Studies on the Microbiology of Fish and Shellfish with Emphasis on Bacteriocin-like Substances to Control *Listeria monocytogenes*

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by

Ngozi Izuchukwu

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SCHOOL OF NATURAL SCIENCES

INSTITUTE OF AQUACULTURE

UNIVERSITY OF
STIRLING

To

The Trinity

DECLARATION

I declare that this thesis has been compiled by myself, and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Ngozi Izuchukwu

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ABBREVIATIONS

ABC	ATP-binding cassette transporters (ABC transporters)
AMP	anti-microbial peptides
APS	ammonium persulphate
APW	alkaline peptone water
ATP	adenosine-triphosphate
AU	arbitrary unit
BCA	bicinchoninic acid
BLIS	bacteriocin like substances
BSA	bovine serum albumen
CAB	Columbia agar base
CAMP	Christie, Atkins, and Munch-Petersen
CFC	Cephaloridine-Fucidin-Centrimide
CFS	cell free supernatant
CFU	colony forming unit
CSS	cold smoked salmon
CTSI	cresol red thallium acetate sucrose inulin
DTT	dithiothreitol
ECPs	extracellular products
EMBA	eosin methylene blue agar
GRAS	generally regarded as safe
HIC	hydrophobic interaction chromatography
HPP	high-pressure processing
kDa	kilo Dalton
KGy	kilogray
LAB	lactic acid bacteria
LPFP	lightly preserved fish product
LPS	lightly preserved seafood
MAP	modified atmosphere-packed
MES	2-(N-morpholino) ethanesulfonic acid
Mg	microgram
Min	minute(s)
Ml	Millilitre
mM	milli Mole
Mpa	megapascal
MRS	de Man Rogosa and Sharpe agar
MW	molecular weight
NAP	nitrite actidion polymyxin
NCIMB	National Collection of Industrial and Marine Bacteria
Nucleotide BLAST	Nucleotide Basic Logical Alignment Search Tool
OD	optical density
RTE	ready to eat
PALCAM	polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PW	peptone water
16S rDNA	16S ribosomal deoxyribonucleic acid

SD	sodium diacetate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide electrophoresis
SL	sodium lactate
SSOs	specific spoilage organisms
TCBS	thiosulphate citrate bile salts sucrose agar
TEMED	tetramethyl-ethylenediamine
TFA	trifluoro-acetic acid
THC	total heterotrophic counts
TMA	trimethylamine
TMA-N	trimethylamine-Nitrogen
TMAO	trimethylamine oxide
TSA	tryptone soya agar
TSB	tryptone soya broth
TNA	tryptone soya agar supplemented with 1% (w/v) sodium chloride
TNB	tryptone soya broth supplemented with 1% (w/v) sodium chloride
TVB-N	total volatile base nitrogen
UVM	University of Vermont Modification Medium
µl	microlitre
VP	vaccum packed

ABSTRACT

Seafood permits the transmission of many bacterial pathogens. In order to reconcile consumer demands with important safety standards, traditional means of regulating microbial spoilage and safety hazards in foods are combined with novel technologies. These include biological antimicrobial systems, such as the use of lactic acid bacteria (LAB) and/or their bacteriocins, such as *Carnobacterium maltaromaticum* CS526 and its bacteriocin piscicocin CS526. The aims of this study were to investigate the presence of *Listeria monocytogenes* in temperate seafood, namely fresh and smoked salmon, fresh and smoked haddock, and fresh mussels and oysters. Additionally, there was an aim to recover, characterise and use bacteriocin-like-substance to control *Listeria monocytogenes* in cold smoked haddock.

Vibrio spp., *Enterobacteriaceae* representatives, total aerobic heterotrophic counts and *Listeria monocytogenes* were isolated from commercially prepared smoked and fresh Atlantic salmon, smoked and fresh haddock, live mussels and oysters using selective media and tryptone soya agar (TSA). *Vibrio* spp. occurred in high densities (>10^6 CFU g^{-1}) in mussels and *Enterobacteriaceae* representatives were recorded at >10^6 CFU g^{-1} in fresh salmon. Total aerobic heterotrophic counts in fresh salmon, live mussels and oysters reached 10^7, > 10^7, and > 10^6 CFU g^{-1}, respectively. *Listeria monocytogenes* was recorded at 5.0 x 10^4 CFU g^{-1} in mussels. In total sixty one bacterial isolates were recovered from the seafood examined. The results revealed 19 genera of bacteria, i.e. *Acinetobacter, Aerococcus, Aeromonas, Bacillus, Brochothrix, Carnobacterium, Citrobacter, Corynebacterium, Enterobacter, Escherichia coli, Moraxella, Micrococcus, Pseudomonas, Psychrobacter, Serratia, Shewanella, Staphylococcus, Vibrio* and *Listeria.* The prominent characteristics of fish spoilage isolates were demonstrated by the ability of the isolates to reduce trimethylamine oxide (TMAO) to

trimethylamine, and to produce H_2S. *Sh. baltica* OS185, *Aeromonas* spp. HB-6, *Sh. baltica*, *Sh. putrefaciens*, *A. hydrophila* HX201006-3, *A. salmonicida* subsp. *achromogenes*, *A. hydrophila*, *C. freundii*, *Enterobacter cloacae* were strong producers of TMA and H_2S. The spoilage microorganisms were tested for potential pathogenicity. The result revealed that 6/15 of the spoilage microorganisms produced proteolytic, lecithinase, blood (β and α haemolysin) and elastinase activity, respectively, whereas 7/15 of the spoilage microorganisms showed lipolytic activity.

Cell free supernatants, ammonium sulphate precipitated supernatants and semi-purified bacteriocin-like substances of *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 producing strains isolated from commercially prepared smoked salmon were investigated for their potential antimicrobial activity against potentially pathogenic and food spoilage microorganisms. Generally, a broad spectrum of activity was revealed against potentially pathogenic and food spoilage microorganisms *in vitro*.

Cold-smoked haddock treated with bacteriocin producing *C. maltaromaticum* MMF-32, *C. piscicola* A9b bac^- phenotype nonbacteriocin producing strain a mutant of *C. piscicola* A9b bac^+, cell free supernatants, ammonium sulphate precipitated supernatants and semi-purified bacteriocin-like substances was challenged with *L. monocytogenes* ATCC 19114 up to 10^3 CFU g^{-1}, respectively. Samples were stored at 4 °C for 10 days. *L. monocytogenes* and total bacterial counts were determined along with changes in total volatile base nitrogen (TVBN) and biogenic amines production as well as texture, colour and odour. Although the study on anti-listerial effects of *C. maltaromaticum* MMF-32 was not successful, this organism did have a positive effect on retention of firmness and sensory perception in cold smoked haddock.

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Chapter 1. General introduction

Introduction

1.1. Potential benefits of seafood

Seafood constitutes all fresh or saltwater finfish, molluscan shellfish, crustaceans, and other forms of aquatic animal life (Okonko *et al*., 2009). Seafood or fishery products make up a significant share of the food for a large portion of the world population, more so in developing countries, where fish forms a cheap source of protein. There has been increased information on the nutritional and health benefits of seafood for the last two to three decades (Din *et al*., 2004; Hellberg *et al*., 2012), and fish and fishery products are regarded as an important part of a healthy diet because of the presence of high quality protein and other vital nutrients, namely omega-3 fatty acid. In addition, fishery products may be low in saturated fat (Okonko *et al*., 2009). Fish proteins are regarded as easily digestible and are abundant in important amino acids (Costa, 2007). Essential vitamins, namely vitamins A, B3, B6, and B12, and D, and the minerals calcium, iron, selenium, and zinc are provided in seafood (USDA, 2010).

Fish products are significant for human nutrition, representing 16.7% of global protein consumption (FAO, 2009). Fish production has increased over many years, and the increase in the last five decades has been due to a rapid growth in the aquaculture sector (FAO, 2012).

Consequently, initial estimates of actual fish intake increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 world per capita per year (Table 1.1). Due to the intense growth in its fish production, especially from aquaculture China has been responsible for most of the development in fish production. The actual fish intake also increased at an average annual rate of 6.0 % in the period 1990–2010 to ~35.1 kg in 2010 per capita per year in China. Yearly per capita fish provided in the rest of the world was about

15.4 kg in 2010 (11.4 kg in the 1960s, and 13.5 kg in the 1990s) (FAO, 2012). Worldwide capture fishery production of 93.7 million tonnes in 2011 was the second highest ever attained with the highest being 93.8 million tonnes in 1996. Besides, apart from anchoveta catches, 2012 revealed (86.6 million tonnes). Worldwide fishery production in marine waters was 82.6 million tonnes in 2011, and 79.7 million tonnes in 2012 (Table 1.1). Worldwide aquaculture production reached an all-time high of 90.4 million tonnes (live weight equivalent) in 2012 (US$144.4 billion), together with 66.6 million tonnes of fish, and 23.8 million tonnes of aquatic algae, with approximations for 2013 of 70.5 million and 26.1 million tonnes, respectively (FAO, 2012).

The integration of worldwide commerce, among other reasons, has led to increased fish consumption around the world. Moreover, healthier life styles and cultural globalization have popularized the consumption of raw fish dishes, which were historically restricted to the oriental countries (Da Silva *et al*., 2010).

Fish consumption is recognised to reduce coronary heart diseases (CHD) (Din *et al*., 2004; Hellberg *et al*., 2012). Two omega-3 polyunsaturated fatty acids (PUFAs) have been recognized to play a key role in CHD reduction and mortality König *et al*. (2005**)**. They are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Mozaffarian 2008). EPA and DHA cannot be produced in considerable amounts by the human body, and must be acquired through the diet, with the chief source being seafood, particularly oily fish (Williams and Burdge, 2006). Additional omega-3 fatty acid found in plant-based oils, alpha-linolenic acid (ALA), is an originator to EPA and DHA, but is transformed at very low rates in the human body (<10%) (Williams and Burdge, 2006). Some health benefits have been linked with EPA and DHA, and fish consumption leads to a reduced risk of cardiovascular disease consequences, such as sudden death (Mozaffarian, 2008) and stroke (Bouzan *et al*., 2005), augmented duration of gestation

(IOM, 2005), and better visual and cognitive development (Brenna and Lapillonne, 2009).

Table 1.1. World fisheries and aquaculture production and utilization (2007-2012)

	2007	2008	2009	2010	2011	2012
			Million	*tonnes*		
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	90.8	90.1	90.1	89.1	93.7	91.3
Aquaculture						
Inland	29.9	33.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	49.9	52.9	55.7	59.0	62.0	66.6
TOTAL WORLD FISHERIES	140.7	143.1	145.8	148.1	155.7	158.0
UTILIZATION						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (*billions*)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (*kg*)	17.6	17.9	18.1	18.5	18.7	19.2

Notes: Excluding aquatic plants. Totals may not match due to rounding of figures. Data for 2012 are provisional estimates (FAO, 2012).

1.2. Potential risks of seafood consumption

Consumption of raw or undercooked seafood is a known health risk to consumers. Fish and fishery products are prone to bacterial contamination, especially filter feeders such as mussels and oysters, which concentrate bacteria in their filtration systems and, therefore, are ideally suited to trap bacteria and viruses, that are present in water (Huss, 1997; Popovic *et al.*, 2010). In addition, seafoods are capable of having surface or tissue contamination arising from the marine environment (Iwamoto, *et al.*, 2010). Contamination of fish with microorganisms may reflect environmental pollution: water temperature, salt content, distance between localization of catch and polluted areas (human and animal faeces), natural occurrence of bacteria in the water, intake of food by fish, methods of catch and chilling conditions (Feldhusen, 2000; Adeyemo, 2003; Goja, 2013). Seafood may also become contaminated during handling, processing and preparation. Other factors include storage, transportation at inappropriate temperatures, cross contamination from infected food handlers, or through contact with contaminated seafood or water (Iwamoto, *et al.*, 2010).

Adak *et al*. (2005) reported the approximation of the yearly impact of foodborne diseases in England and Wales which revealed that seafood was responsible for 116,603 (7%) of cases and 30 (4%) of deaths. The majority of known seafood-related outbreaks are caused by toxins (biotoxins and histamine) and viruses (noroviruses and hepatitis A virus) but fish and shellfish may also be a source of pathogenic bacteria naturally occurring in aquatic environments, indigenous or derived from polluted waters and/ or post capture (WHO, 2000; Gillespie *et al*., 2001; Huss *et al*., 2003; 2009).

Live fish and crustaceans may be contaminated with a number of pathogenic bacteria normally found in the aquatic environment, such as *Clostridium botulinum*, various *Vibrio* spp., *Listeria monocytogenes* and *Aeromonas* spp. (Feldhusen, 2000). With reference to bacterial food poisoning or intoxication, the causative organism multiplies

in the food where it produces its toxins. The distinctive sign of food poisoning is the rapid onset of illness (typical signs are nausea/vomiting) as the toxins are already formed in the food before consumption. Therefore, the intake of viable bacteria is not a condition for the induction of the disease. Most often intoxications require that the toxin producing bacteria have grown to high numbers ($10^5 – 10^8$ CFU g^{-1}) in the food before its ingestion (FAO, 1997). Distinctively, the food merely acts as a vehicle for the causative organism in food-borne infections. The infectious agent may not have multiplied in the food, but the ingested bacteria continue to grow within the host's body to produce typical symptoms (fever and diarrhoea). The number of viable bacterial cells that inevitably cause disease (the Minimum Infective Dose, MID) differs considerably between bacterial species. Thus, the MID are known to be high ($>10^5 – 10^6$ cells) for pathogenic *Vibrio* spp. (Twedt, 1989) and very low for some *Salmonella enterica* serovar Typhi and *Shigella* spp. (Kothary and Babu, 2001).

The Food Standard Agency (2008) described a microbiological survey of retail smoked fish with particular reference to the presence of *Listeria monocytogenes*. The survey was carried out over a period of 4 months between July and November, 2006. The major purpose was to measure the incidence of *L. monocytogenes* in ready-to-eat smoked fish sold in retail premises in the UK. Further tests were carried out to determine the presence of *Salmonella*, *Escherichia coli*, coagulase positive staphylococci and other Enterobacteriaceae representatives. Three thousand, two hundred and twenty six samples of hot and cold smoked RTE fish products were collected, and a total of 3,222 samples were analyzed. The incidence of *Listeria* spp. and *L. monocytogenes* were examined in the samples. Thus, the weighted incidence of *Listeria* spp. in all smoked fish was 10.5% (hot smoked was 5.2%, cold smoked 3.2% and others 2.2%). *L. monocytogenes* was predominant with an incidence of 8.3%, but

99.3% of the samples were below the 100 CFU g^{-1} limit laid down by the Microbiological Criteria Regulation (EC, 2005). The weighted incidence of other *Listeria* spp. was 2.2%. The FSA (2008) reported that the incidence of *Listeria* spp. in different fish species varied regardless of the type of smoked fish. Nevertheless, *L. monocytogenes* was the most dominant type of *Listeria* spp. found in different hot or cold smoked fish species. Ninety six samples revealed the presence of *Listeria* spp. from a total of 1,878 hot smoked fish sample. The weighted incidence of total *Listeria* spp. in hot smoked fish was 5.2%, with *L. monocytogenes* being the most dominant, with the weighted incidence of 3.4%. Other *Listeria* spp. recorded a weighted incidence of 1.8% (FSA 2008). Furthermore, out of 1,344 cold smoked fish samples collected, 282 revealed the presence of total *Listeria* spp. In cold smoked fish, *Listeria* spp. revealed a total weighted incidence of 20.5%; *L. monocytogenes* being the most predominant with a weighted incidence of 17.4%. A weighted incidence of 3.1% was recorded of with other *Listeria* spp. in cold smoked fish samples (FSA, 2008). An aggregate of 1,244 cold smoked salmon samples were collected of which 269 revealed the presence of *Listeria* spp. The weighted incidence of total *Listeria* spp. in cold smoked salmon was 21.0%, whereas *L. monocytogenes* was the most dominant had weighted incidence of 17.6%. Other *Listeria* spp. revealed a weighted incidence of 3.4% (FSA, 2008). A collection of 504 hot smoked salmon samples was collected from which 28 samples revealed the presence of *Listeria* spp. The weighted frequency of total *Listeria* spp. was 5.4%, whereas *L. monocytogenes* was the most dominant with an incidence of 4.7%. An incidence of 0.8% was revealed of other *Listeria* spp. (FSA, 2008).

