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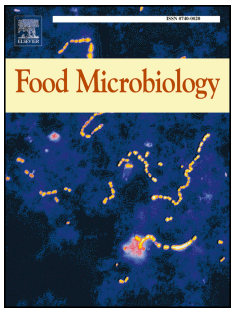

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Spoilage indicator bacteria in farmed Atlantic salmon (*Salmo salar*) stored on ice for 10 days

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Abstract

This study investigated the growth of indicator and spoilage bacteria on whole Atlantic salmon (*Salmo* salar) stored aerobically at 2°C. On days 0, 2, 3, 6, 8 and 10 microbiological analysis was carried out on inner flesh and outer skin samples as well as outer skin swabs (25cm^2 surface areas). Mesophilic total viable counts (TVC_m) on skin, flesh and swab samples increased from 1.9, 1.1 and 2.7 $\log_{10}$ CFUcm^2 to 6.0, 5.1 and 5.7 $\log_{10}$ CFU/cm^2 after 10 days, respectively. Psychrotrophic counts (TVC_p), increased from 2.2, 1.8 and 3.1 $\log_{10}$ CFU/cm^2 to 6.2, 5.3 and 5.9 $\log_{10}$ CFU/cm^2, for skin, flesh and swab samples respectively. Hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Pseudomonas* spp., *Brochothrix thermosphacta* and *Photobacterium* spp. grew well with similar growth rates (mean generation times of 17.2 to 26h). It was concluded that the shelf-life of salmon at 2°C was approximately 10 days and that HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. may be a better indicator of fish spoilage rather than TVC growth, with a count of 5-6 $\log_{10}$ CFU/cm^2 indicating the end of shelf-life.

Keywords: Atlantic salmon, *Salmo salar*, shelf-life, spoilage bacteria.

1. Introduction

Fresh Atlantic salmon (*Salmo salar)* is a very nutritionally and economically beneficial product and year by year global consumption increases (Amanatidou et al., 2000). However all fresh seafood is highly perishable and the quality starts to deteriorate immediately following capture and continues during storage. It has been estimated that 10% of the global seafood harvest is spoiled yearly (Alfaro et al., 2013; Kulawik et al., 2013). Spoilage is a complex process involving enzymatic, chemical and microbiological changes, with the latter

reported as the primary determinant of shelf life (Anacleto et al., 2011). Due to their aquatic nature, fish are constantly exposed to the indigenous microorganisms in their environment (Horsley, 1973; Roeselers et al., 2011) and the natural microflora of fish is therefore determined by the local environment. Microbial growth on seafood is supported by a diverse nutrient composition (Ghanbari et al., 2013) and a favourable pH (6-7) and water activity (a_w) of ~ 0.99 (Boziaris et al., 2013). However if fish are immediately stored at low temperatures, straight from harvest, microbial spoilage can be delayed (Badiani et al., 2013). Thus fresh fish are stored under chilled conditions (temperature approaching that of melting ice), as required in European Commission (EC) 853/2004, to inhibit bacterial growth. Moreover, (EC) 853/2004 lays down specific rules for food business operators (FBOs) and supplements Regulation (EC) 852/2004 by adding specific hygiene requirements for products of animal origin such as fish and fishery products.

Protecting consumer health is reliant on maintaining fish at chilled temperatures and having an appropriate shelf-life, the period of time after which the fish should not be consumed. Approximately 10% of foodborne outbreaks in any given year are associated with the consumption of seafood (EFSA. and ECDC., 2016; Huss et al., 2000). While the majority are allergy-type food poisoning, associated with the biogenic amine, histamine (formed from histidine by the action of bacterial histidine decarboxylase (Ruiz-Capillas and Moral, 2004), pathogenic bacteria such as shiga-toxigenic *Escherichia coli* and *Salmonella* spp. may also cause human illness associated with fish (Costa, 2013; Friesema et al., 2014);.

