



**Shelf-life extension ingredient and processing technologies  
applied to Atlantic salmon (*Salmo salar*)**

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A thesis submitted to University College Dublin for the degree of Doctor of  
Philosophy

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## Abbreviations

ATP	Adenosine triphosphate
ANOVA	Analysis of variance
CO <sub>2</sub>	Carbon dioxide
cm	Centimetre
CFC	Ceftriaxone fucidin cephalosporin
CFU	Colony forming unit
°C	Degrees Celsius
d	Day
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FIRM	Food Institutional Research Measure
g	Gram
>	Greater than
h	hour

kg	Kilogram
kPa	Kilopascal
<	Less than
l	Litre
log <sub>10</sub>	Logarithm to base 10
MRS	de Man Rogosa Sharpe
MRD	Maximum recovery diluent
μl	Microlitre
ml	Millilitre
%	Percent
±	Plus or minus
RNA	Ribonucleic acid
NaCl	Sodium chloride
STAA	Streptomycin-thallos acetate-actidione
TEC	Total <i>Enterobacteriaceae</i> counts
TVC	Total viable counts
v/v	Volume over volume
a <sub>w</sub>	Water activity
w/v	Weight over volume

## **Publications and Conference Presentations**

### **Publications**

- Fogarty, C., Whyte, P., Brunton, N., Lyng, J., Smyth, C., Fagan, J., Bolton, D., (2019). Spoilage indicator bacteria in farmed Atlantic salmon (*Salmo salar*) stored on ice for 10 days. Food Microbiology (2019), 77, 38-42, accepted 2 August 2018.
- Fogarty, C., Burgess, C. M., Cotter, P. D., Cabrera-Rubio, R. , Whyte, P. , Smyth, C. and Bolton, D. (2019), Diversity and composition of the gut microbiota of Atlantic salmon (*Salmo salar*) farmed in Irish waters. Journal of Applied Microbiology. Accepted Author Manuscript. doi:[10.1111/jam.14291](https://doi.org/10.1111/jam.14291)
- Fogarty, C and Smyth, C., Whyte, P., Brunton, N. and Bolton, D., (2019). Sensory and ATP derivative based indicators for assessing the freshness of Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*). Irish Journal of Food and Agricultural Research, Accepted Author Manuscript.

### **Conference and seminar presentations**

- Fogarty, C., Whyte, P., and Bolton, D. (2014). Shelf-life extension of fresh salmon (*Salmo salar*) using organic acids and trisodium phosphate. Oral presentation at the 43<sup>rd</sup> Annual Food Research Conference, University College Dublin (UCD), Belfield, Dublin, 10<sup>th</sup> and 11<sup>th</sup> December 2014.
- Fogarty, C., Whyte, P., and Bolton, D. (2016). Shelf-life extension of fresh salmon (*Salmo salar*) using organic acids and the phenolic compounds present in essential oils. Poster presentation at the 25<sup>th</sup> International ICFMH | FoodMicro Conference,

Held at University College Dublin (UCD), Belfield, Dublin, between 19<sup>th</sup> and 22<sup>nd</sup> July 2016.

- Fogarty, C., Whyte, P., and Bolton, D. (2016). Assessing the quality of raw salmon (*Salmo salar*) using microbiological, sensory and chemical indicators. Poster presentation at the 18<sup>th</sup> International Union of Food Science and Technology (IUFOST) Conference, held at the Royal Dublin Society (RDS), Ballsbridge, Dublin, between 21<sup>st</sup> and 25<sup>th</sup> August 2016.
- Fogarty, C., Whyte, P., and Bolton, D. (2016). Shelf-life extension of fresh salmon (*Salmo salar*) using organic acids and the phenolic compounds present in essential oils. Poster presentation at the 46<sup>th</sup> West European Fish Technologists' Association (WEFTA) conference, held at the Hotel "Park", Split, Croatia, between 12<sup>th</sup> and 14<sup>th</sup> October 2016.
- Fogarty, C., Whyte, P., and Bolton, D. (2017). Effect of the combinations of clean label ingredients and packaging conditions on the shelf-life of fresh fish. poster presentation at the 63rd International Congress of Meat Science and Technology (ICoMST), held at the The Rochestown Park Hotel, Cork City, Cork, between 13<sup>th</sup> and 18<sup>th</sup> August 2017

- Fogarty, C., Smyth, C., Whyte, P., and Bolton, D. (2017). Using natural ingredients and packaging technologies to enhance the shelf-life of cod and salmon. Oral presentation at the 47<sup>th</sup> West European Fish Technologists' Association (WEFTA) conference, held at the Aviva Stadium, Lansdowne Rd, Dublin, between 9<sup>th</sup> and 12<sup>th</sup> October 2017.
- Fogarty, C., Whyte, P., and Bolton, D. (2017). Assessing the quality of raw salmon (*Salmo salar*) using microbiological, sensory and chemical indicators. Poster presentation at the 47<sup>th</sup> West European Fish Technologists' Association (WEFTA) conference, held at the Aviva Stadium, Lansdowne Rd, Dublin, between 9<sup>th</sup> and 12<sup>th</sup> October 2017.
- Fogarty, C., Whyte, P., and Bolton, D. (2017). Assessing the quality of raw salmon (*Salmo salar*) using microbiological, sensory and chemical indicators. Poster presentation at the Walsh Fellowship Seminar, held at the Royal Dublin Society (RDS), Ballsbridge, Dublin, on the 9<sup>th</sup> November 2017.

# **Chapter 1 – General Introduction**

## 1.1. General Introduction

Ireland's location in the clean cold waters of the north Atlantic is the perfect habitat for the growth of a wide range of fish and shellfish. This has led to a worldwide demand for Irish seafood products resulting in the seafood industry becoming a significant contributor to the national economy (Vega et al., 2014). The Irish seafood industry has expanded and is currently responsible for the employment of approximately 14,000 individuals. According to Bord Iascaigh Mhara (BIM, the Irish state agency responsible for developing the Irish marine fishing and aquaculture industries), in 2017 the Irish seafood market was worth €1.15 billion, which was an increase of over 6% from the previous year (BIM, 2018). A large contributor to the overall market value is overseas exports. In 2017, there was a total of 313,600 tonnes (€666 million) exported overseas. Increasing the value of the fish sector is reliant on the development of new export markets. The majority of Ireland's exports are within the EU and United Kingdom (€477 million), however as preservation techniques evolve, international seafood exports towards Asia and Africa continue to grow with exports in 2017 valued at approximately €79 million (10% increase from 2016) and €65 million (47% increase from 2016), respectively.

Atlantic salmon (*Salmo salar*) is Ireland's most valuable seafood product (BIM, 2018). In 2017 salmon exports were valued at €121 million, which was a 69% increase in value from 2016. Not only is salmon Ireland's leading seafood export it also tops domestic sales (€96 million), making up over a third of the retail sales valuation in 2017 (€249 million). Thus, it is important to maintain a product of excellent health and quality.

It is essential to maintain a product of excellent quality; however it is also necessary to improve quality where possible. All fresh seafood is highly perishable with an estimated shelf-life of 9 or 10 days. This has resulted in almost 10% of the global seafood harvest

being lost to spoilage every year (Kulawik et al., 2013). A 24-hour extension in shelf-life will significantly impact on profitability, sustainability and reduce waste (personal communication, John Fagan, BIM).

Spoilage is a complex process that involves both chemical and microbiological changes. Studies have shown that the primary determinant of shelf-life is the behaviour of spoilage microorganism on fish following harvest or capture (*post-mortem*) (Anacleto et al., 2011). Microbial growth and metabolism results in the production of volatile compounds (Chen et al., 2010) that have a negative effect on the sensory attributes of fresh seafood. To maintain a seafood product of the highest quality it is important to understand the microbial diversity associated with spoilage and how they affect the physico-chemical attributes.

Maintaining and improving the quality of fresh fish is primarily reliant on chilled storage temperatures and the use of packaging technologies (modified atmosphere and skin packaging), however, more recently there has been an increased interest in the use of natural antimicrobial derived from plants to extend shelf life (Oliveira et al., 2015; Tajkarimi et al., 2010).

These current studies explore the possibility of improving analytical methods to assess freshness in seafood and to investigate the antimicrobial potential of a range of natural ingredients both alone and in combination with packaging and chilled storage temperatures to extend the shelf life of Atlantic salmon.

**The aims of this research study were;**

- To investigate bacterial growth on Atlantic salmon stored under chilled aerobic conditions thus providing data which may be used to assess which bacterial groups and concentrations are most appropriate for shelf-life determination.
- To characterize the microbiota present in the GI tract of Atlantic salmon, using Miseq Illumina high throughput sequencing
- To develop and validate rapid sensory (QIM and QDA) and ATP derivative based methods for assessing the freshness of Atlantic salmon.
- To investigate the effects of a natural antimicrobial immersion treatment on microbial growth for Atlantic salmon fillets during chilled storage.
- To examine the effects of either a natural antimicrobial immersion or spray treatment on mean bacterial counts in combination with packaging technologies for Atlantic salmon fillets during chilled storage.
- To assess the effects of skin packaging with retail and sub-zero temperatures on the mean bacterial counts for Atlantic salmon fillets.

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## **Chapter 2 – Literature Review**

## **2.1. Atlantic salmon (*Salmo salar*)**

### 2.1.1. Background

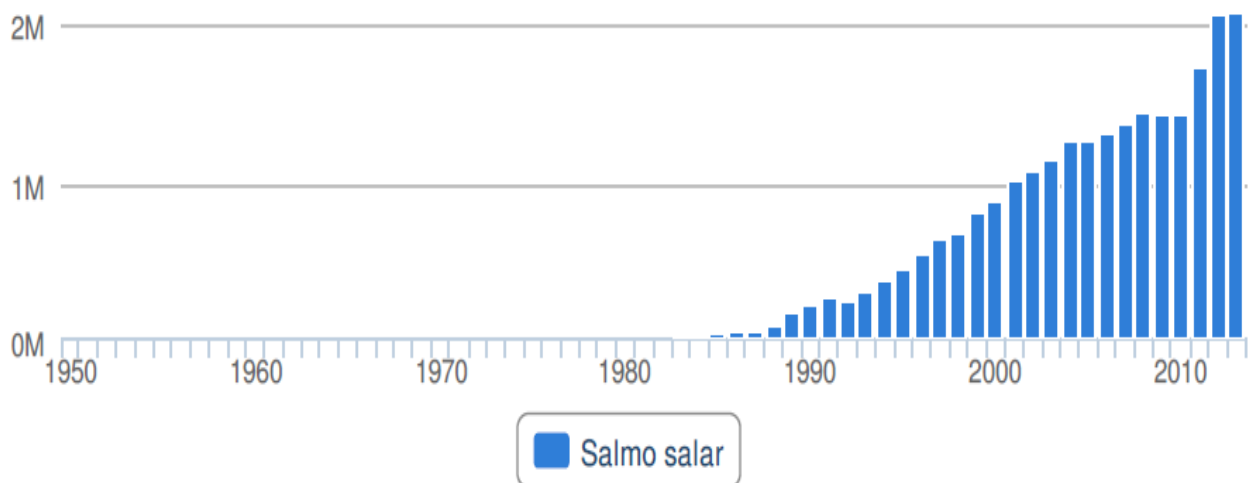
Fresh seafood is a nutritionally and economically beneficial product and year by year global consumption has increased (Amanatidou et al., 2000). In 2017, the average worldwide consumption of fish was 20.3kg per capita per annum. In addition to the desirable organoleptic attributes of fish, consumers are also attracted by the health benefits associated with seafood. Several species of fish, including Atlantic salmon (*Salmo salar*), are rich in polyunsaturated n-3 fatty acids (Foran et al., 2005; Tarvainen et al., 2015). These fatty acids have been reported to be beneficial to humans by decreasing the risk of heart disease, lowering blood pressure and enhancing the immune system (Kulawik et al., 2013; Tarvainen et al., 2015). Countries such as Japan with a high per capita consumption of seafood have less reported cases of obesity and cardiovascular disease, which has resulted in greater life expectancy whereas in countries with substantially lower intake of fish, such as the United States, obesity and cardiovascular disease are more prevalent (Sampels, 2015). These health benefits, as well as health concerns related to red meat protein (Swartz et al., 2010), has led to an increased demand for fresh seafood over the last decade.

### 2.1.2. Aquaculture

As a result of the increased demand, many wild fish populations are in decline due to over exploitation, to the point where fish stocks cannot biologically replenish at the same rate they are being caught (Lotze and Worm, 2009; Perissi et al., 2017). As a result fish farming has become increasingly attractive as a means of satisfying the increased demand

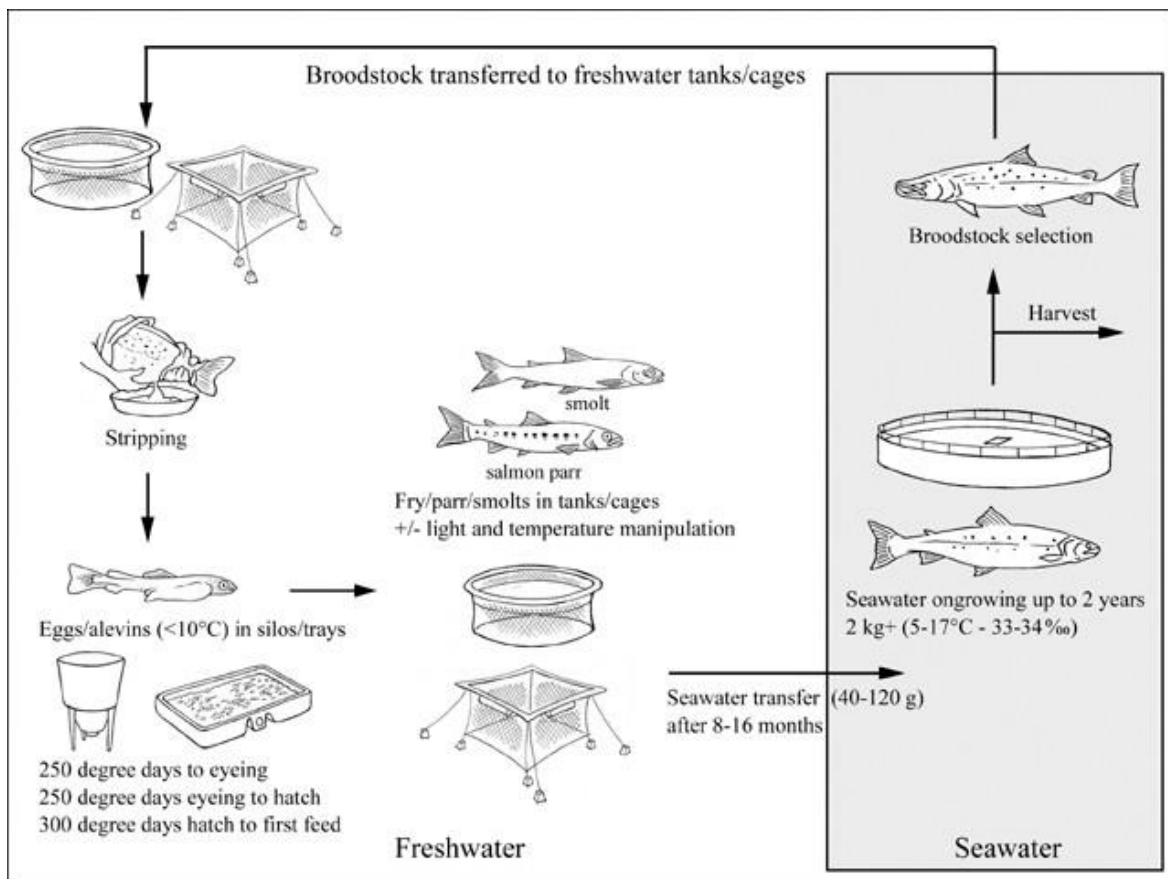
due to its ability to provide seasonally independent supplies of fish (Duun and Rustad, 2007; Llewellyn et al., 2014).

Atlantic salmon is one of the most economically important aquaculture species and currently makes up more than 50% (2 million tonnes/year) of the global salmon production (Figure 2.1) (FAO, 2018; Rotariu et al., 2014; Soon and Baines, 2012) with Norway (53%), Chile (23%) and Scotland (10%) as the leading producers. In 2017, the Irish aquaculture industry was worth €208 million, which was an increase of 24% from the previous year and made up 34% of the total value of seafood cultivated in Ireland (BIM, 2018). According to BIM (2018), production of Atlantic salmon (*Salmo salar*) grew 25% since 2016, up to 20,000 tonnes, with a value of €147 million, making it not only the most valuable aquaculture product, but also Irelands most valuable seafood product overall.



**Figure 2. 1** The global production (tonnes/year) of Atlantic salmon (*Salmo salar*) (FAO, 2018).

The farming process mimics the species life cycle. Being a diadromous species, the Atlantic salmon life cycle takes place in both fresh and salt water environments. It is essential that the farming process incorporates both these life stages (Figure 2.2). From each production stock, a broodstock are selected to supply eggs for the next cycle. These eggs are fertilised and transferred to freshwater hatching trays. Juveniles are usually raised in freshwater inland tanks or ponds until they have smolted, which indicates they are ready to be transferred to the sea. When salmon reach an appropriate size, usually 3-4kg, they are harvested using sweep nets.



**Figure 2. 2** Diagram illustrating aquaculture lifecycle of Atlantic salmon (*Salmo salar*) (FAO, 2018).

Fish can either be slaughtered at sea or they may be transported live to a slaughter plant. Slaughter involves an initial stunning followed by cutting of the gills resulting in rapid death due to blood loss. During harvest and slaughter, stress must be kept to a minimum so as to not accelerate the spoilage process. Fish exposed to excessive stress prior to slaughter can undergo physiological changes, including a release of adrenaline that can lead to a stronger state of rigor mortis resulting in poor fillet texture and yield (Sigholt et al., 1997). It is also possible that fish may consume toxic chemicals or pathogenic bacteria from their feed or the environment. Excessive consumption of these hazardous agents can result in fish spoilage making their consumption potentially hazardous to human health. The European Commission (EC) (2002) has classified hazards as “Biological, chemical or physical agents in, or condition of, food with the potential to cause an adverse health effect”. Farmers are therefore required to enforce a strong hygienic practice and constantly assess the quality of the marine environment where fish are reared (Soon and Baines, 2012). The United Nations Food and Agricultural Organization (FAO) developed a Code of Conduct for Responsible Fisheries as guidance for farmers to reduce the risk of introducing hazards by ensuring the use of safe feed and feed additives (Fairgrieve and Rust, 2003; FAO, 1997).

## **2.2. Seafood Safety**

Most fresh foods are susceptible to changes in pH, nutrient composition and microbial activity, leading to spoilage and large quantities of global food waste (Gram and Dalgaard, 2002). Fresh seafood is a highly perishable product with a relatively short shelf-life (Ghanbari et al., 2013) and it has been estimated that more than 10% of the global seafood harvest is lost yearly to spoilage (Alfaro, Hernández, Balino-Zuazo, 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 resulting in the development of undesirable sensory characteristics (Anacleto et al., 2011; Gram and Huss, 1996).

### **2.2.1. Spoilage**

The initial deterioration in the quality of fresh fish is normally a result of autolytic changes creating a suitable environment for bacterial growth and spoilage. Autolytic changes include the production of substrates such as trimethylamine N-oxide (TMAO), sulphur containing amino acids and inosine monophosphate (Castro et al., 2006; Parlapani et al., 2014). The breakdown of these substrates results in the production of volatile compounds such as trimethyl amine (TMA), biogenic amines (histamine), volatile sulphur compounds and hypoxanthine (Hx) (Chen et al., 2010; Gram and Dalgaard, 2002) resulting in the production of unpleasant odours and the loss of the sweet, creamy and meaty flavours associated with fish (Mørkøre et al., 2010; Schirmer et al., 2009). Previous studies have highlighted that most volatile compounds are produced as a result of microbial growth and metabolism (Gram and Huss, 1996).

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. For example, fish from an environment exposed to human or animal effluent can harbour high levels of *Enterobacteriaceae* (Feldhusen, 2000; Nirmal and Benjakul, 2011). Spoilage bacteria rely on their ability to adapt to storage conditions. Gram negative psychrotrophic bacteria are normally the predominant spoilage organisms found on aerobically stored chilled fish, whereas, under anaerobic conditions, these bacteria are out competed by lactic acid bacteria (LAB) or *Photobacterium* spp. (Gram and Dalgaard, 2002).

Microbial growth on fish is enhanced by suitable growth conditions, including a favourable pH (6- 7) and water activity ( $a_w$ ) of  $\sim 0.99$  (Boziaris et al., 2013; Gram and Huss, 1996). Initial microbial counts at harvest can vary between 2 and 6  $\log_{10}$  CFU/cm<sup>2</sup>. Slattery et al. (1998) suggested that an initial count of 4  $\log_{10}$  CFU/cm<sup>2</sup> was the limiting factor for shelf-life and fish with counts in excess of this level were more susceptible to spoilage. Specific spoilage organisms (SSOs) are selected for their ability to adapt to the changing physical and chemical conditions of the product during processing and storage (Gram and Dalgaard, 2002). *Shewanella* spp., *Pseudomonas* spp. and *Photobacterium* spp., are all ubiquitous in the marine environment (Emborg et al., 2002; Janda, 2014) and possess the ability to colonise the skin, gills or gastrointestinal (GI) tract of fish (Ringø and Holzappel, 2000). These Gram negative psychrotrophic bacteria have all been reported to be the main spoilage organisms for chilled fish stored under various conditions (Emborg et al., 2002; Gram and Huss, 1996; Møretrø et al., 2016).

### 2.2.2. Spoilage Organisms

Fresh seafood provides an ideal environment to support the growth of bacteria due to the diverse nutrient composition (Ghanbari et al., 2013). The microflora is usually determined by environmental conditions and water quality at the site of rearing and catching. These bacteria are either part of the natural microflora (indigenous) or can be present due to contamination either from faecal pollution or the processing environment (non-indigenous). Levels of indigenous pathogens are normally quite low and can be eliminated with adequate cooking or processing. The natural microflora differs from region to region (Feldhusen, 2000). Fish reared and caught from coastal regions are more likely to come in contact with faecal pollution and therefore may have higher levels of *Enterobacteriaceae* present on their surface and in their gut (Gram and Huss, 1996). The National Oceanic and Atmospheric Administration have suggested that marine environments away from coastal regions are an uncommon reservoir for human infection, and fish caught from these regions present a relatively low risk of infection. To date there has been at least ten genera of bacterial pathogens associated with seafood illness (Feldhusen, 2000). Common spoilage organisms are listed and described below;

#### 2.2.2.1. *Shewanella* spp.

*Shewanella* spp. is a Gram negative psychrotolerant spoilage bacterium, ubiquitous in the marine environment. They are a common indigenous species associated with all kinds of marine life including fin fish, shell fish, coral and sponges (Janda, 2014). *Shewanella* spp. belong to the hydrogen sulphide producing bacteria group (HSPB). These bacteria possess the ability to produce hydrogen sulphide as well as other volatile organic compounds such as TMA (Dalgaard, 1995b; van Spreekens, 1977). These volatile organic compounds

contribute to the spoilage of fresh seafood and are responsible for the foul odours associated with spoilt seafood, particularly fish stored aerobically (Møretrø et al., 2016). Several studies have shown that HSPB, such as *Shewanella putrifaciens*, are the predominant spoilage bacteria in stored, chilled seafood; however the growth of this organism can be reduced under anaerobic packaging conditions (Calliauw et al., 2016; Debevere and Boskou, 1996; Nirmal and Benjakul, 2011).

For many years this bacterial genera was only associated with fish spoilage and not with human illness. However, more recently it has been shown that several species within this genus produce decarboxylase enzymes that break down free amino acids to biogenic amines, capable of causing human illness. Up until 1990 each case of foodborne intoxication caused by *Shewanella* spp. was associated with the species *S. putrifaciens*; however Nozue et al. (1992) identified *S. algae* as a leading cause of human illness, which was later confirmed by Janda and Abbott (2014). Although these two species are the predominant species associated with human illness, other species such as *S. haliotis*, *S. xiamenensis* and *S. epidermidis* have all been linked with human disease after being misidentified as other genus such as *Vibrio* spp. and *Pseudomonas* spp..

*Shewanella* spp. foodborne illnesses can cause gastrointestinal infections in humans. Nath et al. (2011) highlighted a case of two patients with bloody diarrhoea approximately 12 hours after they had consumed fish contaminated with *Shewanella* spp. Although *Shewanella* spp. have been associated with foodborne illness, they are most commonly associated with skin and soft tissue infections (SSTIs) as a result of coming into contact with abraded skin surfaces e.g. sea urchin stings (Janda, 2014). As they belong to the group HSPB, *Shewanella* spp. are easily identified as they appear as black colonies on Iron Lyngby agar (NMKL, 2006; Vogel et al., 2005).

#### 2.2.2.2. *Photobacterium* spp.

*Photobacterium* spp. are Gram negative, marine dwelling genus of spoilage bacteria and are a psychrophilic and halophilic producers of volatile organic compounds (Emborg et al., 2002). *Photobacterium phosphoreum* is a problem in the seafood industry as it possesses the ability to produce histamine and TMA anaerobically which can lead to fish spoilage when packed under anaerobic conditions (Dalgaard, 1995a; Debevere and Boskou, 1996). Due to the large size of the bacterium, TMA production is at a considerably faster rate per cell than *S. putrefaciens* (Dalgaard, 1995b; Debevere and Boskou, 1996). Several studies have reported that *P. phosphoreum* are amongst the dominant spoilage organism of fish, including Atlantic salmon and cod (*Gadus morhua*), packed in a modified atmosphere (Dalgaard et al., 1997; Macé et al., 2012; Powell and Tamplin, 2012). This has led to an increased interest in fish being treated with natural antimicrobials prior to packaging (Mejlholm and Dalgaard, 2002).

At both low temperatures (<15°C) and high salt concentrations *P. phosphoreum* produces more histamine than almost any other histamine producing species associated with seafood (Kanki et al., 2004). However reported cases of histamine fish poisoning (HFP) related to *P. phosphoreum* are rare, as many cases go unreported due to symptom being similar to other pathogenic bacteria.

The presence of luminous colonies on Long and Hammer agar indicates the likely presence of *P. phosphoreum* (Dalgaard et al., 1997; NMKL, 2006). Several studies have reported the growth of luminous colonies on Long & Hammer agar for fish stored in a modified atmosphere pack (MAP) or skin pack (SP), however there have also been studies where luminous *P. phosphoreum* colonies grew on fish stored aerobically (Dalgaard et al., 1997).

### 2.2.2.3. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are both beneficial and harmful to the seafood industry. LAB have relatively recently been described as naturally occurring organisms in the marine environment. Genera including *Carnobacterium* spp., *Lactobacillus* spp. and *Lactococcus* spp. have all been associated with freshwater and marine fish (Ghanbari et al., 2013). Towards the end of shelf-life of seafood, the natural microflora is usually dominated by psychrophilic LAB, showing that this group is a dominant spoilage organism (Pothakos et al., 2014). These bacteria are not only associated with aerobic spoilage but are also amongst the dominant spoilage organisms of MAP fish fillets as they are CO<sub>2</sub> tolerant (Parlapani, Haroutounian, et al., 2015; Rudi et al., 2004). LAB are also resistant to the use of chemical and/or organic preservatives, such as essential oils, as they are able to cope with osmotic stress (Burt, 2004), therefore making them one of the more difficult spoilage organisms to inhibit. However high LAB levels (7-8 log<sub>10</sub> CFU/g) have been reported to be present for several weeks before causing any negative sensory effects (Gram and Huss, 1996).

However, LAB may also play a role in the preservation of seafood. *Carnobacterium* spp. possess the ability to produce antimicrobial compounds such as organic acids and bacteriocins. (Matamoros et al., 2009). Bacteriocins are antibacterial proteins that have been shown to inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes*. Bacteriocins are safe for human consumption and biopreservation using LAB is being researched as a possible method of extending the shelf-life of fresh fish.

It has also been suggested that LAB are essential to the wellbeing of fish during their life cycle. Ringø et al. (2010) hypothesized that LAB colonize the GI tract and produce bacteriocins as a form of protection. Previous studies have shown the antimicrobial impact

of LAB genera such as *Carnobacterium* spp. and *Lactobacillus* spp. against pathogenic genera such as *Aliivibrio* spp. and *Vibrio* spp., within the foregut of Atlantic salmon (Ringø, 2008; Ringø et al., 2007; Salinas et al., 2008).

#### 2.2.2.4. *Enterobacteriaceae*

*Enterobacteriaceae* growth is not a leading cause of spoilage for the seafood industry, however there are cases where these organisms have been identified as the dominant group of spoilage bacteria (Reilly and Twiddy, 1992; Saheki et al., 1989). The occurrence of *Enterobacteriaceae* on fish caught in freshwater environments and coastal regions is not uncommon as they are more likely to come in contact with faecal contamination through land sources (Nirmal and Benjakul, 2011). Fish caught away from the coast possess a very low risk of coming in contact with faecal contamination and therefore have insignificant levels of *Enterobacteriaceae* present in their microflora. Farmed fish, such as Atlantic salmon, also have low risk of coming in contact with *Enterobacteriaceae* as farmers are required to enforce strong hygienic practice and constantly assess the quality of the environment where the fish are being reared (Soon and Baines, 2012).

Growth of *Enterobacteriaceae* to high levels (7-8 log<sub>10</sub> CFU/g) will contribute to spoilage and result in an ammonia like off odour/taste as this genera possesses the ability to metabolise TMAO to TMA (Gram and Dalgaard, 2002). MAP can successfully inhibit the growth of *Enterobacteriaceae*; however SP is not as successful. Previous studies by Radetic et al. (2007) and Milijasevic et al. (2015) support this statement. They observed that SP had an increased microaerophilic *Enterobacteriaceae* growth and that MAP with a high CO<sub>2</sub> concentration inhibits the growth of *Enterobacteriaceae*.

#### 2.2.2.5. *Pseudomonas* spp.

*Pseudomonas* spp. are a Gram negative, psychrotrophic, dominant spoilage genera for marine fish stored aerobically at chilled temperatures (Gram and Huss, 1996; Koutsoumanis et al., 1999). Similar to HSPB, the growth of these genera is inhibited in an anaerobic packaging environment. *Pseudomonas* spp. cause spoilage due to their ability to produce numerous volatile compounds such as hypoxanthine (Surette et al., 1988) and sulphides (Parlapani, Verdos, et al., 2015). Production of these volatiles normally results in fruity and putrefactive flavours and odours.

Previous studies by Parlapani, Verdos, et al. (2015) observed that *P. fluorescens* was the predominant pseudomonad on both fresh and spoiled sea bream (*Pagrus major*), leading to the conclusion that this species was a major spoilage organism on aerobically chilled marine fish.

Pseudomonads, such as *P. aeruginosa*, have the ability to convert histidine to histamine and therefore can cause seafood poisoning in humans through intoxication, however cases are rarely reported due to the mild symptoms or misdiagnoses of histamine poisoning as a food allergy (Visciano et al., 2012). *P. aeruginosa* is also known to be a pathogen towards certain species of fish and can cause economic losses in the aquaculture industry (Thomas et al., 2014).

#### 2.2.2.6. *Brochothrix thermosphacta*

*Brochothrix thermosphacta* is a Gram positive, psychrotrophic spoilage bacteria that is capable of growing in a wide range of storage conditions. This bacterium is a dominant spoilage organism in the meat industry and is also recognised as an issue in the seafood

industry. *Br. thermosphacta* can grow under both aerobic and anaerobic conditions (Yesudhasan et al., 2014). Although *Br. thermosphacta* possesses the ability to spoil fish stored aerobically, the bacterium is usually outcompeted by other Gram negative psychrotrophic bacteria such as *S. putrifaciens* and *P. fluorescens* (Gram and Huss, 1996). Previous studies on Atlantic salmon (Rudi et al., 2004), sea bream (Parlapani et al., 2014), European sea bass (*Dicentrarchus labrax*) (Parlapani, Haroutounian, et al., 2015) and Eastern little tuna (*Euthynnus affinis*) (Thiansilakul et al., 2013) have shown that *Br. thermosphacta* is either the dominant spoilage organism or the co-dominant spoilage organism with LAB or *P. phosphoreum*.

*Br. thermosphacta* has the ability to produce several volatile components that can result in a caramel off-odour (Laursen et al., 2006), however these volatile are not believed to cause any seafood poisoning outbreaks in humans.

#### 2.2.2.7. *Listeria monocytogenes*

*Listeria monocytogenes* is a ubiquitous gastrointestinal invasive pathogen that is associated with most cases of listeriosis (Allen et al., 2016; Tang et al., 2013). There is increasing concern worldwide with regards to this pathogen due to the associated high mortality rates reported (20-30%), particularly amongst pregnant women and the elderly. Between 2005-2008 there were 16 reported listeriosis outbreaks in the EU and United States and there are, on average, approximately 1850 cases reported in the United States annually with circa 425 (23%) of these resulting in death (Løvdaal, 2015; Rotariu et al., 2014). For this reason the U.S. government has adopted a zero tolerance of *L. monocytogenes* in ready to eat products, whereas the EU allows for levels approaching 100 CFU/g determined at the end of shelf-life (EC, 2005; Feldhusen, 2000; Løvdaal, 2015). This foodborne infection is

most commonly associated with ready to eat foods including seafood such as smoked salmon. Even though *L. monocytogenes* is widespread in the environment and can be found in soil and faeces, it is also detected in food processing environments as it can create biofilms on solid surfaces, it can grow in both aerobic and anaerobic conditions and it survives at refrigerated temperatures (0-4°C) (Mahmoud, 2012; Tang et al., 2013). As it may be very difficult to completely decontaminate equipment it can persist in food processing environments, including for example, slicing machines and brining areas (Løvdal, 2015). To completely disinfect the machinery used along the process line, plants must implement good hygienic practices and food safety management systems, including the use of hot (80°C) steam, water and air. Vogel et al. (2001) concluded that, even with robust HACCP programmes, as fish are a natural carrier of *Listeria* spp. it is not always possible to prevent this pathogen from entering the processing environment.

### 2.2.3. Seafood Poisoning

Fresh seafood is highly susceptible to spoilage and is frequently associated with foodborne illness outbreaks in humans. This has led to the development of process treatments to improve preservation and shelf-life; however consumers have increasingly looked for high quality and minimally processed fresh fish. However minimally processed foodstuffs are not treated in ways that will effectively eliminate human pathogens leading to increased risk of foodborne outbreaks (Jung et al., 2014). Approximately 13% of foodborne outbreaks in any given year are associated with the consumption of seafood (Huss et al., 2000). An outbreak is defined as an event where illness has occurred in at least two people who have ingested a common food (EFSA. and ECDC., 2017; Wallace et al., 1999). Many outbreaks are associated with gastrointestinal symptoms such as abdominal pain or

diarrhoea and have been caused by the ingestion of high levels of scrombotoxin or biogenic amines such as histamine (Joob and Wiwanitkit, 2015). Biogenic amines are usually formed when decarboxylase enzymes utilise free amino acids within fish muscle (Chen et al., 2010). For example, aerobic Gram negative psychrotrophic bacteria such as *Pseudomonas* spp. and *S. putrefaciens* produce decarboxylase enzymes that convert free histidine to the biogenic amine histamine which is associated with an allergy like form of food poisoning. Histamine fish poisoning (HFP) is a mild illness associated with almost 50% of illness cases associated with fish or fish products (EFSA. and ECDC., 2017). HFP can occur 24hrs after ingestion of spoiled or contaminated fish (Lehane and Olley, 2000) and symptoms include nausea, vomiting, diarrhoea, skin irritation and a rash. HFP mortalities are rare; however, intoxication can be quite serious in individuals taking medication or to those with a pre-existing illness.

Immediately *post-mortem* spoilage bacteria produce the enzyme, histidine decarboxylase, which converts histidine found in the muscle tissues of fish to histamine (Lee et al., 2016; Lehane and Olley, 2000; Özogul et al., 2004). HFP generally requires the ingestion of large amounts of histamine and therefore is most common in fish with large amounts of free histidine within their muscle, for example scromboid fish species such as tuna. During the 1990s it is estimated that >90% of fin fish associated illnesses were related to scromboid fish (Wallace et al., 1999). Although HFP is most frequently associated with scromboid fish, cases have been reported for other non-scromboid species such as mahi-mahi (*Coryphaena hippurus*), bluefish (*Pomatomus saltatrix*), and sardines (*Sardina pilchardus*) (Taylor et al., 1989).

There are over 100 species of bacteria that can produce histidine decarboxylase and the primary histamine producing bacteria usually belong to the family *Enterobacteriaceae*,

however many indigenous bacteria such as *Pseudomonas* spp. and *Photobacterium* spp. also possess the ability (Chang et al., 2008; Chen et al., 2010). Poor storage temperatures and improper handling from the time the fish are caught to the time they are consumed can cause the onset of histamine production (Lehane and Olley, 2000; Visciano et al., 2012). Although the rate of histamine production increases as the storage temperature increases, psychrophilic bacteria such as *Photobacterium* spp. can produce histidine decarboxylase at refrigerated temperatures (0-5°C) (Bjornsdottir-Butler et al., 2016).

Histidine decarboxylase producing bacteria can originate from the marine environment or contamination introduced following death. Post-slaughter handling is a susceptible time for seafood due to blood discharge which may contain pathogens. It is important that *post-mortem* handling reduces the rate of deterioration (Hansen et al., 2012) therefore processing establishments must operate a system that will not jeopardise fish freshness due to poor hygienic standards. To prevent this council directive 93/53/EEC was established to outline minimum requirements to control the spread of certain fish diseases in case of an outbreak. It would also be beneficial for large processors to share their HACCP principles with smaller organisations to reduce the risk of contamination (Rotariu et al., 2014).

#### 2.2.4. European Legislation

As a member of the European Union, Ireland follows requirements set by the European Commission. According to EC (2005) regulation 2073/2005, all Member States must ensure that foodstuffs should not contain microorganism, their toxins or metabolites in quantities that present an unacceptable risk to human health. This includes testing against values set as acceptable limits. EC (2004) regulation 852/2004 states that all members are required to ensure that safety controls are frequently carried out at appropriate stages of the

production, processing and distribution lines. EC (2005) regulation 2073/2005 sets criteria for ready to eat, cooked fish, crustaceans and molluscs, however when it comes to fresh fish the only microbial criterion for fresh is stated in EC (2013) regulation 1019/2013. The directive gives provisions for histamine checks and acceptable levels. It states that the border between acceptable and marginally acceptable (m) begins at 200 mg/kg, whereas the border between marginally acceptable and unacceptable (M) begins at 400 mg/kg.

As the majority of salmon consumed in Ireland is primarily from an aquacultural environment it is necessary that European criteria must be followed to maintain a product of excellent health and quality. EC (2006) regulation 2006/88 lists the necessary requirements for the prevention and control of disease within aquaculture animals. All Member States must follow this regulation and have in place a system that can guarantee seafood products that are not harmful for human consumption. This involves health surveillance, good hygiene practice and knowledge of product history. It states that aquaculture products are an important source of income and that inadequate controls to prevent the spread of pathogens can have a negative economic impact.

### **2.3. Control of Spoilage Microorganisms**

A combination of nutritional benefits and consumer preferences has increased the demand for fresh fish (Cheng et al., 2014). In the EU alone, over 60% of salmon is sold fresh (Rotariu et al., 2014). This has led to increased research activity to develop more effective seafood preservation technologies (Duun and Rustad, 2007; Fernández et al., 2009). As the seafood market expands and transport chains increase in length, preservation techniques must extend shelf-life while maintaining the microbial safety of fresh fish (Alfaro, Hernández, Balino-Zuazo, et al., 2013; Amanatidou et al., 2000). The extent of processing combined with storage temperature and atmosphere can determine the rate at which microbial and biochemical spoilage can occur (Sivertsvik et al., 2002).

#### **2.3.1. Storage Temperature**

Storage temperature is the main factor affecting fish spoilage and plays a major role in the shelf-life of fresh seafood. Immediately after harvest fish may be contaminated with a wide range of microflora and their quality starts to deteriorate, affecting sensory characteristics such as odour, taste, texture and appearance. However if fish are immediately stored at low temperatures following 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, 2004) 853/2004, to inhibit bacterial growth. The European Commission (Regulation (EC) No 854/2004) does not specify a temperature for the storage and transport of fish and only states that the temperature must be of that approaching melting ice (usually interpreted as 0-2°C). Any seafood stored over 7°C results in accelerated spoilage, whereas fish stored at

sub-zero freezing temperatures, is no longer considered fresh and falls under the frozen fish category, which in turn can decrease its value. Moreover, EC (2004) 853/2004 lays down specific rules for food business operators (FBOs) and supplements EC regulation 852/2004 by adding specific hygiene requirements for products of animal origin such as fish and fishery products.

The beneficial effects of other preservation methods, such as packaging, employed to prolong shelf-life will decrease as storage temperatures increase (Alfaro, Hernández, Le Marc, et al., 2013; Reddy et al., 1995; Sigholt et al., 1997). In a study carried out by Silbande et al. (2016), it was observed that modified atmosphere packaging (MAP) and skin packaging (SP) had no significant effect on extending the shelf-life of tropical yellowfin tuna (*Thunnus albacares*). In this study aerobic samples were stored at 0°C throughout the trial, whereas MAP and SP were stored at 4°C for the first week and then 8°C for the remainder of the trial.

### 2.3.2. Modified Atmospheric Packing (MAP) & Skin Packaging (SP)

MAP is a popular packaging system developed to replace the use of chemical preservatives and freeze technology in seafood (Masniyom et al., 2002). MAP requires replacing air from a package with a new fixed gas mixture (Fernández et al., 2009). Gas mixtures are usually made up with different ratios of carbon dioxide (CO<sub>2</sub>), nitrogen (N<sub>2</sub>) and oxygen (O<sub>2</sub>). This method increases the shelf-life of seafood by inhibiting the growth of aerobic spoilage organisms such as *Shewanella putrefaciens* and *Pseudomonas* spp. (Macé et al., 2012). CO<sub>2</sub> is commonly used for this method as it has bacteriostatic properties which make it very important in preservation (Emborg et al., 2002; Fernández et al., 2009). CO<sub>2</sub>

dissolves in flesh and must be used with a filler gas such as N<sub>2</sub> to reduce the risk of package collapse (Schirmer et al., 2009). Sivertsvik et al. (2002) noted four mechanisms responsible for the bacteriostatic effects of CO<sub>2</sub>; cell membrane function alteration, enzyme inhibition, intracellular pH changes and changes in the physio-chemical properties of proteins (Milne and Powell, 2014). Many factors affect the success of MAP inhibiting microbial growth, such as initial product quality, *post-mortem* handling, packaging materials and storage temperature. The most important factor is the amount of available CO<sub>2</sub> to dissolve into the food product. A suitable gas to product volume ratio (g/p), usually greater than 2:1, must be established to be effective. A low g/p ratio can result in package collapse due to volume contraction or no microbial inhibition (Fernández et al., 2010). Sivertsvik et al. (2002) found that when MAP and a storage temperature of 2°C were applied, the shelf-life for Atlantic salmon could be extended by up to 18 days. European Parliament and Council directive No. 95/2 states that if a food product is packed under a protective atmosphere, this must be indicated on the packaging, where the gases must be listed with their corresponding E-number

SP uses a low vacuum to shrink a thin film tightly around the fillet creating an almost complete anaerobic environment (Łopacka et al., 2016; Nassu et al., 2012). It has become an increasingly popular option in recent years, often replacing MAP as it is considered more attractive to the consumer and is believed to result in a longer product shelf-life (Vázquez et al., 2004). For this reason SP is the packaging method used predominantly in the Irish seafood industry (personal communication, Oceanpath, Howth, Co. Dublin). Previous studies on silver carp (*Hypophthalmichthys molitrix*) (Kachele et al., 2017) observed that fillets packaged at 30 kPa had significantly lower pH values and total volatile basic nitrogen (TVBN) contents than those stored aerobically. Bacterial populations were also significantly lower and it was determined that, under these

conditions, the shelf-life was extended from 6 to 11 days, when compared to aerobically stored samples. However studies on fresh seafood including; Atlantic salmon (Amanatidou et al., 2000; Schirmer et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Rodrigues et al., 2016), Atlantic herring (*Clupea harengus*) (Özogul et al., 2000), sardines (Özogul et al., 2004) and (Silbande et al., 2016) have shown that MAP is more successful in inhibiting bacterial growth than SP.

Packaging technologies have limitations in the seafood industry as it is still possible for CO<sub>2</sub> resistant psychrophilic bacteria, such as *Photobacterium phosphoreum*, and lactic acid producing bacteria, such as *Carnobacteriaceae*, to colonise fresh seafood (Emborg et al., 2002; Rudi et al., 2004; Yesudhasan et al., 2014). To more effectively inhibit microbial growth it may be necessary to establish a combination of preservation techniques (Holley and Patel, 2005). Emborg et al. (2002) determined that by eliminating *Photobacterium phosphoreum* from MAP salmon fillets, the shelf-life was extended by almost two weeks. They achieved this by freezing the fillets prior to storage which may not be acceptable to consumers or regulators alike.

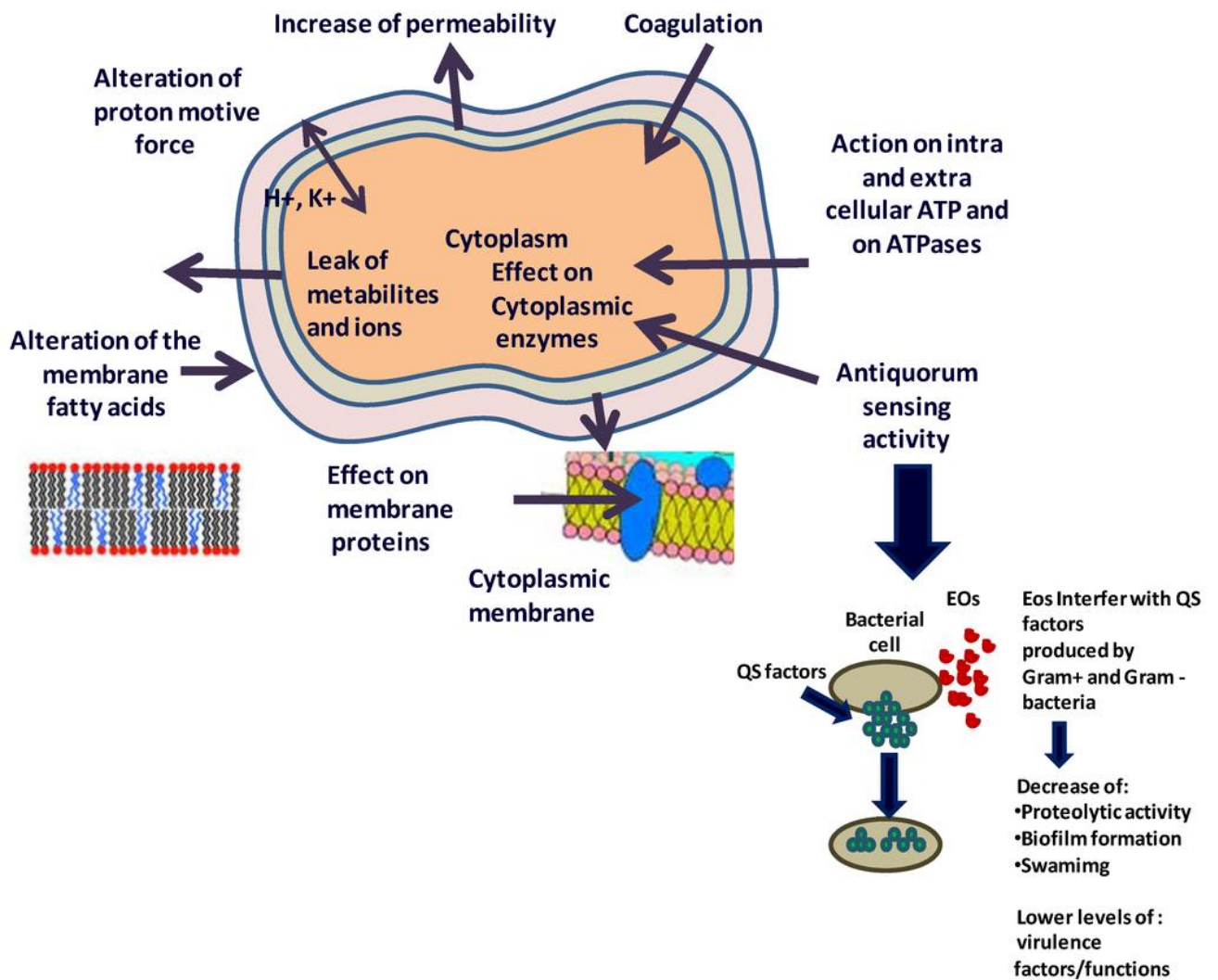
### 2.3.3. Organic Acids and Essential Oils

Antimicrobial resistance has caused a growing demand for alternate means of improving food quality (Rivas et al., 2010). The use of organic acids, such as citric acid and lactic acid, has been suggested as a potential preservation technique to control microbial growth. In their undissociated form an acid molecule can pass through the cell membrane, after which they can dissociate and acidify the cytoplasm (Brul and Coote, 1999; Schirmer et al., 2009). Schirmer et al. (2009) determined that the use of citric (3% w/v) and acetic (1% w/v) acid with CO<sub>2</sub> (MAP) completely inhibited the growth of naturally occurring bacteria

on Atlantic salmon fillets during a 14 day storage period (4°C), whereas García-Soto et al. (2014) reported that both citric (1.25 g/l) and lactic (0.5 g/l) acids significantly lowered the bacterial counts on hake (*Merluccius merluccius*) and megrim (*Lepidorhombus whiffiagonis*) throughout a 15 day storage at 0 to 1°C. Sallam et al. (2007) observed that an immersion treatment using 2.5% aqueous solutions of sodium acetate (NaA), sodium lactate (NaL) or sodium citrate (NaC) increased the shelf-life of chilled salmon fillets (1°C) by up to 7 days, when compared against an untreated control. However there is unease amongst consumers about the use of chemical preservatives. There is a growing trend where consumers want products containing no preservatives or only those that occur naturally (Oliveira et al., 2015), leading to an increased interest in the use of natural antimicrobials derived from plant oils.

As seen in Figure 2.3 essential oils (EO) have several mechanisms of action including increasing the permeability of the cell membrane (through cell wall degradation and damaging cell membrane proteins), disruption of the proton motive force, electron flow and active transport systems and inhibiting enzymes involved in energy regulation and the synthesis of structural component (Nazzaro et al., 2013). The preservative characteristics of these EO dates back to the mummifying processes in ancient Egypt (Mahmoud et al., 2004; Tajkarimi et al., 2010), and it is well documented that they have the potential to extend the shelf-life either alone or in combination with other preservation techniques such as MAP. EO are usually a mixture of multiple components (Holley and Patel, 2005) with the phenolic compounds such as thymol (thyme/oregano), carvacrol (oregano/anethole) and eugenol (clove) largely making up the antimicrobial constituents (Gómez-Estaca et al., 2010; Mahmoud et al., 2004). In particular thymol and carvacrol have been linked to a strong antimicrobial effect against *Photobacterium phosphoreum*, which suggests that oregano could improve the shelf-life of MAP salmon fillets. Mejlholm and Dalgaard

(2002) tested the effect of oregano on MAP cod fillets against other EO and found that oregano (0.05% w/v) had the strongest antimicrobial effect extending the original shelf-life of 11-12 days up to 26 days, however this treatment had no effect on MAP salmon fillets, possibly due to the higher fat content.



**Figure 2. 3** Mechanisms of action of essential oils (EO) against a bacterial cell (Nazzaro et al., 2013).

## 2.4. Assessment of Freshness in Fish

Worldwide there has been an increase in the consumption of fresh seafood as consumers move away from buying products that have been through several processing treatments including the addition of chemical preservatives (Cheng et al., 2014). This has made freshness the single most important attribute when assessing seafood quality (Alasalvar et al., 2001). The three primary methods of assessing freshness implemented in the seafood industry are microbial analysis, sensory analysis and chemical analysis.

### 2.4.1. Microbial Analysis

Fish spoilage may involve different reactions but most quality deterioration is a result of microbial activity (Gram and Huss, 1996). The most common method for culturing spoilage organisms associated with seafood is by culture on growth media. Based on the relationship between microbial log values and spoilage it may be possible to predict shelf-life using SSO log values (Dalgaard et al., 1996). It has been suggested that spoilage usually occurs when total viable counts (TVC) log values reach  $7 \log_{10}$  CFU/cm<sup>2</sup> or when a specific spoilage organism (SSO) reaches between 6-8  $\log_{10}$  CFU/cm<sup>2</sup> (Alfaro, Hernández, Le Marc, et al., 2013; Liston, 1980). However, there is no common consensus on which bacterial count should be used to monitor the shelf-life of fresh fish. Although 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., 2002; Gram and

Dalgaard, 2002). Fresh fish are stored under chilled conditions (temperature approaching that of melting ice (0-3°C)), as required in European Commission (EC, 2004) 853/2004, to inhibit bacterial growth. However these storage conditions are suitable for the growth of psychrophilic aerobic Gram negative spoilage bacteria such as *Shewanella putrefaciens* and *Pseudomonas* spp. (Alfaro, Hernández, Balino-Zuazo, et al., 2013; Emborg et al., 2002). Previous research on sea bream (Parlapani et al., 2014) and European sea bass (Parlapani, Haroutounian, et al., 2015) found that these genera are the dominant spoilage organisms during a period of aerobically chilled storage, suggesting that these bacteria might provide a better predictor of spoilage.