A total of 1,073 hot smoked mackerel samples were collected, of which 55 samples revealed the presence of *Listeria* spp. Total *Listeria* spp. in hot smoked mackerel had an

incidence of 5.2%; *L. monocytogenes* being the most predominant revealing an incidence of 3.1%. Other *Listeria* spp. in hot smoked mackerel revealed a weighted incidence of 2.1% (FSA, 2008). With regard to specific countries, the incidence of total *Listeria* spp. was higher in Northern Ireland, Wales and England than the UK average. Moreover, the incidence of *L. monocytogenes* was higher in England, Wales and Northern Ireland and lower in Scotland (Table 1.2) (FSA, 2008).

Enterobacteriaceae representative in all smoked fish revealed counts extending from <10 to 1.0 x 10^6 CFU g^{-1}. *Enterobacteriaceae* counts in smoked fish was beyond the guidelines for RTE foods as *Enterobacteriaceae* counts should be >10^4 CFU g^{-1}. *Enterobacteriaceae* representatives were recovered from 21.2% of samples that also contained *Listeria* spp. *E. coli* was recovered from 0.3% (7) samples of smoked fish. Coagulase positive staphylococci were recovered in 0.3% (13) samples of smoked fish (FSA, 2008).

Table 1.2. Comparison between *Listeria* species across countries in the UK (sample size = 3,222)

	England		Wales		Scotland		N. Ireland		UK	
	%	(n)	%	(n)	%	(n)	%	(n)	%	(n)
L. monocytogenes										
Prevalence *	84	(223)	14.2	(38)	5.6	(25)	9.2	(16)	8.3	
Lower Cl	7.3		7.1		3.0		4.0		7.4	
Upper Cl	9.4		21.4		8.2		14.5		9.3	
Other *Listeria* spp.										
Prevalence	2.4	(57)	5.6	(14)	0.5	(3)	1.1	(2)	2.2	(76)
Lower Cl	1.8		0.9		0.0		0.0		1.7	
Upper Cl	2.9		10.3		1.3		3.0		2.7	
L. innocua										
Prevalence	0.7	(17)	2.5	(6)	0.0	(0)	0.6	(1)	0.7	(24)
Lower Cl	0.4		0.0		0.0		0.0			
Upper Cl	1.0		5.7		1.0		1.9			
L. seeligeri										
Prevalence	0.4	(8)	0.0	(0)	0.0	(0)	0.0	(0)	0.3	(8)
Lower Cl	0.1		0.0		0.0		0.0		0.1	
Upper Cl	0.6		3.2		1.0		2.5		0.5	
L. welshimeri										
Prevalence	1.3	(32)	3.1	(8)	0.5	(3)	0.6	(1)	1.2	(44)
Lower Cl	0.9		0.0		0.0		0.0		0.9	
Upper Cl	1.7		6.6		1.3		1.9		1.6	
Total *Listeria* spp.										
Prevalence	10.7	(280)	19.8	(52)	6.1	(28)	10.4	(18)	10.5	(378)
Lower Cl	9.6		11.7		3.4		4.9		9.5	
Upper Cl	11.9		28.0		8.8		15.9		11.6	

*****weighted prevalence
(FSA, 2008)

Iwamoto *et al.* (2010) described the epidemiology of seafood-associated infections in the USA, specifically reviewing national seafood-associated outbreaks reported to the Centers for Disease Control and Prevention (CDC) covering 1973 to 2006. Their research on outbreaks of food-borne illnesses was used to define the food vehicles for pathogens that cause illness and to improve their understanding of the epidemiology of these outbreaks. During the period under investigation, 188 outbreaks of seafood-

associated infections resulting in 4,020 illnesses, 161 hospitalizations, and 11 deaths were reported to the Food-Borne Disease Outbreak Surveillance System. Their investigations showed that most of the seafood-associated outbreaks (143[76.1%]) were caused by bacterial agents; 40 (21.3%) outbreaks had a viral aetiology, and 5 (2.6%) had a parasitic cause (Table 1.3). Moreover, Iwamoto *et al.* (2010) in their research reported that seafood-associated outbreaks happened through the year, with a peak during late summer (Fig. 1.1). Bacterial pathogens, especially vibrios, caused more outbreaks during warmer months, whereas outbreaks of seafood-associated norovirus infections occurred more often during colder month. Furthermore, seafood-related outbreaks of infection differed by seafood commodity. Molluscs were involved in 85 (45.2%) outbreaks, followed by fish in 73 (38.8%) outbreaks and crustaceans in 30 (16.0%) outbreaks (Table 1.3).

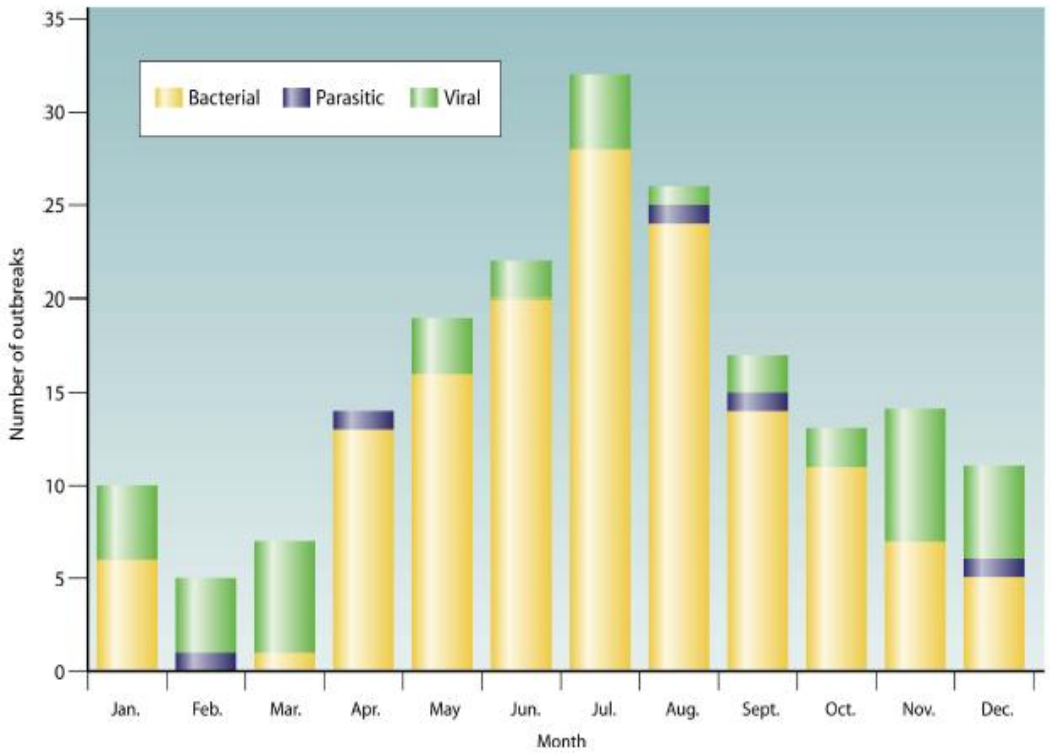


Figure 1.1. Number of confirmed seafood-associated outbreaks, aetiology of occurrence, 1973-2006 (from Iwamoto *et al*., 2010).

Furthermore, Iwamoto *et al*. (2010) reported that bacteria were the etiological agents in 143 (76.1%) outbreaks, causing 2,646 illnesses, 136 hospitalizations, and 11deaths. Nearly all outbreaks associated with fish (89.0%) and crustaceans (93.3%) were caused by bacterial agents; among outbreaks associated with molluscs, 50 outbreaks (58.8%) were caused by bacteria. Reports from their study showed that vibrios were the most common cause of seafood outbreaks. Particularly, *Vibrio parahaemolyticus* caused more outbreaks and illnesses than any other pathogen (45 outbreaks [23.9% of all infectious disease outbreaks], 1,393 illnesses, and no deaths) during the study period. Outbreaks of *Vibrio* illnesses were seasonal, with 44 (81.5%) occurring during the warmer months of May to September. During the period of 1997 to 2006, the largest and increased number of *Vibrio* infection was recorded over the study period (Fig.1.2). In 1998, *V. parahaemolyticus* infections associated with oyster consumption were reported as responsible for the largest seafood-associated outbreak with 416 illnesses among persons in 13 states (Daniel *et al*., 2000; Iwamoto *et al*., 2010).

In addition to causing human health problems, the presence of disease causing agents in seafood led to rejection of seafood consignments resulting in major economic losses to exporting countries in general and to seafood processing industries in particular (Ramaiah, 2004).

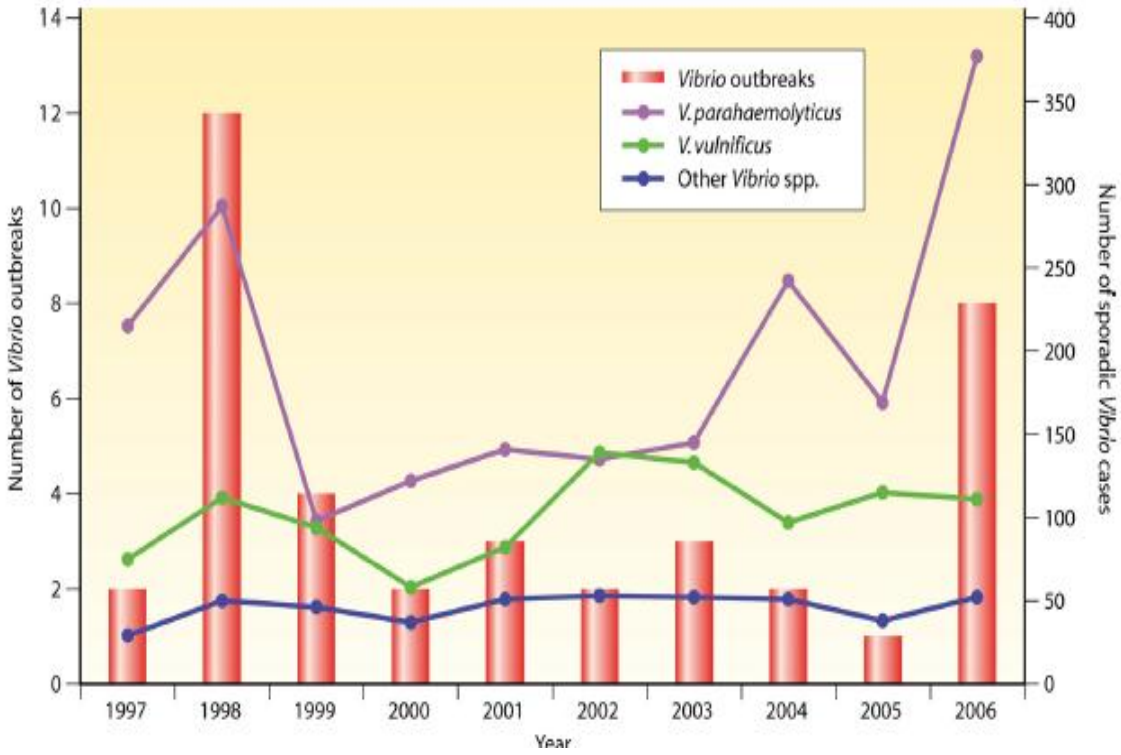


Figure 1.2. Numbers of outbreaks and sporadic cases of *Vibrio* infection, by species and year 1997 to 2006 (Iwamoto *et al*., 2010).

1.2.1. Microbial flora found in seafood

Much research has been conducted showing the presence of non-pathogenic and pathogenic bacteria on the skin, the gills, and intestines of fish, also such bacteria were found in other seafood (Al-Harbi and Uddin, 2005a; Vivekanandhan., *et al*., 2005; Herrera *et al*., 2006; Kim *et al*., 2007; Da Silva *et al*., 2010; Bakr *et al*., 2011; Adebayo-Tayo *et al*., 2012; González *et al*., 2013; Goja, 2013). These studies showed differences in the microbial flora in fish species and seafood collected from different locations in various countries. Bacteria found in these fish species and seafood included *Shewanella putrefaciens, Corynebacterium urealyticum, Flavobacterium* spp., *Aeromonadaceae,*

Enterobacteriaceae and *Pseudomonas* representatives, *Vibrio* spp., *Listeria moncytogenes, Cl. perfringens* and *Carnobacterium* spp.

The microflora of temperate water fish is dominated by psychrotrophic Gram-negative, rod-shaped bacteria, namely *Pseudomonas, Moraxella, Acinetobacter, Alcaligenes, Alteromonas, Shewanella;* members of *Vibrionaceae (Vibrio* spp. and *Aeromonadaceae* (*Aeromonas* spp.), and also Gram-positive bacteria, such as *Micrococcus, Bacillus,* coryneforms, *Carnobacterium* and *Brochothrix* were found in varying proportion (Shewan, 1977; Liston, 1980; Huss, 1995). In addition, *Corynebacterium, Micrococcus, Pseudomonas, Bacillus, Flavobacterium, Escherichia coli, Vibrio, Staphylococcus, Salmonella* and *Shigella* have been isolated from tropical seafood (Kapute *et al*., 2012).

The skin surface of Atlantic salmon (*Salmo salar*) from the U. K. was reported to possess populations of 10^2 - 10^3 CFU cm^{-2} bacteria (Horsley, 1973); Trust (1975) reported a high bacterial population 10^6 CFU g^{-1} from gill tissue; Evelyn and McDermott, (1961), and Toranzo *et al*. (1993) recorded the presence of bacteria in muscle and kidney.