However, there is no consensus on which bacteria should be used to monitor the shelf-life of fresh fish. Although total viable count (TVC) is most commonly applied, the levels reported to indicate the end of shelf-life vary considerably, from 5-6 $\log_{10}$ CFU/g (Robson et al., 2007) to 7 $\log_{10}$ CFU/g (Liston, 1980) and 8-9 $\log_{10}$ CFU/g (Dalgaard et al., 1997). Thus, it has been suggested that specific spoilage bacterial counts might provide a better assessment of

shelf-life than TVC (Alonso-Calleja et al., 2004; Álvarez-Astorga et al., 2002; Emborg et al., 2002a; Gram and Dalgaard, 2002). *Shewanella* spp., *Pseudomonas* spp. and *Photobacterium* spp., for example, are ubiquitous in the marine environment (Emborg et al., 2002b; Janda, 2014) and colonise the fish by the skin, gills or gastrointestinal (GI) tract (Ringø and Holzapfel, 2000). Moreover they are psychrotrophic bacteria and have been reported to be the main spoilage organisms for chilled fish (Emborg et al., 2002b; Gram and Huss, 1996; Møretrø et al., 2016). However, there is a dearth of information on these and other potential spoilage bacteria.

The objective of this study was therefore to investigate bacteria growth (mesophilic TVC (TVC_m), psychrophilic TVC (TVC_p), total *Enterobacteriaceae* (TEC), hydrogen sulphide producing bacteria (HSPB, mainly *Shewanella* spp.), lactic acid bacteria (LAB), *Pseudomonas* spp., *Brochothrix thermosphacta* and *Photobacterium* spp.) on salmon stored under chilled (2°C) aerobic conditions thus providing data which may be used to assess which bacterial count is the most appropriate for shelf-life determination.

2. Materials and Methods

2.1.Fish Samples

Farmed Atlantic salmon were obtained from a local fish monger (Connolly Fish Sales, Rathmines, Dublin 6). Each salmon was a consistent size (3-4kg) and was obtained within 48h of harvest. The fish were transported on ice to the laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15) within an hour. Once on site the salmon were again stored on ice in polystyrene boxes, in a chilled room set at 2°C, for 10 days.

2.2.Microbiological Analysis

On days 0, 2, 3, 6, 8 and 10 microbiological analysis was carried out. On each sampling day the fish was split into two sides. From one side there were two samples (10g) of inner flesh and two samples (10g) of outer skin obtained on each of the sampling days. From the other side the outer skin of the fish was swabbed (25cm^2 surface areas) in duplicate using sterile cellulose acetate sponges pre-moistened with maximum recovery diluent (MRD, Oxoid, Basingstoke, United Kingdom (CM0733)). Each of the meat and skin samples were homogenized (Pulsifier ® PUL100E, Microgen Bioproducts Ltd, Surrey, United Kingdom) for 1 minute in 90ml MRD and ten-fold dilution series prepared up to 10^{-5}. Plate count agar (PCA) (Oxoid, Basingstoke, United Kingdom (CM0325)), with and without 1% NaCl was used to estimate total viable counts (TVC) for both mesophilic (TVC_m, incubated 30°C for 72h) and psychrotrophic (TVC_p, incubated at 6.5°C for 240h) bacteria using standard spread plate techniques. Standard pour plate techniques were used to estimate total *Enterobacteriaceae* counts on violet red bile glucose agar (VRBGA) (Oxoid, Basingstoke, United Kingdom (CM0485)) incubated at 37°C for 24h, HSPB on Iron Lyngby agar incubated at 25°C for 72h, per ingredients used by NMKL (2006) No.184 and lactic acid bacteria (LAB) on de Man Rogosa Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom (CM0361)) incubated at 30°C for 72h. Pseudomonad counts were carried out on *Pseudomonas* Agar Base (Oxoid, Basingstoke, United Kingdom (CM0559)), supplemented with Cetrimide-Fucidin-Cephaloridine (CFC) supplements (Oxoid, Basingstoke, United Kingdom (SR0103)) incubated at 30°C for 72h, *Br. thermosphacta* counts on streptomycin-thallous acetate-actidione (STAA) agar base (Oxoid, Basingstoke, United Kingdom (CM0881)), supplemented with STAA (Oxoid, Basingstoke, United Kingdom (SR0151E)) incubated at 25°C for 72h and Photobacterium spp. on Photobacterium Broth (Sigma Aldrich, Steinheim, Germany (38719-500G-F)), with bacteriological agar (Oxoid, Basingstoke, United Kingdom (LP0011)) added to solidify the media, incubated at 15°C for

168h. All three media were inoculated using standard spread plate techniques. Each meat, skin and swab sample were plated out in duplicate.

