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Hélène L. Lauzon
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Functionality testing of selected Chill-on technologies during a transport-simulation study of palletised cod boxes: qPCR for fish spoilage bacteria, SLP model and QMRA to evaluate pathogen growth in spiked cod

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Hélène L. Lauzon[1]
Björn Margeirsson[1]
Kolbrún Sveinsdóttir[1]
Eyjólfur Reynisson[1]
María Guðjónsdóttir[1]
Emilía Martinsdóttir[1]
Radovan Gospavic[2]
Nasimul Haque[2]
Viktor Popov [2]
Guðrún Ólafsdóttir[3]
Tómas Hafliðason[3]
Einir Guðlaugsson[3]
Sigurður Bogason[3]

[1]Matís ohf
[2]Wessex Institute of Technology
[3]University of Iceland

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Icelandic Food and Biotech R&D

Report summary

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Titill / Title	**Functionality testing of selected Chill-on technologies during a transport-simulation study of palletized cod boxes: qPCR for fish spoilage bacteria, SLP model and QMRA to evaluate pathogen growth in spiked cod**		
Höfundar / Authors	*Hélène L. Lauzon, Björn Margeirsson, Kolbrún Sveinsdóttir, Eyjólfur Reynisson, María Guðjónsdóttir, Emilía Martinsdóttir (Matís); Radovan Gospavic, Nasimul Haque, Viktor Popov (WIT); Guðrún Ólafsdóttir, Tómas Hafliðason, Einir Guðlaugsson, Sigurður Bogason (UoI)*		
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Styrktaraðilar / funding:	*EU IP Chill-on (contract FP6-016333-2)*		
Ágrip á íslensku:	Í þessari rannsókn voru gerðar prófanir á tæknilausnum sem þróaðar voru í EU verkefninu Chill-on þar sem sett var upp hermitilraun til að líkja eftir raunverulegum flutningum á fiski frá Íslandi til Evrópu. Hitastigssveiflur, sem fiskurinn varð fyrir, miðuðu að því að herma eftir flutningi frá Íslandi til Frakklands með skipi. Bretti af þorskhnökkum í frauðplastkössum voru flutt til Vestmannaeyja með skipi og til baka aftur til Matís í Reykjavík. Sýni úr þessum brettum voru síðan borin saman við samanburðarsýni sem geymd höfðu verið við undirkældar aðstæður hjá Matís. Þorskhnökkum var jafnframt pakkað í neytendapakkningar (bakka) strax eftir vinnslu og síðan eftir 6 daga og voru geymdir við undirkældar eða kældar aðstæður. Einnig voru gerðar örveruvaxtartilraunir þar sem *Listeria monocytogenes, Escherichia coli* og *Salmonella Dublin* var bætt út í þorskhnakka sem geymdir voru í frauðplastkössum við aðstæður sem líktust geymslu- og flutningsferli við útflutning. Hitastigsmælingar, skynmat, örveru- og efnafræðilegar mælingar voru notaðar til að setja fram gögn til að prófa og sannreyna QMRA/SLP líkönin og magngreiningu á *Pseudomonas* bakteríum með qPCR tækni.		
Lykilorð á íslensku:	*Þorskur – Geymsluþolsspá – Skemmd – Öryggi - qPCR - Hitastigsstýring - Undirkæling - Sjóflutningur*		

Skýrsluágrip Matís ohf
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Report summary

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Summary in English:

The aim of the cod wet trials and the corresponding shelf life study was to include scenarios to test and demonstrate the functionality of some Chill-on technologies in a simulated cod supply chain. Temperature fluctuations were induced according to the actual scenario in the supply chain of cod from Iceland to France via sea freight. The study included sample groups created at the point of processing after packaging in EPS boxes. The reference group was stored at Matís under superchilled conditions. Simulation trials for downward distribution were performed at Matís upon receipt of the pallets shipped to the Westman Isles from Reykjavik (Iceland-Europe freight simulation) and compared with the reference group. Repackaging of loins in retail trays was performed on days 0 and 6 with storage under superchilled and chilled conditions, respectively. In addition, a pathogen challenge trial was performed by spiking loins (5 kg) with *Listeria monocytogenes, Escherichia coli* and *Salmonella Dublin*, followed by storage in EPS boxes under temperature conditions simulating export and distribution. Temperature recordings along with microbial, chemical and sensory analyses from the groups evaluated provided necessary data to test and validate the QMRA/SLP models and the quantitative molecular (qPCR) method to estimate counts of pseudomonads.

English keywords: *Cod – Predictive microbiology – Shelf life – Spoilage - Safety – Temperature control - Superchilling – Sea freight*

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1. INTRODUCTION

This report is based on experiments made within EU-funded Integrated Research Project CHILL-ON (contract FP6-016333-2). The aim of the cod wet trials and the corresponding shelf life study was to include scenarios to test and demonstrate the functionality of some Chill-on technologies in a simulated supply chain of fish from the company Opale. Temperature fluctuations were induced according to the actual scenario in the supply chain of cod from Iceland to France via sea freight. The study included sample groups created at the point of processing after packaging in EPS boxes as seen in Figure 1. The reference group was stored at Matís under superchilled conditions. Simulation trials for downward distribution were performed at Matís upon receipt of the pallets shipped to the Westman Isles from Reykjavik and compared with the reference group. Repackaging of loins in retail trays was performed on days 0 and 6 with storage under superchilled and chilled conditions, respectively.

In addition, a pathogen challenge trial was performed by spiking a whole EPS box containing 5 kg of loins cut in half with *Listeria monocytogenes*, *Escherichia coli* and *Salmonella Dublin*, to be stored under temperature conditions simulating export and distribution. Temperature recordings along with microbial, chemical and sensory analyses from the groups evaluated provided necessary data to test and validate the QMRA/SLP models, quantitative molecular (qPCR) method to estimate counts of pseudomonads.

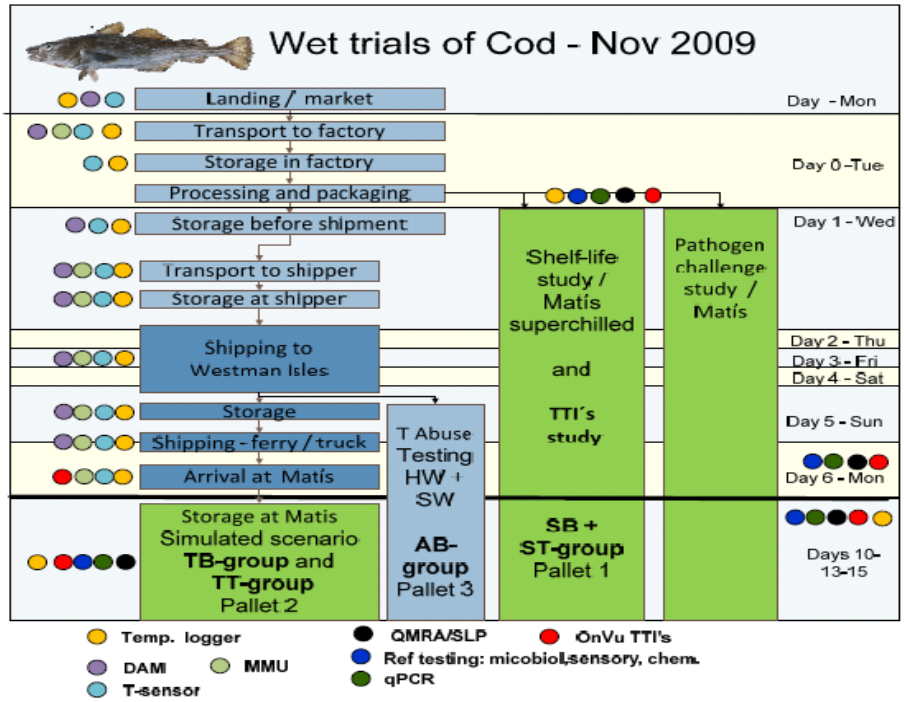


Figure 1. Overview of the experimental design of Opale cod wet trials in November 2009 simulating fish export to Europe (Processing day 0 = 3 days post catch; HW: hardware; SW: software)

2. EXPERIMENTAL DESIGN

2.1 Fish processing and packaging

Three tubs (approximate weight of 1500 kg) of fresh, whole Atlantic cod (*Gadus morhua*), were obtained at the fish market in Grindavík. The fish was caught by Danish Seine by the vessel Páll Helgi IS 142 on November 21st 2009. The fish was bled, gutted, rinsed, sorted (average weight 1.38 kg; average length 0.35 m) and iced in tubs with alternating layers of flake ice. On Nov. 23rd the fish arrived to the processing plant where it was stored overnight in a cooling chamber until processed in the afternoon, 3 days post catch. In the storage study this day is referred to as day 0 for the processed fish products. The ice to fish ratio was appropriate with fish temperature of 0 °C. The fish tub was emptied into a water bath for rinsing at the beginning of the processing line, followed by de-heading, filleting, skinning, trimming and cutting the fillet into loins and tails. Thereafter, the fish loins were chilled in a liquid cooling medium to reach a temperature of -0.5 °C and packed into EPS boxes, each weighing 5 kg. The EPS boxes were packed with 2 absorbent pads, lined with a plastic bag and a 250-g cooling mat (frozen) put on top of the closed bag. Temperature recording devices were inserted aseptically (in sterile plastic bags) between layers of loins in predefined boxes and positions.

a. Ibutton temperature (T) loggers (Figure 2), type DS1922L, with an accuracy of ±0.5 °C, resolution of 0.0625 °C and operating range of -40 to 85 °C (http://www.maxim-ic.com/quick_view2.cfm/qv_pk/4088). The diameter is 17 mm and the thickness 5 mm. The iButton loggers were used to monitor the product temperature.
b. Onset temperature (T) loggers (Figure 3), type UTBI-001 with an accuracy of ±0.2 °C, resolution of 0.02 °C and operating range of -20 to 70 °C. (http://www.onsetcomp-.com/products/data-loggers/utbi-001). The diameter is 30 mm and the thickness 17 mm. Those loggers were used for measuring ambient temperature in climate chambers.

Four iButton loggers were put in each predetermined box and two on the outside of each box. Five Onset temperature loggers were used in each climate chamber at Matís for measuring ambient temperature.

Figure 2. The iButton temperature data loggers

Figure 3. The Onset temperature data loggers

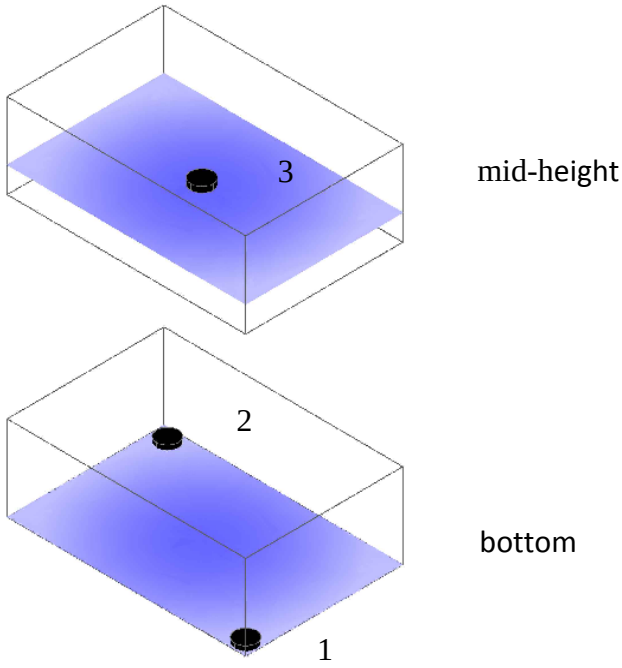


Figure 4. Configuration of temperature data loggers inside EPS boxes

Figure 4 shows the location of temperature loggers inside the boxes. The numbering scheme used in Figure 4 is consistent with Table 1, listing the names used for each logger position hereafter.

