VP) or modified atmosphere (MAP; 60/40% CO_2/N_2) and stored at 4 and 8°C. Un-inoculated pieces of salmon were packed and stored under the same conditions. The mean initial concentrations of *A. salmonicida* SU2 in inoculated samples were 4·9 ± 0·3 log CFU per g and 4·5 ± 0·1 log CFU per g for the VP and MAP samples, respectively. The inoculated strain of *A. salmonicida* was able to grow in both vacuum and in modified

atmosphere at both storage temperatures. For the VP samples, the concentration of the inoculated strain increased by 1·2 and 2·4 log units during the first 2 days at 4 and 8°C, respectively. The maximum growth rate (μ_{max}) was approximately 2 times higher at 8°C compared to storage at 4°C (Table 1). A maximum bacterial concentration (Y_{max}) of approximately 8 log CFU per g was reached at day 4 and 6 for the two temperatures, respectively (Fig. 1). No *Aeromonas* were detected in the VP control samples at any sampling points.

The combination of MAP and cold storage (4°C) resulted in a maximum concentration of the inoculated strain at 6·4 ± 0·3 log CFU per g after 15 days, including a detectable lag-phase. At 8°C, the growth rate of the inoculated strain was 3·6 times higher than the rate at 4°C (Table 1), but the growth flattened out after 9 days at a concentration near 8 log CFU per g (Fig. 1). No presumptive *Aeromonas* was detected in the MA control samples at any temperatures during 21 days of storage. However, colonies that were not typical for *Aeromonas* (small, white) appeared on the SAA plates after 6 and 15 days of MAP storage at 8 and 4°C, respectively. This population continued to increase throughout storage to a maximum concentration of 7·5 log CFU per g (8°C) and 1·1 log CFU per g (4°C) and were later confirmed by sequencing of the 16S rRNA gene as *Yersinia* spp. (graphs not shown). Overall, MAP combined with cold storage (4°C) resulted in a twofold reduction of the *A. salmonicida* growth rate compared to VP.

Quantification of the total aerobic count in the inoculated VP samples resulted in only black colonies (on iron agar). The black colonies, representing H_2S-producing bacteria, were observed in the un-inoculated samples as well, but these samples had a significant proportion of white colonies (Fig. 2a). The H_2S-producing bacteria in the VP control samples appeared at day 10 (1·1 ± 0·01 log CFU per g) and increased to 5·3 ± 0·05 log CFU per g at day 14 at 4°C. The H_2S-producing bacteria appeared earlier (after 8 days) when stored at 8°C but reached a lower maximum concentration (4·3 ± 2·2 log CFU per g) at day 12 (graphs for H_2S-producing bacteria only are

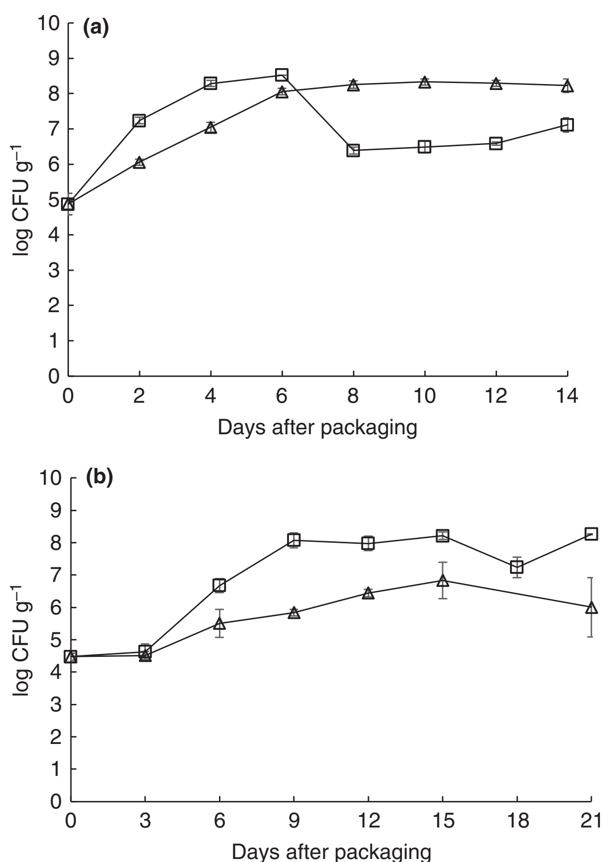

Figure 1 Growth of *Aeromonas salmonicida* strain SU2 in (a) vacuum-packaged salmon stored at 4°C (Δ) and 8°C (□) for 14 days and in (b) MAP salmon (60/40% CO_2/N_2) stored at 4°C (Δ) and 8°C (□) for 21 days. Each sampling point represents the average bacterial count (n = 3, except day 0 where n = 4), and vertical bars indicate ± SE.