However, only 1% of bacteria are detectable using culture dependent techniques (Ghanbari et al., 2015; Ingerslev et al., 2014) meaning it is impossible to completely assess the microbiota of seafood. The need for more accurate information has led to the development of new molecular based methods in the hope of providing a clearer insight into the diversity of microbial communities present on Atlantic salmon and other fish species (Austin, 2006; Ghanbari et al., 2015). Molecular methods allow for the identification of bacteria whether they have the ability to grow on media or not (Navarrete et al., 2009). Next-generation sequence (NGS) analysis of 16S ribosomal RNA gene (rRNA) is a popular non-culture based method for investigating microbial diversity and has been used to characterize several taxonomic groups (Baker et al., 2003; Klindworth et al., 2013; Llewellyn et al., 2014). Illumina high throughput sequencing of the 16S rRNA gene is generally considered a reliable method of NGS used in the characterization of the natural microbiota (Gloor et al., 2010; Reuter et al., 2015). A common observation in most studies is that there is a regular occurrence of genera belonging to the class  $\gamma$ -Proteobacteria, including *Pseudomonas* spp., *Vibrio* spp., *Aliivibrio* spp., *Photobacterium* spp. and

*Shewanella* spp. (Nayak, 2010; Reveco et al., 2014), all of which have been suggested as spoilage bacteria in aerobic and anaerobically stored fish samples.

#### 2.4.2. Chemical Analysis

The demand for higher requirements in fish quality control has led to the development of quick, effective and non-destructive techniques for analysing fish freshness (Cheng et al., 2014; Parlapani, Haroutounian, et al., 2015). Chemical analysis, such as the measurement of microbial metabolites, is a favoured industry method of assessing freshness as it is faster than microbial analysis (Gram and Dalgaard, 2002). Autolytic changes, such as the breakdown of adenosine phosphate molecules (ATP, ADP and AMP) to inosine monophosphate (IMP), occur immediately *post-mortem*. Inosine monophosphate (IMP) is an ATP catabolite believed to be responsible for the pleasant taste and aroma associated with high quality seafood and is therefore used as an indicator of freshness (Aliani et al., 2013; Mørkøre et al., 2010). Stress associated with capture and *post-mortem* handling accelerate the breakdown of ATP to IMP, however IMP is more slowly dephosphorylated to inosine (I), before eventually being degraded to hypoxanthine (Hx) (Chen et al., 2010; Gram and Dalgaard, 2002). The ATP metabolite Hx is responsible for the bitter flavour and unpleasant aroma associated with low quality seafood, and is therefore an indicator of spoilage.

A number of studies have suggested that IMP (Dingle and Hines, 1971; Ehira and Uchiyama, 1974) or I (Bremner et al., 1988; Murata and Sakaguchi, 1988) are biochemical markers suitable for evaluating freshness, although, the concentrations obtained are not always linear over time (Beauchat, 1973; Bremner et al., 1988; Jahns and Rand, 1977; Murata and Sakaguchi, 1988).

### 2.4.3. Sensory Analysis

The freshness of fish can be difficult to assess once it arrives at a processing plant making it difficult to predict the shelf-life. Seafood processing plants therefore require a system that can quickly and accurately assess the freshness of fish. Sensory analysis assesses physical characteristics, such as appearance, odour, taste and texture, and develops grading systems based on attribute deterioration. Sensory characteristics provide immediate quality information to the processor or consumer, and it is therefore essential that all fresh seafood products appear visibly fresh (Alasalvar et al., 2001).

One such system is the quality index method (QIM), a grading system, which can be adapted to each individual fish species (Pons-Sánchez-Cascado et al., 2006). QIM schemes assess the freshness of fish by scoring different sensory attributes (appearance, odour and texture) during storage (Bremner, 1985). QIM requires a trained sensory panel to grade quality attributes on a scale of 0 to 3, taking into account all sensory characteristics. A score of 0 is given to fish that are very fresh, whereas a score of 3 is given to fish with unacceptable characteristics. Before assessment, a complete list of attributes and their scoring descriptors must be developed (Table 2.1). Assuming the QI increases linearly with time, once the total score for fish at the end of their shelf-life is established, the score obtained prior to this can be used to estimate the remaining shelf-life (Martinsdóttir et al., 2001). A similar approach, quantitative descriptive analysis (QDA) is used to assess the sensory status of cooked fish (Sveinsdottir et al., 2002). Sveinsdottir et al. (2002) and Sveinsdottir et al. (2003) recorded that freshness scores for Atlantic salmon stored on ice began to decrease between days 6 and 8. They observed a linear relationship between the QI score and time ( $R^2 = 0.95$ ), however, contrary to studies using microbial analysis, they suggested that salmon was acceptable to consume until storage day 20. Other QIM/QDA

studies have focused less on appearance and reported that off-flavours were the primary determinant of sensory shelf-life (Whittle et al., 1990).

**Table 2. 1** Quality index method (QIM) list of attributes and their scoring descriptors developed for farmed Atlantic salmon (*Salmo salar*) (Sveinsdottir et al., 2003).

<b>Quality parameters</b>	<b>Description</b>	<b>Points</b>
<i>Skin</i>		
<b>Colour/appearance</b>	Pearl-shiny all over the skin	0
	The head is still pearl-shiny, but the rest less, perhaps yellow	1
<b>Mucus</b>	Clear and not clotted	0
	Milky and clotted	1
	Yellow and clotted	2
<b>Odour</b>	Fresh seaweedy, cucumber	0
	Neutral to metal, dry grass, corn	1
	Sour	2
	Rotten	3
<i>Eyes</i>		
<b>Pupils</b>	Clear and black, metal shiny	0
	Dark grey	1
	Mat, grey	2
<b>Form</b>	Flat	0
	Little sunken	1
	Sunken	2
<i>Abdomen</i>		
<b>Blood in abdomen</b>	Blood light red/not present	0
	Blood more brown	1
<b>Odour</b>	Neutral	0
	Corn	1
	Sour	2
	Rotten/rotten kale	3
<i>Gills</i>		
<b>Colour/appearance</b>	Red/dark brown	0
	Light red/brow	1
	Grey-brown, grey, green	2
<b>Mucus</b>	Transparent	0
	Yellow, clotted	1
	Brown	2
<b>Odour</b>	Fresh, seaweed	0
	Metal	1
	Sour	2
	Rotten	3
<i>Texture</i>		
<b>Elasticity</b>	Finger mark disappears immediately	0
	Finger leaves mark over 3 s	1

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**Chapter 3 - Spoilage indicator bacteria in farmed  
Atlantic salmon (*Salmo salar*) stored on ice for 10 days**

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**See Appendix C**

### 3.1. Summary

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<sup>2</sup> surface areas). Mesophilic total viable counts (TVC<sub>m</sub>) on skin, flesh and swab samples increased from 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.0, 5.1 and 5.7 log<sub>10</sub> CFU/cm<sup>2</sup> after 10 days, respectively. Psychrotrophic counts (TVC<sub>p</sub>), increased from 2.2, 1.8 and 3.1 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.2, 5.3 and 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>, 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<sub>10</sub> CFU/cm<sup>2</sup> indicating the end of shelf-life.

### 3.2. 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  $\sim 0.99$  (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, 2004) 853/2004, to inhibit bacterial growth. Moreover, (EC, 2004) 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<sub>10</sub> CFU/g (Robson et al., 2007) to 7 log<sub>10</sub> CFU/g (Liston, 1980) and 8-9 log<sub>10</sub> 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; Gram and Dalgaard, 2002). *Shewanella* spp., *Pseudomonas* spp. and *Photobacterium* spp., for example, are ubiquitous in the marine environment (Emborg et al., 2002; Janda, 2014) and colonise the fish by the skin, gills or gastrointestinal (GI) tract (Ringø and Holzappel, 2000). Moreover they are psychrotrophic bacteria and have been reported to be the main spoilage organisms for chilled fish (Gram and Huss, 1996; Møretro 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<sub>m</sub>), psychrophilic TVC (TVC<sub>p</sub>), 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.

### 3.3. Materials and Methods

#### 3.3.1. 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.

#### 3.3.2. 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<sup>2</sup> 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<sup>-5</sup>. 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<sub>m</sub>, incubated 30°C for 72h) and psychrotrophic (TVC<sub>p</sub>, 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 Ceftrimide-Fucidin-Cephaloridine (CFC) supplements (Oxoid, Basingstoke, United Kingdom (SR0103)) incubated at 30°C for 48h, *Br. thermosphacta* counts on streptomycin-thallos 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.

### 3.3.3. 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 $\pm$ , 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<sup>2</sup>.

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 record skin and core temperatures of the whole salmon.

#### 3.3.4. Data Analysis

Bacterial counts were converted to  $\log_{10}$  CFU/cm<sup>2</sup>. 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:  $G = t/3.3 \log b/B$ , 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  $P < 0.05$  with Tukey's multiple comparison test where applicable.

### 3.4. Results

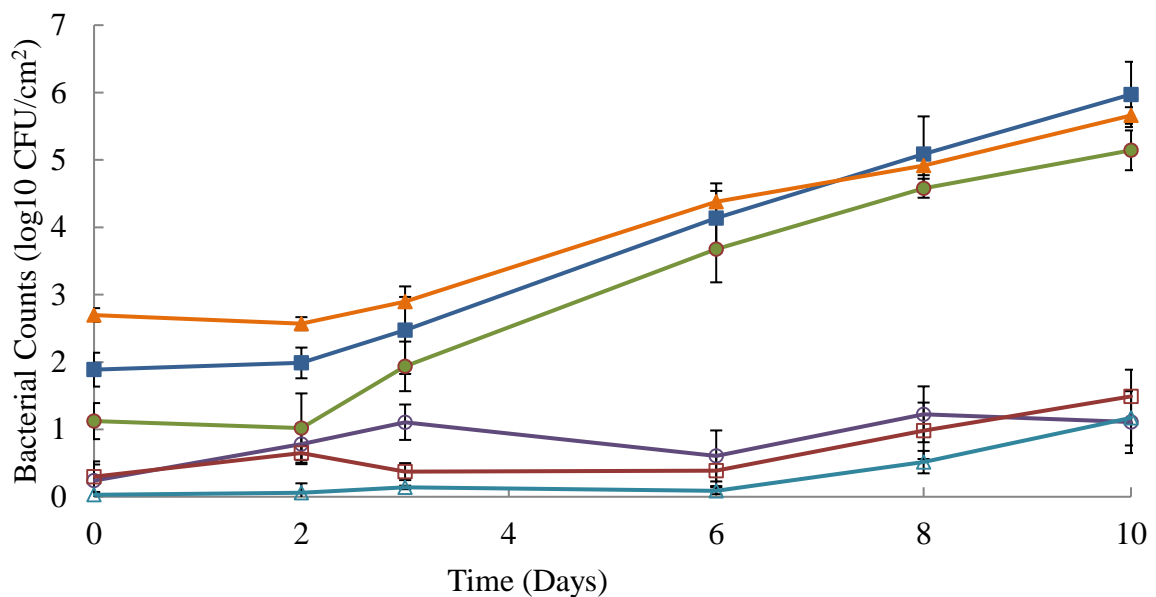
This experiment was repeated on three separate occasions and a mean value was obtained for each data point on each day. These results are presented below. Table 3.1 presents the results for the pH and  $a_w$  obtained over the 10 day trial. The pH of the salmon flesh and skin samples followed a similar 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.

**Table 3. 1** pH and  $a_w$  measurements as determined from skin, flesh and swab samples from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days.

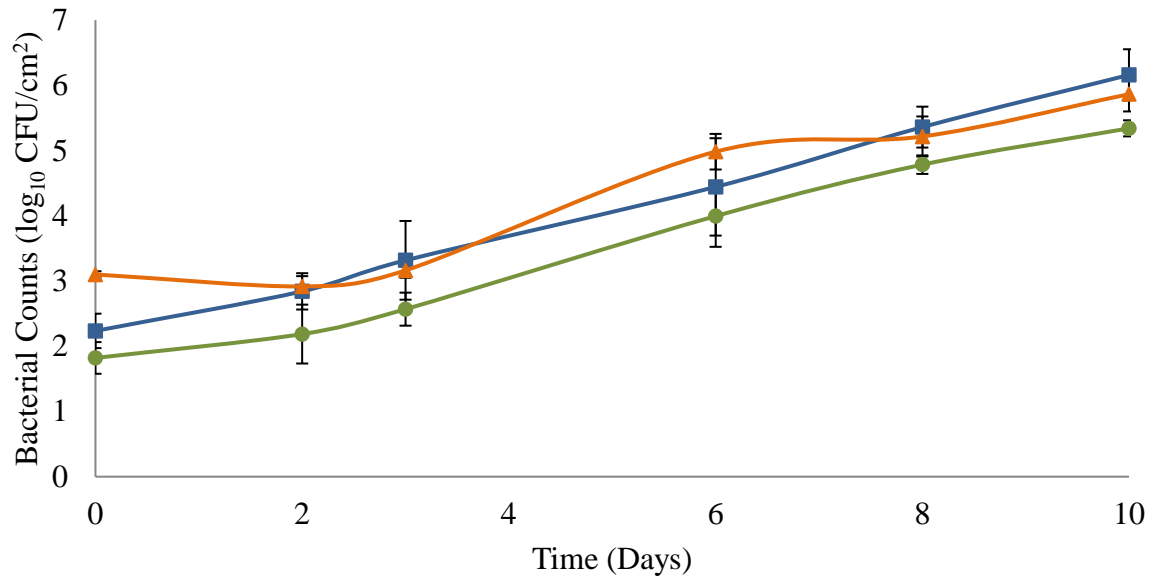
	Day	pH	$a_w$
<b>Flesh</b>	0	7.0 ± 0.1	0.96 ± 0.00
	2	6.8 ± 0.0	0.96 ± 0.01
	3	7.5 ± 0.3	0.97 ± 0.01
	6	7.2 ± 0.2	0.94 ± 0.01
	8	6.6 ± 0.1	0.96 ± 0.00
	10	6.5 ± 0.2	0.96 ± 0.01
<b>Skin</b>	0	7.1 ± 0.1	0.95 ± 0.01
	2	6.9 ± 0.0	0.95 ± 0.01
	3	7.7 ± 0.4	0.96 ± 0.01
	6	8.0 ± 0.6	0.95 ± 0.00
	8	6.8 ± 0.1	0.96 ± 0.00
	10	6.7 ± 0.2	0.96 ± 0.00

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<sub>m</sub> counts on skin, flesh and swab samples on day 0 were 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup> which increased to 6.0, 5.1 and 5.7 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively, after 10 days storage (Figure 3.1). TEC increased from 0.3, 0.2 and 0.02 log<sub>10</sub> CFU/cm<sup>2</sup> on skin, flesh and swab samples to 1.5, 1.2 and 1.2 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively, by day 10. Figure 3.2 shows the growth of TVC<sub>p</sub>, with counts increasing from 2.2, 1.8 and 3.1 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.2, 5.3 and 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>, for skin, flesh and swab samples, respectively.

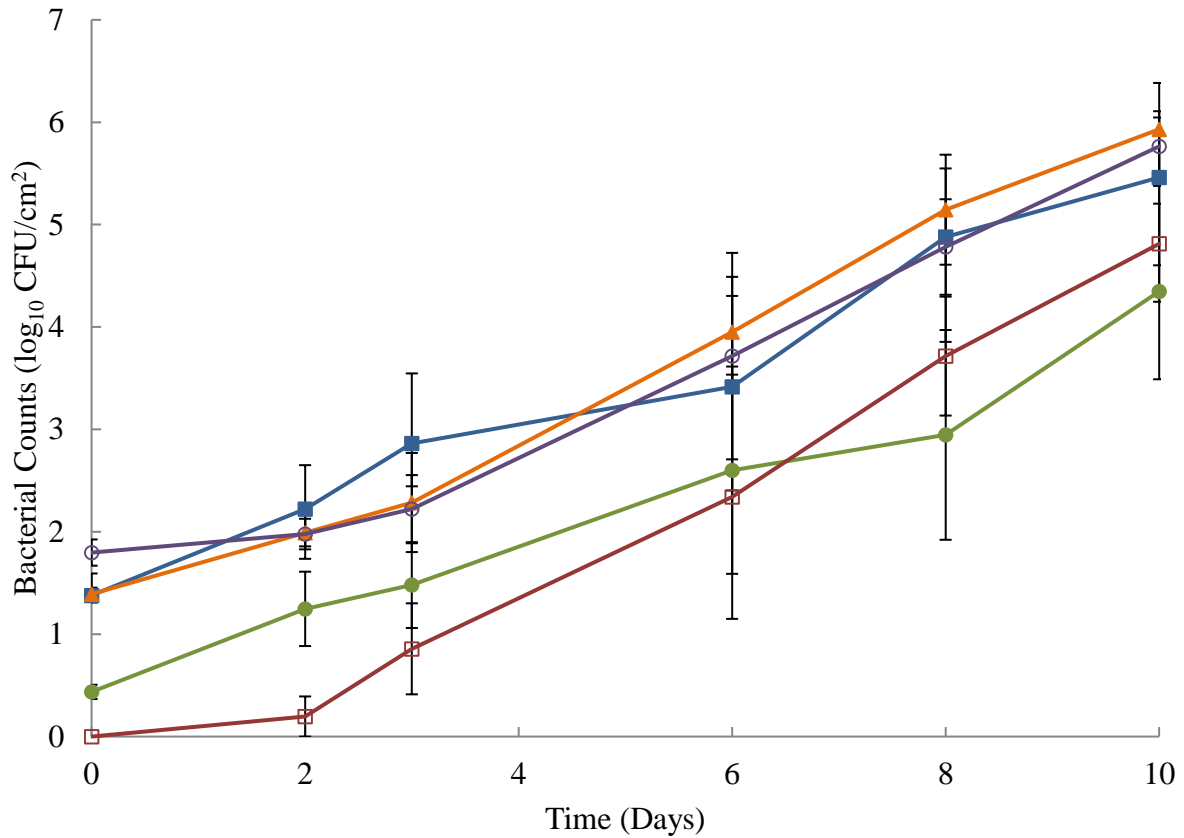


**Figure 3. 1** Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC<sub>m</sub> (■) and TEC (□); flesh TVC<sub>m</sub> (●) and TEC (○) and swab TVC<sub>m</sub> (▲) and TEC (△) samples stored at 2°C for 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

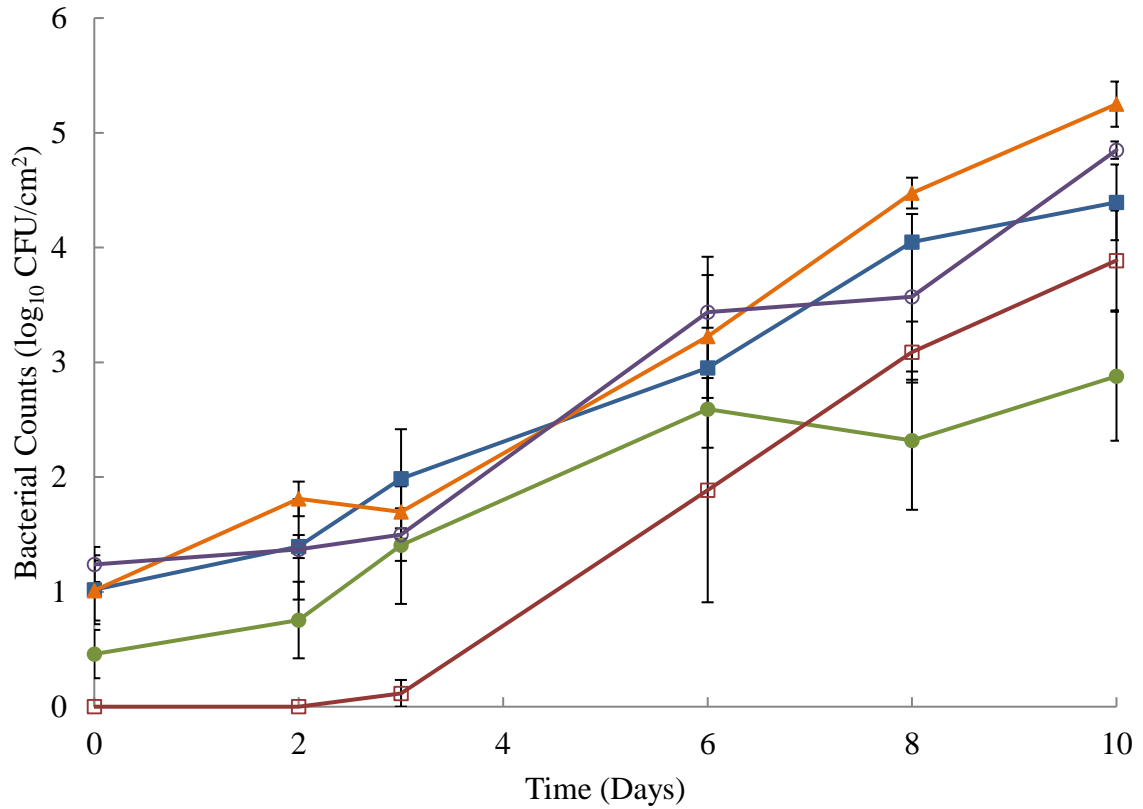


**Figure 3. 2** Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC<sub>p</sub> (■), flesh TVC<sub>p</sub> (●) and swab TVC<sub>p</sub> (▲) samples stored at 2°C for 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

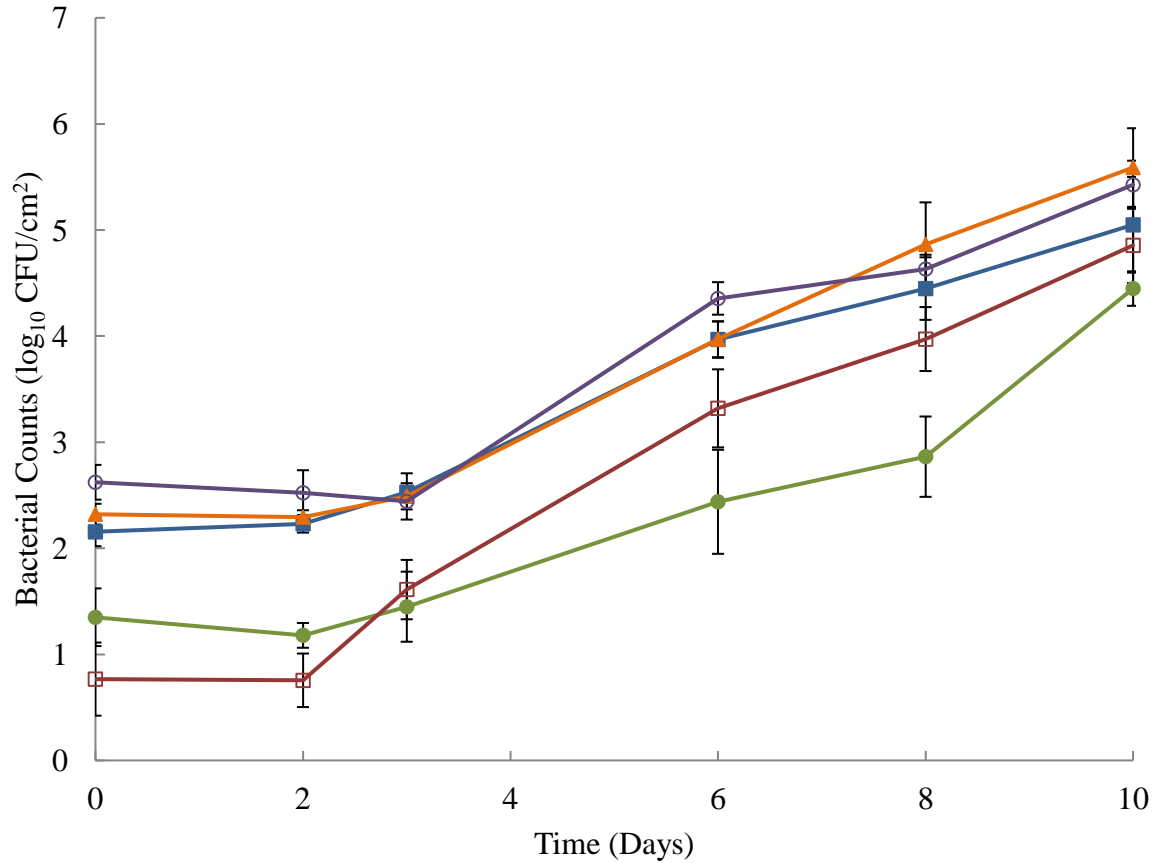
Initial counts of 1.4, 1.4, 1.4, <1.0 and 1.8 log<sub>10</sub> CFU/cm<sup>2</sup> for HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. on skin samples increased to 5.5, 5.9, 5.9, 4.8 and 5.8 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively (Figure 3.3). Corresponding counts on flesh samples were 1.0, 1.0, 1.0, <1.0 and 1.2 log<sub>10</sub> CFU/cm<sup>2</sup> increasing to 4.4, 5.2, 5.2, 3.9 and 4.8 log<sub>10</sub> CFU/cm<sup>2</sup> (Figure 3.4). The data for the swab samples is shown in Figure 3.5. HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. counts increased by 2.8, 3.3, 3.3, 4.1 and 2. log<sub>10</sub> CFU/cm<sup>2</sup>, respectively.



**Figure 3. 3** Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp. (○), on the skin from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days. Each data point and the error bars show the mean of 3 replicates  $\pm$  the standard error.



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



**Figure 3. 5** Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp. (○), in swab samples from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

The growth parameters for all bacteria investigated are shown in Table 3.2. 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.

**Table 3. 2** Growth parameters for bacterial counts (total viable count mesophilic (TVC<sub>m</sub>) and psychrotrophic (TVC<sub>p</sub>), total *Enterobacteriaceae* (TEC), hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp.) as determined from skin, flesh and swab samples from Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days.

<b>Treatment</b>	<b>Initial concentration (log<sub>10</sub> CFU/cm<sup>2</sup>)</b>	<b>Mean generation time (h)<sup>1</sup></b>	<b>μ<sub>max</sub> (generations day<sup>-1</sup>)</b>	<b>Maximum concentration observed (log<sub>10</sub> CFU/cm<sup>2</sup>)</b>
<b>Skin</b>				
<b>TVC<sub>m</sub></b>	1.9	23.5	1.44	6.0
<b>TVC<sub>p</sub></b>	2.2	18.2	0.96	6.2
<b>TEC</b>	0.3	60.5	0.96	1.5
<b>HSPB</b>	1.4	17.7	0.96	5.5
<b>LAB</b>	1.4	16.2	1.20	5.9
<i>Pseudomonas</i> spp.	1.4	16.2	1.20	5.9
<i>Br. thermosphacta</i>	ND	15.2	1.44	4.8
<i>Photobacterium</i> spp.	1.8	18.2	1.20	5.8
<b>Flesh</b>				
<b>TVC<sub>m</sub></b>	1.1	18.2	1.44	5.1
<b>TVC<sub>p</sub></b>	1.8	20.8	1.20	5.3
<b>TEC</b>	0.2	72.7	0.24	1.2
<b>HSPB</b>	1.0	21.4	0.96	4.4
<b>LAB</b>	1.0	17.3	1.20	5.2
<i>Pseudomonas</i> spp.	1.0	17.3	1.20	5.2
<i>Br. thermosphacta</i>	ND <sup>2</sup>	18.6	1.68	3.9

<i>Photobacterium</i> spp.	1.2	20.2	0.96	4.8
		<b>Skin Swab</b>		
<b>TVC<sub>m</sub></b>	2.7	24.2	1.20	5.7
<b>TVC<sub>p</sub></b>	3.1	26.0	0.96	5.9
<b>TEC</b>	0.02	60.5	1.68	1.2
<b>HSPB</b>	2.2	26.0	1.20	5.0
<b>LAB</b>	2.3	22.0	1.20	5.6
<i>Pseudomonas</i> spp.	2.3	22.0	1.20	5.6
<i>Br.</i> <i>thermosphacta</i>	0.08	17.2	1.20	4.9
<i>Photobacterium</i> spp.	2.6	26.0	1.44	5.4

<sup>1</sup> calculated using the formula  $G = t/3.3 \log b/B$ , 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)

<sup>2</sup>ND = Not Detected

### 3.5. Discussion

The initial TVC<sub>m</sub> counts on skin, flesh and swab samples were 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup>. Other studies have reported initial bacterial levels in fresh farmed salmon of approximately 3 log<sub>10</sub> 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<sub>10</sub> CFU/cm<sup>2</sup>, 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<sub>10</sub> CFU/cm<sup>2</sup>), 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 Bozaris, 2016; Parlapani et al., 2013). The relatively high levels (4.8 to 5.8 log<sub>10</sub> CFU/cm<sup>2</sup>) 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<sub>m</sub> ranged from 5.1 to 6.0 log<sub>10</sub> CFU/cm<sup>2</sup>, TVC<sub>p</sub> from 5.3 to 6.2 log<sub>10</sub> CFU/cm<sup>2</sup> and the spoilage bacterial (HSPB, LAB, *Pseudomonas* spp. and *Photobacterium* spp.) counts from 4.8 to 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>. This is in agreement with Robson et al. (2007), who found seafood spoiled when the bacterial count reached 5 to 6 log<sub>10</sub> CFU/cm<sup>2</sup>. In contrast Dalgaard et al. (1997) suggested the end of shelf-life of aerobically stored fish occurs when a bacterial concentration of 8-9 log<sub>10</sub> CFU/cm<sup>2</sup> 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 5-6 log<sub>10</sub> CFU/g or CFU/cm<sup>2</sup>.

From this study, 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<sub>10</sub> CFU/cm<sup>2</sup>, indicating the end of shelf-life.

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**Chapter 4 - Characterization of the microbial  
community present in the gut of Atlantic salmon (*Salmo  
salar*) farmed in Irish waters**

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**See Appendix D**

#### 4.1. Summary

Next-generation sequence analysis of the 16S ribosomal RNA gene (rRNA) is a commonly used non-culture based method for investigating microbial diversity and has been used to characterize microbial communities in a wide range of ecological niches. In this study, the microbiota from the distal and proximal intestine (DI and PI, respectively) in Atlantic salmon (*Salmo salar*) was examined using MiSeq Illumina high throughput sequencing of the 16S rRNA gene. Six phyla were present in the DI samples, dominated by *Tenericutes* (70.9% abundance). These six phyla were also amongst the 12 phyla detected in the PI samples. The PI microbiota was dominated by *Tenericutes* (17.8%), *Firmicutes* (17%), *Bacteroidetes* (15.2%) and *Proteobacteria* (14.2%). There was a greater abundance of species within the PI samples; however comparisons with the DI data suggested that this difference was not significant (Chao 1,  $P=0.0996$ . ACE,  $P=0.0973$ ). Diversity estimates (Shannon Index, Simpson Index) also suggested that there was a more diverse bacterial population within the PI samples and this difference was significant (Shannon,  $P=0.0151$ . Simpson,  $P=0.0111$ ). A core microbiota of 20 operational taxonomic units (OTUs) common to the distal and proximal region was observed.

## 4.2. Introduction

Global consumption of fresh seafood increases year by year due to the associated nutritional and economic benefits (Amanatidou et al., 2000). This increased demand has led to a decline in wild fish stocks, which in turn has resulted in the growth of the aquaculture industry (Llewellyn et al., 2014). In 2017, the Irish aquaculture industry was worth €208 million, an increase of 24% from the previous year (BIM, 2018). According to BIM (2018), the production of Atlantic salmon (*Salmo salar*) grew 25% since 2016, up to 20,000 tonnes, with a value of €147 million. Ensuring optimal health of farmed fish is necessary if the final product is to be of good quality. A balanced gut microbiota is essential to maximise nutrition and prevent infection (Navarrete et al., 2009, Llewellyn et al., 2014).

Microbes normally interact with hosts along mucosal surfaces, the largest of which are found in the intestines (O'Hara and Shanahan, 2006). The gastrointestinal (GI) tract of an animal is home to a large and diverse microbial ecosystem, known as the microbiota (Nayak, 2010). In mammals, the gut acts as an ecological niche for specialized bacteria and the gut microbiota is very important to the health and survival the host organism by promoting nutrient supply and reducing the risk of disease by outcompeting pathogenic bacteria (Dehler et al., 2017, Ghanbari et al., 2015, Nyman et al., 2017, Nayak, 2010). Fish species also harbour a unique and diverse gut microbiota which is essential for maintaining host health. Rawls et al. (2004), for example, demonstrated the involvement of the microbiota in promoting nutrient breakdown and pathogen resistance in zebrafish. However, less research has been carried out on the gut microbiota of fish, when compared to other vertebrates.

The gut surface has different physical and chemical properties throughout, resulting in a unique and diverse microbiota at different locations along the GI tract (Nayak, 2010, Navarrete et al., 2009). Austin and Al-Zahrani (1988) observed that in rainbow trout, for example, the proximal region of the GI tract had a higher microbial diversity when compared to the distal region. However these differences may be less significant in other fish species (Ringø et al., 1995, Nyman et al., 2017). There are many factors which can affect the microbiota of fish such as the environment, feeding habitats and host genetic background (Li et al., 2014). Due to their aquatic nature, fish are constantly exposed to the indigenous microorganisms in their environment (Roeselers et al., 2011, Horsley, 1973) and the natural microflora of fish is primarily determined by the local environment (Ghanbari et al., 2015, Dehler et al., 2017). Thus different farming environments and locations can have a major influence on the GI microbiota (Lyons et al., 2017, Nayak, 2010).

Previously, studies investigating the microbiota of the gut in fish relied on culture based methods. However, it has been estimated that only 1% of bacteria may be detected using these culture dependent techniques (Ghanbari et al., 2015, Ingerslev et al., 2014). The need for more accurate information has led to the development of new molecular based methods to provide a clearer insight into the diversity of microbial communities present in the gut (Ghanbari et al., 2015, Austin, 2006). Molecular methods allow for the identification of bacteria that are undetectable using culture based methods (Navarrete et al., 2009). Next-generation sequence (NGS) analysis of the 16S ribosomal RNA gene (rRNA) is a popular non-culture based method for investigating microbial diversity and has been used to characterize several taxonomic groups (Klindworth et al., 2013, Baker et al., 2003, Llewellyn et al., 2014). Illumina high throughput sequencing of the 16S rRNA gene is

generally considered a reliable method of NGS for the characterization of microbial diversity (Gloor et al., 2010, Reuter et al., 2015).

In this study, the microbiota from the proximal and distal intestine in Atlantic salmon was examined using MiSeq Illumina high throughput sequencing of the 16S rRNA gene. The objectives of this study were to characterize the microbiota in the GI tract of Atlantic salmon farmed in waters off the west coast of Ireland and to investigate whether or not there is a difference in microbiota diversity between the proximal and distal regions of the intestine.

### **4.3. Materials and Methods**

#### **4.3.1. Sample Collection**

A total of 50 proximal and 50 distal intestines, from Atlantic salmon, were obtained from a salmon fish farm operating off the west coast of Ireland (Kilkieran, Galway). As required by the European Commission (EC, 2004) 853/2004, the samples were transported on ice to the laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15) to maintain a storage temperature of that approaching melting ice. Once in the laboratory both the proximal and distal intestines were randomly separated into ten groups of five. The entire intestinal contents for each sample were removed by gently squeezing the intestine tissue with a sterile forceps and the intestinal contents for each group were combined in sterile 50ml tubes. These tubes were then vortexed (Clifton Cyclone vortex mixer, Thermo Fisher Scientific, Ireland) for 30 seconds, leaving ten pools of proximal intestine contents and ten pools of distal intestine contents.

#### **4.3.2. DNA extraction**

Exactly 220mg of intestinal contents, from each pool, was weighed out into a sterile 2ml microtube containing 0.25 g of sterile zirconia beads (0.125 g of 0.1 mm and 0.125 g of 1.0, Stratech Science, Newmarket, UK) and a single 2.5mm sterile bead (Stratech Science, Newmarket, UK). Each sample was then suspended in 1.4ml ASL buffer (Qiagen, Hilden, Germany) after which the samples were disrupted using a bead beater (Vortex-Genie 2, Thermo Fisher Scientific, Ireland) at maximum speed for 4 cycles of 30s. DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) with the following modification to the manufacturer's protocol; the suspension was heated at 90°C for 5min to improve cell lysis. Each extraction was carried out in duplicate, meaning 20

extractions were carried out for both the DI and PI samples. After extraction the DNA concentration of all samples were determined both fluorometrically (Qubit® dsDNA BR Assay Kit, Thermo Fischer Scientific, Ireland) and spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific, Ireland), to ensure DNA concentrations were adequate for sequencing. Samples were stored at -80°C until sequencing was carried out.

#### 4.3.3. Illumina sequencing

The composition of the microbiota within these samples was established by amplicon sequencing. The V<sub>3</sub>-V<sub>4</sub> variable region of the 16s rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina, CA, USA). The sequence specific to the V<sub>3</sub>-V<sub>4</sub> region from the 16S rRNA gene was amplified with the universal primers (forward primer; 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and reverse primer; 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Klindworth et al., 2013), and subsequently sequenced on the Illumina MiSeq platform using V3 sequencing chemistry with 2x250bp paired-end reads (Illumina, 2013). The Illumina reads were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and remove windows of 20 nucleotides with a low average quality) and length (removal of sequences with less than 200bp) with prinseq (Schmieder and Edwards, 2011). Finally paired-end reads (with a minimum overlap of 20bp) were joined using Fastq-join (Aronesty, 2011).

Using clean reads (based on quality and length) a closed-reference Usearch v7.0 algorithm (Edgar, 2010) was applied allowing for sequences to be clustered with 97% identity to obtain operational taxonomic units (OTUs), while also removing chimeric OTUs against

the gold database. The taxonomic assignment of these OTUs was obtained against the Ribosomal database project (Cole et al., 2014). Alpha and Beta-diversity was determined using QIIME (Caporaso et al., 2010). Additionally, the R package Phyloseq was used to compute the core microbiome (McMurdie and Holmes, 2013).

#### 4.3.4. Statistical analysis

Statistical analysis of the data was analysed using an ANOVA to calculate significance in the analysis of the alpha-diversity index. Statistical significance was established at  $P < 0.05$ .

#### 4.4. Results

Out of the 20 DNA extractions carried out on contents from the distal intestine (DI) and a similar number on contents from the proximal intestine (PI), DNA concentration analysis suggested that only ten distal and five proximal samples were suitable for sequence analysis (data not shown).

The mean ( $\pm$  SE) number of sequences per DI and PI samples were  $262,579 \pm 16,786$  and  $160,070 \pm 30,143$ , respectively. These sequences represented an average of  $203 \pm 11$  and  $1223 \pm 134$  OTUs per DI and PI sample, respectively.

Using an abundance cut off point of  $\geq 0.1\%$ , six phyla were identified in the DI samples (Table 4.1). The microbiota of the DI samples was dominated by *Tenericutes* (70.9%), followed by *Firmicutes* (19.1%) and *Spirochaetes* (8.9%). *Bacteroidetes* (0.3%), *Proteobacteria* (0.2%) and *Actinobacteria* (0.1%) were also detected. These six phyla were also found in the PI samples, where twelve phyla were identified (Table 4.2). The microbiota of the PI was dominated by *Tenericutes* (17.8%), *Firmicutes* (17%), *Bacteroidetes* (15.2%) and *Proteobacteria* (14.2%). “Unassignable; Other” bacteria accounted for 31% of all sequences in the PI samples reflecting a group of phyla that could not be identified using the currently available databases.

**Table 4. 1** Summary of bacterial taxa identified in contents of the distal intestine (DI) of Atlantic salmon (*Salmo salar*), with relative abundance (%) of phylum and genera.

<b>Phylum</b>	<b>Class</b>	<b>Order</b>	<b>Family</b>	<b>Genera</b>
Actinobacteria (0.1%)	Actinobacteria	Actinomycetales	<i>Micrococcaceae</i>	<i>Micrococcus</i> (0.1%)
Bacteroidetes (0.3%)	Bacteroidia	Bacteroidales	<i>Bacteroidaceae</i>	Other (0.1%)
Firmicutes (19.1%)	Bacilli	Bacillales	<i>Bacillaceae</i>	Other (17.7%)
		Clostrida	Clostridiales	<i>Clostridiaceae</i>
	<i>Ruminococcaceae</i>			<i>Faecalibacterium</i> (0.1%)
	Other (0.1%)			
Proteobacteria (0.2%)	Betaproteobacteria	Burkholderiales	<i>Comamonadaceae</i>	<i>Delftia</i> (0.1%)
	Gammaproteobacteria	Enterobacteriales	<i>Enterobacteriaceae</i>	<i>Serratia</i> (0.1%)
		Pseudomonadales	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> (0.3%)
		Vibrionales	<i>Vibrionaceae</i>	<i>Aliivibrio</i> (1.3%)
				<i>Photobacterium</i> (1.5%)
Xanthomonadales	<i>Xanthomonadaceae</i>	<i>Stenotrophomonas</i> (0.1%)		
Spirochaetes (8.9%)	Spirochaetia	Spirochaetales	<i>Brevinemataceae</i>	<i>Brevinema</i> (8.6%)
Tenericutes (70.9%)	Mollicutes	Mycoplasmatales	<i>Mycoplasmataceae</i>	Other (68.4%)
Unassignable (0.4%)				

**Table 4. 2** Summary of bacterial taxa identified in contents of the proximal intestine (PI) of Atlantic salmon (*Salmo salar*), with relative abundance (%) of phylum and genera.

<b>Phylum</b>	<b>Class</b>	<b>Order</b>	<b>Family</b>	<b>Genera</b>
Actinobacteria (1.5%)	Actinobacteria	Actinomycetales	Other	Other (0.1%)
			<i>Corynebacteriaceae</i>	<i>Corynebacterium</i> (0.2%)
			<i>Microbacteriaceae</i>	Other (0.4%)
			<i>Micrococcaceae</i>	<i>Micrococcus</i> (0.1%)
			<i>Nocardiaceae</i>	<i>Rhodococcus</i> (0.3%)
		Bifidobacteriales	<i>Bifidobacteriaceae</i>	<i>Bifidobacterium</i> (0.1%)
Bacteroidetes (15.2%)	Other	Other	Other	Other (0.3%)
	Bacteroidia	Bacteroidales	Other	Other (0.1%)
			<i>Bacteroidaceae</i>	<i>Bacteroides</i> (4.3%)
			<i>Porphyromonadaceae</i>	Other (4.1%)
				<i>Barnesiella</i> (0.1%)
				<i>Odoribacter</i> (0.3%)
			<i>Prevotellaceae</i>	<i>Prevotella</i> (1.0%)
	<i>Rikenellaceae</i>	<i>Alistipes</i> (1.3%)		
	Flavobacteriia	Flavobacteriales	Other	Other (0.1%)
			<i>Flavobacteriaceae</i>	Other (1.8%)

	Sphingobacteriia	Sphingobacteriales		<i>Cloacibacterium</i> (0.7%)
				<i>Flavobacterium</i> (0.1%)
			Other	Other (0.1%)
			<i>Saprospiraceae</i>	Other (0.1%)
			<i>Sphingobacteriaceae</i>	<i>Sphingobacterium</i> (0.1%)
Candidatus Saccharibacteria (0.2%)	Other	Other	Other	Other (0.2%)
Chlamydiae (0.1%)	Other	Other	Other	Other (0.1%)
Cyanobacteria (0.1%)	Other	Other	Other	Other (0.1%)
Deferribacteres (0.1%)	Deferribacteres	Deferribacterales	<i>Deferribacteraceae</i>	<i>Mucispirillum</i> (0.1%)
Firmicutes (17%)	Bacilli	Bacillales	Other	Other (1.1%)
			Other	Other (0.6%)
			<i>Bacillaceae</i>	<i>Anoxybacillus</i> (0.5%)
				<i>Geobacillus</i> (0.3%)
			<i>Bacillales Incertae Sedis</i>	<i>Thermicanus</i> (0.7%)
			<i>Planococcaceae</i>	<i>Planococcaceae incertae sedis</i> (0.2%)
				<i>Sporosarcina</i> (0.2%)
			<i>Staphylococcaceae</i>	<i>Staphylococcus</i> (0.1%)
	Lactobacillales	<i>Carnobacteriaceae</i>	<i>Carnobacterium</i> (0.2%)	

			<i>Enterococcaceae</i>	<i>Enterococcus</i> (1.3%)
			<i>Lactobacillaceae</i>	<i>Lactobacillus</i> (3.2%)
			<i>Streptococcaceae</i>	<i>Lactococcus</i> (0.3%)
	Clostridia	Clostridiales	Other	Other (1.3%)
			<i>Clostridiaceae</i>	<i>Clostridium sensu stricto</i> (0.6%)
			<i>Clostridiales Incertae Sedis</i>	<i>Ezakiella</i> (0.1%)
			<i>Lachnospiraceae</i>	Other (3.3%)
				<i>Acetatifactor</i> (0.1%)
				<i>Anaerostipes</i> (0.1%)
				<i>Blautia</i> (0.1%)
				<i>Coprococcus</i> (0.2%)
				<i>Dorea</i> (0.1%)
				<i>Fusicatenibacter</i> (0.2%)
				<i>Lachnospiracea incertae sedis</i> (0.2%)
			<i>Roseburia</i> (0.6%)	
			<i>Ruminococcaceae</i>	Other (2.3%)
				<i>Butyricicoccus</i> (0.1%)

				<i>Clostridium IV</i> (0.1%)
				<i>Faecalibacterium</i> (0.9%)
				<i>Gemmiger</i> (0.1%)
				<i>Oscillibacter</i> (0.1%)
				<i>Ruminococcus</i> (0.7%)
	Erysipelotrichia	Erysipelotrichales	<i>Erysipelotrichaceae</i>	Other (0.1%)
				<i>Allobaculum</i> (0.3%)
Negativicutes	Selenomonadales	<i>Acidaminococcaceae</i>	<i>Phascolarctobacterium</i> (0.1%)	
		<i>Veillonellaceae</i>	<i>Dialister</i> (0.4%)	
Parcubacteria (0.1%)	Other	Other	Other	Other (0.1%)
Proteobacteria (14.2%)	Alphaproteobacteria	Rhizobiales	Other	Other (0.1%)
			<i>Bradyrhizobiaceae</i>	<i>Bosea</i> (0.2%)
			<i>Brucellaceae</i>	<i>Brucella</i> (0.4%)
			<i>Methylobacteriaceae</i>	<i>Methylobacterium</i> (0.1%)
			<i>Rhizobiaceae</i>	Other (0.5%)
		<i>Rhizobium</i> (0.3%)		
		Rhodobacterales	<i>Rhodobacteraceae</i>	Other (0.2%)
				<i>Amaricoccus</i> (0.1%)
			<i>Planktomarina</i> (0.1%)	

		Rhodospirillales	<i>Rhodospirillaceae</i>	Other (0.3%)
				<i>Defluviicoccus</i> (0.1%)
		SAR11	<i>SAR11</i>	<i>Candidatus Pelagibacter</i> (0.5%)
		Sphingomonadales	<i>Sphingomonadaceae</i>	<i>Novosphingobium</i> (0.1%)
				<i>Sphingomonas</i> (0.3%)
	Betaproteobacteria	Other	Other	Other (0.1%)
		Burkholderiales	<i>Comamonadaceae</i>	<i>Curvibacter</i> (0.1%)
				<i>Delftia</i> (0.5%)
				<i>Pelomonas</i> (0.2%)
				<i>Sutterellaceae</i>
				<i>Sutterella</i> (0.1%)
	Hydrogenophilales	<i>Hydrogenophilaceae</i>	<i>Tepidiphilus</i> (0.1%)	
	Deltaproteobacteria	Desulfovibrionales	<i>Desulfovibrionaceae</i>	<i>Bilophilia</i> (0.1%)
	Epsilonproteobacteria	Campylobacterales	<i>Campylobacteraceae</i>	<i>Arcobacter</i>
	Gammaproteobacteria	Other	Other	Other (0.4%)
		Alteromonadales	<i>Shewanellaceae</i>	<i>Shewanella</i> (0.1%)
Enterobacteriales		<i>Enterobacteriaceae</i>	<i>Citrobacter</i> (0.1%)	
			<i>Escherichia/Shigella</i> (0.1%)	

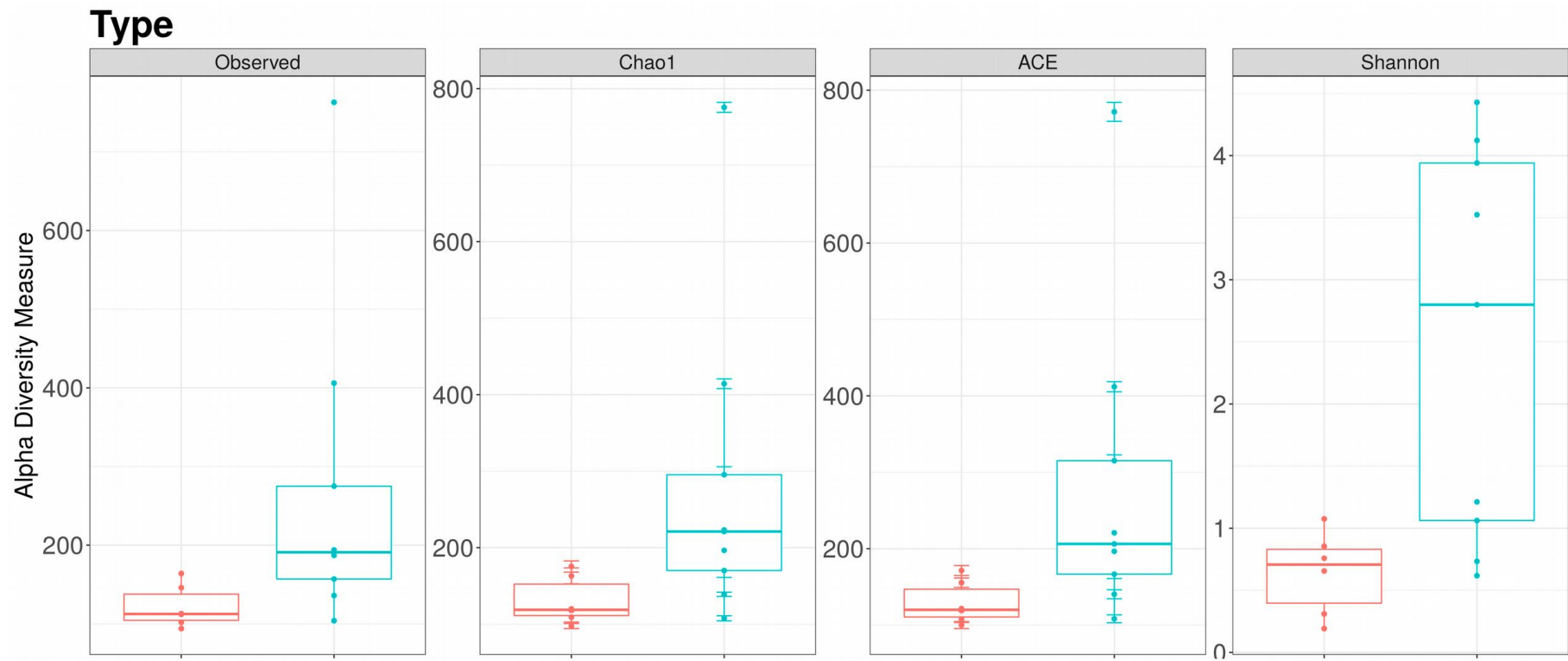
		Pseudomonadales	<i>Moraxellaceae</i>	<i>Acinetobacter</i> (0.1%)
				<i>Enhydrobacter</i> (0.1%)
			<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> (1.2%)
		Vibrionales	<i>Vibrionaceae</i>	<i>Aliivibrio</i> (2.8%)
				<i>Photobacterium</i> (2.9%)
				<i>Vibrio</i> (0.2%)
		Xanthomonadales	<i>Xanthomonadaceae</i>	Other (0.1%)
				<i>Pseudoxanthomonas</i> (0.1%)
				<i>Stenotrophomonas</i> (0.2%)
		Spirochaetes (0.4%)	Spirochaetia	Spirochaetales
Tenericutes (17.8%)	Mollicutes	Mycoplasmatales	<i>Mycoplasmataceae</i>	Other (17.2%)
Verrucomicrobia (2.3%)	Verrucomicrobiae	Verrucomicrobiales	<i>Verrucomicrobiaceae</i>	Other (2.0%)
Unassignable (31%)				

Overall within the phyla obtained in the DI and PI samples, 99 genera were identified. Interestingly, only 11 were shared between the different GI tract locations. The only genera exclusive to the DI samples were *Serratia* spp. or belonged to the family *Bacilliaceae*, while 86 genera were only detected in the PI samples. The most dominant genera present in the DI samples belonged to the families *Mycoplasmataceae* and *Bacilliaceae*, although *Brevinema* spp. were also prevalent. Similarly, genera belonging to the family *Mycoplasmataceae* were also the most dominant throughout the PI samples, followed by *Bacteroides* spp. and “other *Porphyromonadaceae*”. However 30% of all sequences present in the PI samples were accredited as “Unassignable; Other”.

When each sample was examined individually, there were 20 operational taxonomic units (OTUs) common to all DI and PI samples, representing a portion of the core intestinal microbiome in Atlantic salmon. These OTUs included seven *Proteobacteria*, four *Firmicutes*, four *Bacteroidetes*, one *Tenericutes* and one *Spirochaetes*. The most prevalent genera within the samples belonged to the family *Mycoplasmataceae*, with other genera such as *Pseudomonas* spp., *Photobacterium* spp., and *Aliivibrio* spp. also identified. When the DI and PI samples were examined as individual groups there were no additional OTUs exclusive to all DI samples. *Bacilliaceae* and *Serratia* spp. were two genera exclusive to several DI samples, occurring in 90% and 50% of samples, respectively. Out of the 86 exclusive genera in the PI samples, 25 OTUs were observed in all PI samples, including *Ruminococcus* spp. and *Flavobacterium* spp..

Figure 4.1 illustrates the alpha diversity comparisons between the DI and PI samples. Although it is apparent there is greater species richness within the PI samples (Chao 1 and ACE) this difference was not significant (Chao 1,  $P=0.0996$ . ACE,  $P=0.0973$ ). Diversity estimates (Shannon Index, Simpson Index) also suggested that there was a more diverse

bacterial population within the PI samples and this difference was significant (Shannon,  $P=0.0151$ . Simpson,  $P=0.0111$ ). Rarefaction curves suggest that all samples reached saturation and that there were higher levels of diversity in the PI samples (data not shown).