Table 1. Location and numbering scheme of temperature loggers (see Figure 4)

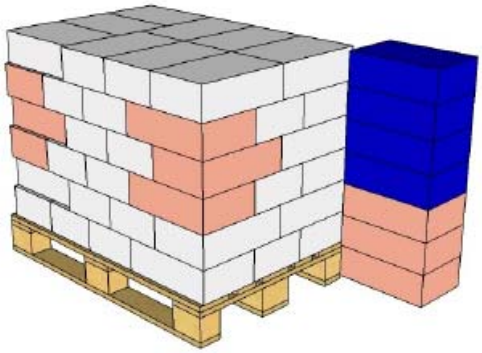
#	Horizontal location	Vertical location	Name used
1	Outer corner	Bottom	Outer corner
2	Inner corner	Bottom	Inner corner
3	Middle	Middle	Middle

2.2 Pallet assembling and group definition

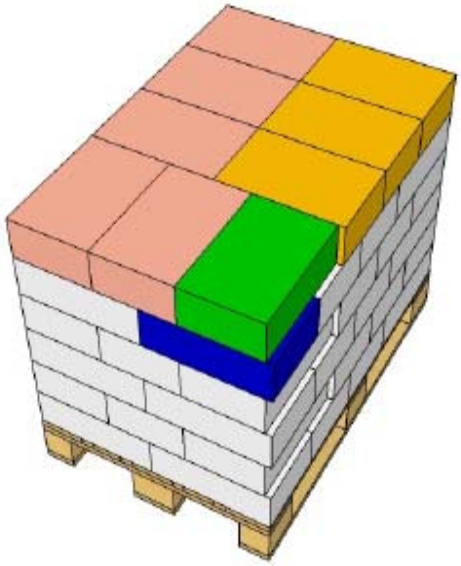
This study included 5 test groups, 3 bulk groups constituted of EPS boxes assembled into 3 pallets (SB, TB, AB) and 2 retail groups (ST, TT) repackaged at Matís in 800-g tray portions. Six loins placed on a polystyrene tray (Linstar E39-34, 225 x 175 mm) were packaged in a plastic vacuum bag (25 x 35 cm x 70 µm, 20PA/50PE ; Samhentir, Iceland) under 40% vacuum on d0 using freshly produced loins (ST, superchilled trays) and d6 using loins from pallet 2 (TT, trays prepared from TB boxes). The 3 pallets were assembled for the study based on a predefined setup, using EPS boxes containing either salt (5 kg) or processed loins. Pallet 1 was stored under superchilled conditions at Matís from day 0 (SB, superchilled boxes). Pallet 2 was shipped in a superchilled container to the Westman Isles, simulating sea freight and distribution to the retailer in France on day 6 but arriving then to Matís (TB, transport-simulated boxes). Pallet 3 was also shipped to the Westman Isles but removed from the superchilled container on day 5, abused for 13 hours (mean environmental temperature around 13 °C) before being shipped to Reykjavik later that day and received at Matís on day 6 (AB, abused boxes). In order to minimize cost, some of the EPS boxes contained salt instead of fish. The specific heat capacity of salt is 0.88 kJ/kg K [1] and its density is around 801 kg/m^3 [2] compared with the specific heat capacity of fish at 0 °C around 3.3 kJ/kg/K and the density of around 1050 kg/m^3.

a. Pallet 1 (54 boxes on 6 layers): 9 boxes with loins layered at 3 corners (levels 2, 3 and 4, counting from the top of the pallet) used for quality analysis and 45 salt boxes. The top layer included only salt boxes. T loggers monitored the temperature of loins in 3 boxes (one corner) of the 9 fish boxes (see pallet 1 of Figure 5).
b. Pallet 2 (90 boxes on 10 layers): 9 boxes with loins layered at 3 corners (levels 2, 3 and 4, counting from the top of the pallet) used for quality analysis and 75 salt boxes. The top layer also included 6 fish boxes with 2 positioned at diagonal corners (containing T-loggers, T-sensors and Controllant T recording system; only T-loggers reported in this report) but other 4 boxes not placed at corners. These 4 boxes were used for retail packaging of loins in trays on day 6 at Matís. T loggers monitored the temperature of loins in 3 boxes (one corner) of the 9 fish boxes (see pallet 2 of Figure 5). Loggers were placed in a salt box (2-10-3) to evaluate the temperature profile compared to that of fish boxes.
c. Pallet 3 (54 boxes on 6 layers): 6 EPS boxes (used for quality analysis) and 3 CP (corrugated plastic) boxes with loins layered at first level and 45 salt boxes. All 9 boxes contained T loggers and one corner box also included a T-sensor and a Controllant device (see pallet 3 of Figure 5).
d. Three single boxes were shipped to Matís for both fish quality analysis (d0) and pathogen spiking experiment (see chapter 5), as well as four boxes for retail packaging of loins in trays (ST, superchilled trays).

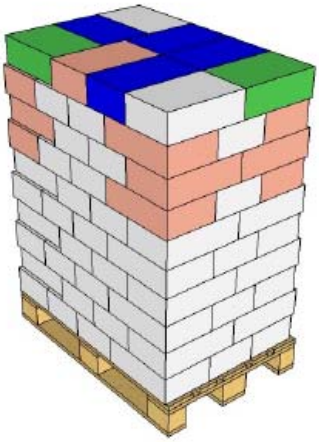
[1] http://physics.info/heat-sensible/
[2] http://www.simetric.co.uk/si_materials.htm



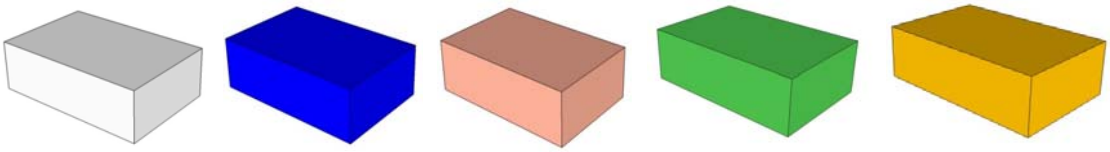
Pallet 1 – Superchilled EPS boxes (SB)



Pallet 3 – Transported and abused (AB)



Pallet 2 – Transported EPS boxes (TB)



Box with salt, simulating cooling media.

Box for retail packaging.

Box with fish for microbial analysis

Box with fish and T-sensor

Corrugated plastic (CP) box with fish

Figure 5. Design of the three pallets and extra EPS boxes (7) used at Matís on day 0 for tray retail packaging (4), initial analysis and pathogen spiking (3). Designed by Tómas Hafliðason, University of Iceland.

3. STORAGE STUDY - ANALYSIS OF SENSORY, MICROBIAL AND CHEMICAL DATA FOR GROUPS SB, ST, TB, TT, AB

3.1 Materials and Methods

3.1.1 *Sampling*

Samples were obtained in triplicate using 3 EPS boxes (selecting 2 loins from the upper fish layer as one sample replicate) or 3 trays for microbiological and chemical analyses. Loins used for sensory analysis were obtained from the 3 EPS boxes sampled (from the upper layer) or 3 new trays, pooling all the fish for each group. Regular sampling was done as described in Table 2.

Table 2. Definition of sample groups evaluated

Sample name	Description	Sampling days
Day 0	Loins from the lot processed	0
SB	Superchilled EPS boxes	6, 10, 13
ST	Superchilled trays	6, 13, 15
TB	Transport-simulated EPS boxes	6, 10, 13
AB	Abused (shipped) EPS boxes	6, 10
TT	Trays packed on d6 from TB boxes	10, 13

3.1.2 *Sensory evaluation*

Five groups of cod loins were evaluated with sensory evaluation. The main purpose was to study differences in shelf life according to sensory evaluation by a trained panel. Quantitative Descriptive Analysis (QDA), as introduced by Stone and Sidel (1985), and Torry freshness score sheet (Shewan *et al*., 1953) were used to assess cooked samples (MA09sky114-116,118-122). Ten panellists participated in the sensory evaluation. They had all been trained according to international standards (ISO 8586, 1993); including detection and recognition of tastes and odours, use of scales and in the development, and use of descriptors. The members of the panel were familiar and experienced in using the QDA method and Torry freshness score sheet for cod. The panel was trained in recognition of sensory characteristics of the samples and describing the intensity of each attribute for a given sample using an unstructured scale (from 0 to 100%). Most of the attributes were defined and described by the sensory panel during other projects (Sveinsdottir *et al.* 2009). The sensory attributes were 30 and are described in Table 3**.**

Portions weighing about 40 g were cut from the loins and placed in aluminium boxes coded with three-digit random numbers. The samples were cooked for 6 minutes in a pre-warmed oven (Convotherm Elektrogeräte GmbH, Eglfing, Germany) at 95-100 °C with air circulation

and steam, and then served to the panel. Each panellist evaluated duplicates of each sample group in a random order in nine sessions (maximum four samples per session). A computerised system (FIZZ, Version 2.0, 1994-2000, Biosystèmes) was used for data recording.

Table 3. Sensory vocabulary for cooked cod

Sensory attribute	Short name	Description of attribute
Odour		
sweet	o-sweet	Sweet odour
shellfish, algae	o-shellfish	Shellfish, algae, characteristic fresh odour
meaty	o-meat	Meaty odour, reminds of boiled meat or halibut
vanilla/warm milk	o-vanilla	Vanilla, sweet heated milk
boiled potatoes	o-potatoes	Reminds of whole, warm, boiled potatoes
frozen storage	o-frozen	Refrigerator, freezer storage odour
table cloth	o-cloth	Reminds of a table cloth (unclean, damp cloth to clean kitchen table, left for 36 h)
TMA	o-TMA	TMA odour, reminds of dried salted fish, amine
sour	o-sour	Sour odour, sour milk, spoilage sour, acetic acid
sulphur	o-sulphur	Sulphur, matchstick, boiled kale
Appearance		
colour	a-dark	Left end: light, white colour. Right end: dark, yellowish, brownish, grey
appearance	a-disc	Left end: homogeneous, even colour. Right end: discoloured, heterogeneous, stains
white precipitation	a-prec	White precipitation in the broth or on the fish surface
Flavour		
salt	f-salt	Salty taste
metallic	f-metallic	Characteristic metallic flavour of fresh cod
sweet	f-sweet	Characteristic sweet flavour of fresh boiled cod
meaty	f-meaty	Meaty flavour, reminds of boiled meat
frozen storage	f-frozen	Reminds of wood which has soaked in refrigerator/freezing flavour
pungent	f-pungent	Pungent flavour, bitter
sour	f-sour	Sour taste, spoilage sour
TMA	f-TMA	TMA flavour, reminds of dried salted fish, amine
off-flavour	f-off	Strength of off-flavour (spoilage flavour/off-flavour)
Texture		
flakiness	t-flakes	The fish portion slides into flakes when pressed with the fork
soft	t-soft	Left end: firm. Right end: soft. Evaluate how firm or soft the fish is during the first bite
juicy	t-juicy	Left end: dry. Right end: juicy. Evaluated after chewing several times: dry - draws juice from the mouth
tender	t-tender	Left end: tough. Right end: tender. Evaluated after chewing several times
mushy	t-mushy	Mushy texture
meaty mouthfeel	t-meaty	Meaty texture, meaty mouthfeel, crude muscle fibers
clammy	t-clammy	Clammy texture, tannin (dry red wine)
rubbery	t-rubbery	Rubbery texture, springy

3.1.3 *Microbiological analysis*

Loins were aseptically minced, assessing 2 pooled loins for each sample (one box or tray). Three replicate samples were evaluated for each group. Minced flesh (20 g) was mixed with 180 mL of cooled Maximum Recovery Diluent (MRD, Oxoid, UK) in a stomacher for 1 minute. Successive 10-fold dilutions were done as required. Total viable psychrotrophic counts (TVC) and counts of H_2S-producing bacteria (black colonies) were obtained using on iron agar (IA), modified from Gram *et al.* (1987) with 1% NaCl and no overlay. Plates were spread-plated and incubated at 17 °C for 5 days. Enumeration of presumptive pseudomonads was performed using modified Cephaloridine Fucidin Cetrimide (mCFC) agar as described by Stanbridge and Board (1994). *Pseudomonas* Agar Base (Oxoid, UK) with CFC selective Agar Supplement (Oxoid) was used and the plates were incubated at 22 °C for 3 days. This

traditional counting method was compared to a quantitative Polymerase Chain Reaction (qPCR) method for estimation of pseudomonad counts (Reynisson *et al.*, 2008). Estimation of *Photobacterium phosphoreum* (Pp) counts was achieved by another qPCR method developed at Matís (E. Reynisson, unpublished) and standardised with the Pp Malthus conductance method (Dalgaard *et al*., 1996; Lauzon, 2003) in earlier trials.

3.1.4 *Chemical analysis: Total Volatile Base Nitrogen (TVB-N), trimethylamine (TMA) and pH*

The method of Malle and Tao (1987) was used for total volatile bases (TVB-N) and trimethylamine (TMA) measurements in the previously prepared mince. TVB-N was measured by steam distillation (Struer TVN distillatory, STRUERS, Copenhagen) and titration, after extracting the fish muscle with 7.5% aqueous trichloroacetic acid (TCA) solution. The distilled TVB-N was collected in boric acid solution and titrated with sulphuric acid solution. TMA was measured in TCA extract by adding 20 ml of 35% formaldehyde, an alkaline binding mono- and diamine, TMA being the only volatile and measurable amine. All chemical analyses were done in triplicate.

The pH was measured in 5 grams of minced loins mixed with 5 mL of deionised water using the Radiometer PHM 80. The pH meter was calibrated using the buffer solutions of pH 7.00 ± 0.01 and 4.01 ± 0.01 (25 °C) (Radiometer Analytical A/S, Bagsvaerd, Denmark).

3.1.5 *Data analysis*

QDA data was corrected for level effects (effects caused by level differences between assessors and replicates) by the method of Thybo and Martens (2000). Principal Component Analysis (PCA) on significant mean values of QDA sensory attributes was performed, using full cross validation. Analysis of variance (ANOVA) was carried out in the statistical program NCSS 2000 (NCSS, Utah, USA) on QDA data (see Appendix 1) as well as for the microbial and chemical data. The program calculates multiple comparisons using Duncan's multiple comparison test. The significance level was set at 5%.