2.3.Water activity (a_w), pH and temperature;

On each sampling day, the pH, water activity (a_w) and storage temperatures were monitored. To measure the pH and a_w, two samples (10g) of both inner flesh and outer skin were obtained on each of the sampling days. The pH was measured using a pH meter (Eutech pH 5+, Thermo Fisher Scientific, Ireland). The a_w of the flesh and skin samples were measured using a Decagon AquaLab LITE water activity meter (Labcell Ltd, Alton, United Kingdom) according the manufacturer's instructions. The thickness, length and width of each skin and flesh sample were also recorded, on each day, so as to determine an average total surface area for the samples. This allowed for the log values to be calculated in CFU/cm^2.

During storage, EL-USB-2 temperature data loggers (Lascar Electronics, Whiteparish, United Kingdom) recorded the ambient temperature of the storage cold room environment while a Testo 175T3 data logger (Testo, Lenzkirch, Germany) was used to recorded skin and core temperatures of the whole salmon.

2.4.Data analysis

The experiment was performed in duplicate and repeated on 3 separate occasions. Bacterial counts were converted to $\log_{10}$ CFU/cm^2. Mean generation times (G) for all bacteria (from time t=0 to the time where the highest bacterial concentration was recorded) were calculated using the formula: G = t/3.3 logb/B, where t = time interval in h, b = number of bacteria at the end of the time interval, and B = number of bacteria at the beginning of the time interval (Koolman et al., 2014). The difference between mean values was compared using a two way analysis of variance (ANOVA). Graph Pad Prism v7.0 software (Graphpad Software Inc., La

Jolla, CA, USA) was used for statistical analysis, and significant differences are reported at $P<0.05$.

3. Results

These results are presented below. Table 1 presents the results for the pH and a_w obtained over the 10 day trial. The pH of the salmon flesh and skin samples followed a similar trend, decreasing from 7.0 and 7.1 to 6.5 and 6.7, respectively. The a_w for both flesh and skin remained constant between 0.95 and 0.96. Over the 10 days storage in a chilled room set at 2°C, the average ambient temperature recorded was 1.6°C. The average skin and core temperature ranged between 2.5 and 3°C, with a minimum temperature of 0°C recorded for both. No difference in growth of TVC grown on PCA with or without 1% NaCl was observed ($P > 0.05$) and therefore only data obtained with 1% NaCl is presented. The initial TVC_m counts on skin, flesh and swab samples on day 0 were 1.9, 1.1 and 2.7 $\log_{10}$ CFU/cm^2 which increased to 6.0, 5.1 and 5.7 $\log_{10}$ CFU/cm^2, respectively, after 10 days storage (Figure 1). TEC increased from 0.3, 0.2 and 0.02 $\log_{10}$ CFU/cm^2 on skin, flesh and swab samples to 1.5, 1.2 and 1.2 $\log_{10}$ CFU/cm^2, respectively, by day 10. Figure 2 shows the growth of TVC_p, with counts increasing from 2.2, 1.8 and 3.1 $\log_{10}$ CFU/cm^2 to 6.2, 5.3 and 5.9 $\log_{10}$ CFU/cm^2, for skin, flesh and swab samples, respectively. Initial counts of 1.4, 1.4, 1.4, <1.0 and 1.8 $\log_{10}$ CFU/cm^2 for HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. on skin samples increased to 5.5, 5.9, 5.9, 4.8 and 5.8 $\log_{10}$ CFU/cm^2, respectively (Figure 3). Corresponding counts on flesh samples were 1.0, 1.0, 1.0, <1.0 and 1.2 $\log_{10}$ CFU/cm^2 increasing to 4.4, 5.2, 5.2, 3.9 and 4.8 $\log_{10}$ CFU/cm^2 (Figure 4). The data for the swab samples is shown in Figure 5. HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. counts increased by 2.8, 3.3, 3.3, 4.1 and 2. $\log_{10}$ CFU/cm^2, respectively.

The growth parameters for all bacteria investigated are shown in Table 2. The mean generation times for TVC ranged from 18.2 to 26 h for both mesophilic and psychrotrophic groups irrespective of sample type. Enterobacteriaceae grew considerably slower with mean generation times of 60.5 to 72.7h. Interestingly the spoilage bacteria, HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. showed similar mean generation times of 17.2 to 26h, regardless of sample type.