not shown). There was a significant correlation between the concentration of *A. salmonicida* quantified on SAA and the H_2S-producing bacteria quantified as black colonies on iron agar at 4°C ($R^2 = 0·90$, $P < 0·001$ for VPI4). The correlation was weaker but also significant at 8°C ($R^2 = 0·51$, $P < 0·05$). The total count in the control samples increased from the initial sampling point and

Table 1 Growth kinetic parameters (maximum growth rate (μ_{max}, day^{-1}), lag phase duration (days) and maximum population density (Y_{max}, log CFU per g) of *A. salmonicida* strain SU2 inoculated to salmon packaged in vacuum or modified atmosphere (60/40% CO_2/N_2) and stored at 4 and 8°C. The parameters were estimated using the primary growth model of Baranyi and Roberts (1994). R^2 is the fit of the model to the dataset and SE is the standard error of the estimate

Packaging	Storage temp (°C)	μ_{max} (day^{-1})	Lag phase (days)	Y_{max} (log CFU per g)	R^2	*SE*
Vacuum	4	0·56 ± 0·04	No lag	8·23 ± 0·05	0·991	0·114
	8	1·19 ± 0·12	No lag	8·42 ± 0·12	0·991	0·162
Modified atmosphere	4	0·24 ± 0·13	2·4 ± 3·4	6·43 ± 0·25	0·814	0·389
	8	0·88 ± 0·69	3·5 ± 2·3	7·95 ± 0·19	0·931	0·414

onwards and reached a maximum density of 7·5–8 log CFU per g for both storage temperatures (Fig. 2a).

The initial concentration of the total aerobic count, represented by H_2S-producing bacteria only, was not significantly different from the quantified concentration of inoculated *A. salmonicida* on SAA in the MAP samples (P = 0·74) and there was a significant correlation between the concentration of H_2S-producing bacteria and bacteria quantified on SAA during the entire storage period (R^2 = 0·73 and P = 0·007 for MAPI4 and R^2 = 0·99 and P < 0·001 for MAPI8), indicating that the black colonies on iron agar represent the inoculated strain. Growth of non-H_2S-producing bacteria was detected in the control samples, and there was a temperature-dependent increase in the total aerobic count. In the MAP control samples stored at 8°C, the concentration of total aerobic bacteria reached 8·6 ± 0·2 log CFU per g after 18 days (Fig. 2b). H_2S-producing bacteria appeared after 9 days (4·5 ± 0·2 log CFU per g) and were relatively stable throughout the storage period. The combination of MAP and refrigeration temperature (4°C) was enough to delay the growth of the total microbiota, and bacterial growth was first detected after 9 days and reached a maximum density of 5·2 ± 0·2 log CFU per g at day 21, but no H_2S-producing bacteria were detected.