Table 1.3. Seafood associated outbreaks of infection, aetiology and seafood commodity, 1973-2006 in the USA

Aetiology	Fish			Crustaceans			Molluscs			All seafood		
	No. (%) of outbreaks	No. (%) of illnesses	No. (%) of hospitalisations	No. (%) of outbreaks	No. (%) of illnesses	No. (%) of hospitalisations	No. (%) of outbreaks	No. (%) of illnesses	No. (%) of hospitalisations	No. (%) of outbreaks	No. (%) of illnesses	No. (%) of hospitalisations
Bacteria												
Bacillus cereus	2 (3)	7 (1)	0 (0)	2 (7)	122 (20)	0 (0)	0 (0)	0 (0)	0 (0)	4 (2)	129 (3)	0 (0)
Campylobacter spp.	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)	5 (0)	0 (0)	2 (1)	5 (0)	0 (0)
Clostridium botulinum	43 (59)	152 (13)	61 (66)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	43 (23)	152 (4)	61 (38)
Clostridium perfringens	1 (1)	46 (4)	0 (0)	1 (3)	55 (9)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	101 (3)	0 (0)
Escherichia coli, enteroaggregative	0 (0)	0 (0)	0 (0)	1 (3)	12 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	12 (0)	0 (0)
E. coli, enterohaemorrhagic	0 (0)	0 (0)	0 (0)	1 (3)	21 (3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	21 (1)	0 (0)
Listeria monocytogenes	0 (0)	0 (0)	0 (0)	1 (3)	2 (0)	1 (8)	0 (0)	0 (0)	0 (0)	1 (1)	2 (0)	1 (1)
Salmonella	10 (14)	261 (22)	15 (16)	4 (13)	81 (13)	8 (62)	4 (5)	32 (1)	5 (9)	18 (10)	374 (9)	28 (17)
Shigella spp.	6 (8)	259 (21)	17 (18)	1 (3)	25 (4)	0 (0)	5 (6)	118 (5)	3 (5)	12 (6)	402 (10)	20 (12)
Staphylococcus aureus	3 (4)	7 (1)	0 (0)	2 (7)	22 (4)	0 (0)	0 (0)	0 (0)	0 (0)	5 (3)	29 (1)	0 (0)
Vibrio cholerae, toxigenic	0 (0)	0 (0)	0 (0)	3 (10)	10 (2)	1 (8)	0 (0)	0 (0)	0 (0)	3 (2)	10 (0)	1 (1)
Vibrio cholerae, nontoxigenic	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (5)	12 (1)	0 (0)	4 (2)	12 (0)	0 (0)
Vibrio parahaemolyticus	0 (0)	0 (0)	0 (0)	12 (40)	234 (38)	1 (8)	33 (39)	1,159 (53)	23 (42)	45 (24)	1,393 (35)	24 (15)
Vibrio vulnificus	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (0)	1 (2)	1 (1)	2 (0)	1 (1)
Vibrio spp., not specified	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	2 (0)	0 (0)	1 (1)	2 (0)	0 (0)
Total	65 (89)	732 (60)	93 (100)	28 (93)	584 (96)	11 (85)	50 (59)	1,330 (60)	32 (58)	143 (76)	2,646 (66)	136 (84)
Viruses												
Norovirus	4 (5)	418 (35)	0 (0)	0 (0)	0 (0)	0 (0)	27 (32)	747 (34)	16 (29)	31 (16)	1,165 (29)	16 (10)
Hepatitis A virus	1 (1)	7 (1)	0 (0)	1 (3)	7 (1)	0 (0)	7 (8)	121 (6)	7 (13)	9 (5)	135 (3)	7 (4)
Total	5 (7)	425 (35)	0 (0)	1 (3)	7 (1)	0 (0)	34 (40)	868 (39)	23 (42)	40 (21)	1,300 (32)	23 (14)
Parasites												
Anisakidae	1 (1)	14 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	14 (0)	0 (0)
Giardia lamblia	1 (1)	29 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	3 (0)	0 (0)	2 (1)	32 (1)	0 (0)
Paragonimus	0 (0)	0 (0)	0 (0)	1 (0)	18 (0)	2 (0)	0 (0)	0 (0)	0 (0)	1 (0)	18 (0)	2 (0)
Diphyllobothrium	1 (1)	10 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	10 (1)	0 (0)
Total	3 (4)	53 (4)	0 (0)	1 (0)	18 (0)	2 (0)	1 (1)	3 (0)	0 (0)	5 (3)	74 (2)	2 (1)
Total (1973-2006)	73 (100)	1,210 (100)	93 (100)	30 (100)	609 (100)	13 (100)	85 (100)	2,201 (100)	55 (100)	188 (100)	4,020 (100)	161 (100)

From Iwamota *et al*. (2010)

Kapute *et al*. (2012) reported that the bacterial populations on the skin surface, muscle, gills, kidney and intestines of Lake Malawi tilapia collected from supermarkets and local markets were 2.4 x 10^2 and 3.3 x 10^2 CFU cm^{-2}, 7.9 x 10^1 and 2.9 x 10^2 CFU g^{-1}, 8.8 x 10^3 and 5.2 x 10^6 CFU g^{-1}, 4.5 x 10^4 and 4.2 x 10^5 CFU g^{-1}, 2.2 x 10^5 and 9.5 x 10^8 CFU g^{-1}, respectively. High bacterial counts were enumerated from gills and intestines of fish from both local and super markets. Muscle and skin surface recorded low bacterial populations.

1.2.2. Pathogenic Microflora found in seafood

Those bacteria that may cause illness in humans are known as potential human pathogens. Usually potentially foodborne pathogenic bacteria are in low numbers among the many different types of micro-organisms found on/in seafood bacteria (FAO, 1997). Pathogenic bacteria associated with seafood can be divided into three groups. These are:

1) bacteria that occur in the marine and estuarine environment (indigenous bacteria, including *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, Listeria monocytogenes, Clostridium botulinum* and *Aeromonas hydrophila.*

2) enteric bacteria, which are present as a result of faecal contamination (non-indigenous bacteria): *Salmonella* spp., *pathogenic Escherichia coli, Shigella* spp. *Campylobacter* spp., and *Yersinia enterocolytica* (very few pathogenic serotypes).

3) bacterial contamination during processing: Examples include *Bacillus cereus, L. monocytogenes, Staphylococcus aureus* and *Cl. perfringens* (Reilly and Käferstein, 1997; Reilly, 1998; Feldhusen, 2000).

1.3. *Listeria monocytogenes*

1.3.1. Characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, motile bacterium that grows well at 37°C, i.e. human body temperature, but which at the same time is psychrotolerant and halotolerant. *L. monocytogenes* may grow under aerobic, microaerophilic, and anaerobic conditions, and under vacuum (ICMSF, 1996). The bacterium is ubiquitous in nature and has been isolated from various sources including soil, water, plants, feeds and silage, as well as from the environment in the food industry and from foods (Farber and Peterkin, 1991; Fugett *et al*., 2007; Vongkamjan *et al*., 2012; Den Bakker *et al*., 2014). The bacterial genus *Listeria* currently comprises ten species, i.e. *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L.* marthii, *L. rocourtiae*, *L. fleischmannii* and *L. weihenstephanensis* (Zhang *et al*., 2007; Halter *et al*., 2013). Listeriosis is a generalized infection that starts after ingestion of *L. monocytogenes* (Finlay, 2001). The organism is catalase positive and oxidase negative and expresses a β-haemolysin, which produces zones of clearing in blood agar. The haemolysin acts synergistically with the β-haemolysin of *S. aureus* on sheep erythrocytes; the substance mediating this effect is known as the CAMP factor after Christie, Atkins, and Munch-Petersen (Christie *et al*., 1944). *L. monocytogenes* possesses peritrichous flagella, which gives it a characteristic tumbling motility at 20 - 25°C (Peel *et al*., 1988). The colonies have a characteristic blue-green sheen by obliquely transmitted light (Henry, 1933; Low and Donachie, 1997). *L. monocytogenes* has the ability to multiply in a wide pH and temperature range, as well as in low water activity (the unbound water to the food molecule). The generation time at 4°C for *L. monocytogenes* is 12 to 36 hours. Whenever food is stored for a long time, at +4°C the bacterial numbers can increase up to 10^6 g^{-1}. *L. monocytogenes* as a halotolerant organism survives up to 100 days at 30.5% salt concentration at +4°C (Kuzmanović *et al.,* 2011; Ghanbari *et al*., 2013).

1.3.2. *Listeria monocytogenes* Infections

L. monocytogenes is a facultative anaerobic opportunistic intracellular bacterial pathogen that has its primary route of transmission by the consumption of contaminated food (Latorre *et al*., 2007). This organism may be an invasive gastrointestinal human pathogen (Lorber, 1997) capable of causing serious illness with high mortality rates (20-30% of total cases) in vulnerable groups, such as pregnant women and the elderly (Bennion *et al*., 2008). In these groups the most regular clinical symptoms are meningitis followed by septicaemia, gastroenteritis and pneumonia, which may be fatal in susceptible populations of the elderly, immunocompromised individuals and newborn babies. The infection of pregnant women causes miscarriage, foetal death, preterm delivery and other symptoms of neonatal septicaemia, as well as meningitis and death; this is known as the invasive form of listeriosis (Vazquez-Boland *et al*., 2001; Denny and McLauchlin, 2008; Allerberger and Wagner, 2010). In healthier subjects, *L. monocytogenes* may lead to episodes of gastroenteritis and fever, referred to as the non-invasive form of listeriosis (Franciosa *et al*., 2001; Piana *et al*., 2005; Allerberger and Wagner, 2010). The occurrence patterns of human listeriosis comprise a background level of sporadic cases with occasional outbreaks (Thomas *et al*., 2012). Reports show that fish (0% to 12.5%) and shellfish (4%) products may be contaminated with *L. monocytogenes* (Abeyta, 1983; Pinto *et al*., 2006; Pao *et al*., 2008). Once ingested, *L. monocytogenes* can penetrate the intestinal endothelial barrier, the placenta or blood-brain barrier (Goldfine and Shen, 2007). Nevertheless compared with listeriosis outbreaks associated with other foods, a low number of cases have been linked to seafood (European Food Safety Authority, 2013).

In European countries, the number of reported listeriosis cases has increased in recent years (EFSA, 2012), and this illness is the most important cause of mortality from

foodborne infections. Listeriosis is a low prevalence disease, and in 2007, the reported incidence rates were 0.3 cases per 100 000 population in EU, and specifically 0.5 cases per 100 000 population in the Czech Republic (EFSA Zoonosis Report, 2009). In developing countries, this species is one of the most important causes of death from foodborne infections (Jemmi and Stephan, 2006). In spite of that, the gravity of this food–borne zoonosis lies in high case fatality rates reaching up to 30% (Denny and Mclauchlin, 2008). The greatest threat of this pathogen is linked to refrigerated products that have a long shelf life and products that are generally eaten with little or no prior heating (Bremer *et al*., 2003). Seafood is the first among these high risk, ready-to-eat (RTE) products (Rocourt *et al*., 2003; Reji and Den Aantrekker, 2004).

Phenotypic characterization through subtyping analysis allows possible identification of the sources of infection (Wagner and Allerberger, 2003). *L. monocytogenes* is grouped into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (FDA, 2011). Nevertheless, this classification yields limited discrimination during epidemiological investigations because the majority of human listeriosis cases are associated with 3 serotypes, namely 4b, 1/2a, and 1/2b (Liu, 2006). Large outbreaks have been primarily linked to serotype 4b, whereas serotype 1/2a has been associated with sporadic cases (Lianou and Koutsoumanis, 2013). Serotypic classification is important for the tracking of *L. monocytogenes* strains linked to disease outbreaks. Only the haemolytic species of *Listeria*, *L. monocytogenes* and *L. ivanovii*, are associated with human pathogenicity. However, *L. monocytogenes* is the only species of *Listeria* that has been involved in food-borne outbreaks of disease (USMI, 2014). Ericsson *et al.* (1997) documented a listeriosis outbreak traced to consumption of vacuum packed (VP) cold-smoked and "gravad" (= filleted fish, salt, sugar, dill and sometimes pepper and fennel are added and the fillets are allowed to mature for 1 to 3 days) (Jami *et al*., 2014)

rainbow trout (*Oncorhynchus mykiss*). The research conducted on this particular outbreak showed that the strain belonged serotype 4b with variable levels of isolates ranging from < 100 to 2.5 x 10^6 CFU g^{-1}. Another outbreak, the first to be reported on fibrile gastroenteritis caused by *L. monocytogenes*, was reported by Miettinen *et al*. (1999). Epidemiology showed serotype 1/2a in VP cold-smoked rainbow trout at levels reaching 1.9 x 10^5 CFU g^{-1}. In the period between 2005 and 2008, 16 outbreaks involving *L. monocytogenes* in RTE foods (deli meats, ham, dairy products and fish) were reported in several EU countries and the USA (Todd and Notermans, 2011). Also, many listeriosis outbreaks have been associated with smoked fish, especially cold smoked salmon (Garrido *et al*., 2008).

There is no agreement on the "acceptable levels" of *L. monocytogenes* in food (Jami *et al*., 2014). Several risk assessments (Buchanan *et al*., 1997; FAO/WHO, 2001; FDA, 2001) have concluded that although even low number of cells carry some risk of infection, the majority of cases (>99%) are caused by food products with high levels of the organism. Thus, the real risk is the growth of the organism in the product rather than its mere presence. Despite this knowledge and the understanding that low levels are unlikely to cause disease, some countries, such as the USA and UK have regulations calling for so-called zero tolerance, i.e. that the organism must not be detected in 25 grams of food. Achieving a zero count of *L. monocytogenes* in ready-to eat (RTE) smoked fish is technologically difficult. This has brought inconsistencies in standards set by several countries for RTE fish products. On an international scope, the Codex Alimentarius Commission has set a "zero tolerance" for products that provide favourable environments for the growth of *L. monocytogenes* (they are high risk foods that are likely to be ingested by immunocompromised people), and a limit of 100 CFU g^{-1} for foods that do not provide favourable conditions for growth (based on a shelf life

of less than 5 days) **(**EC No. 2073/2005; FAO, 2012). Similarly, there are guidelines that have been put in place by EU mandate that a zero tolerance must be observed by food operators for RTE fish as long as these products are under their immediate control (European Commission, 2005).

1.3.3. Pathogenicity of *Listeria monocytogenes*

Listeriosis is a complex disease in which the bacteria multiply in the cytoplasm and spread to other cells (Kuhn and Goebel, 1999, Camejo *et al.,* 2011). *Listeria* enters the enterocytes, as well as M cells or micro-fold cells in the vicinity of Peyer's patches. At this time, multiplication starts in the phagocytic cells that are beneath the enterocytes (Decatur and Portnoy, 2000; Camejo *et al.,* 2011). The next step is the transport of *Listeria* hidden in macrophages into the blood stream and lymph, finally reaching the liver and spleen. The vast majority of *Listeria* cells are destroyed by neutrophils in the vicinity of kupffer cells. Nevertheless, if cellular immunity of the host is compromised or inadequate, *Listeria* can multiply in the hepatocytes, as well as the macrophages. In such cases, bacteria can be transported by the blood to other organs, particularly the brain and/ or placenta (Lecuit, 2007). During each of the phases in *L. monocytogenes* pathogenesis, bacteria synthesize several virulence factors: internal (enabling penetration of non-phagocyte cells: epithelial cells, hepatocytes); superficial protein p104 (bacterial adhesion to the intestinal cells); listeriolysin O (cytoplasm vacuola membrane lysis, enabling the bacteria to escape into the cytoplasm of the host cell); ActaA protein (polymerization of the globular actin molecules in order to form the actin tail that enables bacteria to move to the cell membrane where *Listeria* form listeriopods that attack neighbouring cells) (Camejo, *et al*., 2011). In such a way, *Listeria* spreads locally with no possibility to get into contact with anti-*Listeria* antibodies and other

immunoactive molecules, such as phopholipases, metalloproteases, C1p proteases and ATP-ases; protein p60 (Dons *et al*., 1999; Jin *et al*., 2001; Kuzmanović *et al*., 2011).

1.3.4. Detection of *Listeria monocytogenes*

The traditional method of detecting *L. monocytogenes* in food involves selective enrichments and subsequent culturing on a selective medium to obtain colonies, followed by biochemical and/or serological confirmation and differentiation of *Listeria* species (Lovett, 1988; Bhat *et al*., 2012). These methods are demanding and usually require many days to produce results (Fluit *et al*., 1993; Jamali *et al*. (2013). Food processing companies depend increasingly on rapid tests, which deliver results within a short time and which allow release of food batches dependent on such results (Stephan *et al*., 2003). Advances in biotechnology have led to the development of rapid methods that reduce analysis time and offer great sensitivity and specificity in the detection of *L. monocytogenes* (Olsen *et al*., 1995; Dwivedi and Jaykus, 2011). Among these methods are polymerase chain reaction (PCR) based methods with suitable methods for DNA extraction (Amagliani *et al*., 2007), and analysis of PCR products obtained by denaturing gradient gel electrophoresis (DGGE) (Cocolin *et al*., 2002). Immunologic methods, such as those based on the enzyme-linked immunosorbent assay, (ELISA) offer faster and specific alternatives (Mattinggly *et al.*, 1988; Kerr *et al*., 1990). Fourier transform infrared (FT-IR) spectroscopy has been used for differentiation of pure cultures of different bacterial species, as well as intact compared with sonication-injured *L. monocytogenes* cells (Lin *et al*., 2004). FT-IR spectroscopy was successfully used to detect sub-lethal heat injury in *Salmonella enterica* serotype Typhimurium and *L. monocytogenes* (Al-Qadiri *et al*., 2008; Nyarko *et al*., 2014).

1.3.5. Isolation of *Listeria monocytogenes*

Present microbiological culture methods depend on growth in culture media followed by isolation, and biochemical and serological identification. Nevertheless, the detection of *L. monocytogenes* in food by these standard culture methods is made difficult by sporadic or low levels of *Listeria* contamination (< 100 CFU g^{-1}), by the presence of a high level of background microflora and competitor organisms that could cover the presence of *L. monocytogenes*, and by interference due to food matrix components (Norton *et al*., 2001).