**Figure 4. 1** Alpha diversity comparisons (Chao 1, ACE, Shannon) of the distal intestine (DI) contents (red) and the proximal intestine (PI) contents (Blue).

#### 4.5. Discussion

In this study the distal (DI) and proximal (PI) intestinal microbiota from Atlantic salmon farmed off the west coast of Ireland was investigated using high throughput sequencing of the 16S rRNA gene. Our data suggested there was a greater bacterial diversity in the PI region. Varying bacterial diversity and prevalence along the piscine GI tract has been reported previously (Reveco et al., 2014). In contrast Navarrete et al. (2009) found that the same bacteria were evenly distributed throughout the GIT. Although, it is not certain as to why greater bacterial diversity may be associated with the proximal region, it has been suggested that the close proximity to the stomach may support a broader range of bacteria which in turn aid digestion (Li et al., 2014, Reveco et al., 2014, Navarrete et al., 2013).

Regardless, more diverse microbial populations are associated with increased competition for nutrients and adhesion sites which provides protection against pathogenic organisms (Ringø et al., 2010, Ringø et al., 2004, Dillon et al., 2005, Vasemägi et al., 2017). Interestingly, *Lactobacillus* spp. (3.2%), *Enterococcus* spp. (1.3%), *Lactococcus* spp. (0.3%) and *Carnobacterium* spp. (0.2%) were present in relatively high concentrations in 80% of PI samples in this study. These bacteria have been previously shown to have a protective effect against pathogenic genera such as *Aliivibrio* spp. and *Vibrio* spp. within the foregut of Atlantic salmon (Ringø, 2008, Ringø et al., 2007, Salinas et al., 2008).

Previous studies on the intestinal microbiota of Atlantic salmon have reported a dominance of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Navarrete et al., 2009, Navarrete et al., 2010, Green et al., 2013). In the current study these three phyla were all present in both the DI and PI samples, however the predominant phyla in both was *Tenericutes*, with *Mycoplasmataceae* the dominant family present. This was not unexpected as a number of previous studies have observed a similar pattern in a range of fish species (Lyons et al.,

2017, Dehler et al., 2017, Llewellyn et al., 2016), including Atlantic salmon (Green et al., 2013, Abid et al., 2013). Several genera belonging to the *Mycoplasmataceae* family, including *Mycoplasma mobile*, have been associated with necrosis in freshwater fish but not those reared in seawater (Adan-Kubo et al., 2012).

A common observation in all fish microbiota studies is that the majority of *Proteobacteria* detected belong to the class  $\gamma$ -*Proteobacteria*, including *Pseudomonas* spp., *Vibrio* spp., *Aliivibrio* spp., *Photobacterium* spp. and *Shewanella* spp., all of which are commonly found in the environment (Nayak, 2010, Reveco et al., 2014). In this study *Pseudomonas* spp., *Aliivibrio* spp. and *Photobacterium* spp. were present in both the DI and PI, whereas *Vibrio* spp. and *Shewanella* spp. were only present in the latter. All five genera possess strains pathogenic to fish, but can also be responsible for the *post-mortem* deterioration of fish quality through the production of volatile compounds which may also be hazardous if consumed by humans (Gram and Huss, 1996).

In this study sequences were clustered into operational taxonomic units (OTUs) according to their similarity with the threshold set at 97% similarity. All samples, regardless of location within the GIT, have 20 common OTUs. Similar results were observed by Dehler et al. (2017), who obtained 19 common OTUs in samples from the GIT of juvenile Atlantic salmon. There were three common OTUs between this study and the study carried out by Dehler et al. (2017). These OTUs represented “Other *Mycoplasmataceae*”, “Other *Ruminococcaceae*” and *Delftia* spp..

Overall, it was not surprising that many of the genera detected belong to the orders *Bacteriodales*, *Flavobacteriales* and *Sphingobacteriales*, which are widespread in the marine environment. However, other genera present include fish pathogens such as *Allivibrio* spp., *Delftia* spp., *Micrococcus* spp., *Photobacterium* spp., *Pseudomonas* spp.,

*Serratia* spp., *Stentrophomonas* spp, *Brucella* spp., *Planococcaceae* “*incertae sedis*”, *Rhodococcus* spp., *Shewanella* spp., *Staphylococcus* spp. and *Vibrio* spp. (Austin and Austin, 1999) that are of particular concern to the salmon industry.

It was concluded that the PI region had greater diversity of bacteria than the distal area. Although phyla diversity may have a protective effect inhibiting pathogens, several genera were detected which contain species that are pathogenic to Atlantic salmon. This study contributes to previous research on the microbiota of fish and provides further insight into the type of bacteria present in the GIT. Further work is now required to identify salmon microbiota at the species level.

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**Chapter 5 - Sensory and ATP derivative based  
indicators for assessing the freshness of Atlantic salmon  
(*Salmo salar*)**

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## 5.1. Summary

In order to estimate the shelf-life of fresh fish, the processor must know the period of time between catch/harvest and arrival at the processing plant. This information is not always available, necessitating the provision of methods to estimate the age of the fish. The objectives of this study were therefore to develop sensory and ATP derivative based methods for rapidly assessing the freshness of fish. A quality index method (QIM) (raw fish) and quantitative descriptive analysis (QDA) (cooked fish) were developed and validated (against bacterial count (total viable count (TVC)) and time) for Atlantic salmon (*Salmo salar*). The production of inosine monophosphate (IMP), inosine (I) and hypoxanthine (Hx) and associated ratios (IMP/Hx, K1 value or H value) were also investigated for use as freshness markers. There was a linear relationship between QIM and TVC ( $R^2 = 0.93$ ), QIM and time ( $R^2 = 0.96$ ), QDA and TVC ( $R^2 = 0.93$ ) and QDA and time ( $R^2 = 0.94$ ), suggesting the QIM and QDA schemes developed could be used to monitor/assess freshness. The H value also increased linearly with TVC ( $R^2 = 0.88$ ) and time ( $R^2 = 0.93$ ). It was therefore concluded that both the QIM/QDA approach and monitoring ATP degradation, specifically expressed as the H value, could be used as rapid methods to assess the freshness of salmon arriving at the processing plant.

## 5.2. Introduction

Fresh seafood is perishable with a short shelf-life (Ghanbari et al., 2013) and approximately 10% is lost due to spoilage each year (Alfaro et al., 2013; Kulawik et al., 2013). Chemical and enzymic autolytic processes commence immediately following death, resulting in the loss of the 'fresh' flavours of fish. Unpleasant odours and tastes are then produced by the metabolic activities of spoilage bacteria (Mørkøre et al., 2010; Schirmer et al., 2009). Thus spoilage is a complex process involving enzymatic, chemical and microbiological changes, but the latter is the primary determinant of shelf-life (Anacleto et al., 2011).

The quality index method (QIM) is a scheme used to assess the freshness of fish based on scoring different sensory attributes (appearance, odour and texture) during storage (Bremner, 1985). Assuming the QI increases linearly with time, once the total score for fish at the end of their shelf-life is established, the score obtained prior to this can be used to estimate the remaining shelf-life (Martinsdóttir et al., 2001). A similar approach, quantitative descriptive analysis (QDA) is used to assess the sensory status of cooked fish (Sveinsdottir et al., 2002). Although QIM schemes have been developed for several fish species, (Luten et al., 2000), Irish fish processors are still seeking QIM and QDA schemes for salmon (personal communication, Keohanes, Co. Cork).

The autolytic processes that commence immediately *post-mortem* include the deamination of adenosine phosphate molecules (ATP, ADP and AMP) to inosine monophosphate (IMP) before being more slowly dephosphorylated to inosine (I) and eventually degraded to hypoxanthine (Hx) (Chen et al., 2010; Gram and Dalgaard, 2002). The concentration of I and Hx, expressed as the IMP/Hx ratio or the modified  $K_1$ -value  $((I + Hx)/(IMP + I + Hx)) \times 100$  or the H-value  $((Hx)/(IMP + I + Hx)) \times 100$  have been proposed as indicators

of freshness for fish but their formation varies greatly depending on the fish species and storage conditions. Several studies have demonstrated a linear relationship between Hx concentration and time in a range of fish species (Beauchat, 1973; Dingle and Hines, 1971; Jahns and Rand, 1977; Jones et al., 1964; Kassemarn et al., 1963). However, a number of alternative studies have concluded that IMP (Dingle and Hines, 1971; Ehira and Uchiyama, 1974) or I (Bremner et al., 1988; Murata and Sakaguchi, 1988) are better biochemical markers for evaluating freshness, although, the concentrations obtained are not always linear over time (Beauchat, 1973; Bremner et al., 1988; Jahns and Rand, 1977; Murata and Sakaguchi, 1988). Research is therefore required to determine which, if any, of these values are suitable for use in assessing the freshness of Atlantic salmon (*Salmo salar*).

The objectives of this study were therefore to develop and validate rapid sensory (QIM and QDA) and ATP derivative based methods for assessing the freshness of Atlantic salmon arriving at the processing plant.

### **5.3. Materials and Methods**

#### **5.3.1. Fish Samples**

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

#### **5.3.2. Sensory Analysis**

A quality index method (QIM) was developed for scoring attributes of the whole fish and raw fillets for salmon (Table 5.1). The fish were graded by a trained panel of 12 people, all from Teagasc Food Research Centre (Ashtown, Dublin, Ireland). The panel underwent two 1.5 hour training sessions, where they became familiar with different terminology and descriptive language for the grading scheme. From these sessions, it became apparent what attributes needed to be included in the QIM grading scheme, which included skin colour, eyes, gills, texture, stiffness and mucus (whole fish); flesh colour, odour, texture, stiffness and mucus (raw fillet). The QIM grading was carried out on days 0, 2, 3, 6, 8 and 10. On each of these days the panel was presented with a whole fish and a raw fillet. There were descriptions for each attribute that related to a score of 0, 1, 2 or 3 (with the lower score indicating a fresher sample) and each panellist graded the fish by indicating which description they thought best described the physical attribute.

**Table 5. 1** Quality index method (QIM) scheme used to evaluate the sensory characteristics of Atlantic salmon (*Salmo salar*) stored at 2°C for 10 days.

<b>Parameter</b>	<b>Description</b>	<b>Score</b>
<b>QIM – whole salmon</b>		
<b>Skin colour</b>	Shiny, bright without blemishes, iridescent pigmentation, silver	0
	Rather dull	1
	Dull, slimy, gritty, grey, lack of pigmentation	2
<b>Mucus</b>	Uniform, thin, transparent	0
	Little thicker, opaque	1
	Clotted, thick, yellowish	2
<b>Slime</b>	Transparent, white	0
	Off-white	1
	Yellowish, grey-brown	2
<b>Eye</b>	Bright, full, clear	0
	Cloudy, dull, sunken	1
<b>Stiffness</b>	Firm	0
	Not quite firm	1
	Soft	2

<b>Texture</b>	Quick rebound from finger pressure	0
	Slow response to finger pressure	1
	Persistence of finger imprint	2
<b>Back</b>	Firm, full, unblemished	0
	Slightly soft	1
	Soft, mushy, blemishing, sunken	2
<b>Belly</b>	Firm, full, unblemished, intact	0
	Slightly soft	1
	Faded, sunken, bruised, battered, grazed	2
<b>Blood</b>	Bright red, not present	0
	Dull red	1
	Shadowy, brown	2
<b>Gills</b>	Bright Red, Full	0
	Brown, shrivelled	1
<b>QIM – salmon fillets</b>		
<b>Colour</b>	Orange, Bright	0
	Some white, Pale	1
	Overall Pale	2
<b>Texture</b>	Firm	0
	Rather soft	1

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	Very soft	2
<b>Brightness</b>	Transparent, bluish	0
	Opaque	1
	Milky	2
<b>Odour</b>	Fresh, neutral	0
	Seaweed, marine, grass	1
	Sour milk	2
	Acetic, ammonia, offensive, unpleasant	3
<b>Stiffness</b>	Rigour	0
	Post rigour	1
<b>Mucus</b>	Absent	0
	Some evidence	1
	Excessive	2
<b>Gaping</b>	No gaping, one longitudinal gaping at the neck part of the fillet	0
	Slight gaping less than 25% of the fillet	1
	Slight gaping, 25-75% of the fillet	2
	Deep gaping or slight gaping over 75% of the fillet	3

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Quantitative descriptive analysis (QDA) was carried out alongside the QIM to score the characteristics of the cooked product. Similar to the QIM grading scheme, panellists were asked to grade the sensory attributes (colour, odour, taste, mouth feel, juiciness) of the cooked fish on a scale ranging from 0 to 2, where a lower score indicated a fresher sample (Table 5.2). Samples were steam cooked at 99°C for 7 minutes (Sveinsdottir et al., 2002) (Rational SCC WE 61E Electric Combi Oven, Rational, Landsberg am Lech, Germany), after which the panellists assessed the cooked fillet. Each panellist was given a small piece of cooked fish and asked to grade it. As with the QIM, a scoring sheet was provided with descriptions relating to a score of 0, 1 or 2 for each physical attribute. All panellists graded the fish by indicating which description they thought best described the physical attribute. Each QIM score was scored out of 33 and each QDA was scored out of 12. These scores were then converted to a percentage, which in turn was used as the freshness score.

**Table 5. 2** Quantitative descriptive analysis (QDA) scheme used to evaluate the sensory characteristics of cooked Atlantic salmon (*Salmo salar*).

<b>Parameter</b>	<b>Description</b>	<b>Score</b>
<b>QDA-cooked salmon</b>		
<b>Colour</b>	Orange, Bright	0
	Orange, some off-white	1
	Pale, Dull	2
<b>Odour</b>	Fresh, seaweed odour	0
	Stronger, fishy	1
	Strong fishy, offensive, unpleasant	2
<b>Taste</b>	Mild, fishy, pleasant	0
	Moderately fishy	1
	Strong fishy, offensive, unpleasant	2
<b>Mouth-feel</b>	Dissolve, melt in the mouth, crumbly, soft	0
	Slightly chewy	1
	Chewy, rubbery, tough	2
<b>Moist</b>	Moist, pleasant	0
	Mildly moist	1
	Dry, very dry	2
<b>Sticky/gluey</b>	Does not stick to or coat the palate or the teeth	0
	Some-what sticky	1
	Coats the palate and teeth	2

### 5.3.3. ATP Derivative Analysis

ATP degradation was measured on days 0, 2, 3, 6, 8 and 10. On each of these days two flesh samples (5g) were aseptically removed from the whole fish. The degradation of ATP was measured using a microplate PRECICE® K-Freshness Assay Kit following the manufacturer's methodology (Novocib, Lyon, France). This kit measures the progressive conversion of IMP, I and Hx to NADH<sub>2</sub>, using specific dehydrogenase enzymes, provided in the kit. The NADH<sub>2</sub> is then quantified by measuring specific absorbance at 340nm (Multiskan Go, Thermo Fisher Scientific, Ireland) in accordance with the manufacturer's instructions. The concentration of I and Hx, could also be expressed in the following ways; the IMP/Hx ratio, the modified K<sub>1</sub>-value  $((I + Hx)/(IMP + I + Hx)) \times 100$  or the H-value  $((Hx)/(IMP + I + H)) \times 100$ .

### 5.3.4. 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 obtained on each sampling days. From the other side the outer skin of the fish was swabbed (25cm<sup>2</sup> 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 swab 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<sup>-5</sup>. Plate count agar (PCA) (Oxoid, Basingstoke, United Kingdom (CM0325)), with and without 1% NaCl, was used to estimate the total mesophilic viable count (TVC<sub>m</sub>, 30°C for 72h).

#### 5.3.5. Temperature Analysis

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

#### 5.3.6. Data Analysis

All experiments were undertaken in duplicate and repeated on three separate occasions and a mean value was obtained for each data point on each day. The equation of best fit and the correlation coefficients (R) of QIM and QDA against TVC (flesh and skin swab), storage time in ice, IMP concentration (mg/g), Hx concentration (mg/g), I concentration (mg/g), the IMP/Hx ratio, the K1 value (%) and the H value (%Hx) were calculated using Microsoft® Excel 2010 (Microsoft Corporation, Redmond, Wash., U.S.A.).

## 5.4. Results

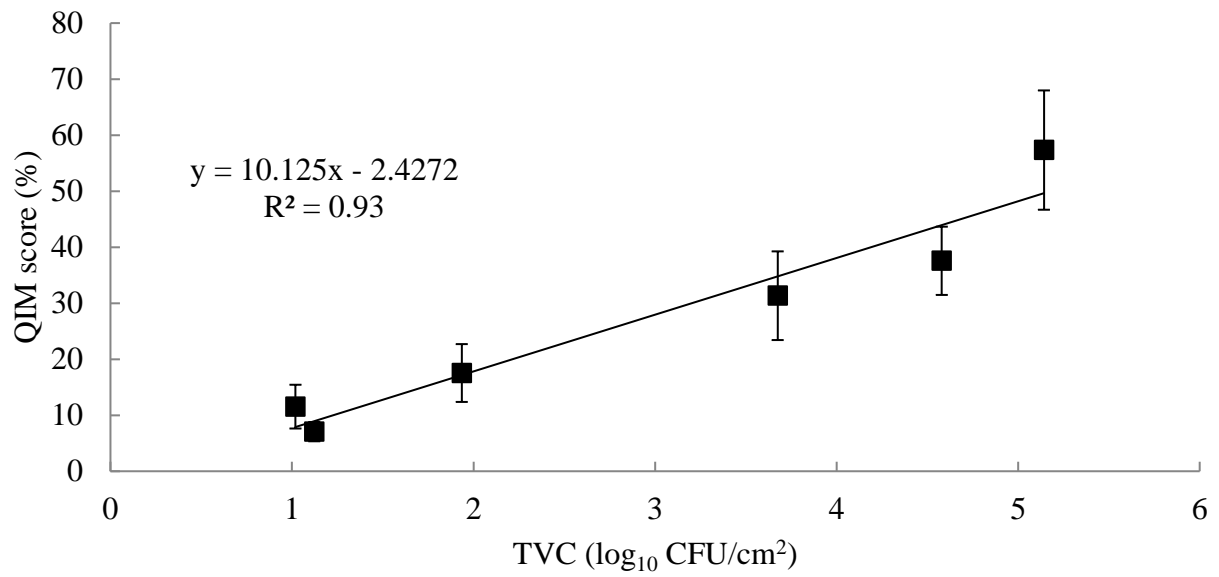
Over the 10 days storage on ice in a chilled room set at 2°C, the average ambient temperature recorded was 1.6°C. The average skin and core temperature for salmon ranged between 1.5 and 2°C. The salmon recorded a minimum temperature of 0°C for both skin and core readings. Throughout the 10 day storage period, TVC for salmon increased from 1.1 and 2.7 to 5.1 and 5.7 log<sub>10</sub> CFU/cm<sup>2</sup> on flesh and skin swab samples, respectively.

For salmon fillets, *post-rigour* 'stiffness' was the first attribute associated with a lack of freshness by the majority of panellists after 3 days and all by day 6. For the whole fish, 'cloudy, dull, sunken' eyes and 'brown, shrivelled' gills also provided early indicators as loss of freshness, with all panellists indicating the maximum score at day 8 and day 6, respectively. With the exception of odour (maximum QIM score after 10 days), none of the other attributes reached their maximum score over the course of the experiment.

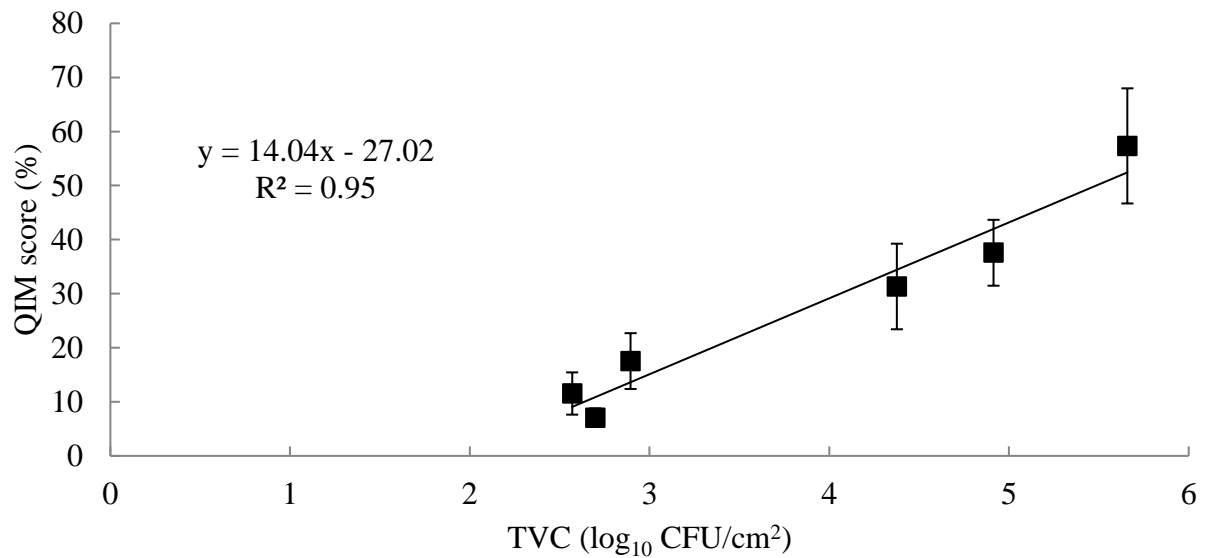
Interestingly, the QDA for cooked salmon suggested colour was the most important attribute in the early indication of spoilage with the majority of panellists giving it a top score after 8 days and all by 10 days. Most of the panellists did not consider the taste of the fish to be 'strong, fishy, offensive and unpleasant' or the fish to be 'dry or very dry' until day 10. The other attributes had not reached the maximum demerit score by the end of the experiment. Overall, the panel suggested that salmon was spoiled on day 10.

Regression analysis suggested a strong relationship between the QIM scores for salmon and TVC<sub>m</sub> concentrations in flesh ( $R^2 = 0.93$ ) and skin swabs ( $R^2 = 0.95$ ), with approximately 10 to 14 sensory units lost for each 1 log<sub>10</sub> CFU/cm<sup>2</sup> increase in bacterial counts (Figure 5.1).

(A)

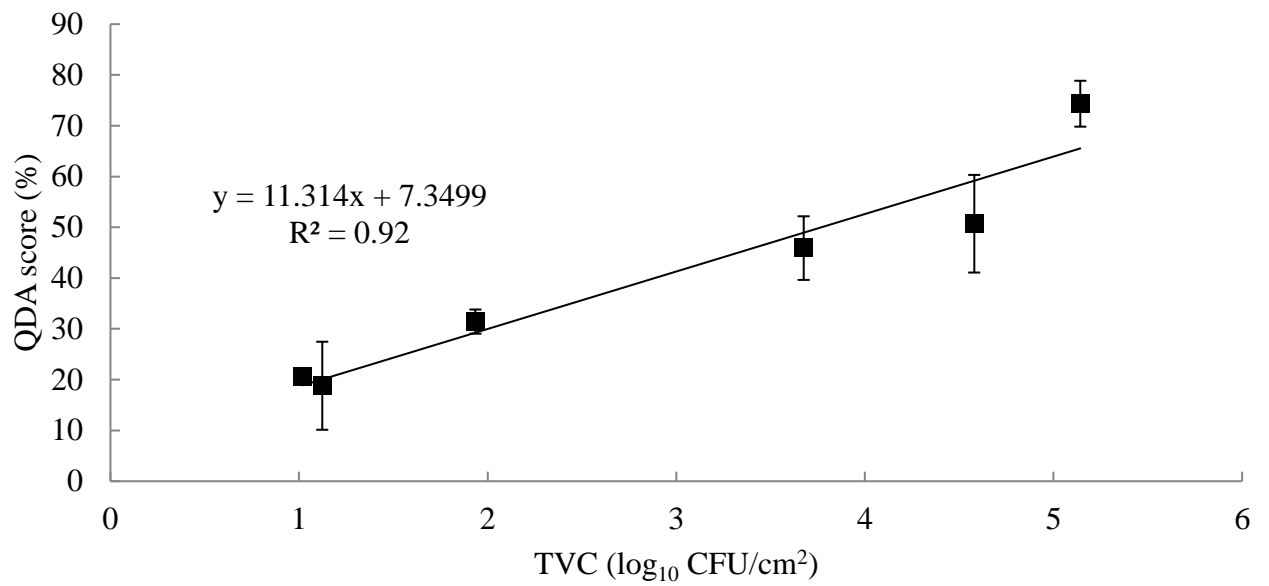


(B)

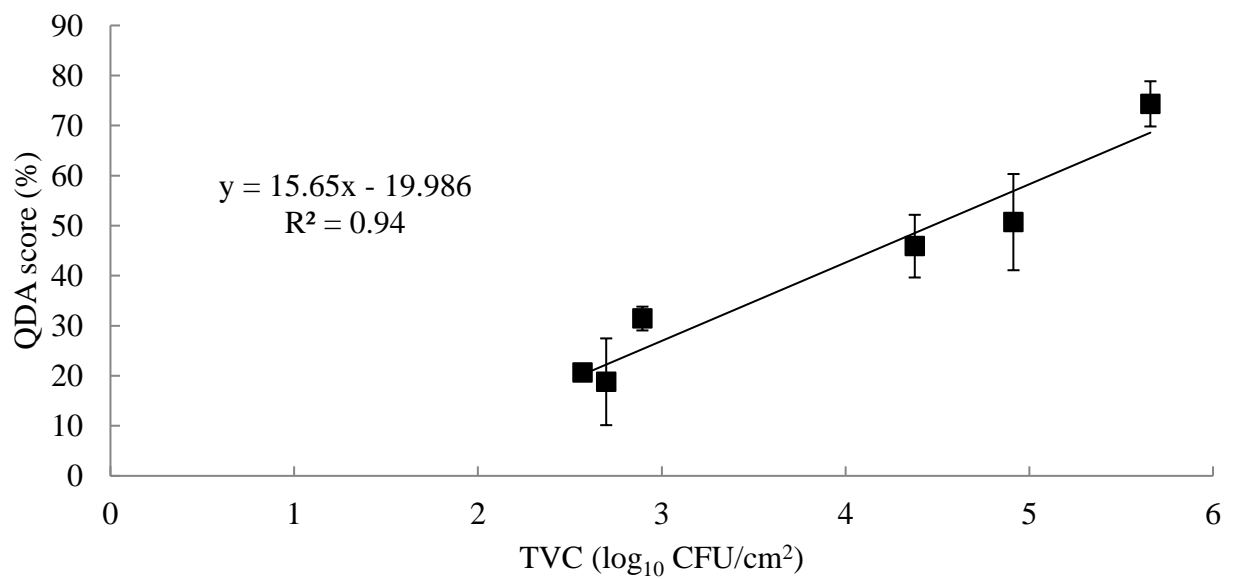


**Figure 5. 1** Relationship between the quality index method (QIM) score and the mesophilic total viable count (TVC<sub>m</sub>) for Atlantic salmon (*Salmo salar*) (A) flesh and (B) skin swab samples stored aerobically on ice in a chilled room at 2°C for 10 days. Each point corresponds to 30 data values.

(A)



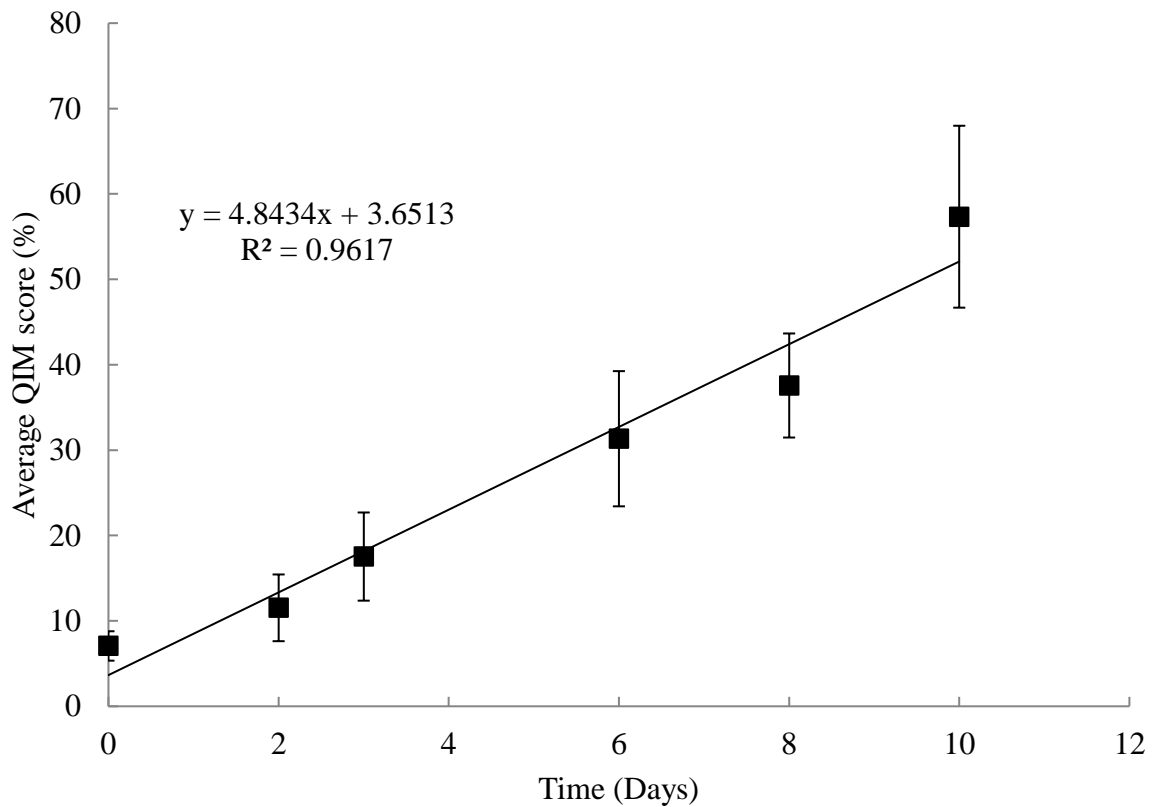
(B)



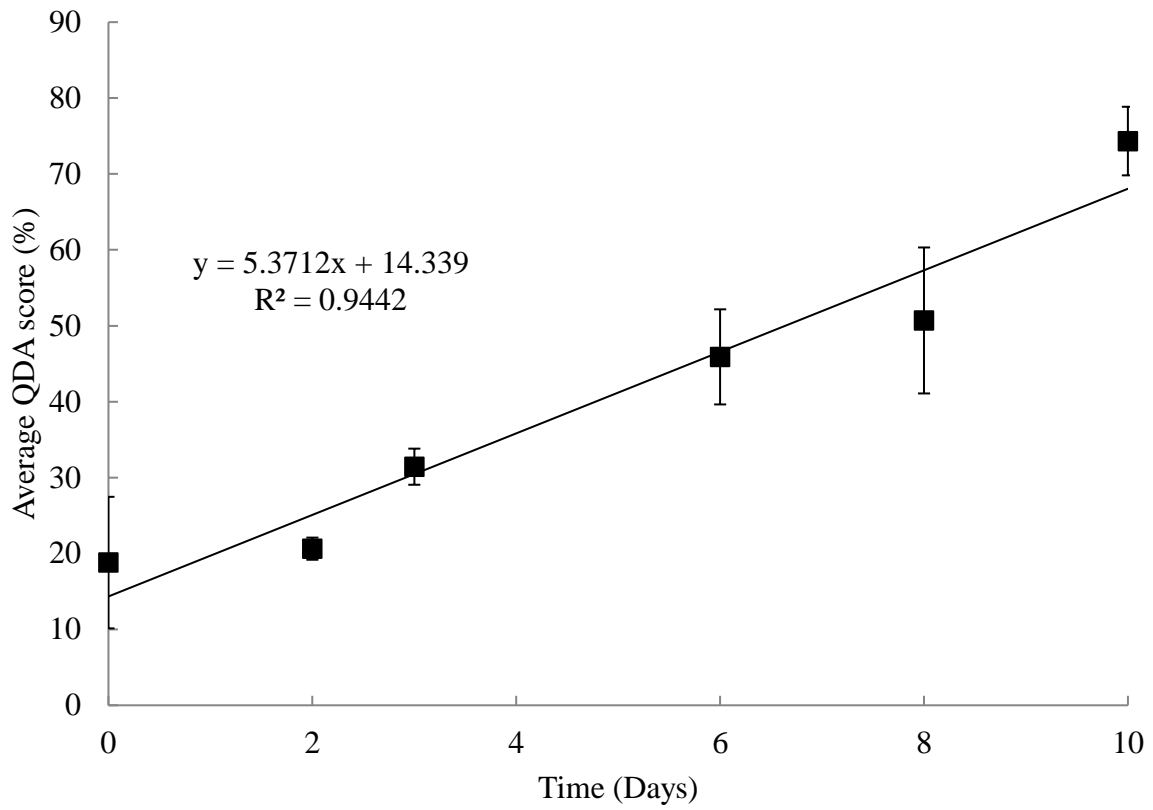
**Figure 5. 2** Relationship between the quantitative descriptive analysis (QDA) score and the mesophilic total viable count (TVC<sub>m</sub>) for Atlantic salmon (*Salmo salar*) (A) flesh and (B) skin swab stored aerobically on ice in a chilled room at 2°C for 10 days. Each point corresponds to 30 data values.

Similar results were obtained for QDA scores versus time with  $R^2$  values of 0.92 (salmon-flesh TVC) and 0.94 (salmon-skin swab TVC) (Figure 5.2). It was suggested that approximately 11 to 16 sensory units were lost for each  $1 \log_{10}$  CFU/cm<sup>2</sup> increase in TVC<sub>m</sub>.

Regression analysis also suggested a strong relationship between time and both the salmon QIM (Figure 5.3) and QDA (Figure 5.4) scores for salmon ( $R^2 = 0.96$  and 0.94, respectively).



**Figure 5. 3** Relationship between the quality index method (QIM) score and time (days) for Atlantic salmon (*Salmo salar*) stored aerobically on ice in a chilled room at 2°C for 10 days. Each point corresponds to 30 data values.



**Figure 5. 4** Relationship between the quantitative descriptive analysis (QDA) score and time (days) for Atlantic salmon (*Salmo salar*) stored aerobically on ice in a chilled room at 2°C for 10 days. Each point corresponds to 30 data values.

After death, adenosine phosphate was immediately degraded to inosine monophosphate (IMP) and then more slowly to inosine (I) and hypoxanthine (Hx). The IMP, I and Hx concentrations at each sampling time as well as the IMP/Hx ratio,  $K_1$  and H values, for salmon are shown in Tables 5.3.

**Table 5. 3** Summary of inosine monophosphate (IMP), inosine (I) and hypoxanthine (Hx) concentrations as well as the IMP/Hx ratio,  $K_1$  and H values for Atlantic salmon (*Salmo salar*) stored aerobically at 2°C for 10 days.

<b>Time (d)</b>	<b>IMP (mg/g)</b>	<b>I (mg/g)</b>	<b>Hx (mg/g)</b>	<b>IMP/Hx ratio</b>	<b><math>K_1</math> value (%)<sup>1</sup></b>	<b>H value (%Hx)</b>
<b>0</b>	2.8	3.6	1.0	2.8	82.2	13.5
<b>2</b>	1.9	3.9	1.2	1.6	72.9	17.1
<b>4</b>	0.6	3.8	1.2	0.5	89.3	21.4
<b>6</b>	0.7	3.2	1.3	0.5	86.5	25.0
<b>8</b>	0.6	3.2	1.3	0.5	88.2	25.5
<b>10</b>	0.3	3.5	1.5	0.2	94.3	28.3
<b>R<sup>2</sup> Value<sup>2</sup></b>	0.71	0.38	0.89	0.69	0.51	0.93

<sup>1</sup>  $K_1$  value =  $((I + Hx)/(IMP + I + Hx)) \times 100$ ; H value =  $((Hx)/(IMP + I + Hx)) \times 100$

<sup>2</sup> R<sup>2</sup> Value represents correlation between time and the ratio for each corresponding column

On day 0 the concentration of IMP in salmon samples was 2.8 mg/g which decreased to 0.6 mg/g after 4 days before decreasing to 0.3 mg/g after 10 days. The initial Hx concentration was 1.0 mg/g which showed a slight increase to 1.5 mg/g after 10 days. The concentration of I remained relatively constant (3.2 to 3.9 mg/g) throughout the experiment. With the exception of H-values ( $R^2 = 0.93$ ) the relationship between time and IMP, I or Hx concentration or between time and IMP/Hx ratio and K1-value were non-linear.

## 5.5. Discussion

The initial TVC ranged from 1.1 to 2.7 log<sub>10</sub> CFU/cm<sup>2</sup> suggesting the fish were fresh and from clean waters (Gram, 1992; Gram and Huss, 1996) and of good microbiological quality (Briones et al., 2010; Li et al., 2017; Schubring, 2003). Moreover, as the TVC never exceeded 7 log<sub>10</sub> CFU/cm<sup>2</sup>, the fish was safe to eat throughout the course of the study (Ellis et al., 2002). By the end of the sensory shelf-life (10 days) experiment, the TVC<sub>m</sub> ranged from 5.1 to 5.7 log<sub>10</sub> CFU/cm<sup>2</sup>. This is in agreement with Robson et al. (2007), who found seafood spoiled when the bacterial count reached 5 to 6 log<sub>10</sub> CFU/cm<sup>2</sup>.

The QIM developed for Atlantic salmon provided a good description of the sensory changes that occurred during aerobic chilled storage and the linear relationship between QIM scores and both TVC and time suggested this scheme could be used to assess fish freshness. This was complemented by the QDA for cooked fish. Other studies have also reported a linear relationship between QIM score and time for salmon (Sveinsdottir et al., 2003; Sveinsdottir et al., 2002), blackspot seabream (Sant'Ana et al., 2011) and rainbow trout (Diler and Genç, 2018).

In this study the IMP concentration decreased in the salmon (2.8 to 0.3 mg/g) over the 10 days aerobic storage at 2°C, however inosine levels did not change and only a minor increase was observed in the Hx concentration (1.0 to 1.5mg/g). Similar data from other fish studies is limited and that which is available focuses on Hx. Burns et al. (1985) found initial Hx concentrations of 0.12 mg/g and 0.15 mg/g in mackerel and cod, respectively. Whittle et al. (1990) reported that Hx levels increased from 2.4 mg/100g to 8.8 mg/100g in cod stored on ice for 10 days. Other studies observed increases from 3.3 mg/100g to 16.6 mg/100g in striped bass fillets stored at 4°C for a similar time period (Karahadian et al.,

1997) and from 0.09 mg/g to 0.41 mg/g in salmon stored at 1°C for 20 days (Sallam, 2007).

However, rather than simply monitoring the concentration of ATP derivatives, Karahadian et al. (1997) proposed that the loss of freshness should be expressed as a IMP/Hx ratio,  $K_1$  value  $\left(\frac{(I + Hx)}{(IMP + I + Hx)} \times 100\right)$  or H value  $\left(\frac{(Hx)}{(IMP + I + Hx)} \times 100\right)$ , arguing that these ratios are a better indicator of freshness as they take into account the concentrations of all the ATP derivatives. In our study the IMP/Hx ratio,  $K_1$  and H values for salmon changes from initial values of 2.8, 82.2% and 13.5% to 0.2, 94.3% and 28.3%, respectively. Although there is a dearth of similar data in the scientific literature, Karahadian et al. (1997) reported a  $K_1$  value of 37.8 at time  $t = 0$  for striped bass which did not exceed 90% until day 9.

Although the pattern of increase of H-value occurred linearly for both TVC and time, the relationships between IMP/HX ratio and  $K_1$  values were non-linear. Other studies on the best ATP derivative/ratio for monitoring fish freshness are contradictory (Bremner, 1985; Murata and Sakaguchi, 1988; Sallam, 2007; Whittle et al., 1990). This was not unexpected as nucleotide degradation rates depend on a range of factors including fish maturity, muscle type, stress during capture and storage conditions (Erikson et al., 1997; Huss, 1995; Luong et al., 1992).

It was concluded that the QIM and QDA schemes developed in this study may be used as a rapid sensory based tool for assessing the freshness of salmon. Moreover, 'cloudy, dull and sunken' eyes and 'brown shrivelled' gills providing early indicators of loss of freshness of whole fish. The H-value may be a suitable ATP derivative ratio for assessing Atlantic salmon freshness but, given the conflicts reported in the literature, further studies should be undertaken to confirm this finding.

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**Chapter 6 - Investigating the antimicrobial effect of a  
range of compounds on the bacteriology of salmon  
(*Salmo salar*) during chilled storage**

## 6.1. Summary

The immediate and storage effects of immersion treatments (30 seconds at 20°C) with 5% (w/v) citric acid, 5% (v/v) lactic acid and 12% (w/v) trisodium phosphate (experiment 1) and 1% (v/v) citral, 1% (v/v) carvacrol, 1% (w/v) thymol and 1% (v/v) eugenol (experiment 2) on total viable count (mesophilic and psychrophilic TVC) , total *Enterobacteriaceae* count (TEC), hydrogen disulphide producing bacteria (HSPB), *Pseudomonas* spp., lactic acid bacteria (LAB), *Brochothrix thermosphacta* and *Photobacterium* spp. on Atlantic salmon (*Salmo salar*) fillets (stored aerobically at 2°C) was investigated. Untreated fillets and samples dipped in sterile distilled water (SDW) were used as controls. Initial reductions ranged from 0.2 to 1.4 log<sub>10</sub> CFU/cm<sup>2</sup> as compared to the untreated control. However, after 18 days storage, statistically similar (P >0.05) bacterial counts were obtained regardless of the treatment. It was concluded that these organic compounds were not effective antibacterial treatments for aerobically stored salmon fillets when used at the above concentrations.

## 6.2. Introduction

According to Bord Iascaigh Mhara (BIM), seafood is worth €1.2bn annually to the Irish economy and Atlantic salmon (*Salmo salar*) is the most valuable product worth €121 million per annum (BIM, 2018). However salmon is highly perishable with a relatively short shelf-life of 10-12 days when stored under aerobic conditions. This, coupled with growing export market necessitating longer transport chains, has created an interest in preservation methods that extend shelf-life while maintaining microbial safety (Alfaro et al., 2013). At present, maintaining the quality of fresh fish is primarily reliant on storage at chilled temperatures. The European Commission (Regulation EC No. 853/2004) (EC, 2004) does not specify a temperature for the storage and transport of fish and only states that the temperature must be of that approaching melting ice (usually interpreted as 0-2°C). However, psychrophilic bacteria can grow at these temperatures and any further reduction in bacterial growth may necessitate the combination of low temperature with other preservation methods such as chemical preservatives, packaging or the use of natural antimicrobial compounds derived from plants (Burt, 2004; García-Soto et al., 2014; Schirmer et al., 2009).

Chemical preservatives such as phosphates are commonly used in the meat industry (Ritz et al., 2012) to extend shelf-life and in the fish industry to improve functionality (Masniyom et al., 2005). A study by Kilinc et al. (Kilinc et al., 2009) found that treating frozen-thawed sea bass (*Dicentrarchus labrax*) and saithe (*Pollachius virens*) with 5% sodium monophosphate, sodium diphosphate and sodium triphosphate decreased bacterial loads and reduced spoilage rates. However, there are concerns about the impact of phosphates in food on human health and the environment (Ritz et al., 2012) and as a result there has been a growing interest in the use of natural antimicrobials derived from plants (Oliveira et al., 2015; Tajkarimi et al., 2010; Tarvainen et al., 2015). Previous studies on essential oils

have shown that components such as citral, carvacrol, thymol and eugenol possess antimicrobial properties capable of causing structural breakdown of the bacterial cell membrane (Holley and Patel, 2005; Tajkarimi et al., 2010). Other natural antimicrobials include organic acids, such as citric and lactic acid, which are readily available at low cost (García-Soto et al., 2014). In the undissociated form the acid molecule can pass through the bacterial cell membrane where it then dissociates and acidifies the cytoplasm thereby inhibiting cellular functions (Brul and Coote, 1999; Schirmer et al., 2009). Both essential oils and organic acids have been previously tested on fish (Metin et al., 2001; Schirmer et al., 2009) however information on their application, including immediate and storage effects on indicator and spoilage bacteria, is limited. The objectives of this study were therefore to investigate the immediate and storage effects of dip treatment with; [1] 5% (v/v) citric acid (CA), 5% (v/v) lactic acid and 12% (w/v) trisodium phosphate (TSP) (experiment 1) and [2] 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) and 1% (v/v) eugenol (EUG) (experiment 2), on total viable mesophilic and psychrotrophic counts (TVC<sub>m</sub> & TVC<sub>p</sub>), total *Enterobacteriaceae* counts (TEC), hydrogen disulphide producing bacteria (HSPB), *Pseudomonas* spp., lactic acid bacteria (LAB), *Brochothrix thermosphacta* and *Photobacterium* spp. on salmon fillets during chilled (2°C) storage.

## 6.3. Materials and Methods

### 6.3.1. 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 of purchase.

### 6.3.2. Fish Fillet Preparation and Treatment

The salmon were divided into mini-fillets (n = 200 and 168 for experiment 1 and 2, respectively) of approximately 10g each. For experiment 1 the mini fillets were divided into groups (n = 40) before being treated using a 2 litre immersion (20°C) for 30 seconds with one of the following solutions; sterile distilled water (SDW), 5% (v/v) CA (Sigma Aldrich, Steinheim, Germany), 5% (v/v) LA (Sigma Aldrich, Steinheim, Germany), 12% (w/v) TSP (Sigma Aldrich, Steinheim, Germany). In experiment 2 the samples groups (n = 28) were treated with one of the following solutions; SDW, 1% (v/v) citral (Sigma Aldrich, Steinheim, Germany), 1% (v/v) carvacrol (Sigma Aldrich, Steinheim, Germany), 1% (w/v) thymol (Sigma Aldrich, Steinheim, Germany) and 1% (v/v) eugenol (Sigma Aldrich, Steinheim, Germany). The concentrations of all treatments were selected based on preliminary sensory analysis that found concentrations in excess of these values affected the organoleptic (appearance, odour and/or taste) properties of the fish and previous studies that found essential oils could be applied at concentrations of up to 1% (v/v) without adversely affecting the sensory properties (Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007; Govaris, Solomakos, Pexara, & Chatzopoulou, 2010; Karabagias, Badeka, & Kontominas, 2011; Petrou, Tsiraki, Giatrakou, & Savvaidis, 2012; Sánchez-

Escalante, Torrescano, Djenane, Beltrán, & Roncalés, 2003; Skandamis & Nychas, 2001). After each immersion the treated samples were left to drain for 15 seconds before being immersed in 2 litres of SDW for 30 seconds. This second immersion allowed for any excess treatment residue to be washed away. After each wash, the samples were once again left to drip for 15 seconds, before being stored aerobically at 2°C for 18 days. Untreated samples and dipping in sterile distilled water (SDW) were used as controls n = 40 and 28 for experiment 1 and 2, respectively).

### 6.3.3. Microbiological Analysis

For analysis, treatment groups were divided (n = 20 and 14 for experiment 1 and 2, respectively) for microbial analysis and for pH/a<sub>w</sub> measurements. In experiment 1, microbiological analysis was performed at time (t) = 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 days following treatment. For experiment 2, testing was performed at t = 0, 3, 6, 9, 12, 15 and 18 days. At each sampling time, 2 mini-fillets from each treatment group were randomly selected, placed in a stomacher bag to which 90ml maximum recovery diluent (MRD, Oxoid, Basingstoke, United Kingdom (CM0733)) was added and the solution homogenized (Pulsifier ® PUL100E, Microgen Bioproducts Ltd, Surrey, United Kingdom) for 1 minute. Thereafter a 10-fold serial dilution was prepared using maximum recovery diluent (MRD, Oxoid, Basingstoke, United Kingdom (CM0733)). Plate count agar (PCA) (Oxoid, Basingstoke, United Kingdom (CM0325)) was used to estimate total viable counts (TVC) for both mesophilic (TVC<sub>m</sub>, incubated 30°C for 72h) and psychrotrophic (TVC<sub>p</sub>, incubated 6.5°C for 240h) bacteria using a standard spread plate technique. Standard pour plate techniques were used to enumerate total enterobacteriaceae counts (TEC) using violet red bile glucose agar (VRBGA) (Oxoid, Basingstoke, United

Kingdom (CM0485)) incubated at 37°C for 24h, hydrogen sulphide producing bacteria (HSPB) on Iron Lyngby agar incubated at 25°C for 72h, 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 obtained on Pseudomonas Agar Base (Oxoid, Basingstoke, United Kingdom (CM0559)), supplemented with Ceftrimide-Fucidin-Cephaloridine (CFC) supplements (Oxoid, Basingstoke, United Kingdom (SR0103)) incubated at 30°C for 48h. *B. thermosphacta* were enumerated using streptomycin-thallos 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. tested using Long & Hammer Agar incubated at 15°C for 168h.

#### 6.3.4. Water Activity ( $a_w$ ) and pH

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) were randomly selected 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 each sample was 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 sample were also recorded, on each day, so as to determine an average total surface area for the samples. This allowed for  $\log_{10}$  values to be calculated in CFU/cm<sup>2</sup>.

#### 6.3.5. Data Analysis

Each experiment was performed in duplicate and repeated on 3 separate occasions. Bacterial concentrations were converted to  $\log_{10}$  CFU/cm<sup>2</sup> and the mean calculated. The difference between mean values was compared using a two way analysis of variance (ANOVA) with significance defined as  $P < 0.05$  with Tukey's multiple comparison test where applicable. Graph Pad Prism v7.0 software (Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

#### **6.4. Results**

The mean pH and  $a_w$  of the salmon fillets treated with 5% (v/v) CA, LA or 12% (w/v) TSP and stored at 2°C for 18 days (experiment 1) are shown in Table 6.1. The mean pH of the untreated control samples was 6.8, which decreased to between 6.0-6.4 when treated with the organic acids. TSP treated samples had an initial pH of 7.3. During storage the pH increased to between 7.8 and 8.3, regardless of treatment. The  $a_w$  values ranged from 0.95 to 0.98 regardless of treatment or sampling time. The corresponding pH and  $a_w$  values for samples treated with 1% (v/v) CIT, CAR, THY or EUG are provided in Table 6.2. The pH of treated samples ranged from 6-6.4) which were similar to untreated controls (6.8), and increased to 7.5-7.8 during storage. The  $a_w$  values (0.98 to 0.99) were also unaffected by treatment with essential oils.

**Table 6. 1** Mean pH and  $a_w$  measurements for salmon fillets treated with sterile distilled water (SDW), 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA) or 12% (w/v) trisodium phosphate (TSP) and stored at 2°C for 18 days.

Time (d)	Treatment				
	CTL <sup>1</sup>	SDW	CA	LA	TSP
	<b>pH</b>				
<b>0</b>	6.8 ± 0.4 <sup>A</sup>	6.9 ± 0.5 <sup>A</sup>	6.0 ± 0.2 <sup>A</sup>	6.4 ± 0.1 <sup>A</sup>	7.3 ± 0.5 <sup>B</sup>
<b>2</b>	6.8 ± 0.6 <sup>A</sup>	6.4 ± 0.6 <sup>A</sup>	6.3 ± 0.5 <sup>A</sup>	6.5 ± 0.1 <sup>A</sup>	6.7 ± 0.2 <sup>A</sup>
<b>4</b>	6.9 ± 0.0 <sup>A</sup>	6.8 ± 0.1 <sup>A</sup>	6.7 ± 0.0 <sup>A</sup>	6.6 ± 0.0 <sup>A</sup>	6.8 ± 0.0 <sup>A</sup>
<b>6</b>	6.9 ± 0.1 <sup>A</sup>	6.8 ± 0.1 <sup>A</sup>	6.5 ± 0.1 <sup>A</sup>	6.5 ± 0.1 <sup>A</sup>	6.6 ± 0.0 <sup>A</sup>
<b>8</b>	6.6 ± 0.6 <sup>A</sup>	6.5 ± 0.6 <sup>A</sup>	6.4 ± 0.5 <sup>A</sup>	6.4 ± 0.4 <sup>A</sup>	6.5 ± 0.5 <sup>A</sup>
<b>10</b>	7.2 ± 0.1 <sup>A</sup>	7.1 ± 0.1 <sup>A</sup>	6.9 ± 0.1 <sup>A</sup>	6.8 ± 0.1 <sup>A</sup>	7.0 ± 0.1 <sup>A</sup>
<b>12</b>	7.6 ± 0.3 <sup>A</sup>	7.5 ± 0.3 <sup>A</sup>	7.5 ± 0.2 <sup>A</sup>	7.2 ± 0.2 <sup>A</sup>	7.3 ± 0.2 <sup>A</sup>
<b>14</b>	7.4 ± 0.3 <sup>A</sup>	7.4 ± 0.3 <sup>A</sup>	7.4 ± 0.3 <sup>A</sup>	7.3 ± 0.2 <sup>A</sup>	7.4 ± 0.1 <sup>A</sup>
<b>16</b>	7.5 ± 0.3 <sup>A</sup>	7.4 ± 0.2 <sup>A</sup>	7.6 ± 0.3 <sup>A</sup>	7.6 ± 0.2 <sup>A</sup>	7.5 ± 0.2 <sup>A</sup>
<b>18</b>	8.1 ± 0.2 <sup>A</sup>	8.3 ± 0.3 <sup>A</sup>	7.8 ± 0.1 <sup>A</sup>	7.9 ± 0.2 <sup>A</sup>	8.3 ± 0.6 <sup>A</sup>
	<b><math>a_w</math></b>				
<b>0</b>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>
<b>2</b>	0.97 ± 0.00 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>
<b>4</b>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>
<b>6</b>	0.96 ± 0.02 <sup>A</sup>	0.95 ± 0.02 <sup>A</sup>	0.96 ± 0.02 <sup>A</sup>	0.95 ± 0.02 <sup>A</sup>	0.95 ± 0.02 <sup>A</sup>
<b>8</b>	0.97 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>
<b>10</b>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>
<b>12</b>	0.98 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>
<b>14</b>	0.96 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>

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**Continuation of Table 6. 2 Mean pH and  $a_w$  measurements for salmon fillets treated with sterile distilled water (SDW), 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA) or 12% (w/v) trisodium phosphate (TSP) and stored at 2°C for 18 days.**

<b>16</b>	0.98 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>
<b>18</b>	0.97 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>

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<sup>A, B</sup> Different superscripts within each row denote statistical significance between treatments ( $P < 0.05$ ).