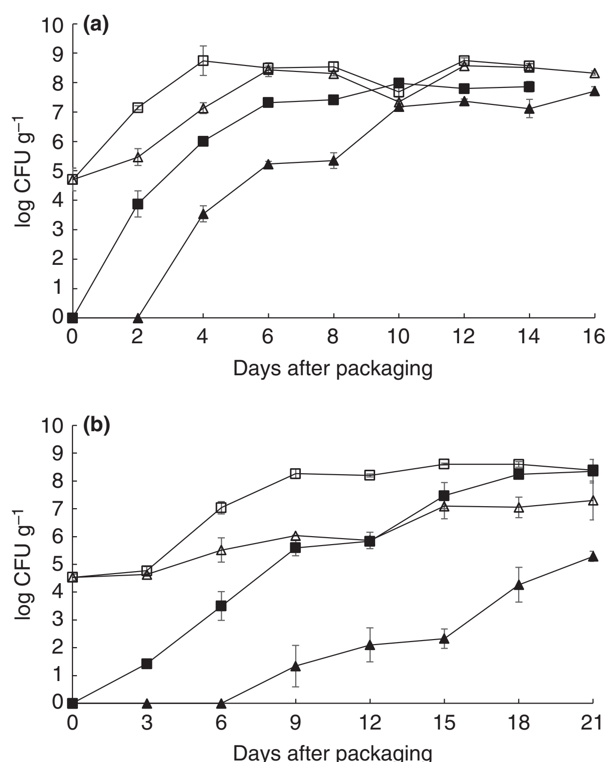

Figure 2 Total aerobic plate count (white and black colonies) in (a) vacuum-packaged salmon stored at 4°C (▲) and 8°C (■), and in inoculated samples stored at 4°C (Δ) and 8°C (□) and in (b) MAP salmon stored at 4°C (▲) and 8°C (■), and in inoculated samples stored at 4°C (Δ) and 8°C (□).

Quantitative analysis of metabolites with spoilage potential in salmon

A subset of water-soluble metabolites implicated in fish spoilage were detected in VP and MAP samples stored at 4 and 8°C using ^{1}H-NMR. In the present study, post-mortem changes in the metabolite composition in control and inoculated samples were analysed at day 0, 6 and day 14 (VP) and day 15 (MAP) after packaging.

The average concentrations of TMAO and TMA in the salmon fillets before packaging were 40 ± 7 and 0·2 ± 0·04 mg 100 g^{-1}, respectively (TMA showed in Table 2). A correlation between the decrease in TMAO concentration and the increase in TMA concentration was observed during storage (R^2 = 0·75, P < 0·05). The main effect analysis shown that all variables, storage time, inoculation with *A. salmonicida*, storage temperature and packaging atmosphere, had a significant effect on the concentration of TMA (GLM, P < 0·001 for all factors), but there were no interaction effects (P > 0·05 for all variables). Storage time was the factor that contributed most to the observed variance in TMA concentration (effect size 35%), followed by packaging atmosphere (effect size 15%). Packaging in MAP resulted in a significantly lower TMA level compared to VP (P = 0·001) and higher TMA concentrations were detected in samples stored for 14/15 days compared with the earlier sampling points (P < 0·001).

Moreover, the concentration of TMA was significantly higher in samples inoculated with *A. salmonicida* compared to untreated control samples (P = 0·012) and in samples stored at 8°C compared to 4°C (P = 0·027). For samples stored in vacuum, inoculation with *A. salmonicida* resulted in a higher TMA concentration compared to untreated control samples (P = 0·043), but the inoculation effect disappeared after 14 days for samples stored at 8°C. At this time point, there was no difference in TMA level between control and inoculated samples stored at 8°C (25·4 ± 2·9 mg 100 g^{-1} and 28·0 ± 2·0 mg 100 g^{-1}, respectively), nor between inoculated samples stored at 4 and 8°C (P> 0·05) (27·3 ± 1·7 mg 100 g^{-1} and 28·0 ± 2·0 mg 100 g^{-1}, respectively). MAP packaging, on the other hand, kept the TMA concentration relatively low during storage, although the TMA level increased somewhat with storage temperature and storage time (P < 0·05). A strong inoculation effect was observed at the last sampling point (15 days) in samples stored at 8°C (TMA = 25·6 ± 2·3 mg 100 g^{-1}) compared to the control at the same temperature (2·9 ± 0·9 mg 100 g^{-1}; Table 2).

Table 2 Concentration of analysed metabolites (TMA: trimethylamine, IMP: inosine monophosphate, HxR: inosine and Hx: hypoxanthine) by ^{1}H-NMR (mg 100 g^{-1}) in vacuum-packaged (VP) and MAP salmon stored at 4 and 8°C and inoculated with *A. salmonicida* stored at 4°C and 8°C on days 0, 6 and day 14/15 after packaging. Mean value (n = 3) ± 1 SD is presented for each metabolite. ND = not detected. Different superscript letters indicate significant differences between different groups in each column (P < 0·05)