4. Discussion

The initial TVC_m counts on skin, flesh and swab samples were 1.9, 1.1 and 2.7 $\log_{10}$ CFU/cm^2. Other studies have reported initial bacterial levels in fresh farmed salmon of approximately 3 $\log_{10}$ CFU/g (Briones et al., 2010; Schubring, 2003). However, Møretrø et al. (2016) found that psychrotrophic bacteria species, such as *Shewanella* spp. (HSPB) and *Pseudomonas* spp., were the most prevalent spoilage organisms found on fresh salmon fillets and in the processing plant environment. The initial HSPB count, obtained in this study, ranged from 1.0 to 2.2 $\log_{10}$ CFU/cm^2, similar to that obtained previously on salmon (Briones et al., 2010). These relatively low counts are considered indicative of fish of good microbiological quality (Li et al., 2017). This is supported by the relatively low TEC (0.02 to 0.3 $\log_{10}$ CFU/cm^2), suggesting the salmon was farmed in clean waters.

The initial HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. were similar to the TVC on each of the sample types (skin, flesh and swab), but considerably higher than the initial TEC. Moreover, the HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. grew more rapidly (mean generation times 17.3 to 21.4h on flesh) than the *Enterobacteriaceae* (mean generation time 72.7h) suggesting these were the main spoilage bacteria. This was not unexpected as these bacteria are common in the

low temperature waters where the salmon was farmed (Briones et al., 2010; Cruz-Romero et al., 2008) and the storage conditions (aerobic and approximately 2°C) in this study favour their growth (Linton et al., 2003; Parlapani and Boziaris, 2016; Parlapani et al., 2013). The relatively high levels (4.8 to 5.8 $\log_{10}$ CFU/cm^2) of *Photobacterium* spp. after 10 days was particularly significant as these bacteria produce trimethylamine (TMA), a key determinant of fish spoilage as determined by sensory evaluation (Dalgaard, 1995). *Shewanella* spp. and *Pseudomonas* spp. also produce volatile organic compounds which contribute to fish spoilage, resulting in a negative effect on fish flavour (Møretrø et al., 2016).

By the end of shelf life (10 days), the TVC_m ranged from 5.1 to 6.0 $\log_{10}$ CFU/cm^2, TVC_p from 5.3 to 6.2 $\log_{10}$ CFU/cm^2 and the spoilage bacterial (HSPB, LAB, *Pseudomonas* spp. and *Photobacterium* spp.) counts from 4.8 to 5.9 $\log_{10}$ CFU/cm^2. This is in agreement with Robson et al. (2007), who found seafood spoiled when the bacterial count reached 5 to 6 $\log_{10}$ CFU/cm^2. In contrast Dalgaard et al. (1997) suggested the end of shelf life of aerobically stored fish occurs when a bacterial concentration of 8 to 9 $\log_{10}$ CFU/cm^2 is achieved. This apparent difference may be explained by differences in the proportion of the total bacterial population that is composed of spoilage bacteria, specifically the higher the proportion of spoilage bacteria the lower the TVC associated with the end of shelf life (Gram and Huss, 1996). Thus HSPB, LAB, *Pseudomonas* spp. or *Photobacterium* spp. counts may be a better microbiological indicator of shelf life than general bacterial counts such as TVC, with the fish spoiled when these reach 5 to 6 $\log_{10}$ CFU/g or CFU/cm^2.

5. Conclusion

It was concluded that HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. all contributed to the spoilage of salmon stored aerobically at 2°C and

that the growth of these organisms may be a better indicator of fish spoilage, rather than TVC growth, with a count of 5-6 $\log_{10}$ CFU/cm^2, indicating the end of shelf-life.