3.2 Results and Discussion

3.2.1 *Results from temperature monitoring*

The temperature profiles of three EPS product groups were followed by T loggers during the simulation trial along with two retail product groups created from the EPS products at days 0 and 6 post-processing (Table 4 and Figure 6 to Figure 11).

Table 4. Average temperature (± SD °C) of the different product groups and their environment during the storage period

Opale cod loins processed on Nov. 24th 2009	Group	Storage period (days)	$T_{product}$ (°C, center*) during storage	$T_{ambient}$ (°C) during storage
EPS 5 kg-P1	SB	13	-0.7 +/- 0.1	-4.8 +/- 2.5
EPS 5 kg-P1	SB**	13	-1.2 +/- 0.2	-4.8 +/- 2.5
EPS 5 kg-P2	TB	13	-0.1 +/- 0.3	0.4 +/- 0.8
EPS 5 kg-P3	AB	10	0.1 +/- 0.4	1.4 +/- 3.9
Trays-P1 (800 g)	ST	15	-2.9 +/- 1.2	-4.7 +/- 2.6
Trays-P2 (800 g)	TT	13	-0.2 +/- 0.3	0.4 +/- 0.8

* Logger positioned in the box center; average of 3 replicate boxes
** Logger positioned in the box bottom-corner, average of 3 replicate boxes

The SB group was intended to be stored at a constant superchilled temperature of -1 °C but resulted in an ambient temperature of -4.8 ± 2.5 °C for the 13-day period due to failure of the temperature control of the air climate chamber. Differences in product temperature (Δ 0.5 °C) were observed based on the loggers' position in the SB boxes, with the bottom-corner loggers being considerably influenced by the low environmental temperature (Figure 6). The transport-simulated pallet (P2) underwent a milder storage condition with a slightly higher product temperature (Δ 0.6 °C) for TB (Figure 8) than SB group (Figure 7). Similar temperature was observed inside the box containing salt as in the boxes containing fish (Figure 8). The abused (AB) group had the highest product temperature (0.1 ± 0.4 °C) due to the average ambient temperature of 1.4 ± 3.9 °C (Figure 9). Unlike the EPS boxes, the retail trays were unprotected and their average product temperature was much influenced by the environmental conditions, explaining the lowest temperature observed for ST trays (-2.9 ± 1.2 °C; Figure 10) while the average TT-product temperature (Figure 11) was similar to that of the TB group.

The ambient temperature profiles are shown in Figure 6, Figure 10 and Figure 11 for all the groups. The abusive treatment (13 °C for 13 h) performed on day 5 (AB) to mimic

distribution in Europe resulted in an increase in average product temperature of 0.2 °C (based on TB).

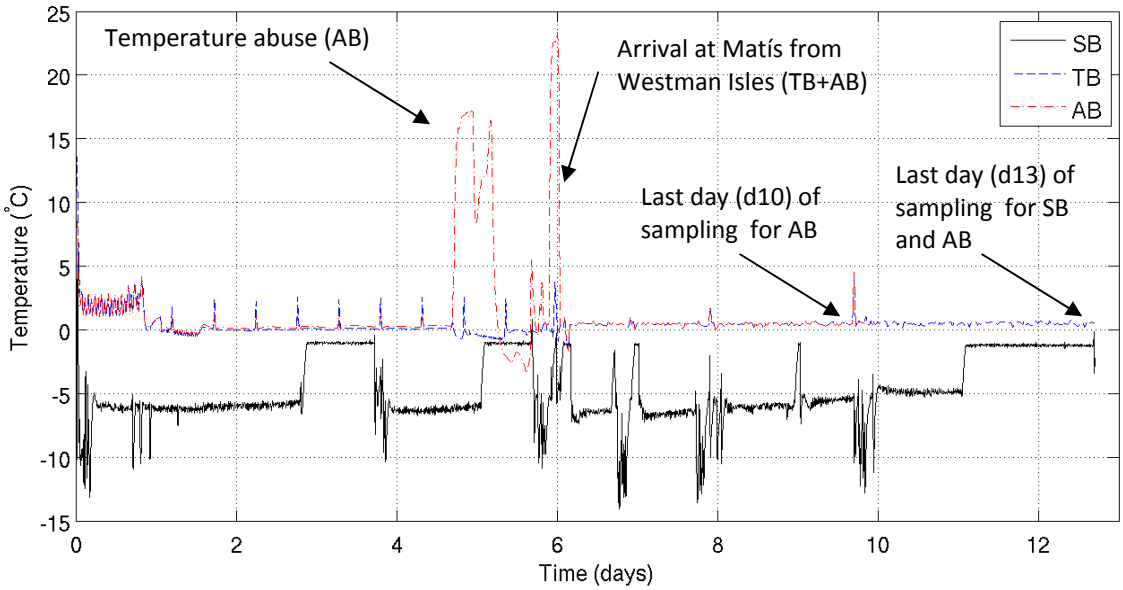


Figure 6. Environmental temperature for the three EPS groups (SB, superchilled boxes; TB, transport-simulated boxes; and AB, transport-simulated and abused boxes)

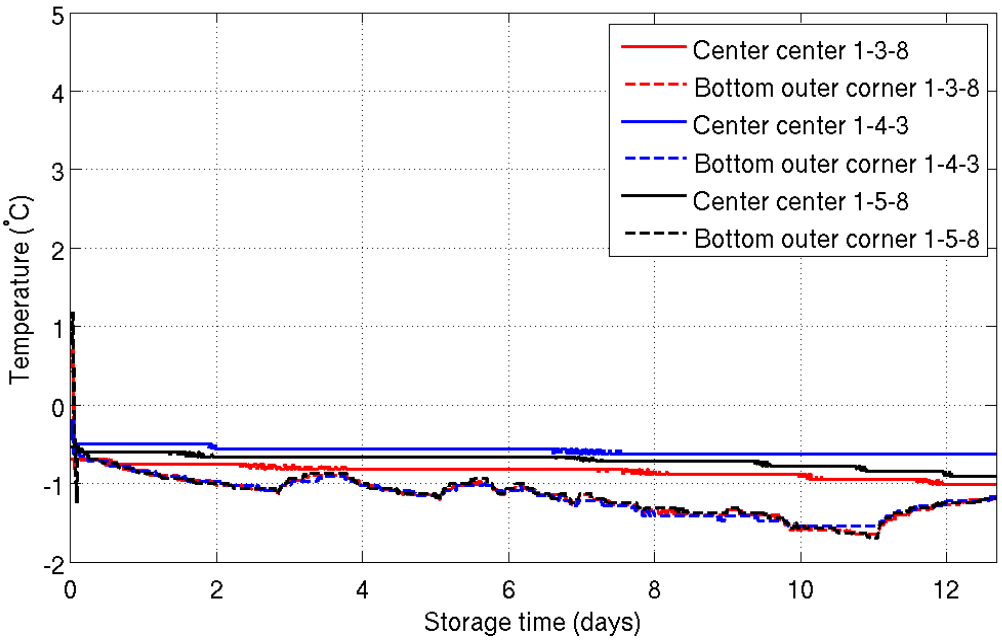


Figure 7. Product temperature of SB group (pallet 1, superchilled) in three boxes positioned at the corner on three pallet levels (3rd, 4th, 5th) during the 13-day storage period. Center-center refers to the middle part of the box (least influenced by environmental T); bottom outer corner refers to a thermally sensitive point in the box (allowing "environmental heat" to flow in).

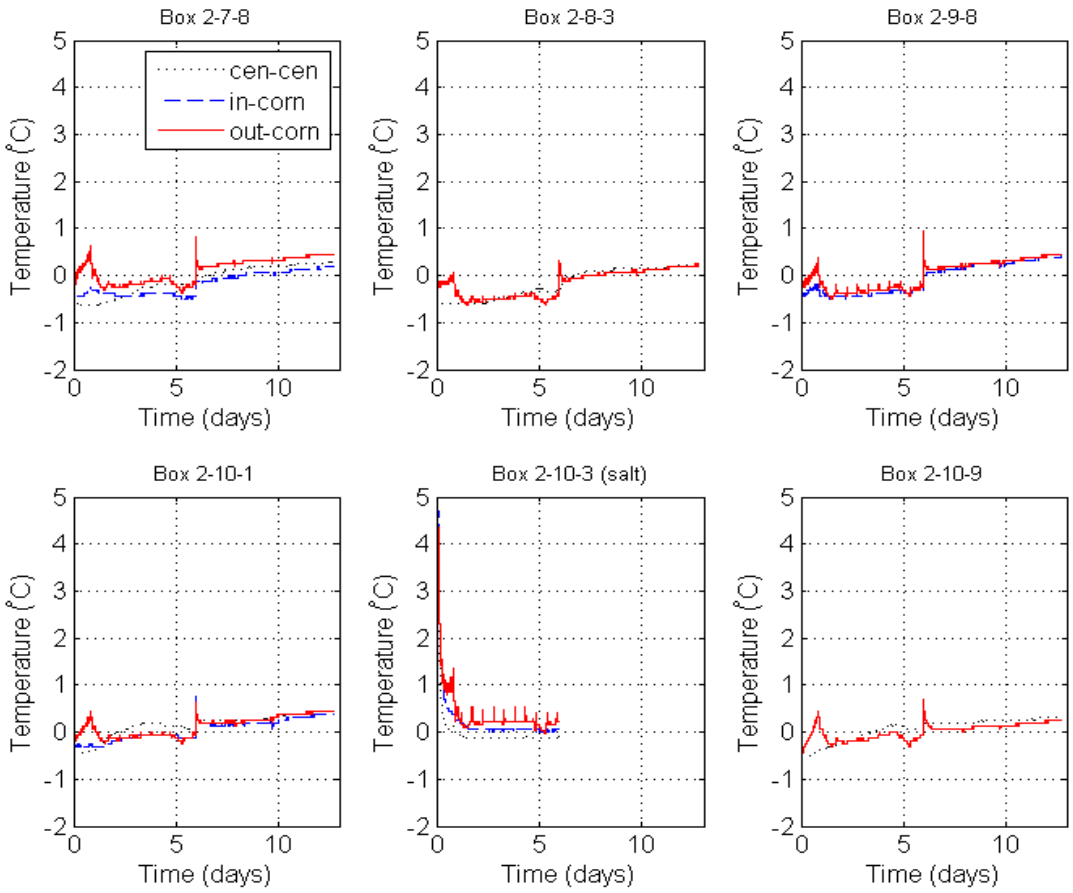


Figure 8. Product temperature of TB group (pallet 2, transport-simulated) during the 13-day storage period in three boxes positioned at the corner on three pallet levels (7^{th}, 8^{th}, 9^{th}) and two others from the top layer (10^{th}). Center-center refers to the middle part of the box (least influenced by environmental T); in-corner refers to the corner closest to the middle of the pallet; out-corner refers to the corner pointing outward on the pallet.

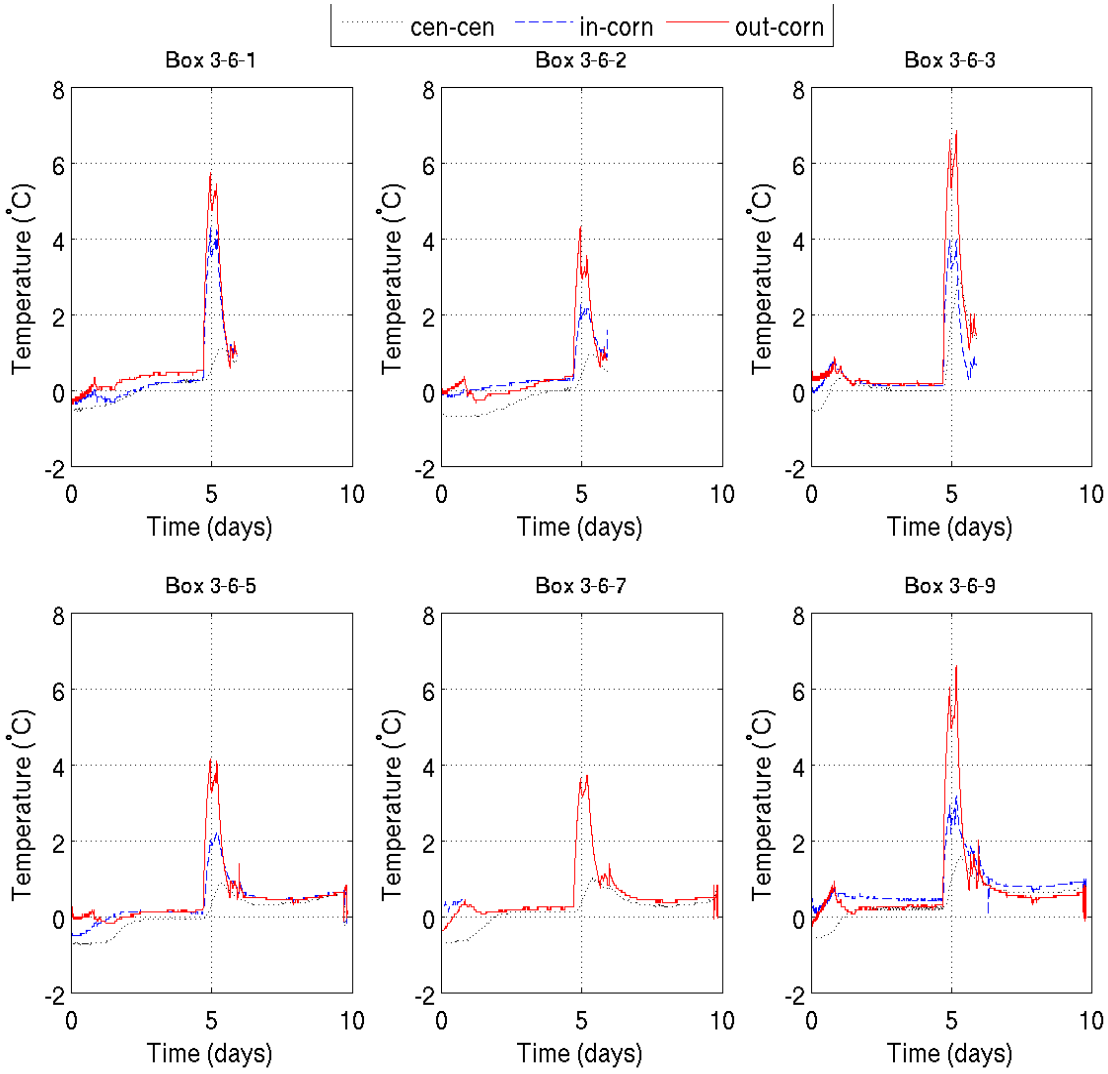


Figure 9. Product temperature of AB group (pallet 3, transport-simulated and abused) during the 10-day storage period in six boxes positioned at the top of the pallet (6th)

