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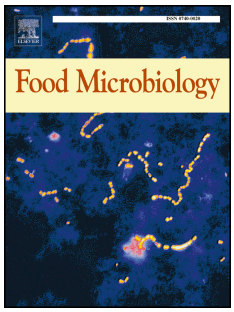

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Superchilling in combination with modified atmosphere packaging resulted in long shelf-life and limited microbial growth in Atlantic cod (*Gadus morhua* L.) from capture-based-aquaculture in Greenland

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Abstract

Sensory, chemical and microbial changes for Atlantic cod (*Gadus morhua* L.) filets from capture-based-aquaculture in Greenland were studied. The objective was to determine shelf-life and indices of spoilage for iced or superchilled fillets when stored in air, or modified atmosphere packed (MAP; 40% CO_2 and 60% N_2). MAP iced storage extended the sensory shelf-life from 15 days to 21 days compared to storage in air. With superchilling at -1.7 $^{\circ}$C sensory shelf-life was above 32 days, and no formation of total volatile nitrogen (TVN) was observed irrespective of storage in air or MAP. pH of ≥7.0, TVN (≥35 mg-N/100g) and trimethylamine (≥20 mg-N TMA/100g) were promising indices of spoilage. Aerobic viable counts were less valuable indices of spoilage as the dominating microbiota of cod in air (*Pseudomonas* spp., *Photobacterium* spp., *Shewanella* spp., *Acinetobacter* spp.) changed to *Photobacterium* spp. in MAP cod. Spoilage activity determined as the yield factor for TVN formation was 6-200 folds higher for *Photobacterium* spp. compared to *Shewanella* spp. and *Pseudomonas* spp. *Photobacterium carnosum* was responsible for TVN formation in iced cod irrespective of storage in air or MAP, and it was identified at the specific spoilage organism that limited iced product shelf-life.

Keywords: Storage trial, indices of spoilage, total volatile nitrogen (TVN), 16S rRNA gene amplicon sequencing, specific spoilage organisms (SSO), spoilage activity.

1. Introduction

Fisheries make-up more than 90 % of the total export value from Greenland and Atlantic cod (*Gadus morhua* L.) has been an important species (Statistics Greenland, 2018). After a collapse of stocks for Atlantic cod in the 1990s (Buch et al., 1994; Storr-Paulsen and Wieland, 2006) this fishery is now regaining volume, primarily with in-shore catches by smaller fishing vessels (Statistics Greenland, 2019). Traditionally whitefish, including cod, from Greenland were shipped frozen to the primary export market in Europe. The frozen distribution was appropriate due to variable fish landings and typical transit time by ship of approximated two weeks.

Capture-based aquaculture (CBA) was newly introduced as a fishery and processing technic for cod in Greenland, with full scale production starting in 2015. The cod was caught by pound nets near the costal line and kept alive up to about two weeks in net enclosure next to the fishing ground and without additional feeding. Live cod, where then transported by well-boat to a processing plant where the fish was kept in large nets for two-four days. Cod were electrically stunned, slaughtered by machine decapitation, viscera removed, bleed and fileted within two hours from the time fishes are pumped from nets to the processing line. The combination of CBA and fast processing gives new options for distribution, which include shipment of non-frozen cod with reduced energy consumption and carbon footprint compared to frozen shipment. One option was superchilling at about -2°C with the potential benefit that cod do not need to be covered with iced and therefore more fish can be transported per volume of ship hull. This reduce the carbon footprint for transport (Claussen et al., 2011; Hoang et al., 2016) and provide a potential to meet market demands for non-frozen fish (Altintzoglou et al., 2012). However, for non-frozen cod from Greenland to be transported and marketed in Europe a shelf-life above 19 days is needed with about 10 days for superchilled transport by ship, seven days for ground distribution including display in supermarkets at +2 $^{\circ}$C and one to two day of consumer's storage at +5 $^{\circ}$C (James and James, 2014; Koutsoumanis and Gougouli, 2015).

Shelf-life and quality changes of Atlantic cod from Europe and the North-East Atlantic have been extensively studied. During chilled storage, quality changes were characterised by a loss of freshness due to autolytic reactions followed by sensory spoilage resulting from microbial activity including the reduction of trimethylamine-oxide (TMAO) to trimethylamine (TMA) by specific spoilage organisms (SSO) that grow to high concentrations. For iced cod the sensory shelf-life of aerobically stored and MAP fillets were typical of 10-14 days and 14-26 days, respectively, with indices of spoilage including total volatile nitrogen (TVN), TMA and concentrations of SSO (Dalgaard, 2000; DeWitt and Oliveira, 2016; Sivertsvik et al., 2002). For aerobic storage in ice, H_2S-producing *Shewanella* and *Photobacterium phosphoreum* have been identified as the SSO responsible for spoilage and TMA formation. *P. phosphoreum* with high resistance to CO_2 and pronounced TMA formation was the SSO in chilled MAP cod. Growth of H_2S-producing *Shewanella* was markedly reduced by CO_2, and therefore they were not responsible for spoilage and TMA formation in iced MAP cod (Dalgaard, 2006; Hovda et al., 2007; Kuuliala et al., 2018; Olafsdottir et al., 2005; Reynisson et al., 2009). The microbiota of live Atlantic cod differs for fish from the Baltic sea, the Norths sea and the North-East Atlantic ocean (Wilson et al., 2008). In Greenland, the low seawater temperature of less than 1°C to 5 °C (Buch, 2002) may select for a microbiota that differs from those of cod caught in warmer waters, but we have found no previous studies of this or of the potential effect on product spoilage and shelf-life.

Compared to iced storage, superchilling of Atlantic cod has been little studied. However, shelf-life of from 12 to >42 days has been observed for storage at -0.9 to -2.2 °C (Duun and Rustad, 2007; Eliasson et al., 2019; Lauzon et al., 2009; Olafsdottir et al., 2006; Wang et al., 2008). The combination of superchilling and MAP with 50% CO_2, 5% O_2 and 45% N_2 resulted in shelf-life of 21 to >24 days for Atlantic cod from Iceland and similar results have been found for other fish species including wolfish and salmon from Norway (Lauzon et al., 2009; Rosnes et al., 2006; Sivertsvik et al., 2003; Wang et al., 2008). Thus, superchilling may provide sufficient shelf-life of Atlantic cod for non-frozen transport from Greenland to Europe.

The objective of the present study was to determine shelf-life and indices of spoilage of iced and superchilled Atlantic cod from CBA in Greenland and thereby to evaluate the feasibility of non-frozen transportation to Europe. Firstly, sensory, chemical and microbial changes were studied in a storage trial with aerobically or MAP stored cod. The spoilage microbiota was studied by culture-dependent techniques and by *16S rRNA* gene amplicon sequencing. Secondly, to point out SSO and evaluate indices of spoilage the spoilage potential and the spoilage activity of isolates from the spoilage microbiota were determined.