<sup>1</sup>CTL - Control

**Table 6. 3** pH and  $a_w$  measurements for salmon fillets treated with sterile distilled water (SDW), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) and stored at 2°C for 18 days.

Time (d)	Treatment					
	CTL <sup>1</sup>	SDW	CIT	CAR	THY	EUG
<b>pH</b>						
<b>0</b>	6.8 ± 0.0 <sup>A(2)</sup>	6.6 ± 0.1 <sup>A</sup>	6.4 ± 0.0 <sup>B</sup>	6.4 ± 0.1 <sup>AB</sup>	6.3 ± 0.1 <sup>B</sup>	6.3 ± 0.1 <sup>B</sup>
<b>3</b>	6.8 ± 0.0 <sup>A</sup>	6.8 ± 0.2 <sup>A</sup>	6.5 ± 0.2 <sup>AB</sup>	6.5 ± 0.1 <sup>AB</sup>	6.4 ± 0.1 <sup>AB</sup>	6.4 ± 0.1 <sup>B</sup>
<b>6</b>	6.9 ± 0.1 <sup>A</sup>	6.7 ± 0.1 <sup>A</sup>	6.7 ± 0.1 <sup>A</sup>	6.5 ± 0.1 <sup>A</sup>	6.6 ± 0.1 <sup>A</sup>	6.5 ± 0.0 <sup>A</sup>
<b>9</b>	7.1 ± 0.0 <sup>A</sup>	7.1 ± 0.1 <sup>A</sup>	7.1 ± 0.1 <sup>A</sup>	7.0 ± 0.1 <sup>A</sup>	6.9 ± 0.1 <sup>A</sup>	7.0 ± 0.1 <sup>A</sup>
<b>12</b>	7.3 ± 0.1 <sup>A</sup>	7.5 ± 0.1 <sup>A</sup>	7.5 ± 0.0 <sup>A</sup>	7.2 ± 0.1 <sup>A</sup>	7.2 ± 0.0 <sup>A</sup>	7.2 ± 0.0 <sup>A</sup>
<b>15</b>	7.6 ± 0.1 <sup>A</sup>	7.6 ± 0.1 <sup>A</sup>	7.7 ± 0.1 <sup>A</sup>	7.4 ± 0.1 <sup>A</sup>	7.5 ± 0.1 <sup>A</sup>	7.4 ± 0.1 <sup>A</sup>
<b>18</b>	7.5 ± 0.1 <sup>A</sup>	7.6 ± 0.1 <sup>A</sup>	7.8 ± 0.1 <sup>A</sup>	7.5 ± 0.1 <sup>A</sup>	7.7 ± 0.2 <sup>A</sup>	7.5 ± 0.2 <sup>A</sup>
<b><math>a_w</math></b>						
<b>0</b>	0.98 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>
<b>3</b>	0.98 ± 0.01 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>
<b>6</b>	0.98 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>
<b>9</b>	0.98 ± 0.01 <sup>A</sup>	0.99 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.97 ± 0.01 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>
<b>12</b>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>
<b>15</b>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.98 ± 0.00 <sup>A</sup>	0.99 ± 0.01 <sup>A</sup>
<b>18</b>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.99 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>	0.98 ± 0.01 <sup>A</sup>	0.99 ± 0.00 <sup>A</sup>

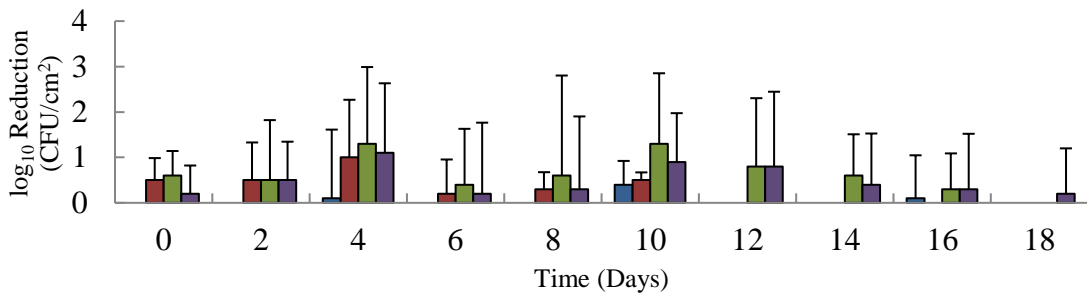
<sup>A, B</sup> Different superscripts within each row denote statistical significance between treatments ( $P < 0.05$ ).

<sup>1</sup>CTL – Control

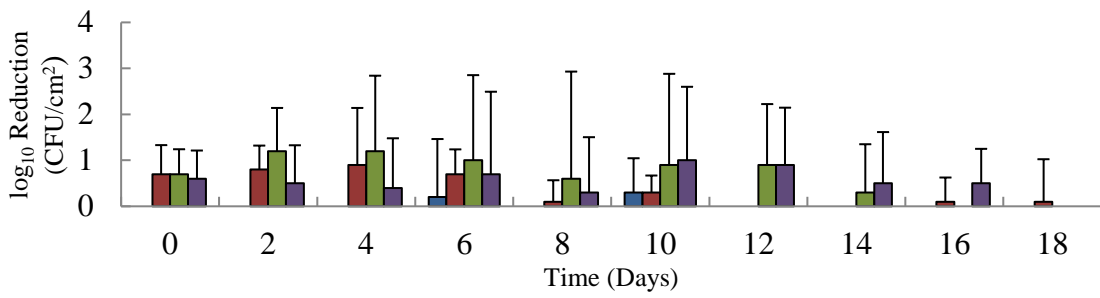
The mean bacterial (TVC<sub>m</sub>, TVC<sub>p</sub>, TEC, HSPB, LAB, *Pseudomonas* spp., *Br. Thermosphacta* and *Photobacterium* spp.) reductions on salmon fillets treated with 5% (w/v) CA, LA and 12% (w/v) trisodium phosphate (TSP) are shown in Figure 6.1 (Supplementary Table 1, Appendix A). TVC<sub>m</sub> counts were statistically similar ( $P > 0.05$ ) to the control at each sampling time, regardless of treatment, and there were no significant reductions. A similar pattern was observed for the other bacterial groups with the exception of the following combinations; HSPB with CA (t = 0, 2, 4, 6, 8 and 10) and LA (t = 0, 2, 4, 6, 8, 10, 12 and 14); LAB with CA (t = 0) and LA (t = 0 and 2), *Pseudomonas* spp. with CA (t = 0) and LA (t = 0, 2 and 4) and *Br. thermosphacta* with CA (t = 2) and LA (t = 2, 4 and 6), where significantly lower ( $P < 0.05$ ) counts were obtained (Supplementary Table 1, Appendix A).

The mean bacterial (TVC<sub>m</sub>, TVC<sub>p</sub>, TEC, HSPB, LAB, *Pseudomonas* spp., *Br. Thermosphacta* and *Photobacterium* spp.) reductions on salmon fillets treated with 1% (v/v) CIT, CAR, THY or EUG and stored at 2°C for 18 days are shown in Figure 6.2 (Supplementary Table 2, Appendix A).. The bacterial counts obtained were statistically similar ( $P > 0.05$ ) as compared to the control for each bacterial group with the following exceptions; TVC<sub>m</sub> with EUG (t = 0); TEC with CAR (t = 3); HSPB with CIT (t = 0), CAR (t = 0, 3, and 9) and EUG (t = 3 and 9); LAB with CIT (t = 0), CAR (t = 0 and 3); *Pseudomonas* spp. with CIT, CAR and EUG (t = 0); *Br. thermosphacta* with CIT (t = 0), CAR (t = 0 and 3) and EUG (t = 0) and *Photobacterium* spp. with CIT (t = 0) and CAR (t = 0).

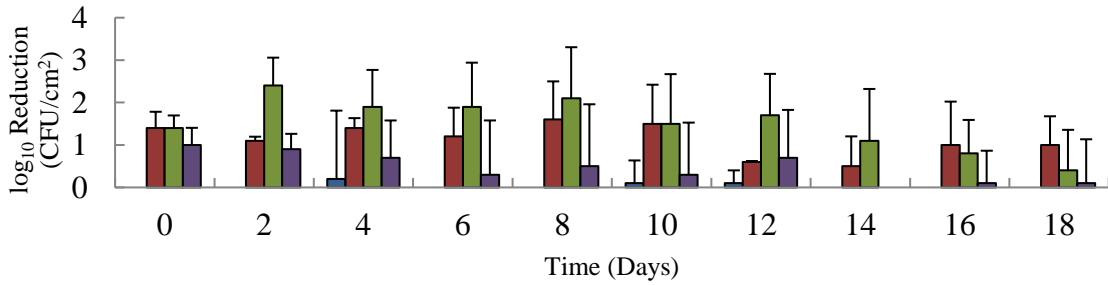
(a) TVC<sub>m</sub>



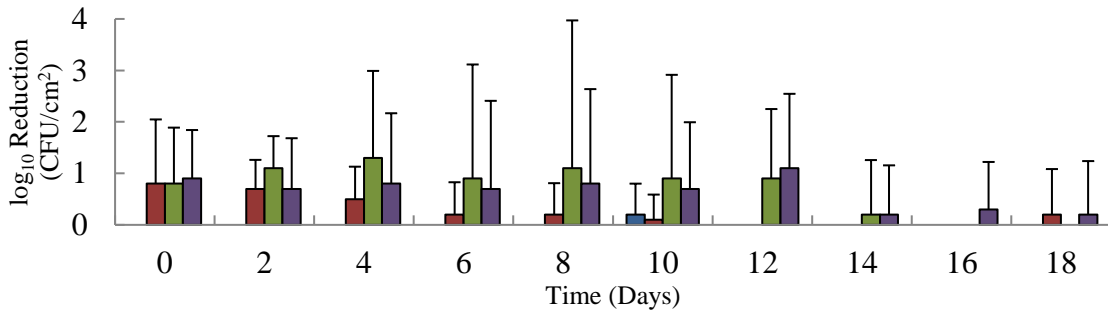
(b) TVC<sub>p</sub>



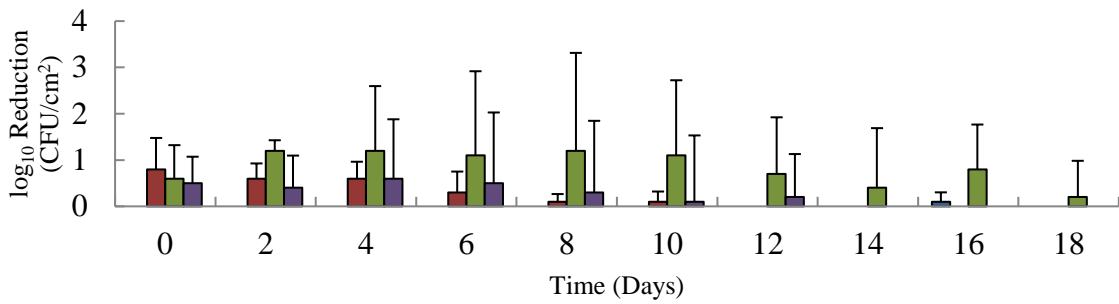
(c) Hydrogen sulphide producing bacteria (HSPB)



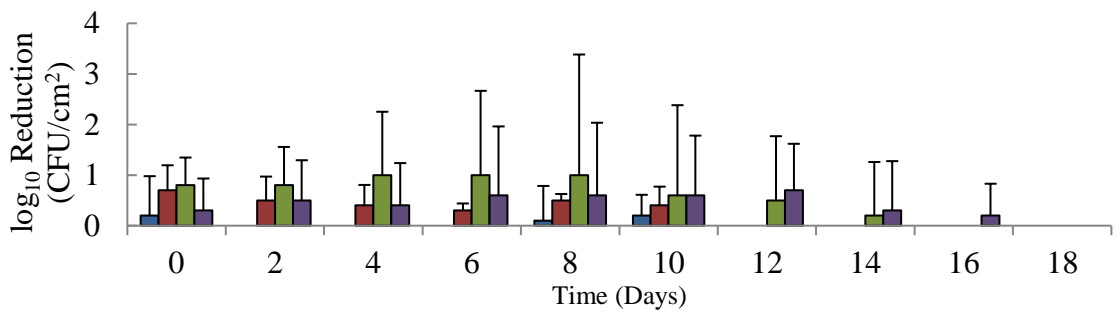
(d) *Pseudomonas* spp.



(e) *Brochothrix thermosphacta*



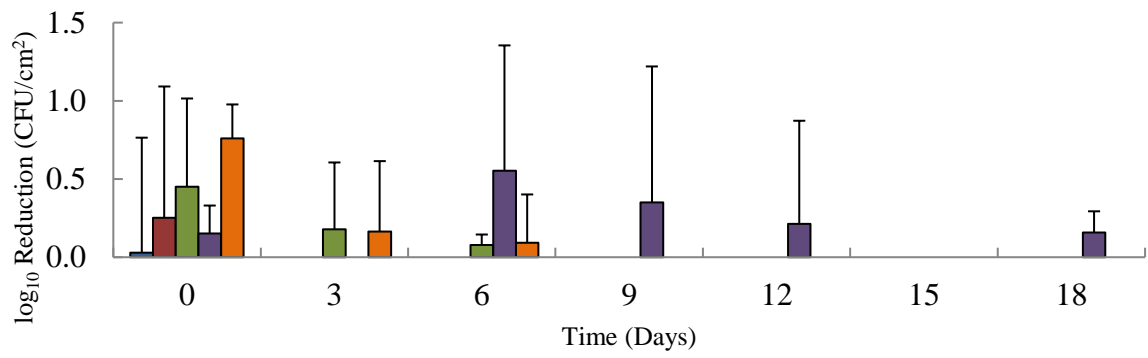
(f) *Photobacterium* spp.



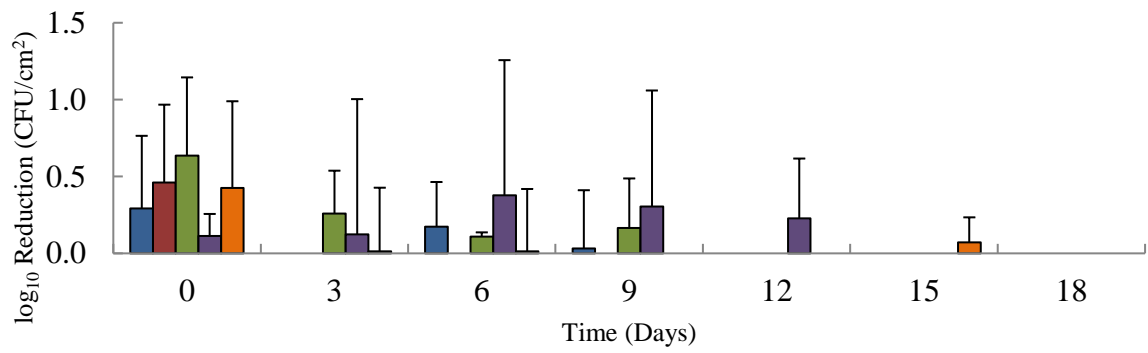
**Figure 6. 1** Mean bacterial log<sub>10</sub> reductions (CFU/cm<sup>2</sup>) on salmon mini-fillets treated with; sterile distilled water (SDW), 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA) or 12% (w/v) trisodium phosphate (TSP) and stored at 2°C for 18 days. ■ SDW, ■ CA, ■ LA,

■ TSP.

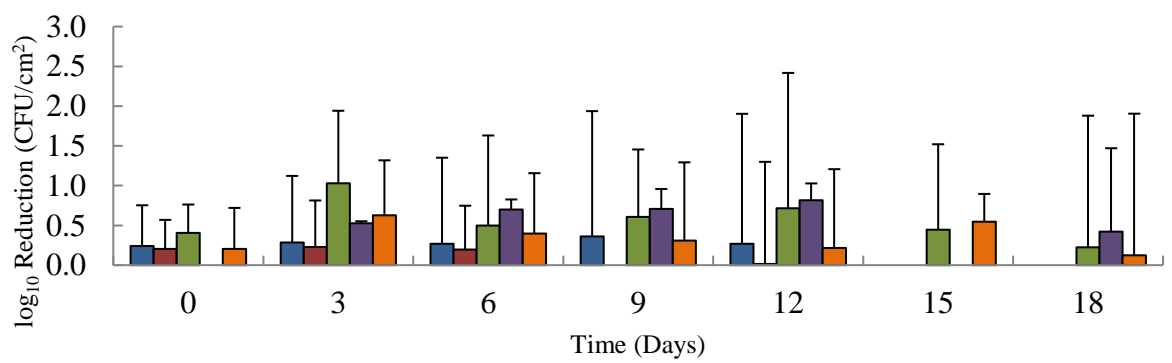
(a) TVC<sub>m</sub>



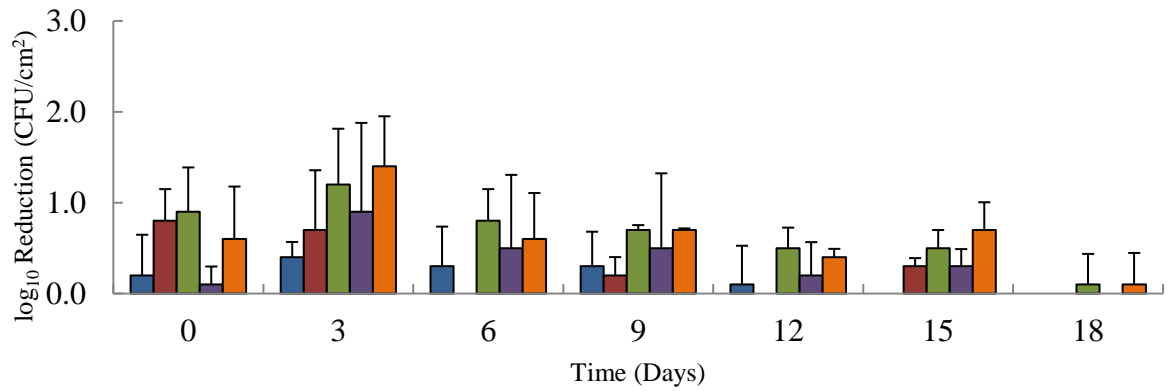
(b) TVC<sub>p</sub>



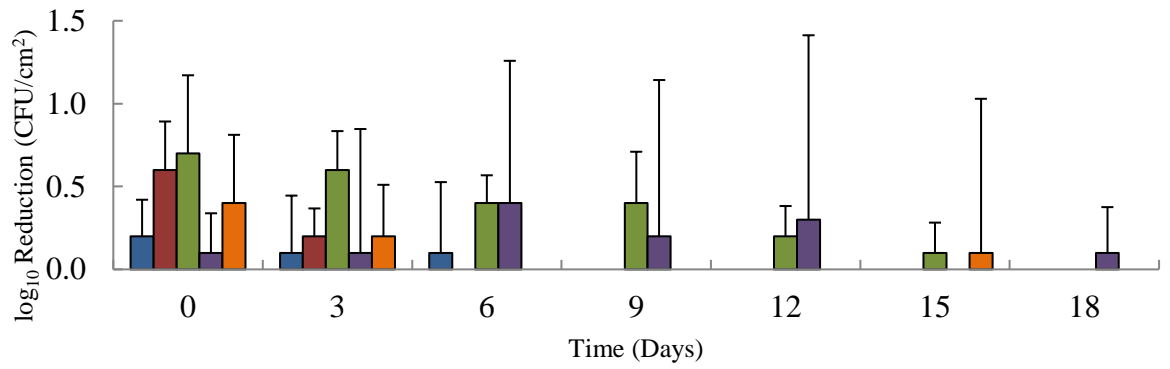
(c) TEC



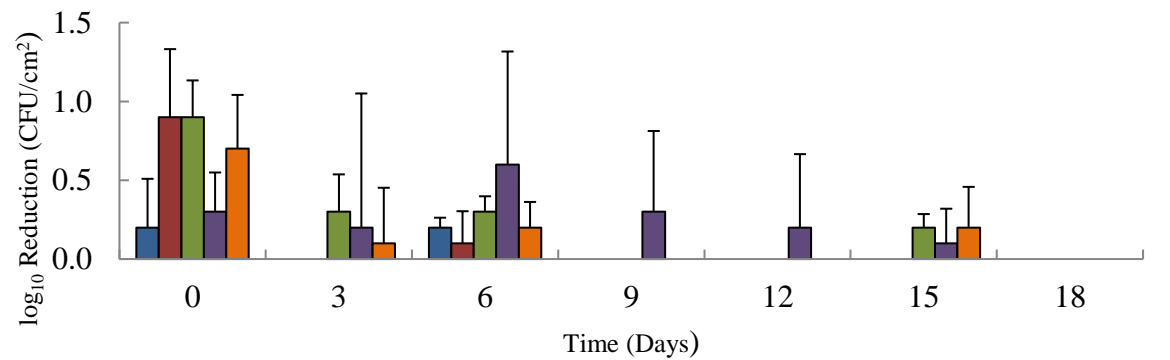
**(d) Hydrogen sulphide producing bacteria**



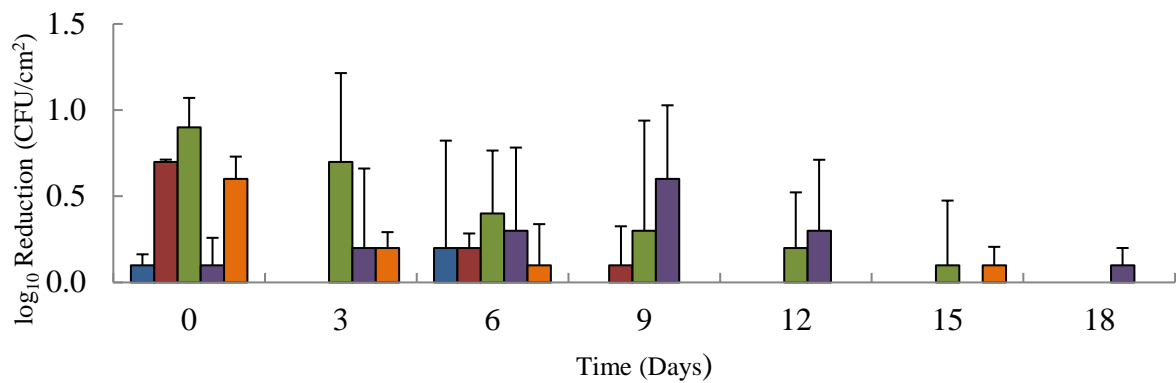
**(e) Lactic acid bacteria**



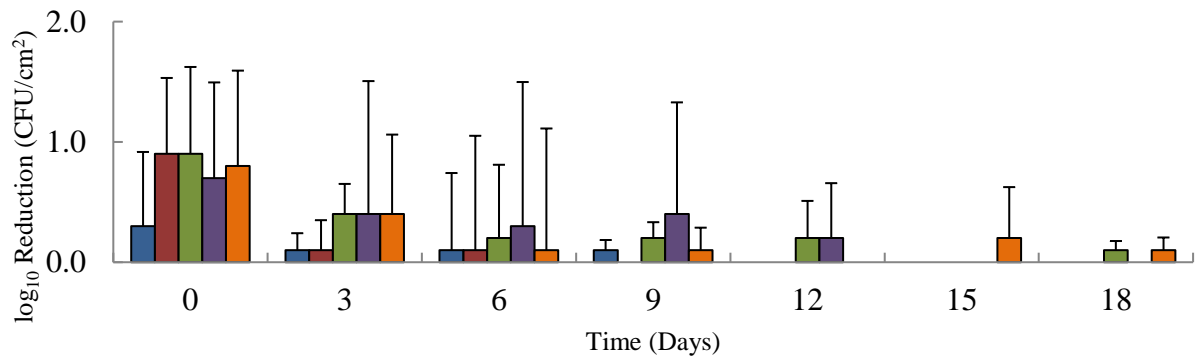
**(f) *Pseudomonas* spp.**



(g) *Brochothrix thermosphacta*



(h) *Photobacterium* spp.



**Figure 6. 2** Mean bacterial log<sub>10</sub> reductions (CFU/cm<sup>2</sup>) on salmon mini-fillets treated with; sterile distilled water (SDW), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) and stored at 2°C for 18 days.

■ SDW, ■ CIT, ■ CAR, ■ THY, ■ EUG.

## 6.5. Discussion

The initial TVC<sub>m</sub> of samples (approximately 3.5 log<sub>10</sub> CFU/cm<sup>2</sup>) was similar to that reported in other fish studies, suggesting good microbiological quality (Li et al., 2017). The low initial levels of *Enterobacteriaceae* also suggested that the fish was farmed in clean, unpolluted waters (Gram, 1992). Moreover, assuming a microbiological (TVC<sub>m</sub>) acceptability limit of 7 log<sub>10</sub> CFU/cm<sup>2</sup> (Ojagh et al., 2010), the shelf-life of our salmon was approximately 8 days which is consistent with that reported for fresh fish stored under chilled aerobic conditions (Li et al., 2012; Sallam, 2007). The initial pH of the control samples (6.8) was statistically similar to the samples treated with CA (6.0), LA (6.4) and CAR (6.4) but significantly different ( $P < 0.05$ ) to samples dipped in TSP (7.3), CIT (6.4), THY (6.3) and EUG (6.3). This is consistent with previous fish decontamination studies (Kim et al., 1995; Williams et al., 1995) pH increases observed during storage (Sallam, 2007).

Neither CA nor LA at 5% (v/v) significantly ( $P > 0.05$ ) reduced the TVC<sub>m</sub>, TVC<sub>p</sub> or TEC on salmon fillets in our study. In contrast García-Soto et al. (2014) reported that both citric (1.25 g/l) and lactic (0.5 g/l) acids significantly lowered these bacterial counts on hake (*Merluccius merluccius*) and megrim (*Lepidorhombus whiffiagonis*) and this effect was maintained during 15 days storage at 0 to 1°C. Metin et al. (2001) also observed a significant ( $P < 0.05$ ) reduction in TVC on chub mackerel dip treated with 2% and 4% LA solutions and stored in vacuum packages at 0 to 4°C for 12 days. These differences in the observed effect of organic acid treatments on fish may be due to the methods used to apply the acid solutions or more specifically the impact on pH and thus the degree of dissociation of the acid molecules. In our study the fish was dip treated for 30 seconds followed by a 30 second rinse with SDW to remove the acid in the hope to limit the any potential negative effects on the physical attributes of the fish. Metin et al. (2001) also used a dip treatment

but for 30 minutes and without a subsequent washing step, while García-Soto et al. (2014) applied the acids in an ice-slurry. The pH of our fish was reduced to 6.0 from 6.4. In the other studies the pH was reduced to as low as pH 4.7, and thus the organic acid molecules were in an undissociated form capable of diffusing across the bacterial cell membrane and disrupting cellular processes (Dibner and Buttin, 2002) resulting in reduced bacterial loads and inhibition of growth (Metin et al., 2001). The post-treatment wash may also explain the failure of 12% TSP to remove and/or inhibit bacterial growth. To the best of our knowledge there are no other studies reporting the effect of TSP on fish but this alkaline product has been shown to be an effective decontaminant of poultry (Meredith et al., 2013). However, the bactericidal effect may be lower if the product is rinsed with water after treatment (Slavik et al., 1994).

These differences in the reported data raise important issues about treatment design. In the EU no chemical decontamination products have yet been approved for use in fresh fish and according to the European Food Safety Authority (EFSA), data to be used for assessing future applications will require studies with a rinsing step (EFSA, 2011; Meredith et al., 2013).

Although CA and LA failed to significantly reduce the growth of indicator bacteria, they significantly reduced HSPB growth for the majority of the 18 day storage trial (CA (t = 2, 4, 6, 8 and 10), LA (t = 2, 4, 6, 8, 10, 12 and 14)). This is significant as the group HSPB is largely made up of *Shewanella* spp., a bacterial genera largely responsible for the spoilage of aerobically stored fish (Møretrø et al., 2016). This genus of bacteria is ubiquitous in the marine environment and therefore may not commonly come into contact with organic acids such as CA and LA. As a result of this *Shewanella* spp. may not possess the genetic

machinery to combat these acids which in turn would make their cells susceptible to damage when treated.

Both CIT and CAR caused a significant ( $P < 0.05$ ) reduction in all the spoilage bacteria (HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp.) immediately following treatment. Moreover, significantly reduced bacterial populations were observed during storage with HSPB (9 days), LAB (6 days) and *Br. thermosphacta* (3 days). This is consistent with previous studies which reported reduced growth rates for spoilage bacteria on rainbow trout (*Onchorynchus mykiss*) treated with oregano (Mexis et al., 2009) and cinnamon essential oils (Andevvari and Rezaei, 2011) and shrimps treated with thymol (Mastromatteo et al., 2010). Essential oils have several mechanisms of action including increasing the permeability of the cell membrane (through cell wall degradation and damaging cell membrane proteins), disruption of the proton motive force, electron flow and active transport systems and inhibiting enzymes involved in energy regulation and the synthesis of structural components (Jayasena and Jo, 2013). These properties are related to their phenolic chemical structure and essential oils have successfully been used to inhibit bacteria in a range of fish (Gómez-Estaca et al., 2010; Harpaz et al., 2003) and other meat products (Fратиanni et al., 2010; Gill and Holley, 2006).

Overall, CIT, CAR, THY and EUG did not significantly reduce bacterial concentrations for the majority of treatment combinations. As with the organic acids, this observation may be related to the pH of the treated fish (6.3 to 6.6) as bacterial susceptibility increases with decreasing pH (at acidic pH values the hydrophobicity of the essential oils increases promoting dissolution across the bacterial membrane) (Burt, 2004). The relatively low storage temperature (Friedman et al., 2004), atmospheric oxygen levels (Burt, 2004) and single (rather than mixed) treatments (Mahmoud et al., 2004) may also have adversely

affected efficacy. Regardless, it is also possible that the relatively high fat content of salmon renders these fish unsuitable for essential oil treatment. Mejlholm and Dalgaard (2002), for example, showed that oregano oil is more effective against *Photobacterium phosphoreum* on cod fillets than salmon attributing the difference to the relatively high fat content in the latter.

In conclusion, this study suggested that overall CA, LA, TSP, CIT, CAR, THY and EUG were not effective antibacterial treatments for salmon fillets when used at the concentrations above which the sensory properties of the fish may be a problem. Arguably the success, although limited, with CA, LA, CIT and CAR against a selection of spoilage bacteria warrants further investigation, perhaps focused on combinations of antimicrobials with other preservation technologies such as anaerobic packaging.

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**Chapter 7 - Investigating the antimicrobial effect of a  
range of compounds combined with packaging  
technologies on the bacteriology of Atlantic salmon  
(*Salmo salar*) during chilled storage**

## 7.1. Summary

Microbial spoilage is a major problem for the seafood sector. This problem could be addressed using multiple hurdle technologies (dip or spray with antimicrobial treatments plus packaging plus chilled storage) to retard bacterial growth. The objective of this study was to investigate the immediate and storage effects of either a dip or spray treatment of; 1 & 5% (w/v) citric acid (CA), 1 & 5% (v/v) lactic acid (LA), 0.5 & 1% (v/v) citral (CIT), 0.5 & 1% (v/v) carvacrol (CAR), 0.5 & 1% (w/v) thymol (THY) and 0.5 & 1% (v/v) eugenol (EUG) on mean bacterial (total viable counts (TVC), total *Enterobacteriaceae* counts (TEC), hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Photobacterium* spp., and *Br. Thermosphacta*) reductions on modified atmosphere packed (MAP) and skin packed (SP) Atlantic salmon (*Salmo salar*) after 9 and 18 days storage at 2°C. Both MAP and SP significantly inhibited microbial growth ( $P < 0.05$ ) when compared to the control; however CA, LA, CIT, CAR, THY and EUG were not significantly effective antimicrobial treatments ( $P > 0.05$ ). Both the 5% CA and LA treatments achieved limited significant antimicrobial success against spoilage organisms which may warrant further investigation.

## 7.2. Introduction

The Irish seafood market was worth €1.15 billion in 2017, an increase of over 6% from the previous year (BIM, 2018). In Ireland, Atlantic salmon (*Salmo salar*) is the most valuable seafood product with exports in 2017 valued at €121 million euro (BIM, 2018). However seafood is a highly perishable product with a relatively short shelf-life of 10-12 days. With a large consumer demand for fresh fish and increasingly longer transport chains, there is a need for preservation techniques that extend shelf life while maintaining microbial safety (Alfaro et al., 2013; Duun and Rustad, 2007; Fernández et al., 2009). Low storage temperature are used as one of the main methods to extend shelf-life (Sigholt et al., 1997). Due to the high perishability of seafood most preservation techniques are only effective if stored below 4°C. The European Commission Regulation 853/2004 (EC, 2004) does not specify a maximum temperature for the storage and transport of fish but states that the temperature must be close to that of melting ice (usually interpreted as 0-2°C). Any seafood stored over 7°C results in accelerated spoilage. However to further retard bacterial growth and extend shelf life it is necessary to combine low temperature storage with other preservation methods such as chemical preservatives, packaging or the use of natural antimicrobials derived from plants (Burt, 2004; García-Soto et al., 2014; Schirmer et al., 2009). Modified atmosphere packaging (MAP) and skin packaging (SP) are the two leading packaging systems used in the meat and seafood industry (Masniyom et al., 2002; McMillin, 2017). MAP replaces air from a package with a controlled gas mixture consisting of carbon dioxide (CO<sub>2</sub>), nitrogen (N<sub>2</sub>) and/or oxygen (O<sub>2</sub>) (Fernández et al., 2009; Sivertsvik et al., 2002). The addition of CO<sub>2</sub> creates a more acidic environment inhibiting the growth of aerobic spoilage organisms (Fernández et al., 2009; Macé et al., 2012; Nassu et al., 2012), however depending on the mixture used, studies have shown increased growth of aerobic organisms on products compared to those stored in SP packs

(Arvanitoyannis and Stratakos, 2012). SP uses a low vacuum to shrink a thin film tightly around the fillet creating an anaerobic environment (Łopacka et al., 2016; Nassu et al., 2012). It has also become more popular than MAP as it is considered more attractive to the consumer and is believed to result in a longer product shelf life (Vázquez et al., 2004). Even with the incorporation of these packaging technologies, previous studies have reported growth of spoilage bacteria such as lactic acid bacteria (LAB), *Shewanella* spp., *Photobacterium phosphoreum* and *Brochothrix thermosphacta* (Dalgaard, 1995; Macé et al., 2012; Powell and Tamplin, 2012; Rudi et al., 2004). It may be necessary to further extend the shelf-life of fresh salmon using combinations of packaging technologies and natural antimicrobials derived from plants, in order to prevent or retard the growth of spoilage organisms. A variety of essential oils and organic acids with antimicrobial properties have been used to inhibit the growth of pathogenic and spoilage bacteria. In the undissociated form an acid molecule can pass through the cell membrane, after which they can dissociate and acidify the cytoplasm (Brul and Coote, 1999; Schirmer et al., 2009). Essential oil components such as citral, carvacrol, thymol and eugenol are phenolic compounds capable of causing structural breakdown of the bacterial cell membrane (Holley and Patel, 2005; Tajkarimi et al., 2010). The aim of this study was to (1) investigate the immediate and storage effects of either a dip or spray treatment of; 1 & 5% (w/v) citric acid (CA), 1 & 5% (v/v) lactic acid (LA), 0.5 & 1% (v/v) citral (CIT), 0.5 & 1% (v/v) carvacrol (CAR), 0.5 & 1% (w/v) thymol (THY) and 0.5 & 1% (v/v) eugenol (EUG) on mean bacterial (total viable counts (TVC), total *Enterobacteriaceae* (TEC), hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Photobacterium* spp., and *Br. Thermosphacta*) reductions on Atlantic salmon (*Salmo salar*) after 9 and 18 days storage at 2°C, and (2) investigate the immediate and storage effects of both MAP

and SP on mean bacterial reductions on Atlantic salmon (*Salmo salar*) after 9 and 18 days storage at 2°C.

## 7.3. Materials and Methods

### 7.3.1. 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.

### 7.3.2. Sample Treatment

All chemical solutions were diluted to the following concentrations; 1% and 5% (w/v) citric acid (CA), 1% and 5% (v/v) lactic acid (LA), 0.5 & 1% v/v citral (CIT), 0.5 & 1% (v/v) carvacrol (CAR), 0.5 & 1% (w/v) thymol (THY) and 0.5 & 1% (v/v) eugenol (EUG) (Sigma Aldrich, Steinheim, Germany). The salmon was divided into 10g samples (n = 252). Samples were subjected to the following treatments; [1] 30 second immersion in a 2 litre volume or [2] sprayed using a 1l trigger sprayer bottle (Garden Plus) using one of the following treatments; 1% (w/v) CA, 5% (w/v) CA, 1% (v/v) LA, 5% (v/v) LA, 0.5% (v/v) CIT, 1% (v/v) CIT, 0.5% (v/v) CAR, 1% (v/v) CAR, 0.5% (w/v) THY, 1% (w/v) THY, 0.5% (v/v) EUG, 1% (v/v) EUG or sterile distilled water (SDW) (control sample). Each sample was sprayed once on each surface, with the bottle approximately a distance of 5cm away. After each initial immersion or spray, treated samples were left to drain for 15 seconds before being immersed in 2 litres of SDW for 30 seconds. This immersion allowed for excess treatment residue to be washed away. After each rinse, samples were allowed to drain for 15 seconds. In total there was a combined 28 dip and spray treatments. From each treatment, 3 samples (total, n = 84) were immediately stored aerobically at 2°C for 18

days. The remaining 168 samples were transported immediately under refrigeration to a packaging facility (Multivac Ireland, Dublin, Ireland) for vacuum skin and MAP.

### 7.3.3. Sample Packaging

A total of 3 samples from each treatment (n = 84) were packed using skin or MAP. Skin pack and MAP were carried out using a T-200 semi-automatic tray sealer (Multivac Ireland, Dublin, Ireland). The packaging gas mix used for MAP was 60% CO<sub>2</sub>: 40% N<sub>2</sub> (Air Products, Dublin, Ireland). The MAP trays were R-PET/PE (Holfeld Plastics, Wicklow, Ireland) and the film used was PET/PE/EVOH (Südpack, Ochsenhausen, Germany). The O<sub>2</sub> permeability for the film was 2.5 cm<sup>3</sup>/m<sup>2</sup> d bar. Skin pack trays were made of R-PET (Holfeld Plastics, Wicklow, Ireland) and the film used was PE/EVOH (Südpack, Ochsenhausen, Germany). The O<sub>2</sub> permeability for the film was ≤2 cm<sup>3</sup>/m<sup>2</sup> d bar. Once packaging was complete the 84 skin pack and 84 MAP samples were transported back to the laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15) under refrigeration. All samples were then stored at 2°C for 18 days.

### 7.3.4. Microbiological Analysis

Microbiological analysis was carried out on days 0, 9 and 18. Each of the meat 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<sup>-7</sup>. Samples were inoculated in duplicate. Plate count agar (PCA) (Oxoid, Basingstoke, United Kingdom (CM0325)), with 1% NaCl was used for total viable counts (TVC, incubated 30°C for 72h) using a standard spread plate techniques. A standard pour plate method was used for total *Enterobacteriaceae* counts (TEC) 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)) at 30°C for 72h. *Photobacterium* spp. was enumerated using Long & Hammer agar, per ingredients used by NMKL (2006) No.184, incubated at 15°C for 168h and *Br. thermosphacta* counts on streptomycin-thallos 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. A standard spread plate method was used for the latter two organisms.

#### 7.3.5. Statistical Analysis

Duplicate plates for each sample and bacterial group were prepared and the experiment was carried out in triplicate. Mean difference ( $\log_{10}$  CFU/cm<sup>2</sup>) were compared using a two way analysis of variance (ANOVA) with Tukey's multiple comparison test where applicable. Graph Pad Prism v7.0 software (Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analysis with significance defined as  $P < 0.05$ .

#### 7.4. Results

The effect of multiple hurdles (antimicrobial treatments (CA, LA, CIT, CAR, THY or EUG) plus packaging (air, MAP or SP) in combination with storage at 2°C on the TVC of salmon after 9 and 18 day are shown in Table 7.1. Initial reductions, immediately after treatment (time  $t = 0$ ) were not significant ( $P > 0.05$ ), regardless of treatment compared to controls (data not shown). The combinations of antimicrobial treatment, air storage and 2°C achieved reduced TVC of up to  $0.5 \log_{10} \text{CFU/cm}^2$  after 9 and 18 days, but none of these were significant ( $P > 0.05$ ) when compared to the control samples (SDW & air storage at 2°C). In contrast, MAP achieved reductions of up to  $4.1 \log_{10} \text{CFU/cm}^2$  after 9 days (1% LA or 0.5% CIT sprays) (Supplementary Table 1, Appendix B) and up to  $4.4 \log_{10} \text{CFU/cm}^2$  after 18 days (0.5% THY dip) (Supplementary Table 5, Appendix B). Moreover, TVC for the majority of hurdle combinations that included MAP packaging were significantly ( $P < 0.05$ ) lower than controls (Supplementary Table 1, 3, 5 & 7, Appendix B). A similar pattern was observed when SP was used with the highest reduction achieved with 1% CA dip after 18 days (Supplementary Table 5, Appendix B).

The corresponding data for TEC in salmon are shown in Table 7.2. TEC were significantly reduced by up to  $1 \log_{10} \text{CFU/cm}^2$  after 9 days aerobic storage at 2°C for 0.5% CIT, CAR and EUG spray treatments (Supplementary Table 1, Appendix B). All treatments, combined with MAP, were up to  $4.0 \log_{10} \text{CFU/cm}^2$  lower after 18 days compared to control samples. A similar pattern was not observed when SP was used with only the higher concentration spray treatments significantly reducing growth after 9 days (Supplementary Table 3, Appendix B). The highest recorded reduction after 18 days was  $2.5 \log_{10} \text{CFU/cm}^2$  (1 & 5% LA dip) (Supplementary Table 5 & 7, Appendix B).

**Table 7. 1** Mean differences in TVC<sub>m</sub> (log<sub>10</sub> CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

Treatment	Packaging technology and Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	3.3 <sup>B/A</sup>	3.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.7 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.0 <sup>A/A</sup>	3.5 <sup>B/A</sup>	2.9 <sup>B/A</sup>	0.3 <sup>A/A</sup>	4.3 <sup>B/A</sup>	4.0 <sup>B/B</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.0 <sup>A/A</sup>	3.7 <sup>B/A</sup>	3.2 <sup>B/A</sup>	0.2 <sup>A/A</sup>	4.2 <sup>C/A</sup>	3.2 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.1 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	3.6 <sup>B/A</sup>	3.6 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	0.3 <sup>A/A</sup>	3.1 <sup>B/A</sup>	2.2 <sup>B/A</sup>	0.1 <sup>A/A</sup>	4.3 <sup>C/A</sup>	3.2 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.2 <sup>A/A</sup>	3.7 <sup>C/A</sup>	2.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	4.4 <sup>B/A</sup>	3.3 <sup>B/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.0 <sup>A/A</sup>	3.5 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	4.3 <sup>B/A</sup>	3.7 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	3.6 <sup>C/A</sup>	2.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>1% CA-spray</b>	0.0 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.1 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.3 <sup>C/A</sup>	2.2 <sup>B/A</sup>
<b>1% LA-spray</b>	0.5 <sup>A/A</sup>	4.1 <sup>C/A</sup>	3.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>C/A</sup>	2.3 <sup>B/A</sup>
<b>0.5% CIT-spray</b>	0.2 <sup>A/A</sup>	4.1 <sup>C/A</sup>	2.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	2.2 <sup>C/A</sup>
<b>0.5% CAR-spray</b>	0.3 <sup>A/A</sup>	3.9 <sup>C/A</sup>	2.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.4 <sup>C/A</sup>	2.1 <sup>B/A</sup>
<b>0.5% THY-spray</b>	0.2 <sup>A/A</sup>	3.5 <sup>C/A</sup>	2.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.8 <sup>B/A</sup>
<b>0.5% EUG-spray</b>	0.2 <sup>A/A</sup>	3.7 <sup>C/A</sup>	2.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.0 <sup>B/A</sup>

**Continuation of Table 7. 2** Mean differences in TVC<sub>m</sub> (log<sub>10</sub> CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	2.3 <sup>B/A</sup>	2.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.7 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>5% CA-dip</b>	0.4 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.8 <sup>B/A</sup>	0.3 <sup>A/A</sup>	3.0 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>5% LA-dip</b>	0.4 <sup>A/A</sup>	2.7 <sup>B/A</sup>	3.3 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.3 <sup>B/A</sup>	3.5 <sup>B/A</sup>
<b>1% CIT-dip</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.2 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.9 <sup>B/A</sup>
<b>1% CAR-dip</b>	0.4 <sup>A/A</sup>	1.7 <sup>B/A</sup>	2.9 <sup>C/A</sup>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>1% THY-dip</b>	0.1 <sup>A/A</sup>	2.7 <sup>B/A</sup>	3.0 <sup>B/A</sup>	0.3 <sup>A/A</sup>	2.7 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.1 <sup>A/A</sup>	2.5 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.3 <sup>A/A</sup>	2.5 <sup>B/A</sup>	3.2 <sup>C/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.3 <sup>B/A</sup>
<b>5% CA spray</b>	0.0 <sup>A/A</sup>	2.7 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>5% LA spray</b>	0.0 <sup>A/A</sup>	2.7 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>1% CIT-spray</b>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.4 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>1% THY-spray</b>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.2 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.6 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	2.4 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

**Table 7. 3** Mean differences in TEC ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

Treatment	Packaging technology/Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	1.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	1.5 <sup>B/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.0 <sup>A/A</sup>	2.0 <sup>B/A</sup>	1.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.7 <sup>B/A</sup>	2.4 <sup>B/A</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	1.3 <sup>A/A</sup>	0.4 <sup>A/A</sup>	3.7 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.1 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	1.4 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	0.1 <sup>A/A</sup>	1.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	0.2 <sup>A/A</sup>	4.0 <sup>C/A</sup>	1.4 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	0.6 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	1.1 <sup>A/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	0.7 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.2 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	0.1 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.6 <sup>B/A</sup>	0.2 <sup>A/A</sup>
<b>1% CA-spray</b>	0.2 <sup>A/A</sup>	2.9 <sup>B/A</sup>	0.1 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>
<b>1% LA-spray</b>	0.1 <sup>A/A</sup>	3.2 <sup>B/A</sup>	0.3 <sup>A/A</sup>	0.1 <sup>A/A</sup>	3.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>
<b>0.5% CIT-spray</b>	0.8 <sup>A/B</sup>	3.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.2 <sup>A/A</sup>	3.6 <sup>B/A</sup>	0.2 <sup>A/A</sup>
<b>0.5% CAR-spray</b>	0.9 <sup>A/B</sup>	3.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.1 <sup>A/A</sup>	3.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>
<b>0.5% THY-spray</b>	0.6 <sup>A/A</sup>	2.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.1 <sup>A/A</sup>	2.9 <sup>B/A</sup>	0.1 <sup>A/A</sup>
<b>0.5% EUG-spray</b>	1.0 <sup>A/B</sup>	3.3 <sup>A/A</sup>	0.0 <sup>A/A</sup>	0.1 <sup>A/A</sup>	3.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>

**Continuation of Table 7. 4** Mean differences in TEC ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.4 <sup>A/A</sup>	0.0 <sup>A/A</sup>	2.0 <sup>B/A</sup>	1.4 <sup>A/A</sup>
<b>5% CA-dip</b>	0.6 <sup>A/A</sup>	2.1 <sup>B/A</sup>	1.3 <sup>A/A</sup>	0.1 <sup>A/A</sup>	2.7 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>5% LA-dip</b>	0.5 <sup>A/A</sup>	2.2 <sup>B/A</sup>	1.5 <sup>A/A</sup>	0.6 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>1% CIT-dip</b>	0.0 <sup>A/A</sup>	1.1 <sup>B/A</sup>	0.9 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>1% CAR-dip</b>	0.5 <sup>A/A</sup>	2.1 <sup>B/A</sup>	0.6 <sup>A/A</sup>	0.1 <sup>A/A</sup>	2.2 <sup>B/A</sup>	0.9 <sup>A/A</sup>
<b>1% THY-dip</b>	0.3 <sup>A/A</sup>	2.8 <sup>C/A</sup>	1.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.2 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.6 <sup>A/A</sup>	2.4 <sup>B/A</sup>	0.2 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.7 <sup>A/A</sup>	1.8 <sup>A/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	1.6 <sup>A/A</sup>	1.4 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.8 <sup>A/A</sup>	1.8 <sup>A/A</sup>
<b>5% CA spray</b>	0.0 <sup>A/A</sup>	1.9 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	1.8 <sup>B/A</sup>
<b>5% LA spray</b>	0.2 <sup>A/A</sup>	2.4 <sup>B/A</sup>	1.9 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	2.3 <sup>B/A</sup>
<b>1% CIT-spray</b>	0.0 <sup>A/A</sup>	1.8 <sup>B/A</sup>	1.6 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	1.9 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>1% THY-spray</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	1.6 <sup>B/A</sup>	0.2 <sup>A/A</sup>	2.4 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.2 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

Comparisons made between the different packaging conditions (keeping the antimicrobial treatment constant) showed significant reductions ( $P < 0.05$ ) in spoilage bacteria growth for both MAP and SP after 9 and 18 days. After 9 days, LAB growth (Table 7.3) was significantly reduced under MAP conditions for all combinations with the exceptions of the 0.5% CAR dip treatment, however after 18 days 5% CA, 1% CIT and 1% CAR spray treatments failed to maintain these significant reductions (Supplementary Table 2, 4, 6 & 8, Appendix B). When combined with SP conditions, both the lower and higher concentration spray treatments significantly reduced LAB growth on day 9 but only the higher concentration combination maintained these reductions until day 18 (Supplementary Table 2 & 4, Appendix B). After 9 days the only dip treatments to significantly reduce LAB growth was 5% LA, 1% THY, 1% EUG (Supplementary Table 8, Appendix B) and 0.5% EUG (Supplementary Table 6, Appendix B). All dip treatments combined with SP significantly reduced LAB growth on day 18 with the exception of 1% CAR (Supplementary Table 8, Appendix B). Both MAP and SP significantly reduced HSPB growth (Table 7.4) on days 9 and 18; however SP combinations with lower concentration spray treatments did not record significant reductions on day 18 (Supplementary Table 2, Appendix B). All antimicrobial treatments combined with MAP and SP showed significant *Photobacterium* spp. and *Br. thermosphacta* reductions ( $P < 0.05$ ) on days 9 and 18 (Table 7.5 & 7.6) (Supplementary Table 2, 4, 6 & 8, Appendix B).