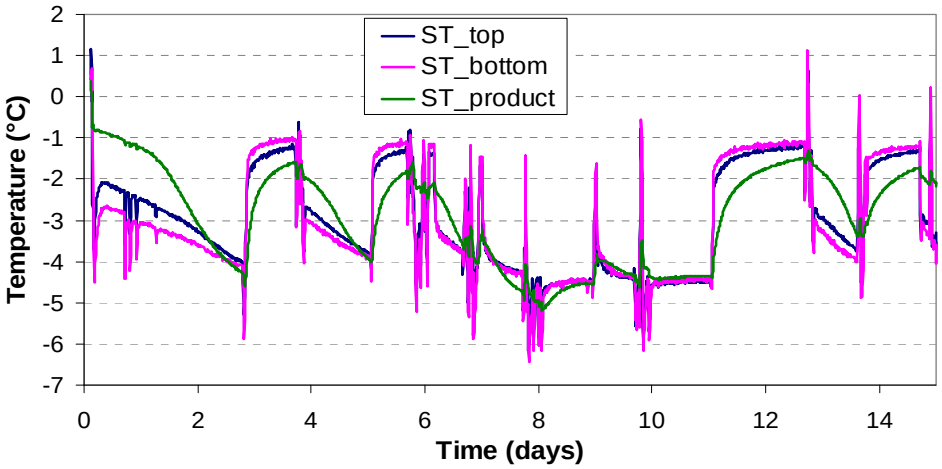


Figure 10. Environmental (top and bottom of tray) and product temperature of ST group during the storage period

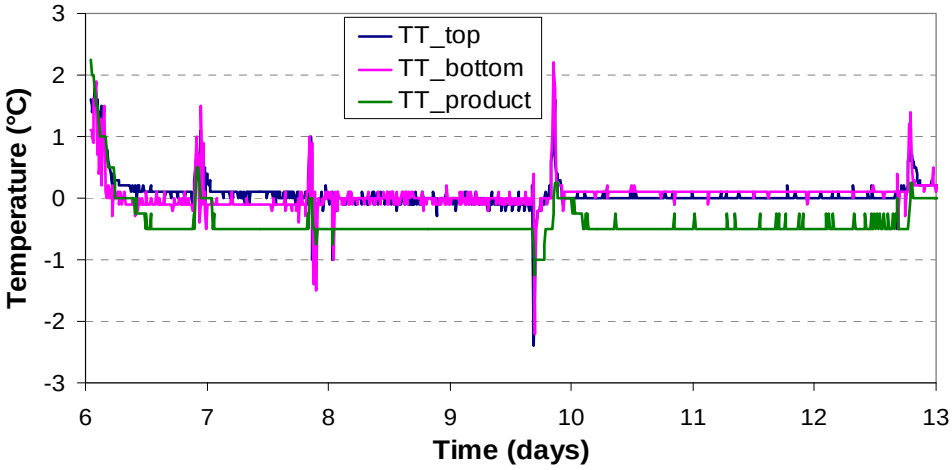


Figure 11. Environmental (top and bottom of tray) and product temperature of TT group during the storage period

3.2.2 *Sensory evaluation*

Figure 12 shows how the samples were characterised by the sensory attributes. Altogether 79% of the sensory variation was explained in the first two principal components. The main variation (54%) between the samples was due to differences explained by storage time along the horizontal axis of Figure 12. Sensory attributes characteristic for cod at the beginning of storage, such as shellfish odour, sweet and meaty odours and flavours are located to the left in Figure 12 describing samples during the first days of storage (Day 0, SB-d06, AB-d06, TB-d06 and ST-d06). As storage time progressed, these sensory attributes became less evident but the sensory attributes describing the cod at the end of storage, such as sour and TMA flavour, were more evident. Table cloth odour and off flavour were pronounced at the end of storage time, especially for sample SB-d13.

The samples were also different with regard to texture and appearance which is clearly seen along the vertical axis of Figure 12. Especially the ST group, stored at undesirable conditions ($T_{product}$ = -2.9 ± 1.2 °C), was described with dark colour, precipitation and discolouration, more rubbery texture and less juicy, tender, flaky, soft and mushy texture than other groups. This trend became more obvious within this group as storage time progressed (ST-d13 and ST-d15). This was opposite to the other samples which had a higher product temperature ranging from -0.7 to 0.1 °C. Interactions between lipid oxidation products and proteins cause undesirable property changes of proteins including texture deterioration and change in colour. Conformational changes in the protein structure can significantly affect functional properties important to final products (Xiong and Decker, 1995).

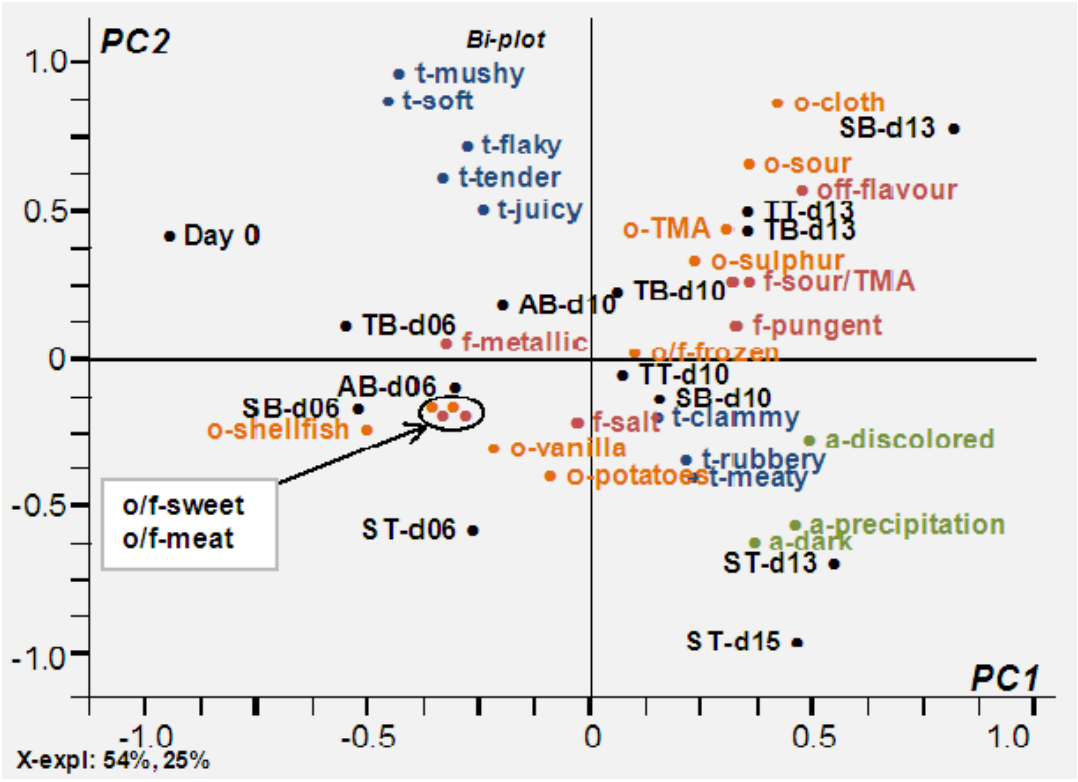


Figure 12. PCA describing sensory quality, odour (o-), appearance (a-), flavour (f-) and texture (t-) of the sample groups with storage time (d). PC1 VS PC2 (X-expl.: 54% and 25%). Bi-plot of scores (samples) and loadings (sensory attributes).

Most of the sensory panellists detect spoilage attributes when the average Torry score is around 5.5 and this limit has been used as the limit for consumption at Matís (Martinsdóttir *et al.*, 2001). According to this criterion, all groups had a shelf life of 10 days, except for ST group which had a slightly longer shelf life, or 11 days (Figure 13). End of shelf life is usually determined when sensory attributes related to spoilage become evident. When the average QDA score for those attributes is above the value 20 (on the scale 0 to 100) most panellists detect them (Bonilla *et al.* 2007; Magnússon *et al*. 2006). Hints of table cloth odour and off flavour were evident of AB after 10 days, indicating it was approaching end of shelf life (Figure 14). A similar trend was seen for SB after 10 days of storage. According to odour and flavour attributes describing spoilage, ST had not reached the end of shelf life after 15 days, though hints of spoilage attributes were detected on days 13 and 15. However, due to the low temperature during storage ($T_{product}$ = -2.9 ± 1.2 °C), the loins were half-frozen part of the time, resulting in changes in appearance and texture which negatively affected the product. TB was close to end of shelf life on day 10, with obvious table cloth odour, with hints of TMA odour and off odour. This was observed for TT as for TB. A summary of sensory shelf life estimation for the different products is shown in Table 5, based on either Torry or QDA score.

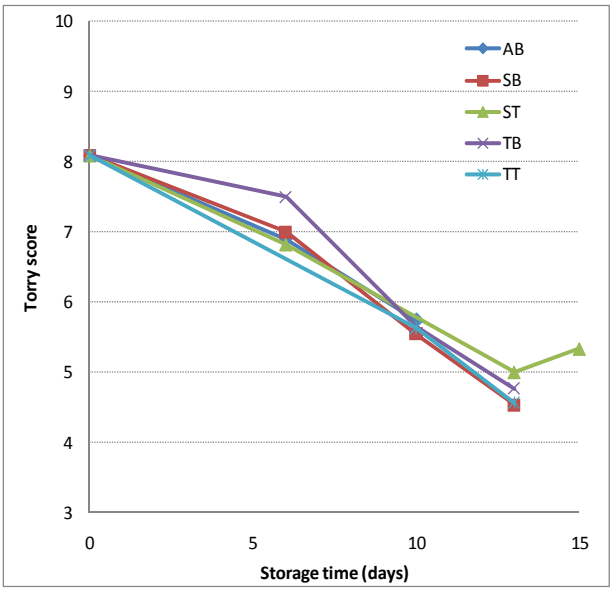


Figure 13. Average Torry freshness scores

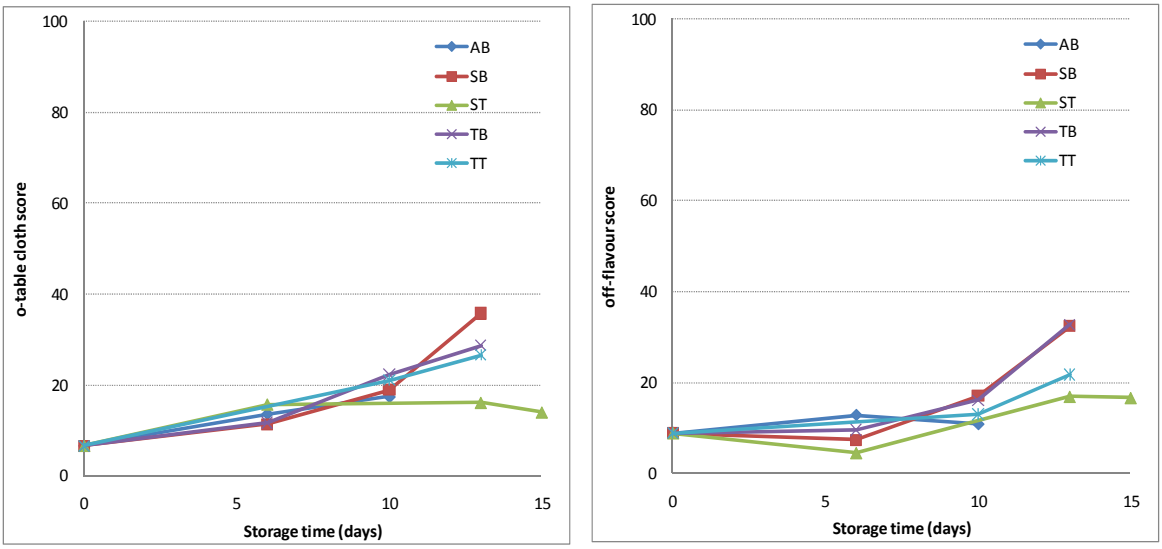


Figure 14. Average sensory scores (on the scale 0-100) for table cloth odour and off flavour