2. Materials and methods

2.1 Storage trial with fresh Atlantic cod from capture-based aquaculture.

2.1.1 Fish raw material, packaging and storage conditions.

Atlantic cod (*Gadus morhua* L.) were captured inshore by pound net at NAFO fishing ground 1C during September 2018, kept alive in net enclosures next to the fishing ground, transported by well-boat to a fish factory in Maniitsoq, Greenland and kept alive in net enclosures until the time of processing. The cod was slaughtered by decapitation and rinsed before machine filleting. One hundred fourteen filets were taken directly from the production line and cut by hand into 342 pieces, each with a weight of approximated 100 grams. Between the cuttings of each filet, cutting boards and knives were rinsed with 96% ethanol to avoid cross-contamination of microorganism between fillets. Each piece of cod was packed individually in plastic bags of 70 µm thick polyethylene film with high permeability of >6 g m^{-2} d^{-1} for water vapour, >3,000 cm^3 m^{-2} d^{-1} atm^{-1} for O_2 and >14,000 cm^3 m^{-2} d^{-1} atm^{-1} for CO_2 (H902, Topiplast A/S, Greve, Denmark) and transported by aeroplane to DTU Food, Denmark in polystyrene boxes where the fish was cooled with gel ice packs (Sorbatek, Sorbafreeze, Glenrothes, UK). At DTU Food, the pieces of cod were randomly divided into four treatments, with 90 pieces for each treatment except treatment (i) with 63 pieces. In addition, nine pieces were used for sensory, chemical and microbial evaluation of fresh cod prior

110 to initiation of storage (See 2.1.2-2.1.5). For the four treatments, each cod piece was placed in plastic trays
111 (71-51A hvid/PS, Færch Plast, Holstebro, Denmark).

112 A storage trial with four treatments were carried out including (i) aerobic storage in ice; (ii)
113 aerobic superchilled storage in slurry ice; (iii) MAP (40% CO_2 and 60% N_2) storage in ice and (iv) superchilled
114 MAP (40% CO_2 and 60% N_2) storage in slurry ice. Trays with cod were packed using bags of a 117 ± 6 µm
115 laminate film with low gas permeability of 0.45 $cm^3\ m^{-2}\ d^{-1}\ atm^{-1}$ for O_2 and 1.8 $cm^3\ m^{-2}\ d^{-1}\ atm^{-1}$ for CO_2
116 (NEN 40 HOB/LLPDE 75, Amcore, Horsens, Denmark). The gas to product ratio was about fifteen to one for
117 both aerobic and MAP treatments with the large ratio selected to ensure relatively stable gas composition
118 during storage. Bags for aerobic storage were sealed, without altering the composition of the air inside the
119 bags, by using a Multivac C500 packaging machine (Multivac A/S, Vejle, Denmark). Bags for MAP storage
120 were prepared by removal of air (20 mbar), followed by injection a 40% CO_2 and 60% N_2 gas mixture (AGA,
121 Copenhagen, Denmark) to atmospheric pressure and finalised by sealing of the bags (Multivac C500,
122 Multivac A/S, Vejle, Denmark). All samples (n = 342) were stored in a chilled room. Iced samples, both
123 aerobic (n = 63) and MAP (n = 90), were entirely covered with flake ice which was regularly refilled during
124 storage, as the ice melted. For superchilling, bags (n = 180) were submerged in slurry ice, produced by
125 mixing sodium chloride, ice flakes and water to obtain a target temperature of -2.0 °C. The temperature
126 was recorded every 30 min. during transport of the cod from Greenland to Denmark and during storage of
127 all treatment by using a minimum of two temperature loggers for each treatment (TinyTag Plus, Gemini
128 Data Loggers Ltd., Chichester, UK). After processing in Maniitsoq, triplicate samples for aerobic viable
129 counts (See 2.1.4) were taken, and at DTU Food, before dividing the cod pieces into the four treatment,
130 fresh samples were analysed using sensory, chemical and microbial (enumeration and amplicon
131 sequencing) methods (See 2.1.2-2.1.5). For each treatment, sampling was performed with intervals of two
132 to four days during a total storage period of 21 days in ice to 32 days for superchilling. At each sampling
133 time, three randomly picked bags, from each treatment, were analysed for microbiological and chemical
134 changes. Five other randomly picked bags, from each treatment, were chosen for sensory evaluation.

135

136 2.1.2 Sensory changes during iced and superchilled storage

137 Sensory evaluation was performed by using the Quality Index Method (QIM) for thawed
138 Atlantic cod filets, including scores for “Texture”, “Odour”, “Colour”, “Bloodstains” and “Parasites” (Archer,
139 2010). Compared to the original scheme, the odour attribute was expanded from the original score of 0, 1
140 and 2 to include “Acetic, ammonia” with a score value of 3. At each day of analyses, five pieces of cod from
141 each treatment were each given a random three digits code and placed on cooling plates, to avoid changing
142 sensory scores during the session. Samples were presented under artificial daylight (6500K, L 36W 965
143 Lumilux De Luxe, Osram, Germany) to a tested and trained panel consisting of four to seven assessors per
144 session. At each sensory session during the storage trial, six mock samples were included randomly to
145 prevent assessors from guessing the evolution of QI scores. The mock samples were prepared by thawing
146 cod from the same fishing ground and by storing these samples at the same conditions as the real samples,
147 but with different storage times. The results of the mock samples were not included in the presented data.
148 End of shelf-life was determined after completion of the storage trial and based on the evolution of scores
149 for the four treatments as well as variability of scores for the mock samples.

150

151 2.1.3 Chemical changes

152 Chemical changes as potential indices of spoilage were determined throughout the storage
153 trial: Trimethylamine-oxide (TMAO), trimethylamine (TMA) and total volatile nitrogen (TVN) was
154 determined in duplicate for each bag by a modified Conway and Byrne method (Conway and Byrne, 1933).
155 pH was recorded for each sample as part of the Conway and Byrne protocol by using a pH meter (HQ411D
156 Benchtop Meter, HACH Company, Loveland, USA). Lactic and acetic acids were determined, with duplicate
157 extract of each of the three cod pieces from each treatment, by HPLC with external standards for
158 identification and quantification (Dalgaard and Jørgensen, 2000). Headspace gas composition was

159 determined on each bag for microbiological and chemical analysis by using a gas analyser to measure CO_2
160 and O_2 concentrations (Checkmate3, MOCON Dansensor®, Ringsted, Denmark).