Isolation and detection of *L. monocytogenes* in food is usually performed using methods approved by the International Standard Organization (ISO) (ISO 11290), US Food and Drug Administration (FDA) and US Department of Agriculture (USDA). The choice of methods depends on the food matrix. Although different enrichment broths can be used in these methods, only limited selective/differential agars are available for the detection of *L. monocytogenes*. *Listeria* cells grow slowly and can be quickly out-grown by competitors, and for this reason bacteriostatic agents, such as acriflavin and nalidixic acid that specifically act to suppress competing microflora, have been introduced into the enrichment media or selective agar (Welshimer, 1981). These two agents are incorporated into many methods used to isolate *Listeria* from food and environmental samples (Gasanov *et al*., 2005).

Listeria enrichment medium base is used for the selective cultivation and isolation of *L. monocytogenes* from clinical samples. The medium, originally formulated by Donnelly and Baigent (1986), was later modified by decreasing the nalidixic acid concentration in the selective supplements and subsequently increasing the amount of acriflavin (McClain and Lee, 1988). University of Vermont Modification Medium (UVM) uses a two-step selective enrichment medium resulting in a higher isolation rate of *L. monocytogenes* from meat products within 3-4 days. UVM broth is recommended as a

primary enrichment broth for recovery of heat-injured *Listeria* (Bailey *et al*., 1990). UVM broth comprises casein enzymic hydrolysate, proteose peptone, beef extract and yeast extract, which provide necessary nutrients whereas aesculin offers differential properties to the medium by detection of β-D-glucosidase activity by *Listeria*, causing a blackening of the medium. Nalidixic acid and acriflavin hydrochloride together with higher concentration of phosphate render the medium selective for *Listeria*. Gram-positive and Gram-negative organisms are inhibited by nalidixic acid and acriflavin hydrochloride, respectively (Donnelly and Baigent, 1986).

The information and concern surrounding the presence of *L. monocytogenes* in food has resulted in the development of many media for its isolation (In't Veld and de Boer, 1991; Gunasinghe *et al*., 1994). The formulation of PALCAM medium (Oxoid) was described by Van Netten *et al*., (1991), and recommended for isolation of *L. monocytogenes* in foods. PALCAM contains Columbia blood agar, which provides the nutrients and cofactors required for growth of *Listeria*. Addition of a selective supplement containing lithium chloride, ceftazidime, polymyxin B and acriflavine hydrochloride makes the medium highly selective and suppresses growth of most non-*Listeria* spp. present in foods and clinical specimens. It allows the easy differential diagnosis of *L. monocytogenes* by utilising the double indicator system: (1) aesculin and ferrous iron, and (2) mannitol and phenol red (PALCAM). On this medium, *L. monocytogenes* hydrolyses aesculin resulting in the formation of a black halo around colonies. The product of hydrolyses, aesculetin (=6, 7-dihydroxycoumarin), reacts with the ferric ions to form a brown to black complex. Thus, typical *Listeria* spp. form colonies that are approximately 2 mm in diameter, grey-green in colour with a black sunken centre and a black halo against a cherry-red medium background. *L. monocytogenes* does not ferment mannitol so easy differentiation from contaminants,

such as enterococci and staphylococci, can be achieved as these will ferment mannitol. This formulation produces a change from red to yellow in pH indicator phenol red. Occasionally, enterococci and staphylococci develop on this medium as grey colonies with blue green haloes or yellow colonies with a yellow halo. Strict aerobes, such as *Bacillus* spp. and *Pseudomonas* spp. that might appear on the medium, are inhibited by incubating under microaerophilic conditions. A modification of PALCAM medium in which incubated plates are overlaid with medium containing blood enables haemolytic *Listeria* spp. to be differentiated and enumerated (Van Netten *et al*., 1991).

Furthermore, Curtis *et al*. (1989) described the formulation of Listeria selective medium (Oxford formulation) which is recommended for the detection of *L. moncytogenes* from clinical and food specimens. The medium uses the selective inhibitory components lithium chloride, acriflavin, colistin sulphate, cefotetan, cycloheximide or amphotericin B and fosfomycin combined with the indicator system aesculin and ferrous iron (Curtis *et al*., 1989). In this medium, *L. monocytogenes* hydrolyses aesculin, producing black zones around the colonies due to the formation of black iron phenolic compounds derived from aglucon. On this medium, Gram-negative bacteria are completely inhibited. Most unwanted Gram-positive species are suppressed, but some strains of enterococci grow poorly and show a weak aesculin reaction, usually after 40 h of incubation. A few staphylococci may grow as aesculin-negative colonies (Curtis *et al*., 1989).

CHROMagar™ *Listeria* is able to differentiate *L. monocytogenes* and *L. ivanovii* from other *Listeria* species through formation of phospholipase-C (El Marrakchi *et al*., 2005). Suspected colonies are purified on TSA, and pure colonies are confirmed as *L. monocytogenes* showing the following micro-morphological characteristics: Gram-positive rods, aerobic and facultatively anaerobic, non-spore forming, catalase-positive,

oxidase-negative and motile at 25 °C. Further confirmation as *L. monocytogenes* is by β-haemolysis and fermentation of glucose and xylose (Aygun and Pehlivanlar, 2006). The Christie-Atkins-Munch-Petersen (CAMP) test (Aygun and Pehlivanlar, 2006) can be used to differentiate between the haemolytic *Listeria* species. The test is carried out by streaking a β-haemolysin-producing *Staphylococcus aureus* strain and *Rhodococcus equi* parallel to each other on a blood agar plate with incubation at 35 °C for 24-48 h. On examination for haemolysis, *L. monocytogenes* and *L. seeligeri* are haemolytic, with the haemolytic zone being effectively active by *S. aureus* (Aygun and Pehlivanlar, 2006).

1.3.6. *Listeria monocytogenes* in seafood

Listeria spp. are part of the microflora in surface water and other water bodies associated with rivers. For this reason, these microorganisms are usually present on the external surface of fish that swim in contaminated water. *L. monocytogenes* has been detected on the fish surface and in the stomach lining, gills and intestines, but the flesh is usually free of the organism unless it has been contaminated from different sources. Fish may be contaminated with *Listeria* from two possible routes: (1) spread from the intestinal contents to other fish tissues (including muscle) particularly if the period between death and visceral removal is greater than a few hours*;* (2) cross-contamination (due to manipulation of fish using contaminated equipment and inappropriate transport) (Souza *et al*., 2008). *L. monocytogenes* occurs widely in raw fresh fish in several countries, but the level of contamination tends to be low, and varies between 0% and approximately 30% of the products (Miettinen and Wirtanen, 2005; Thomas *et al*., 2012). Studies on raw fish samples entering the processing plants were reviewed by Gram (2001), who detected 1% to 34% samples positive with *L. monocytogenes*. Davies *et al*. (2001) observed that *L. monocytogenes* was present in 3% of European

fish. Yucel and Balci (2010) reported that the distribution of *Listeria* spp. was 30% in fresh fish samples and 10.4% in marine fish samples in Turkey. *L. monocytogenes* (44.5%) and *L. murrayi* (83.5%) were the most commonly isolated species from fresh water and marine fish samples, respectively (Jami *et al* 2014). *L. monocytogenes* contamination rate of fresh fish (including salmon and tilapia) as observed by Wang *et al*. (2011) was 4.1% (4.8% in salmon and 4.3% in tilapia). At retailers, wholesalers and importers, fresh and repackaged fish products may be easily affected by frequent *L. monocytogenes* contamination. Food contact surfaces and/ or secondary contamination from site equipment are sources of the pathogen and the prevalence of this type of contamination varies from very low levels to 14% (Mena *et al*., 2004; Handa *et al*., 2005; Parihar *et al*., 2008). Detailed examination of minced tuna collected from retail stores in Japan between 2002 and 2003, *L. monocytogenes* was detected in 14.3% of the raw material (Handa *et al.,* 2005). The distribution of *L. monocytogenes* has ranged from 0% to 12.5% in some US market surveys (Abeyta, 1983; Pao *et al*., 2008).

The filter feeding habit of shellfish helps in accumulating bacteria from polluted aquatic environments. Consequently, pathogens, such as *Listeria*, may often be found in shellfish (Berry *et al*., 1994; Ghanbari *et al*., 2013). The transmission of *L. monocytogenes* through shellfish, either as a carrier or a direct source, is a part of proposed cycle for infection of humans with *L. monocytogenes* (Norhana *et al*., 2010). Cordano and Rocourt (2001) reported the presence *L. monocytogenes* in 28% of the fresh shrimp in Chile. In Iceland, *Listeria* was detected in 20.9% of the fresh shrimp studied (cold water) (Gudmundsdottir *et al*., 2006). Nevertheless, some workers (Dhanashree *et al*., 2003; Moharem *et al*., 2007; Jalali and Abedi, 2008) did not detect *L. monocytogenes* in fresh shrimp samples (tropical). As a matter of fact these products might contain *L. monocytogenes* as they do not constitute a risk to the greater number of

consumers because of cooking before consumption, which is similar to the case of other aquatic food products (Jami *et al*., 2014).

The presence of *L. monocytogenes* in raw and whole crawfish (freshwater lobsters) and in cooked and crawfish meat in 2 processing plants was monitored by Thimothe *et al*., (2002). These workers observed that only 3 of the 78 raw material samples (3.8%) were positive for *L. monocytogenes,* and all the processed products sampled were negative for the pathogen. The workers inferred that heat treatment of raw material during processing and practices to prevent post processing contamination could significantly decrease *Listeria* spp. contamination in the final product.

For effective development of in-package thermal treatment processes for meat products, at numerous processing temperatures, it is necessary to determine the degree of inactivation of target microorganisms such as *L. monocytogenes*. To produce pathogen-free ready-to-eat meat products, effective heat processing treatments as decimal reduction values (D-values) and z-values must be determined (McCormick *et al*., 2003).

D-values are prerequisite time in seconds to attain a log reduction in the bacterial population at chosen temperature (McCormick *et al*., 2003), whereas *z*-values are defined as the numbers of ºF or ºC necessary for a thermal death time curve to pass through 1 log cycle (Rippen *et al*., 1993). McCormick *et al*. (2003) reported the *D*-values of *Listeria monocytogenes* at various surface pasteurization temperatures were determined for low-fat turkey bologna. The results of the above mentioned workers revealed that the *D*-value for *L. monocytogenes* at 61°C was 124 s, but at 65°C the *D*-value decreased to 16.2 s. The z-value was 4.4°C. Carlier *et al*. (1996) demonstrated that the thermal resistance of *L. monocytogenes* in meat products revealed *D*-values from 0.75 to 28 min and *z*-values that range from 4.6 to 7.4°C.

With shellfish, mussels are the prevailing source of *L. monocytogenes* (Laciar and de Centrobi, 2002; Pinto *et al*., 2006). Pinto *et al*. (2006) detected *L. monocytogenes* from 4% of the bivalve mollusc samples marketed in Portugal. Nevertheless in a study carried out in Japan, most of the live mussels examined were *Listeria* negative (Handa *et al*., 2005).

1.3.7. *Listeria monocytogenes* in lightly preserved seafood products (LPSPs)

Lightly preserved seafood products (LPSPs) comprise a large group of chilled, RTE foods with a pH > 5.0 and < 6% NaCl in the water phase of the product (Lyhs *et al*., 2002). During the last ten years, *L. monocytogenes* has been often isolated from RTE and LPSPs, including cold- and hot-smoked salmon, gravad salmon, fermented fish and fish salads. This observation points out the high risk of transmission of *Listeria* spp. (Jami *et al*., 2014). On account of *L. monocytogenes* being an important food safety concern, the presence of *L. monocytogenes* in smoked fish products in Europe has been widely studied. The presence of *L. monocytogenes* in cold-smoked fish has been examined by legislators, researchers and food-control agencies. Altogether, reported prevalence rate of *L. monocytogenes* in cold-smoked salmon is approximately 10% (colder region) (Azevedo *et al*., 2005; Gudmundsdottir *et al*., 2005; Miettinen and Wirtanen, 2006); nevertheless, other workers including Vitas *et al*., (2004) and Di Pinto *et al*. (2010) detected much higher prevalence (warmer region) (28% and 34.1%, respectively). The overall rate is consistent with studies (with reported incidence levels between 5% and 35%) in specific EU member states (European Food Safety Authority, 2013).

Various fish species and seafood products have different intrinsic characteristics (pH, water activity, salt concentration etc) that affect the presence and resistance of *L. monocytogenes* (Uyttendaele *et al*., 2009); these characteristics might underlie the

differences in prevalence. The levels of contamination in smoked fish are low (<10 CFU g^{-1}), but retail samples might occasionally contain viable bacterial counts ranging from 10^4 to 10^6 CFU g^{-1} (Gombas *et al*., 2003).

"Gravad" fish is another high-risk RTE food that can support the growth of *L. monocytogenes* (Tham *et al*., 2000). This type of fish product is lightly preserved with NaCl content of 3% to 6% (w/v) and pH greater than 5, and products are typically consumed without heat treatment (Lyhs *et al*., 2002). Loncarevic *et al*. (1996) isolated *L. monocytogenes* from 21% (12/58) of gravad fish samples. Mejlholm (2007) observed that 31.6% of gravad products (*n* = 403) contained *L. monocytogenes*. Among the contaminated samples, gravad trout had the highest contamination rate (36.6%), followed by gravad salmon (14%). The high prevalence of *L. monocytogenes* in cold-smoked and gravad fish draws attention to the need for improved in-house control and vigilant supervision to get rid of this bacterial hazard and to reduce the risk of human listeriosis (Jami *et al*., 2014).

1.3.8. *Listeria monocytogenes* in the processing environment

Although raw materials are the main origin of potential *L. monocytogenes* contamination in seafood products (Eklund *et al*., 1995), the processing plant environment might play an important role particularly in transmission of the pathogen (Rorvik *et al*., 1995, 2000; Reji and Den Aantrekker, 2004). Floors and drains are notably difficult to clean and maintain *Listeria*-free. Rorvik *et al*. (1997) noted the 33% of salmon smokehouse plants (*n* = 40) were contaminated with *L. monocytogenes* mainly due to the drains representing a niche of contamination. Nevertheless, these authors reported the presence of *Listeria* spp. in 15.4% of the drains (n = 6/39) and in 5.1% of the employee contact surfaces (gloves and apron) (2/39) but in none of the samples from the food contact surfaces. Gubjornsdottir *et al*. (2004) reported that raw

material and specific locations in a cold-smoked salmon processing plant, specifically the floors and drains, were potential sources of *L. monocytogenes* in final products. It was reported by Srey *et al*. (2013) that *L. monocytogenes* produces strong biofilms on plastic surfaces (for example, the conveyor belts used in fish factories). Eklund *et al*. (1995) observed that the external surfaces of fresh and frozen fish might act as inoculating agents to introduce *L. monocytogenes* into the producing plant; this contamination is prolonged by operations such as filleting, rinsing and brining.

1.3.9. Mitigation processes for *Listeria monocytogenes* in seafoods

Essentially, cleaning and sanitizing procedures can eliminate the pathogen from the processing line and equipment, but recontamination can occur after processing (Eklund *et al*., 1995). In their review, Carpentier and Cerf (2011) proposed important conclusions concerning the control of *Listeria* spp. in manufacturing environments. An important rule is to avoid water in sites of product exposure; this restriction is not easy during the processing of raw materials, but it may be possible for RTE products after processing. One more important rule is to clean the floors surrounding the equipment to avoid contamination by bacteria dislodged from the floors. In addition, bacterial growth must be prevented by temperature reduction, and limitation of soiling and drying.