Table 5. Estimation of shelf life (in days) according to sensory evaluation with Torry freshness scale and QDA odour and flavour attributes

Opale cod loins processed on Nov. 24th 2009	Group	$T_{ambient}$ (°C) based on SSL	$T_{product}$ (°C, center*) based on SSL	Shelf life in days (Torry)	Shelf life in days (QDA)
EPS 5 kg-P1	SB	-5.4 +/- 2.3	-0.7 +/- 0.1	10-11	10-11
EPS 5 kg-P2	TB	0.4 +/- 0.9	-0.2 +/- 0.3	10-11	10-11
EPS 5 kg-P3	AB	1.4 +/- 3.9	0.1 +/- 0.4	10-11	ca 10
Trays-P1 (800 g)	ST	-5.4 +/- 2.3	-3.1 +/- 1.3	11	15+**
Trays-P2 (800 g)	TT	0.4 +/- 0.9	-0.2 +/- 0.3	10-11	10-11

* Logger positioned in the box center; average of 3 replicate boxes

** Appearance and texture attributes indicated that the shelf life was shorter (11 d)

3.2.3 *Microbiological analysis*

The initial microbial load (TVC) on the cod loins was in average log 5.1 ± 0.2 colony-forming units (CFU) g^{-1}. Contamination by specific spoilage organisms (SSO) was dominated by pseudomonads (log 3.5 ± 0.1 CFU g^{-1}), followed by H_2S-producing bacteria (log 2.3 ± 1.0 CFU g^{-1}) with low levels of *Photobacterium phosphoreum* (Pp). TVC and counts of H_2S-producing bacteria progressed more slowly in SB and ST groups and became significantly lower than TB and TT groups on day 13 (P<0.05) (Figure 15). In fact ST group had significantly lowest counts (TVC and SSO) compared to all groups (Figure 15 and Figure 16). Pp growth was significantly slower in SB than TB and AB groups on day 6. Pseudomonads developed at a slower rate in ST and SB groups, being significant for SB on day10 and for ST on days 6, 13 and 15. Overall, the little difference in average product temperature resulted in similar behaviour of SSO in TB, TT and AB groups, while slowest growth was observed in ST group. Pseudomonads rapidly prevailed in all groups, followed by H_2S-producing bacteria. Pp apparently had a lesser importance in the spoilage process of the products studied in this trial. Average data and statistical differences are provided in Appendix II.

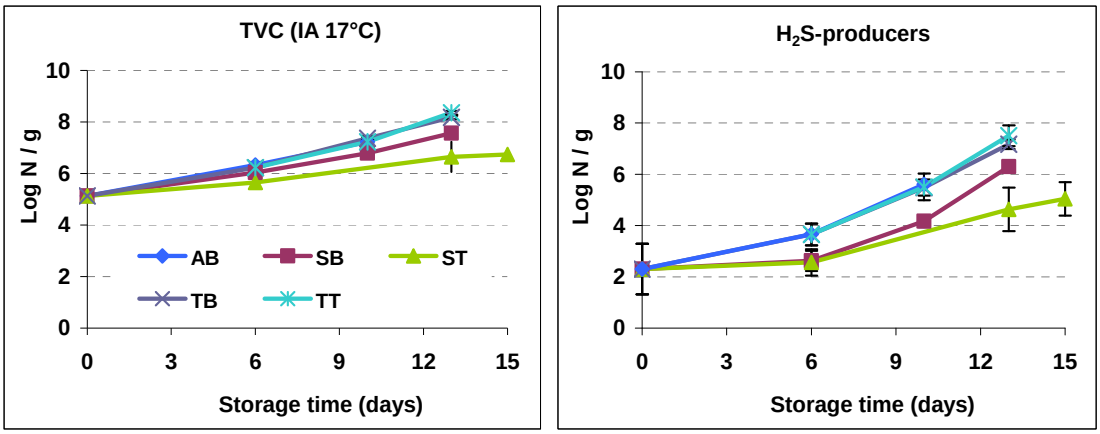


Figure 15. Total viable psychrotrophic counts (TVC) and counts of H_2S-producing bacteria in differently stored cod loins (ave ± SD, n=3)

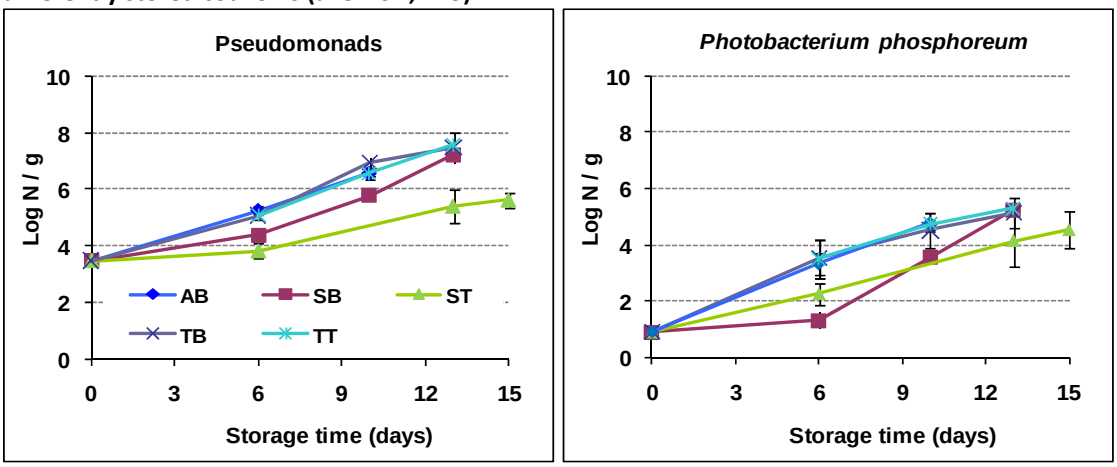


Figure 16. Growth of presumptive pseudomonads and *Photobacterium phosphoreum* in differently stored cod loins (ave ± SD, n=3)

Comparison of estimated pseudomonad counts by the qPCR method and the traditional plate count one is presented in Figure 17. It is noteworthy to mention that the samples had been frozen and thawed three times due to problems encountered during DNA extraction. It follows that the qPCR results may underestimate the pseudomonad levels present since DNA may have been lost. This is well illustrated by Figure 14 with the greatest deviations being observed at early storage when lower pseudomonad levels were present. By setting the intercept to zero, it is observed that PCR results must be multiplied by a factor of 0.9154 to reach CFC counts. The marked data point of Figure 17 could be considered as an outlier. If it is excluded the correlation is improved considerably resulting in a trend line with the equation y=0.8932x+0.0826 and R^2=0.8332.

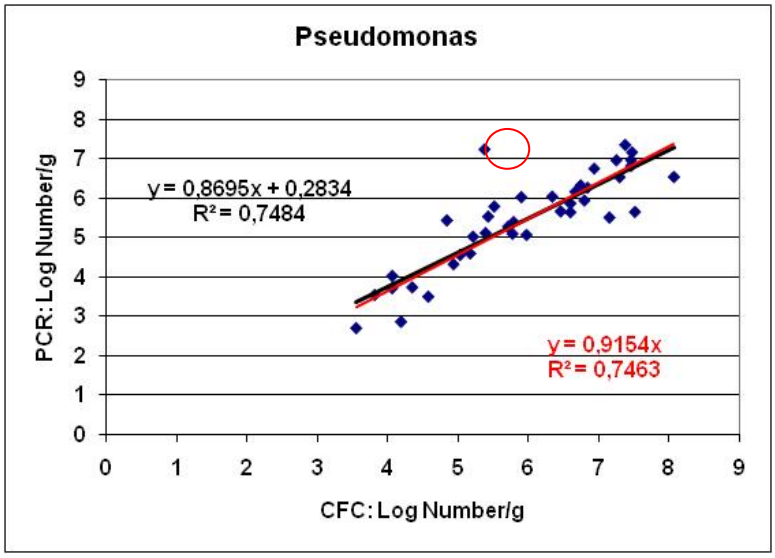


Figure 17. Comparison of conventional enumeration (modified CFC) to qPCR analysis of pseudomonads

3.2.4 *Chemical analysis: TVB-N, TMA and pH*

No significant increase was observed in TVB-N and TMA content during the first 10 days of storage (Figure 18). Little change was detected in ST loins during the storage period while a slight increase was observed in AB loins on day 10. After day 10, a rapid development was seen in TT, TB and SB groups. This TVB-N and TMA increase may be attributed to more rapid SSO growth observed in these groups (TT, TB and AB). TMA is mainly a bacterial metabolite derived from the reduction of trimethylamine oxide (TMAO). This reaction is expected to proceed at a faster rate at conditions of low oxygen tension (Huss, 1972) e.g. under vacuum. Therefore, an atmosphere poor in O_2 should contribute to TMA production. Both *Shewanella putrefaciens* (a H_2S-producer) and *P. phosphoreum* (Pp) can produce TMA (Dalgaard, 1995). Indeed they proliferated more rapidly in TT, TB and AB than ST and SB groups (Figure 15 and

Figure 16). However, the more rapid TMA development observed in TT loins could be explained by the 40% vacuum condition used at packaging and the storage temperature for TT group. On day 13, TT group had reached TVB-N levels higher than proposed EU limits for gadoid fish (TVB-N=35 mgN/100 g) while TB and SB groups were almost there.

Flesh pH is shown in Figure 19 and the increase observed mostly corresponds to the accumulation of basic nitrogen compounds reported above. However, the increased TVB-N and TMA content of SB loins was not confirmed with the steady pH observed which could be due to a concomitant production of acidic metabolites under superchilled conditions.

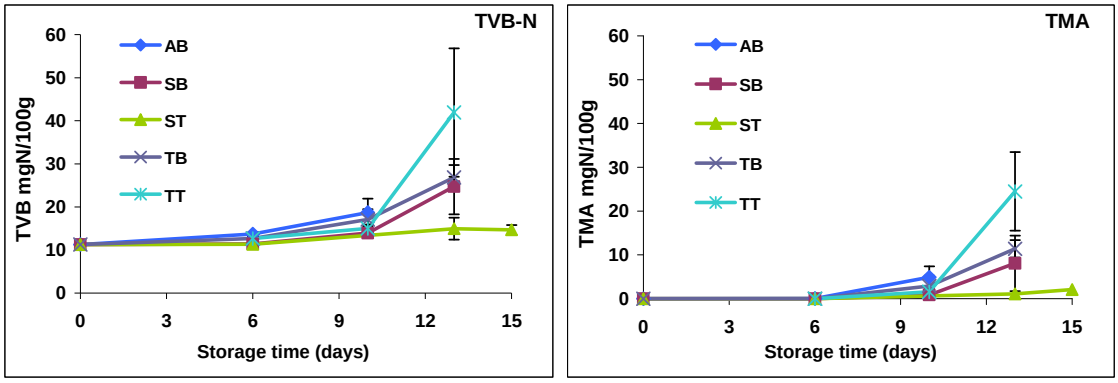


Figure 18. Total Volatile Base Nitrogen (TVB-N) and trimethylamine (TMA) in differently stored cod loins (ave ± SD, n=3)

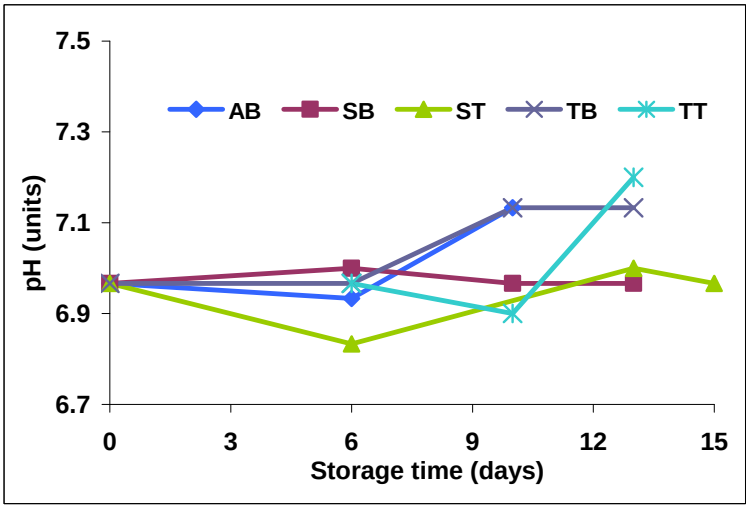


Figure 19. Measurements of pH in differently stored cod loins (ave ± SD, n=3)

3.3 Conclusion

The ST group (superchilled trays) had the longest shelf life according to sensory evaluation of flavour and odour. However, because of the negative influence of the too low temperature on the appearance and texture of this group, the shelf life was concluded to be 11 days. The

ST group differed from other groups in appearance and texture attributes, as it was more described with dark colour, precipitation and discolouration, more rubbery texture and less juicy, tender, flaky, soft and mushy texture than other groups. This indicates that chemical and enzymatic spoilage rather than bacterial spoilage occurred at this sub-zero condition. Based on these results, the total shelf life of the ST group did not differ from other groups in shelf life which had a shelf life of 10-11 days according to Torry and QDA. Overall, the spoilage profile was represented by a low TMA production at sensory rejection, explained by the dominance of *Pseudomonas* spp. in the product microbiota.