161

162 2.1.4 Culture-depended microbiology

163 The microbiota was quantified in triplicate, i.e. three separate bags, for each treatment and
164 for each sampling time by diluting 20.0 grams of cod without skin tenfold in chilled physiological saline with
165 0.1% peptone (PSP) (NMKL, 2006) followed by homogenisation for 60 seconds in a Stomacher 400 (Seward
166 Medical, London, UK). Further 10-fold dilutions with PSP were performed as required. Aerobic viable counts
167 (AVC) was determined by spread plating on chilled Long and Hammer (LH) agar with 1% NaCl (7 d; 15°C)
168 (NMKL , 2006). *Pseudomonas* spp. was determined by spread plating on Pseudomonads agar (CM0559,
169 Oxoid, Basingstoke, UK) with CFC selective supplement (SR0103, Oxoid, Basingstoke, UK) and incubation for
170 48 h at 25°C. H_2S-producing *Shewanella* spp. was determine as black colonies by pour plating in Iron Agar
171 Lyngby (CM0964, Oxoid, Basingstoke, UK) with L-cysteine hydrochloride and incubation for three days at 25
172 °C (NMKL, 2006). *Photobacterium phosphoreum* was enumerated by using a conductance method with
173 incubation at 15 °C (Dalgaard et al., 1996). Lactic Acid Bacteria (LAB) were quantified by pour plating in
174 nitrite actidione polymyxin (NAP) agar and counted after incubation for four days at 25 °C (Davidson and
175 Cronin, 1973).

176 To identify the dominating microbiota for each treatment, all countable colonies on LH
177 plates with the highest dilution factor (3 plates for each treatment) were divided into groups based on
178 colony characteristics (size, profile, elevation, boundary, colour). 13 colonies for iced cod in air, nine
179 colonies for iced cod in MAP, 28 colonies for superchilled cod in air and 16 colonies for superchilled cod in
180 MAP were divided into groups and for each group of cololonies, their proportion of the concentration of
181 countable colonies was calculated. To identify the groups of colonies present for each treatment, ten
182 colonies were isolated from LH plates (highest dilutions) at the time of sensory spoilage or at the end of the

storage period, with the exception of iced cod in MAP, where only eight isolates were sequenced. For identification of isolates these were pure-cultured using the GMB medium (Dalgaard et al., 1994) and LH plates. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany), the *16S rRNA* gene was targeted by PCR reactions with specific primers (Forward (27F): AGAGTTTGATCMTGGCTCAG, Reverse (1492R): ACCTTGTTACGACTT) and the PCR products were purified by using the MinElute PCR Purification Kit (Qiagen, Germany). The purified PCR products were sent to Eurofins Genomic for sequencing (Mix2Seq, Eurofins Genomics). Sequences were trimmed with the CLC workbench, by removing part of the sequences with low quality score (limit of 0.05) and the trimmed sequences were not allowed to have more than two ambiguous nucleotides (CLC workbench 8.1, Qiagen, Aarhus, Denmark). The trimmed sequences were assembled to reads with a minimum of 50 aligned base pairs. For identification, the *16S rRNA* gene reads of the isolates were compared to the NCBI 16S ribosomal RNA sequence Database using their website service tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Isolates identified as *Photobacterium* spp. by *16S rRNA* gene sequencing, were further analysed by partial sequencing of their *gyrB* gene (Forward (22fVf): GAAGTTATCATGACGGTACTTC, Reverse (1240rVf): AGCGTACGAATGTGAGAACC) (Ast and Dunlap, 2004) and speciated by Maximum Likelihood Phylogeny, constructed based on the Neighbour-joining methods and by using a general time-reversible nucleotide substitution model. Bootstrap values were calculated based on 1,000 replicates (CLC workbench 8.1, Qiagen, Aarhus, Denmark).

A subsection (n=19) of the isolates (3x10+8) were chosen to represent the different identified species and analysed for spoilage potential and spoilage activity (Table 1, See 2.2).

2.1.5 Culture-independent microbiology

DNA amplicon sequencing was performed to analyse the microbiota at the start of the storage period, and at the point of sensory spoilage or at the end of the storage period for the different

treatments. DNA amplicon sequencing was performed in triplicate resulting in 15 samples with three samples before storage and three samples taken after storage for each of the four treatments. One gram of filet meat was sampled and stored at -80 ^{o}C until the end of the experiment. Samples were shipped covered in dry ice to Eurofins Genomics for DNA extraction and amplicon Illumina MiSeq sequencing of the V1-V3 region in the *16S rRNA* gene (Forward: AGAGTTTGATCATGGCTCAG, Reverse: GTATTACCGCGGCTGCTG) (Leser et al., 2002; Weisburg et al., 1991). Eurofins performed quality control check, and the primer sequences were removed from the sequences. Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen et al., 2018) using the DADA2 pipeline (Callahan et al., 2016) and following the standard operating procedure (https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2018.6)) were used to assign Amplicon Sequence Variants (ASV) from reads. The DADA2 approach was selected for identification of real biological variants, and the term ASV was used to separate these from Operational Taxonomic Units (OTUs) (Callahan et al., 2016). To minimise sequencing carry-over contamination between MiSeq runs, ASV with an abundance of less than 0.1% of the total observations were filtered out, and the sampling depth of the analysis was based on the number of reads in the sample with fewest reads. To assign taxonomy for the ASVs, a classifier was generated based on the SILVA 132 SSU Ref NR 99 database (Quast et al., 2013). The V1-V3 region based on the amplicon sequencing primers were extracted from the database to minimise false-positive and used as the classifier. Merged reads were deposited at the NIH NCBI Sequence Read Archive with the accession number PRJNA565897.

The total number of ASVs were used for each treatment to represent the species richness and Faith phylogenetic diversity (Faith, 1992) was used as a measure of phylogenetic differences within a treatment. Phylogenetic beta-diversities were calculated using the unweighted UniFrac matrix and used for pairwise comparison of microbial composition between treatments with values ranging from 0.0 for complete similarity to 1.0 for complete dissimilarity (Lozupone and Knight, 2005).