Packaging/group	Storage temp (°C)	Days after packaging	TMA (mg 100 g^{-1})	IMP (mg 100 g^{-1})	HxR (mg 100 g^{-1})	Hx (mg 100 g^{-1})
VP Control*	4, 8	0	0·2 ± 0·0^{a}	65·1 ± 9·3^{c}	69·5 ± 8·7^{b}	7·8 ± 0·6^{a}
VP Control	4	6	0·2 ± 0·0^{a}	24·0 ± 6·3^{ab}	138·8 ± 6·1^{gh}	15·5 ± 0·6^{abc}
VP Control	4	14	12·4 ± 3 .7^{b}	5·9 ± 1·6^{a}	104·8 ± 13·6^{cdef}	34·7 ± 5·6^{g}
VP Inoculated*	4, 8	0	0·3 ± 0·0^{a}	116·4 ± 7·8^{d}	92·5 ± 1·7^{bcde}	11·3 ± 0·6^{ab}
VP Inoculated	4	6	8·8 ± 3·2^{b}	27·5 ± 15·8^{ab}	138·0 ± 6·2^{gh}	16·5 ± 0·9^{abcd}
VP Inoculated	4	14	27·3 ± 1·7^{c}	4·6 ± 1·3^{a}	85·0 ± 5·3^{bc}	44·4 ± 2·6^{h}
VP Control	8	6	4·3 ± 0·2^{a}	3·5 ± 1·6^{a}	118·9 ± 3·0^{defg}	24·8 ± 0·5^{def}
VP Control	8	14	25·4 ± 2·9^{c}	2·0 ± 0·2^{a}	35·1 ± 13·1^{a}	66·0 ± 3·3^{i}
VP Inoculated	8	6	27·2 ± 2·1^{c}	2·7 ± 0·9^{a}	89·6 ± 11·8^{bcd}	34·4 ± 3·3^{g}
VP Inoculated	8	14	28·0 ± 2·0^{c}	2·5 ± 0·8^{a}	32·1 ± 9·2^{a}	67·6 ± 8·8^{i}
MAP Control*	4, 8	0	0·2 ± 0·0^{a}	65·1 ± 9·3^{c}	69·5 ± 8·7^{b}	7·8 ± 0·6^{a}
MAP Control	4	6	0·2 ± 0·0^{a}	33·0 ± 17·2^{b}	146·8 ± 3·2^{h}	16·3 ± 1·3^{abcd}
MAP Control	4	15	4·1 ± 3·6^{a}	4·6 ± 0·5^{a}	97·7 ± 22·9^{bcde}	20·4 ± 1·8^{bcd}
MAP Inoculated*	4, 8	0	0·3 ± 0·0^{a}	116·4 ± 7·8^{d}	92·5 ± 1·7^{bcde}	11·3 ± 0·6^{ab}
MAP Inoculated	4	6	0·2 ± 0·0^{a}	38·4 ± 21·6^{b}	142·3 ± 11·9^{gh}	22·7 ± 3·4^{cde}
MAP Inoculated	4	15	1·8 ± 0·7^{a}	5·1 ± 0·4^{a}	118·3 ± 4·3^{defg}	29·6 ± 3·5^{efg}
MAP Control	8	6	1·3 ± 1·1^{a}	4·9 ± 0·6^{a}	138·5 ± 2·4^{gh}	21·0 ± 1·6^{cde}
MAP Control	8	15	2·9 ± 0·9^{a}	4·9 ± 0·4^{a}	95·4 ± 7·7^{bcde}	33·4 ± 3·5^{fg}
MAP Inoculated	8	6	3·5 ± 0·8^{a}	3·7 ± 0·3^{a}	129·1 ± 5·1^{fgh}	25·2 ± 1·6^{def}
MAP Inoculated	8	15	25·6 ± 2·3^{c}	5·3 ± 1·6^{a}	73·6 ± 3·2^{b}	43·0 ± 1·5^{gh}

*The control samples at day 0 were analysed before packaging and storage and are thereby only separated by inoculation/no inoculation.