4. SHELF LIFE PREDICTION MODEL FOR FRESH COD

4.1 Development of the shelf life prediction (SLP) model for fresh cod by WIT

Predictive microbiology uses mathematical models to predict the growth and concentration of the microbial organisms in the food under dynamical environmental conditions. There are two basic types of the mathematical models for microbial growth: deterministic and stochastic. In the case of deterministic models, the outcome is one single growth curve or one single quantity for microbial concentration at any instant of time. On the other hand, the stochastic models include the probability component in the analysis and the outcome is the probability or probability density function (PDF) to have certain microbial concentration at any instant of time. Some of the commonly used deterministic models are the Baranyi and Roberts, and Gompertz (Baranyi *et al*., 1995; Zwietering *et al*., 1990). The deterministic models cannot take into account this probabilistic component in the microbial growth.

A SLP model for fresh cod was developed by WIT and implemented by AFI in the TRACECHILL Server. The model is explained in detail in D1.18 Chill-On report (Popov *et al*., 2009), and here only a short outline is presented. Briefly, a dynamic growth model under variable temperature conditions was developed using Matís raw data for microbial growth of cod SSO (*Photobacterium phosphoreum*, pseudomonads and H_2S-producing bacteria) under aerobic conditions. The primary Baranyi model was implemented using measurement data under a set of fixed temperatures. In the Baranyi model, the maximum specific growth rate and the lag phase at constant environmental conditions are expressed in exact form and it has been shown that, in limit case when maximal cells concentration is much higher than the initial concentration, the maximum specific growth rate is approximately equal to the specific growth rate. The model parameters were determined in a temperature range of -1 to +11 °C. As a secondary model, the square root model was used for the estimation of the maximum specific growth rate, assuming that the initial physiological state of the inoculum

is constant and independent of the environmental parameters. For prediction of the remaining shelf life, the deterministic model is used. The confidence interval for the growth rate is used to calculate upper and lower limits for the remaining shelf life. The shelf life is assessed based on the intersection of the threshold level or rejection level and growth curves which correspond to the upper, lower and predicted value of the growth rate.

The SLP model has had nine modifications in the year following the wet trial in November 2009, which is reported in this document. The most important update related to this report is the inclusion of automatic calibration of the model by using the laboratory results for SSO counts. The problem that the calibration of the model addresses is the physiological state of the cells which is an unknown initial parameter. To calibrate the model, data on microbial growth under variable environmental conditions is required. This is usually provided by two species counts at the beginning of the supply chain, e.g. samples taken on day 1 and day 2. The adjustment function, which takes into account the lag phase during which the bacterial population adapts to the new environment is denoted by *α(t)*. The natural logarithm of the cell concentration is denoted by *y*. The connection between the specific growth rate, lag phase and parameter h is given by the following equation:

$$h = t_{lag} \cdot \mu_0, \tag{1}$$

where lag time is denoted by t_{lag}. The parameter *h* is used instead of the initial physiological state because of the numerical stability. Using the raw data and minimising the mean square root error (MSRE), the model is calibrated and the initial value for physiological state, i.e. parameter *h*, is obtained (Gospavic *et al*., 2008).

4.2 Validation of SLP model for fresh cod

One of the aims was to validate the SLP model developed for fresh cod by applying the product temperature profiles to obtain predicted SSO (*P. phosphoreum*, pseudomonads and H_2S-producing bacteria) growth curves and compare them to observed proliferation of spoilage bacteria in the products during the trial (data from 3.2.3). Also, SSO limits for product rejection (SSO counts from log 7 to 7.5 CFU/g) were evaluated and rejection time compared to the shelf life determined by sensory analysis (data from 3.2.3).

Table 6 summarizes the observed and predicted shelf life values obtained for the different cod products during the wet trial based on the rejection SSO count of log 7/g. The control group (Pallet 1, P1) was shipped to Matís soon after its production and stored under superchilled conditions (SB). Some of the boxes were repackaged as trays (ST) and stored in the same chamber where the temperature profile deviated from the planned one (-1°C). The

temperature recorded by the logger situated on top of pallet 1 was -4.8 ± 2.5 °C. As the cod loin temperature went beyond -1 °C, additional deteriorative changes occurred even though bacterial spoilage was delayed (Figure 15 and Figure 16). This explains the disagreement between the sensory shelf life (10-11 days) and the SSO predicted one for groups ST (17.5 days) and SB (12.9 days). It is important to emphasize here that the system must specify the range of conditions suitable for the models developed. Outside this temperature range (SLP model T range: -1.3°C to 11.1°C), it is not possible to rely on the prediction. The prediction of the other groups (TB, TT and AB) fitted well the sensory data. It is noteworthy that the error in shelf life prediction increases with storage time, resulting in a possible deviation of about 1.5d on day 10 for SB-Pseudomonad plot but 2 days at day 15. Overall, pseudomonads were the SSO reaching first log 7/g, hence the prevailing spoilage bacteria in these products. This could be expected since their initial levels were highest. Figure 20 compares predicted SSO growth to the observed counts.

Indeed, as the for correctly predicted shelf life for groups AB, TB and TT, the predicted SSO growth curves coincided generally well with the observed SSO counts as illustrated in Figure 20. The low initial level of *P. phosphoreum* (PP) in the cod products and the low sampling frequency at early storage caused the shifting of the curve to the right, likely to deviate slightly PP growth curve to what should be observed. But still growth fitting was generally satisfactory.

Table 6. Comparison of sensory shelf life to predicted values by SLP model based on SSO limit of log 7/g for fresh cod products

Opale cod loins processed on Nov. 24th 2009	Group	$T_{product}$ (°C, center*) during storage	Sensory shelf life in days ** (Torry)	Predicted SL based on SSO counts of log 7/g		
				Pp mean	Pseud mean	H2S mean
EPS 5 kg-P1	SB	-0.7 +/- 0.1	10-11	14.5	**12.9**	15.8
EPS 5 kg-P2	TB	-0.1 +/- 0.3	10-11	15.0	**10.5**	12.9
EPS 5 kg-P3	AB	0.1 +/- 0.4	10-11	12.6	**10.6**	12.8
Trays-P1 (800 g)	ST	-2.9 +/- 1.2	11	**17.5**	18.4	18.4
Trays-P2 (800 g)	TT	-0.2 +/- 0.3	10-11	14.5	**11.1**	13.5

* Logger positioned in the box center; average of 3 replicate boxes.
** Days from processing/packaging.

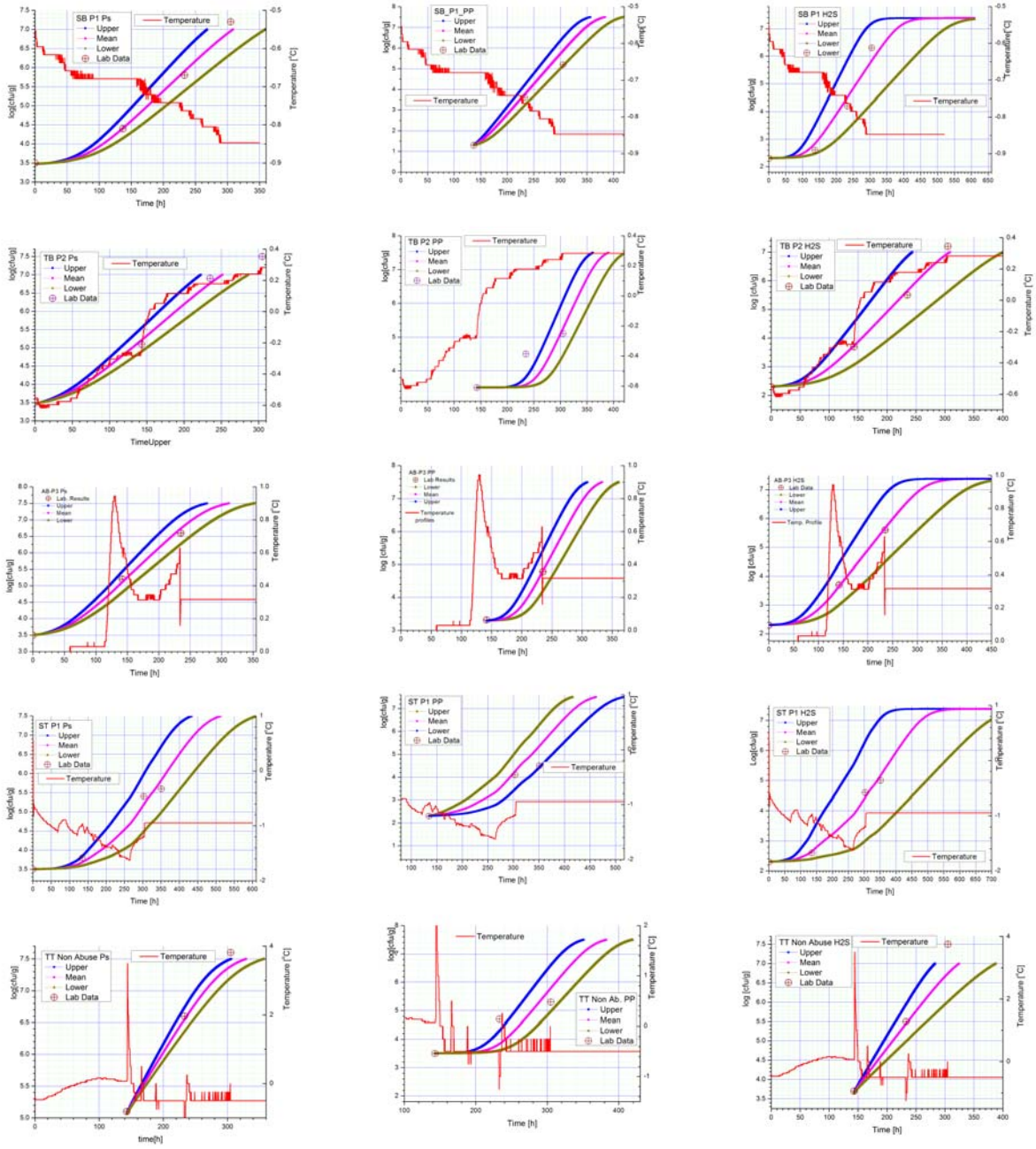


Figure 20. Predicted growth curves for SSOs (Ps, pseudomonads; PP, *Photobacterium phosphoreum*; H2S, H_2S-producing bacteria) over the storage period produced by applying to the SLP models the average temperature profile of cod products that undergone 5 different treatments. Average temperature profile shown in red (right y-axis, °C) and observed SSO counts indicated by a pink circle (left y-axis, log CFU/g). Upper, mean and lower SSO predicted growth curves shown in blue, pink and green, respectively. SB, superchilled boxes; TB, transport-simulated boxes; AB, transport-simulated and abused boxes; ST, superchilled trays packed on day 0 from processing; TT, trays packed from TB boxes on day 6 post processing and stored chilled; P1, pallet 1; P2, pallet 2; P3, pallet 3.

5. QMRA AND PATHOGEN CHALLENGE TEST COMPARISON

A challenge study was performed to evaluate the growth of pathogens (*Escherichia coli, Listeria monocytogenes* and *Salmonella Dublin*) under dynamic conditions added to cod loins at low levels to validate the QMRA model.

5.1 Materials and Methods

5.1.1 *Preparation of pathogenic strains*

Three strains (*L. monocytogenes* [*Lm*] isolated from fish flesh in Iceland, *E.coli* DSM 30083 [*Ec*] and *S. Dublin* [*Salm*] from Livsmedelsverket, Uppsala, Sweden) were cultured separately at 37°C (24 h) in tryptic soy broth (TSB, Difco). Fifty µl were then transferred to 5 ml TSB and culture at 22°C for 48 h. Prior to inoculation, 0.333 ml of each culture was added to 9 ml chilled MRD and mix, after which serial dilutions were prepared and plated on the selective media to estimate the level of each pathogen added to the cod loins. A low-level pathogen solution was to be reached (10^{2-3}cells/ml) by adding 1 ml of 10^{4-5} cells/ml dilution to 99 ml of chilled MRD.