231 2.2 Spoilage potential and spoilage activity of the dominating microbiota

232 To identify the bacteria responsible for spoilage of cod in different treatments, the
233 qualitative spoilage potential and the quantitative spoilage activity was determined (Dalgaard, 1995).
234 Spoilage potential was determined as the ability of isolates to produce off-odours when growing in cod
235 muscle blocks (MB) at 0 °C. Spoilage activity was quantified as the yield factor for, respectively, TVN
236 ($Y_{TVN/CFU}$, mg-N/CFU) and TMA ($Y_{TMA/CFU}$, mg-N/CFU) formation of isolates growing in cod MB at 0 °C.
237 Spoilage potential and spoilage activity were determined in duplicate for 19 different isolates (Table 1).
238 Each isolate was pre-cultured in GMB medium at 15 °C (Dalgaard et al., 1994) and thawed cod MB were
239 then inoculated with 4 log CFU/g. This high inoculum was chosen to ensure that the background microbiota
240 was not contributing to off-odour or amine formation which was further evaluated for non-inoculated MB.
241 MB was, respectively, stored in the same atmosphere as used for the treatments in the storage trial where
242 the different isolates were isolated from (Table 1). After storage at 0°C during ten days in air or 14 days in
243 MAP, the MB were placed in plastic trays at room temperature for 15 minutes before off-odour evaluation
244 by five assessors. The assessors provided a score based on off-odour attributes; (i) no off-odour, (ii) weak
245 off-odour or (iii) strong off-odour. An isolate was determined to have a spoilage potential if the average
246 score was above 2.0.

247 The same 19 isolates and the same inoculation and storage conditions were used to
248 determine both spoilage potential and spoilage activity. For each isolate, the cell concentration and the
249 concentrations of TVN and TMA were determined after inoculation of MB and at the end of the storage
250 period. Cell concentrations were determined using LH (See 2.1.4), and the volatile amines were quantified
251 by using Conway titration (See 2.1.3). The yield factors (mg-N/CFU) were calculated using the equation
252 presented by Dalgaard, (1995), with the modification that yield factors for both TVN formation and TMA
253 formation were determined (Eq. 1).

254 $$Y_{TVN/CFU} = \frac{\left(TVN_{Final}\left({}^{mg-N}/_{100\ g}\right) - TVN_{\ Initial}\left({}^{mg-N}/_{100\ g}\right)\right)\times\frac{1}{100}}{10^{\log(CFU/g)_{Final}} - 10^{\log(CFU/g)_{Initial}}} \quad (1)$$

The yield factor for a group of isolates was calculated as the average of the log-transformed yield factor values for the isolates within the group. These average yield factors for each bacterial group were used to determine calculated concentrations of TVN (mg-N/100g) during the storage period for the four treatments analysed in the storage trial (See 2.1). Calculated concentrations of TVN (mg-N/100g) were determined from measured concentrations ($10^{\log CFU/g}$) of *Pseudomonas* spp., H_2S-producing *Shewanella* and *Photobacterium* spp. (See 2.1.4) as shown in Eq. (2). The initial TVN concentration of 14 mg-N TVN/100g in cod was added to the amount of TVN formed by the three groups of bacteria (Eq. 2).

$$Calculated_{TVN}\ (mg-N/100g) = Initial\ _{TVN} + \left(10^{\frac{logCFU\ (Pseudomonas)}{g}} \times Y_{TVN\ (Pseudomonas)} + 10^{\frac{logCFU\ (Shewanella)}{g}} \times Y_{TVN\ (Shewanella)} + 10^{\frac{logCFU}{g}(Photobacterium)} \times Y_{TVN\ /Photobacterium}\right) \times 100 \quad (2)$$

2.2 Statistical analyses

Statistical analyses for the difference between the initial values of pH or lactic acid and the values at sensory spoilage or at the end of the storage trial were performed using with a two-tailed homoscedastic distribution t-Test (Microsoft Excel 2016, Microsoft Corp., Redmond, WA, USA). Maximum specific growth rate (μ_{max}, h^{-1}) of *P. carnosum* were determined by fitting the results of the conductance methods to the log-transformed 3-parameter logistic model (Dalgaard et al., 1997b).

3. Results

3.1 Storage trial

3.1.1 Storage conditions

During transport for 38 hours from Maniitsoq in Greenland to the laboratory at DTU Food in Denmark, the pieces of cod were at 1.5 ± 1.1°C. The average temperatures for the following iced or

277 superchilled storage were, respectively, 0.4 ± 0.06°C and -1.7 ± 0.08°C (Table 2). During storage, the
278 headspace gas composition surrounding the cod changed with the major changes occurring after the point
279 of sensory spoilage, i.e. CO_2 and O_2 concentrations changed in the iced and aerobically stored cod from the
280 point of spoilage on day 15 to the end of the storage period on day 21 (Table 2).

281

282 3.1.2 Sensory changes

283 Scores of odour attributes resulted in a shelf-life for iced cod of 15 days when stored in air
284 and of 22 days in MAP. The end of shelf-life was set to a score value of 1.0. Superchilled cod did not
285 reached sensory spoilage within the storage period of 32 days, irrespective of storage in air or MAP (Fig. 1,
286 Table 3). With the applied QIM scheme, the total QI scores did not show a clear development during the
287 storage period (Results not shown).

288

289 3.1.3 Chemical changes

290 The cod meat pH of 6.7 ± 0.4, measured 1.6 days after processing in Greenland, increased
291 during storage to 7.3 ± 0.4 and 7.4 ± 0.6 for iced cod in air and MAP, respectively (Table 4). Using pH of 7.0
292 as an index of spoilage, it took 15 days for iced cod in air and 24 days for cod in MAP to reach this value
293 (Table 2). For superchilled cod, pH did not reach 7.0 (Table 4). A similar development was observed for the
294 concentration of volatile amines where TVN reached the EU limit for Gadidae of 35 mg-N TVN/100 g fish
295 muscle (EC, 2008) after 14 and 20 days, respectively, for iced cod in air or MAP (Fig. 2; Table 3). Superchilled
296 cod showed no significant increase in TVN concentrations ($p > 0.05$; linear regression (slope), Fig. 2). The
297 initial TMAO concentration of 73 ± 16 mg-N TMAO/100 g fish muscle was reduced when TMA was
298 produced during iced storage. TMA concentrations increased with the same rate as TVN, from zero to 59
299 mg-N TMA/100 g and to 63 mg-N TMA/100 g for iced cod in air or MAP, respectively. The concentration of

300 TMA in superchilled MAP samples remained below ten mg-N TMA/100 g during storage. The initial lactic
301 acid concentration of 3,209 ± 373 ppm was reduced to 1,805 ± 316 ppm during iced aerobic storage
302 whereas significant changes were not observed ($p > 0.05$, t-test) for other storage conditions (Table 5).
303 Other chemical changes included an increased level of isobutyric acid (tentatively identified by HPLC
304 retention time of 1.54 relative to lactic acid) for iced cod in air (Results not shown).