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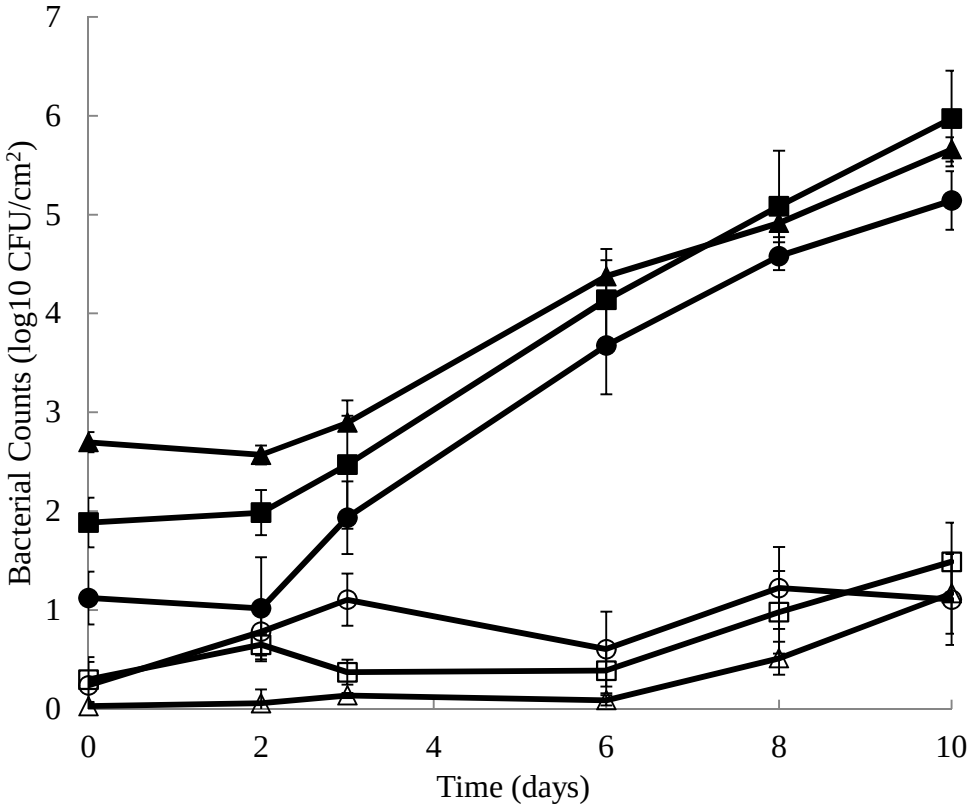

320

321 Figure 1. Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC_m (■) and TEC (□);
322 flesh TVC_m (●) and TEC (O) and swab TVC_m (▲) and TEC (Δ) samples stored at 2°C for
323 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

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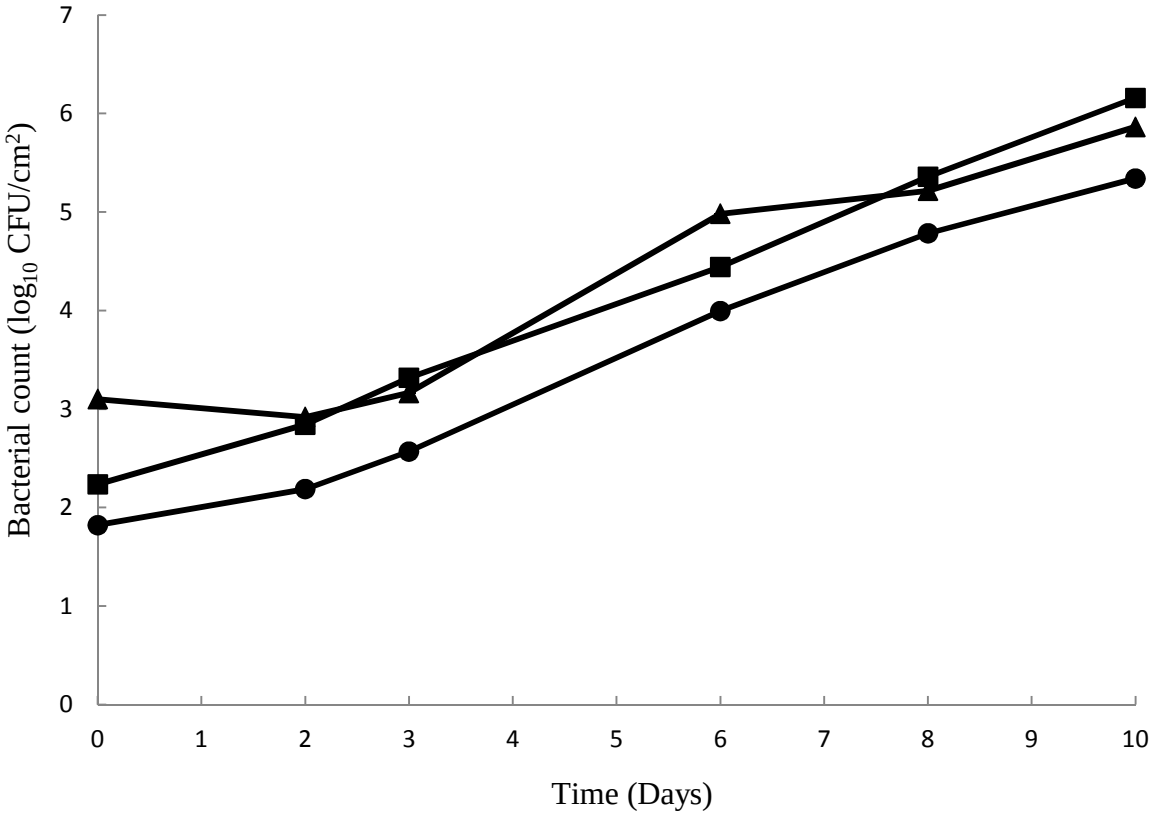