5.1.2 *Spiking pathogenic bacteria to cod loins*

Following initial sampling of the cod loins from 3 EPS boxes, 15 loins were cut into half aseptically to obtain 30 pieces. The standardised contamination process was conducted as follows. Each half loin was put into a sterile plastic bag, weighed (152 ± 26 g) and the necessary inoculum added (1 ml on both sides, i.e. 2 ml for 150 g cod piece) to be distributed evenly on the fish surface by massaging the bag gently for 1 min, ensuring that all liquid had been absorbed into the fish. The inoculum levels aimed to reach 10^{0-1} cells/g. To do so, 1-ml pathogen solution had to contain at least 100-1000 cfu/ml of each bacterium (*Lm, Ec, Salm*). When all cod pieces had been contaminated, they were transferred to a doubled layer plastic bag used to line the EPS box reaching a total load of 4.6 kg, the lid put on and the box incubated at the pre-determined temperature cycle. Two temperature loggers were stuck outside the lining bag about 5 cm below the upper level of the EPS box, measuring the temperature of the upper/inner box section, and one logger was put outside the box to monitor the environmental temperature.

5.1.3 *Storage and sampling of contaminated cod loins*

Storage temperature had been profiled to simulate sea freight transport, product retailing and distribution at destination. The storage time was set to 13 days with 5 sampling points (d1-3-7-10-13) and triplicate samples analysed each time. To avoid contamination around the lab during sampling, one fish piece (one sample) was cut aseptically, mixed and about 25 g of cuts weighed into a stomacher bag, adding chilled MRD accordingly and stomaching for 120 s.

5.1.4 *Microbiological analysis*

Presence of the three pathogens (*Lm, Ec, Salm*) was assessed in the cod loins initially using the detection methods for *Listeria* and *Salmonella*, while MPN test was done for fecal coliforms (see below). Evaluation of total viable counts (TVC, 17 °C) using iron agar (IA) as previously described as well as detection/MNP and direct plating of *Lm*, *Ec* and *Salm* were done throughout the experiment.

Listeria detection (presence or absence in 25 g): For quantitative detection methods, a 50-ml portion is removed and the following is done. Primary enrichment in LB 1 (30 g of fish mince in 270 ml; 30 °C, 24 h) removing a 50-ml portion of stomached mixture prior to supplementation; secondary enrichment in Fraser broth (0.1 ml in 10 ml; 37 °C, 48 h). Isolation is done both from primary and secondary broth cultures onto Oxford Agar (OX; 37 °C for up to 48 h) and Ocla agar (37 °C for up to 48 h). Growth from black colonies on Oxford agar and green-blue colonies on Ocla agar is streaked onto Tryptone soya agar (37 °C, 24 h), selecting five typical colonies from Ocla agar and five from Oxford agar, and then onto Horse Blood agar (HBA; 37 °C for up to 24 h). After incubation, confirmation tests are carried out using pure cultures from TSA or HBA Confirmation tests include Gram-staining and catalase testing. Species identification includes haemolysis on HBA and testing by using API Listeria (System for the identification of *Listeria*, bioMérieux SA/France). Reference: NMKL no 136: 4. ed. 2007

Listeria enumeration with direct plating (DP): From the portion of LB1 primary enrichment broth (before addition of selective agents) kept aside, inoculation onto *Listeria* selective media (Ocla and OX agar) was performed. Further dilutions were done in LB 1 broth and 0.1 ml plated out. After incubation (37 °C, up to 48 h) typical colonies are counted. Confirmation tests are carried out as in the detection part. Detection limit: 10 CFU/g. Reference: NMKL no 136: 4. ed. 2007

E.coli enumeration with VRBA technique: The tenfold MRD-diluted sample prepared earlier was used. Pre-incubation was done in Tryptone Soya Agar (TSA) for 1 hour (20 – 25°C; pour-plating 1 ml of above tenfold MRD-diluted sample). Then Violet Red Bile Agar is poured on top of the inoculated TSA layer (44.0°C, 24 h). Ratio of TSA:VRBA in petri dishes should be 1:2. At least five suspicious colonies of each type are confirmed by inoculation in EC-broth (44.0°C, 24 h). Culture from gas producing tubes is inoculated into Tryptone water (44.0°C, 24 h). Confirmation of *E.coli* is done by testing of indole production with Kovac's reagent. Detection limit: 10 CFU/g

Reference: NMKL no 125: 4. ed. 2005 and FDA: 2002, chapter 4.

E.coli enumeration with MPN technique (3 x 3 series): Three tubes are used for each dilution. Pre-enrichment is done in Lauryl Sulfate Tryptose (LST) broth (1 ml of tenfold MRD-diluted sample from above in 10 ml LST; 37°C, 48 h). Confirmation test for faecal coliforms is done in EC broth, transferring 3 drops from LST positive tubes to 10 ml EC (44.0°C water bath, 24 h). Positive EC tubes are inoculated into Tryptone water (TW), transferring 3 drops to 5 ml TW (44.0°C, 24 h). Confirmation of *E. coli* is done by testing of indole production with Kovac's reagent. Detection limit: 3 MPN/g. Reference: NMKL no 96: 3. ed. 2003

Salmonella detection: The mince prepared above is used. First enrichment is done in Buffered Peptone Water (25 g in 225 ml; 37°C 18 h); second enrichment in RV broth (0.1 in 10 ml; 41.5°C, 24 h) and Tetrathionate broth (1 ml in 10 ml; 41.5°C, 24 h). From these broths a loopful is streaked onto two solid media: XLD and BG agar (37°C, 24 hours). Typical colonies (2-4 or as needed) are inoculated onto TSI-agar and LI-agar slants (37°C, 24 h). Finally slants are tested for O and H antigens. Species identification is carried out by serological methods.

Detection limit: presence or absence in 25 g

Reference: NMKL no 71: 5. ed. 1999

Salmonella counts: BG plates are spread-plated with **the tenfold MRD-diluted sample** and corresponding serially diluted tubes (37°C, 24h).

Detection limit: 10 CFU/g (distributing 1 ml of first dilution on 2 plates)

5.2 Results and Discussion

Temperature recordings along with microbial analyses from these groups were collected. The dynamic pathogen challenge trial was conducted for 13 days but the fish (5-kg EPS box) was obviously spoiled on day 10 due to the excessive temperature abuses during the storage period. The three pathogenic strains were added simultaneously at low levels (2-20 cells /g)

to cod loins to simulate naturally occurring contamination. This low initial cell concentration led to a very slow growth as shown in Figures 21 and 22 for *Lm* and *E. coli*, respectively.

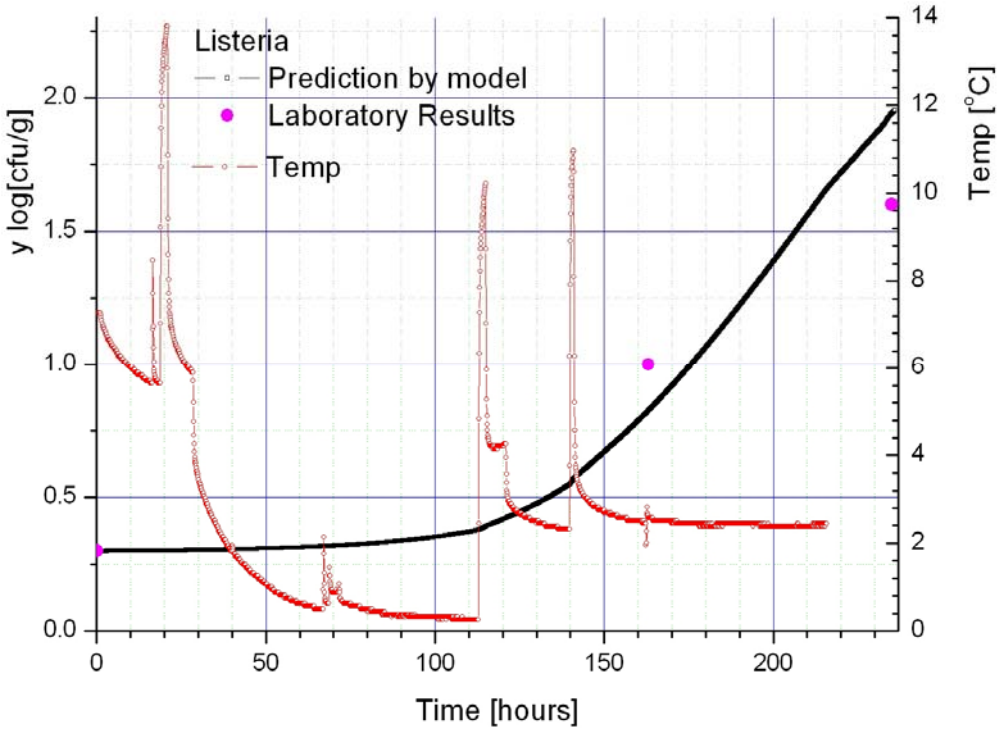


Figure 21. Comparison of predicted Lm growth (black line) based on fish temperature profile (red line) to measured Lm concentrations (pink dots)

The temperature profile with abusive temperatures is shown as well, and the average product temperature resulting was 1.9 ± 2.2 °C. *Salmonella* was detected by the conventional methods but no growth occurred to allow for its enumeration above the detection limit (20 cells/g). This corresponds to the "no growth" obtained by the *Salmonella* model (data not shown). Similarly, *E.coli* counts were below this detection limit but it was shown to be present in the product by the conventional method. In fact, very little growth was predicted by the model.

This could be expected for both pathogens since their growth in fish muscle below 10°C has been shown to be little or none during data collection trials. The results therefore show that *Lm* growth is more "problematic" than that of the other pathogens tested in chilled cod products. It also demonstrates that at inoculation levels probably slightly above reality, level of *Lm* at the end of shelf life (<240 h) is still just below the EC limit (100 CFU/g) for ready-to-eat products. Finally, this dynamic trial confirms the possibility to predict growth of at least *Lm*, and likely *E. coli*, in chilled cod products.

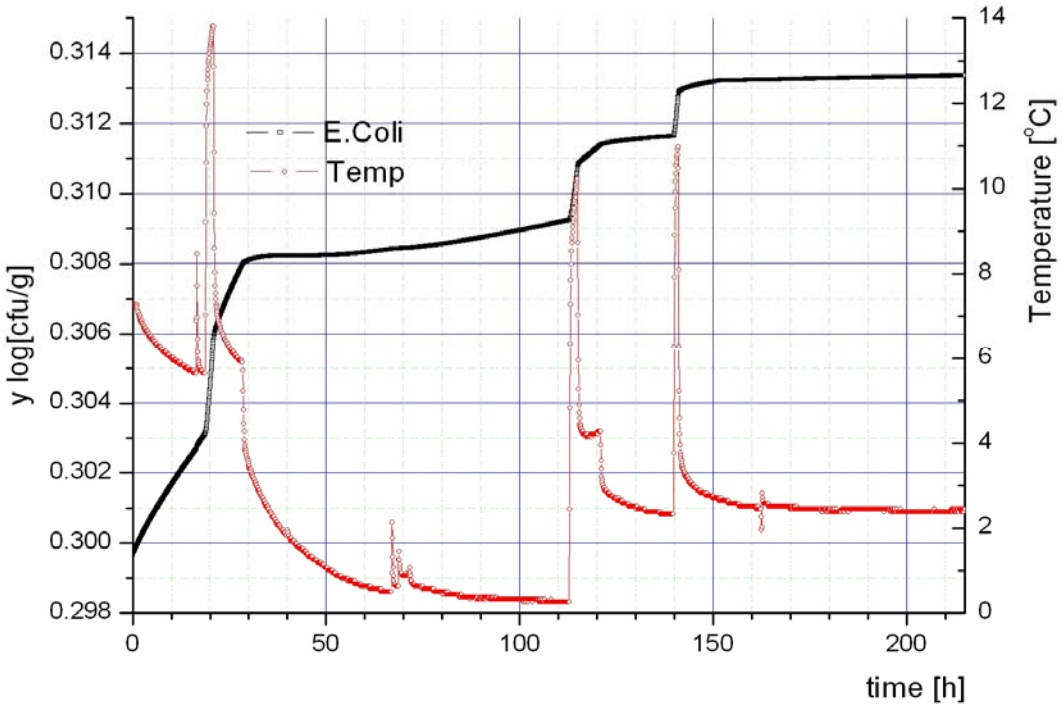


Figure 22. Predicted *E.coli* growth (black line) based on fish temperature profile (red line)

6. CONCLUDING REMARKS

This cod wet trial included scenarios to test and demonstrate the functionality of some Chill-on technologies in a simulated cod supply chain via sea freight. This report considered the following technologies: pseudomonads enumeration by a qPCR method, shelf life prediction (SLP) and pathogen growth models for fresh cod products. Comparison of estimated pseudomonad counts by the rapid qPCR method and the traditional plate count has been done earlier in several Matís trials, showing good agreement of the methods (Reynisson *et al*., 2008) as in this study. However DNA extraction problems were encountered in this trial, leading to repeated analyses and DNA loss in some refrozen-thawed samples. SLP model evaluation demonstrated that it is important to consider the temperature limits of the model, with the prediction domain ranging between -1 and +11 °C. Outside these limits, a successful prediction cannot be expected as observed in the heavily superchilled groups. For the correctly predicted shelf life of other groups tested, the predicted SSO growth curves coincided generally well with the observed SSO counts. Finally, a challenge study was performed to evaluate the growth of pathogens, *Escherichia coli, Listeria monocytogenes* and *Salmonella Dublin* added to cod loins at low levels and stored under dynamic conditions. The results indicated that *Lm* growth is more likely to occur than that of the other pathogens

tested in chilled cod products. It also demonstrated that the level of *Lm* at the end of shelf life (<240 h) was just below the EC limit (100 CFU/g) for ready-to-eat products even though the inoculation level was probably slightly above reality. This should not be of concern for cod products to be cooked, but the possibility that consumers may use the fish for raw consumption cannot be excluded. Finally, this dynamic trial confirmed the possibility to predict growth of at least *Lm*, and likely *E. coli*, in chilled cod products. Salmonella requires higher temperature (>10 °C; H.L. Lauzon, unpublished data) to grow in cod, which will inevitably spoil rapidly.