**Table 7. 5** Mean differences in LAB ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

CLI treatment	Packaging technology/Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	1.4 <sup>B/A</sup>	1.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.1 <sup>A/A</sup>	0.5 <sup>A/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.3 <sup>A/A</sup>	1.5 <sup>B/A</sup>	1.2 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.3 <sup>A/A</sup>	2.0 <sup>B/A</sup>	1.2 <sup>A/A</sup>	0.2 <sup>A/A</sup>	1.3 <sup>B/A</sup>	1.4 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.2 <sup>A/A</sup>	1.1 <sup>B/A</sup>	0.5 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.2 <sup>B/A</sup>	0.8 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	0.6 <sup>A/A</sup>	1.4 <sup>A/A</sup>	0.4 <sup>A/A</sup>	0.1 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.9 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.0 <sup>A/A</sup>	1.7 <sup>B/A</sup>	0.7 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.4 <sup>B/A</sup>	1.0 <sup>B/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.4 <sup>B/A</sup>	1.2 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	0.7 <sup>A/A</sup>
<b>1% CA-spray</b>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	1.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.3 <sup>A/A</sup>
<b>1% LA-spray</b>	0.2 <sup>A/A</sup>	2.7 <sup>B/A</sup>	2.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.3 <sup>A/A</sup>
<b>0.5% CIT-spray</b>	0.6 <sup>A/A</sup>	2.8 <sup>C/A</sup>	1.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	0.7 <sup>B/A</sup>
<b>0.5% CAR-spray</b>	0.4 <sup>A/A</sup>	2.6 <sup>C/A</sup>	1.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	0.6 <sup>A/A</sup>
<b>0.5% THY-spray</b>	0.2 <sup>A/A</sup>	2.0 <sup>B/A</sup>	1.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	0.2 <sup>A/A</sup>
<b>0.5% EUG-spray</b>	0.3 <sup>A/A</sup>	2.1 <sup>C/A</sup>	1.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.4 <sup>B/A</sup>	0.1 <sup>A/A</sup>

**Continuation of Table 7. 6** Mean differences in LAB ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	0.8 <sup>B/A</sup>	0.7 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.1 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>5% CA-dip</b>	0.0 <sup>A/A</sup>	1.2 <sup>B/A</sup>	0.8 <sup>A/A</sup>	0.0 <sup>A/A</sup>	1.2 <sup>B/A</sup>	1.4 <sup>B/A</sup>
<b>5% LA-dip</b>	0.6 <sup>A/A</sup>	2.0 <sup>B/B</sup>	1.2 <sup>B/A</sup>	0.5 <sup>A/A</sup>	1.8 <sup>B/A</sup>	1.8 <sup>B/A</sup>
<b>1% CIT-dip</b>	0.0 <sup>A/A</sup>	0.8 <sup>B/A</sup>	0.7 <sup>A/A</sup>	0.1 <sup>A/A</sup>	1.0 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>1% CAR-dip</b>	0.4 <sup>A/A</sup>	1.2 <sup>B/A</sup>	0.5 <sup>A/A</sup>	0.2 <sup>A/A</sup>	1.1 <sup>B/A</sup>	1.0 <sup>B/A</sup>
<b>1% THY-dip</b>	0.3 <sup>A/A</sup>	1.6 <sup>B/A</sup>	1.1 <sup>B/A</sup>	0.3 <sup>A/A</sup>	1.1 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.2 <sup>A/A</sup>	1.1 <sup>B/A</sup>	0.9 <sup>B/A</sup>	0.3 <sup>A/A</sup>	1.1 <sup>B/A</sup>	1.3 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	1.4 <sup>B/A</sup>	1.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.6 <sup>A/A</sup>	0.7 <sup>A/A</sup>
<b>5% CA spray</b>	0.1 <sup>A/A</sup>	1.3 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.5 <sup>A/A</sup>	1.3 <sup>B/A</sup>
<b>5% LA spray</b>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.9 <sup>A/A</sup>	1.3 <sup>B/A</sup>
<b>1% CIT-spray</b>	0.0 <sup>A/A</sup>	1.6 <sup>B/A</sup>	1.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.8 <sup>A/A</sup>	1.0 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.1 <sup>A/A</sup>	1.7 <sup>B/A</sup>	1.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.7 <sup>A/A</sup>	0.9 <sup>A/A</sup>
<b>1% THY-spray</b>	0.0 <sup>A/A</sup>	1.7 <sup>B/A</sup>	1.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.0 <sup>B/A</sup>	1.1 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.0 <sup>A/A</sup>	1.8 <sup>B/A</sup>	1.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	0.9 <sup>A/A</sup>	1.1 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

**Table 7. 7** Mean differences in HSPB ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

CLI treatment	Packaging technology/Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	4.2 <sup>B/A</sup>	3.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.6 <sup>A/A</sup>	4.5 <sup>B/A</sup>	3.9 <sup>B/A</sup>	0.4 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.2 <sup>B/A</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.1 <sup>A/A</sup>	4.2 <sup>B/A</sup>	3.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.7 <sup>A/A</sup>	4.2 <sup>C/A</sup>	2.9 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	1.5 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	1.0 <sup>A/A</sup>	3.7 <sup>B/A</sup>	2.8 <sup>B/A</sup>	0.3 <sup>A/A</sup>	2.5 <sup>B/A</sup>	2.4 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.4 <sup>A/A</sup>	4.2 <sup>C/A</sup>	3.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.5 <sup>A/A</sup>	4.0 <sup>B/A</sup>	3.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.3 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.8 <sup>C/A</sup>	1.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	0.3 <sup>A/A</sup>
<b>1% CA-spray</b>	0.1 <sup>A/A</sup>	3.6 <sup>C/A</sup>	1.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.7 <sup>B/A</sup>	0.1 <sup>A/A</sup>
<b>1% LA-spray</b>	0.3 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.3 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>
<b>0.5% CIT-spray</b>	0.1 <sup>A/A</sup>	3.6 <sup>C/A</sup>	1.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.4 <sup>B/A</sup>	0.3 <sup>A/A</sup>
<b>0.5% CAR-spray</b>	0.0 <sup>A/A</sup>	3.7 <sup>C/A</sup>	1.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>B/A</sup>	0.5 <sup>A/A</sup>
<b>0.5% THY-spray</b>	0.0 <sup>A/A</sup>	2.9 <sup>C/A</sup>	1.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>B/A</sup>	0.2 <sup>A/A</sup>
<b>0.5% EUG-spray</b>	0.6 <sup>A/A</sup>	3.6 <sup>C/A</sup>	1.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>

**Continuation of Table 7. 4** Mean differences in HSPB (log<sub>10</sub> CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	2.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.0 <sup>A/A</sup>	1.1 <sup>B/A</sup>
<b>5% CA-dip</b>	1.0 <sup>A/A</sup>	4.8 <sup>C/B</sup>	3.2 <sup>B/B</sup>	1.2 <sup>A/B</sup>	2.6 <sup>B/B</sup>	2.5 <sup>B/B</sup>
<b>5% LA-dip</b>	0.7 <sup>A/A</sup>	5.4 <sup>C/B</sup>	3.6 <sup>B/B</sup>	0.9 <sup>A/A</sup>	2.5 <sup>B/B</sup>	2.7 <sup>B/B</sup>
<b>1% CIT-dip</b>	0.5 <sup>A/A</sup>	3.9 <sup>C/A</sup>	2.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.9 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>1% CAR-dip</b>	0.4 <sup>A/A</sup>	3.5 <sup>C/A</sup>	1.8 <sup>B/A</sup>	0.3 <sup>A/A</sup>	1.7 <sup>B/A</sup>	1.5 <sup>B/A</sup>
<b>1% THY-dip</b>	0.1 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	1.3 <sup>B/A</sup>	1.9 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.2 <sup>A/A</sup>	4.0 <sup>C/A</sup>	2.8 <sup>B/A</sup>	0.1 <sup>A/A</sup>	1.6 <sup>B/A</sup>	1.5 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	3.1 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	1.7 <sup>B/A</sup>
<b>5% CA spray</b>	1.1 <sup>A/A</sup>	4.3 <sup>B/A</sup>	4.7 <sup>B/B</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>5% LA spray</b>	1.1 <sup>A/A</sup>	4.6 <sup>B/B</sup>	3.9 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>1% CIT-spray</b>	0.5 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	1.8 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.5 <sup>A/A</sup>	3.4 <sup>B/A</sup>	3.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>1% THY-spray</b>	0.6 <sup>A/A</sup>	3.5 <sup>B/A</sup>	2.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	1.6 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.1 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.2 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

When effects of the various antimicrobial treatments within the same packaging system were assessed, relatively few resulted in significant reductions in levels of spoilage bacteria. Under MAP conditions, 5% LA significantly retarded the growth of LAB (dip) by day 9 (Table 7.3) (Supplementary Table 8, Appendix B) and *Br. thermosphacta* (Table 7.6) was significantly lower after 18 days by 1% CA (dip) (Supplementary Table 6, Appendix B) and 0.5% CAR (spray) (Supplementary Table 2, Appendix B) compared to SDW treated samples. Under SP conditions, *Br. thermosphacta* growth was significantly reduced on day 9 (5% LA (dip)) and day 18 (1% CIT (dip)) (Supplementary Table 8, Appendix B). As seen in Table 7.5, *Photobacterium* spp. was also significantly lower on salmon mini fillets treated with 5% LA dip and spray by day 18 (Supplementary Table 4 & 8, Appendix B). Significant reductions in HSPB were observed on salmon stored under both MAP and SP conditions (Table 7.4) using 5% (w/v) CA and LA dips (t = 9 and 18) (Supplementary Table 8, Appendix B).

**Table 7. 8** Mean differences in *Photobacterium* spp. ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

CLI treatment	Packaging technology/Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	2.7 <sup>C/A</sup>	1.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.0 <sup>A/A</sup>	2.5 <sup>B/A</sup>	1.7 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.4 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.1 <sup>A/A</sup>	2.9 <sup>C/A</sup>	1.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	3.8 <sup>B/A</sup>	2.8 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.0 <sup>A/A</sup>	2.9 <sup>C/A</sup>	1.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.5 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	0.4 <sup>A/A</sup>	2.9 <sup>C/A</sup>	1.6 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.4 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	1.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.3 <sup>B/A</sup>	2.8 <sup>B/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	1.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.7 <sup>C/A</sup>	1.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	2.0 <sup>B/A</sup>
<b>1% CA-spray</b>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	1.8 <sup>B/A</sup>
<b>1% LA-spray</b>	0.1 <sup>A/A</sup>	3.1 <sup>B/A</sup>	2.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.8 <sup>B/A</sup>
<b>0.5% CIT-spray</b>	0.2 <sup>A/A</sup>	3.0 <sup>C/A</sup>	1.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>C/A</sup>	1.9 <sup>B/A</sup>
<b>0.5% CAR-spray</b>	0.2 <sup>A/A</sup>	3.3 <sup>C/A</sup>	1.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.0 <sup>B/A</sup>
<b>0.5% THY-spray</b>	0.2 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.9 <sup>C/A</sup>	1.6 <sup>B/A</sup>
<b>0.5% EUG-spray</b>	0.0 <sup>A/A</sup>	3.2 <sup>C/A</sup>	1.6 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.3 <sup>B/A</sup>	1.6 <sup>B/A</sup>

**Continuation of Table 7. 9** Mean differences in *Photobacterium* spp. ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	2.3 <sup>A/A</sup>	2.2 <sup>A/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>A/A</sup>	3.1 <sup>A/A</sup>
<b>5% CA-dip</b>	0.3 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.5 <sup>B/A</sup>	0.6 <sup>A/A</sup>	3.4 <sup>B/A</sup>	3.4 <sup>B/A</sup>
<b>5% LA-dip</b>	0.6 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.2 <sup>B/A</sup>	0.3 <sup>A/A</sup>	3.7 <sup>B/A</sup>	3.7 <sup>B/B</sup>
<b>1% CIT-dip</b>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.5 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.9 <sup>B/A</sup>
<b>1% CAR-dip</b>	0.2 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.0 <sup>B/A</sup>	0.7 <sup>A/A</sup>	3.3 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>1% THY-dip</b>	0.1 <sup>A/A</sup>	2.5 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.8 <sup>A/A</sup>	3.1 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.0 <sup>A/A</sup>	1.7 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.7 <sup>A/A</sup>	3.1 <sup>B/A</sup>	3.2 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	2.9 <sup>A/A</sup>	2.1 <sup>A/A</sup>	0.0 <sup>A/A</sup>	2.9 <sup>A/A</sup>	2.3 <sup>A/A</sup>
<b>5% CA spray</b>	0.1 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>5% LA spray</b>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	3.1 <sup>B/B</sup>
<b>1% CIT-spray</b>	0.0 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.4 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.8 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.2 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.8 <sup>B/A</sup>
<b>1% THY-spray</b>	0.1 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.6 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.4 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.8 <sup>B/A</sup>	2.7 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

**Table 7. 10** Mean differences in *Br. thermosphacta* ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C compared to the control (treated with SDW and stored aerobically at 2°C).

CLI treatment	Packaging technology/Storage time					
	9 days			18 days		
	Air	MAP	SP	Air	MAP	SP
	Log	Log	Log	Log	Log	Log
<b>SDW<sup>1</sup></b>	0.0 <sup>A/A</sup>	3.5 <sup>B/A</sup>	2.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	3.1 <sup>B/A</sup>
<b>1% CA<sup>2</sup>-dip</b>	0.0 <sup>A/A</sup>	3.6 <sup>B/A</sup>	3.3 <sup>B/A</sup>	0.2 <sup>A/A</sup>	4.0 <sup>C/B</sup>	2.9 <sup>B/A</sup>
<b>1% LA<sup>3</sup>-dip</b>	0.1 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.2 <sup>B/A</sup>	0.5 <sup>A/A</sup>	3.3 <sup>B/A</sup>	3.3 <sup>B/A</sup>
<b>0.5% CIT<sup>4</sup>-dip</b>	0.1 <sup>A/A</sup>	3.3 <sup>B/A</sup>	2.4 <sup>B/A</sup>	0.3 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>0.5% CAR<sup>5</sup>-dip</b>	0.1 <sup>A/A</sup>	3.4 <sup>B/A</sup>	2.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	3.3 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>0.5% THY<sup>6</sup>-dip</b>	0.0 <sup>A/A</sup>	3.1 <sup>C/A</sup>	2.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>0.5% EUG<sup>7</sup>-dip</b>	0.0 <sup>A/A</sup>	2.9 <sup>B/A</sup>	2.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>B/A</sup>	2.5 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	3.5 <sup>B/A</sup>	3.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.7 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>1% CA-spray</b>	0.2 <sup>A/A</sup>	3.7 <sup>B/A</sup>	3.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>C/A</sup>	2.0 <sup>B/A</sup>
<b>1% LA-spray</b>	0.4 <sup>A/A</sup>	3.9 <sup>B/A</sup>	3.1 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>C/A</sup>	1.9 <sup>B/A</sup>
<b>0.5% CIT-spray</b>	0.5 <sup>A/A</sup>	4.2 <sup>C/A</sup>	2.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.1 <sup>C/A</sup>	1.7 <sup>B/A</sup>
<b>0.5% CAR-spray</b>	0.4 <sup>A/A</sup>	4.0 <sup>C/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.6 <sup>C/B</sup>	2.1 <sup>B/A</sup>
<b>0.5% THY-spray</b>	0.2 <sup>A/A</sup>	3.7 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>C/A</sup>	1.8 <sup>B/A</sup>
<b>0.5% EUG-spray</b>	0.3 <sup>A/A</sup>	3.7 <sup>C/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.3 <sup>C/A</sup>	1.8 <sup>B/A</sup>

**Continuation of Table 7. 11** Mean differences in *Br. thermosphacta* ( $\log_{10}$  CFU/cm<sup>2</sup>) in salmon treated with various antimicrobial treatments, packed in air, modified atmosphere packaging (MAP) or skin packaging (SP) and stored at 2°C.

<b>SDW</b>	0.0 <sup>A/A</sup>	2.3 <sup>B/A</sup>	2.2 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.3 <sup>B/A</sup>	2.6 <sup>B/A</sup>
<b>5% CA-dip</b>	0.6 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.8 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.7 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>5% LA-dip</b>	0.8 <sup>A/A</sup>	3.6 <sup>B/B</sup>	3.5 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.6 <sup>B/A</sup>	3.4 <sup>B/A</sup>
<b>1% CIT-dip</b>	0.1 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.1 <sup>A/A</sup>	2.0 <sup>B/A</sup>	3.4 <sup>C/B</sup>
<b>1% CAR-dip</b>	0.6 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.3 <sup>A/A</sup>	2.3 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>1% THY-dip</b>	0.1 <sup>A/A</sup>	2.5 <sup>B/A</sup>	3.0 <sup>B/A</sup>	0.4 <sup>A/A</sup>	1.9 <sup>B/A</sup>	3.0 <sup>B/A</sup>
<b>1% EUG-dip</b>	0.1 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.7 <sup>B/A</sup>	0.2 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.7 <sup>B/A</sup>
<b>SDW</b>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.1 <sup>B/A</sup>
<b>5% CA spray</b>	0.1 <sup>A/A</sup>	3.5 <sup>B/A</sup>	3.4 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.2 <sup>B/A</sup>	2.9 <sup>B/A</sup>
<b>5% LA spray</b>	0.4 <sup>A/A</sup>	3.2 <sup>B/A</sup>	3.3 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.6 <sup>B/A</sup>	2.9 <sup>B/A</sup>
<b>1% CIT-spray</b>	0.0 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.5 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.1 <sup>B/A</sup>	2.4 <sup>B/A</sup>
<b>1% CAR-spray</b>	0.5 <sup>A/A</sup>	3.0 <sup>B/A</sup>	2.8 <sup>B/A</sup>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	2.6 <sup>B/A</sup>
<b>1% THY-spray</b>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	2.9 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.3 <sup>B/A</sup>
<b>1% EUG-spray</b>	0.0 <sup>A/A</sup>	3.2 <sup>B/A</sup>	3.0 <sup>B/A</sup>	0.0 <sup>A/A</sup>	2.4 <sup>B/A</sup>	2.7 <sup>B/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. Second superscripted letters denote statistical significance between treatments within the same packaging system and sampling time. (P > 0.05).

<sup>1</sup> SDW – Sterile Distilled Water, <sup>2</sup> CA – Citric Acid, <sup>3</sup> LA – Lactic Acid, <sup>4</sup> CIT – Citral

<sup>5</sup> CAR – Carvacrol, <sup>6</sup> THY – Thymol, <sup>7</sup> EUG – Eugenol

## 7.5. Discussion

The initial TVC reported for each control sample ranged from 2.9 to 4.6 log<sub>10</sub> CFU/cm<sup>2</sup> (Supplementary Table 1, 3, 5 & 7) and was similar to that reported in other fish studies, suggesting an acceptable microbial quality (Parlapani et al., 2015; Yesudhason et al., 2014). The low initial *Enterobacteriaceae* levels also suggested that the fish were farmed in clean unpolluted waters (Gram, 1992).

Throughout the 18 day storage trial, both CA and LA failed to significantly ( $P > 0.05$ ) inhibit the growth of TVC and TEC, irrespective of application method or concentration applied. However both the 5% CA and LA dip treatments achieved significant antimicrobial success against HSPB (*Shewanella* spp.) at days 9 and 18, under MAP and SP conditions. HSPB are amongst the dominant spoilage organisms found in aerobically stored seafood, however their growth is inhibited in an anaerobic environment (Calliauw et al., 2016; Nirmal and Benjakul, 2011; Parlapani et al., 2015). The CO<sub>2</sub> rich atmosphere may exert a stress on the bacterial cell, making these bacteria more susceptible to treatment with organic acids. This is consistent with previous studies by Schirmer et al. (2009) who observed reduced HSPB growth on salmon treated with a MAP + 3% CA combination.

The pattern of antimicrobial inconsistency was also observed for each essential oil concentration and application method. As with the organic acids the lower concentrations had little or no effect on the indicator or spoilage bacteria. The lack of consistent antimicrobial activity associated with essential oils under examination is supported by previous studies. Fernández et al. (2009) observed that the addition of natural additives to salmon stored under MAP conditions at 2°C, had no significant effect on microbial growth, whereas Thiansilakul et al. (2013) made similar observations when treating Eastern little tuna (*Euthynnus affinis*) slices with phenolic compounds under MAP. Other studies

have reported effective antimicrobial activity associated with oregano oil (0.1% v/v) on aerobically stored grass carp (*Ctenopharyngodon idellus*) (Huang et al., 2018) and cinnamon oil (0.1% v/v) on vacuum packed common carp (*Cyprinus carpio*) (Zhang et al., 2017). However both studies employed different methods of application. In our study the fish was immersed for 30 seconds followed by a post-treatment 30 second rinse with SDW to remove any essential oil residue. Both Zhang et al. (2017) and Huang et al. (2018) also used an immersion treatment; however their samples were left submerged for 10 and 30 minutes, respectively. Neither study carried out a rinsing step after the samples were treated. These differences in the reported data raise important issues about treatment design. In the EU no chemical decontamination products have yet been approved for use in fresh fish and according to the European Food Safety Authority (EFSA), data to be used for assessing future applications will require studies with a rinsing step (EFSA, 2011; Meredith et al., 2013).

There may be several explanations as to why the essential oil treatments were not consistently effective throughout this trial. Burt (2004) suggested that certain spoilage organisms, such as LAB, are resistant to essential oil treatments due to their ability to adapt to osmotic stresses, however it is also possible that the high fat content of salmon renders these fish unsuitable for essential oil treatment. For example, Mejlholm and Dalgaard (2002) observed that oregano oil had a greater antimicrobial effect against *Photobacterium phosphoreum* on cod (*Gadus morhua*) fillets than salmon, attributing the difference to the relatively high fat content in the latter.

Throughout this study, both MAP and SP were successful in inhibiting the growth of TVC. Using  $7 \log_{10}$  CFU/cm<sup>2</sup> as the upper level of microbial acceptability (Calliauw et al., 2016; Liston, 1980), neither MAP nor SP samples had reached spoilage by day 18, whereas

aerobically stored samples had reached this level before day 9. TVC growth on MAP fillets was significantly lower than SP ( $P < 0.05$ ). This has also been reported in previous studies on seafood such as; Atlantic salmon (Amanatidou et al., 2000; Schirmer et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Rodrigues et al., 2016), Atlantic herring (*Clupea harengus*) (Özogul et al., 2000), sardines (*Sardina pilchardus*) (Özogul et al., 2004) and yellow fin tuna (*Thunnus albacares*) (Silbande et al., 2016).

On days 9 and 18, MAP successfully inhibited the growth of TEC; however SP was not as successful which is in agreement with previous studies by Radetic et al. (2007) and Milijasevic et al. (2015). They observed that SP may initially be microaerophilic favouring the growth of *Enterobacteriaceae* while MAP with a high CO<sub>2</sub> concentration reduced the rate growth of *Enterobacteriaceae* for a longer time period.

Although MAP and SP can successfully inhibit bacterial growth, it is still possible for anaerobic psychrophilic bacteria to grow. Spoilage organisms such as LAB, *Br. thermosphacta* and *Photobacterium* spp. have all been reported to grow in a CO<sub>2</sub> rich environment (Emborg et al., 2002; Rudi et al., 2004; Yesudhasan et al., 2014). The results of this study suggest that LAB and *Br. thermosphacta* were the fastest growing spoilage organisms in salmon fillets. This has also been observed in studies carried out by Parlapani et al. (2015), who observed that LAB were co-dominant with *Br. thermosphacta* on European sea bass (*Dicentrarchus labrax*) fillets stored under MAP at 2°C.

The presence of luminous colonies on Long and Hammer agar indicated the presence of *Photobacterium phosphoreum* a common spoilage organism of MAP fish (Dalgaard et al., 1997; NMKL, 2006). The luminous colonies were most prevalent in the CO<sub>2</sub> rich atmospheres of MAP and SP.

Even though MAP showed greater success in reducing microbial growth, the Irish seafood industry mostly uses SP technology (personal communication, Oceanpath, Howth, Co. Dublin). Reasons for this include lower cost and effectiveness in reducing oxidation (Kachele et al., 2017), however the primary reason is that SP maintain a better physical appearance throughout storage (Silbande et al., 2018). A disadvantage of MAP is that a suitable gas to product ratio (g/p) must be established to be effective. Failure to optimise the gas ratio can result in little or no inhibition of bacterial growth, package collapse or product discoloration (Fernández et al., 2010; Stenstrom, 1985).

It was concluded that both MAP and SP were successful at inhibiting microbial growth. MAP was significantly more successful than SP, which suggests that the Irish seafood industry should consider adopting MAP technologies into current processing methods. Overall CA, LA, CIT, CAR, THY and EUG were not effective antimicrobial treatments, however the limited success of the 5% (w/v) CA and LA treatments observed may warrant further investigation.

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**Chapter 8 - Investigating the effect of sub-zero storage  
on the microbial shelf-life of skin packed Atlantic salmon  
(*Salmo salar*)**

### **8.1. Summary**

While the majority of fish processors store processed fish fillets in skin-packs at approximately 2°C, a small number use sub-zero temperatures (approximately -2°C) to enhance microbial shelf-life. The objective of this study was to investigate the effect of these 2 storage temperatures on the microbiology (mesophilic and psychrophilic total viable counts, total *Enterobacteriaceae*, hydrogen sulphide producing bacteria, lactic acid bacteria, *Photobacterium* spp., *Br. Thermosphacta* and *Clostridium* spp.) of skin-packed salmon (*Salmo salar*) fillets. The data obtained in this study suggests that storing skin packed salmon fillets at -2°C as compared to 2°C considerably retards ( $P < 0.05$ ) bacterial growth and extends shelf-life.

## 8.2. Introduction

In 2017, the Irish seafood market was worth €1.15 billion, an increase of over 6% from the previous year (BIM, 2018). In 2017, Irish seafood retail sales amounted to €249 million with domestic sales of Atlantic salmon (*Salmo salar*) valued at €96 million (BIM, 2018). With increasing consumer demand for fresh fish and ever extending transport chains, there is a need for preservation techniques that inhibit microbial growth (Alfaro, Hernández, Balino-Zuazo, et al., 2013; Duun and Rustad, 2007; Fernández et al., 2009). Storage temperature is a key method to preserve fresh fish (Sigholt et al., 1997) however; to further reduce the growth rates of spoilage bacteria and extend shelf life it is necessary to combine low temperature storage with other preservation methods such as packaging. In the Irish seafood industry, skin pack (SP) technology is predominantly used to inhibit microbial growth and prolong the shelf-life of fresh fish (personal communication, John Fagan, BIM). SP uses a low vacuum to shrink a thin film tightly around the fillet creating an anaerobic environment (Łopacka et al., 2016; Nassu et al., 2012). Moreover, skin packs are more attractive to the consumer and are generally considered to enhance shelf-life (Vázquez et al., 2004). Indeed, previous studies have shown that SP is successful in prolonging the shelf-life of several fish species, including; Atlantic salmon (Duun and Rustad, 2008), sardines (Özogul et al., 2004) and silver carp (Kachele et al., 2017).

Due to the perishability of seafood most packaging techniques are only effective if stored below 4°C as any beneficial effects will decrease as storage temperatures increase (Alfaro, Hernández, Le Marc, et al., 2013; Reddy et al., 1995; Sigholt et al., 1997). The European Commission (Regulation (EC) No. 853/2004) does not specify a temperature for the storage and transport of fish and only states that the temperature must be of that approaching melting ice (usually interpreted as 0-2°C), however some processors are

storing products at sub-zero temperatures (approx.. -2°C) in the belief that it can substantially enhance shelf-life.

The aim of this study was to investigate the immediate and storage effects of two temperatures on mean bacterial (mesophilic total viable counts (TVC<sub>m</sub>), psychrophilic total viable counts (TVC<sub>p</sub>), total *Enterobacteriaceae* (TEC), hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Photobacterium* spp., *Br. Thermosphacta* and *Clostridium* spp.) counts on salmon stored in SP for 30 days.

### **8.3. Materials and Methods**

#### **8.3.1. Fish Samples**

Fresh Atlantic salmon fillets were obtained from a local fish processing plant (Oceanpath, Howth, Dublin). The salmon were of a consistent size (3-4kg) and were obtained within 48hrs of capture. The fish were transported on ice to the laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15) within one hour.

#### **8.3.2. Sample Packaging**

Prior to packaging, fillets were aseptically divided into 50g mini fillets (n=60). Skin packaging was carried out using a T-200 semi-automatic tray sealer (Multivac Ireland, Dublin, Ireland). Skin pack trays were made of R-PET (Holfeld Plastics, Wicklow, Ireland) and the film used consisted of PE/EVOH (Südpack, Ochsenhausen, Germany). The O<sub>2</sub> permeability for the film was  $\leq 2$  cm<sup>3</sup>/m<sup>2</sup> d bar. Once packaging was complete the samples were transported under refrigeration to the laboratory (Teagasc Food Research Centre, Ashtown, Dublin 15). SP fillets were stored as follows; 30 samples at 2°C (control samples) and 30 samples at sub-zero (-2°C).

#### **8.3.3. Microbiological Testing**

On days 0, 3, 6, 9, 12, 15, 18, 21, 24 and 30 microbiological analysis was carried out. Each of the fish samples (25g) was homogenized (Pulsifier ® PUL100E, Microgen Bioproducts Ltd, Surrey, United Kingdom) for 1 minute in 225ml MRD and a ten-fold dilution series prepared up to 10<sup>-7</sup>. Samples were plated in duplicate. Plate count agar (PCA) (Oxoid,

Basingstoke, United Kingdom (CM0325)), with 1% NaCl was used to calculate total viable counts (TVC) for both mesophilic (TVC<sub>m</sub>, incubated at 30°C for 72h) and psychrotrophic (TVC<sub>p</sub>, incubated at 6.5°C for 240h) bacteria using a standard spread plate techniques. A standard pour plate technique was used to enumerate 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)) at 30°C for 72h. *Br. thermosphacta* were enumerated on streptomycin-thallos acetate-actidione (STAA) agar base (Oxoid, Basingstoke, United Kingdom (CM0881)), supplemented with STAA (Oxoid, Basingstoke, United Kingdom (SR0151E)) incubated at 25°C for 72h, *Photobacterium* spp. was tested using Long & Hammer agar, per ingredients used by NMKL (2006) No.184, incubated at 15°C for 168h and *Clostridium* spp. counts were incubated, anaerobically, on reinforced clostridial agar (RCA) (Oxoid, Basingstoke, United Kingdom (CM0151B)) at 30°C for 72h using AnaeroGen sachets (Oxoid, Basingstoke, United Kingdom (AN0035A)) and a GENbox Jar 7.0L (Biomérieux Ltd, Basingstoke, United Kingdom). All four media were inoculated using standard spread plate techniques.

#### 8.3.4. 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  $a_w$ , 10g of fillet was 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 fillet samples was measured using a Decagon AquaLab LITE water

activity meter (Labcell Ltd, Alton, United Kingdom) according to the manufacturer's instructions. The thickness, length and width of each 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<sup>2</sup>.

During storage, EL-USB-2 temperature data loggers (Lascar Electronics, Whiteparish, United Kingdom) recorded the ambient temperature of the storage environment.

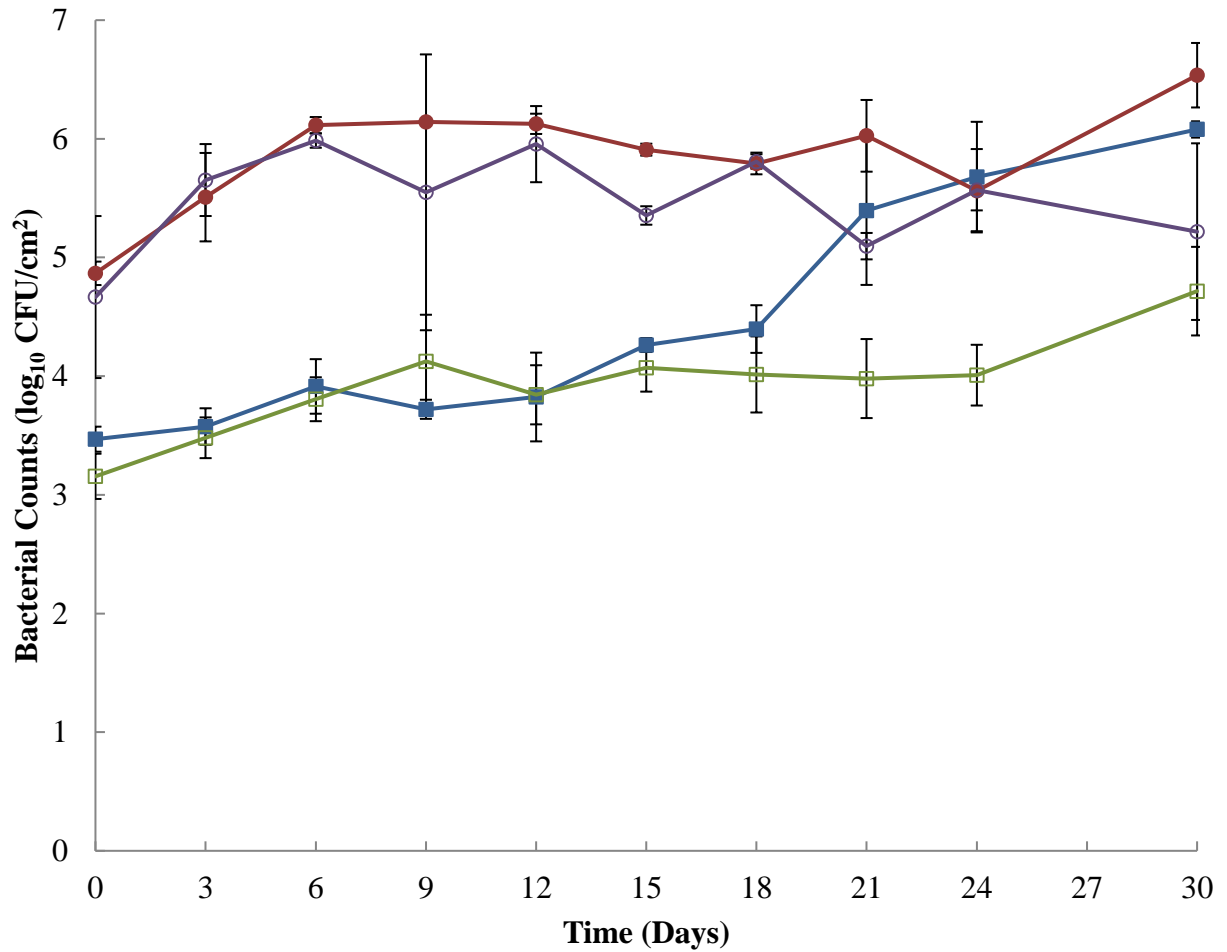
#### 8.3.5. Statistical Analysis

Each sample was plated in duplicate for each of the bacterial groups examined and the experiment was carried out in triplicate. Bacterial counts were converted to log<sub>10</sub> CFU/cm<sup>2</sup>. 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:  $G = t/3.3 \log b/B$ , 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). Differences between mean values were compared using a two way analysis of variance (ANOVA) with significance defined as P < 0.05, with Tukey's multiple comparison test where applicable. Graph Pad Prism v7.0 software (Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

#### 8.4. Results

The initial mean pH of the salmon fillets was 6.3 (data not shown). After 30 days storage, the pH decreased to approximately pH 5.8, for both storage temperature. The  $a_w$  remained between 0.98 and 1.0, regardless of the storage temperature (data not shown).

The mean TVC (mesophilic and psychrophilic) on skin packed salmon fillets are shown in Figure 8.1. After 30 days storage, the mean TVC<sub>m</sub> increased from 3.2 log<sub>10</sub> CFU/cm<sup>2</sup> (-2°C) and 3.5 log<sub>10</sub> CFU/cm<sup>2</sup> (2°C) to 4.7 and 6.0 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively. From 21 days storage onwards, significant differences in counts were observed between the two storage temperatures ( $P < 0.05$ ). The initial mean TVC<sub>p</sub> increased by 1.3 log<sub>10</sub> CFU/cm<sup>2</sup> at -2°C and 1.6 log<sub>10</sub> CFU/cm<sup>2</sup> at 2°C throughout the 30 day storage period. Mean TVC<sub>p</sub> were significantly ( $P < 0.05$ ) lower on salmon stored at -2°C at times  $t = 15, 21$  and 30 days.



**Figure 8. 1** Mean mesophilic total viable counts ( $\log_{10}$  CFU/cm<sup>2</sup>) on Atlantic salmon (*Salmo salar*) stored at 2°C (■) and -2°C (□), mean psychrophilic total viable counts on Atlantic salmon stored at 2°C (●) and -2°C (○).

The growth parameters (initial and maximum bacterial concentrations as well as the mean generation times) for TEC, hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. thermosphacta*, *Photobacterium* spp. and *Clostridium* spp. on salmon are presented in Table 8.1. For skin packed salmon fillets the growth parameters were similar regardless of storage temperature, with the exception of the mean generation times for *Clostridium* spp which were 3.7 days at 2°C and 7.6 days at -2°C.

**Table 8. 1** Growth parameters for TEC, hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. thermosphacta*, *Photobacterium* spp. and *Clostridium* spp. as determined from Atlantic salmon (*Salmo salar*) stored at 2°C and sub-zero temperatures for 30 days.

<b>Treatment</b>	<b>Initial concentration (log<sub>10</sub> CFU/cm<sup>2</sup>)</b>	<b>Mean generation time (d)</b>	<b>Maximum concentration observed (log<sub>10</sub> CFU/cm<sup>2</sup>)</b>
<b>TEC</b>			
2°C	1.0	7.0	3.1
-2°C	0.9	8.4	2.1
<b>HSPB</b>			
2°C	2.5	1.5	4.0
-2°C	2.4	1.0	4.0
<b>LAB</b>			
2°C	1.3	2.0	4.7
-2°C	1.2	1.3	4.0
<b><i>Br. thermosphacta</i></b>			
2°C	1.7	4.4	3.2
-2°C	1.8	1.6	3.4
<b><i>Photobacterium</i> spp.</b>			
2°C	5.1	1.1	6.6
-2°C	4.9	1.4	6.3
<b><i>Clostridium</i> spp.</b>			
2°C	1.9	3.7	4.9
-2°C	1.8	7.6	4.0

## 8.5. Discussion

At each storage temperature, the initial TVC<sub>m</sub> counts on salmon ranged between 3.2 and 3.4 log<sub>10</sub> CFU/cm<sup>2</sup>. Other studies have reported initial bacterial levels in fresh salmon of approximately 3 log<sub>10</sub> CFU/g (Briones et al., 2010; Schubring, 2003). These counts are considered indicative of fish of good microbiological quality originating from clean waters (Li et al., 2017), which is also supported by the relatively low TEC (< 1.1 log<sub>10</sub> CFU/cm<sup>2</sup>). Our data suggests that super-chilling at -2°C retarded but did not prevent bacterial growth on salmon as the TVC<sub>m</sub> increased by 1.5 log<sub>10</sub> CFU/cm<sup>2</sup> during the course of the study. Although the TVC<sub>m</sub> at -2°C were significantly lower after 21 days storage than those obtained at 2°C on salmon, these findings are likely of little practical significance.

However, other studies have reported significant inhibition in the growth of spoilage bacteria and our TEC data suggests storage at -2°C may prolong shelf-life. Thus, the increase in TEC of 1.2 log<sub>10</sub> CFU/cm<sup>2</sup> obtained on salmon at -2°C was approximately half that recorded for fillets stored at 2°C. Thus, our *Enterobacteriaceae* data would suggest that while skin packaging may extend the shelf-life of fish, this can be considerably enhanced by combining with super-chilling temperatures, a cheaper alternative to MAP, which is currently used retard the *Enterobacteriaceae* on fish (Milijasevic et al., 2015; Radetic et al., 2007).

Both *Pseudomonas* spp. and HSPB growth remained low under both storage temperatures, and were not significantly different. It was no surprise that HSPB growth was low and not affected by a reduced temperature as these organisms are primarily associated with aerobic spoilage and struggle to grow in an anaerobic environment (Calliauw et al., 2016; Nirmal and Benjakul, 2011). However, *Br. thermosphacta* has been shown to be the dominant or the co-dominant spoilage organism with LAB or *P. phosphoreum* on fish stored

anaerobically (Parlapani et al., 2014; Thiansilakul et al., 2013), yet observed growth in the current study was low in samples stored at both the 2°C and -2°C. Previous studies have concluded that, under aerobic conditions, *Br. thermosphacta* may be outcompeted by other psychrotrophic spoilage organisms (Gram and Huss, 1996). It is possible that in this study *Br. thermosphacta* was out competed by LAB and *Photobacterium* spp., which were the dominant spoilage organisms present at the end of the trial.

Anaerobic, psychrophilic bacteria such as *Photobacterium* spp. and LAB are an issue in the food industry as they have the ability to colonise anaerobically packed, fresh seafood (Emborg et al., 2002; Rudi et al., 2004; Yesudhason et al., 2014). Throughout our study sub-zero temperatures successfully inhibited the growth of LAB on salmon (t = 3, 6, 21, 24 and 30). The presence of luminous colonies on Long and Hammer agar indicated the presence of *Photobacterium phosphoreum*, a common spoilage organism of anaerobically packed fish (Dalgaard et al., 1997; NMKL, 2006). These luminous colonies were prevalent under all storage temperatures; however growth was significantly reduced on salmon (t = 15, 21, 24 and 30) stored at -2°C when compared to equivalent samples stored at 2°C.

Throughout this trial, *Clostridium* spp. growth rates were lower on salmon fillets stored under sub-zero conditions, but were not statistically different to those stored at the higher temperature.

In conclusion results from this trial suggest that sub-zero storage temperatures are suitable for inhibiting microbial growth on salmon fillets. This was also observed by Duun and Rustad (2008), who found that SP salmon fillets were more suitable to sub-zero storage than SP cod fillets. Sub- zero storage temperatures combined with SP were successful at inhibiting microbial growth. Problematic spoilage bacteria such as LAB and

*Photobacterium* spp. were significantly reduced, which may suggest that the Irish seafood industry should consider incorporating these conditions into the current processing methods.

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## **Chapter 9 – General Discussion**

## 9.1. General Discussion

Seafood is a nutritionally and economically beneficial product and consumer demand has been increasing. As a result the Irish seafood market value is growing year by year and in 2017 the overall seafood GDP grew by 6.4% to €1.15 billion (BIM, 2018). However, fresh seafood is highly perishable and consequently has a short shelf-life of approximately 9-10 days (Alfaro et al., 2013; Kulawik et al., 2013). This limits Ireland's export market potential. To maintain growth in the seafood sector it will be necessary to develop technologies to extend the shelf-life of fish which will allow expansion into new export markets. A 24 hour extension in shelf-life could enhance exports by allowing product to be conveyed *via* several hubs throughout Europe and by providing additional time for chilled retail display. It would also result in lower levels of product wastage and maximise utilisation of the catch (personal communication, John Fagan, BIM). Therefore it is essential to not only maintain a product of excellent quality, but to explore new analytical and processing methods to enhance product quality and shelf-life

As Atlantic salmon is Ireland's most valuable seafood product, topping both exports (€121m) and domestic retail sales (€96m) in 2017 (BIM, 2018), the current study explored potential approaches for improving analytical methods for assessing freshness employed by seafood processors and also looked at the effectiveness of a range of natural ingredients both alone and in combination with packaging and chilled storage temperatures to inhibit spoilage organisms.

In order to maximise product quality, it is essential to understand what bacteria are involved in the spoilage of fresh salmon. The first part of this research aimed to characterise the microflora of salmon stored under chilled (2°C) aerobic conditions thus

providing data which could be used to identify the most appropriate bacteria for shelf-life determination.

In the current study culture based methods were used and it was observed that initial levels of HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. were similar to the TVC but considerably higher than initial levels of TEC. Moreover, 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 low temperature waters where the salmon are 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). By the end of shelf-life (10 days), TVC ranged from 5.1 to 6.0 log<sub>10</sub> CFU/cm<sup>2</sup> and spoilage bacterial (HSPB, LAB, *Pseudomonas* spp. and *Photobacterium* spp.) counts ranged from 4.8 to 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>. This is in agreement with Robson et al. (2007), who found seafood spoiled when the bacterial count reached 5 to 6 log<sub>10</sub> CFU/cm<sup>2</sup>.

Using MiSeq Illumina high throughput sequencing of the 16S rRNA gene, it was observed that spoilage bacteria belonging to the phylum Proteobacteria such as; *Pseudomonas* spp. and *Photobacterium* spp. were present in both the DI and PI, whereas *Shewanella* spp. was only present in the latter. All genera possess strains pathogenic to fish, but are also responsible for the post mortem degradation of fish quality through the production of volatile compounds. These spoilage organisms are ubiquitous in the marine environment and possess the ability to colonise the gastrointestinal (GI) tract of salmon, which was also a common observation in previous studies (Nayak, 2010; Revenco et al., 2014). Thus

HSPB, LAB, *Pseudomonas* spp. or *Photobacterium* spp. counts may be a better microbiological indicator of shelf-life than total bacterial counts, with fish considered to be spoiled when these spoilage bacteria reach 5 to 6 log<sub>10</sub> CFU/g or CFU/cm<sup>2</sup>. However it is still unclear as to the function of the bacteria within the GIT of salmon. It is unlikely that spoilage organisms colonising the GIT play a part in quality degradation as the GIT tends to be removed immediately *post-mortem*.

It has been suggested that the close proximity to the stomach may support a broader range of bacteria which in turn aids digestion. Firmicutes were common in DI and PI samples. It is generally accepted that bacteria belonging to this phylum play an important role in the conversion of dietary carbohydrates to short chain fatty acids such as acetate, propionate and butyrate which may be used by the fish as an energy source. However, more diverse microbial populations may be associated with an increased competition for nutrients and adhesion sites which in turn may provide protection against pathogenic organisms. *Lactobacillus* spp., *Enterococcus* spp., *Lactococcus* spp. and *Carnobacterium* spp. were present in relatively high concentrations in 80% of PI samples in this study. These lactic acid bacteria (LAB) have been previously shown to have a protective effect against pathogenic genera such as *Aliivibrio* spp. and *Vibrio* spp. within the foregut of Atlantic salmon (Ringø et al. 2007; Ringø 2008).

The dominant phyla, regardless of GIT sample, were the Firmicutes. This phylum has been shown to be an important constituent of the gut microbiome of salmon (Holben et al., 2002). Our data supports the hypothesis that salmon are a specific host for these bacteria, regardless of geographical location (Lyons et al., 2016). This finding may be of concern for fish producers, as several genera belonging to the *Mycoplasmataceae* family, including

*Mycoplasma mobile*, have been associated with necrosis in fish (Adan-Kubo et al. 2012) while other *Mycoplasma* species are human pathogens (Holben et al., 2002).

The presence of *Pseudomonas* spp., *Shewanella* spp. and *Photobacterium* spp. was particularly significant as these bacteria produce volatile organic compounds which contribute to fish spoilage, resulting in a negative effect on the sensory attributes of fish (Møretrø et al., 2016). Therefore, the second part of this study aimed to develop and validate rapid sensory (QIM and QDA) and ATP derivative based methods for assessing the freshness of salmon.

The QIM developed for salmon provided a good description of the sensory changes that occurred during aerobic chilled storage with ‘cloudy, dull, sunken’ eyes and ‘brown, shrivelled’ gills early indicators as loss of freshness. There was a linear relationship between QIM scores and both TVC and time which suggested this scheme would be suitable to assess fish freshness. This was complemented by the QDA for cooked fish. Other studies have also reported a linear relationship between QIM score and time for salmon for salmon (Sveinsdottir et al., 2003; Sveinsdottir et al., 2002).

In this study the IMP concentration decreased over the 10 days aerobic storage at 2°C. Inosine levels did not change and only a minor increase was observed in the Hx concentration. However Karahadian et al. (1997) suggested that the use of the IMP/Hx ratio, K1 value  $\left(\frac{I + Hx}{IMP + I + Hx}\right) \times 100$  or H value  $\left(\frac{Hx}{IMP + I + Hx}\right) \times 100$  ratios were a better indicator of freshness as they take into account the concentrations of all the ATP derivatives. In our study the H-value increase occurred linearly for both TVC and time, whereas the relationships between IMP/HX ratio and K1 values were non-linear. Other studies on the best ATP derivative/ratio for monitoring fish freshness are contradictory (Bremner, 1985; Sallam, 2007). This was not unexpected as nucleotide

degradation rates depend on a range of factors including fish maturity, muscle type, stress during capture and storage conditions (Huss, 1995; Luong et al., 1992). This suggests that ATP ratio methods for monitoring fish freshness may not be ideal as the rates of stress may differ from batch to batch. This author believes that it is not possible to ensure that each individual fish is reared, fed, caught, killed and stored under the exact same conditions. Each fish may experience stress at different levels and therefore the production of ATP catabolites will differ. These differences may be too large to be a reliable representative of the batch. It is of this author's opinion that freshness analysis should be based on a factor that all fish are exposed to equally i.e. bacteria and their toxins or metabolites (TVBN)

The research then moved onto extending shelf-life using natural antimicrobials derived from plants. The immediate and storage effects of organic acid (CA and LA) and essential oil (CIT, CAR, THY and EUG) dip treatments (30 seconds at 20°C) on mean bacterial counts on Atlantic salmon (*Salmo salar*) fillets (stored at 2°C aerobically) was investigated.

In this study, neither CA nor LA at 5% (v/v) significantly ( $P > 0.05$ ) reduced the TVC<sub>m</sub>, TVC<sub>p</sub> or TEC on the salmon fillets. This is in contrast to previous studies on hake and megrim (García-Soto et al., 2014), and chub mackerel (Metin et al., 2001) where the use of organic acids significantly reduced TVC over the course 12-15 days. These differences may be due to the different methods of acid application. In our study the fish were immersed in organic acid solutions for no more than 30 seconds whereas Metin et al. (2001) used a dip treatment for 30 minutes, and García-Soto et al. (2014) applied the acids in an ice-slurry. Although CA and LA failed to significantly reduce the growth of indicator bacteria, they significantly reduced HSPB growth for the majority of the 18 day storage

period (CA (t = 2, 4, 6, 8 and 10), LA (t = 2, 4, 6, 8, 10, 12 and 14)). This is an important observation as the HSPB group includes *Shewanella* spp., a bacterial genera largely responsible for the spoilage of aerobically stored fish (Mørretrø et al., 2016). Commonly occurring in marine environments, *Shewanella* spp. is likely not exposed to acidic conditions and may not possess the genetic machinery to adapt to these organic acids making them more susceptible when treated.

Both CIT and CAR caused a significant ( $P < 0.05$ ) reduction in all the spoilage bacteria immediately following treatment, however these reductions were not maintained during storage. Overall, CIT, CAR, THY and EUG did not significantly reduce the bacterial concentration for the majority of treatment combinations.

The limited success of CA, LA, CIT and CAR warranted further investigation with the potential of greater success when paired with packaging technologies. Therefore the next study investigated the antimicrobial effect of a range of natural compounds combined with packaging technologies on the microflora of salmon fillets during chilled storage.

Firstly, the study looked at combining the use of antimicrobial treatments with packaging technologies (modified atmosphere packaging (MAP) and skin packaging (SP)) on salmon fillets stored at 2°C for 18 days. Bacterial growth on both MAP and SP salmon fillets was significantly reduced throughout the storage trial. However when statistically analysed it was apparent that within each packaging type, the antimicrobial treatments had very little if no additional effect. Both 5% CA and LA dip treatments had a significant additive effect on HSPB growth under both MAP and SP conditions. This was expected as HSPB are most commonly associated with aerobic spoilage. It is possible that the anaerobic packaging conditions induced stress that may have made the HSPB cells more susceptible to acidic treatments.

Bacterial growth on MAP fillets was significantly lower than SP which may suggest that the Irish seafood industry should incorporate MAP technologies into the current processing methods. Overall CA, LA, CIT, CAR, THY and EUG were not effective antimicrobial treatments. It is possible the relatively high fat content of salmon renders these fish unsuitable for essential oil treatment. Mejlholm and Dalgaard (2002) showed that oregano oil is more effective against *Photobacterium phosphoreum* on cod fillets than salmon attributing the difference to the relatively high fat content in the latter.

As packaging was the only significantly successful treatment, the final part of this study looked to further optimise SP by combining with a reduced (sub-zero) storage temperature. Sub-zero storage temperatures combined with SP successfully inhibited the growth of indicator bacteria and also significantly reduced the growth of anaerobic spoilage bacteria such as LAB and *Photobacterium* spp. which may suggest that the Irish seafood industry should incorporate these conditions into the current processing methods. This may provide the solution needed to help the Irish seafood export market to grow

## 9.2. Main Findings and Conclusions

- Hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. all contribute to the spoilage of salmon stored aerobically at 2°C and the growth of these organisms may be a better indicator of fish spoilage with a count of 5-6 log<sub>10</sub> CFU/cm<sup>2</sup>, indicating the end of shelf-life (Chapter 3).
- Spoilage bacteria were found in all regions of the GI tract of salmon; however there was a greater microbial diversity in the proximal region rather than the distal region (Chapter 4).
- There were 20 common operational taxonomic units, regardless of location within the GI tract, which suggests the presence of a core microbiome (Chapter 4).
- QIM and QDA schemes developed in this study may be used as a rapid sensory-based tool for assessing the freshness of salmon with ‘cloudy, dull and sunken’ eyes and ‘brown shrivelled’ gills providing early indicators of loss of freshness of whole fish (Chapter 5).
- The concentrations of ATP derivatives were not reliable assessors of freshness; however the H-value may be a suitable ATP derivative ratio for assessing salmon freshness (Chapter 5).
- CA, LA, TSP, CIT, CAR, THY and EUG were not effective antibacterial treatments for salmon fillets when used at concentrations above which the sensory properties of the fish may be affected (Chapter 6).
- MAP and SP significantly reduced bacterial growth on salmon fillets stored at 2°C for 18 days (Chapter 7)

- The combination of CA, LA, CIT, CAR, THY and EUG with packaging technologies did not provide any additional significant inhibitory effects (Chapter 7).
- Sub-zero (-2°C) storage temperatures combined with SP was successful inhibiting microbial growth over a 30 day storage trial (Chapter 8).

### **9.3. Future Work**

As the QIM developed for salmon provided a good description of the sensory changes that occurred during aerobic chilled storage with ‘cloudy, dull, sunken’ eyes and ‘brown, shrivelled’ gills early indicators as loss of freshness, going forward it may be suggested that this method should be incorporated into the Irish seafood industry . The linear relationship between QIM scores and both TVC and time suggest that this scheme would be suitable to assess fish freshness. It is also a non-time consuming method of freshness analysis which is essential for maximising shelf-life.

As it is still unclear as to the function of the bacteria within the GIT of salmon, further research is required as to what role the microbiome plays in fish quality and health. Next generation sequencing methods should be used to analyse the flora down to species level in the hope that it may shed some insight into the function of the microbiome.

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**Appendix A – Chapter 6 Tables for mean bacterial  
counts on salmon fillets treated with organic acids,  
essential oil components and trisodium phosphate (TSP)**

**Table 1.** Mean TVC<sub>m</sub>, TVC<sub>p</sub>, TEC, HSPB, LAB, *Pseudomonas* spp., *Br. Thermosphacta* and *Photobacterium* spp. counts (log<sub>10</sub> CFU/cm<sup>2</sup>) on salmon fillets treated with 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA) or 12% (w/v) trisodium phosphate (TSP) and stored at 2°C for 18 days.