Contamination of food products by *L. monocytogenes* has led to product recalls (Zhu *et al*., 2005; Norhana *et al*., 2010). Therefore to improve the control of this pathogen in smoked fish products, there is a need to apply thermal and non-thermal techniques (Tocmo *et al*., 2014). Miladi *et al*. (2008) reported that frozen storage conditions of salmon slices inoculated with *L. monocytogenes* (-20 °C) over a period of 10 months had no effect on the potential of this pathogen to survive. In one study, *L. monocytogenes* (6 log CFU g^{-1}) spread on surfaces of catfish was reduced by 4 to 5 log CFU within 2 min of microwave heating at 1250 W (Sheen *et al*., 2012). Cold-smoked

salmon fillets, aerobically or vacuum packaged and exposed to gamma-irradiation (3kGy), revealed 6.59 log CFU g^{-1} reduction of *L. monocytogenes* growth. No microbial growth was observed after 6 weeks of refrigeration storage (4 ± °C). *L. monocytogenes* elimination in fish products have been performed recently using X-ray irradiation. Inoculated hot-smoked catfish fillets treated at different intensities (0.1 to 2.0 kGy, 22 °C, 55% to 60% relative humidity) revealed no detectable (<1.0 log CFU g^{-1}) *L. monocytogenes* growth at 2.0 kGy throughout a 35 d storage at 5 °C (Mahmoud *et al*., 2012). Su *et al*. (2004) demonstrated the use of Electronic-beam (E-beam) irradiation in prevention of *L. monocytogenes* growth in cold-smoked salmon with an initial load of 3.6 log CFU g^{-1}. A 2.5 log CFU g^{-1} reduction was observed at an E-beam dose of 1.0 kGy after 8 d storage at 5 °C, whereas complete elimination was achieved at E-beam doses of 2.0 and 4.0 kGy. Lasagabaster and de-Maraňó (2012) studied the effect of pulsed UltraViolet–light treatment against 6 *Listeria* isolates in lightly preserved fish products under refrigeration, distribution and storage temperature of 4°C. Treatments below 0.3 J cm^{-2} revealed the highest detectable inactivation (6 to 6.5 log). Using *L. innocua,* Gudbjornsdottir *et al*. (2010) demonstrated that the bacterial population (4500 CFU g^{-1}) in VP cold-smoked salmon decreased to a nondetectable level (<0.3 CFU g^{-1}) with the application of high-pressure processing (HPP) 700 to 900 megapascal (Mpa) for 10 s. Nevertheless, it was observed that salmon fillets became lighter in colour.

Chlorine and chlorine-based sanitizers are probably the most commonly used chemical sanitizers in fish processing due to their broad antimicrobial spectrum (Ray, 2003). A 99.79% reduction of *L. monocytogenes* population was observed by rinsing the surface of inoculated raw salmon fish with a solution containing 200 ppm free chlorine. Neetoo *et al*. (2008) investigated the efficacy of sodium lactate (SL), sodium diacetate (SD) and their combination against *L. monocytogenes* in smoked salmon pâté and fillets. It was

observed that SD (0.25%), alone and the combined treatment using SL (2.4%) with SD (0.125%) were effective in inhibiting *L. monocytogenes* growth in samples stored at 4 °C for 3 wk. Essential oil (EO) obtained from *Bunium persium* (black zira) inhibited growth of *L. monocytogenes* when used together with smoke and sodium chloride, as well as low temperature storage (Rabiey *et al*., 2013). Montiel *et al*. (2012) investigated the combined effect of high pressure processing (HPP) and the lactoperoxidase system (LPS) against *L. monocytogenes* in cold-smoked salmon. These authors found that a HPP treatment (450 Mega pascals (Mpa) for 10 min) combined with LPS from bovine milk resulted in a 3.84 log CFU g^{-1} reduction in the *L. monocytogenes* H66a count after 35-d storage (5 °C).

Carnobacterium is a group of lactic acid bacteria (LAB) widely studied for its biopreservative properties. In particular, *Carnobacterium divergens* V41 showed inhibitory effect against *L. monocytogenes* without adverse effects on the sensory attributes of cold-smoked salmon (Brillet *et al*., 2005). Bacteriocins can likewise be detected in bioactive packaging application of VP fish products. Alginate films incorporated with LAB and nisin (Nisaplin) suppressed *L. monocytogenes* growth when wrapped around VP cold-smoked salmon after 28 d of storage at 4 °C, revealing counts being 3 to 4 log lower than in the control (Concha-Meyer *et al*., 2011).

1.4. Microbial spoilage of fish

Fresh fish is easily affected by spoilage, which can be as a result of both chemical reaction and microbiological growth (Serio *et al.,* 2014). Fish spoilage can be swift after harvest (Adebowale *et al*., 2008). Most fish species are degraded due to digestive enzymes and lipases, microbial spoilage from surface bacteria and oxidation (AMEC, 2003). The process of fish spoilage results in breakdown of various components and the formation of new compounds (Ghaly *et al*., 2010). The spoilage compounds of seafood

products comprise of ammonia from breakdown of amino acids, sulphides formed from sulphur-containing amino acids (Herbert and Shewan, 1975), trimethylamine resulting from bacterial reduction of trimethylamine oxide, and esters that may arise from degradation of phospholipids (Gram, 2009). Manifestation of microbial spoilage is evidenced in production of off-odour and off-flavour, slime formation, pigmented and non-pigmented colonies, gas production and discolouration (Gram and Dalsgaard, 2002).

1.4.1. Spoilage microorganisms and spoilage processes in fresh fish

Microorganisms found in fish at the point of spoilage are known as the spoilage microflora, whereas those that are able to produce off-odour and off-flavours are the specific spoilage organisms (SSOs) (Gram and Dalsgaard, 2002). Seafood spoilage is dynamic with changes and interactions between different microbial spoilage groups (Gram and Dalsgaard, 2002; Tsigarida *et al*., 2003). The SSOs and suitably the metabolites produced are function of the storage condition and microbial contents of the water in which the fish lives (Drosinos and Nychas, 1997; Gram and Huss, 2000). The most commonly reported fish and fish products spoilage bacteria are *Shewanella putrefaciens*, *Photobacterium phosphoreum*, lactobacilli (Rudi *et al*., 2004) and *Pseudomonas* spp. (Gennari *et al*., 1999). Shewanellae and pseudomonads are known to dominate warm tropical freshwater fish and in fish caught in cold, marine waters (Gram *et al*., 1990; Koutsoumanis and Nychas, 1999). *Aeromonas* spp. are typical of freshwater fish (Gram *et al*., 1990). In marine fish growth of these psychrotrophic bacteria is accompanied by the production of trimethylamine (TMA) caused by *Shewanella, Vibrio and Photobacterium* metabolism. The nature of fish microflora is altered dramatically during spoilage (Gram and Huss, 1996). The spoilage of iced freshwater finfish is dominated by *Pseudomonas* spp. (Gram, 1989; Gelman *et al*.,

2001; Chytiri *et al*., 2004) the spoilage off-odour are fruity and onion-like. At temperature range of (20 - 25 °C), the microflora at the point of spoilage is dominated by mesophilic *Vibrionaceae* and, especially if the fish is caught in polluted waters, *Enterobacteriacea*e (Len, 1987; Liston, 1992; Gram and Huss, 1996; Ghaly *et al*., 2010).

Trimethylamine oxide is reduced to TMA by these bacteria to produce several sulphides. During the stages of deterioration autolytic enzymes reduce textural quality but do not produce the characteristic spoilage off-odours and off-flavours and which are related to the breakdown of adenosine triphosphate (ATP). Lipid hydrolysis and oxidation may continue in fatty fish species and add towards the development of unpleasant off-odours and off-flavours. Nevertheless, the offensive off-odours and off-flavours leading to spoilage at a low temperature are a result of bacterial action (Gram, 2009). Decrease in temperature results to change in the microflora of the product and at 0-2 °C, the spoilage microflora which reach 10^8 - 10^9 CFU g^{-1} after 2-4 weeks is dominated by pseudomonads and shewanellae even though other psychrotrophic microbes sometimes may grow (Gram, 2009). Grigorakis *et al*. (2004) and Lalitha *et al*. (2005) reported the growth of Gram-positive bacterium *Brochothrix thermosphacta* in iced finfish but their numbers are two orders of magnitude lower than the numbers of *Shewanella* (Koutsoumanis and Nychas, 1999).

1.4.2. Spoilage microorganisms and spoilage processes in salted cold-smoked fish

Salmon, but to some extent trout, cod and halibut are processed as lightly salted, cold-smoked products. The salt concentration is typically between 3 and 6% (as water-phase salt) and the cold-smoking process is never above 28-30 °C (Siskos *et al*., 2005). Packaging of cold–smoked product is done under vacuum and retail distributed at refrigeration temperature (Gram, 2009). The shelf life of vacuum-packaged, cold-

smoked fish differs between species and factories and is connected to the degree of drying, smoking and the amount of salt added (Leroi and Joffraud, 2000). The sensory shelf life differs between 3 and 9 weeks (Jorgensen, Huss *et al*., 2000a); nevertheless, the longer shelf lives may not be accepted from a safety point of view. Gram (2001) reported *L. monocytogenes* and sometimes *Clostridum botulinum* may be able to growth in this product.

During refrigerated storage (4-5 °C), the total bacterial count of vacuum-packaged, cold-smoked fish increases and the spoilage microflora is frequently dominated by lactic acid or a combination of lactic acid bacteria and fermentative Gram-negative bacteria (*Enterobacteriaceae* or *Ph. phosphoreum*) (Hansen *et al*., 1998; Leroi *et al*., 1998). Lactic acid bacteria counts characteristically increase to 10^6- 10^8 CFU g^{-1} during a few weeks of storage (Jorgensen, Dalsgaard *et al*., 2000b; Leroi and Joffraud, 2000). Several studies have shown that the microflora of cold-smoked product is dominated by carnobacteria (Paludan-Muller *et al*., 1998) and other trials revealed different types of *Lactobacillus* being dominant, such as *Lb. curvatus, Lb. sakei* and *Lb, plantarum* with each of the three processing plants having their own composition of the flora (Hansen and Huss, 1998). The odour profile of cold-smoked fish differs and potentially odorous volatile compounds are produced during spoilage, and include alcohols, aldehydes, ester ketones and phenols (Jorgensen *et al.*, 2001).

1.5. Biogenic amines in seafood

Biogenic amines are low molecular weight organic bases with biological activity that are produced in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases (Zhai *et al*., 2012). The most significant biogenic amines, histamine, tyramine, tryptamine, putrescine and cadavarine are produced from free amino acids namely histidine,

tyrosine, tryptophane, ornithine and lysine, respectively. Spermidine and spermine arise from putrescine (Zarei *et al*., 2011). They are significant from the perspective of food intoxication and also as chemical indicators of fish spoilage (Visciano *et al*., 2012). Cadaverine, putrescine, and histamine are diamines that may be produced *post mortem* from the decarboxylation of specific free amino acids in fish or shellfish tissue (Silla Santos, 1996). Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscles tissue, and histamine forming bacteria will start to grow, leading to the formation of biogenic amines (Erkan *et al*., 2006; Food and Drug Administration (FDA), 2011). Biogenic amine production in seafood is significant as histamine, and possibly other biogenic amines, are responsible for scombrotoxic fish poisoning (Erkan, 2004). Putrescine, spermidine, spermine and cadavarine have no contrary health effect, but may react with nitrite to form carcinogenic nitrosoamines and also have been proposed as indicators of spoilage (Eerola *et al*., 1997; Hernandez-Jover *et al*., 1997). Tryptamine has toxic effects on human such as blood pressure increase and therefore causes hypertension (Shalaby, 1996). The maximum allowable level of tryramine in food as proposed by Nout (1994) is 100-800 mg kg^{-1}. The US Food and Drug Administration guidance level of 50 mg kg^{-1} and European Union (EU) acceptable level of 100 mg kg^{-1} has been established for histamine in fish in tuna and other fish belonging to the Scombridae and Scomberesocidae families (CE, 1991). High levels of putrescine and cadavarine have been identified as potentiators of histamine or tyramine toxicity, but no recommendation about levels have been suggested (Park *et al.*, 2010).

Several bacterial species of the *Enterobacteriaceae* family are known to have histidine decarboxylase activity and have the ability to form histamine, including the species *Morganella morganii, Klebsiella pneumoniae, Hafnia alvei, Proteus vulgaris, Proteus*

mirabilis, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Serratia liquefaciens*, *Raoultella* (formerly *Klebsiella*) *planticola*, *Raoultella ornithinolytica*, *Prov- idencia stuartii*, and *Citrobacter freundii* (Kim *et al.*, 2003). In addition to the enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lwoffii*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have also been reported as histamine producers (Chen *et al*., 2010). *Morganella psychrotolerans*, a strong histamine-producer has been identified as a novel psychrotolerant bacterium (Emborg *et al*., 2006). Histamine formed in fermented products, such as wine (Lonvaud-Funel and Joyeux, 1994), cheese (Leuschner *et al*., 1998) and fish sauce (Satomi *et al*., 1997; Kimura *et al*., 2001) is produced by Gram-positive lactic acid bacteria whereas histamine is produced in raw fish products is formed primarily by Gram-negative enteric bacteria (Gingerich *et al*., 1999; Kim *et al*., 2001).

The levels of histamine in freshly caught fish are generally low, usually below 1 mg kg^{1} (Auerswald *et al*., 2006). Erkan and Özden (2008) determined the initial histamine values of ungutted and gutted sardine in ice as 12.30 mg kg^{-1}. The maximum levels of histamine were 52.80 mg kg^{-1} for ungutted and 45.80 mg kg^{-1} for gutted after 7 days of storage in ice. At any time, exposure of certain fish to high temperatures after the catch and before consumption can result in formation of histamine from histidine by bacterial histidine decarboxylases. Several studies agree that histamine formation is negligible in fish stored at 0˚C or below (Rossano *et al*., 2006). Nevertheless, biogenic amines are more likely to be formed in fish when decomposition occurs at harvest or in the first stages of handling on fishing vessels, rather than later in the distribution chain (Staruszkiewicz *et al*., 2004).

Baixas-Nogueras *et al*. (2005) used biogenic amine index consisting of a combination of biogenic amines for evaluation of iced Mediterranean hake (*Merluccius merluccius*) in the chilling condition as applied in the merchandising chain. Putrescine and cadavarine were the main amines accumulated, whereas histamine and tyramine were less abundant. The authors proposed a biogenic amines index limit of acceptability in a range of 15–20 μg g^{-1}. Similarly, Jorgensen, Huss *et al*. (2000a) demonstrated the production of biogenic amines in cold-smoked salmon as a mark of spoilage. Several different combinations of biogenic amines can be found at the point of spoilage (Table 1.4) (Jorgensen, Dalsgaard *et al*. 2000b) some are probably the result of metabolism by mixed bacterial communities. For example, the majority of spoilage bacteria produce only low concentrations (max 10 μg 10 g^{-1}) of putrescine when grown as single cultures (Jorgensen, Huss *et al*., 2000a). The spoiling product is characterized by much higher putrescine concentrations and co-cultures of lactic acid bacteria and Gram-negative bacteria give rise to the concentrations equivalent of the spoiling product. Tahiri *et al*. (2009) demonstrated the production of tyramine in smoked salmon inoculated with *Carnobacterium* spp. and uninoculated with values of ~ 130 μg g^{-1} and 47 μg g^{-1} at day 0, respectively. *C. divergens* strains M35 and ATCC 35677 produced tyramine progressively during storage and reached 130 and 135 mg g^{-1}, respectively, after 21 days. Production of spermidine was discovered at low levels by both strains at day 21. Nevertheless, no production of methylamine, tryptamine, putrescine, cadaverine, histidine and spermine beyond acceptable levels was detected in *Carnobacterium*-treated smoked salmon after the three weeks of storage.

Table 1.4. Production of biogenic amines by pure and mixed cultures of bacteria isolated from spoiled cold-smoked salmon

		Biogenic amines µg g^{-1}				
Species/group	No of strains or batches	Agmatine	Cadavarine	Histamine	Putrescine	Tyramine
Photobacterium phosphoreum	3	200	400	180	9	90
Aeromonas	1	<	50	<	<	<
Serratia liquefaciens	2	<	400	<	9	<
Enterobacter	2	13	5	<	10	<
Hafnia	1	<	180	<	5	<
Lactobacillus curvatus (I)	5	<	<	<	6	0-200
Lactobacillus curvatus (IV)	2	<	5	<	6	0-100
Lactobacillus sakei	3	<	<	<	5	<
Carnobacterium divergens	2	<	<	<	4	95
S. liquefaciens or *Hafnia* + *C. divergens* or *Lb. sakei*					25-80	
C. divergens + Gram-negative bacteria						80-130
Spoiling product (I)	6	90-270	150-350	100-240	3-35	80-140
Spoiling product (II)	3	2-30	100-135	3-50	8-40	130-180
Spoiling product (III)	2	2-25	180-300	10-15	190-380	225-335
Spoiling product (IV)	1	20	35	20	30	200

(Jorgensen, Dalsgaard *et al*., 2000b; Jorgensen, Huss *et al*., 2000a)

1.6. Production of Total Volatile Base Nitrogen (TVB-N) and Trimethylamine-Nitrogen (TMA-N) in seafood

Determination of the freshness of aquatic products is the most significant test when assessing quality (Boziaris *et al*., 2011). Freshness is assessed using sensory, microbiological and chemical methods (Jaffrès *et al*., 2011). Sensory estimation is subjective and necessitates the service of highly trained personnel to be reliable. Thus, it is not predisposed for routine examination (Dainty, 1996). Furthermore, microbiological results are retrospective, thus the determination of chemical spoilage parameters related to microbial growth, is more practical for use (Dainty, 1996). Total Volatile Base Nitrogen (TVB-N) and Trimethylamine-Nitrogen (TMA-N) has been proposed as chemical indicators in fish (Olafsdottir *et al*., 1997).