7. ACKNOWLEDGEMENTS

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9. APPENDIX I: STATISTICAL ANALYSIS OF SENSORY DATA

	Torry score	o-sweet	o-shellfish	o-meat	o-vanilla	o-potatoes	o-frozen	o-cloth	o-TMA	o-sour	o-sulphur
p-value	0,0000	0,0009	0,0000	0,0001	0,0260	0,1105	0,2343	0,0000	0,0000	0,0000	0,0000
0-point	8,1 ^{a}	29	35 ^{a}	23 ^{a}	23 ^{a}	28	1	7 ^{d}	2 ^{c}	1 ^{b}	0 ^{b}
AB-D06	6,9 ^{ab}	24	22 ^{bcd}	18	16	24	1	14 ^{d}	6 ^{c}	7 ^{b}	1 ^{b}
AB-D10	5,8 ^{c}	19	17 ^{ce}	12 ^{bc}	18	23	2	17 ^{bcd}	9 ^{bc}	5 ^{b}	2 ^{b}
SB-D06	7,0 ^{a}	26	29 ^{ab}	22 ^{ab}	27 ^{a}	24	0	11 ^{d}	6 ^{c}	2 ^{b}	1 ^{b}
SB-D10	5,6 ^{bc}	20	18 ^{ce}	13 ^{bc}	19	24	2	19 ^{bcd}	8 ^{bc}	9 ^{b}	2 ^{b}
SB-D13	4,5 ^{c}	13	12 ^{e}	9 ^{c}	12 ^{b}	19	2	36 ^{a}	21 ^{a}	28 ^{a}	12 ^{a}
ST-D06	6,8 ^{ab}	25	23 ^{bcd}	19	23	28	1	16 ^{d}	9 ^{bc}	4 ^{b}	1 ^{b}
ST-D13	5,0 ^{c}	17	17 ^{ce}	13 ^{bc}	22	32	2	16 ^{bcd}	11	13 ^{b}	3 ^{b}
ST-D15	5,3 ^{c}	13	16 ^{ce}	10 ^{bc}	15	32	2	14 ^{cd}	8 ^{c}	8 ^{b}	3 ^{b}
TB-D06	7,5 ^{a}	28	26 ^{bc}	17	20	20	1	12 ^{d}	6 ^{c}	5 ^{b}	1 ^{b}
TB-D10	5,6 ^{c}	17	18 ^{ce}	13 ^{bc}	21	30	2	22 ^{bcd}	12	10 ^{b}	4 ^{b}
TB-D13	4,8 ^{c}	16	17 ^{ce}	12 ^{bc}	18	27	3	29 ^{ab}	15	16 ^{b}	8 ^{b}
TT-D10	5,6 ^{bc}	19	17 ^{ce}	10 ^{bc}	19	22	3	21 ^{bcd}	9 ^{bc}	6 ^{b}	3 ^{b}
TT-D13	4,6 ^{c}	14	15 ^{de}	11 ^{bc}	18	23	2	27 ^{ac}	19 ^{ab}	27 ^{a}	13 ^{a}

	a-dark	a-discol.	a-prec.	t-flaky	t-soft	t-juicy	t-tender	t-mushy	t-meaty	t-clammy	t-rubbery
p-value	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0224	0,0002	0,0000
0-point	23 ^{bc}	20 ^{b}	20 ^{d}	64 ^{a}	81 ^{a}	64 ^{a}	77 ^{a}	70 ^{a}	12	6	4 ^{c}
AB-D06	19 ^{c}	30 ^{b}	32 ^{ce}	62 ^{ab}	69 ^{bc}	61	71 ^{ab}	48 ^{ab}	21	13	8 ^{bc}
AB-D10	19 ^{c}	26 ^{b}	33 ^{ce}	54 ^{ab}	70 ^{ab}	59 ^{ab}	65 ^{ac}	55 ^{ab}	21	11	7 ^{c}
SB-D06	21 ^{c}	25 ^{b}	43 ^{ac}	60 ^{ab}	73 ^{ab}	63 ^{ab}	73 ^{ab}	53 ^{ab}	12	7 ^{de}	6 ^{c}
SB-D10	26 ^{bc}	37 ^{b}	45 ^{ac}	50 ^{ab}	67 ^{bd}	54 ^{ab}	63	53 ^{ab}	20	15 ^{ac}	8 ^{c}
SB-D13	27 ^{bc}	41 ^{b}	51 ^{ab}	54 ^{ab}	63 ^{be}	54 ^{ab}	66 ^{ac}	57 ^{ab}	16	15	7 ^{c}
ST-D06	23 ^{bc}	32 ^{b}	45 ^{ac}	60 ^{ab}	56 ^{cdf}	53 ^{bc}	60	43 ^{bc}	24	10 ^{bcd}	7 ^{c}
ST-D13	44 ^{a}	52 ^{a}	53 ^{a}	41 ^{bc}	53 ^{dg}	49 ^{bc}	54 ^{ce}	47 ^{bc}	24	13	14 ^{ab}
ST-D15	35 ^{b}	38 ^{b}	46 ^{ac}	37 ^{c}	50 ^{fg}	41 ^{c}	50 ^{de}	33 ^{d}	19	20 ^{a}	16 ^{a}
TB-D06	20 ^{c}	25 ^{b}	33 ^{ce}	60 ^{ab}	73 ^{ab}	63 ^{ab}	73 ^{ab}	56 ^{ab}	12	9 ^{ce}	4 ^{c}
TB-D10	21 ^{bc}	29 ^{b}	35 ^{ce}	52 ^{ab}	67 ^{be}	58 ^{ab}	64 ^{ac}	56 ^{ab}	19	14	5 ^{c}
TB-D13	23 ^{bc}	41 ^{b}	47 ^{ac}	51 ^{ab}	71 ^{ab}	56 ^{ab}	68 ^{ac}	59 ^{ab}	14	11	6 ^{c}
TT-D10	21 ^{bc}	29 ^{b}	36 ^{bc}	55 ^{ab}	67 ^{bd}	54	60 ^{bcd}	50 ^{ab}	25	16 ^{ab}	10
TT-D13	27 ^{bc}	33 ^{b}	36 ^{ce}	58 ^{a}	64 ^{be}	51 ^{ab}	66 ^{ac}	50 ^{ab}	19	10	6 ^{c}

	f-salt	f-metallic	f-sweet	f-meat	f-frozen	f-pungent	f-sour	f-TMA	f-off
p-value	0,1551	0,0000	0,0000	0,0000	0,0023	0,0001	0,0000	0,0000	0,0000
0-point	8	28 ^{a}	25 ^{a}	22 ^{a}	2	5 ^{bc}	2 ^{c}	6 ^{bcd}	9 ^{de}
AB-D06	9	15 ^{bc}	15	14 ^{bc}	2	5 ^{bc}	3 ^{c}	4 ^{de}	13 ^{ce}
AB-D10	5	17 ^{bc}	14 ^{b}	10 ^{bc}	2	12	4 ^{c}	6 ^{bcd}	11 ^{ce}
SB-D06	10	16 ^{bc}	17	16	1 ^{c}	5 ^{bc}	3 ^{c}	5 ^{ce}	7 ^{ce}
SB-D10	6	15 ^{bc}	14 ^{b}	11 ^{bc}	4	12	8 ^{bc}	8 ^{bcd}	17 ^{bcd}
SB-D13	4	12 ^{bc}	9 ^{b}	6 ^{c}	4 ^{ab}	16 ^{a}	20 ^{a}	19 ^{a}	32 ^{a}
ST-D06	14	16 ^{bc}	23 ^{a}	20 ^{ab}	3	6 ^{bc}	2 ^{c}	4 ^{ce}	4 ^{ce}
ST-D13	5	16 ^{bc}	13 ^{b}	12 ^{bc}	5 ^{a}	12	13	13 ^{ac}	17 ^{bcd}
ST-D15	6	8 ^{c}	9 ^{b}	7 ^{bc}	5	15	11 ^{bc}	10	17 ^{bcd}
TB-D06	10	19 ^{b}	19	13 ^{bc}	1 ^{bc}	3 ^{c}	3 ^{c}	3 ^{de}	10 ^{ce}
TB-D10	6	13 ^{bc}	11 ^{b}	8 ^{bc}	3	10	8 ^{bc}	8 ^{bcd}	16 ^{bcd}
TB-D13	2	11 ^{bc}	10 ^{b}	10 ^{bc}	5	10	17 ^{ab}	12	33 ^{ab}
TT-D10	7	16 ^{bc}	13 ^{b}	9 ^{bc}	2	8 ^{bc}	6 ^{bc}	7 ^{bcd}	13 ^{ce}
TT-D13	4	15 ^{bc}	14 ^{b}	11 ^{bc}	2	13 ^{ab}	12	16 ^{ab}	22 ^{ac}

10. APPENDIX II: STATISTICAL ANALYSIS OF MICROBIAL AND CHEMICAL DATA

Groups	TVC-IA	H2S-prod.	PCR-Pp	*Pseud.*	pH	TVB-N	TMA
t0	5.1 ± 0.2 a	2.3 ± 1.0 a	0.9 ± 0.1 a	3.5 ± 0.1 a	7.0 ± 0.1 b	11.3 ± 1.0 a	0.0 ± 0.0 a
SB-d6	6.0 ± 0.3 b	2.6 ± 0.4 ab	**1.3 ± 0.7 a**	4.4 ± 0.2 bc	7.0 ± 0.0 b	11.5 ± 1.0 a	0.0 ± 0.0 a
ST-d6	5.6 ± 0.1 ab	2.6 ± 0.5 ab	2.3 ± 0.4 ab	**3.8 ± 0.3 ab**	6.8 ± 0.1 a	11.3 ± 0.5 a	0.0 ± 0.0 a
TB-d6	6.2 ± 0.1 bc	3.7 ± 0.4 bc	3.5 ± 0.8 bc	5.1 ± 0.1 cd	7.0 ± 0.1 b	12.7 ± 0.6 a	0.0 ± 0.0 a
AB-d6	6.3 ± 0.1 bc	3.7 ± 0.2 bc	3.3 ± 0.4 bc	5.2 ± 0.1 d	6.9 ± 0.1 a	13.7 ± 0.4 ab	0.1 ± 0.1 a
SB-d10	6.8 ± 0.1 cd	4.2 ± 0.2 cd	3.6 ± 0.3 bc	**5.8 ± 0.0 d**	7.0 ± 0.1 ab	13.9 ± 0.9 ab	0.9 ± 0.3 a
TT-d10	7.2 ± 0.1 de	5.5 ± 0.5 d	4.7 ± 0.2 c	6.6 ± 0.1 e	6.9 ± 0.0 a	15.0 ± 0.9 ab	1.6 ± 0.2 a
TB-d10	7.4 ± 0.1 de	5.5 ± 0.3 d	4.5 ± 0.7 c	6.9 ± 0.2 ef	7.1 ± 0.1 bc	17.1 ± 2.4 ab	2.9 ± 2.3 ab
AB-d10	7.2 ± 0.2 de	5.6 ± 0.5 d	4.8 ± 0.3 c	6.6 ± 0.2 e	7.1 ± 0.1 bc	18.7 ± 3.2 ab	4.9 ± 2.5 ab
SB-d13	**7.6 ± 0.1 e**	**6.3 ± 0.0 e**	5.2 ± 0.5 d	7.2 ± 0.3 ef	7.0 ± 0.1 ab	**24.7 ± 6.4 ab**	8.1 ± 6.4 ab
ST-d13	**6.6 ± 0.6 c**	**4.6 ± 0.9 cd**	**4.1 ± 1.0 c**	**5.4 ± 0.6 d**	7.0 ± 0.0 b	**15.0 ± 2.5 ab**	1.1 ± 1.2 a
TB-d13	8.2 ± 0.1 f	7.2 ± 0.2 f	5.1 ± 0.5 d	7.5 ± 0.0 f	7.1 ± 0.1 bc	26.9 ± 2.8 b	11.4 ± 2.0 b
TT-d13	8.4 ± 0.1 f	7.5 ± 0.4 f	5.3 ± 0.0 d	7.6 ± 0.4 f	7.2 ± 0.0 c	41.9 ± 14.9 c	24.5 ± 9.0 c
ST-d15	6.7 ± 0.1 c	5.0 ± 0.6 cd	**4.5 ± 0.7 c**	**5.6 ± 0.3 d**	7.0 ± 0.2 b	**14.7 ± 1.1 ab**	2.1 ± 1.0 ab

Different letters within a column indicate significant difference among samples ($P < 0.05$).