305

306 3.1.4 Culture-based microbial changes

307 At the time of filleting in Greenland, cod had AVC of 2.7 ± 0.6 log CFU/g. For storage in ice,
308 AVC reached 7.0 log CFU/g after 13 days of aerobic storage and after 17 days in MAP (Fig. 3, Table 3).
309 Superchilled cod in air showed a slower growth rate for AVC and reached 7.0 log CFU/g after 23 days, and
310 for superchilled MAP cod, no clear microbial growth was observed with AVC reaching 3.9 ± 1.8 log CFU/g
311 after 32 days of storage (Fig. 3a, Table 3). Based on concentrations of bacteria as determined by using
312 selective media, *Photobacterium* spp. dominated the microbiota of iced cod in air during the period of from
313 six to 14 days of storage (Fig. 3b, c and d). From 14 days of storage the microbiota were also dominated by
314 *Pseudomonas* spp. and H_2S-producing *Shewanella* as they reached concentrations of 7.7 log CFU/g and
315 similar to those of *Photobacterium* spp. (Fig. 3c, b, d). For superchilled cod in air, *Pseudomonas* spp.
316 dominated the microbiota with H_2S-producing *Shewanella* and *Photobacterium* spp. being at slightly lower
317 concentrations (Fig. 3). However the cod was not sensory spoiled (Fig. 1, Table 3). *Photobacterium* spp.
318 dominated the spoilage microbiota of iced MAP cod and at the time of sensory spoilage (22 d), their
319 concentration was 4 log CFU/g higher than those of both *Pseudomonas* spp. and H_2S-producing *Shewanella*
320 (Fig. 3b, c, d). This was reflected by identification of the isolated bacteria where 100 % were
321 *Photobacterium* spp. (Table 6). Finally, for superchilled MAP cod growth of the studied groups of
322 microorganisms were limited, and no particular group of microorganisms seemed to dominate the

323 microbiota (Table 6). For LAB limited growth was observed and exclusively for iced cod in air where they
324 reached 4.7 log CFU/g (Results not shown).

325

326 3.1.5 Identification of isolates

327 Thirty-eight isolates from the dominating microbiota on L&H agar plates were identified by
328 *16S rRNA* gene sequencing (Table 6). For cod in air, *Pseudomonas* spp. dominated the microbiota with 92 %
329 and 67 % in iced and superchilled cod, respectively. These percentages were calculated from identified
330 isolates and from the proportion of their colonies in the microbiota (See 2.1.4). For MAP storage,
331 *Photobacterium* spp. dominated the microbiota with 100 % and 85 % of the isolates from iced cod (n = 8)
332 and superchilled cod (n = 7), respectively. Of the 15 isolates of *Photobacterium* spp. from the two MAP
333 treatments (Table 6), 11 had their *gyr*B gene successfully sequenced and used for identification. Ten of
334 these isolates were identified as *P. carnosum,* and one isolate (M0.6) may belong to a not yet described
335 *Photobacterium* species (Fig. 4).

336

337 3.1.6 Analyses of microbiota by *16S rRNA* gene amplicon sequencing

338 Of the 15 analysed samples, 13 samples passed the performed quality control. One of the
339 triplicate fresh cod samples (before storage) and one superchilled MAP sample did not pass the quality
340 control. The 13 samples resulted in a total of 923,177 individual reads. After rarefication, the sampling
341 depth was chosen to 9,300 reads per sample, at this depth, all rarefication curves had levelled off. Species
342 richness was lowest for iced MAP cod with an average of 34 ASVs compared to 98 ASVs for fresh samples
343 (Fig. 5). Within treatments, the fresh sample had the highest phylogenetic differences with Faith
344 phylogenetic diversity of 23.6, followed by superchilled MAP cod with limited microbial growth (5.8), iced
345 cod in air (2.3), superchilled cod in air (2.0) and iced cod in MAP (1.2). The phylogenetic differences were

346 also shown by the composition of genera for the treatments of cod (Table 6) and this corresponded to
347 number of ASVs (Fig. 5). Between treatments, the unweighted UniFrac distance matrix showed the
348 microbial composition of fresh samples to differ from samples at the time of sensory spoilage or at the end
349 of experiments (0.92 - 0.95) although the value for superchilled MAP cod was slightly lower (0.83; Table 6).
350 Compared to iced cod in air with shelf-life of 15 days, MAP iced cod with shelf-life of 22 days changed the
351 microbial composition (UniFrac distance of 0.60) more than superchilled cod in air with shelf-life > 32 days
352 (UniFrac distance of 0.43) (Table 7).

353

354 3.2 Spoilage potential and activity

355 Common for isolates of *Pseudomonas*, *Shewanella* and *Photobacterium*, was that the
356 majority of the isolates had spoilage potential (Table. 8). The *Photobacterium carnosum* isolates had six
357 time's higher yield-factor for TVN and TMA formation than the *Shewanella baltica* and *Shewanella*
358 *putrefaciens* isolates and 200 times higher yield-factor for TVN formation than *Pseudomonas* spp. (Table 8).
359 For non-inoculated MB, cell concentrations remained below 6.1 log CFU/g, and less than 5.0 mg-N
360 TVN/100g was formed indicating that the TVN concentration used for yield factor determination was
361 formed by the studied isolates (Results not shown).

362 For iced cod in air, the TVN-concentrations calculated from enumerated bacteria and yield
363 factors were close to TVN-concentrations observed in the storage trial both concerning the final
364 concentration and changes during the storage time (Fig. 6A). For iced MAP cod, the observed and
365 calculated TVN-concentrations were in close agreement until the end of sensory shelf-life (22 d). After 28
366 days of storage the calculated TVN-concentration was lower than the observed TVN-concentration and this
367 may reflect an underestimation of the concentration of *Photobacterium* spp. (Fig. 2B, Fig. 6). For both
368 treatments, *Photobacterium* spp. were responsible for at least 97 % of the calculated TVN formation (Table
369 9). For iced cod in air, *Photobacterium* spp. made up 65 % of the spoilage microbiota based on enumeration

370 by selective media (Fig. 3) and 24.1 % based on amplicon sequencing, but they contributed 97 % of the
371 produced TVN whereas *Pseudomonas* spp. contribute less than one percent of the formed TVN
372 concentrations, which was significantly less than their large proportion of the microbiota (Table 9).

373

374 **4. Discussion**

375 For Atlantic cod from Iceland, Wang et al. (2008) and Lauzon et al. (2009) found shelf-life of
376 24-26 days for superchilled MAP products at -0.9 or -2.0 °C an d these shelf-lives included 3-5
377 days of iced storage before packaging and superchilling, these results were similar to the shelf-life of
378 superchilled MAP cod from Greenland (Table 3). However, they found sensory shelf-lives of 14-17 days for
379 aerobic superchilled cod and these were markedly shorter than the > 32 days observed in the present
380 study. To evaluate distribution of cod at changing temperatures, we calculated 32 days at -1.7 °C as
381 equivalent to 22 days at 0 °C according to the relative rate of spoilage (RRS) model suggested by Dalgaard
382 and Huss (1997) for fresh fish from temperate waters. By using this RRS model, as included in the FSSP
383 software (FSSP, 2014), a distribution chain with ten days superchilled transportation from Greenland to
384 Europe at -1.7 °C followed by seven days chilled distribution at +2 °C e.g. through supermarkets and one
385 day consumer storage at +5 °C corresponds to 19.2 days at 0 °C. The remaining shelf-life of > 2.8 days at 0
386 °C suggests it would be possible to ship superchilled cod from Greenland to Europe with a final chilled
387 distribution on land. This distribution, however, requires careful temperature management. The remaining
388 shelf-life of > 2.8 days would be reduced to zero if the ten days of superchilling was carried out at -0.1 °C
389 instead of -1.7 °C or the seven days of chilled distribution was at +3.6 °C instead of +2.0 °C.