325
326 Figure 2. Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC_p (■), flesh TVC_p
327 (●) and swab TVC_p (▲) samples stored at 2°C for 10 days. Each data point and the error
328 bars show the mean of 3 replicates ± the standard error.

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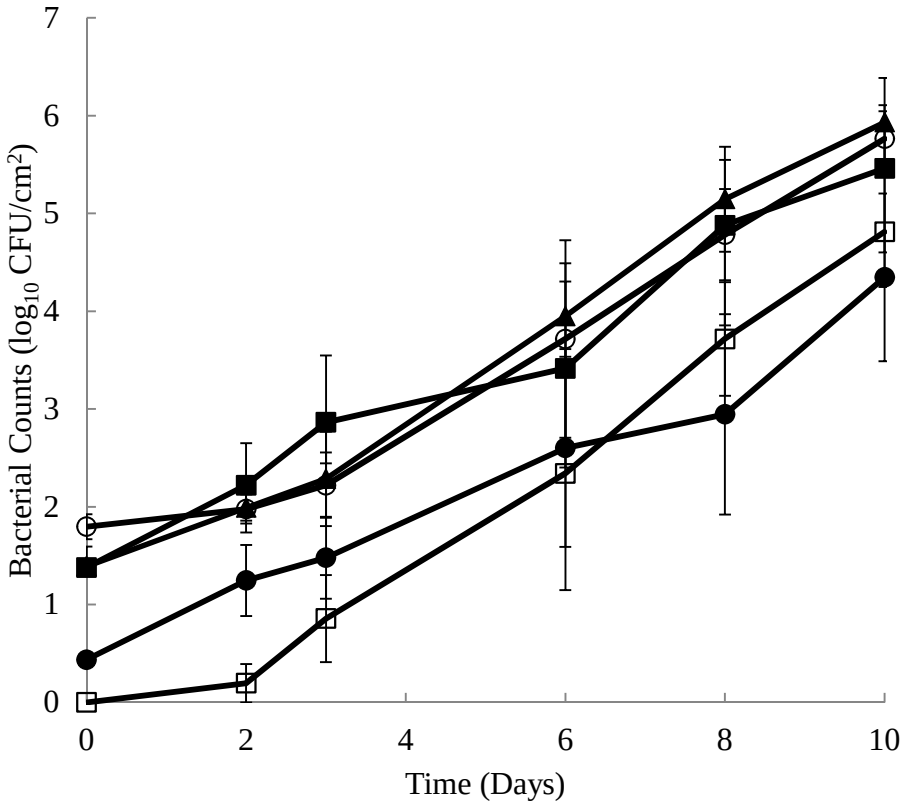

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331 Figure 3. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid

332 bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp.

333 (O), on the skin from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days. Each data

334 point and the error bars show the mean of 3 replicates ± the standard error.