TVB-N is commonly considered as a fish spoilage indicator instead of a freshness indicator (Anastasio *et al*., 1999; Baixas-Nogueras *et al*., 2003; Baixas-Nogueras *et al*., 2009). TVB-N is a term that comprises measurement of trimethylamine, dimethylamine, ammonia and other compounds connected with seafood spoilage (Ocaño-Higuera *et al*., 2011). The concentration of TVB-N in freshly caught fish is typically between 5 and 20 mg 100 g^{-1} flesh, whereas levels of 30-35 mg 100 g^{-1} flesh are generally regarded as the limit of acceptability for iced stored cold water fish (Gökodlu *et al*., 1998).

Brillet *et al*. (2005) demonstrated the production of total volatile base nitrogen in commercial batches of cold-smoked salmon alone and in the presence of *C. divergens* V41 stored under vacuum. Regarding TVBN production, the above workers observed a low production ranging from 18 ± 2 and 21 ± 2 mg-N 100 g^{-1} in the control samples of high hygienic quality, respectively after 4 weeks of storage. The high hygienic samples

in the presence of *C. divergens* V41 revealed a slightly significant increase of 29 ± 0 and 25 ± 2 mg-N 100 g^{-1} of TVB-N production, respectively after 4 weeks of storage.

In the second batch, in the control samples of poor hygenic quality, a TVB-N production of 42 ± 2 and 51 ± 9 mg-N 100 g^{-1} was detected, respectively at the end of the storage, the inoculated samples did not reveal a statistically significant difference with the values of 45 ± 1 and 50 ± 9, respectively.

The initial TVB-N values of ungutted and gutted sardines stored in ice determined by Erkan and Özden (2008) were 11.11 mg 100 g^{-1} and 10.18 mg 100 g^{-1}, respectively. TVB-N values of an ungutted group of samples increased according to time of storage. At the expiration of the storage period of 9 days, TVB-N values reached 29.23 mg 100 g^{-1}, respectively. The legal limits of 35 mg 100 g^{-1} for TVBN were not exceeded throughout the storage in gutted sardine samples. Furthermore in their study, Erkan and Özden (2008) determined the TVB-N value of whole sardine higher than the eviscerated sardine flesh because of the enzymatic and bacterial activity in internal organs. The Monterey sardine (*Sardinops sagax caerulea*), like other small pelagic fish, is susceptible to quick autolytic degradation of abdominal tissue after capture; this process being caused mainly by proteases from digestive tract (Castillo-Yañez *et al*., 2004). Erkan (2005) reported the initial TVB-N value of fresh mussels stored at 4 ± 1 °C as 12.38 mg 100 g^{-1}. On the fourth day of storage, the TVB-N level of chilled mussels was 15 mg 100 g^{-1} which is at the border line of acceptability, and increased to 22.55 mg 100 g^{-1} after the sixth day of storage, acceptable limits of 15mg per 100 g for TVB-N in mussels was exceeded. Kim *et al*. (2002) demonstrated TVB-N values between 19 and 35 mg 100 g^{-1} for oysters packed in Low-density polyethylene (LDPE) pouches after 12 days of refrigerated storage.

TMA-N is frequently used as an index in estimating the shelflife and quality of fishery and aquaculture products because it rapidly accumulates in the muscles under refrigerated conditions. The TMA production in fish tissue during cold storage could be used as an indicator of bacterial activity, which is an accepted deterioration measure (Ocaño-Higuera *et al*., 2011). The pungent odour of spoiled fish has been associated to TMA tissue levels, also with the number of spoiling organisms present in many fish species (El Marrackchi *et al*., 1990). TMA is produced by the reduction of TMAO, caused by bacterial activity of *Sh. putrefaciens*, *Ph. phosphoreum*, *Vibrio* spp., *Aeromonas* spp. and psychrotolerant *Enterobacteriaceae* (Dalsgaard *et al*., 1993; Gram and Dalsgaard, 2002). This reaction reveals that these microorganisms can grow in microaerophilic or anaerobic conditions when the oxygen is poor or absent in muscle (Hobbs and Hodgkiss, 1982).

It has been recommended that quality cold-water fish should contain less than 1.5 mg TMA-N 100 g^{-1} fish, and 10-15 mg TMA-N 100 g^{-1} are generally considered as the limit of acceptability for human consumption (Huss, 1988). Erkan and Özden (2008) demonstrated the initial average values of 2.5 and 2.6 mg of TMA-N 100 g^{-1} muscle for ungutted and gutted sardine samples (*Sardina pilchardus*), respectively, with final values of 4.16 and 2.36 mg of TMA-N 100 g^{-1} of muscle for 9 days. In their study, the TMA-N value of gutted sardine was found to be lower than that of whole ungutted sardine flesh because of uneviscerated internal organs. Boziaris *et al.* (2011) revealed that the level of TMA-N was not detected or found to be low in Norwegian lobster (*Nephrops norvegicus*) even after long storage at various temperatures. Similar results have been reported for other fish and Norway lobster caught in Mediterranean waters (Koutsoumanis and Nychas, 1999; Papadopoulos *et al*., 2003; Aubourg *et al*., 2007). This shows low levels of TMAO in the flesh and/or low levels of *Sh. putrefaciens*

(Drosinos *et al*., 1997; Koutsoumanis and Nychas, 1999; Ocaňo-Higuera *et al*., 2011). For the above reasons, Boziaris *et al.* (2011) concluded that TMA-N could not be used as a spoilage indicator for their work. Usually, TMA-N is produced in much higher amounts when fish is stored under low oxygen conditions due to domination of primary *Ph. phosphoreum* and secondary *Sh. putrefaciens* (based on CO_2 concentration), which can use TMAO in place of oxygen, as final electron acceptor in respiratory metabolism (Boskou and Debevere, 1997; Sivertsvik, 2007). Gökodlu *et al*. (1998) demonstrated for *Sardina pilchardus* kept at +4 °C, that TMA-N levels at day 0 and 10 were 1.45 and 10.1 mg TMA-N 100 g^{-1} muscle, respectively.

Ocaňo-Higuera *et al*. (2011) in their analysis of filleted ray fish (*Dasyatis brevis*) stored in ice reported that TMA-N content at day 0 was 2.90 ± 0.04 mg TMA-N 100 g^{-1} of muscle whereas 5.0 ± 1.47 mg of TMA-N was detected on day 18; their results agreeing with those in the literature (Ocaňo-Higuera *et al*., 2009). Initial values were in excess of 0.62 ±0.07 mg of TMA-N 100 g^{-1} above that reported by Ocaňo-Higuera *et al*. (2009) for another elasmobranch, i.e. cazon (*Mustelus lunulatus*) at the onset of storage, whereas final values were similar to those demonstrated by Barnett *et al*. (1991) for pink salmon, of 4.5 mg TMA-N at 15 days of storage at 2 °C. The later values were higher than those reported by Pacheco-Aguilar *et al*. (2000) who reported for the winter Monterey sardine muscle (*Sardinops sagax caerulea*), initial values of 0.818 ± 0.33 mg 100 g^{-1} at 0 °C whereas at the end of storage (day 15) a value of 1.62 mg of TMA-N 100 g^{-1} of the muscle was reached. The results of Ocaňo-Higuera *et al*. (2011) revealed that the ray fish (*Dasyatis brevis*) muscle maintained an edible quality during at least 15 days of storage whereas the TMA-N level on the day 18 was similar to rejection limits cited in the literature (Huss, 1988). These workers went further to suggest that microbial activity was not responsible for the initial value of TMA-N.

1.7. Lactic acid bacteria (LABs)

LABs comprise a large group of non sporulating Gram positive, catalase and oxidase negative rods and cocci that ferment carbohydrate to form mainly lactic acid. They are anaero/aerotolerant and commonly have complex nutritional requirements especially for amino acids and vitamins. They also have low proportions of guanine-cytosine G+C in their DNA bases (this refers to the fraction of nitrogenous bases on DNA fragment known as guanine or cytosine (< 55%) (Françoise *et al*., 2010). LABs comprise the genera *Lactococcus, Streptococcus, Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Carnobacterium, Aerococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella* (Ghanbari *et al*., 2010). LABs show attractive physiological properties and technological applications such as proteolytic activity, lactose and citrate fermentation, production of polysaccharides, high resistance to freezing and lyophilization, capacity for adhesion and colonization of the digestive mucosa, and production of antimicrobial substances (Ananou *et al*., 2007).

LABs are widely distributed in nature and used in a variety of fermentation processes (Ennahar *et al*., 1999; Ishizaki *et al*., 2001; Nakayama *et al*., 2007). They are commonly used in the food industry and mainly contribute to the safety, stability, flavour and structure of products. LABs are beneficial in the food biopreservation and medical fields because of the production of antimicrobial compounds such as acetic acid, phenyl lactic acid, cyclic dipeptides, 3-hydroxy fatty acids, peptides and bacteriocin (Atanassova *et al*. 2003; Broberg *et al*., 2007). It has been discovered that many strains of LABs in fermented foods and starter cultures produce bacteriocins, ribosomally synthesized antimicrobial peptides with inhibitory activity against bacteria closely related or similar bacterial strain(s) and against foodborne spoilage and pathogenic bacteria (Sawa *et al*., 2009; Masuda *et al*., 2012; Martinez *et al*., 2013b).

Various strains of LABs are considered to be probiotics. Probiotics may be defined as a product containing viable distinct microorganisms in sufficient numbers to change the microflora (by implantation or colonization) in an area of the host and that exert beneficial health effects in the host (De Vuyst *et al*., 2004; Nithya *et al*., 2012). By this definition, LABs can be considered as effective probiotics (Reid, 1999; Vázquez *et al*., 2005). The properties of LABs include the ability to (1) adherence to cells; (2) exclude or reduce pathogenic adherence; (3) compete for essential nutrients; (4) stimulate the immunity of the host; (5) persist and multiply; (6) produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; (7) resist vaginal microbicides, including spermicides (for terrestrial animals); (8) be safe and therefore noninvasive, noncarcinogenic, and nonpathogenic; (9) coaggregate and form a normal, balanced flora. Species belonging to the genera *Lactobacillus* and *Bifidobacterium,* which have been isolated from the human gastrointestinal tract have been used as probiotics for man. Nevertheless, strains belonging to species of the other LABs have been used previously as probiotics as well, such as *Enterococcus faecium, Enterococcus faecalis, Streptococcus thermophilus, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteriodes* and *Pediococcus acidilactici* (Moreno *et al*., 2006).

1.7.1. Characteristics of LAB bacteriocins

Bacteriocins are antimicrobial peptides produced by LABs and other bacteria for the purpose of killing other bacteria. The production of bacteriocins provides a competitive advantage in their environment by eliminating competitors to gain resources. Lactic acid bacteria producing bacteriocins (*Lactococcus lactis*) are generally regarded as safe substances (GRAS), are not toxic to eukaryotic cells and are post translationally modified (Cotter *et al*., 2005; Drider e*t al*., 2006; Elayaraja *et al*., 2014). The nature of bacteriocins enables them to be inactivated by proteases in the gastrointestinal tract.

Some of the LAB bacteriocins are thermostable cationic molecules having up to 60 amino acid residues and hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding, forming of pores and killing of the cells after causing lethal damage and autolysin activation to digest the cellular wall (Gálvez *et al*., 1990; Abee, 1995). Some studies have shown that *Lactobacillus fermentum* UN01 produced by *Lactobacillus fermentum* was antagonistic against *E. coli, Salmonella enterica* serovar Typhi, *Bacillus cereus, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Udhayashree *et al*., 2012). Meanwhile bacteriocins carnobacteriocin X and Carnolysins A1 and A2, produced by *Carnobacterium maltaromaticum* C2 isolated from smoked fish had activity against *L. monocytogenes, L. innocua, Lactobacillus sakei* and non-bacteriocin producing *C. maltaromaticum* A9b bac^{-} is the phenotype of bacteriocin producing strain *C. maltaromaticum* A9b bac^{+} (Tulini *et al*., 2014).

Bacteriocin production always takes place during late log phase or early stationary phase, and is commonly influenced by a quorum sensing mechanism or by any sign of stress (Martinez *et al*., 2013a). The LAB bacteriocins exhibit some interesting attributes that make them appropriate candidates for use as food preservatives such as:

- Proteinaceous nature, inactivation by proteolytic enzymes of gastrointestinal tract,
- Non-toxic to laboratory animals tested and generally non-immunogenic,
- Inactive against eukaryotic cells,
- Generally thermoresistant (can maintain antimicrobial activity after pasteurization and sterilization),

- Broad bactericidal activity affecting most of the Gram-positive bacteria and some, Gram-negative bacteria including various pathogens, such as *L. monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella* spp.
- Ability of the bacteriocin producing strain to grow at low temperatures (Sarika *et al*., 2012) reported the growth of LAB strains at 4 °C.

With the above-mentioned reasons, the use of bacteriocins in recent years has drawn significant attention to their use as biopreservatives in food, which has resulted in an increasing potential for these peptides. Without doubt, the most widely studied bacteriocin is nisin, which has gained extensive applications in the food industry. This American Food and Drug Administration (FDA) approved bacteriocin is produced by the GRAS microorganism *Lactococcus lactis* and is used as a food additive in at least 48 countries, especially in processed cheese, dairy products and canned foods. Nisin is active against food-borne pathogens such as *L. monocytogenes* and many other Gram-positive spoilage microorganisms (Delves-Broughton *et al*., 1996; Turtell and Delves-Broughton, 1998; Thomas and Delves-Broughton, 2001; Collins *et al*., 2010).

Pediocin produced from strains of *Pediococcus acidilactici*, *Pediococcus parvulus* and *L. plantarum* WHE92, has been used as a food preservative (Enan *et al.,* 1996; Wang and Wang, 2014). Quest International, Sarasota, Florida has produced a commercial pediocin – ALTA 2341® from *P. acidilactici* that is used as a medium component for fermentation processes (Papagianni and Anastasiadou, 2009). As ALTA 2341® presented high inhibitory action on *L. monocytogenes*, the producer has applied for its approval by FDA (Chen *et al*., 2004).

Nevertheless, some aspects have to be taken into consideration when using the bacteriocin producing strain.

In seafood products, the production of histamine must be checked as it is regulated in fish rich in histidine for the allergic problems it can cause (Leroi, 2010).

The presence of virulent factors and the possibility of multiple antibiotic resistance have to be ruled out before its use as biopreservative (Campos *et al*., 2013).

Some *Enterococcus* strain has been associated with virulence (Khan *et al*., 2010).

Safety of using bacteriocin in foods (Javed, 2009).

1.7.2. Classification of bacteriocins

Notwithstanding the differences in spectrum of activity and in biochemical and genetic determinants of bacteriocins from LABs, some distinctive qualities allow them to be divided into four classes, based on primary structure, molecular weight, heat stability and molecular organization (Cotter *et al*., 2005; Heng *et al*., 2007a).