As an indicator of fish freshness, the ATP degradation products, inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx), were quantified in VP and MAP samples (Table 2). The main effect analysis showed that the observed differences in the concentration of IMP was mainly influenced by storage time (GLM, P < 0·001, effect size 62%) and by inoculation with *Aeromonas* (GLM, P = 0·006, effect size 4%), and there was no significant effect of packaging atmosphere nor temperature on the IMP concentration (GLM, P> 0·05 for both factors). There were no differences in the concentration of inosine between inoculated and control samples (P > 0·05) and the observed differences in inosine concentration could only be explained by packaging atmosphere (P = 0·032) and storage temperature (P = 0·006). For hypoxanthine, the main effect analysis showed that sampling day, inoculation, packaging atmosphere and storage temperature contributed to the observed differences in the concentration of Hx (GLM, P < 0·01 for all factors). Sampling day was the factor that contributed most to the observed variance in Hx concentration (effect size 62%), and the Hx concentration increased significantly throughout storage, from 9·5 ± 2·0 mg 100 g^{-1} at day 0 to 42·4 ± 17·3 mg 100 g^{-1} at day 14/15 (P < 0·001).

The concentrations of 2,3-butanediol, ethanol and glucose were not affected by inoculation of *A. salmonicida* (P> 0·05) (Table 3). However, MAP resulted in a lower concentration of 2,3-butanediol (P < 0·001) and EtOH (P = 0·022) compared to VP. Correspondently, the concentration of glucose was also higher in MAP samples (P = 0·001). Whereas the glucose concentration was not affected by temperature in MAP samples, VP samples stored at 4°C had a significantly higher concentration of glucose compared to VP samples stored at 8°C (P = 0·012).

The biogenic amines (putrescine, tyramine and cadaverine) were not initially detected in any sample (day 0). Moreover, there was no putrescine detected in any VP samples stored at 4°C nor in any MAP samples. The NMR spectra indicated that cadaverine was present in all VP samples at days 6 and 14 and higher concentrations of cadaverine in VP compared to MAP samples. However, due to a high degree of overlapping signals and thereby an uncertain quantification, these results are not addressed in more detail.

Discussion

In this study, the growth kinetic parameters and possible spoilage metabolite production of *A. salmonicida* were studied in inoculated salmon under various packaging atmospheres (MAP and vacuum) and storage

Table 3 Concentration of analysed metabolites (2,3-butanediol, ethanol and glucose) by ^{1}H-NMR (mg 100 g^{-1}) in vacuum-packaged (VP) and MAP salmon stored at 4°C and 8°C and inoculated with *A. salmonicida* stored at 4°C and 8°C on days 0, 6 and 14/15 after packaging. Mean value (n = 3) ± 1 SD is presented for each metabolite. ND = not detected. Different superscript letters indicate significant differences between different groups in each column (P < 0·05)

Packaging/group	Storage temp (°C)	Days after packaging	2,3-butanediol (mg 100 g^{-1})	Ethanol (mg 100 g^{-1})	Glucose (mg 100 g^{-1})
VP Control*	4, 8	0	0·5 ± 0·0^{a}	2·9 ± 2·3^{ab}	38·7 ± 9·1^{bcd}
VP Control	4	6	ND	6·9 ± 2·5^{bc}	51·9 ± 6·0^{cde}
VP Control	4	14	25·8 ± 12·2^{b}	1·8 ± 0·4^{ab}	44·7 ± 8·0b^{cde}
VP Inoculated*	4, 8	0	0·7 ± 0·1^{a}	2·6 ± 2·0^{a}	50·1 ± 5·2^{cde}
VP Inoculated	4	6	1·6 ± 0·8^{a}	8·5 ± 1·5^{b}c	54·9 ± 8·7^{cde}
VP Inoculated	4	14	29·6 ± 3·2^{b}	2·0 ± 0·3^{ab}	22·5 ± 6·0^{ab}
VP Control	8	6	1·8 ± 0·3^{a}	5·9 ± 1·0^{abc}	44·2 ± 7·2^{bcde}
VP Control	8	14	48·3 ± 11·5^{c}	1·6 ± 1·3^{ab}	3·0 ± 0·6^{a}
VP Inoculated	8	6	28·2 ± 4·5^{b}	17·3 ± 2·8^{d}	19·9 ± 3·7^{ab}
VP Inoculated	8	14	44·6 ± 12·8^{c}	2·0 ± 0·6^{ab}	5·5 ± 1·6^{a}
MAP Control*	4, 8	0	0·5 ± 0·0^{a}	2·9 ± 2·3^{ab}	38·7 ± 9·1^{bcd}
MAP Control	4	6	0·6 ± 0·2^{a}	4·0 ± 0·4^{ab}	68·6 ± 3·9^{e}
MAP Control	4	15	1·9 ± 0·6^{a}	0·9 ± 0·2^{a}	44·7 ± 3·6^{bcde}
MAP Inoculated*	4, 8	0	0·7 ± 0·1^{a}	2·6 ± 2·0^{a}	50·1 ± 5·2^{cde}
MAP Inoculated	4	6	0·6 ± 0·2^{a}	4·2 ± 0·9^{ab}	65·1 ± 16·3de
MAP Inoculated	4	15	1·5 ± 0·0^{a}	1·4 ± 0·3^{a}	56·8 ± 7·9^{cde}
MAP Control	8	6	1·6 ± 0·5^{a}	3·2 ± 0·5^{ab}	50·4 ± 3·3^{cde}
MAP Control	8	15	3·3 ± 0·7^{a}	1·6 ± 0·7^{ab}	50·1 ± 1·1^{cde}
MAP Inoculated	8	6	0·9 ± 0·1^{a}	3·0 ± 0·6^{ab}	62·6 ± 17·3de
MAP Inoculated	8	15	9·7 ± 0·4^{a}	1·6 ± 0·2^{ab}	33·1 ± 11·8^{bc}