Time (d)	Treatment									
	CTL		SDW		CA		LA		TSP	
	<b>TVC<sub>m</sub></b>									
	<b>Log</b>	<b>SE<sup>1</sup></b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>
<b>0</b>	3.5 <sup>A</sup>	0.2	3.5 <sup>A</sup>	0.5	3.0 <sup>A</sup>	0.3	2.9 <sup>A</sup>	0.3	3.3 <sup>A</sup>	0.4
<b>2</b>	4.0 <sup>A</sup>	0.5	4.3 <sup>A</sup>	0.8	3.5 <sup>A</sup>	0.5	3.5 <sup>A</sup>	0.8	3.5 <sup>A</sup>	0.5
<b>4</b>	5.4 <sup>A</sup>	0.8	5.3 <sup>A</sup>	0.9	4.4 <sup>A</sup>	0.7	4.1 <sup>A</sup>	1.0	4.3 <sup>A</sup>	0.9
<b>6</b>	5.3 <sup>A</sup>	0.7	6.0 <sup>A</sup>	0.8	5.1 <sup>A</sup>	0.4	4.9 <sup>A</sup>	0.7	5.1 <sup>A</sup>	0.9
<b>8</b>	6.5 <sup>A</sup>	0.9	6.8 <sup>A</sup>	0.8	6.2 <sup>A</sup>	0.2	5.9 <sup>A</sup>	1.3	6.2 <sup>A</sup>	0.9
<b>10</b>	7.5 <sup>A</sup>	0.5	7.1 <sup>A</sup>	0.3	7.0 <sup>A</sup>	0.1	6.2 <sup>A</sup>	0.9	6.6 <sup>A</sup>	0.6
<b>12</b>	7.3 <sup>A</sup>	0.5	7.4 <sup>A</sup>	0.4	7.5 <sup>A</sup>	0.3	6.5 <sup>A</sup>	0.9	6.5 <sup>A</sup>	1.0
<b>14</b>	7.7 <sup>A</sup>	0.3	8.0 <sup>A</sup>	0.5	7.7 <sup>A</sup>	0.4	7.1 <sup>A</sup>	0.5	7.3 <sup>A</sup>	0.7
<b>16</b>	8.0 <sup>A</sup>	0.4	7.9 <sup>A</sup>	0.5	8.0 <sup>A</sup>	0.2	7.7 <sup>A</sup>	0.5	7.7 <sup>A</sup>	0.7
<b>18</b>	7.9 <sup>A</sup>	0.2	8.5 <sup>A</sup>	0.4	8.0 <sup>A</sup>	0.5	8.0 <sup>A</sup>	0.5	7.7 <sup>A</sup>	0.6
	<b>TVC<sub>p</sub></b>									
<b>0</b>	3.2 <sup>A</sup>	0.3	3.3 <sup>A</sup>	0.6	2.5 <sup>A</sup>	0.4	2.5 <sup>A</sup>	0.3	2.6 <sup>A</sup>	0.4
<b>2</b>	3.6 <sup>A</sup>	0.5	4.1 <sup>A</sup>	0.9	2.8 <sup>A</sup>	0.3	2.4 <sup>A</sup>	0.5	3.1 <sup>A</sup>	0.5
<b>4</b>	4.8 <sup>A</sup>	0.5	5.2 <sup>A</sup>	1.1	3.9 <sup>A</sup>	0.7	3.6 <sup>A</sup>	0.9	4.4 <sup>A</sup>	0.6
<b>6</b>	6.1 <sup>A</sup>	0.6	5.9 <sup>A</sup>	0.7	5.4 <sup>A</sup>	0.3	5.1 <sup>A</sup>	1.1	5.4 <sup>A</sup>	1.0

<b>8</b>	6.7 <sup>A</sup>	0.7	7.2 <sup>A</sup>	0.5	6.6 <sup>A</sup>	0.3	6.1 <sup>A</sup>	1.3	6.4 <sup>A</sup>	0.7
<b>10</b>	7.7 <sup>A</sup>	0.5	7.4 <sup>A</sup>	0.4	7.4 <sup>A</sup>	0.2	6.8 <sup>A</sup>	1.1	6.7 <sup>A</sup>	0.9
<b>12</b>	7.7 <sup>A</sup>	0.2	7.8 <sup>A</sup>	0.2	7.9 <sup>A</sup>	0.2	6.8 <sup>A</sup>	0.8	6.8 <sup>A</sup>	0.7
<b>14</b>	8.1 <sup>A</sup>	0.4	8.4 <sup>A</sup>	0.6	8.3 <sup>A</sup>	0.0	7.8 <sup>A</sup>	0.6	7.6 <sup>A</sup>	0.6
<b>16</b>	8.2 <sup>A</sup>	0.2	8.2 <sup>A</sup>	0.3	8.1 <sup>A</sup>	0.3	8.2 <sup>A</sup>	0.5	7.7 <sup>A</sup>	0.4
<b>18</b>	8.0 <sup>A</sup>	0.4	8.2 <sup>A</sup>	0.4	7.9 <sup>A</sup>	0.5	8.1 <sup>A</sup>	0.5	8.0 <sup>A</sup>	0.6
<b>TEC</b>										
<b>0</b>	1.6 <sup>A</sup>	0.3	1.3 <sup>A</sup>	0.5	1.4 <sup>A</sup>	0.3	1.0	0.5	1.1	0.3
<b>2</b>	1.5 <sup>A</sup>	0.6	1.8 <sup>A</sup>	0.7	1.5 <sup>A</sup>	0.5	2.2	0.4	1.7	0.2
<b>4</b>	2.2 <sup>A</sup>	0.3	2.9 <sup>A</sup>	0.4	2.2 <sup>A</sup>	0.5	2.2	0.3	1.8	0.2
<b>6</b>	2.4 <sup>A</sup>	0.2	3.2 <sup>A</sup>	0.8	2.5 <sup>A</sup>	0.3	2.6	0.5	2.6	0.4
<b>8</b>	2.9 <sup>A</sup>	0.3	3.5 <sup>A</sup>	0.8	3.0 <sup>A</sup>	0.3	3.1	0.8	3.0	0.8
<b>10</b>	3.4 <sup>A</sup>	0.3	3.5 <sup>A</sup>	0.4	3.7 <sup>A</sup>	0.3	3.2	0.9	3.4	0.5
<b>12</b>	2.8 <sup>A</sup>	0.0	3.2 <sup>A</sup>	0.0	4.8 <sup>B</sup>	0.8	2.3 <sup>A</sup>	0.6	2.7 <sup>A</sup>	0.5
<b>14</b>	3.1 <sup>A</sup>	0.3	4.0 <sup>AB</sup>	0.3	4.8 <sup>B</sup>	0.5	3.6 <sup>AB</sup>	1.2	3.8 <sup>AB</sup>	0.9
<b>16</b>	3.7 <sup>A</sup>	0.3	4.0 <sup>A</sup>	0.3	4.3 <sup>A</sup>	0.5	3.5 <sup>A</sup>	1.0	3.3 <sup>A</sup>	0.8
<b>18</b>	4.2 <sup>A</sup>	0.6	5.3 <sup>A</sup>	0.4	4.7 <sup>A</sup>	0.2	4.8 <sup>A</sup>	0.5	4.9 <sup>A</sup>	0.6
<b>HSPB</b>										
<b>0</b>	3.0 <sup>A</sup>	0.4	3.0 <sup>A</sup>	0.6	1.6 <sup>B</sup>	0.2	1.6 <sup>B</sup>	0.2	2.0 <sup>A</sup>	0.2
<b>2</b>	4.0 <sup>A</sup>	0.2	4.0 <sup>A</sup>	0.6	2.9 <sup>B</sup>	0.1	1.6 <sup>C</sup>	0.4	3.1 <sup>AB</sup>	0.2
<b>4</b>	4.9 <sup>A</sup>	0.3	4.7 <sup>A</sup>	0.9	3.5 <sup>B</sup>	0.1	3.0 <sup>B</sup>	0.5	4.2 <sup>AB</sup>	0.5
<b>6</b>	5.7 <sup>A</sup>	0.6	6.1 <sup>A</sup>	0.5	4.5 <sup>B</sup>	0.4	3.8 <sup>B</sup>	0.6	5.4 <sup>A</sup>	0.7
<b>8</b>	6.5 <sup>A</sup>	0.7	6.5 <sup>A</sup>	0.6	4.9 <sup>A</sup>	0.5	4.4 <sup>A</sup>	0.7	6.0 <sup>A</sup>	0.8

<b>10</b>	6.8 <sup>A</sup>	0.6	6.7 <sup>A</sup>	0.3	5.3 <sup>B</sup>	0.5	5.3 <sup>B</sup>	0.7	6.5 <sup>A</sup>	0.7
<b>12</b>	7.2 <sup>A</sup>	0.2	7.1 <sup>A</sup>	0.2	6.6 <sup>A</sup>	0.0	5.5 <sup>B</sup>	0.6	6.5 <sup>A</sup>	0.7
<b>14</b>	6.9 <sup>AB</sup>	0.4	7.4 <sup>A</sup>	0.6	6.4 <sup>AB</sup>	0.4	5.8 <sup>B</sup>	0.7	6.9 <sup>AB</sup>	0.5
<b>16</b>	7.0 <sup>A</sup>	0.4	7.1 <sup>A</sup>	0.2	6.0 <sup>A</sup>	0.6	6.2 <sup>A</sup>	0.5	6.9 <sup>A</sup>	0.4
<b>18</b>	7.1 <sup>A</sup>	0.4	7.3 <sup>A</sup>	0.3	6.1 <sup>A</sup>	0.4	6.7 <sup>A</sup>	0.6	7.0 <sup>A</sup>	0.6
<b>LAB</b>										
<b>0</b>	3.2 <sup>A</sup>	0.3	3.2 <sup>A</sup>	0.0	2.6 <sup>B</sup>	0.2	2.6 <sup>B</sup>	0.1	2.9 <sup>AB</sup>	0.1
<b>2</b>	3.2 <sup>A</sup>	0.2	3.8 <sup>A</sup>	0.4	2.7 <sup>AB</sup>	0.2	2.3 <sup>B</sup>	0.1	2.7 <sup>AB</sup>	0.1
<b>4</b>	3.5 <sup>A</sup>	0.2	4.0 <sup>A</sup>	0.9	3.3 <sup>A</sup>	0.2	3.0 <sup>A</sup>	0.1	3.4 <sup>A</sup>	0.2
<b>6</b>	4.4 <sup>A</sup>	0.2	4.8 <sup>A</sup>	0.6	4.1 <sup>A</sup>	0.1	3.6 <sup>A</sup>	0.4	4.1 <sup>A</sup>	0.5
<b>8</b>	4.9 <sup>A</sup>	0.4	5.2 <sup>A</sup>	0.6	5.0 <sup>A</sup>	0.1	4.3 <sup>A</sup>	0.8	4.9 <sup>A</sup>	0.8
<b>10</b>	5.5 <sup>A</sup>	0.2	5.8 <sup>A</sup>	0.5	5.8 <sup>A</sup>	0.4	4.7 <sup>A</sup>	0.7	5.5 <sup>A</sup>	0.5
<b>12</b>	5.7 <sup>AB</sup>	0.2	5.5 <sup>AB</sup>	0.1	6.6 <sup>A</sup>	0.0	4.7 <sup>B</sup>	0.6	5.5 <sup>AB</sup>	0.5
<b>14</b>	5.7 <sup>A</sup>	0.1	6.4 <sup>A</sup>	0.3	6.0 <sup>A</sup>	0.3	5.5 <sup>A</sup>	0.9	5.8 <sup>A</sup>	0.4
<b>16</b>	6.1 <sup>A</sup>	0.2	6.2 <sup>A</sup>	0.1	6.0 <sup>A</sup>	0.1	5.7 <sup>A</sup>	0.8	6.0 <sup>A</sup>	0.4
<b>18</b>	6.2 <sup>A</sup>	0.2	6.2 <sup>A</sup>	0.0	6.1 <sup>A</sup>	0.1	6.2 <sup>A</sup>	0.7	6.4 <sup>A</sup>	0.3
<b><i>Pseudomonas</i> spp.</b>										
<b>0</b>	3.1 <sup>A</sup>	0.5	3.1 <sup>A</sup>	0.7	2.3 <sup>B</sup>	0.7	2.3 <sup>B</sup>	0.6	2.2 <sup>B</sup>	0.5
<b>2</b>	3.9 <sup>A</sup>	0.6	4.4 <sup>A</sup>	0.9	3.2 <sup>AB</sup>	0.3	2.8 <sup>B</sup>	0.4	3.2 <sup>AB</sup>	0.6
<b>4</b>	5.3 <sup>AB</sup>	0.6	5.6 <sup>A</sup>	0.7	4.8 <sup>AB</sup>	0.4	4.0 <sup>B</sup>	1.0	4.5 <sup>AB</sup>	0.8
<b>6</b>	6.1 <sup>A</sup>	0.6	6.1 <sup>A</sup>	0.6	5.9 <sup>A</sup>	0.4	5.2 <sup>A</sup>	1.3	5.4 <sup>A</sup>	1.0
<b>8</b>	7.3 <sup>A</sup>	0.9	7.3 <sup>A</sup>	0.5	7.1 <sup>A</sup>	0.4	6.2 <sup>A</sup>	1.7	6.5 <sup>A</sup>	1.1
<b>10</b>	7.8 <sup>A</sup>	0.5	7.6 <sup>A</sup>	0.3	7.7 <sup>A</sup>	0.3	6.9 <sup>A</sup>	1.2	7.1 <sup>A</sup>	0.7

<b>12</b>	7.8 <sup>A</sup>	0.2	7.9 <sup>A</sup>	0.2	8.1 <sup>A</sup>	0.2	6.9 <sup>A</sup>	0.8	6.7 <sup>A</sup>	0.8
<b>14</b>	7.8 <sup>A</sup>	0.4	7.9 <sup>A</sup>	0.3	8.2 <sup>A</sup>	0.2	7.6 <sup>A</sup>	0.6	7.6 <sup>A</sup>	0.6
<b>16</b>	8.4 <sup>A</sup>	0.4	8.4 <sup>A</sup>	0.4	8.4 <sup>A</sup>	0.4	8.4 <sup>A</sup>	0.6	8.1 <sup>A</sup>	0.5
<b>18</b>	8.0 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.2	7.8 <sup>A</sup>	0.5	8.3 <sup>A</sup>	0.5	7.8 <sup>A</sup>	0.6
<b><i>Br. Thermosphacta</i></b>										
<b>0</b>	1.7 <sup>A</sup>	0.3	2.1 <sup>A</sup>	0.7	0.9 <sup>A</sup>	0.4	1.1 <sup>A</sup>	0.4	1.2 <sup>A</sup>	0.3
<b>2</b>	2.6 <sup>A</sup>	0.3	3.2 <sup>A</sup>	0.9	2.0 <sup>B</sup>	0.2	1.4 <sup>B</sup>	0.1	2.2 <sup>AB</sup>	0.4
<b>4</b>	3.9 <sup>AB</sup>	0.5	4.4 <sup>AB</sup>	0.9	3.3 <sup>AB</sup>	0.2	2.7 <sup>B</sup>	0.8	3.3 <sup>AB</sup>	0.7
<b>6</b>	4.8 <sup>AB</sup>	0.4	5.2 <sup>A</sup>	0.8	4.5 <sup>AB</sup>	0.3	3.7 <sup>B</sup>	1.0	4.3 <sup>AB</sup>	0.9
<b>8</b>	5.6 <sup>A</sup>	0.4	5.7 <sup>A</sup>	0.5	5.5 <sup>A</sup>	0.1	4.4 <sup>A</sup>	1.2	5.3 <sup>A</sup>	0.9
<b>10</b>	6.1 <sup>A</sup>	0.4	6.1 <sup>A</sup>	0.5	6.0 <sup>A</sup>	0.1	5.0 <sup>A</sup>	0.9	6.0 <sup>A</sup>	0.8
<b>12</b>	6.0 <sup>A</sup>	0.1	6.1 <sup>A</sup>	0.1	6.6 <sup>A</sup>	0.1	5.3 <sup>A</sup>	0.7	5.8 <sup>A</sup>	0.5
<b>14</b>	6.5 <sup>A</sup>	0.2	7.1 <sup>A</sup>	0.4	6.6 <sup>A</sup>	0.0	6.1 <sup>A</sup>	0.7	6.6 <sup>A</sup>	0.3
<b>16</b>	6.8 <sup>A</sup>	0.4	6.7 <sup>A</sup>	0.1	6.8 <sup>A</sup>	0.1	6.0 <sup>A</sup>	0.6	6.8 <sup>A</sup>	0.4
<b>18</b>	6.7 <sup>A</sup>	0.1	6.7 <sup>A</sup>	0.1	6.9 <sup>A</sup>	0.2	6.5 <sup>A</sup>	0.5	6.9 <sup>A</sup>	0.3
<b><i>Photobacterium spp.</i></b>										
<b>0</b>	3.6 <sup>A</sup>	0.3	3.4 <sup>A</sup>	0.4	2.9 <sup>A</sup>	0.3	2.8 <sup>A</sup>	0.3	3.3 <sup>A</sup>	0.4
<b>2</b>	3.5 <sup>A</sup>	0.3	3.9 <sup>A</sup>	0.7	3.0 <sup>A</sup>	0.3	2.7 <sup>A</sup>	0.4	3.0 <sup>A</sup>	0.5
<b>4</b>	4.8 <sup>A</sup>	0.4	5.0 <sup>A</sup>	0.9	4.4 <sup>A</sup>	0.2	3.8 <sup>A</sup>	0.7	4.4 <sup>A</sup>	0.5
<b>6</b>	5.8 <sup>A</sup>	0.3	5.9 <sup>A</sup>	0.5	5.5 <sup>A</sup>	0.1	4.8 <sup>A</sup>	1.0	5.2 <sup>A</sup>	0.8
<b>8</b>	6.9 <sup>A</sup>	0.5	6.8 <sup>A</sup>	0.4	6.4 <sup>A</sup>	0.1	5.9 <sup>A</sup>	1.4	6.3 <sup>A</sup>	0.8
<b>10</b>	7.4 <sup>A</sup>	0.4	7.2 <sup>A</sup>	0.2	7.0 <sup>A</sup>	0.2	6.8 <sup>A</sup>	1.0	6.8 <sup>A</sup>	0.7
<b>12</b>	7.5 <sup>A</sup>	0.1	8.0 <sup>A</sup>	0.1	7.7 <sup>A</sup>	0.0	7.0 <sup>A</sup>	0.7	6.8 <sup>A</sup>	0.5

<b>14</b>	7.7 <sup>A</sup>	0.4	8.0 <sup>A</sup>	0.6	7.8 <sup>A</sup>	0.2	7.5 <sup>A</sup>	0.6	7.4 <sup>A</sup>	0.6
<b>16</b>	7.8 <sup>A</sup>	0.2	7.9 <sup>A</sup>	0.2	8.1 <sup>A</sup>	0.3	8.0 <sup>A</sup>	0.4	7.6 <sup>A</sup>	0.4
<b>18</b>	7.5 <sup>A</sup>	0.3	8.0 <sup>A</sup>	0.3	7.7 <sup>A</sup>	0.4	8.0 <sup>A</sup>	0.4	7.8 <sup>A</sup>	0.4

<sup>A, B</sup> Different superscripts within each row denote statistical significance between treatments ( $P < 0.05$ ).

<sup>1</sup>SE – Standard Error

**Table 2.** Mean TVC<sub>m</sub>, TVC<sub>p</sub>, TEC, HSPB, LAB, *Pseudomonas* spp., *Br. Thermosphacta* and *Photobacterium* spp. counts (log<sub>10</sub> CFU/cm<sup>2</sup>) on salmon fillets treated with 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) and stored at 2°C for 18 days.

Time (d)	Treatment											
	CTL		SDW		CIT		CAR		THY		EUG	
	<b>TVC<sub>m</sub></b>											
	<b>Log</b>	<b>SE<sup>1</sup></b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>	<b>Log</b>	<b>SE</b>
<b>0</b>	3.6 <sup>A</sup>	0.3	3.6 <sup>A</sup>	0.4	3.4 <sup>AB</sup>	0.5	3.2 <sup>AB</sup>	0.3	3.5 <sup>AB</sup>	0.1	2.9 <sup>B</sup>	0.1
<b>3</b>	4.8 <sup>A</sup>	0.4	5.1 <sup>A</sup>	0.1	5.1 <sup>A</sup>	0.2	4.6 <sup>A</sup>	0.2	4.8 <sup>A</sup>	0.5	4.6 <sup>A</sup>	0.3
<b>6</b>	6.4 <sup>A</sup>	0.0	6.4 <sup>A</sup>	0.1	6.4 <sup>A</sup>	0.1	6.3 <sup>A</sup>	0.0	5.8 <sup>A</sup>	0.5	6.3 <sup>A</sup>	0.2
<b>9</b>	7.3 <sup>A</sup>	0.3	7.3 <sup>A</sup>	0.3	7.4 <sup>A</sup>	0.1	7.3 <sup>A</sup>	0.2	7.0 <sup>A</sup>	0.5	7.3 <sup>A</sup>	0.2
<b>12</b>	7.7 <sup>A</sup>	0.3	8.0 <sup>A</sup>	0.3	8.1 <sup>A</sup>	0.1	8.0 <sup>A</sup>	0.1	7.5 <sup>A</sup>	0.4	7.9 <sup>A</sup>	0.2
<b>15</b>	7.8 <sup>A</sup>	0.2	7.9 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.3	8.1 <sup>A</sup>	0.3	8.1 <sup>A</sup>	0.2	7.8 <sup>A</sup>	0.0
<b>18</b>	8.3 <sup>A</sup>	0.3	8.6 <sup>A</sup>	0.3	8.6 <sup>A</sup>	0.1	8.5 <sup>A</sup>	0.2	8.2 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.2
	<b>TVC<sub>p</sub></b>											
<b>0</b>	3.9 <sup>A</sup>	0.2	3.6 <sup>A</sup>	0.3	3.4 <sup>A</sup>	0.3	3.2 <sup>A</sup>	0.3	3.7 <sup>A</sup>	0.1	3.4 <sup>A</sup>	0.3
<b>3</b>	5.1 <sup>A</sup>	0.4	5.2 <sup>A</sup>	0.1	5.1 <sup>A</sup>	0.3	4.8 <sup>A</sup>	0.2	5.0 <sup>A</sup>	0.5	5.1 <sup>A</sup>	0.2
<b>6</b>	6.8 <sup>A</sup>	0.1	6.6 <sup>A</sup>	0.2	6.9 <sup>A</sup>	0.1	6.7 <sup>A</sup>	0.0	6.4 <sup>A</sup>	0.5	6.8 <sup>A</sup>	0.2

<b>9</b>	7.9 <sup>A</sup>	0.1	7.9 <sup>A</sup>	0.2	8.2 <sup>A</sup>	0.1	7.8 <sup>A</sup>	0.2	7.6 <sup>A</sup>	0.4	8.1 <sup>A</sup>	0.1
<b>12</b>	8.2 <sup>A</sup>	0.0	8.3 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.1	8.2 <sup>A</sup>	0.1	8.0 <sup>A</sup>	0.2	8.2 <sup>A</sup>	0.1
<b>15</b>	8.4 <sup>A</sup>	0.2	8.5 <sup>A</sup>	0.2	8.6 <sup>A</sup>	0.2	8.5 <sup>A</sup>	0.2	8.5 <sup>A</sup>	0.2	8.3 <sup>A</sup>	0.1
<b>18</b>	8.6 <sup>A</sup>	0.3	8.9 <sup>A</sup>	0.4	8.8 <sup>A</sup>	0.1	8.7 <sup>A</sup>	0.3	8.7 <sup>A</sup>	0.2	8.7 <sup>A</sup>	0.3
<b>TEC</b>												
<b>0</b>	0.6 <sup>A</sup>	0.3	0.4 <sup>A</sup>	0.3	0.4 <sup>A</sup>	0.2	0.2 <sup>A</sup>	0.2	0.8 <sup>A</sup>	0.4	0.4 <sup>A</sup>	0.3
<b>3</b>	1.5 <sup>A</sup>	0.2	1.2 <sup>A</sup>	0.5	1.3 <sup>A</sup>	0.3	0.5 <sup>B</sup>	0.5	1.0 <sup>AB</sup>	0.0	0.9 <sup>AB</sup>	0.4
<b>6</b>	2.3 <sup>A</sup>	0.4	2.0 <sup>A</sup>	0.6	2.1 <sup>A</sup>	0.3	1.8 <sup>A</sup>	0.7	1.6 <sup>A</sup>	0.1	1.9 <sup>A</sup>	0.4
<b>9</b>	3.0 <sup>A</sup>	0.6	2.6 <sup>A</sup>	0.9	3.1 <sup>A</sup>	0.5	2.4 <sup>A</sup>	0.5	2.3 <sup>A</sup>	0.1	2.7 <sup>A</sup>	0.6
<b>12</b>	3.1 <sup>A</sup>	0.9	2.8 <sup>A</sup>	0.9	3.1 <sup>A</sup>	0.7	2.4 <sup>A</sup>	1.0	2.3 <sup>A</sup>	0.1	2.9 <sup>A</sup>	0.6
<b>15</b>	3.4 <sup>A</sup>	0.6	3.5 <sup>A</sup>	0.9	3.7 <sup>A</sup>	0.7	3.0 <sup>A</sup>	0.6	3.5 <sup>A</sup>	0.6	2.9 <sup>A</sup>	0.2
<b>18</b>	3.5 <sup>A</sup>	0.9	4.0 <sup>A</sup>	1.0	3.9 <sup>A</sup>	0.8	3.3 <sup>A</sup>	1.0	3.1 <sup>A</sup>	0.6	3.4 <sup>A</sup>	1.0
<b>HSPB</b>												
<b>0</b>	3.0 <sup>A</sup>	0.3	2.8 <sup>AB</sup>	0.3	2.2 <sup>B</sup>	0.2	2.1 <sup>B</sup>	0.3	2.9 <sup>AB</sup>	0.1	2.4 <sup>AB</sup>	0.3
<b>3</b>	5.3 <sup>A</sup>	0.0	4.9 <sup>AB</sup>	0.1	4.6 <sup>AB</sup>	0.4	4.1 <sup>B</sup>	0.4	4.4 <sup>AB</sup>	0.6	3.9 <sup>B</sup>	0.3
<b>6</b>	6.0 <sup>A</sup>	0.0	5.7 <sup>AB</sup>	0.3	6.0 <sup>A</sup>	0.1	5.2 <sup>B</sup>	0.2	5.5 <sup>AB</sup>	0.5	5.4 <sup>AB</sup>	0.3
<b>9</b>	6.9 <sup>A</sup>	0.1	6.6 <sup>AB</sup>	0.2	6.7 <sup>AB</sup>	0.1	6.2 <sup>B</sup>	0.0	6.4 <sup>AB</sup>	0.5	6.2 <sup>B</sup>	0.0
<b>12</b>	6.7 <sup>A</sup>	0.3	6.6 <sup>A</sup>	0.2	6.7 <sup>A</sup>	0.1	6.2 <sup>A</sup>	0.1	6.5 <sup>A</sup>	0.2	6.3 <sup>A</sup>	0.1

<b>15</b>	7.1 <sup>A</sup>	0.3	7.1 <sup>A</sup>	0.2	6.8 <sup>A</sup>	0.1	6.6 <sup>A</sup>	0.1	6.8 <sup>A</sup>	0.1	6.4 <sup>A</sup>	0.2
<b>18</b>	6.7 <sup>A</sup>	0.2	6.9 <sup>A</sup>	0.2	6.9 <sup>A</sup>	0.1	6.6 <sup>A</sup>	0.2	6.7 <sup>A</sup>	0.1	6.6 <sup>A</sup>	0.2
<b>LAB</b>												
<b>0</b>	2.0 <sup>A</sup>	0.2	1.8 <sup>AB</sup>	0.1	1.4 <sup>B</sup>	0.2	1.3 <sup>B</sup>	0.3	1.9 <sup>AB</sup>	0.1	1.6 <sup>AB</sup>	0.2
<b>3</b>	3.1 <sup>A</sup>	0.3	3.0 <sup>AB</sup>	0.2	2.9 <sup>AB</sup>	0.1	2.5 <sup>B</sup>	0.1	3.0 <sup>AB</sup>	0.4	2.9 <sup>AB</sup>	0.2
<b>6</b>	4.3 <sup>A</sup>	0	4.2 <sup>A</sup>	0.2	4.3 <sup>A</sup>	0.1	3.9 <sup>B</sup>	0.1	3.9 <sup>AB</sup>	0.5	4.3 <sup>A</sup>	0.1
<b>9</b>	5.0 <sup>A</sup>	0.1	5.0 <sup>A</sup>	0.3	5.2 <sup>A</sup>	0.1	4.6 <sup>A</sup>	0.2	4.8 <sup>A</sup>	0.5	5.2 <sup>A</sup>	0.2
<b>12</b>	5.5 <sup>A</sup>	0.1	5.7 <sup>A</sup>	0.2	5.8 <sup>A</sup>	0	5.3 <sup>A</sup>	0.1	5.2 <sup>A</sup>	0.6	5.8 <sup>A</sup>	0.2
<b>15</b>	5.9 <sup>A</sup>	0.1	6.0 <sup>A</sup>	0.1	6.1 <sup>A</sup>	0.1	5.8 <sup>A</sup>	0.1	6.2 <sup>A</sup>	0.1	5.8 <sup>A</sup>	0.5
<b>18</b>	6.1 <sup>A</sup>	0.1	6.3 <sup>A</sup>	0.1	6.3 <sup>A</sup>	0.1	6.2 <sup>A</sup>	0.1	6.0 <sup>A</sup>	0.2	6.3 <sup>A</sup>	0.1
<i>Pseudomonas spp.</i>												
<b>0</b>	4.0 <sup>A</sup>	0.2	3.8 <sup>AB</sup>	0.2	3.1 <sup>B</sup>	0.2	3.1 <sup>B</sup>	0.1	3.7 <sup>AB</sup>	0.1	3.3 <sup>B</sup>	0.2
<b>3</b>	5.3 <sup>A</sup>	0.3	5.4 <sup>A</sup>	0.2	5.3 <sup>A</sup>	0.2	5.0 <sup>A</sup>	0.1	5.1 <sup>A</sup>	0.5	5.2 <sup>A</sup>	0.2
<b>6</b>	7.0 <sup>A</sup>	0.1	6.8 <sup>A</sup>	0.0	6.9 <sup>A</sup>	0.1	6.7 <sup>A</sup>	0.1	6.4 <sup>A</sup>	0.4	6.8 <sup>A</sup>	0.1
<b>9</b>	7.7 <sup>A</sup>	0.1	7.8 <sup>A</sup>	0.2	7.9 <sup>A</sup>	0.2	7.7 <sup>A</sup>	0.1	7.4 <sup>A</sup>	0.3	7.8 <sup>A</sup>	0.1
<b>12</b>	8.2 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.1	8.3 <sup>A</sup>	0.1	8.2 <sup>A</sup>	0.0	8.0 <sup>A</sup>	0.3	8.3 <sup>A</sup>	0.0
<b>15</b>	8.8 <sup>A</sup>	0.1	8.8 <sup>A</sup>	0.1	8.8 <sup>A</sup>	0.1	8.6 <sup>A</sup>	0.0	8.7 <sup>A</sup>	0.1	8.6 <sup>A</sup>	0.1
<b>18</b>	8.8 <sup>A</sup>	0.2	8.9 <sup>A</sup>	0.3	9.1 <sup>A</sup>	0.1	9.0 <sup>A</sup>	0.2	8.8 <sup>A</sup>	0.1	8.8 <sup>A</sup>	0.2

<i>Br. Thermosphacta</i>												
<b>0</b>	2.8 <sup>A</sup>	0.2	2.7 <sup>AB</sup>	0.0	2.1 <sup>B</sup>	0.0	1.9 <sup>B</sup>	0.1	2.7 <sup>AB</sup>	0.1	2.2 <sup>AB</sup>	0.1
<b>3</b>	4.3 <sup>A</sup>	0.2	4.4 <sup>A</sup>	0.3	4.3 <sup>A</sup>	0.1	3.6 <sup>B</sup>	0.3	4.1 <sup>AB</sup>	0.3	4.1 <sup>AB</sup>	0.1
<b>6</b>	5.7 <sup>A</sup>	0.2	5.5 <sup>A</sup>	0.4	5.5 <sup>A</sup>	0.0	5.3 <sup>A</sup>	0.2	5.4 <sup>A</sup>	0.3	5.6 <sup>A</sup>	0.1
<b>9</b>	6.6 <sup>A</sup>	0.2	6.8 <sup>A</sup>	0.3	6.5 <sup>A</sup>	0.1	6.3 <sup>A</sup>	0.4	6.0 <sup>A</sup>	0.2	6.6 <sup>A</sup>	0.2
<b>12</b>	6.8 <sup>A</sup>	0.1	7.1 <sup>A</sup>	0.3	7.2 <sup>A</sup>	0.3	6.6 <sup>A</sup>	0.2	6.5 <sup>A</sup>	0.2	7.2 <sup>A</sup>	0.2
<b>15</b>	7.1 <sup>A</sup>	0.2	7.3 <sup>A</sup>	0.3	7.5 <sup>A</sup>	0.4	7.0 <sup>A</sup>	0.2	7.2 <sup>A</sup>	0.2	7.0 <sup>A</sup>	0.1
<b>18</b>	7.1 <sup>A</sup>	0.2	7.5 <sup>A</sup>	0.2	7.5 <sup>A</sup>	0.1	7.2 <sup>A</sup>	0.2	7.0 <sup>A</sup>	0.1	7.3 <sup>A</sup>	0.2
<i>Photobacterium spp.</i>												
<b>0</b>	4.6 <sup>A</sup>	0.3	4.3 <sup>A</sup>	0.4	3.7 <sup>B</sup>	0.4	3.7 <sup>B</sup>	0.4	3.9 <sup>AB</sup>	0.5	3.8 <sup>AB</sup>	0.5
<b>3</b>	5.7 <sup>A</sup>	0.3	5.6 <sup>A</sup>	0.1	5.6 <sup>A</sup>	0.1	5.3 <sup>A</sup>	0.1	5.3 <sup>A</sup>	0.8	5.3 <sup>A</sup>	0.4
<b>6</b>	6.7 <sup>A</sup>	0.6	6.6 <sup>A</sup>	0.5	6.6 <sup>A</sup>	0.7	6.5 <sup>A</sup>	0.4	6.4 <sup>A</sup>	0.8	6.6 <sup>A</sup>	0.7
<b>9</b>	8.2 <sup>A</sup>	0.1	8.1 <sup>A</sup>	0.0	8.3 <sup>A</sup>	0.1	8.0 <sup>A</sup>	0.1	7.8 <sup>A</sup>	0.5	8.1 <sup>A</sup>	0.1
<b>12</b>	8.4 <sup>A</sup>	0.1	8.7 <sup>A</sup>	0.2	8.4 <sup>A</sup>	0.1	8.2 <sup>A</sup>	0.2	8.2 <sup>A</sup>	0.3	8.4 <sup>A</sup>	0.1
<b>15</b>	8.9 <sup>A</sup>	0.1	9.1 <sup>A</sup>	0.2	9.0 <sup>A</sup>	0.1	9.0 <sup>A</sup>	0.1	8.9 <sup>A</sup>	0.1	8.7 <sup>A</sup>	0.2
<b>18</b>	9.0 <sup>A</sup>	0.1	9.1 <sup>A</sup>	0.2	9.2 <sup>A</sup>	0.2	8.9 <sup>A</sup>	0.0	9.0 <sup>A</sup>	0.0	8.9 <sup>A</sup>	0.1

<sup>A, B</sup> Different superscripts within each row denote statistical significance between treatments (P < 0.05)

<sup>1</sup>SE - Standard Error

**Appendix B - Chapter 7 Tables for mean bacterial counts on salmon fillets treated with organic acids or essential oil components and packed in a modified atmosphere or skin pack**

**Table 1.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for total viable mesophilic (TVC<sub>m</sub>) and psychrophilic (TVC<sub>p</sub>) counts and total *Enterobacteriaceae* (TEC) as determined from salmon stored at 2°C for 18 days and treated with a spray treatment of either 1% (w/v) citric acid (CA), 1% (v/v) lactic acid (LA), 0.5% (v/v) citral (CIT), 0.5% (v/v) carvacrol (CAR), 0.5% (w/v) thymol (THY) or 0.5% (v/v) eugenol (EUG) in combination with different packaging conditions.

Time (days)	TVC <sub>m</sub>			TVC <sub>p</sub>			TEC		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>								
<b>0</b>	3.3 ± 0.1 <sup>A/A</sup>	3.7 ± 0.4 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>	3.2 ± 0.2 <sup>AB/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.4 ± 0.2 <sup>A/A</sup>	1.8 ± 0.1 <sup>A/A</sup>	1.6 ± 0.2 <sup>A/A</sup>	0.2 ± 0.2 <sup>A/B</sup>
<b>9</b>	8.3 ± 0.0 <sup>A/A</sup>	4.7 ± 0.1 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/C</sup>	8.4 ± 0.0 <sup>A/A</sup>	5.1 ± 0.1 <sup>A/B</sup>	5.7 ± 0.3 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/A</sup>	1.4 ± 0.1 <sup>A/B</sup>	3.9 ± 0.3 <sup>A/A</sup>
<b>18</b>	8.7 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.0 ± 0.3 <sup>A/B</sup>	8.6 ± 0.0 <sup>A/A</sup>	5.7 ± 0.0 <sup>A/B</sup>	6.5 ± 0.2 <sup>A/C</sup>	4.8 ± 0.1 <sup>A/A</sup>	1.2 ± 0.2 <sup>A/B</sup>	4.6 ± 0.1 <sup>A/A</sup>
	<b>CA</b>								
<b>0</b>	3.2 ± 0.2 <sup>A/A</sup>	3.1 ± 0.3 <sup>AB/A</sup>	2.9 ± 0.2 <sup>A/A</sup>	3.0 ± 0.2 <sup>AB/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.5 ± 0.2 <sup>A/A</sup>	1.9 ± 0.2 <sup>A/A</sup>	1.4 ± 0.2 <sup>A/AB</sup>	0.7 ± 0.3 <sup>AB/B</sup>
<b>9</b>	8.3 ± 0.0 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	5.2 ± 0.2 <sup>A/B</sup>	8.2 ± 0.1 <sup>A/A</sup>	4.6 ± 0.1 <sup>AB/B</sup>	5.5 ± 0.1 <sup>A/C</sup>	3.8 ± 0.0 <sup>A/A</sup>	1.1 ± 0.2 <sup>A/B</sup>	3.9 ± 0.1 <sup>A/A</sup>
<b>18</b>	8.8 ± 0.1 <sup>A/A</sup>	5.4 ± 0.2 <sup>A/B</sup>	6.5 ± 0.2 <sup>A/C</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.7 ± 0.1 <sup>A/B</sup>	6.8 ± 0.1 <sup>A/C</sup>	5.3 ± 0.2 <sup>A/A</sup>	1.0 ± 0.5 <sup>A/B</sup>	5.0 ± 0.2 <sup>A/A</sup>
	<b>LA</b>								
<b>0</b>	3.3 ± 0.2 <sup>A/A</sup>	3.0 ± 0.2 <sup>AB/A</sup>	3.1 ± 0.2 <sup>A/A</sup>	3.1 ± 0.3 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.8 ± 0.2 <sup>A/A</sup>	1.6 ± 0.2 <sup>A/A</sup>	1.6 ± 0.1 <sup>A/A</sup>	1.4 ± 0.1 <sup>B/A</sup>
<b>9</b>	7.8 ± 0.1 <sup>A/A</sup>	4.2 ± 0.2 <sup>A/B</sup>	5.3 ± 0.2 <sup>A/C</sup>	8.0 ± 0.1 <sup>A/A</sup>	4.3 ± 0.1 <sup>B/B</sup>	5.4 ± 0.2 <sup>A/C</sup>	3.8 ± 0.1 <sup>A/A</sup>	0.8 ± 0.3 <sup>A/B</sup>	3.7 ± 0.2 <sup>A/A</sup>
<b>18</b>	8.8 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/C</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/C</sup>	4.7 ± 0.0 <sup>A/A</sup>	1.2 ± 0.3 <sup>A/B</sup>	4.9 ± 0.3 <sup>A/A</sup>
	<b>CIT</b>								
<b>0</b>	2.6 ± 0.2 <sup>A/A</sup>	3.1 ± 0.1 <sup>AB/A</sup>	3.1 ± 0.3 <sup>A/A</sup>	2.6 ± 0.2 <sup>A/A</sup>	2.6 ± 0.3 <sup>A/A</sup>	2.8 ± 0.2 <sup>A/A</sup>	1.3 ± 0.2 <sup>A/A</sup>	1.0 ± 0.4 <sup>A/A</sup>	1.6 ± 0.4 <sup>B/A</sup>
<b>9</b>	8.1 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/B</sup>	5.8 ± 0.0 <sup>A/C</sup>	8.1 ± 0.1 <sup>A/A</sup>	4.6 ± 0.2 <sup>AB/B</sup>	5.9 ± 0.1 <sup>A/C</sup>	3.2 ± 0.0 <sup>A/A</sup>	0.6 ± 0.2 <sup>A/B</sup>	4.1 ± 0.1 <sup>A/A</sup>
<b>18</b>	9.1 ± 0.5 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.5 ± 0.1 <sup>A/C</sup>	9.2 ± 0.4 <sup>A/A</sup>	5.7 ± 0.1 <sup>A/B</sup>	6.8 ± 0.1 <sup>A/C</sup>	4.6 ± 0.5 <sup>A/A</sup>	1.2 ± 0.4 <sup>A/B</sup>	4.6 ± 0.2 <sup>A/A</sup>

<b>CAR</b>									
<b>0</b>	2.6 ± 0.3 <sup>A/A</sup>	2.7 ± 0.3 <sup>B/A</sup>	3.0 ± 0.2 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.5 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>	0.9 ± 0.2 <sup>A/A</sup>	1.5 ± 0.2 <sup>A/A</sup>
<b>9</b>	8.0 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/C</sup>	8.1 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>AB/B</sup>	6.0 ± 0.1 <sup>A/C</sup>	3.1 ± 0.2 <sup>A/A</sup>	0.4 ± 0.4 <sup>A/B</sup>	4.6 ± 0.0 <sup>A/C</sup>
<b>18</b>	8.7 ± 0.0 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/B</sup>	6.6 ± 0.0 <sup>A/C</sup>	8.8 ± 0.1 <sup>A/A</sup>	5.8 ± 0.0 <sup>A/B</sup>	6.7 ± 0.0 <sup>A/C</sup>	4.7 ± 0.1 <sup>A/A</sup>	1.4 ± 0.4 <sup>A/B</sup>	4.8 ± 0.2 <sup>A/A</sup>
<b>THY</b>									
<b>0</b>	3.5 ± 0.1 <sup>A/A</sup>	3.2 ± 0.2 <sup>AB/A</sup>	3.0 ± 0.2 <sup>A/A</sup>	3.5 ± 0.2 <sup>B/A</sup>	2.8 ± 0.1 <sup>A/AB</sup>	2.7 ± 0.0 <sup>A/B</sup>	2.0 ± 0.1 <sup>A/A</sup>	1.6 ± 0.2 <sup>A/A</sup>	1.3 ± 0.0 <sup>A/A</sup>
<b>9</b>	8.1 ± 0.1 <sup>A/A</sup>	4.8 ± 0.0 <sup>A/B</sup>	5.8 ± 0.0 <sup>A/C</sup>	8.2 ± 0.0 <sup>A/A</sup>	5.0 ± 0.1 <sup>AB/B</sup>	5.9 ± 0.1 <sup>A/C</sup>	3.4 ± 0.3 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/B</sup>	4.4 ± 0.0 <sup>A/A</sup>
<b>18</b>	8.9 ± 0.2 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.9 ± 0.2 <sup>A/C</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.8 ± 0.0 <sup>A/B</sup>	7.0 ± 0.0 <sup>A/C</sup>	4.7 ± 0.2 <sup>A/A</sup>	1.9 ± 0.2 <sup>A/B</sup>	4.7 ± 0.0 <sup>A/A</sup>
<b>EUG</b>									
<b>0</b>	3.1 ± 0.1 <sup>A/A</sup>	3.0 ± 0.1 <sup>AB/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	2.8 ± 0.0 <sup>AB/A</sup>	2.7 ± 0.1 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	1.5 ± 0.2 <sup>A/A</sup>	1.3 ± 0.2 <sup>A/A</sup>	1.3 ± 0.2 <sup>A/A</sup>
<b>9</b>	8.1 ± 0.0 <sup>A/A</sup>	4.6 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/C</sup>	8.1 ± 0.1 <sup>A/A</sup>	4.7 ± 0.1 <sup>AB/B</sup>	6.1 ± 0.2 <sup>A/C</sup>	3.0 ± 0.0 <sup>A/A</sup>	0.7 ± 0.2 <sup>A/B</sup>	4.2 ± 0.3 <sup>A/C</sup>
<b>18</b>	9.0 ± 0.0 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/B</sup>	7.7 ± 0.6 <sup>A/C</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	7.1 ± 0.1 <sup>A/C</sup>	4.7 ± 0.2 <sup>A/A</sup>	1.3 ± 0.3 <sup>A/B</sup>	5.2 ± 0.2 <sup>A/A</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 2.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. Thermosphacta* and *Photobacterium* spp. counts as determined from salmon stored at 2°C for 18 days and treated with a spray treatment of either 1% (w/v) citric acid (CA), 1% (v/v) lactic acid (LA), 0.5% (v/v) citral (CIT), 0.5% (v/v) carvacrol (CAR), 0.5% (w/v) thymol (THY) or 0.5% (v/v) eugenol (EUG) in combination with different packaging conditions.

Time (days)	HSPB			LAB			<i>Photobacterium</i> spp.			<i>Br. thermosphacta</i>		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>											
<b>0</b>	2.8±0.2 <sup>AB/A</sup>	2.4±0.2 <sup>A/A</sup>	1.3±0.4 <sup>A/B</sup>	2.5±0.1 <sup>A/A</sup>	2.0±0.1 <sup>A/A</sup>	1.7±0.1 <sup>A/A</sup>	3.4±0.1 <sup>A/A</sup>	3.3±0.1 <sup>A/A</sup>	3.0±0.3 <sup>A/A</sup>	2.4±0.2 <sup>A/A</sup>	2.3±0.1 <sup>A/A</sup>	1.0±0.2 <sup>A/B</sup>
<b>9</b>	7.3±0.1 <sup>A/A</sup>	4.5±0.1 <sup>A/B</sup>	5.9±0.3 <sup>A/C</sup>	6.9±0.1 <sup>A/A</sup>	4.8±0.1 <sup>A/B</sup>	5.2±0.2 <sup>A/B</sup>	8.2±0.0 <sup>A/A</sup>	5.5±0.1 <sup>A/B</sup>	6.4±0.3 <sup>A/C</sup>	7.5±0.1 <sup>A/A</sup>	4.0±0.1 <sup>A/B</sup>	4.3±0.4 <sup>A/B</sup>
<b>18</b>	7.4±0.1 <sup>A/A</sup>	4.2±0.3 <sup>A/B</sup>	7.1±0.1 <sup>A/A</sup>	6.9±0.1 <sup>A/A</sup>	5.6±0.1 <sup>A/B</sup>	6.2±0.2 <sup>A/B</sup>	8.8±0.1 <sup>A/A</sup>	5.8±0.1 <sup>A/B</sup>	6.8±0.1 <sup>A/C</sup>	7.6±0.1 <sup>A/A</sup>	4.8±0.0 <sup>A/B</sup>	5.4±0.1 <sup>A/B</sup>
	<b>CA</b>											
<b>0</b>	2.7±0.1 <sup>AB/A</sup>	2.0±0.3 <sup>A/A</sup>	1.0±0.2 <sup>A/B</sup>	2.5±0.1 <sup>A/A</sup>	1.7±0.1 <sup>A/A</sup>	2.0±0.2 <sup>A/A</sup>	3.4±0.1 <sup>A/A</sup>	3.0±0.2 <sup>A/A</sup>	3.1±0.2 <sup>A/A</sup>	2.4±0.1 <sup>A/A</sup>	2.0±0.2 <sup>A/A</sup>	0.8±0.1 <sup>A/B</sup>
<b>9</b>	7.2±0.1 <sup>A/A</sup>	3.7±0.2 <sup>A/B</sup>	6.0±0.1 <sup>A/C</sup>	6.9±0.1 <sup>A/A</sup>	4.5±0.1 <sup>A/B</sup>	5.1±0.2 <sup>A/B</sup>	8.2±0.1 <sup>A/A</sup>	5.2±0.1 <sup>A/B</sup>	6.0±0.1 <sup>A/C</sup>	7.3±0.0 <sup>A/A</sup>	3.8±0.1 <sup>A/B</sup>	4.0±0.1 <sup>A/B</sup>
<b>18</b>	7.4±0.1 <sup>A/A</sup>	3.7±0.0 <sup>A/B</sup>	7.3±0.0 <sup>A/A</sup>	6.9±0.1 <sup>A/A</sup>	5.3±0.1 <sup>A/B</sup>	6.6±0.1 <sup>A/A</sup>	9.1±0.1 <sup>A/A</sup>	5.8±0.1 <sup>A/B</sup>	7.0±0.1 <sup>A/C</sup>	7.7±0.0 <sup>A/A</sup>	4.3±0.2 <sup>A/B</sup>	5.6±0.1 <sup>A/C</sup>
	<b>LA</b>											
<b>0</b>	2.7±0.4 <sup>AB/A</sup>	2.1±0.2 <sup>A/AB</sup>	1.2±0.2 <sup>A/B</sup>	2.4±0.3 <sup>AB/A</sup>	2.1±0.2 <sup>A/A</sup>	2.0±0.1 <sup>A/A</sup>	3.3±0.2 <sup>A/A</sup>	3.3±0.1 <sup>A/A</sup>	3.4±0.1 <sup>A/A</sup>	2.6±0.2 <sup>A/A</sup>	2.2±0.0 <sup>A/A</sup>	0.9±0.2 <sup>A/B</sup>
<b>9</b>	7.0±0.1 <sup>A/A</sup>	4.2±0.4 <sup>A/B</sup>	6.0±0.1 <sup>A/C</sup>	6.7±0.1 <sup>A/A</sup>	4.2±0.1 <sup>A/B</sup>	4.9±0.3 <sup>A/B</sup>	8.1±0.0 <sup>A/A</sup>	5.1±0.2 <sup>A/B</sup>	5.8±0.2 <sup>A/B</sup>	7.1±0.0 <sup>A/A</sup>	3.6±0.1 <sup>A/B</sup>	4.4±0.1 <sup>A/B</sup>
<b>18</b>	7.3±0.1 <sup>A/A</sup>	3.7±0.0 <sup>A/B</sup>	7.2±0.1 <sup>A/A</sup>	7.0±0.0 <sup>A/A</sup>	5.3±0.1 <sup>A/B</sup>	6.7±0.1 <sup>A/A</sup>	9.0±0.0 <sup>A/A</sup>	5.7±0.1 <sup>A/B</sup>	7.0±0.1 <sup>A/C</sup>	7.7±0.1 <sup>A/A</sup>	4.4±0.0 <sup>A/B</sup>	5.7±0.0 <sup>A/C</sup>
	<b>CIT</b>											
<b>0</b>	2.3±0.3 <sup>AB/A</sup>	2.0±0.4 <sup>A/A</sup>	2.0±0.3 <sup>A/A</sup>	1.8±0.2 <sup>B/A</sup>	2.2±0.2 <sup>A/A</sup>	2.1±0.2 <sup>A/A</sup>	3.0±0.3 <sup>A/A</sup>	2.9±0.2 <sup>A/A</sup>	3.5±0.2 <sup>A/A</sup>	2.1±0.2 <sup>A/A</sup>	1.9±0.2 <sup>A/A</sup>	1.1±0.2 <sup>A/B</sup>
<b>9</b>	7.2±0.1 <sup>A/A</sup>	3.7±0.1 <sup>A/B</sup>	6.2±0.0 <sup>A/C</sup>	6.3±0.1 <sup>A/A</sup>	4.1±0.1 <sup>A/B</sup>	5.2±0.1 <sup>A/C</sup>	8.0±0.0 <sup>A/A</sup>	5.2±0.2 <sup>A/B</sup>	6.6±0.0 <sup>A/C</sup>	7.0±0.1 <sup>A/A</sup>	3.3±0.1 <sup>A/B</sup>	5.0±0.2 <sup>A/C</sup>
<b>18</b>	7.7±0.3 <sup>A/A</sup>	4.0±0.3 <sup>A/B</sup>	7.1±0.3 <sup>A/A</sup>	7.0±0.1 <sup>A/A</sup>	5.6±0.1 <sup>A/B</sup>	6.3±0.2 <sup>A/B</sup>	9.4±0.4 <sup>A/A</sup>	5.8±0.1 <sup>A/B</sup>	6.9±0.1 <sup>A/C</sup>	7.8±0.2 <sup>A/A</sup>	4.5±0.3 <sup>A/B</sup>	5.9±0.2 <sup>A/C</sup>