Class I comprises the lantibiotics (lanthionine-containing peptides with antibiotic activity). They are small peptides differentiated from other bacteriocins by their content in dehydroamino acids and thioether amino acids (Ananou *et al*., 2007). Class I (lantibiotics): consists of linear (type A) and globular (type B) peptides, with low molecular weight (< 5 kDa, with approximately 19 to 38 amino acids). They are post-translationally modified peptides that contain unusual amino acids such as lanthionine and derivatives (Drider *et al*., 2006; Todorov, 2009). Nisin is the first and most popularly known lantibiotic (Ghrairi *et al*., 2012). Other lantibiotics include lacticin 481 of *L. lactis* (Piard *et al*., 1992), cytolysin of *E. faecalis* (Gilmore, 1990) and lacticin 3147 of *L. lactis* (Ryan *et al*., 1999), among others.

Class II composed of (< 10 kDa, with approximately 37 to 48 amino acids) thermostable non-lantibiotic linear peptides, which according to Ananou *et al*. (2007) are divided into three subclasses on the basis of distinctive N-terminal sequence. Class IIa the pediocin-like bacterioicins, this subclass has become one of the most interesting

groups for use in food preservation (inhibiting the growth of gram-positive food spoilage and pathogenic bacteria such as *Bacillus cereus, Clostridium perfringens, Staphylococcus aureus* and *L. monocytogenes*) as well as in medicine (as antibiotic complements in treating infectious diseases or as antiviral agents) (Drider *et al*., 2006). Class IIb are bacteriocins requiring the union of two peptides to completely exert antibacterial effect e.g., lactocin G. By definition, their antibacterial activity depends on the action of two different peptides, where their cationic nature is important, facilitating the initial contact between bacteriocins and the negatively charged membranes of target organisms via electrostatic interactions (Diep *et al*., 2009). Class IIc are bacteriocins having a covalent bond between the C and N terminal, resulting in a cyclic structure (Blaciunas *et al*., 2013). Examples of the three subclasses include pediocin PA-1/AcH produced by Pediococci (Henderson *et al*., 1992a), enterocin EJ97 by *E. faecalis* (Sanchez-Hidalgo *et al*., 2003) and enterocin L50A by *E. faecalis* (Cintas *et al*., 2000), respectively.

Class III includes the large (> 30 kDa) heat-labile bacteriocins that are not well characterized (Ananou, 2007). Examples are helveticin J, acidophilicin A and lactacins A and B (Heng *et al*., 2007b).

Class IV are composed of complex bacteriocins that contain carbohydrate or lipid moieties, in addition to the protein portion (Heng *et al*., 2007b). Also they are circular antibacterial peptides, an intriguing and novel type of antimicrobial substance produced not only by bacteria but also by plants and mammalian cells (Ananou, *et al*., 2007). Example is enterocin AS-48 (Maqueda *et al*., 2004). Nevertheless, Cleveland *et al*. (2001) proposed that these complexes are by products of partial purification and not a new class of bacteriocins.

1.7.3. Biosynthesis and assay of class II bacteriocins

Drider *et al.* (2006) reported that there are four genes needed for the production and secretion of bacteriocins. In particular, they are (i) the structural bacteriocin gene, encoding a prebacteriocin or antimicrobial peptides (AMP) precursor (ii) an immunity gene encoding the immunity protein that that protects the bacteriocin producer from being killed by its own bacteriocin (iii) a gene encoding a membrane-associated ATP-binding cassette transporters (ABC transporter) that transfers the bacteriocin across the membrane occurring along with the removal of leader peptide sequence (iv) a gene encoding an accessory protein of unknown function, although it appears to be necessary for the secretion of bacteriocin. According to Bauer *et al*. (2005), genes encoding bacteriocin production are usually located on plasmids. Tagg *et al*. (1976) suggested that genes coding for bacteriocin produced by different LABs are plasmid linked and the Bac+ phenotype can be eliminated by using standard technique for plasmid curing.

The majority of class II bacteriocins are synthesized in the form of a pre-peptide or a biologically inactive pre-bacteriocin, which carries an N-terminal leader peptide attached to the C-terminal propeptide. This compound or the precusor contains a sequence from 18 to 27 amino acids most often of double glycine at the N-terminus. This sequence keeps the bacteriocin inactive until it has been secreted from the cell and serves as a recognition signal for the transport system involving the ABC transporter proteins and accessory proteins (Savadago *et al*., 2006; Kanmani *et al*., 2013). The double glycines present in the sequence are responsible for recognition by the pre-bacteriocin transport system (Moll *et al*., 1999). After recognition the pre-peptide, the leader amino acid sequence of bacteriocin is removed and then the active peptide/bacteriocin is secreted into the extracellular medium (Ehrmann *et al*., 2000).

1.7.4. Mode of action of bacteriocins

Considering the mode of action, various mechanisms have been put forward for bacteriocins. Such mechanisms are directly dependent on factors related to bacterial species and their growth conditions, bacteriocin dose used and purification degree (Parada *et al*., 2007). Normally, pore formation, which leads to change of the cytoplasm membrane potential due to the hydrogen ion exchange between the inner and outer membrane surfaces, is the main mechanism by which most of the bacteriocins from LABs exert their antilisterial effect (Ghrairi *et al*., 2012). In the class II, this mechanism is activated when bacteriocin binds to a protein-receptor on the cell membrane of the target bacteria (Da Silva Sabo *et al*., 2014). As it is with most membrane permeabilizing bacteriocins, the class II bacteriocins are cationic and partly amphiphilic and or hydrophobic. Their positive charges presumably facilitate interactions with the negatively charged bacterial phospholipid-containing membranes and/or acidic bacterial cell walls, whereas their amphipilic/hydrophobic character enables membrane-permeabilization (Fimland *et al*., 2005).

1.7.5. Bacteriocin production and Quorum sensing

Understanding the relationship between cell growth and bacteriocin production is important for effective application and maximum production of bacteriocin (Settanni *et al*., 2008; Arauza *et al*., 2009). During the growth conditions of *Lactococcus lactis* subsp. *lactis* ST1, the exponential phase commenced after 2 h and the maximum biomass was realised after about 14 h of incubation, which remained constant up to the end of incubation (Taheri *et al*., 2012). They reported that the bacteriocin production of *Lactococcus lactis* subsp. *lactis* ST1 commenced immediately the cells entered the exponential phase. They reported that the bacteriocin activity increased rapidly and the maximum activity was reached within 12 to 14 h of incubation.

Quorum sensing (QS) is defined as a bacterial cell–cell message device that controls gene expression by means of secreted signalling molecules. Quorum sensing at least partially controls most important roles such as virulence, competence, conjugation or sporulation. In Gram-positive bacteria, the signalling molecules are regularly small secreted peptides (bacterioicins) that are vigorously released into the extracellular environment. When they reach a threshold concentration, they accrue and are detected by a sensor protein. Their recognition leads bacterial cells to co-ordinate gene expression at the population level (Monnet and Gardan, 2015).

1.7.6. Factors affecting effectiveness of bacteriocins in food systems

It known that the production and activity of bacteriocins in foods can be influenced by the physical and chemical conditions of foods. These are factors that can interfere on the bacteriocin production by LABs, such as unsuitable process conditions (pH, temperature, nutrients, among others), spontaneous cell loss on producing bacteriocin, infection of the cell by phage and presence of competitive microorganisms in the medium (Schillinger *et al*., 1996). In addition, the effectiveness of bacteriocin would also be influenced by the presence of bacteriocin-resistant microorganisms, enzymes (like proteases), occurrence of oxidation-reduction reactions, interactions with component of food formula (fats, proteins, preservatives, pH, for example), diffusion restraints due to high salt concentration, inactivation by other additives. However, it can be affected by the presence of nitrate and nitrite and low water activity (the unbound water to the food molecule), which may result to inadequate distribution of bacteriocin throughout the food matrix (Schillinger *et al*., 1996; Alves *et al*., 2006). Reports from Kristo *et al*. (2008) revealed that bacteriocin may present a higher effectiveness when added into films and not directly incorporated to the product. In fact, the production of

bacteriocin by *Lactobacillus plantarum* was higher in cellulose derivative films when compared with protein films (Sánchez-Gonźlez *et al*., 2013).

1.8. Purification of bacteriocins

Since bacteriocins are secreted into the culture medium, most patterns of purification start with a step to concentrate the bacteriocins from the culture supernatant, using for instance diatomite calcium silicate (Coventry *et al*., 1996) or ammonium sulphate precipitation (Yang *et al*., 1992). In fact these procedures are used mainly to concentrate the working volume but they do not yield a high degree of purification (Guyonnet, 2000). Consequently, the following steps by using preparative isoelectric focusing and/or multiple chromatographic separations, including cation exchange, gel filtration, hydrophobic interaction and reverse-phase liquid chromatography are indispensable to achieving significant purification of bacteriocins (Parada *et al*., 2007). Usually, but not always, the yields obtained are low. This is probably due to the high number of steps in the protocol, resulting in time consuming processes (Parada *et al*., 2007). An ideal protocol for bacteriocin production should be one that is relevant to large-scale purification, resulting to bacteriocin yields higher than 50% and purity around 90% (Schöbitz *et al*., 2006).

In general, for the non-lantibiotic bacteriocins, the procedures involve growth in a suitable liquid nutrient medium under most efficient states for bacteriocin production, removal of the cells followed by fractionated precipitation of the protein culture supernatant by addition of ammonium sulphate. The precipitated proteins are subsequently dissolved in deionized water or a weak buffer, and bacteriocin molecules are separated by use of various methods including hydrophobic, ion-exchange, size exclusion chromatography. Although these techniques have made easy production of

highly purified bacteriocin preparations the final yield has generally been below 20% (Jack *et al*., 1995).

Bacteriocins produced by many *Lactobacillus* species have been purified using ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic interaction and reverse-phase HPLC (Parada *et al*., 2007). Purification of a bacteriocin produced by *Lactobacillus acidophilus* 30SC was partially purified by ammonium sulphate precipitation and hydrophobic interaction chromatography using octyl-sepharose CL-4B (Oh *et al*., 2000). A bacteriocin-like substance produced by *Carnobacterium piscicola* L103 was partially purified by ammonium sulphate precipitation and gel filtration on Sephadex G-25, followed by lyophilization, for the purpose of activity test against *L. monocytogenes* in vacuum-packaged meat (Schöbitz *et al*., 1999).

1.9. Application of LABs, bacteriocins and effect on pathogen control in seafood

LABs of different genera, such as *Lactococcus, Enterococcus* and *Carnobacterium,* have been used as protective cultures. The effectiveness of listerial inhibition is sustained mainly on the adequate selection of: (i) bacteriocin or the bacteriocinogenic strain, in the case of *in situ* production, (ii) the form of application, (iii) the interaction with food components and/ or other conventional factors (Campos *et al*., 2013).

The comparison of the activity of different bacteriocins or their producer strains have been studied by various workers. Tomé *et al*. (2008) demonstrated the ability of five bacteriocin producing strains *Enterococcus faecium* ET *ET105, Lactococcus curvatus ET106, Lactococcus curvatus ET30, Lactobacillus delbreucki ET32* and *Pediococcus acidilactici ET34,* to grow and inhibit of *Listeria* spp. in the conditions applied for salmon processing. The bacteriocin producing strains were added to salmon fillets by immersion and subjected to cold smoking process. Results obtained from the analysis

revealed that ET105 was the best biopreservative for controlling *Listeria* spp. whereas ET106 and ET34 exerted a bacteriostatic action. *Enterococcus faecium* ET *ET105* in the study revealed the maximum decrease of *Listeria innocua* 2030c numbers at all sampling times. In a study by Hisar *et al*. (2005) growth of *L. monocytogenes* was significantly inhibited ($P < 0.05$) by *Lactobacillus sakei* Lb706 in rainbow trout fillets stored under vacuum at 4 °C during 10 days of storage while bacteriocin negative Lb706-B did not affect the growth of *L. monocytogenes*. In the presence of the sakacin A-producing strain of *L. sakei* (Lb706), the growth of *L. monocytogenes* was significantly reduced ($P < 0.05$) in the first three days of storage at 10 °C, after which its counts increased to 10^7 CFU g^{-1}. Inhibition of *Pseudomonas* spp. and *Photobacterium phosphoreum* in vacuum packed fresh plaice fillets determined by Altieri *et al*. (2005) at low temperatures by using a *Bifidobacterium bifidum* as starter culture and extending the shelf-life, was successful especially under modified atmosphere packed (MAP).

The efficacy of bacteriocins to control the growth of *L. monocytogenes* in vacuum packed cold-smoked salmon (CSS) has been demonstrated by several researchers. Among them, Duffes *et al*. (1999) isolated *Carnobacterium divergens* and *Carnobacterium maltaromaticum* strains that exhibited listericidal activity in a model experiment with cold-smoked fish. Their work revealed that *Carnobacterium piscicola* V1 inhibited *L. monocytogenes* by the *in situ* production of bacteriocin in vacuum-packed stored at 4 °C and 8 °C. In contrast, another related species, namely, *Carnobacterium divergens* and its divergicin V41 only showed a bacteriostatic effect on the target microorganism. Two strains of *Carnobacterium maltaromaticum* isolated from CSS determined their efficiency to limit the growth of *L. monocytogenes* in vacuum packed CSS during 31 days at 5 °C (Nilsson *et al*., 1999).

The influence of the form of bacteriocin application and its effectiveness has also been determined in several studies. Ghalfi *et al*. (2006) analyzed different strategies for the addition of the bacteriocin producing strain *Lactobacillus curvatus* CWBI-B28: *in situ* production, spraying with partially purified bacteriocin, packaging in bacteriocin coated plastic film and immobilization onto the producer cell. All the approaches were useful for the inactivation, but the efficacy was different. Samples treated with either partially purified or *in situ* bacteriocin production resulted in reduction of *Listeria* spp. counts 0.7 log CFU cm^{-2} within the first week but one log cycle (one log cycle is the time and temperature to reduce the number of the target microorganism by 90%) increase was observed after 14 days. The bioactive packaging yielded a slower inactivation of the pathogen but prohibited any ensuing increase in the CFU throughout 22 days of storage at 4°C. The most effective treatment was immobilization of the bacteriocin onto the producer cell since a complete inactivation was ascertained within 3 days at 4 °C and no increase in *Listeria* growth was observed up to 22 days of storage. The adsorption of the bacteriocin to the producing strain (the activity of unbound bacteriocin in the supernatant being determined by the critical dilution method) does not need premarket approval since it can be regarded as a conditioning method of lactic acid bacteria which has the status of generally regarded as safe. Tahiri *et al*. (2009) likewise studied different application strategies they used *Carnobacterium divergens*, the producing strain of the bacteriocin divergicin M35, purified divergicin, and culture supernatants of *Carnobacterium divergens* for *Listeria* spp. inhibition in CSS. Different patterns of inhibition were obtained depending on whether the producer strain, the purified bacteriocin or the culture supernatants were used. Application of bacteriocin producing strain progressed, slower but more pronounced reduction of *Listeria* spp. counts than the one revealed for the purified bacteriocin or the culture supernatant. The later

produced quicker and continuous inactivation than divergicin M35. This trend is associated with the possible degradation of divergicin M35 by endogenous proteases or by molecular interaction with food matrix. Nevertheless, in other studies it has been revealed that purified form was more effective than the producer strain (Vaz-Velho *et al*., 2005; Calo-Mata *et al*., 2008).

1.10. Aims of the study

The aims of this study were to investigate the presence of *L. monocytogenes* in temperate seafood, namely fresh and smoked salmon, fresh and smoked haddock, and fresh mussels and oysters. Additionally, the work sought to recover, characterise and use bacteriocin-like-substance to control *L. monocytogenes* populations in cold smoked haddock.