*The control samples at day 0 were analysed before packaging and storage and are thereby only separated by inoculation/no inoculation.

temperatures (4 and 8°C) and compared with uninoculated control samples over a period corresponding to the expected shelf-life (14 days for vacuum and 21 days for MAP).

The initial headspace CO_2 concentration significantly decreased until equilibrium was obtained for both storage temperatures applied. CO_2 is generally highly soluble in the water phase of the fish muscle and in fluid lipids (Gill 1988; Sivertsvik *et al.* 2004) and diffusion of CO_2 from headspace into the fish muscle occur until equilibrium is reached. The headspace CO_2 concentration of samples stored at 8°C stabilized as expected at a higher level than samples stored at 4°C, indicating less dissolved CO_2 in the fish muscle. The solubility of CO_2 in the product is highly temperature-dependent (Sivertsvik *et al.* 2004), and high storage temperature results in less dissolved CO_2 in the product and consequently loss of inhibitory activity towards micro-organisms. In addition, the overall diffusion of CO_2 into the food matrix depends on several factors, for example, initial gas content, package surface/volume, mass of food, geometry and structure (Chaix *et al.* 2014). Moreover, production of CO_2 resulting from microbial growth could contribute to the higher level of CO_2 in the headspace of inoculated samples compared to control samples.

The present study demonstrated that an atmosphere of 60% CO_2/40% N_2 in combination with refrigeration (4°C) was not efficient to inhibit growth of *A. salmonicida* in salmon. To our knowledge, no comparable growth data exist on *A. salmonicida* in MAP salmon, but Provincial *et al.* (2013) previously showed that an atmosphere containing 70% CO_2 combined with strict temperature control was necessary to inhibit the growth of *A. hydrophila* in sea bream (*S. aurata*). Although the combination of the applied MAP condition and refrigeration did not prevent the growth of *A. salmonicida* in our setup, the growth rate was affected by both storage temperature and packaging atmosphere. In vacuum, the inoculated strain was able to multiply without any lag-phase regardless of storage temperature. Packaging in MA resulted in a twofold reduction of the μ_{max} and a 2-log reduction in the maximum concentration of *Aeromonas* in salmon stored at 4°C. However, storage temperature had the greatest impact on the growth rate of *A. salmonicida*, which was 2·1 (VP) and 3·6 (MAP) times higher at 8°C than at 4°C, and in accordance with previously reported data from aerobically stored salmon (Hoel *et al.* 2018). This clearly demonstrates that the combination of an optimal packaging atmosphere and temperature control is needed to prevent the growth of *A. salmonicida* in salmon.