<b>CAR</b>												
<b>0</b>	1.9 ± 0.3 <sup>A/A</sup>	1.7 ± 0.2 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>	1.7 ± 0.2 <sup>B/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	2.1 ± 0.3 <sup>A/A</sup>	3.0 ± 0.2 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>	3.3 ± 0.1 <sup>A/A</sup>	2.0 ± 0.3 <sup>A/A</sup>	1.9 ± 0.1 <sup>A/A</sup>	1.2 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.3 ± 0.1 <sup>A/A</sup>	3.6 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/C</sup>	6.5 ± 0.1 <sup>A/A</sup>	4.3 ± 0.2 <sup>A/B</sup>	5.5 ± 0.2 <sup>A/C</sup>	8.0 ± 0.1 <sup>A/A</sup>	4.9 ± 0.0 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/C</sup>	7.1 ± 0.1 <sup>A/A</sup>	3.5 ± 0.1 <sup>A/B</sup>	5.2 ± 0.3 <sup>A/C</sup>
<b>18</b>	7.4 ± 0.0 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	6.9 ± 0.0 <sup>A/A</sup>	6.9 ± 0.1 <sup>A/A</sup>	5.3 ± 0.0 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/A</sup>	8.9 ± 0.1 <sup>A/A</sup>	6.0 ± 0.0 <sup>A/B</sup>	6.8 ± 0.0 <sup>A/C</sup>	7.6 ± 0.0 <sup>A/A</sup>	4.0 ± 0.1 <sup>A/B</sup>	5.5 ± 0.0 <sup>A/C</sup>
<b>THY</b>												
<b>0</b>	3.0 ± 0.1 <sup>B/A</sup>	2.3 ± 0.1 <sup>A/A</sup>	1.8 ± 0.1 <sup>A/B</sup>	2.6 ± 0.1 <sup>A/A</sup>	1.9 ± 0.2 <sup>A/A</sup>	2.0 ± 0.0 <sup>A/A</sup>	3.6 ± 0.1 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	3.3 ± 0.1 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>	2.4 ± 0.1 <sup>A/A</sup>	1.0 ± 0.1 <sup>A/B</sup>
<b>9</b>	7.4 ± 0.2 <sup>A/A</sup>	4.4 ± 0.2 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/C</sup>	6.7 ± 0.0 <sup>A/A</sup>	4.9 ± 0.0 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/B</sup>	8.0 ± 0.1 <sup>A/A</sup>	5.1 ± 0.1 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/C</sup>	7.3 ± 0.0 <sup>A/A</sup>	3.8 ± 0.0 <sup>A/B</sup>	4.8 ± 0.2 <sup>A/C</sup>
<b>18</b>	7.6 ± 0.1 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	7.2 ± 0.0 <sup>A/A</sup>	7.3 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	9.0 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/C</sup>	7.8 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/C</sup>
<b>EUG</b>												
<b>0</b>	2.5 ± 0.2 <sup>AB/A</sup>	2.0 ± 0.1 <sup>A/A</sup>	2.4 ± 0.3 <sup>A/A</sup>	2.0 ± 0.0 <sup>AB/A</sup>	1.9 ± 0.0 <sup>A/A</sup>	2.0 ± 0.1 <sup>A/A</sup>	3.4 ± 0.1 <sup>A/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	3.4 ± 0.1 <sup>A/A</sup>	2.4 ± 0.0 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/A</sup>	1.1 ± 0.1 <sup>A/B</sup>
<b>9</b>	6.7 ± 0.1 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/B</sup>	6.2 ± 0.0 <sup>A/A</sup>	6.6 ± 0.1 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/C</sup>	8.2 ± 0.0 <sup>A/A</sup>	5.0 ± 0.0 <sup>A/B</sup>	6.6 ± 0.2 <sup>A/C</sup>	7.2 ± 0.0 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/C</sup>
<b>18</b>	7.4 ± 0.0 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	7.2 ± 0.0 <sup>A/A</sup>	7.1 ± 0.1 <sup>A/A</sup>	5.5 ± 0.2 <sup>A/B</sup>	6.8 ± 0.2 <sup>A/A</sup>	9.1 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/C</sup>	7.7 ± 0.0 <sup>A/A</sup>	4.3 ± 0.3 <sup>A/B</sup>	5.8 ± 0.0 <sup>A/C</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 3.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for total viable mesophilic (TVC<sub>m</sub>) and psychrophilic (TVC<sub>p</sub>) counts and total *Enterobacteriaceae* (TEC) as determined from salmon stored at 2°C for 18 days and treated with a spray treatment of either 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) in combination with different packaging conditions

Time (days)	TVC <sub>m</sub>			TVC <sub>p</sub>			TEC		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>								
<b>0</b>	4.6 ± 0.3 <sup>A/A</sup>	4.5 ± 0.6 <sup>A/A</sup>	4.7 ± 0.4 <sup>A/A</sup>	4.6 ± 0.4 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/A</sup>	4.5 ± 0.4 <sup>A/A</sup>	2.0 ± 0.7 <sup>A/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	3.0 ± 0.3 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.0 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/B</sup>	5.5 ± 0.1 <sup>A/B</sup>	8.5 ± 0.1 <sup>A/A</sup>	5.1 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	4.9 ± 0.1 <sup>A/A</sup>	3.3 ± 0.1 <sup>A/B</sup>	3.5 ± 0.2 <sup>A/B</sup>
<b>18</b>	8.1 ± 0.1 <sup>A/A</sup>	5.9 ± 0.0 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/B</sup>	8.3 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/A</sup>	3.9 ± 0.3 <sup>A/B</sup>	3.9 ± 0.2 <sup>A/B</sup>
	<b>CA</b>								
<b>0</b>	4.3 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.4 ± 0.4 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/A</sup>	4.4 ± 0.0 <sup>A/A</sup>	4.3 ± 0.5 <sup>A/A</sup>	1.9 ± 0.4 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.5 ± 0.4 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.0 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/B</sup>	8.4 ± 0.1 <sup>A/A</sup>	5.1 ± 0.2 <sup>A/B</sup>	5.5 ± 0.3 <sup>A/B</sup>	5.1 ± 0.2 <sup>A/A</sup>	3.0 ± 0.2 <sup>A/B</sup>	2.8 ± 0.3 <sup>A/B</sup>
<b>18</b>	8.2 ± 0.2 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	6.3 ± 0.1 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/B</sup>	6.0 ± 0.2 <sup>A/A</sup>	4.1 ± 0.3 <sup>A/B</sup>	3.9 ± 0.1 <sup>A/B</sup>
	<b>LA</b>								
<b>0</b>	4.2 ± 0.1 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/A</sup>	4.6 ± 0.2 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/A</sup>	1.6 ± 0.0 <sup>A/A</sup>	2.2 ± 0.2 <sup>A/A</sup>	2.8 ± 0.5 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.0 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/B</sup>	8.3 ± 0.1 <sup>A/A</sup>	5.3 ± 0.2 <sup>A/B</sup>	5.8 ± 0.2 <sup>A/B</sup>	4.7 ± 0.2 <sup>A/A</sup>	2.5 ± 0.2 <sup>A/B</sup>	3.0 ± 0.2 <sup>A/B</sup>
<b>18</b>	8.3 ± 0.1 <sup>A/A</sup>	5.3 ± 0.2 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/B</sup>	8.7 ± 0.0 <sup>A/A</sup>	5.9 ± 0.2 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/B</sup>	5.8 ± 0.2 <sup>A/A</sup>	3.1 ± 0.0 <sup>A/B</sup>	3.4 ± 0.0 <sup>A/B</sup>
	<b>CIT</b>								
<b>0</b>	4.7 ± 0.2 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.3 ± 0.2 <sup>A/A</sup>	1.3 ± 0.3 <sup>A/A</sup>	2.0 ± 0.3 <sup>A/A</sup>	2.1 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.8 ± 0.1 <sup>A/A</sup>	5.2 ± 0.2 <sup>A/B</sup>	5.5 ± 0.0 <sup>A/B</sup>	8.4 ± 0.1 <sup>A/A</sup>	5.3 ± 0.2 <sup>A/B</sup>	6.0 ± 0.2 <sup>A/B</sup>	5.1 ± 0.3 <sup>A/A</sup>	3.1 ± 0.1 <sup>A/B</sup>	3.3 ± 0.1 <sup>A/B</sup>
<b>18</b>	8.2 ± 0.0 <sup>A/A</sup>	5.7 ± 0.1 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/B</sup>	8.5 ± 0.0 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	5.6 ± 0.0 <sup>A/A</sup>	3.3 ± 0.2 <sup>A/B</sup>	3.6 ± 0.0 <sup>A/B</sup>

<b>CAR</b>									
<b>0</b>	4.6 ± 0.2 <sup>A/A</sup>	4.8 ± 0.2 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/A</sup>	4.5 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	1.9 ± 0.3 <sup>A/A</sup>	2.2 ± 0.2 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/B</sup>
<b>9</b>	7.9 ± 0.2 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/B</sup>	5.3 ± 0.2 <sup>A/B</sup>	7.1 ± 1.5 <sup>B/A</sup>	5.5 ± 0.2 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/A</sup>	2.7 ± 0.3 <sup>A/B</sup>	3.2 ± 0.1 <sup>A/B</sup>
<b>18</b>	8.1 ± 0.0 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.6 ± 0.1 <sup>A/B</sup>	8.7 ± 0.1 <sup>A/A</sup>	6.1 ± 0.0 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	5.5 ± 0.3 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/B</sup>	3.6 ± 0.1 <sup>A/B</sup>
<b>THY</b>									
<b>0</b>	4.6 ± 0.2 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	5.0 ± 0.2 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	2.5 ± 0.2 <sup>A/A</sup>
<b>9</b>	8.1 ± 0.2 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/B</sup>	5.4 ± 0.2 <sup>A/B</sup>	8.4 ± 0.2 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.2 ± 0.1 <sup>A/B</sup>	5.5 ± 0.0 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/B</sup>	3.3 ± 0.1 <sup>A/B</sup>
<b>18</b>	8.0 ± 0.3 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.9 ± 0.0 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/B</sup>	5.5 ± 0.0 <sup>A/A</sup>	3.3 ± 0.2 <sup>A/B</sup>	4.1 ± 0.3 <sup>A/B</sup>
<b>EUG</b>									
<b>0</b>	4.2 ± 0.1 <sup>A/A</sup>	4.3 ± 0.3 <sup>A/A</sup>	4.1 ± 0.3 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/A</sup>	4.6 ± 0.2 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	2.3 ± 0.2 <sup>A/A</sup>	2.3 ± 0.2 <sup>A/A</sup>
<b>9</b>	8.1 ± 0.3 <sup>A/A</sup>	4.8 ± 0.0 <sup>A/B</sup>	5.3 ± 0.2 <sup>A/B</sup>	8.6 ± 0.1 <sup>A/A</sup>	5.5 ± 0.2 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/B</sup>	3.2 ± 0.3 <sup>A/B</sup>
<b>18</b>	8.3 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.7 ± 0.0 <sup>A/B</sup>	8.6 ± 0.1 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	5.5 ± 0.1 <sup>A/A</sup>	3.5 ± 0.1 <sup>A/B</sup>	3.5 ± 0.0 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 4.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. Thermosphacta* and *Photobacterium* spp. counts as determined from salmon stored at 2°C for 18 days and treated with a spray treatment of either 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) in combination with different packaging conditions.

Time (days)	HSPB			LAB			<i>Photobacterium</i> spp.			<i>Br. thermosphacta</i>		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>											
<b>0</b>	3.4 ± 0.1 <sup>A/A</sup>	3.2 ± 0.1 <sup>A/A</sup>	3.4 ± 0.3 <sup>A/A</sup>	3.3 ± 0.5 <sup>A/A</sup>	3.7 ± 0.1 <sup>A/A</sup>	3.5 ± 0.4 <sup>A/A</sup>	5.0 ± 0.0 <sup>A/A</sup>	5.3 ± 0.2 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/A</sup>	3.2 ± 0.4 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.0 <sup>A/A</sup>	4.5 ± 0.1 <sup>AB/B</sup>	4.9 ± 0.1 <sup>A/B</sup>	6.3 ± 0.0 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/B</sup>	5.3 ± 0.1 <sup>A/B</sup>	8.4 ± 0.0 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.3 ± 0.2 <sup>A/C</sup>	7.1 ± 0.0 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	4.8 ± 0.1 <sup>A/B</sup>
<b>18</b>	6.9 ± 0.2 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/B</sup>	5.2 ± 0.3 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/A</sup>	5.7 ± 0.2 <sup>A/A</sup>	8.8 ± 0.1	5.9 ± 0.1 <sup>A/B</sup>	6.5 ± 0.1 <sup>A/B</sup>	7.1 ± 0.2 <sup>A/A</sup>	4.9 ± 0.0 <sup>A/B</sup>	5.0 ± 0.1 <sup>A/B</sup>
	<b>CA</b>											
<b>0</b>	2.9 ± 0.1 <sup>A/A</sup>	3.2 ± 0.1 <sup>A/A</sup>	2.9 ± 0.5 <sup>A/A</sup>	3.4 ± 0.0 <sup>A/A</sup>	3.3 ± 0.3 <sup>AB/A</sup>	3.2 ± 0.5 <sup>A/A</sup>	5.1 ± 0.2 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/A</sup>	4.9 ± 0.3 <sup>A/A</sup>	2.5 ± 0.4 <sup>A/A</sup>	2.8 ± 0.0 <sup>A/A</sup>	2.8 ± 0.4 <sup>A/A</sup>
<b>9</b>	6.5 ± 0.2 <sup>A/A</sup>	3.3 ± 0.2 <sup>AB/B</sup>	2.9 ± 0.4 <sup>B/B</sup>	6.2 ± 0.1 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/B</sup>	4.6 ± 0.3 <sup>A/B</sup>	8.3 ± 0.0 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	7.0 ± 0.1 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/B</sup>	3.7 ± 0.2 <sup>A/B</sup>
<b>18</b>	7.3 ± 0.1 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/B</sup>	3.9 ± 0.1 <sup>A/B</sup>	6.5 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.1 ± 0.1 <sup>A/B</sup>	8.9 ± 0.0 <sup>A/A</sup>	6.2 ± 0.1 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/B</sup>	7.4 ± 0.0 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/B</sup>	4.2 ± 0.1 <sup>A/B</sup>
	<b>LA</b>											
<b>0</b>	3.4 ± 0.1 <sup>A/A</sup>	2.9 ± 0.1 <sup>A/A</sup>	3.3 ± 0.2 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	3.4 ± 0.1 <sup>AB/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	4.8 ± 0.0 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>	3.0 ± 0.1 <sup>A/A</sup>	2.9 ± 0.0 <sup>A/A</sup>
<b>9</b>	6.5 ± 0.2 <sup>A/A</sup>	3.0 ± 0.1 <sup>A/B</sup>	3.7 ± 0.5 <sup>AB/B</sup>	6.3 ± 0.0 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/B</sup>	4.6 ± 0.2 <sup>A/B</sup>	8.4 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	6.1 ± 0.2 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	3.9 ± 0.3 <sup>A/B</sup>	3.8 ± 0.3 <sup>A/B</sup>
<b>18</b>	7.1 ± 0.2 <sup>A/A</sup>	3.0 ± 0.3 <sup>A/B</sup>	3.8 ± 0.3 <sup>A/B</sup>	6.6 ± 0.1 <sup>A/A</sup>	5.5 ± 0.3 <sup>A/B</sup>	5.1 ± 0.1 <sup>A/B</sup>	8.8 ± 0.0 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/B</sup>	7.3 ± 0.1 <sup>A/A</sup>	4.5 ± 0.2 <sup>A/B</sup>	4.2 ± 0.1 <sup>A/B</sup>
	<b>CIT</b>											
<b>0</b>	3.7 ± 0.1 <sup>A/A</sup>	3.3 ± 0.0 <sup>A/A</sup>	3.5 ± 0.1 <sup>A/A</sup>	3.1 ± 0.2 <sup>A/A</sup>	2.8 ± 0.2 <sup>B/A</sup>	2.9 ± 0.1 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/A</sup>	2.8 ± 0.2 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.1 ± 0.1 <sup>A/A</sup>	4.6 ± 0.2 <sup>B/B</sup>	4.9 ± 0.1 <sup>A/B</sup>	6.3 ± 0.0 <sup>A/A</sup>	4.7 ± 0.3 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/B</sup>	8.4 ± 0.0 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/B</sup>	4.6 ± 0.3 <sup>A/B</sup>
<b>18</b>	7.3 ± 0.1 <sup>A/A</sup>	4.4 ± 0.4 <sup>A/B</sup>	5.1 ± 0.0 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/A</sup>	5.7 ± 0.1 <sup>A/A</sup>	8.7 ± 0.0 <sup>A/A</sup>	6.0 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>

<b>CAR</b>												
<b>0</b>	3.4 ± 0.1 <sup>A/A</sup>	3.2 ± 0.1 <sup>A/A</sup>	3.4 ± 0.1 <sup>A/A</sup>	3.3 ± 0.3 <sup>A/A</sup>	3.0 ± 0.2 <sup>AB/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	5.2 ± 0.1 <sup>A/A</sup>	5.1 ± 0.1 <sup>A/A</sup>	4.9 ± 0.0 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.7 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.1 ± 0.0 <sup>A/A</sup>	4.2 ± 0.2 <sup>AB/B</sup>	4.5 ± 0.1 <sup>AB/B</sup>	6.2 ± 0.1 <sup>A/A</sup>	4.6 ± 0.0 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>	8.2 ± 0.1 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/B</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.6 ± 0.2 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/B</sup>	4.3 ± 0.2 <sup>A/B</sup>
<b>18</b>	7.5 ± 0.1 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/B</sup>	4.8 ± 0.1 <sup>A/B</sup>	6.5 ± 0.1 <sup>A/A</sup>	5.7 ± 0.2 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/B</sup>	8.8 ± 0.2 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.0 ± 0.0 <sup>A/B</sup>	7.1 ± 0.0 <sup>A/A</sup>	3.9 ± 1.0 <sup>A/B</sup>	4.5 ± 0.1 <sup>A/B</sup>
<b>THY</b>												
<b>0</b>	3.5 ± 0.1 <sup>A/A</sup>	3.2 ± 0.1 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/A</sup>	3.0 ± 0.1 <sup>A/A</sup>	3.2 ± 0.1 <sup>AB/A</sup>	3.4 ± 0.1 <sup>A/A</sup>	5.0 ± 0.0 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>	2.9 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.0 ± 0.1 <sup>A/A</sup>	4.1 ± 0.2 <sup>AB/B</sup>	5.0 ± 0.1 <sup>A/B</sup>	6.3 ± 0.0 <sup>A/A</sup>	4.6 ± 0.1 <sup>A/B</sup>	5.1 ± 0.1 <sup>A/B</sup>	8.3 ± 0.0 <sup>A/A</sup>	5.6 ± 0.0 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/A</sup>	7.2 ± 0.1 <sup>A/A</sup>	3.9 ± 0.1 <sup>A/B</sup>	4.2 ± 0.3 <sup>A/B</sup>
<b>18</b>	7.6 ± 0.1 <sup>A/A</sup>	4.3 ± 0.3 <sup>A/B</sup>	5.3 ± 0.0 <sup>A/B</sup>	6.5 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	5.3 ± 0.3 <sup>A/B</sup>	8.8 ± 0.1 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/B</sup>	6.2 ± 0.2 <sup>A/B</sup>	7.2 ± 0.0 <sup>A/A</sup>	4.7 ± 0.1 <sup>A/B</sup>	4.8 ± 0.3 <sup>A/B</sup>
<b>EUG</b>												
<b>0</b>	3.1 ± 0.1 <sup>A/A</sup>	3.2 ± 0.0 <sup>A/A</sup>	3.3 ± 0.1 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	3.5 ± 0.1 <sup>AB/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	5.2 ± 0.0 <sup>A/A</sup>	4.9 ± 0.0 <sup>A/A</sup>	5.1 ± 0.1 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.9 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.5 ± 0.0 <sup>A/A</sup>	3.7 ± 0.1 <sup>AB/B</sup>	4.5 ± 0.1 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/A</sup>	4.5 ± 0.0 <sup>A/B</sup>	5.1 ± 0.1 <sup>A/B</sup>	8.5 ± 0.0 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.0 ± 0.0 <sup>A/B</sup>	7.3 ± 0.0 <sup>A/A</sup>	3.9 ± 0.1 <sup>A/B</sup>	4.1 ± 0.2 <sup>A/B</sup>
<b>18</b>	7.4 ± 0.1 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/A</sup>	5.5 ± 0.2 <sup>A/B</sup>	5.3 ± 0.0 <sup>A/B</sup>	8.7 ± 0.0 <sup>A/A</sup>	6.0 ± 0.0 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/B</sup>	4.4 ± 0.2 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 5.** Mean log<sub>10</sub>CFU/cm<sup>2</sup> values for total viable mesophilic (TVC<sub>m</sub>) and psychrophilic (TVC<sub>p</sub>) counts and total *Enterobacteriaceae* (TEC) as determined from salmon stored at 2°C for 18 days and treated with a dip treatment of either 1% (w/v) citric acid (CA), 1% (v/v) lactic acid (LA), 0.5% (v/v) citral (CIT), 0.5% (v/v) carvacrol (CAR), 0.5% (w/v) thymol (THY) or 0.5% (v/v) eugenol (EUG) in combination with different packaging conditions

Time (days)	TVC <sub>m</sub>			TVC <sub>p</sub>			TEC		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>								
<b>0</b>	2.9 ± 0.2 <sup>A/A</sup>	2.3 ± 0.2 <sup>A/A</sup>	3.1 ± 0.3 <sup>A/A</sup>	3.1 ± 0.5 <sup>AB/A</sup>	3.0 ± 0.1 <sup>A/A</sup>	3.6 ± 0.1 <sup>A/A</sup>	1.0 ± 0.1 <sup>A/A</sup>	0.7 ± 0.1 <sup>A/A</sup>	1.0 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.4 ± 0.1 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/B</sup>	7.2 ± 0.3 <sup>AB/A</sup>	4.6 ± 0.0 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/B</sup>	3.7 ± 0.5 <sup>A/A</sup>	1.2 ± 0.3 <sup>A/B</sup>	1.9 ± 0.2 <sup>A/B</sup>
<b>18</b>	9.0 ± 0.1 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	8.9 ± 0.2 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/B</sup>	5.1 ± 0.2 <sup>A/A</sup>	2.1 ± 0.4 <sup>A/B</sup>	3.6 ± 0.1 <sup>A/C</sup>
	<b>CA</b>								
<b>0</b>	2.5 ± 0.1 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/A</sup>	2.7 ± 0.3 <sup>A/A</sup>	3.1 ± 0.4 <sup>AB/A</sup>	3.6 ± 0.2 <sup>A/A</sup>	3.5 ± 0.3 <sup>A/A</sup>	0.3 ± 0.3 <sup>A/A</sup>	0.3 ± 0.2 <sup>A/A</sup>	0.7 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.5 ± 0.1 <sup>A/A</sup>	3.9 ± 0.4 <sup>A/B</sup>	4.5 ± 0.2 <sup>A/B</sup>	7.4 ± 0.1 <sup>AB/A</sup>	4.7 ± 0.2 <sup>A/B</sup>	5.6 ± 0.2 <sup>A/B</sup>	4.2 ± 0.1 <sup>A/A</sup>	1.7 ± 0.8 <sup>A/B</sup>	2.2 ± 0.0 <sup>A/B</sup>
<b>18</b>	8.7 ± 0.1 <sup>A/A</sup>	4.7 ± 0.3 <sup>A/B</sup>	5.0 ± 0.4 <sup>B/B</sup>	8.7 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	5.6 ± 0.0 <sup>A/B</sup>	5.0 ± 0.2 <sup>A/A</sup>	1.4 ± 0.7 <sup>A/B</sup>	2.7 ± 0.7 <sup>A/B</sup>
	<b>LA</b>								
<b>0</b>	2.7 ± 0.0 <sup>A/A</sup>	2.5 ± 0.1 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/A</sup>	3.2 ± 0.3 <sup>A/A</sup>	3.6 ± 0.4 <sup>A/A</sup>	0.2 ± 0.2 <sup>A/A</sup>	0.4 ± 0.4 <sup>A/A</sup>	0.3 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.4 ± 0.0 <sup>A/A</sup>	3.7 ± 0.1 <sup>A/B</sup>	4.2 ± 0.2 <sup>A/B</sup>	7.0 ± 0.1 <sup>AB/A</sup>	4.6 ± 0.2 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/B</sup>	3.7 ± 0.1 <sup>A/A</sup>	0.8 ± 0.1 <sup>A/B</sup>	2.4 ± 0.4 <sup>A/A</sup>
<b>18</b>	8.8 ± 0.0 <sup>A/A</sup>	4.8 ± 0.2 <sup>A/B</sup>	5.8 ± 0.3 <sup>AB/C</sup>	8.7 ± 0.1 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/A</sup>	1.4 ± 0.4 <sup>A/B</sup>	2.6 ± 0.3 <sup>A/B</sup>
	<b>CIT</b>								
<b>0</b>	2.8 ± 0.4 <sup>A/A</sup>	2.3 ± 0.3 <sup>A/A</sup>	2.8 ± 0.3 <sup>A/A</sup>	3.2 ± 0.3 <sup>AB/A</sup>	3.1 ± 0.5 <sup>A/A</sup>	3.1 ± 0.1 <sup>A/A</sup>	1.3 ± 0.1 <sup>A/A</sup>	0.5 ± 0.4 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.3 ± 0.1 <sup>A/A</sup>	4.5 ± 0.3 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>	7.1 ± 0.0 <sup>AB/A</sup>	4.7 ± 0.4 <sup>A/B</sup>	5.9 ± 0.1 <sup>A/C</sup>	4.0 ± 0.2 <sup>A/A</sup>	2.1 ± 0.7 <sup>A/B</sup>	3.6 ± 0.1 <sup>A/A</sup>
<b>18</b>	8.8 ± 0.1 <sup>A/A</sup>	5.4 ± 0.2 <sup>A/B</sup>	5.4 ± 0.2 <sup>AB/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/A</sup>	2.1 ± 0.6 <sup>A/B</sup>	3.7 ± 0.3 <sup>A/C</sup>

<b>CAR</b>									
<b>0</b>	2.4 ± 0.1 <sup>A/A</sup>	2.2 ± 0.1 <sup>A/A</sup>	3.1 ± 0.4 <sup>A/A</sup>	2.9 ± 0.4 <sup>AB/A</sup>	3.3 ± 0.3 <sup>A/A</sup>	3.3 ± 0.5 <sup>A/A</sup>	0.4 ± 0.0 <sup>A/A</sup>	0.1 ± 0.1 <sup>A/A</sup>	0.7 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.1 ± 0.0 <sup>A/A</sup>	4.3 ± 0.2 <sup>A/B</sup>	4.9 ± 0.0 <sup>A/B</sup>	6.9 ± 0.0 <sup>A/A</sup>	4.8 ± 0.3 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	3.6 ± 0.4 <sup>A/A</sup>	2.2 ± 0.2 <sup>A/B</sup>	3.6 ± 0.1 <sup>A/A</sup>
<b>18</b>	8.9 ± 0.1 <sup>A/A</sup>	4.7 ± 0.1 <sup>A/B</sup>	5.8 ± 0.4 <sup>AB/C</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/B</sup>	5.9 ± 0.1 <sup>A/B</sup>	4.9 ± 0.1 <sup>A/A</sup>	1.1 ± 0.1 <sup>A/B</sup>	3.7 ± 0.5 <sup>A/A</sup>
<b>THY</b>									
<b>0</b>	2.7 ± 0.1 <sup>A/A</sup>	2.9 ± 0.3 <sup>A/A</sup>	2.5 ± 0.0 <sup>A/A</sup>	3.1 ± 0.3 <sup>AB/A</sup>	3.5 ± 0.3 <sup>A/A</sup>	3.6 ± 0.1 <sup>A/A</sup>	0.8 ± 0.2 <sup>A/A</sup>	1.3 ± 0.1 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.2 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/C</sup>	7.5 ± 0.2 <sup>AB/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/B</sup>	4.1 ± 0.1 <sup>A/A</sup>	1.3 ± 0.3 <sup>A/B</sup>	3.1 ± 0.3 <sup>A/A</sup>
<b>18</b>	8.9 ± 0.0 <sup>A/A</sup>	4.6 ± 0.3 <sup>A/B</sup>	5.7 ± 0.4 <sup>AB/B</sup>	9.1 ± 0.2 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/B</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.4 ± 0.3 <sup>A/A</sup>	1.9 ± 0.7 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/A</sup>
<b>EUG</b>									
<b>0</b>	2.7 ± 0.1 <sup>A/A</sup>	3.0 ± 0.2 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>	2.5 ± 0.1 <sup>B/A</sup>	3.6 ± 0.1 <sup>A/B</sup>	2.9 ± 0.4 <sup>A/AB</sup>	0.7 ± 0.1 <sup>A/A</sup>	0.8 ± 0.2 <sup>A/A</sup>	0.9 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.2 <sup>A/A</sup>	3.9 ± 0.2 <sup>A/B</sup>	4.8 ± 0.1 <sup>A/B</sup>	8.0 ± 0.0 <sup>B/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	6.0 ± 0.0 <sup>A/B</sup>	4.1 ± 0.3 <sup>A/A</sup>	1.1 ± 0.2 <sup>A/B</sup>	3.0 ± 0.2 <sup>A/A</sup>
<b>18</b>	9.1 ± 0.1 <sup>A/A</sup>	4.7 ± 0.5 <sup>A/B</sup>	5.3 ± 0.1 <sup>AB/B</sup>	9.1 ± 0.1 <sup>A/A</sup>	5.9 ± 0.0 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	5.3 ± 0.1 <sup>A/A</sup>	2.1 ± 0.6 <sup>A/B</sup>	2.9 ± 0.2 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 6.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. Thermosphacta* and *Photobacterium* spp. counts as determined from salmon stored at 2°C for 18 days and treated with a dip treatment of either 1% (w/v) citric acid (CA), 1% (v/v) lactic acid (LA), 0.5% (v/v) citral (CIT), 0.5% (v/v) carvacrol (CAR), 0.5% (w/v) thymol (THY) or 0.5% (v/v) eugenol (EUG) in combination with different packaging conditions.

Time (days)	HSPB			LAB			<i>Photobacterium</i> spp.			<i>Br. thermosphacta</i>		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>											
<b>0</b>	1.6 ± 0.2 <sup>A/A</sup>	1.2 ± 0.2 <sup>A/A</sup>	1.4 ± 0.2 <sup>A/A</sup>	1.3 ± 0.1 <sup>AB/A</sup>	0.9 ± 0.1 <sup>A/A</sup>	1.0 ± 0.1 <sup>A/A</sup>	3.4 ± 0.5 <sup>AB/A</sup>	3.8 ± 0.3 <sup>A/A</sup>	3.2 ± 0.4 <sup>A/A</sup>	1.3 ± 0.1 <sup>A/A</sup>	1.3 ± 0.3 <sup>AB/A</sup>	0.9 ± 0.1 <sup>A/A</sup>
<b>9</b>	6.9 ± 0.3 <sup>A/A</sup>	2.7 ± 0.0 <sup>A/B</sup>	3.6 ± 0.3 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/A</sup>	3.8 ± 0.3 <sup>A/B</sup>	3.9 ± 0.1 <sup>A/B</sup>	7.8 ± 0.3 <sup>A/A</sup>	5.1 ± 0.2 <sup>A/B</sup>	6.4 ± 0.3 <sup>A/C</sup>	5.7 ± 0.4 <sup>A/A</sup>	2.2 ± 0.4 <sup>A/B</sup>	2.9 ± 0.3 <sup>A/B</sup>
<b>18</b>	6.2 ± 0.3 <sup>A/A</sup>	4.0 ± 0.1 <sup>A/B</sup>	4.6 ± 0.1 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/A</sup>	5.0 ± 0.2 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/A</sup>	9.0 ± 0.2 <sup>A/A</sup>	5.7 ± 0.2 <sup>A/B</sup>	6.0 ± 0.2 <sup>A/B</sup>	7.0 ± 0.3 <sup>A/A</sup>	4.4 ± 0.3 <sup>A/B</sup>	3.9 ± 0.2 <sup>A/B</sup>
	<b>CA</b>											
<b>0</b>	1.1 ± 0.3 <sup>A/A</sup>	1.0 ± 0.2 <sup>A/A</sup>	1.2 ± 0.2 <sup>A/A</sup>	1.1 ± 0.0 <sup>AB/A</sup>	0.8 ± 0.2 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>	3.2 ± 0.6 <sup>AB/A</sup>	3.9 ± 0.3 <sup>A/A</sup>	3.0 ± 0.1 <sup>A/A</sup>	0.9 ± 0.2 <sup>A/A</sup>	0.7 ± 0.0 <sup>B/A</sup>	0.7 ± 0.0 <sup>A/A</sup>
<b>9</b>	6.3 ± 0.1 <sup>A/A</sup>	2.4 ± 0.3 <sup>A/B</sup>	3.0 ± 0.3 <sup>A/B</sup>	4.9 ± 0.1 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/B</sup>	4.0 ± 0.4 <sup>AB/A</sup>	7.8 ± 0.0 <sup>A/A</sup>	5.3 ± 0.6 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/A</sup>	2.1 ± 0.3 <sup>A/B</sup>	2.4 ± 0.4 <sup>A/B</sup>
<b>18</b>	5.8 ± 0.0 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/B</sup>	6.2 ± 0.1 <sup>A/A</sup>	4.8 ± 0.2 <sup>A/B</sup>	4.8 ± 0.1 <sup>A/B</sup>	8.9 ± 0.0 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	6.8 ± 0.3 <sup>A/A</sup>	3.0 ± 0.3 <sup>A/B</sup>	4.1 ± 0.2 <sup>A/C</sup>
	<b>LA</b>											
<b>0</b>	1.1 ± 0.4 <sup>A/A</sup>	0.8 ± 0.5 <sup>A/B</sup>	1.1 ± 0.2 <sup>A/A</sup>	0.7 ± 0.4 <sup>A/A</sup>	1.2 ± 0.5 <sup>AB/A</sup>	1.0 ± 0.1 <sup>A/A</sup>	4.2 ± 0.0 <sup>A/A</sup>	3.2 ± 0.3 <sup>A/A</sup>	3.7 ± 0.6 <sup>A/A</sup>	0.9 ± 0.2 <sup>A/A</sup>	0.8 ± 0.1 <sup>AB/A</sup>	0.7 ± 0.0 <sup>A/A</sup>
<b>9</b>	6.8 ± 0.1 <sup>A/A</sup>	2.7 ± 0.0 <sup>A/B</sup>	3.2 ± 0.3 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/B</sup>	4.0 ± 0.1 <sup>AB/A</sup>	7.7 ± 0.1 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/C</sup>	5.6 ± 0.1 <sup>A/A</sup>	1.8 ± 0.1 <sup>A/B</sup>	2.5 ± 0.0 <sup>A/B</sup>
<b>18</b>	6.0 ± 0.2 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/B</sup>	3.7 ± 0.0 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>	8.8 ± 0.1 <sup>A/A</sup>	5.2 ± 0.1 <sup>A/B</sup>	6.2 ± 0.2 <sup>A/B</sup>	6.5 ± 0.3 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	3.7 ± 0.0 <sup>A/B</sup>
	<b>CIT</b>											
<b>0</b>	2.0 ± 0.1 <sup>A/A</sup>	1.4 ± 0.3 <sup>A/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	1.6 ± 0.1 <sup>B/A</sup>	1.2 ± 0.4 <sup>AB/A</sup>	1.5 ± 0.1 <sup>A/A</sup>	3.2 ± 0.3 <sup>AB/A</sup>	2.9 ± 0.3 <sup>A/B</sup>	2.9 ± 0.1 <sup>A/B</sup>	1.1 ± 0.4 <sup>A/A</sup>	1.0 ± 0.2 <sup>AB/A</sup>	0.8 ± 0.1 <sup>A/A</sup>
<b>9</b>	6.2 ± 0.0 <sup>A/A</sup>	2.7 ± 0.0 <sup>A/B</sup>	4.0 ± 0.0 <sup>A/C</sup>	5.0 ± 0.2 <sup>A/A</sup>	4.1 ± 0.3 <sup>A/B</sup>	4.7 ± 0.1 <sup>B/A</sup>	7.8 ± 0.1 <sup>A/A</sup>	4.9 ± 0.3 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/C</sup>	5.6 ± 0.1 <sup>A/A</sup>	2.4 ± 0.2 <sup>A/B</sup>	3.3 ± 0.3 <sup>A/B</sup>
<b>18</b>	6.6 ± 0.1 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	4.7 ± 0.1 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/B</sup>	5.3 ± 0.1 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	5.9 ± 0.0 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	4.3 ± 0.1 <sup>A/B</sup>
	<b>CAR</b>											

<b>0</b>	1.0 ± 0.4 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>	1.1 ± 0.6 <sup>A/A</sup>	1.1 ± 0.2 <sup>AB/A</sup>	1.4 ± 0.2 <sup>AB/A</sup>	1.5 ± 0.1 <sup>A/A</sup>	2.7 ± 0.1 <sup>B/A</sup>	3.5 ± 0.3 <sup>A/A</sup>	3.2 ± 0.5 <sup>A/A</sup>	0.8 ± 0.1 <sup>A/A</sup>	0.9 ± 0.2 <sup>AB/A</sup>	0.9 ± 0.2 <sup>A/A</sup>
<b>9</b>	5.9 ± 0.3 <sup>A/A</sup>	3.2 ± 0.0 <sup>A/B</sup>	4.1 ± 0.1 <sup>A/B</sup>	4.6 ± 0.2 <sup>A/AB</sup>	3.8 ± 0.2 <sup>A/B</sup>	4.8 ± 0.0 <sup>B/A</sup>	7.4 ± 0.1 <sup>A/A</sup>	4.9 ± 0.1 <sup>A/B</sup>	6.2 ± 0.0 <sup>A/C</sup>	5.6 ± 0.1 <sup>A/A</sup>	2.3 ± 0.1 <sup>A/B</sup>	3.2 ± 0.1 <sup>A/B</sup>
<b>18</b>	5.9 ± 0.1 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	3.8 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/A</sup>	4.5 ± 0.1 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.9 ± 0.2 <sup>A/B</sup>	6.9 ± 0.1 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	4.3 ± 0.0 <sup>A/B</sup>
<b>THY</b>												
<b>0</b>	1.6 ± 0.1 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>	1.6 ± 0.1 <sup>A/A</sup>	1.5 ± 0.0 <sup>AB/A</sup>	1.8 ± 0.1 <sup>B/A</sup>	1.6 ± 0.1 <sup>A/A</sup>	2.9 ± 0.1 <sup>B/A</sup>	3.8 ± 0.4 <sup>A/A</sup>	3.4 ± 0.3 <sup>A/A</sup>	1.1 ± 0.3 <sup>A/A</sup>	1.8 ± 0.2 <sup>A/A</sup>	1.0 ± 0.2 <sup>A/A</sup>
<b>9</b>	6.5 ± 0.4 <sup>A/A</sup>	2.7 ± 0.0 <sup>A/B</sup>	3.9 ± 0.6 <sup>A/C</sup>	5.3 ± 0.1 <sup>A/A</sup>	3.5 ± 0.2 <sup>A/B</sup>	4.5 ± 0.3 <sup>AB/A</sup>	7.9 ± 0.0 <sup>A/A</sup>	5.7 ± 0.0 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	6.3 ± 0.2 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/B</sup>	3.7 ± 0.4 <sup>A/C</sup>
<b>18</b>	6.7 ± 0.1 <sup>A/A</sup>	3.7 ± 0.0 <sup>A/B</sup>	4.6 ± 0.4 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/A</sup>	4.7 ± 0.2 <sup>A/B</sup>	5.1 ± 0.0 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.7 ± 0.1 <sup>A/B</sup>	6.2 ± 0.3 <sup>A/B</sup>	7.0 ± 0.0 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/B</sup>	4.5 ± 0.5 <sup>A/B</sup>
<b>EUG</b>												
<b>0</b>	1.3 ± 0.1 <sup>A/A</sup>	1.5 ± 0.1 <sup>A/A</sup>	1.5 ± 0.1 <sup>A/A</sup>	1.5 ± 0.1 <sup>AB/A</sup>	1.4 ± 0.2 <sup>AB/A</sup>	1.5 ± 0.1 <sup>A/A</sup>	2.8 ± 0.1 <sup>B/A</sup>	3.7 ± 0.4 <sup>A/A</sup>	3.2 ± 0.3 <sup>A/A</sup>	1.2 ± 0.3 <sup>A/A</sup>	1.6 ± 0.1 <sup>AB/A</sup>	1.4 ± 0.1 <sup>A/A</sup>
<b>9</b>	6.4 ± 0.3 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/B</sup>	3.9 ± 0.1 <sup>A/B</sup>	5.3 ± 0.0 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/B</sup>	4.5 ± 0.1 <sup>AB/AB</sup>	8.2 ± 0.0 <sup>A/A</sup>	5.7 ± 0.0 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/A</sup>	6.4 ± 0.0 <sup>A/A</sup>	2.8 ± 0.1 <sup>A/B</sup>	3.6 ± 0.1 <sup>A/B</sup>
<b>18</b>	6.5 ± 0.1 <sup>A/A</sup>	3.9 ± 0.2 <sup>A/B</sup>	4.1 ± 0.1 <sup>A/B</sup>	6.3 ± 0.0 <sup>A/A</sup>	4.7 ± 0.5 <sup>A/B</sup>	4.9 ± 0.1 <sup>A/B</sup>	9.2 ± 0.1 <sup>A/A</sup>	5.8 ± 0.0 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	7.3 ± 0.0 <sup>A/A</sup>	3.9 ± 0.2 <sup>A/B</sup>	4.5 ± 0.1 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 7.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for total viable mesophilic (TVC<sub>m</sub>) and psychrophilic (TVC<sub>p</sub>) counts and total *Enterobacteriaceae* (TEC) as determined from salmon stored at 2°C for 18 days and treated with a dip treatment of either 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) in combination with different packaging conditions

Time (days)	TVC <sub>m</sub>			TVC <sub>p</sub>			TEC		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP
<b>SDW</b>									
<b>0</b>	3.8 ± 0.1 <sup>AB/A</sup>	3.7 ± 0.3 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/A</sup>	3.9 ± 0.2 <sup>A/A</sup>	3.9 ± 0.2 <sup>A/A</sup>	4.0 ± 0.1 <sup>A/A</sup>	1.7 ± 0.0 <sup>A/A</sup>	1.8 ± 0.3 <sup>A/A</sup>	1.9 ± 0.3 <sup>AB/A</sup>
<b>9</b>	7.7 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>AB/B</sup>	5.1 ± 0.0 <sup>AB/B</sup>	8.2 ± 0.0 <sup>A/A</sup>	6.0 ± 0.1 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/B</sup>	3.4 ± 0.2 <sup>A/A</sup>	1.8 ± 0.1 <sup>AB/B</sup>	3.0 ± 0.2 <sup>A/A</sup>
<b>18</b>	9.1 ± 0.2 <sup>A/A</sup>	6.4 ± 0.1 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/B</sup>	9.3 ± 0.4 <sup>A/A</sup>	6.1 ± 0.1 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/A</sup>	3.4 ± 0.1 <sup>AB/B</sup>	4.0 ± 0.2 <sup>A/B</sup>
<b>CA</b>									
<b>0</b>	3.2 ± 0.3 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/A</sup>	4.1 ± 0.3 <sup>A/A</sup>	3.4 ± 0.3 <sup>A/A</sup>	3.8 ± 0.3 <sup>A/A</sup>	3.9 ± 0.3 <sup>A/A</sup>	1.3 ± 0.2 <sup>A/A</sup>	1.7 ± 0.2 <sup>A/A</sup>	1.5 ± 0.2 <sup>AB/A</sup>
<b>9</b>	7.3 ± 0.1 <sup>A/A</sup>	5.5 ± 0.2 <sup>AB/B</sup>	4.9 ± 0.0 <sup>AB/B</sup>	7.5 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.1 ± 0.1 <sup>A/B</sup>	2.8 ± 0.2 <sup>A/A</sup>	1.3 ± 0.1 <sup>AB/B</sup>	2.1 ± 0.2 <sup>AB/A</sup>
<b>18</b>	8.8 ± 0.1 <sup>A/A</sup>	6.1 ± 0.4 <sup>A/B</sup>	6.1 ± 0.3 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/B</sup>	5.7 ± 0.4 <sup>A/B</sup>	5.3 ± 0.0 <sup>A/A</sup>	2.7 ± 0.0 <sup>A/B</sup>	3.8 ± 0.7 <sup>A/B</sup>
<b>LA</b>									
<b>0</b>	3.6 ± 0.4 <sup>AB/A</sup>	3.1 ± 0.3 <sup>A/A</sup>	3.4 ± 0.3 <sup>A/A</sup>	3.6 ± 0.4 <sup>A/A</sup>	4.0 ± 0.6 <sup>A/A</sup>	3.4 ± 0.3 <sup>A/A</sup>	1.0 ± 0.3 <sup>A/A</sup>	1.2 ± 0.1 <sup>A/A</sup>	1.2 ± 0.2 <sup>AB/A</sup>
<b>9</b>	7.3 ± 0.0 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/B</sup>	4.3 ± 0.0 <sup>A/B</sup>	7.5 ± 0.0 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/B</sup>	2.9 ± 0.5 <sup>A/A</sup>	1.2 ± 0.1 <sup>AB/B</sup>	1.9 ± 0.1 <sup>AB/AB</sup>
<b>18</b>	9.0 ± 0.3 <sup>A/A</sup>	5.8 ± 0.8 <sup>A/B</sup>	5.6 ± 0.3 <sup>A/B</sup>	9.2 ± 0.1 <sup>A/A</sup>	5.9 ± 0.2 <sup>A/B</sup>	5.3 ± 0.2 <sup>A/B</sup>	4.8 ± 0.0 <sup>A/A</sup>	2.5 ± 0.5 <sup>A/B</sup>	2.9 ± 0.1 <sup>A/B</sup>
<b>CIT</b>									
<b>0</b>	3.8 ± 0.1 <sup>AB/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.1 ± 0.3 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	4.2 ± 0.1 <sup>A/A</sup>	1.7 ± 0.1 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>	1.8 ± 0.2 <sup>AB/A</sup>
<b>9</b>	7.7 ± 0.0 <sup>A/A</sup>	5.6 ± 0.1 <sup>AB/B</sup>	5.6 ± 0.1 <sup>B/B</sup>	8.4 ± 0.0 <sup>A/A</sup>	6.0 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/B</sup>	3.4 ± 0.0 <sup>A/A</sup>	2.3 ± 0.1 <sup>A/B</sup>	2.5 ± 0.1 <sup>AB/A</sup>
<b>18</b>	8.9 ± 0.2 <sup>A/A</sup>	6.3 ± 0.1 <sup>A/B</sup>	6.2 ± 0.2 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	6.4 ± 0.0 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	5.8 ± 0.2 <sup>A/A</sup>	4.1 ± 0.2 <sup>B/B</sup>	4.1 ± 0.1 <sup>A/B</sup>

<b>CAR</b>									
<b>0</b>	3.4 ± 0.1 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/A</sup>	3.5 ± 0.1 <sup>A/A</sup>	3.2 ± 0.0 <sup>A/A</sup>	3.8 ± 0.2 <sup>A/A</sup>	0.9 ± 0.1 <sup>A/A</sup>	1.2 ± 0.2 <sup>A/A</sup>	1.1 ± 0.1 <sup>B/A</sup>
<b>9</b>	7.3 ± 0.1 <sup>A/A</sup>	6.0 ± 0.0 <sup>B/B</sup>	4.8 ± 0.2 <sup>AB/C</sup>	8.0 ± 0.2 <sup>A/A</sup>	6.0 ± 0.2 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	2.9 ± 0.0 <sup>A/A</sup>	1.3 ± 0.1 <sup>AB/B</sup>	2.8 ± 0.0 <sup>AB/A</sup>
<b>18</b>	8.8 ± 0.1 <sup>A/A</sup>	6.6 ± 0.0 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/B</sup>	8.9 ± 0.2 <sup>A/A</sup>	6.2 ± 0.1 <sup>A/B</sup>	6.3 ± 0.0 <sup>A/B</sup>	5.3 ± 0.2 <sup>A/A</sup>	3.2 ± 0.2 <sup>AB/B</sup>	4.5 ± 0.2 <sup>A/A</sup>
<b>THY</b>									
<b>0</b>	4.3 ± 0.3 <sup>B/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.1 ± 0.0 <sup>A/A</sup>	1.8 ± 0.3 <sup>A/A</sup>	1.8 ± 0.3 <sup>A/A</sup>	2.2 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.0 <sup>A/A</sup>	5.0 ± 0.1 <sup>A/B</sup>	4.8 ± 0.1 <sup>AB/B</sup>	8.3 ± 0.0 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.1 ± 0.0 <sup>A/B</sup>	3.1 ± 0.1 <sup>A/A</sup>	0.6 ± 0.3 <sup>B/B</sup>	1.9 ± 0.4 <sup>B/C</sup>
<b>18</b>	8.8 ± 0.1 <sup>A/A</sup>	6.4 ± 0.1 <sup>A/B</sup>	6.0 ± 0.2 <sup>A/B</sup>	8.8 ± 0.1 <sup>A/A</sup>	6.3 ± 0.0 <sup>A/B</sup>	6.2 ± 0.2 <sup>A/B</sup>	5.4 ± 0.2 <sup>A/A</sup>	3.3 ± 0.2 <sup>AB/B</sup>	3.2 ± 0.1 <sup>A/B</sup>
<b>EUG</b>									
<b>0</b>	3.9 ± 0.1 <sup>AB/A</sup>	3.8 ± 0.2 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/A</sup>	3.6 ± 0.3 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/B</sup>	1.7 ± 0.1 <sup>A/A</sup>	1.6 ± 0.1 <sup>A/A</sup>	1.9 ± 0.2 <sup>AB/A</sup>
<b>9</b>	7.6 ± 0.1 <sup>A/A</sup>	5.2 ± 0.2 <sup>A/B</sup>	5.0 ± 0.1 <sup>AB/B</sup>	8.3 ± 0.1 <sup>A/A</sup>	6.1 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	2.8 ± 0.2 <sup>A/A</sup>	1.0 ± 0.1 <sup>B/B</sup>	3.2 ± 0.8 <sup>A/A</sup>
<b>18</b>	8.8 ± 0.0 <sup>A/A</sup>	6.6 ± 0.3 <sup>A/B</sup>	5.9 ± 0.1 <sup>A/C</sup>	8.9 ± 0.1 <sup>A/A</sup>	6.4 ± 0.0 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	5.2 ± 0.2 <sup>A/A</sup>	3.7 ± 0.4 <sup>AB/B</sup>	3.6 ± 0.1 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

**Table 8.** Mean log<sub>10</sub> CFU/cm<sup>2</sup> values for hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Br. Thermosphacta* and *Photobacterium* spp. counts as determined from salmon stored at 2°C for 18 days and treated with a dip treatment of either 5% (w/v) citric acid (CA), 5% (v/v) lactic acid (LA), 1% (v/v) citral (CIT), 1% (v/v) carvacrol (CAR), 1% (w/v) thymol (THY) or 1% (v/v) eugenol (EUG) in combination with different packaging conditions.