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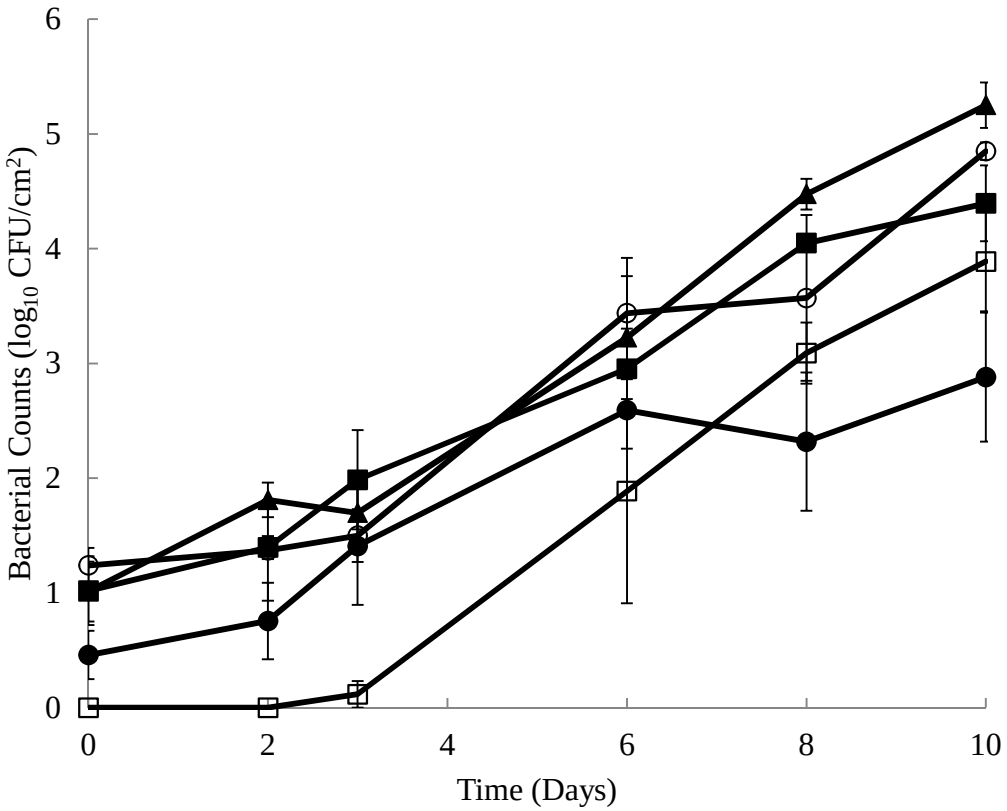
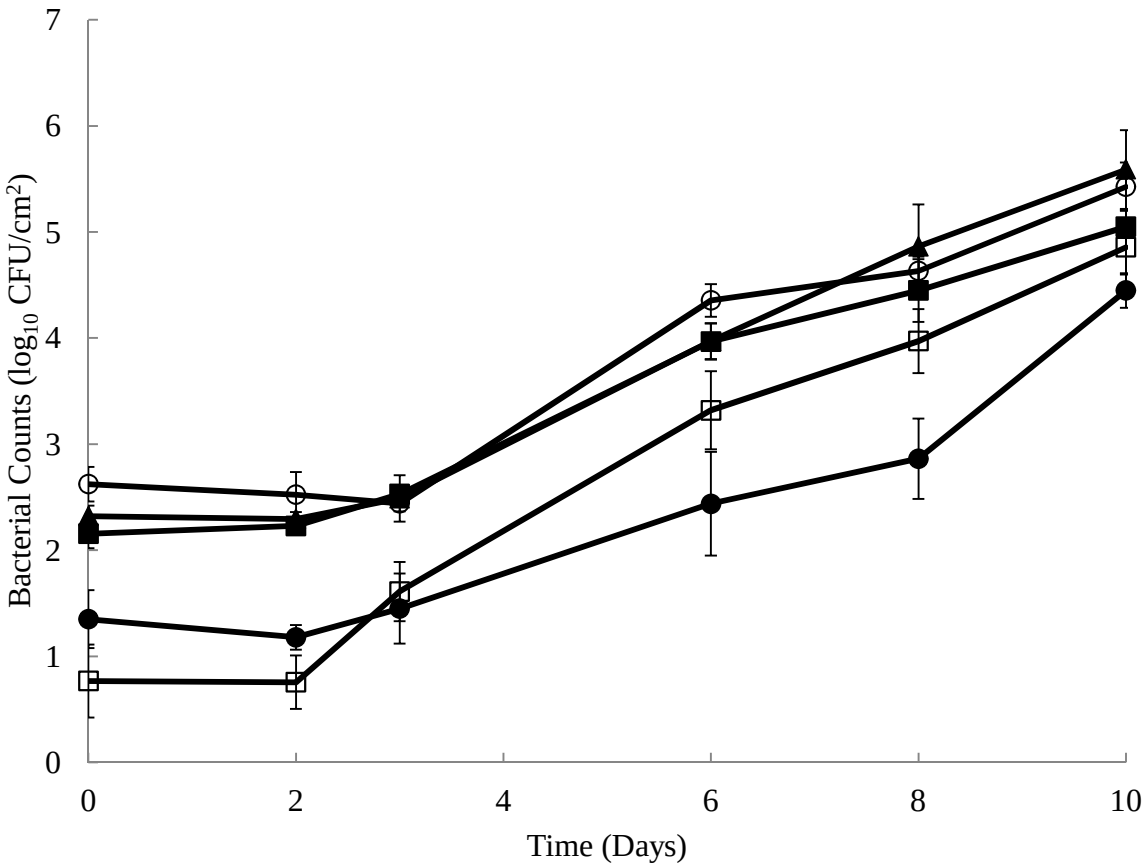