There are no microbiological criteria stating the maximum acceptable number of bacteria in seafood, nor does it exist a fixed correlation between the total APC and the remaining shelf-life of a product. Quality control and shelf-life estimation are often estimated based on the total aerobic psycrotrophic count (Broekaert *et al.* 2011), and standards and guidelines generally accept an APC of <7 log CFU per g for satisfactory microbiological quality (Food and Environmental Hygiene Department of Hong Kong (HKSAR) 2007; Food Safety Authority of Ireland (FSAI) 2016). Some studies have indicated that an APC of 6–8 log CFU per g corresponds to a detectable sensory spoilage in fish (Dalgaard 1995; Gram and Dalgaard 2002; Boziaris and Parlapani 2017) but differences owing to fish species and SSO must be considered. Sensory evaluation was not performed in the present study because of the potentially pathogenicity of the inoculated strain (Hoel *et al.* 2017). A level of 7 log CFU g^{-1} is therefore applied as the limit for satisfactory microbiological quality for the evaluation of packaging and storage.

Our results demonstrated that vacuum packaging did not preserve the microbial quality of the packaged salmon throughout the whole storage period of 14 days. At refrigerated storage, the bacterial count exceeded the limit of 7 log CFU g^{-1} after 10 days. Inoculation with *A. salmonicida* reduced the microbiological shelf-life by 6 days compared to the control owing to the increased initial bacterial load. As expected, MAP gave a longer microbiological shelf-life than VP, and the samples inoculated with *A. salmonicida* stored at 4 and 8°C reached 7 log CFU per g at days 15 and 6, respectively. The control samples stored at 4°C never exceeded the limit of acceptance and the microbiological shelf-life of MAP salmon with a low initial contamination stored under constant refrigeration could under these conditions be at least 21 days. Although the concentration of APC can be used as a general indicator of microbiological quality, the concentration of APC does not indicate the presence of SSO for the specific food under the specific packaging conditions that might better predict spoilage (Fogarty *et al.* 2019). The level of H_2S-producing bacteria was generally low in the control samples and only detected sporadically in concentrations below 2 log CFU g^{-1} the first 12 days, which is expected as the most common H_2S-producing genera, *Shewanella,* is inhibited by a CO_2 atmosphere (Broekaert *et al.* 2011). The H_2S-producing bacteria detected in the inoculated samples were most probably the inoculated *Aeromonas* strain, and the fact that no white colonies were observed on the iron agar for these samples suggest that the inoculated strain was the dominating species. *P. phosphoreum* and *B. thermosphacta* could also be spoilers of VP and MAP salmon (Fogarty *et al.* 2019), and it not unlikely that one or both were present but did not grown on the iron agar (Broekaert *et al.* 2011).

TMA was the metabolite that was most affected by inoculation of *A. salmonicida*. In general, variations in the quantity of the other analysed metabolites were more influenced by the variables storage time, temperature and atmosphere. TMA is produced by some bacteria capable of using TMAO in anaerobic respiration and contributes to the characteristic ammonia-like and fishy off-flavours in spoiled fish (Gram and Dalgaard 2002). The TMAO content in fish depends on fish species, the age, size and diet of the fish, and season (Vanwaarde 1988). In the present study, the initial level was approximately at the same level as reported by Kirkholt *et al.* (2019). Our results demonstrated that TMA was produced in samples inoculated with a strain of *A. salmonicida* and stored under vacuum and modified atmosphere. As expected, there was an effect of storage time, temperature and atmosphere on the TMA concentration in the salmon samples. The ability of *A. salmonicida* to produce TMA is previously demonstrated in large yellow croaker (*Pseudosciaena crocea*) (Liu *et al.* 2018), in Common carp (Beaz-Hidalgo *et al.* 2015) and in MAP shrimps (*P. vannamei*) (Macé *et al.* 2014). However, there are probably strain differences as well as substrate differences as demonstrated by the lack of TMA production by strains of the species *A. trota* and *A. allosaccharophila* in Common carp and in silver carp (*Hypophtalmichthys molitrix*) (Beaz-Hidalgo *et al.* 2015; Jia *et al.* 2019). The level of TMA found in fresh fish rejected by sensory panels is typically around 10–15 mg 100 g^{-1} (anaerobically stored fish) (Huss 1995). The spoilage criterion of TMA is suggested by Dalgaard (1995) to be 30 mg 100 g^{-1} for fresh MAP cod. However, due to the lower content of TMAO in salmon, concentration exceeding 12 mg 100 g^{-1} is proposed as a limit for spoiled salmon (Macé *et al.* 2012). Thus, in our study, all VP samples, except for the control samples stored at 4°C, exceeded the spoilage criterion of TMA at day 14. Among the MAP samples, only inoculated samples stored at 8°C could be characterized as spoiled due to evaluated level of TMA. Furthermore, Mikš-Krajnik *et al.* (2016) found that the level of TMA was 11·5 mg 100 g^{-1} at the time when the APC reached 7 log CFU per g. In the present study, a weak but significant correlation between the APC and TMA concentrations was found ($R^2 = 0{\cdot}638$, $P < 0{\cdot}01$), which is expected as increased bacterial densities will result in more TMA if the organisms can reduce the available TMAO. Inoculation with *A. salmonicida* resulted in a higher TMA concentration compared to the uninoculated control at the same sampling point ($P < 0{\cdot}05$).