390 For storage of Atlantic cod in ice Olafsdottir et al. (2006) and Wang et al. (2008) found 27-49
391 mg-N TVN/100g at sensory spoilage after 9-13 days. MAP extended shelf life to 14->24 days with >36-46
392 mg-N TVN/100g at sensory spoilage. This TVN formation and shelf-lives corresponded reasonably to iced
393 cod in the present study (Table 3). However, these Icelandic studies found 37-59 mg-N TVN/100g at sensory

394 spoilage after 12-17 days for superchilled cod in air. The markedly slower TVN-development (Fig. 2) and
395 longer shelf-life for superchilled cod in the present study (Table 3) could, at least partly, be explained by a
396 slightly lower storage temperature of -1.7 °C compared to -0.9 °C for Wang et al. (2008) and -1.3 °C for
397 Olafsdottir et al. (2006).

398 The initial pH of 6.7 for Atlantic cod from CBA in Greenland (Table 4) was similar to
399 previously reported values of 6.3-6.7 for wild-caught cod (Debevere and Boskou, 1996; Rustad, 1992) and in
400 more than 1,900 cod from an area ranging from West Greenland, Spitzbergen and Aberdeen, only 44
401 individuals had a pH below 6.25 (Love, 1979). However, Atlantic cod from aquaculture typically have much
402 lower pH of 6.10-6.13 (Duun and Rustad, 2007; Hansen et al., 2007; Sivertsvik, 2007). Common for all
403 studies, the pH increased during storage and at the time of sensory spoilage Wang et al. (2008) and
404 Olafsdottir et al., (2006) found pH of 6.7-7.2. These findings support the use of >7.0 as an index for spoilage
405 of Atlantic cod from CBA or wild-caught (Table 3).

406 For fresh and spoiled MAP cod (20 % CO_2 and 80 % N_2) Chaillou et al. (2015) found a 79 %
407 reduction of OTUs from 225 to 48 and Kuuliala et al. (2018) reported OTUs being reduced at the end of
408 storage for salted cod. This corresponds well with the 65 % reduction of ASVs between fresh and spoiled
409 MAP cod in the present study (Fig. 5). 4). A reduction of OTUs during storage for fresh and lightly preserved
410 food have been observed for different seafood and meat products (Chaillou et al. 2015) and this is in
411 agreement with the hypothesis of SSOs being selected during storage (Dalgaard, 2000).

412 The observed shift in the dominating spoilage microbiota from *Pseudomonas* spp.*,*
413 *Photobacterium* spp., *Shewanella* spp. and *Acinetobacter spp.* under aerobic storage to *Photobacterium*
414 spp. in MAP cod (Table 6) also seemed in agreement with several previous studies of cod. *P. phosphoreum*
415 made up less than 1 % of the spoilage microbiota in iced aerobic Atlantic cod from Denmark whereas they
416 made up more than 90 % of the spoilage microbiota in MAP cod from Denmark and Iceland (Dalgaard et al.
417 1997c). By cultivation, Reynisson et al. (2009) found 29 % *Pseudomonas* spp. and 6 % *P. phosphoreum* in

418 the spoilage microbiota for cod in air and these values changed to < 1 % *Pseudomonas* spp. and 21-99 % *P.*
419 *phosphoreum* for MAP storage. However, by using *16S rRNA* clone analysis and t-RFLP Reynisson et al.
420 (2009) found higher percentages (84-100 %) of *P. phosphoreum* for both aerobic and MAP storage.
421 Similarly, based on *16S rRNA* gene amplicon sequencing data, *Photobacterium* spp. made up 81.2-92.5 % of
422 the microbiota in chilled vacuum-packed cod and 96.3-97.5 % in chilled MAP cod (Hansen et al., 2016).

423 > 7.0 log CFU/g has been suggested as the microbial concentration where spoilage of fresh
424 fish generally are detected (ICMSF, 2011) and more recently Eliasson et al. (2019) found this to be
425 appropriate for iced and superchilled Atlantic cod in air. However, this microbial index underestimated
426 sensory shelf-life in the present study (Table 3) as also observed by Olafsdottir et al. (2006) and Wang et al.
427 (2008) where AVC of 7.4-8.1 log CFU/g were found at the time of sensory spoilage for both aerobic and
428 MAP stored cod. With the concentration of TVN being a reasonable index of sensory spoilage (Table 3) and
429 with the spoilage microbiota being selected depending on storage conditions (Table 6) it must be expected
430 that microbial concentrations at the time of spoilage will depend on product storage. This is due to the
431 marked difference in TVN formation by different groups of bacteria. That *Photobacterium* spp. in the
432 present study produced 6-10 time more TVN and TMA per cell than H_2S-producing *Shewanella* (Table 8) is
433 in close agreement with previous studies of their yield-factors (Dalgaard, 1995). The quantitatively very low
434 TVN-formation per cell by *Pseudomonas* spp. (Table 8) also corresponded to available data for this
435 organism (Koutsoumanis and Nychas, 2000; Xie et al., 2018). With 35 mg-N TVN/100g as index of spoilage,
436 the calculated yield-factors for TVN suggest minimal spoilage levels of 7.2 log CFU/g for *Photobacterium*
437 spp., 7.8 log CFU/g for H_2S-producing *Shewanella* spp. and 9.5 log CFU/g for *Pseudomonas* spp. These
438 microbial indices of spoilage were calculated as the concentration required for each group of bacteria to
439 produce 35 mg-N TVN/100g in cod.

440 Based on their pronounced TVN-formation *Photobacterium* spp. were identified as the SSO
441 responsible for spoilage of both aerobically stored and MAP Atlantic cod from CBA in Greenland (Table 8).