Time (days)	HSPB			LAB			<i>Photobacterium</i> spp.			<i>Br. thermosphacta</i>		
	Aer <sup>1</sup>	MAP <sup>2</sup>	SP <sup>3</sup>	Aer	MAP	SP	Aer	MAP	SP	Aer	MAP	SP
	<b>SDW</b>											
<b>0</b>	2.2 ± 0.3 <sup>A/A</sup>	2.6 ± 0.3 <sup>A/A</sup>	3.5 ± 0.3 <sup>A/B</sup>	2.4 ± 0.1 <sup>A/A</sup>	2.5 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>AB/A</sup>	3.9 ± 0.3 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/A</sup>	4.0 ± 0.2 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.6 ± 0.2 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>
<b>9</b>	8.0 ± 0.0 <sup>A/A</sup>	5.0 ± 0.0 <sup>A/B</sup>	6.0 ± 0.2 <sup>A/C</sup>	6.1 ± 0.2 <sup>A/A</sup>	5.3 ± 0.0 <sup>A/B</sup>	5.4 ± 0.2 <sup>A/AB</sup>	8.5 ± 0.0 <sup>A/A</sup>	6.2 ± 0.1 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	6.8 ± 0.0 <sup>A/A</sup>	4.5 ± 0.1 <sup>A/B</sup>	4.6 ± 0.2 <sup>A/B</sup>
<b>18</b>	6.6 ± 0.3 <sup>A/A</sup>	5.6 ± 0.2 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	7.0 ± 0.0 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/B</sup>	9.5 ± 0.4 <sup>A/A</sup>	6.2 ± 0.1 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/B</sup>	7.4 ± 0.1 <sup>A/A</sup>	5.1 ± 0.2 <sup>A/B</sup>	4.8 ± 0.1 <sup>A/B</sup>
	<b>CA</b>											
<b>0</b>	1.4 ± 0.2 <sup>A/A</sup>	1.8 ± 0.1 <sup>A/A</sup>	3.1 ± 0.3 <sup>AB/B</sup>	2.3 ± 0.1 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/A</sup>	3.4 ± 0.5 <sup>A/B</sup>	3.4 ± 0.3 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/A</sup>	4.1 ± 0.4 <sup>A/A</sup>	2.2 ± 0.0 <sup>A/A</sup>	2.3 ± 0.2 <sup>A/A</sup>	2.2 ± 0.1 <sup>A/A</sup>
<b>9</b>	7.0 ± 0.2 <sup>A/A</sup>	3.2 ± 0.1 <sup>BC/B</sup>	4.8 ± 0.3 <sup>B/C</sup>	6.2 ± 0.1 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/B</sup>	5.3 ± 0.3 <sup>A/B</sup>	8.2 ± 0.1 <sup>A/A</sup>	5.6 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/B</sup>	6.2 ± 0.0 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	4.0 ± 0.4 <sup>A/B</sup>
<b>18</b>	5.4 ± 0.1 <sup>B/A</sup>	4.0 ± 0.1 <sup>C/B</sup>	4.1 ± 0.2 <sup>BC/C</sup>	7.1 ± 0.1 <sup>A/A</sup>	5.8 ± 0.1 <sup>A/B</sup>	5.6 ± 0.2 <sup>A/B</sup>	8.9 ± 0.1 <sup>A/A</sup>	6.1 ± 0.2 <sup>A/B</sup>	6.1 ± 0.2 <sup>A/B</sup>	7.3 ± 0.0 <sup>A/A</sup>	4.7 ± 0.0 <sup>A/B</sup>	4.4 ± 0.3 <sup>A/B</sup>
	<b>LA</b>											
<b>0</b>	2.1 ± 0.3 <sup>A/A</sup>	2.4 ± 0.2 <sup>A/A</sup>	2.4 ± 0.3 <sup>B/A</sup>	2.2 ± 0.2 <sup>A/A</sup>	1.9 ± 0.1 <sup>A/A</sup>	2.1 ± 0.2 <sup>B/A</sup>	3.6 ± 0.3 <sup>A/A</sup>	3.9 ± 0.4 <sup>A/A</sup>	3.3 ± 0.3 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>	2.0 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.3 ± 0.2 <sup>A/A</sup>	2.6 ± 0.2 <sup>C/B</sup>	4.4 ± 0.3 <sup>B/C</sup>	5.5 ± 0.1 <sup>A/A</sup>	4.1 ± 0.2 <sup>A/B</sup>	4.9 ± 0.2 <sup>A/A</sup>	7.9 ± 0.0 <sup>A/A</sup>	5.7 ± 0.0 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	6.0 ± 0.1 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/B</sup>	3.3 ± 0.2 <sup>A/B</sup>
<b>18</b>	5.7 ± 0.4 <sup>AB/A</sup>	4.1 ± 0.2 <sup>BC/B</sup>	3.9 ± 0.1 <sup>C/B</sup>	6.5 ± 0.1 <sup>A/A</sup>	5.2 ± 0.2 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/B</sup>	9.2 ± 0.1 <sup>A/A</sup>	5.8 ± 0.2 <sup>A/B</sup>	5.8 ± 0.1 <sup>A/B</sup>	7.3 ± 0.0 <sup>A/A</sup>	4.8 ± 0.1 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/B</sup>
	<b>CIT</b>											
<b>0</b>	1.8 ± 0.2 <sup>A/A</sup>	2.9 ± 0.1 <sup>A/B</sup>	3.2 ± 0.3 <sup>AB/B</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.9 ± 0.2 <sup>A/A</sup>	2.8 ± 0.3 <sup>AB/A</sup>	4.5 ± 0.3 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.3 ± 0.2 <sup>A/A</sup>	1.9 ± 0.1 <sup>A/A</sup>	2.9 ± 0.1 <sup>A/B</sup>	2.6 ± 0.1 <sup>A/AB</sup>
<b>9</b>	7.5 ± 0.0 <sup>A/A</sup>	4.1 ± 0.2 <sup>AB/B</sup>	5.2 ± 0.3 <sup>AB/C</sup>	6.1 ± 0.0 <sup>A/A</sup>	5.3 ± 0.3 <sup>A/B</sup>	5.4 ± 0.1 <sup>A/AB</sup>	8.6 ± 0.1 <sup>A/A</sup>	6.4 ± 0.1 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/B</sup>	6.7 ± 0.0 <sup>A/A</sup>	4.7 ± 0.0 <sup>A/B</sup>	4.1 ± 0.2 <sup>A/B</sup>
<b>18</b>	6.7 ± 0.0 <sup>A/A</sup>	4.7 ± 0.1 <sup>AB/B</sup>	5.0 ± 0.1 <sup>AB/B</sup>	6.9 ± 0.1 <sup>A/A</sup>	6.0 ± 0.1 <sup>A/B</sup>	5.7 ± 0.1 <sup>A/A</sup>	9.0 ± 0.1 <sup>A/A</sup>	6.5 ± 0.0 <sup>A/B</sup>	6.6 ± 0.4 <sup>A/B</sup>	7.3 ± 0.0 <sup>A/A</sup>	5.4 ± 0.1 <sup>A/B</sup>	4.0 ± 0.3 <sup>A/C</sup>

<b>CAR</b>												
<b>0</b>	1.7 ± 0.2 <sup>A/A</sup>	2.2 ± 0.3 <sup>A/A</sup>	2.9 ± 0.1 <sup>AB/B</sup>	2.3 ± 0.1 <sup>A/A</sup>	2.1 ± 0.4 <sup>A/A</sup>	2.7 ± 0.3 <sup>AB/A</sup>	3.8 ± 0.1 <sup>A/A</sup>	3.7 ± 0.3 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.2 ± 0.1 <sup>A/A</sup>	2.3 ± 0.0 <sup>A/A</sup>
<b>9</b>	7.6 ± 0.1 <sup>A/A</sup>	4.5 ± 0.4 <sup>A/B</sup>	6.2 ± 0.1 <sup>A/C</sup>	5.7 ± 0.1 <sup>A/A</sup>	4.9 ± 0.2 <sup>A/B</sup>	5.6 ± 0.0 <sup>A/AB</sup>	8.3 ± 0.1 <sup>A/A</sup>	6.1 ± 0.2 <sup>A/B</sup>	6.5 ± 0.0 <sup>A/B</sup>	6.2 ± 0.1 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	4.5 ± 0.0 <sup>A/B</sup>
<b>18</b>	6.3 ± 0.1 <sup>AB/A</sup>	4.9 ± 0.1 <sup>AB/B</sup>	5.1 ± 0.3 <sup>AB/B</sup>	6.8 ± 0.0 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	6.0 ± 0.0 <sup>A/AB</sup>	8.8 ± 0.1 <sup>A/A</sup>	6.2 ± 0.1 <sup>A/B</sup>	6.8 ± 0.3 <sup>A/B</sup>	7.1 ± 0.1 <sup>A/A</sup>	5.1 ± 0.2 <sup>A/B</sup>	4.7 ± 0.0 <sup>A/B</sup>
<b>THY</b>												
<b>0</b>	2.3 ± 0.2 <sup>A/A</sup>	2.6 ± 0.3 <sup>A/A</sup>	3.6 ± 0.2 <sup>A/B</sup>	2.6 ± 0.3 <sup>A/A</sup>	2.3 ± 0.1 <sup>A/A</sup>	3.2 ± 0.2 <sup>A/A</sup>	4.2 ± 0.3 <sup>A/A</sup>	4.1 ± 0.1 <sup>A/A</sup>	4.6 ± 0.2 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	3.1 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.9 ± 0.2 <sup>A/A</sup>	4.1 ± 0.1 <sup>AB/B</sup>	5.0 ± 0.1 <sup>AB/B</sup>	5.8 ± 0.1 <sup>A/A</sup>	4.5 ± 0.2 <sup>A/B</sup>	5.0 ± 0.1 <sup>A/B</sup>	8.4 ± 0.0 <sup>A/A</sup>	6.0 ± 0.0 <sup>A/B</sup>	6.4 ± 0.1 <sup>A/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	4.3 ± 0.1 <sup>A/B</sup>	3.8 ± 0.3 <sup>A/B</sup>
<b>18</b>	6.7 ± 0.2 <sup>A/A</sup>	5.3 ± 0.0 <sup>A/B</sup>	4.7 ± 0.2 <sup>ABC/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	5.9 ± 0.1 <sup>A/B</sup>	5.7 ± 0.2 <sup>A/B</sup>	8.7 ± 0.1 <sup>A/A</sup>	6.4 ± 0.0 <sup>A/B</sup>	6.5 ± 0.3 <sup>A/B</sup>	7.0 ± 0.2 <sup>A/A</sup>	5.5 ± 0.1 <sup>A/B</sup>	4.4 ± 0.3 <sup>A/C</sup>
<b>EUG</b>												
<b>0</b>	2.3 ± 0.2 <sup>A/A</sup>	2.1 ± 0.1 <sup>A/A</sup>	3.5 ± 0.1 <sup>A/B</sup>	2.7 ± 0.2 <sup>A/A</sup>	2.2 ± 0.1 <sup>A/A</sup>	2.8 ± 0.2 <sup>AB/A</sup>	3.8 ± 0.1 <sup>A/A</sup>	3.8 ± 0.1 <sup>A/A</sup>	4.0 ± 0.1 <sup>A/A</sup>	2.5 ± 0.1 <sup>A/A</sup>	2.6 ± 0.1 <sup>A/A</sup>	2.7 ± 0.2 <sup>A/A</sup>
<b>9</b>	7.8 ± 0.1 <sup>A/A</sup>	4.0 ± 0.1 <sup>AB/B</sup>	5.2 ± 0.2 <sup>AB/C</sup>	5.9 ± 0.2 <sup>A/A</sup>	5.0 ± 0.2 <sup>A/B</sup>	5.2 ± 0.1 <sup>A/AB</sup>	8.5 ± 0.1 <sup>A/A</sup>	6.8 ± 0.2 <sup>A/B</sup>	6.4 ± 0.0 <sup>A/B</sup>	6.7 ± 0.0 <sup>A/A</sup>	4.4 ± 0.1 <sup>A/B</sup>	4.1 ± 0.2 <sup>A/B</sup>
<b>18</b>	6.5 ± 0.0 <sup>A/A</sup>	5.0 ± 0.1 <sup>AB/B</sup>	5.1 ± 0.1 <sup>AB/B</sup>	6.7 ± 0.1 <sup>A/A</sup>	5.9 ± 0.0 <sup>A/AB</sup>	5.7 ± 0.1 <sup>A/B</sup>	8.8 ± 0.1 <sup>A/A</sup>	6.4 ± 0.1 <sup>A/B</sup>	6.3 ± 0.1 <sup>A/B</sup>	7.2 ± 0.1 <sup>A/A</sup>	5.3 ± 0.1 <sup>A/B</sup>	4.7 ± 0.0 <sup>A/B</sup>

**Statistical Analysis X/Y** – first superscripted letters denote statistical significance between the different antimicrobial treatments within the same packaging system and sampling time. Second superscripted letters denote statistical significance between packaging system (air v MAP v SP) within a given treatment and sampling time. (P > 0.05).

<sup>1</sup> Aer - Aerobically stored, <sup>2</sup> MAP - Modified atmosphere packaging, <sup>3</sup> SP - Skin packaging

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

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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 (25 cm<sup>2</sup> surface areas). Mesophilic total viable counts (TVC<sub>m</sub>) on skin, flesh and swab samples increased from 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.0, 5.1 and 5.7 log<sub>10</sub> CFU/cm<sup>2</sup> after 10 days, respectively. Psychrotrophic counts (TVC<sub>p</sub>), increased from 2.2, 1.8 and 3.1 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.2, 5.3 and 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>, 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 17.2–26 h). 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<sub>10</sub> CFU/cm<sup>2</sup> indicating the end of shelf-life.

### 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<sub>w</sub>) of ~0.99 (Bozaris 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-toxicogenic *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<sub>10</sub> CFU/g (Robson et al., 2007) to 7

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$\log_{10}$  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<sub>m</sub>), psychrophilic TVC (TVC<sub>p</sub>), 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 (3–4 kg) and was obtained within 48 h 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 (10 g) of inner flesh and two samples (10 g) of outer skin obtained on each of the sampling days. From the other side the outer skin of the fish was swabbed (25 cm<sup>2</sup> 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<sup>®</sup> PUL100E, Microgen Bioproducts Ltd, Surrey, United Kingdom) for 1 min in 90 ml MRD and ten-fold dilution series prepared up to 10<sup>-5</sup>. 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<sub>m</sub>, incubated 30 °C for 72 h) and psychrotrophic (TVC<sub>p</sub>, incubated at 6.5 °C for 240 h) 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 24 h, HSPB on Iron Lyngby agar incubated at 25 °C for 72 h, 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 72 h. *Pseudomonas* counts were carried out on *Pseudomonas* Agar Base (Oxoid, Basingstoke, United Kingdom (CM0559)), supplemented with Cetrinide-Fucidin-Cephaloridine (CFC) supplements (Oxoid, Basingstoke, United Kingdom (SR0103)) incubated at 37 °C for 48 h, *Br. thermosphacta* counts on streptomycin-thallos acetate-actidione (STAA) agar base (Oxoid, Basingstoke, United Kingdom (CM0881)), supplemented with STAA (Oxoid, Basingstoke, United Kingdom (SR0151E)) incubated at 25 °C for 72 h 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 168 h. 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<sub>w</sub>), pH and temperature

On each sampling day, the pH, water activity (a<sub>w</sub>) and storage temperatures were monitored. To measure the pH and a<sub>w</sub>, two samples (10 g) 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<sub>w</sub> of the flesh and skin samples were measured using a Decagon AquaLab LITE water activity meter (Labcell Ltd, Alton, United Kingdom) according to 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<sup>2</sup>.

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 record 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<sup>2</sup>. 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:  $G = t/3.3 \log_{10} b/B$ , 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

Table 1 presents the results for the pH and a<sub>w</sub> obtained over the 10 day trial. The pH of the salmon flesh and skin samples followed a similar trend, decreasing from 7.0 to 6.5 and 6.7, respectively. The a<sub>w</sub> 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

**Table 1**  
pH and a<sub>w</sub> measurements as determined from skin, flesh and swab samples from Atlantic salmon (*Salmo salar*) stored at 2 °C for 10 days.

	Day	pH	a <sub>w</sub>
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

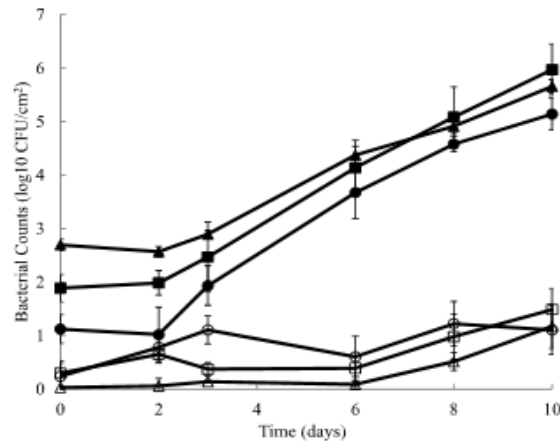


Fig. 1. Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC<sub>m</sub> (■) and TEC (□); flesh TVC<sub>m</sub> (●) and TEC (○) and swab TVC<sub>m</sub> (▲) and TEC (△) samples stored at 2 °C for 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

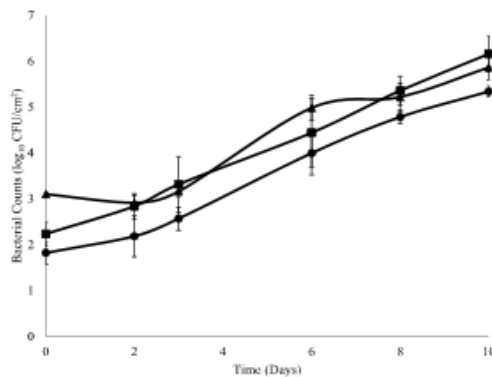


Fig. 2. Bacterial counts on Atlantic salmon (*Salmo salar*); skin TVC<sub>m</sub> (■), flesh TVC<sub>m</sub> (●) and swab TVC<sub>m</sub> (▲) samples stored at 2 °C for 10 days. Each data point and the error bars show the mean of 3 replicates ± the standard error.

only data obtained with 1% NaCl is presented. The initial TVC<sub>m</sub> counts on skin, flesh and swab samples on day 0 were 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup> which increased to 6.0, 5.1 and 5.7 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively, after 10 days storage (Fig. 1). TEC increased from 0.3, 0.2 and 0.02 log<sub>10</sub> CFU/cm<sup>2</sup> on skin, flesh and swab samples to 1.5, 1.2 and 1.2 log<sub>10</sub> CFU/cm<sup>2</sup>, respectively, by day 10. Fig. 2 shows the growth of TVC<sub>m</sub> with counts increasing from 2.2, 1.8 and 3.1 log<sub>10</sub> CFU/cm<sup>2</sup> to 6.2, 5.3 and 5.9 log<sub>10</sub> CFU/cm<sup>2</sup>, for skin, flesh and swab samples, respectively. Initial counts of 1.4, 1.4, 1.4, < 1.0 and 1.8 log<sub>10</sub> CFU/cm<sup>2</sup> 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<sub>10</sub> CFU/cm<sup>2</sup>, respectively (Fig. 3). Corresponding counts on flesh samples were 1.0, 1.0, 1.0, < 1.0 and 1.2 log<sub>10</sub> CFU/cm<sup>2</sup> increasing to 4.4, 5.2, 5.2, 3.9 and 4.8 log<sub>10</sub> CFU/cm<sup>2</sup> (Fig. 4). The data for the swab samples is shown in Fig. 5. HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. counts increased by 2.8, 3.3, 3.3, 4.1 and 2.0 log<sub>10</sub> CFU/cm<sup>2</sup>, 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

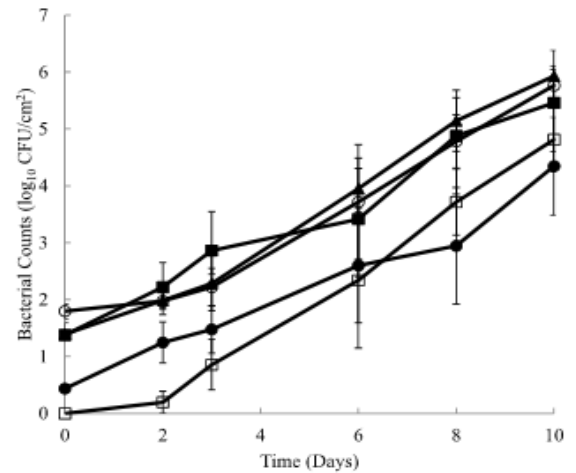


Fig. 3. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp. (○), on the skin from Atlantic salmon (*Salmo salar*) stored at 2 °C for 10 days. Each data point and the error bars show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

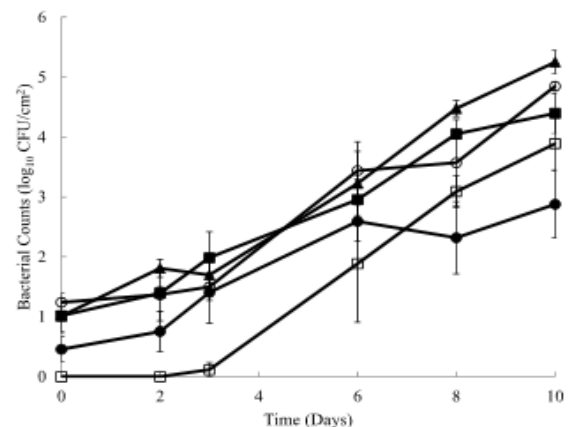


Fig. 4. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp. (○), on Atlantic salmon (*Salmo salar*) flesh stored at 2 °C for 10 days. Each data point and the error bars show the mean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

for both mesophilic and psychrotrophic groups irrespective of sample type. Enterobacteriaceae grew considerably slower with mean generation times of 60.5–72.7 h. Interestingly the spoilage bacteria, HSPB, LAB, *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. showed similar mean generation times of 17.2–26 h, regardless of sample type.

#### 4. Discussion

The initial TVC<sub>m</sub> counts on skin, flesh and swab samples were 1.9, 1.1 and 2.7 log<sub>10</sub> CFU/cm<sup>2</sup>. Other studies have reported initial bacterial levels in fresh farmed salmon of approximately 3 log<sub>10</sub> CFU/g (Briones et al., 2010; Schubring, 2003). However, Mjølretz et al. (2016) found

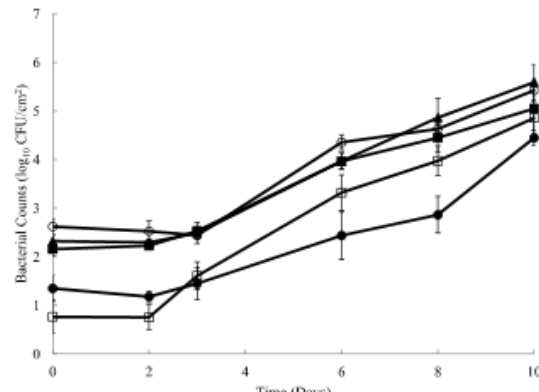


Fig. 5. Bacterial counts; hydrogen sulphide producing bacteria (HSPB) (■), lactic acid bacteria (LAB) (●), *Pseudomonas* spp. (▲), *Br. thermosphacta* (□) and *Photobacterium* spp. (○), in swab samples from Atlantic salmon (*Salmo salar*) stored at 2 °C for 10 days. Each data point and the error bars show the mean of 3 replicates + the standard error.

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<sup>2</sup>, 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 (0.02–0.3  $\log_{10}$  CFU/cm<sup>2</sup>), suggesting the salmon was farmed in clean waters.

Table 2

Growth parameters for total viable count mesophilic (TVC<sub>m</sub>) and psychrotrophic (TVC<sub>p</sub>), TEC, hydrogen sulphide producing bacteria (HSPB), lactic acid bacteria (LAB), *Pseudomonas* spp., *Br. thermosphacta* and *Photobacterium* spp. as determined from skin, flesh and swab samples from Atlantic salmon (*Salmo salar*) stored at 2 °C for 10 days.

Treatment	Initial concentration ( $\log_{10}$ CFU/cm <sup>2</sup> )	Mean generation time (h) <sup>a</sup>	$\mu_{max}$ (generations day <sup>-1</sup> )	Maximum concentration observed ( $\log_{10}$ CFU/cm <sup>2</sup> )
<b>Skin</b>				
TVC <sub>m</sub>	1.9	23.5	1.44	6.0
TVC <sub>p</sub>	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
<i>Pseudomonas</i> spp.	1.4	16.2	1.20	5.9
<i>Br. thermosphacta</i>	ND	15.2	1.44	4.8
<i>Photobacterium</i> spp.	1.8	18.2	1.20	5.8
<b>Flesh</b>				
TVC <sub>m</sub>	1.1	18.2	1.44	5.1
TVC <sub>p</sub>	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
<i>Pseudomonas</i> spp.	1.0	17.3	1.20	5.2
<i>Br. thermosphacta</i>	ND	18.6	1.68	3.9
<i>Photobacterium</i> spp.	1.2	20.2	0.96	4.8
<b>Skin Swab</b>				
TVC <sub>m</sub>	2.7	24.2	1.20	5.7
TVC <sub>p</sub>	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
<i>Pseudomonas</i> spp.	2.3	22.0	1.20	5.6
<i>Br. thermosphacta</i>	0.08	17.2	1.20	4.9
<i>Photobacterium</i> spp.	2.6	26.0	1.44	5.4

<sup>a</sup> Calculated using the formula  $G = t/3.3 \log b/B$ , 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).

$\log_{10}$  CFU/g or CFU/cm<sup>2</sup>.

## 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<sup>2</sup>, indicating the end of shelf-life.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.fm.2018.08.001>.

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**Appendix D – Diversity and Composition of the Gut  
Microbiota of Atlantic Salmon (*Salmo salar*) Farmed in  
Irish Waters**

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**Diversity and composition of the gut microbiota of Atlantic salmon (*Salmo salar*)  
farmed in Irish waters**

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Running head: The microbiome of Atlantic salmon (*Salmo salar*)

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## Abstract

### Aims:

Information on the gut microbiota of salmon is essential for optimising nutrition while maintaining host health and welfare. This study's objectives were to characterize the microbiota in the GI tract of Atlantic salmon (*Salmo salar*) farmed in waters off the west coast of Ireland and to investigate whether there is a difference in microbiota diversity between the proximal and distal regions of the intestine.

### Methods and Results:

The microbiota from the proximal and distal intestine (PI and DI, respectively) of Atlantic salmon was examined using MiSeq Illumina high throughput sequencing of the 16S rRNA gene. The PI region had greater bacterial diversity than the DI region. Six phyla were present in the DI samples, dominated by Tenericutes and Firmicutes. These six phyla were also amongst the 12 phyla detected in the PI samples. The PI microbiota was dominated by Tenericutes, Firmicutes, Bacteroidetes and Proteobacteria. A core microbiota of 20 operational taxonomic units (OTUs) common to both regions was observed.

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Conclusions:

It was concluded that Tenericutes were the dominant phylum in both PI and DI samples and the PI region had greater Shannon and Simpson diversity of bacteria. However further work is required to identify the functionality of the salmon microbiota.

Significance and impact of the study:

Our study determined the composition and diversity of the intestinal microbiota in adult salmon from a commercial fishery and provides data to improve our understanding of their contributions to the nutrition, health and welfare of Atlantic salmon farmed in Irish waters..

Keywords: aquaculture, *Salmo salar*, gut, intestine, microbiome, sequencing.

## 1. Introduction

The growing demand for fish coupled with a decrease in wild stocks has resulted in the growth of the aquaculture industry (FAO, 2010). In 2017, the Irish aquaculture industry was worth €208 million, an increase of 24% from the previous year (BIM 2018). According to Bord Iascaigh Mhara (BIM, the Irish state agency responsible for developing the Irish marine fishing and aquaculture industries) (BIM 2018), the production of Atlantic salmon (*Salmo salar*) grew 25% since 2016, up to 20,000 tonnes, with a value of €147 million. This compares to an overall value of €687m for the whole European Salmon farming sector in Europe (Anon, 2018). The quality of the final product is reliant on producing healthy fish which, in turn, is reliant on the fish having a balanced microbiota that maximises nutrition and prevents infection (Navarrete et al., 2009; Llewellyn et al., 2014).

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Microbes normally interact with hosts along a mucosal surface, the largest of which are the intestines (O'Hara and Shanahan, 2006). The gastrointestinal (GI) tract of an animal is home to a large and diverse microbial ecosystem, known as the microbiota (Nayak 2010). In mammals, the gut acts as an ecological niche for specialized bacteria and the gut microbiota is very important to the health and survival of its host organism by promoting nutrient supply and reducing the risk of disease by outcompeting pathogenic bacteria (Nayak 2010; Ghanbari et al. 2015; Dehler et al. 2017; Nyman et al. 2017). Fish species also harbour a unique and diverse gut microbiota which is essential for maintaining host health (Lowery et al., 2015; Llewellyn et al., 2016). Rawls et al. (2004), for example, demonstrated the involvement of the microbiota in promoting nutrient breakdown and pathogen resistance in zebrafish. However, less research has been carried out on the gut microbiota of fish, when compared to other vertebrates.

The gut surface has different physical and chemical properties throughout, resulting in a unique and diverse microbiota composition at different stages along the GI tract (Navarrete et al. 2009; Nayak 2010). Gajardo et al. (2016), for example, reported microbial differences in different parts of the salmon intestine. Austin and Al-Zahrani (1988) reported greater microbial diversity in the proximal region of the GI tract of rainbow trout, a finding more recently supported by the work of Lowrey et al. (2015). However these differences may be less significant in other fish species (Ringø et al. 1995; Nyman et al. 2017). There are many factors which can affect the microbiota of fish such as diet (Ringo et al., 2010; Hovda et al., 2007; Desai et al., 2012; Harvey et al., 2016), freshwater-to-saltwater transition (Rudi et al., 2018), sex (Dhanasiri et al., 2011), captive state (Dhanasiri et al., 2011), phylogeny (Miyake et al., 2015), trophic level (Claesson et al., 2012; Serino et al., 2012; Miyake et al., 2015; Li et al., 2017), season (Hovda et al., 2012) and developmental stage (Hansen and Olafsen, 1999). Moreover, fish are constantly exposed to the indigenous microorganisms in their

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environment (Horsley 1973; Roeselers et al. 2011) and hence the natural microbiota of fish is also influenced by their habitat (Ghanbari et al. 2015; Dehler et al. 2017).

Culture based methods typically detect only 1% of bacteria (Ingerslev et al. 2014; Ghanbari et al. 2015). Molecular based methods provide a clearer insight into the diversity of microbial communities present in the gut (Austin 2006; Ghanbari et al. 2015 ; Navarrete et al. 2009). Next-generation sequence (NGS) analysis of the 16S ribosomal RNA gene (rRNA) is, for example, a widely used method to investigate microbial diversity and to characterize several taxonomic groups (Baker et al. 2003; Gloor et al., 2010; Klindworth et al. 2013; Llewellyn et al. 2014; Reuter et al., 2015; Gajardo et al., 2016).

In this study, the microbiota from the proximal and distal intestine in Atlantic salmon was examined using MiSeq Illumina high throughput sequencing of the 16S rRNA gene. The objectives of this study were to characterize the microbiota in the GI tract of Atlantic salmon farmed in waters off the west coast of Ireland and to investigate whether or not there is a difference in microbiota diversity between the proximal and distal regions of the intestine.

## **2. Materials & Methods**

### **2.1. Sample Collection**

The salmon used in this study were farmed for approximately 18 months in open water cages in Kilkieran Bay (Galway, Ireland). They were fed on a diet of compound feed (commercial dry pellets composed of vegetable matter and fish meal/fish oils in a 70:30 ratio) and were approximately 4Kg at the time of harvest. A total of 50 proximal and 50 distal intestines, were obtained at the processing stage. The 50 fish were eviscerated and the entire intestines were removed in the fish processing plant using a knife sanitised in water at approximately

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82°C. These were transported to a local laboratory in ice boxes (approximately 25 minutes) where the small intestines were extracted under aseptic conditions (including the use of separate sterile forceps and scalpels for each sample). The small intestine samples were then transported in an ice box, at approximately 2 to 4°C (for approximately 3 hours) to our research centre in Dublin. Using separate sterile forceps and scalpels, the proximal and distal portions were then extracted aseptically from each of the 50 small intestine samples and the entire intestinal contents for each sample were removed by gently squeezing the intestine tissue with a sterile forceps. Both the proximal (n = 50) and distal (n=50) samples were randomly separated into ten groups of five (n=5) in sterile 50ml tubes. These tubes were then vortexed (Clifton Cyclone vortex mixer, Thermo Fisher Scientific, Ireland) for 30 seconds.

## 2.2. DNA extraction

The DNA was extracted immediately from the fresh contents. Exactly 220mg of intestinal contents, from each pool, was weighed out into a sterile 2ml microtube containing 0.25g of sterile zirconia beads (0.125g of 0.1 mm and 0.125g of 1.0mm, Stratech Science, Newmarket, UK) and a single 2.5mm sterile bead (Stratech Science, Newmarket, UK). Each sample was then suspended in 1.4ml ASL buffer (Qiagen, Hilden, Germany) after which the samples were disrupted using a bead beater (Vortex-Genie 2, Thermo Fisher Scientific, Ireland) at maximum speed for 4 cycles of 30s. The samples were kept on ice between cycles to prevent any increase in temperature during this process. DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) with the following modification to the manufacturer's protocol; the suspension was heated at 90°C for 5min to improve cell lysis. Each extraction was carried out in duplicate, meaning 20 extractions were carried out for both the DI and PI samples. After extraction the DNA concentration of all samples were determined both fluorometrically (Qubit® dsDNA BR Assay Kit, Thermo Fischer Scientific,

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Ireland) and spectrophotometrically (NanoDrop 1000, Thermo Fisher Scientific, Ireland), to ensure DNA concentrations were adequate for sequencing. Samples were stored at -80°C until sequencing.

### 2.3. Illumina sequencing

DNA was normalized and 16S metagenomic libraries were prepared using primers to amplify the V3-V4 region of the 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16S Metagenomic Library Preparation guide with the following exceptions; the first PCR (2720 Thermal cycler, Applied Biosystems) reaction was performed in a total volume of 50µl instead of 25µl, and the number of cycles used in the first PCR was increased from 25 cycles to 30. The primers used were the universal primers (forward primer; 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and reverse primer; 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Klindworth et al. 2013). The volume of AMPure XP beads (New England Biolabs.) used in the initial clean-up was scaled up in accordance with the increase in PCR volume. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled in equimolar amounts. The pooled libraries were assessed using the Agilent high sensitivity DNA kit and quantified by qPCR using the Kapa Quantification kit for Illumina (Kapa Biosystems), according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced on the Illumina MiSeq using the V3 600 cycle kit according to Illumina protocols at Teagasc Food Research Centre Moorepark.

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#### 2.4 Bioinformatic analysis

The Illumina reads were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and removal of windows of 20 nucleotides with a low average quality) and length (removal of sequences with less than 200bp) with PRINSEQ-lite 0.20.3 (Schmieder and Edwards 2011). Finally, paired-end reads (with a minimum overlap of 20bp) were joined using Fastq-join (Aronesty 2011). In both processes the singletons were eliminated.

Using clean reads (based on quality and length) a closed-reference Usearch v7.0 algorithm (Edgar 2010) was applied allowing for sequences to be clustered with 97% identity to obtain operational taxonomic units (OTUs), while also removing chimeric OTUs against the gold database. The taxonomic assignment of these OTUs was obtained against the Ribosomal database project with RDP Classifier (RDP Naïve Bayesian rRNA Classifier Version 2.11). Alpha diversity (Chao1, Accumulated Cyclone Energy (ACE) and Shannon Index, Beta-diversity and additional analysis was undertaken using the R package Phyloseq (McMurdie and Holmes 2013). For all analyses, OTUs were normalized and with a relative abundance lower than 0.1% were eliminated.

#### 2.5 Statistical analysis

To determine the significance in the analysis of the alpha-diversity index, in order to study the normality of the samples and determine which method was the best to determine the P value, the "Shapiro.test" was performed. It showed that the test needed for this set of samples would be a statistical parametric distribution analysis. Due to the amount of parameters, the two groups were analysed by the t test method, using the R statistical package version 3.4.4 (<https://www.r-project.org/>). The limit for the P value was established at 0.05.

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### 3. Results

Of the 20 DNA extractions carried out on contents from the distal intestine (DI) and a similar number on contents from the proximal intestine (PI), DNA concentration analysis suggested that only ten distal and five proximal samples were suitable for sequence analysis (data not shown).

The mean ( $\pm$  SE) number of sequences per DI and PI samples were  $262,579 \pm 16,786$  and  $160,070 \pm 30,143$ , respectively. These sequences represented an average of  $203 \pm 11$  and  $1223 \pm 134$  OTUs per DI and PI sample, respectively. In total, 792 OTUs were identified, with 778 present in both DI and PI samples, 523 only present in DI samples and 4,491 OTUs only present in PI samples. Rarefaction curves indicated that the samples from both DI and PI reached the saturation phase (Supplementary Figure 1). Analysis of the alpha diversity demonstrated that DI samples had significantly ( $P < 0.005$ ) lower richness (Chao1 and ACE) and Shannon's diversity than PI samples (Figure 1). Permanova analysis of the two sample sets indicated a significant difference in beta diversity between the samples and the Bray-Curtis metrics indicated that the PI samples clustered together, separately to the DI samples (Figure 2).

Using an abundance cut off point of  $\geq 0.1\%$ , six phyla were identified in the DI samples (Figure 3(A)). The microbiota of the DI samples was dominated by Tenericutes (70.9%), followed by Firmicutes (19.1%) and Spirochaetes (8.9%). Bacteroidetes (0.3%), Proteobacteria (0.2%) and Actinobacteria (0.1%) were also detected. These six phyla were also found in the PI samples, where twelve phyla were identified. The microbiota of the PI was dominated by Tenericutes (17.8%), Firmicutes (17%), Bacteroidetes (15.2%) and Proteobacteria (14.2%).

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The most abundant (>1% of the total genera detected) in the distal and proximal samples are shown in Table 1, with all data provided in supplementary Tables 1 and 2. Figure 3(B) indicates the microbial composition at Family level. Overall within the phyla obtained in the DI and PI samples, 99 genera were identified. Interestingly, only 11 were shared between the different GI tract locations. The only genera exclusive to the DI samples were *Serratia* spp. or belonged to the family *Bacillaceae*, while 86 genera were only detected in the PI samples. The most dominant genera present in the DI samples belonged to the families *Mycoplasmataceae* and *Bacillaceae*, although *Brevinema* spp. were also prevalent. Similarly, genera belonging to the family *Mycoplasmataceae* were also the most dominant throughout the PI samples, followed by *Bacteroides* spp. and “Other *Porphyromonadaceae*”.

When each sample was examined individually, there were 20 operational taxonomic units (OTUs) common to all the DI and PI samples, representing a portion of the core intestinal microbiome in Atlantic salmon. These OTUs included seven *Proteobacteria*, four *Firmicutes*, four *Bacteroidetes*, one *Tenericutes* and one *Spirochaetes*. The most prevalent genera within the samples belonged to the family *Mycoplasmataceae*, with other genera such as *Pseudomonas* spp., *Photobacterium* spp., and *Aliivibrio* spp. also identified. When the DI and PI samples were examined as individual groups there were no additional OTUs exclusive to all DI samples. *Bacillaceae* and *Serratia* spp. were two genera exclusive to several DI samples, occurring in 90% and 50% of samples, respectively. Out of the 86 exclusive genera in the PI samples, 25 OTUs were observed in all PI samples, including *Ruminococcus* spp. and *Flavobacterium* spp.

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#### 4. Discussion

In this study the proximal (PI) and distal (DI) intestinal microbiota from Atlantic salmon farmed off the west coast of Ireland was investigated using high throughput sequencing of the 16S rRNA gene. Our data suggested there was a greater bacterial diversity in the PI region, with greater diversity observed at phylum, family and genus levels as compared to the DI samples. Moreover, the relative abundance of different bacteria varied considerably, depending on the location in the GI tract. Spirochaetes, for example, made up 8.9% of DI but less than 1% of PI samples while the Bacteroides were more prevalent in the PI (15.2%) than the DI (0.3%). Varying bacterial diversity and prevalence along the piscine GI tract has been reported previously (Reveco et al. 2014). Llewellyn et al. (2014) also found the microbial community varied along the fish GIT. A similar observation was made in a study of Atlantic salmon gut digesta and mucosal samples (Gajardo et al, 2016). This is probably due to the variation in physiological environments in the different sections of the digestive tract (Clements et al., 2014). In contrast Navarrete et al. (2009) found that the same bacteria were evenly distributed throughout the GI tract. Although, it is not certain as to why greater bacterial diversity may be associated with the proximal region, it has been suggested that the close proximity to the stomach may support a broader range of bacteria which in turn aids digestion (Navarrete et al. 2013; Li et al. 2014; Reveco et al. 2014). Regardless, more diverse microbial populations are associated with an increased competition for nutrients and adhesion sites which provides protection against pathogenic organisms (Ringø et al. 2004; Dillon et al. 2005; Ringø et al. 2010; Vasemägi et al. 2017). Interestingly, *Lactobacillus* spp. (3.2%), *Enterococcus* spp. (1.3%), *Lactococcus* spp. (0.3%) and *Carnobacterium* spp. (0.2%) were present in relatively high concentrations in 80% of PI samples in this study. These lactic acid bacteria (LAB) have been previously shown to have a protective effect against pathogenic

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genera such as *Aliivibrio* spp. and *Vibrio* spp. within the foregut of Atlantic salmon (Ringø et al. 2007; Ringø 2008; Salinas et al. 2008)

In addition to the region of the gastrointestinal tract sampled, there are several other interacting factors that influence the gut microbiota, including habitat (Bano et al., 2007; Llewellyn et al., 2016), diet (Ringo et al., 2010; Hovda et al., 2007; Desai et al., 2012; Harvey et al., 2016), freshwater-to-saltwater transition (Rudi et al., 2018), sex (Dhanasiri et al., 2011), captive state (Dhanasiri et al., 2011), phylogeny (Miyake et al., 2015), trophic level (Claesson et al., 2012; Serino et al., 2012; Miyake et al., 2015; Li et al., 2017), season (Hovda et al., 2012) and developmental stage (Hansen and Olafsen, 1999). There is also considerable variability within a group of fish even when they are reared in the same habitat. Schmidt et al. (2016) reported a single Lactobacillales OTU represented 96% of the microbiome of one fish which compared to a mean of only 3.5% relative abundance in the other fish from the same shoal.

The dominant phyla, regardless of GIT sample, were the Tenericutes (70.9% in DI and 17.8% in PI samples). The Tenericutes are a phylum of Gram-negative bacteria with a plasma membrane but no cell wall. They are fastidious organisms that are difficult to culture in the laboratory. Thus, it was not until the advent of molecular methods that they were first discovered to be important constituents of the gut microbiome of salmon (Holben et al., 2002). Several more recent studies have confirmed this finding (Abid et al., 2013; Green et al., 2013; Zarkasi et al. 2014; Lowrey et al., 2015; Ozorio et al., 2015). However, some studies have not reported its presence in salmon gut compartments (Gajrdo et al, 2016). Nonetheless, Tenericutes have also been reported as a major component of the gut microflora of other fish species including the silver drummer fish (*Kyphosus sydneyanus*) (Moran et al., 2005), the Californian mudsucker (*Gillichthys mirabilis*) (Bano et al., 2007) and rainbow

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trout (*Oncorhynchus mykiss*) (Kim et al., 2007; Lowery et al., 2015; Lyons et al., 2016; Huyben et al., 2018). Our data therefore provides further evidence of the importance of these bacteria in salmon and supports the hypothesis that salmon are a specific host for these bacteria, regardless of geographical location (Lyons et al., 2016). This finding may be of concern for fish producers, as several genera belonging to the *Mycoplasmataceae* family, including *Mycoplasma mobile*, have been associated with necrosis in fish (Adan-Kubo et al. 2012) while other *Mycoplasma* species are human pathogens (Holben et al., 2002).

Firmicutes were also common in DI (19.1%) and PI (17%) samples. Similarly, Gajardo et al (2016) reported a high level of Firmicutes in salmon digesta (38%). Lyons et al. (2016) reported that this phylum was the second most prevalent in farm (6.98%) and aquarium (8.43%) raised rainbow trout. Indeed it is generally accepted that bacteria belonging to this phylum play an important role in the conversion of dietary carbohydrates to short chain fatty acids such as acetate, propionate and butyrate which may be used by the fish as an energy source. The Proteobacteria represented 0.2% and 14.2% relative phyla abundance in the DI and PI samples, respectively. A previous salmon study reported higher levels of Proteobacteria in digesta (47%), with an almost complete dominance in mucosal samples (Gajardo et al, 2016) The ratio of Firmicutes to Proteobacteria in this study was approximately 100:1 in the DI and 1:1 in the PI region. Previous research has shown that this ratio is greatly influenced by diet with high fat feeds increasing the ratio (Kim et al. 2012).

In our study the Bacteroidetes (15.2%) were the third most abundant phyla followed by the Proteobacteria (14.2%). This finding is in agreement with other similar studies (Navarrete et al. 2009; Navarrete et al. 2010; Green et al. 2013). In contrast Llewellyn et al. (2014) reported that while these bacteria are abundant in returning adults, smolt and par, they occur at negligible levels in marine adults. However, their study differed from the other research as it focused on wild salmon. In a Norwegian study on farmed salmon Gajardo et al. (2016) also

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reported that Bacteroidetes were present in their samples in minimal relative abundance, demonstrating the potential impact of location and feed composition. The Proteobacteria are of particular interest and in this, as in other studies, the majority of Proteobacteria detected belong to the class  $\gamma$ -Proteobacteria, including *Pseudomonas* spp., *Vibrio* spp., *Aliivibrio* spp., *Photobacterium* spp. and *Shewanella* spp., all of which are commonly found in the environment (Nayak 2010; Reveco et al. 2014). In this study *Pseudomonas* spp., *Aliivibrio* spp. and *Photobacterium* spp. were present in both the DI and PI, whereas *Vibrio* spp. and *Shewanella* spp. were only present in the latter. All five genera possess strains pathogenic to fish, but are also responsible for the post mortem degradation of fish quality through the production of volatile compounds which are also hazardous if consumed by humans (Gram and Huss 1996).

The Verrucomicrobia had a low prevalence (2.3%) and were only found in the PI samples. This corroborates similar findings in other salmon microbiota studies (Rawls et al., 2006; Llewellyn et al., 2016) even though these bacteria are common in soil, fresh water and the marine environment (Freitas et al., 2012). Acinetobacter were also detected but at very low levels. Holben et al. (2002) reported this genera had a relative abundance of 2% in Scottish farmed salmon but 55% in fish farmed in Norway. Candidatus Saccharibacteria, Chlamydiae, Cyanobacteria, Deferribacteres, Parcubacteria, Spirochaetes were also detected at levels below 1% prevalence. This was not unexpected as many of these contain bacterial genera commonly found in the marine environment. However, other genera present included fish pathogens such as *Allivibrio* spp., *Delftia* spp., *Micrococcus* spp., *Photobacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Stentrophomonas* spp *Brucella* spp., *Planococcaceae* "incertae sedis", *Rhodococcus* spp., *Shewanella* spp., *Staphylococcus* spp. and *Vibrio* spp. (Austin and Austin 1999) that are of particular concern to the salmon industry. One aspect not considered in this study was the potential for contamination from laboratory reagents which

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has been reported to influence sequencing based studies, particularly with lower microbial biomasses (Salter et al, 2014). Considering the intestinal material examined would have a high microbial biomass laboratory reagent contamination would not be expected to have a particular influence on the results but should be included in future such studies.

All samples, regardless of location within the GIT, had 20 common OTUs. Similar results were observed by Dehler et al. (2017), who obtained 19 common OTUs in samples from the GIT of juvenile Atlantic salmon. There were three common OTUs between this study and the study carried out by Dehler et al. (2017). These OTUs represented “Other *Mycoplasmataceae*”, “Other *Ruminococcaceae*” and *Delftia* spp.

This study is of particular importance as it was undertaken with adult salmon from a commercial fish farm and hence is more representative of the salmon gut microflora as influenced by natural water ecosystems. It was concluded that the PI region had greater diversity of bacteria than the distal area. The relatively high abundance of bacteria belonging to the Tenericutes, Firmicutes, Bacteroidetes and Proteobacteria suggests that these bacteria belong to the autochthonous microbiota that colonise the mucosal surface of the digestive tract. Indeed, a study which examined the mucosa associated microbial community reported a substantial dominance of Proteobacteria (Gajardo et al, 2016). The bacteria of other phyla such as Acinetobacter, Candidatus Saccharibacteria, Chlamydiae, Cyanobacteria, Deferribacteres, Parcubacteria, Spirochaetes are most likely allochthonous, free living in the marine environment that form part of the transient microbiota in the digestive tract. Although phyla diversity may have a protective effect inhibiting pathogens, several genera were detected which contain species that are pathogenic to salmon. This study contributes to previous research on the microbiota of fish and provides further insight into the type of bacteria present in the GIT. Further work is now required to identify salmon microbiota at the

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species level and to better understand the influence of gut microbiota on fish growth and overall health.

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#### **Conflict of Interest**

The authors declare no conflict of interest.

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Table 1. The most abundant (>1% of the total genera detected) in the distal and proximal samples.

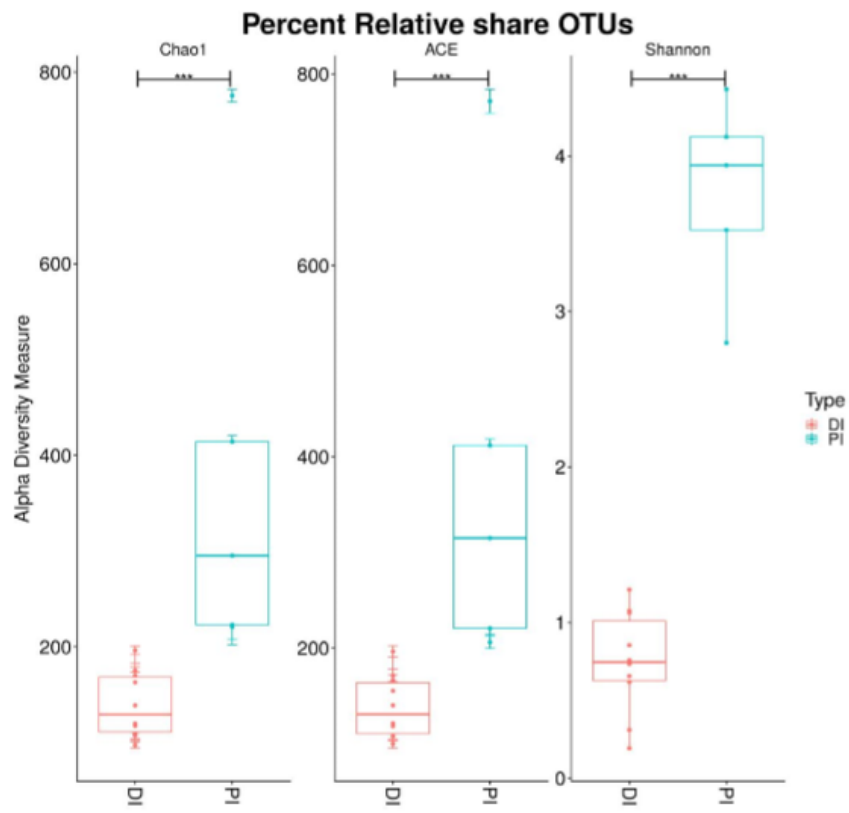
Phylum	Class	Order	Family	Genera
<b>Distal samples</b>				
Firmicutes (19.1%)	Bacilli	Bacillales	<i>Bacillaceae</i>	Other (17.7%)
Proteobacteria (0.2%)	Gammaproteobacteria	Vibrionales	<i>Vibrionaceae</i>	<i>Aliivibrio</i> (1.3%) <i>Photobacterium</i> (1.5%)
Spirochaetes (8.9%)	Spirochaetia	Spirochaetales	<i>Brevinemataceae</i>	<i>Brevinema</i> (8.6%)
Tenericutes (70.9%)	Mollicutes	Mycoplasmatales	<i>Mycoplasmataceae</i>	Other (68.4%)
<b>Proximal samples</b>				
Bacteroidetes (15.2%)	Bacteroidia	Bacteroidales	<i>Bacteroidaceae</i>	<i>Bacteroides</i> (4.3%)
			<i>Porphyromonadaceae</i>	Other (4.1%)
			<i>Rikenellaceae</i>	<i>Alistipes</i> (1.3%)
	Flavobacteriia	Flavobacteriales	<i>Flavobacteriaceae</i>	Other (1.8%)
Firmicutes (17%)	Other	Other	Other	Other (1.1%)
		Lactobacillales	<i>Enterococcaceae</i>	<i>Enterococcus</i> (1.3%)
			<i>Lactobacillaceae</i>	<i>Lactobacillus</i> (3.2%)
	Clostridia	Clostridiales	Other	Other (1.3%)
			<i>Lachnospiraceae</i>	Other (3.3%)
			<i>Ruminococcaceae</i>	Other (2.3%)
	Gammaproteobacteria	Pseudomonadales	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> (1.2%)
		Vibrionales	<i>Vibrionaceae</i>	<i>Aliivibrio</i> (2.8%) <i>Photobacterium</i> (2.9%)
Tenericutes (17.8%)	Mollicutes	Mycoplasmatales	<i>Mycoplasmataceae</i>	Other (17.2%)

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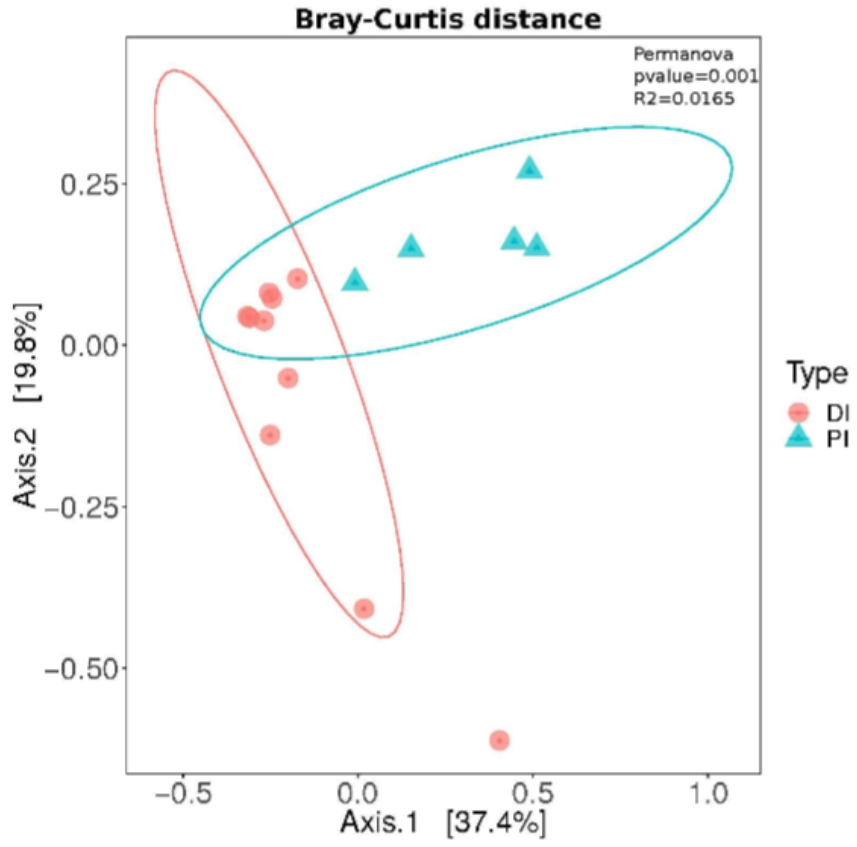
**Supplementary Information**

Supplementary Table 1. Breakdown of bacterial taxa observed in contents from the distal intestine (DI) of Atlantic salmon (*Salmo salar*), with relative abundance (%) of phylum and genera.

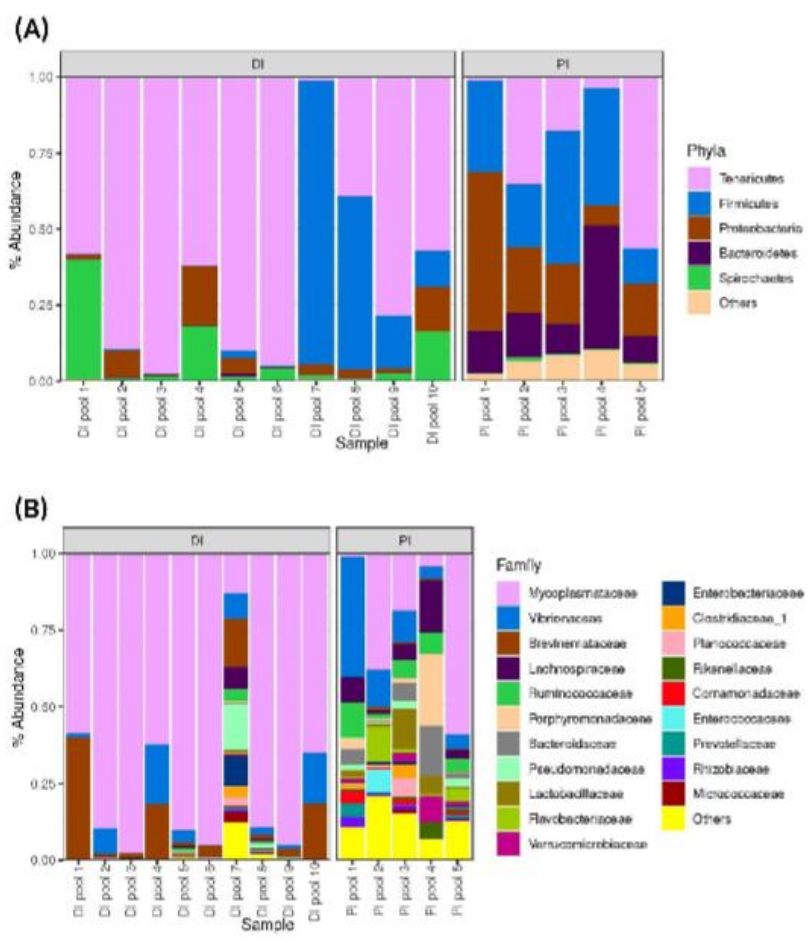
Supplementary Table 2. Breakdown of bacterial taxa observed in contents from the proximal intestine (PI) of Atlantic salmon (*Salmo salar*), with relative abundance (%) of phylum and genera.



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