The development of TMA is in some fish species paralleled by the production of Hx. Hx is an ATP degradation

product formed by the autolytic breakdown of nucleotides but it can also be formed by bacteria such as *Pseudomonas* spp., *S. putrefaciens* and *P. phosphoreum*, and the rate of bacterial Hx formation is higher than the autolytic breakdown (Jorgensen *et al.* 1988; Huss 1995). The accumulation of Hx is responsible for the progressive loss of desirable flavour and will eventually lead to a bitter taste (Hong *et al.* 2017). In the present study, Hx was the only ATP degradation product that showed increased quantities following inoculation with *A. salmonicida*, and thus there were a correlation between the TMA and Hx formed ($R^2 = 0{\cdot}844$; $P < 0{\cdot}001$). Moreover, the presence of *A. salmonicida* had no detectable effect on the concentration of 2,3-butanediol and ethanol in VP nor in MAP, indicating that these metabolites are not produced by *Aeromonas* under the tested conditions. It is however important to point out that the potential spoilage metabolites following growth of *A. salmonicida* was investigated in a natural system containing its endogenous microbiota. No *Aeromonas* was initially present (nor detected during storage) which enables the analysis of the contribution of the inoculated strain, but the non-*Aeromonas* bacterial count reached almost 8 log CFU g^{-1} (VP) and we do not know the growth kinetics nor the spoilage potential of the different bacterial fractions. Food spoilage is a complex bacterial process and a result of interactions between organisms in microbial communities (Gram and Dalgaard 2002). To reduce the contribution from an un-characterized background microbiota, further approaches should aim to study the effect of *A. salmonicida* in an uncontaminated system. This could be achieved by fillet freshly caught whole salmon in-house to reduce post-harvest contamination (Hoel *et al.* 2018), by sterilizing the fish by gamma irradiation (Macé *et al.* 2013), by submerging the fish in methanol (5%) (Li *et al.* 2017), or to study the bacterial metabolites in a liquid model system such as a sterilized fish extract (Wiernasz *et al.* 2017). Furthermore, the data on metabolite production resulting from growth of *Aeromonas* in an uncontaminated system must be accompanied by a sensory analysis to further explore the role of *Aeromonas* as a potential spoilage organism in salmon. Thus, the data presented here should be regarded as preliminary with respect to the spoilage potential of *A. salmonicida* in salmon products. Nevertheless, the relative differences in metabolites produced in inoculated samples compared to the control can be identified and generate new hypotheses of the role of *A. salmonicida* as a potential spoilage organism in chilled, packaged salmon.

Furthermore, to better understand the spoilage potential of *Aeromonas* in MAP seafood, a broader approach to study tolerance to CO_2 should be performed; both regarding range of CO_2 levels applied and inter-species heterogeneity of *Aeromonas* with respect to growth kinetics and production of metabolites at different CO_2 levels.

In conclusion, this study presents the preliminary results on *A. salmonicida* as a potential spoilage organism in vacuum-packaged salmon during cold storage due to its ability to grow relatively uninhibited. Moreover, increased production of TMA and Hx in inoculated samples was observed. The combination of refrigeration (4°C) and a MAP atmosphere consisting of 60% CO_2 reduced the growth rate of *A. salmonicida* and prevented the formation of TMA. Further studies on the effect of CO_2 concentrations on the growth and production of spoilage metabolites following inoculation in a controlled model system accompanied by sensory evaluation as well as studies of bacterial interactions in complex food systems are needed to elucidate the actual role of *A. salmonicida* as a spoilage organism in salmon products.

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Conflict of Interest

The authors declare no conflicts of interest regarding the submitted manuscript. All authors have approved the submission.

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