442 *Photobacterium* spp. did not completely dominate the spoilage microbiota of the aerobically stored cod but
443 concentrations of other groups of bacteria, including H_2S-producing *Shewanella*, could not form the
444 concentration of TVN that correlated with sensory spoilage. A similar situation was previously reported for
445 vacuum-packed and MAP cod fillets where both *P. phosphoreum* and *S. putrefaciens* were present and
446 where the pronounced TMA-formation by *P. phosphoreum* made it the SSO (Dalgaard, 1995). Those highly
447 TMA producing cells were bioluminescent and non-bioluminescent *P. phosphoreum* with some variability in
448 their characteristics (Dalgaard, 1995; Dalgaard et al., 1997a). The non-bioluminescent *P. carnosum* isolates
449 (n = 10) and the potentially new *Photobacterium* species (n = 1) from the present study (Fig. 4) also belongs
450 to the *Photobacterium phosphoreum* clade (Labella et al., 2018; Le Doujet et al., 2019). *P. carnosum* is a
451 recently described species that have been isolated from MAP poultry meat (Hilgarth et al., 2018) and it is an
452 important part of the gut microbiota for Atlantic cod (Le Doujet et al., 2019).

453 *P. carnosum* was enumerated in cod by using a conductance-based method developed for
454 bioluminescent and non-bioluminescent *P. phosphoreum* (Fig. 2; Dalgaard et al., 1996), and found *P.*
455 *carnosum* to have the same yield factor for TMA-formation as the previously studied bioluminescent and
456 non-bioluminescent *P. phosphoreum* isolates (Table 8; Dalgaard, 1995). However, shelf-life of cod from CBA
457 in Greenland was longer than reported for Atlantic cod from other regions were bioluminescent, and non-
458 bioluminescent *P. phosphoreum* was the SSO. The longer shelf-life could be due to low initial
459 concentrations of *P. carnosum* or that they grow slower in cod than bioluminescent, and non-
460 bioluminescent *P. phosphoreum.* The initial concentration of 1.0 log CFU/g for *P. carnosum* in CBA cod from
461 Greenland was similar to initial concentrations of *P. phosphoreum* in Icelandic iced cod in air (Olafsdottir et
462 al., 2006) and Danish iced MAP cod (Dalgaard et al., 1997b). However, the shelf-life of, respectively, 15 days
463 and 22 days for CBA cod from Greenland (Fig. 2b; Table 3) was longer than the 11-14 days and 16-17 days
464 for cod from Iceland and Denmark. At 0.4 $^{\circ}$C and with 0 % or 35 % CO_2 in MAP the maximum specific
465 growth rate of *P. carnosum* in cod from Greenland was, respectively, 0.079 h^{-1} and 0.040 h^{-1} (Fig 2b). For
466 these conditions the growth rate of *P. phosphoreum* was 0.092 h^{-1} and 0.066 h^{-1} as predicted by the growth

model of Dalgaard et al. (1997b) for MAP cod. Thus, *P. carnosum* may grow slower than *P. phosphoreum* previously identified as SSO in cod. Bioluminescent and non-bioluminescent *P. phosphoreum* previously determined as part of the spoilage microbiota for cod and other fresh fishes included *P. aquimaris, P. iliopiscarium, P. kishitanii, P. phosphoreum*, and *P. piscicola* (Ast and Dunlap, 2004; Dalgaard, 1995; Dalgaard et al., 1997a; Figge et al., 2014; Poirier et al., 2018). The present study suggests *P. carnosum* and probably other *Photobacterium* species are part of this group of spoilage bacteria. Further studies are needed to evaluate their occurrence and importance for spoilage of various foods.

5. Conclusions

For cod fillets from CBA in Greenland a lowering of the storage temperature from +0.4 °C to -1.7 °C had a more pronounced effect on shelf-life extension compared to changing the storage atmosphere from air to MAP with 35 % CO_2 at equilibrium. Superchilling increased the sensory shelf-life to more than 32 days. The combination of superchilling and MAP inhibited microbial growth and TVN formation during the studied 32 days of storage. Based on the determination of spoilage activity, *P. carnosum* was pointed out as the specific spoilage organism that limited shelf-life of iced cod irrespective of storage in air or MAP conditions. Further studies are suggested to evaluate the long shelf-life of cod fillets from CBA in Greenland and of the occurrence and importance for *P. carnosum* in food spoilage.

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Table 1: Isolates[a] evaluated for spoilage potential and spoilage activity.

	Number of isolates	
Species	Air	MAP
Pseudomonas spp.	11	0
Shewanella spp.	2	1
Photobacterium spp.	0	5

[a] Isolates originated from iced and superchilled cod in air or modified atmosphere packaging (MAP).

Table 2: Temperature and gas composition of iced and superchilled cod during storage in air or modified atmosphere packaging (MAP).

	Temperature	Gas composition					
	°C (Avg. ± SD)	% CO_2 (Avg. ± SD)			% O_2 (Avg. ± SD)		
	Average during storage trial	Start	Sensory spoilage	End of storage trial	Start	Sensory spoilage	End of storage trial
Iced cod in air[b]	0.4 ± 0.06	1.3 ± 1.1	2.8 ± 1.7	10.8 ± 3.5	19.4 ± 0.3	17.7 ± 2.0	7.8 ± 2.5
Iced cod in MAP[c]	0.4 ± 0.06	35.8 ± 1.1	34.2 ± 4.4	31.3 ± 1.0	≤ 0.1	≤ 0.1	≤ 0.1
Superchilled cod in air[d]	-1.7 ± 0.08	0.8 ± 1.1	-[a]	1.7 ± 1.7	19.4 ± 0.3	-[a]	18.5 ± 1.3
Superchilled cod in MAP[d]	-1.7 ± 0.08	35.4 ± 1.1	-[a]	34.0 ± 0.8	≤0.1	-[a]	0.1 ± 0.02

[a] Product did not reach point of sensory spoilage.
[b] Storage trial ended after 21 days.
[c] Storage trial ended after 28 days.
[d] Storage trial ended after 32 days.

Table 3: Shelf-life of Atlantic cod based on sensory evaluation and indices of spoilage.

	Shelf-life (days)			
	Iced cod		Superchilled cod	
	Air	MAP	Air	MAP
Sensory shelf-life "Odour"	15	22	> 32	> 32
Shelf-life from indices of spoilage				
pH ≥ 7.0	15	24	> 32	> 32
TVN ≥ 35 mg-N/100g	14	20	> 32	> 32
TMA ≥ 20 mg-N/100g	14	19	> 32	> 32
AVC ≥ 7.0 Log CFU/g	13	17	23	> 32

Table 4: pH changes during storage of iced and superchilled cod in air or modified atmosphere packaging (MAP).

	pH (Avg. ± SD)		
	Start	Sensory spoilage	End of storage trial
Iced cod in air[c]	6.7 ± 0.4[a]	7.0 ± 0.5	7.3 ± 0.4 **[f]
Iced cod in MAP[d]		6.9 ± 0.2	7.4 ± 0.6 *[f]
Superchilled in air[e]		-[b]	6.8 ± 0.5
Superchilled in MAP[e]		-[b]	6.7 ± 0.4

[a] Common start value for all treatments.
[b] Products did not reach sensory spoilage within 32 days.
[c] Storage trial ended after 21 days.
[d] Storage trial ended after 28 days.
[e] Storage trial ended after 32 days.
[f] * indicate $p < 0.05$; ** $p < 0.01$, tested between start and end of storage trial (Students t-Test).