336

337 Figure 4. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid
338 bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp.
339 (O), on Atlantic salmon (*Salmo salar*) flesh stored at 2°C for 10 days. Each data point and the
340 error bars show the mean of 3 replicates ± the standard error.

341



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343 Figure 5. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid

344 bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp.

345 (O), in swab samples from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days. Each

346 data point and the error bars show the mean of 3 replicates + the standard error.

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349 Table 1. pH and a_w measurements as determined from skin, flesh and swab samples from

350 Atlantic salmon (Salmo salar) stored at 2°C for 10 days.

	Day	pH	a_w
Flesh	0	7.0	0.96
	2	6.8	0.96
	3	7.5	0.97
	6	7.2	0.94
	8	6.6	0.96
	10	6.5	0.96
Skin	0	7.1	0.95
	2	6.9	0.95
	3	7.7	0.96
	6	8.0	0.95
	8	6.8	0.96
	10	6.7	0.96

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354 Table 2. Growth parameters for total viable count mespohilic (TVC_m) and psychrotrophic
355 (TVC_p), TEC, hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB),
356 *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. as determined from skin,
357 flesh and swab samples from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days.

Treatment	Initial concentration (log_{10} CFU/cm^2)	Mean generation time (h)[1]	µmax (generations day^{-1})	Maximum concentration observed (log_{10} CFU/cm^2)
	Skin			
TVC_m	1.9	23.5	1.44	6.0
TVC_p	2.2	18.2	0.96	6.2
TEC	0.3	60.5	0.96	1.5
HSPB	1.4	17.7	0.96	5.5
LAB	1.4	16.2	1.20	5.9
Pseudomonas spp.	1.4	16.2	1.20	5.9
Br. thermosphacta	ND	15.2	1.44	4.8
Photobacterium spp.	1.8	18.2	1.20	5.8
	Flesh			
TVC_m	1.1	18.2	1.44	5.1
TVC_p	1.8	20.8	1.20	5.3
TEC	0.2	72.7	0.24	1.2
HSPB	1.0	21.4	0.96	4.4
LAB	1.0	17.3	1.20	5.2
Pseudomonas spp.	1.0	17.3	1.20	5.2

Br. thermosphacta	ND	18.6	1.68	3.9
Photobacterium spp.	1.2	20.2	0.96	4.8
	Skin Swab			
TVC_m	2.7	24.2	1.20	5.7
TVC_p	3.1	26.0	0.96	5.9
TEC	0.02	60.5	1.68	1.2
HSPB	2.2	26.0	1.20	5.0
LAB	2.3	22.0	1.20	5.6
Pseudomonas spp.	2.3	22.0	1.20	5.6
Br. thermosphacta	0.08	17.2	1.20	4.9
Photobacterium spp.	2.6	26.0	1.44	5.4

[1] calculated using the formula G = t/3.3 logb/B, where t = time interval in h to when the late lag phase was reached, b=number of bacteria at the end of the time interval, and B = number of bacteria at the beginning of the time interval (Koolman et al., 2014)

The microbiology of farmed Atlantic salmon (*Salmo salar*) stored on ice for 10 days

Colin Fogarty, Paul Whyte, Nigel Brunton, James Lyng, Conor Smyth, John Fagan and Declan Bolton

Highlights

1. Chilled and aerobically stored salmon had a shelf-life of approximately 10 days.
2. HSPB, LAB, Pseudomonas spp., *Br. thermosphacta* and *Photobacterium* spp. were a better indicator of fish spoilage.
3. Spoilage bacterial counts of 5-6 $\log_{10}$ CFU/cm^2 indicated the end of shelf-life