Table 5: Changes in lactic acid concentrations during storage of iced and superchilled cod in air or modified atmosphere packaging (MAP).

	Lactic acid in fish (ppm, Avg. ± SD)	
	Start	End of storage trial
Iced cod in air[b]	3209 ± 373[a]	1805 ± 316 **[e]
Iced cod in MAP[c]		2939 ± 905
Superchilled in air[d]		3049 ± 751
Superchilled in MAP[d]		3299 ± 846

[a] Common start value for all treatments.
[b] Storage trial ended after 21 days.
[c] Storage trial ended after 28 days.
[d] Storage trial ended after 32 days.
[e] ** indicate $p < 0.01$, tested between start and end of storage trial (Students t-Test).

Table 6: Microbiota at point of spoilage for iced and superchilled (SC) cod in air or modified atmosphere packaging (MAP). Microbiota were characterised by *16S rRNA* gene sequencing of isolates and *16S rRNA* gene amplicon MiSeq sequencing.

	Microbiota based on isolates (%)				Microbiota based on amplicon MiSeq sequencing (%)				
	Iced cod in air	Iced cod in MAP	SC cod in air	SC cod in MAP	Iced cod in air	Iced cod in MAP	SC cod in air	SC cod in MAP	Fresh cod
Numbers of isolates	10	8	10	10					
Log CFU/g					8.5	7.3	7.8	3.9	2.4
Pseudomonas spp.	92	-[a]	67	-[a]	36.2	0.1	86.4	31.6	34.1
Shewanella spp.	8[b]	-[a]	17	3[c]	15.5	0.3	3.4	4.2	0.1
Moellerella spp.	-[a]	-[a]	16[d]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
Photobacterium spp.[e]	-[a]	100	-[a]	85	24.1	98.5	2.7	40.5	5.5
Janthinobacterium spp.	-[a]	-[a]	-[a]	12[f]	9.3	-[a]	1.2	0.3	0.5
Acinetobacter spp.	-[a]	-[a]	-[a]	-[a]	14.0	0.1	-[a]	12.5	38.3
Psychrobacter spp.	-[a]	-[a]	-[a]	-[a]	0.1	-[a]	5.9	1.4	-[a]
Delftia spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	1.4	2.0
Ralstonia spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	1.5	2.7
Aliivibrio spp.	-[a]	-[a]	-[a]	-[a]	0.3	1.0	0.1	-[a]	1.1
Arthrobacter spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	1.0	1.0
Stenotrophomanas spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	0.7	0.9
Pelomonas spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	0.7	0.7
Flavobacterium spp.	-[a]	-[a]	-[a]	-[a]	0.5	-[a]	-[a]	0.2	1.2
Carnobacterium spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	0.9	0.2
Brevundimonas spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	0.2	1.1
Enhydrobacter spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	2.5
Enterobacter spp.	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	0.7
Unassigned					-[a]	-[a]	-[a]	-[a]	6.6

[a] Species either not present or below < 0.1 % in relative abundance.
[b] Identified as *Shewanella baltica*.
[c] Identified as *Shewanella putrefaciens*.
[d] Identified as *Moellerella wisconsensis*.
[e] 10 out 11 isolates were identified as *Photobacterium carnosum* by *gyrB* sequencing (See 3.1.5).
[f] Identified as *Janthinobacterium svalbardensis*.

Table 7: Pairwise differences between microbial communities in iced and superchilled (SC) cod in air or modified atmosphere packaging (MAP).

	Unweighted UniFrac distance matrix[a]				
	Iced cod in air	Iced cod in MAP	SC cod in air	SC cod in MAP	Fresh cod
Iced cod in air	0.20				
Iced cod in MAP	0.60	0.26			
SC cod in air	0.43	0.54	0.30		
SC cod in MAP	0.71	0.82	0.72	0.30	
Fresh cod	0.92	0.95	0.93	0.83	0.30

[a] Distance between two communities, calculated as the fraction of the branch length in a phylogenetic tree that leads to descendants in one, but not both, of the two communities. 1.00 indicate complete difference and 0.00 indicate an identical community. The distances were calculated as the average between triplicate samples from each treatment.

Table 8: Spoilage potential and spoilage activity of isolates from the dominating microbiota.

	Spoilage potential[a]	Spoilage activity, yield factor[b] (Avg ± SD)	
Species	Isolates (positive/total)	TVN	TMA
Pseudomonas spp.	7/11	-10.2 ± 0.3	-[c]
Shewanella spp.	2/3	-8.8 ± 0.8	-9.0 ± 0.8
Photobacterium spp.	3/5	-8.0 ± 0.3	-8.0 ± 0.4

[a] Spoilage potential was determined as the ability of isolates to produce off-odours when growing in cod muscle blocks at 0 °C.
[b] Yield factor values were log transformed prior to calculating the average and standard deviation for easier interpretation.
[c] TMA formation were not different from the TMA formation in the non-inoculated samples.

Table 9: Calculated total volatile nitrogen (TVN) based on measured concentrations of bacteria.

	Concentration[a] and corresponding percentage[b] of bacteria			Calculated percentage of TVN[c]		
	Pseudomonas	*Shewanella*	*Photobacterium*	*Pseudomonas*	*Shewanella*	*Photobacterium*
Iced cod in air	6.9 log CFU/g 27.0 %	6.3 log CFU/g 8.0 %	7.3 log CFU/g 65.0 %	0.2 %	2.8 %	97.0 %
Iced cod in MAP	3.5 log CFU/g 0.0 %	4.2 log CFU/g 0.1 %	7.0 log CFU/g 99.8 %	0.0 %	0.0 %	100.0 %

[a] CFU/g as enumerated by selective media descripted, in section 2.1.4.
[b] The relative percentage of each species were calculated based on their concentration in CFU/g ($10^{logCFU/g}$).
[c] TVN was calculated as described by Eq. 2.

- Shelf-life of superchilled Atlantic cod was more than 32 days
- Superchilling in combination with MAP limited microbial growth
- *Photobacterium carnosum* was the SSO responsible for spoilage of iced cod
- TVN, TMA and pH were promising indices of spoilage for Atlantic cod

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jonas Steenholdt Sørensen, Niels Bøknæs and Ole Mejlholm is employed by Royal Greenland.