Chapter 2. Isolation, identification and characterisation of microorganisms from seafood

2.1. Introduction

2.1.1. Potential health benefits and risks involving the consumption of seafood

Fishery products are significant for human nutrition, worldwide (Kromhout *et al*., 1985; Darlington and Stone, 2001; Iwamoto *et al*., 2010), but may also serve as a source of foodborne pathogens (Herrera *et al*., 2006). The consumption of long chain n-3 polyunsaturated fatty acids (LCn3-PUFA) in fish has been connected with good health (Marckmann and Grønbæk, 1999; Ruxton *et al*., 2007). In particular, coronary heart disease is influenced by the consumption of a diet containing sufficient levels of Omega-3 PUFAs (Gibson, 1988). The Omega-3 PUFAs reduce plasma cholesterol, very low density lipoproteins (VLDL) plus low density lipoproteins (LDL) and elevated triglycerides (Gibson, 1988; Schmidt, 1997). Omega-3 PUFAs have the ability to slightly increase the plasma high density lipoproteins (HDL) (Schmidt, 1997). Scientific findings have shown that the consumption of fish containing Omega-3 PUFAs decreases mild hypertension (Toft *et al*., 1995), prevents certain cardiac arrhythmias (Nair *et al*., 1996), lowers the incidence of diabetes (Malasanos and Stacopoole, 1991; Rustan *et al*., 1997), and helps in the neurological development and physiological functions of the brain (Burdge, 1998). Added nutritional advantages associated with fish intake are due to the presence of proteins of high biological value, unsaturated essential fatty acids, minerals (e.g. calcium, iron, selenium and zinc) and vitamins, namely A, B3 (nicotinamide), B6 (pyridoxine), B12 (cobalamine), E (d-tocopherol) and D (Kirpal, 2003). Nevertheless, together with the nutrients and advantages obtained from eating seafood come the risks associated with contamination.

Chemicals, metals, marine toxins, and infectious agents have been detected at various times in seafood. Infective agents related with food-borne illness include bacteria, viruses, and parasites, and the illnesses caused by these agents vary from mild gastroenteritis to life-threatening diseases (Sobel and Painter, 2005; Ayers *et al*., 2008). Seafood accounts for a significant ratio of food-borne illness and outbreaks specifically in the USA but also worldwide. Seafood serves as a significant route for marine toxins and chemical contamination (Sobel and Painter**,** 2005; Ayers *et al*., 2008). These contaminants include the presence of carcinogenic (e.g., DDT, dieldrin, heptachlor, PCBs and dioxins) and non-carcinogenic (e.g., methyl mercury) environmental contaminants in fish tissues (Kirpal, 2003).

Seafood comprises molluscs (*e.g*. oysters, clams, and mussels), finfish (*e.g*. salmon and tuna), marine mammals (*e.g*. seal and whale), fish eggs (roe), and crustaceans (*e.g*. shrimp, crab and lobster) (Iwamoto *et al*., 2010). Contamination of fish tissues with microorganisms may reflect the presence of pollutants in the aquatic environment (Adeyemo, 2003). Therefore, the microbial flora associated with fish is a reflection of the nature of environment from which they are harvested. The contamination of fish by pathogenic bacteria poses a risk to human health (Goja, 2013). Specifically, the consumption of raw or undercooked seafood is especially problematic to human health. Filter feeders, such as mussels, and oysters, are particularly troublesome in terms of microbial contamination insofar as the animals collect bacteria in their filtering systems. Thus, they entrap pathogenic and non-pathogenic bacteria and viruses that occur in the aquatic environment (Huss, 1997; Popovic *et al*., 2010).

2.1.2. Aquatic micro-organisms occurring in or on seafood

Many studies of the bacterial flora from the surface of freshwater fish have been reported, and list the presence of *Acinetobacter johnsonii* (Gonzalez *et al.,* 2001),

aeromonads (notably *Aeromonas hydrophila, A. bestiarum, A. caviae, A. jandaei, A. schubertii,* and *A. veronii* biovar sobria (Gonzalez *et al*., 2000b), *Alcaligenes piechaudii, Enterobacter aerogenes, Escherichia coli, Flavobacterium* (Zmyslowska *et al*., 2001), *Flexibacter* spp., *Micrococcus luteus, Moraxella* spp., *Pseudomonas fluorescens*, psychrobacters (Gonzalez *et al.,* 2001) and *Vibrio fluvialis* (Allen *et al.,* 1983; Diler *et al*., 2000). The following organisms have been recovered from the surface of marine fish:

Acinetobacter calcoaceticus, Alcaligenes faecalis, Bacillus cereus, B. firmus, coryneforms, *Cytophaga/Flexibacter, Escherichia coli, Photobacterium angustum, Ph. logei, Pseudomonas fluorescens, Ps. marina*, and *Vibrio* spp. (including *V. albensis, V. anguillarum, V. harveyi, V. splendidus* biotype I, *V. fischeri, V. ordalii*, and *V. scophthalmi* (Austin, 1982; Austin, 1983; Montes *et al*., 1999). In addition, the result from a survey of samples of fresh marine fish, *i.e*. conger, swordfish, sole, grouper, and whiting over a period of 5 months, revealed the presence of pathogenic microorganisms such as *V. parahaemolyticus, Aeromonas* spp., *Listeria monocytogenes* and *Clostridium perfringens* (Herrera *et al*., 2006). In some situations, the contamination of seafood by pathogenic bacteria, such as *E. coli, Salmonella* spp. and *Shigella* spp., is a result of the presence of animal or human faeces being introduced into water bodies (Goja, 2013). Outbreaks of seafood-associated illness linked to polluted waters have been caused by calicivirus, hepatitis A virus, and *Salmonella enterica* serotype Typhi (Portnoy *et al*., 1975; Morse *et al*., 1986; Desenclos *et al.,* 1991; Tauxe and Pavia 1998). Seafood may be contaminated from various sources, which includes overboard sewage discharge into harvest areas, illegal harvesting from sewage contaminated waters and sewage runoff from points inland after heavy rains or flooding (Iwamoto *et al*., 2010). In addition, seafood quality is associated with bacterial load, which depends on the state of

transport, handling and processing. After harvesting, the storage of seafood at inappropriate temperatures, washing of seafood with water contaminated with pathogenic and/or spoilage organisms, use of contaminated ice for preserving of seafood and contamination by infected food-handlers are all contributory factors to quality deterioration (Jannat *et al*., 2007).

2.1.3. Microbial flora found in cold smoked salmon, fresh salmon, fresh haddock, mussels and oysters

The microbial spoilage flora in LPFP is not well characterized, nevertheless some studies have revealed that the microflora prominent in this type of product include lactobacilli (LABs; Hansen *et al*., 1998), frequently involve Gram negative fermentative bacteria *Photobacterium phosphoreum* and psychrotrophic *Enterobacteriaceae* (Gram and Dalsgaard, 2002). *Brochotrix thermosphacta*, *Aeromonas* spp., and, in some cases, moulds and yeast (Leroi *et al*., 1998) have been recovered from spoiled CSS, representing a comparatively complicated and variable microflora in this type of product. Concerning CSS from Norway, the Faroe Islands and Chile, Hansen and Huss (1998) found that *Lactobacillus curvatus* dominated among LABs, while *Lactobacillus sakei*, *Lactobacillus plantarum*, *Carnobacterium* spp., and *Leuconostoc* spp. existed in smaller numbers. Leroi *et al*. (1998) discovered that *Carnobacterium maltaromaticum* (Mora *et al*., 2003) dominated the LABs in Norwegian farmed salmon smoked in France.

Marine *Vibrio* spp., as well as *Photobacterium phosphoreum*, dominated in the bacterial flora of emerging spoilage conditions in normal dry-salted salmon, while a mixture of LABs and *Enterobacteriaceae* were prominent in the injection brined salmon (Hansen *et al*., 1996). The occurrence of *Listeria monocytogenes* in retail CSS is highly variable.

Earlier reviews report differences from 0 to 100%, but studies prior to year 2000 revealed occurrence between 15 and 40% (Ben Embarek, 1994; Gram, 2001).

The following bacteria are related with health hazards in CSS; i) *Aeromonas hydrophila* (Hudson and Mott, 1993). The occurrence (proportion) of *Aeromonas* spp. in retail and RTE salmon products in Europe is 0.13% (Tusevljak *et al*., 2012). ii) Pathogenic *Vibrio* spp. and *Plesiomonas shigelloides*; typical mesophilic and found only in warm environments and tropical areas (Huss *et al*., 1995). iii) Exogenous bacteria such as *Salmonella* spp., pathogenic *Escherichia coli* (i.e. O157:H7), and *Staphylococcus aureus*; are not naturally found in raw fish material, but could be contaminants during processing as a consequence of poor hygiene or water supply (Friesema *et al*., 2014).

Macé *et al*. (2012), characterise the spoilage related to microbiota of raw salmon using 3 raw salmon batches stored for 3 days at chilled temperature in modified atmosphere packaging (MAP) (50% CO2/50% N2) or under vacuum. Thirteen different genera or species were identified by phenotypic and molecular tests: *Serratia* spp., *Photobacterium phosphoreum*, *Yersinia intermedia*, *Hafnia alvei*, *Buttiauxella gaviniae*, *Pseudomonas* sp., *Carnobacterium maltaromaticum*, *Carnobacterium divergens*, *Lactococcus piscium*, *Lactobacillus fuchuensis*, *Vagococcus carniphilus*, *Leuconostoc gasicomitatum* and *Brochothrix thermosphacta.* Nespolo *et al*. (2012) reported the presence of *Staphylococcus aureus* in one sample out of 31 (16 cooled and 15 frozen) samples of salmon collected in the retail market network of a few cities in the State of São Paulo, Brazil.

Olafsdottir *et al*. (2006) recovered *Photobacterium phosphoreum*, *Pseudomonas* spp. and H_2S-producing bacteria and *Shewanella putrefaciens* from haddock fillets as they analysed for proliferation of specific spoilage organisms (SSO) and quality changes

were evaluated in haddock fillets stored in Styrofoam boxes at 0, 7 and 15 °C and under temperature fluctuations.

Mus *et al.* (2014) reported the presence of 28 *V. parahaemolyticus* isolates, 25 were found in fish samples and three in mussels from seafood samples purchased from retail outlets in Bursa city (Turkey). DePaola *et al*. (2010) revealed the presence of *V. parahaemolyticus* and *V. vulnificus* levels distributed seasonally and geographically by harvest region from two samples of market oysters, mainly from retail institutions, collected twice each month in each of nine states during 2007. Furthermore they reported that pathogens commonly related with faecal pollution were discovered periodically or not at all (toxigenic *Vibrio cholerae*, 0%; *Salmonella* spp., 1.5%; norovirus, 3.9%; hepatitis A virus, 4.4%).

2.1.4. Microbial spoilage of fish and fishery products

Fish and fishery products are some of the most significant protein sources in human nutrition (Gram, 2009). However, fish products are highly perishable, and rapidly deteriorate leading to difficulties with preservation (Musa *et al*., 2010; Dewi *et al*., 2011). Declining fish quality is attributed to a complex series of actions in which physical, chemical and microbiological changes occur (Sallam, 2007b). Spoilage of fresh and lightly preserved fish is mostly caused by microbial action together with their metabolites (Gram and Huss, 1996; Gram, 2009). Microbial spoilage includes growth by bacteria, yeast or fungi to high densities with the resulting production of metabolites giving rise to sensory changes perceived as spoilage (Lyhs *et al*., 2004). There are two significant characteristics associated with bacteria that are capable of causing spoilage. Foremost, the bacteria are often psychrotrophic and so increase in number at refrigeration temperatures. In addition, micro-organisms attack different substances in the fish tissue to produce compounds related to off-flavours and off-odours

(Aberoumand, 2010a). Generally, the off-flavours and off-odours originate from the decomposition of low molecular weight compounds by micro-organisms, and include ammonia from the deamination amino acids, sulphides formed from sulphur-containing amino acids (Herbert and Shewan, 1975), TMA resulting from bacterial reduction of TMAO, and esters that may arise from degradation of phospholipids (Gram, 2009). Bacteria are found on the gill, skin and intestines of fish, but generally not in muscle (Aberoumand, 2010a). At death, bacteria penetrate into the flesh/muscle of the fish leading to the production of (bacterial) enzymes that diffuse widely (Aberoumand, 2010a). Different spoilage bacteria are found in freshwater and marine fish, and in temperate and tropical water fish (Doyle, 2007). The specific spoilage microflora of fresh fish and seafood products stored aerobically at refrigeration temperature are mainly psychrotrophic Gram-negative species including *Pseudomonas* spp., *Photobacterium* spp., *Flavobacterium* spp. (Gram and Huss, 2000; Chytiri *et al*., 2004; Matamoros *et al*., 2006), *Aeromonas* spp., *Shewanella* spp., *Acinetobacter* spp. and *Moraxella* spp. (Joffraud *et al*., 2001; Franzetti *et al*., 2003; Cardinal *et al*., 2004). At higher temperatures (15-30 °C), *Vibrionaceae*, *Enterobacteriaceae* and Gram positive bacteria may cause spoilage (Gram and Huss, 1996; Gram and Huss, 2000).

2.1.5. Characteristics of fish spoilage bacteria

Rich microbial substrates or electron acceptors, such as nonprotein nitrogen and amino acids, nucleotides, and TMAO, are found in fish muscle (Gram, 2009). Fish spoilage is a result of the products of microbial metabolites including extracellular polysaccharide (dextran), carbon dioxide, trimethylamine (TMA), ammonia, hydrogen sulphide and dimethyldisulphide (Seibel and Walsh, 2002; Sadok *et al*., 2003). TMAO acts as an osmoregulant in fish (Gram and Dalsgaard, 2002), and has a fishy odour, which is characteristic of spoiling gadoid fish species, i.e. cod, haddock, whiting, and pollock

(Gram, 2009). Various bacterial species have the ability to use TMAO as an electron acceptor in anaerobic respiration and the reduced compound, trimethylamine (TMA), is the most capable of conveying a "fishy" odour (Huss and Larsen, 1980; Yancey, 2005). Bacteria such as *Shewanella putrefaciens, Aeromonas* spp., psychrotolerant *Enterobacteriaceae*, *Photobacterium phosphoreum*, fluorescent pseudomonads and *Vibrio* spp. can obtain energy by reducing TMAO to TMA creating the ammonia-like off-flavours (Gram and Dalsgaard, 2002). *Sh. putrefaciens* and *Vibrionaceae* representatives produce H_2S from the sulphur containing amino-acid L-cysteine (Gram *et al*., 1987; Stenström and Molin, 1990). *Pseudomonas* spp. do not produce H_2S and TMA, but are characterized by fruity, rotten odours and flavours in spoiling ice fish. Some volatile aldehydes, ketones, esters, and sulphides are produced by *Pseudomonas* spp. (Ghaly *et al*., 2010).

Production of many proteolytic and hydrolytic enzymes by *Pseudomonas putrefaciens*, fluorescent pseudomonads and other spoilage bacteria increase rapidly during the initial stages of spoilage (Shewan, 1961). Fish and fishery products are abundant in protein, and proteolytic bacteria are important in the spoilage process (Gram, 2009). Extracellular proteases, which degrade the proteins in muscle, are secreted by the growth of microorganisms on muscle tissue (Venugopal, 1990; Jackson *et al*., 1997).

2.1.6. Reduction of the incidence of foodborne diseases in seafood supply

Seafood safety is very important as it concerns the health of the consumers. Seafood due to their components, environment where they are harvested, transportation, handling and storage processes are prone to bacterial contamination. Several processes have been used by some workers to reduce bacterial contamination in seafood.

Safety concerns for the survival of psychrotrophic pathogens have been expressed for chill-stored products mildly heated in hermetically sealed packages or packed without

recontamination. A heat treatment of 90 °C for 10 min has been recommended for a six-decimal reduction in the numbers of psychrotrophic *Clostridium botulinum* spores (Gould, 1999). Moir and Szabo (1998) reported that a heat treatment of 70 °C for 2 min will completely destroy *Listeria monocytogenes* if it is the only pathogen of concern.

Low numbers of pathogens are normally present in non-heated ready to eat food such as cold smoked salmon. Foods containing 100 or fewer *Listeria monocytogenes* g^{-1} do not constitute a health hazard for individuals who are not immunocompromised as stated by International Commission on Microbial Specification for Foods (ICMSF). Nevertheless, it is very necessary that low level pathogens be considered because of the possible growth and toxin production in the final products (Van Schothorst, 1996**).**

A microbial reduction of 0.7 log CFU cm^{-2} was achieved on tilapia inoculated with *Escherichia coli* when soaked into electrolyzed oxidizing water (EO water) for up to 10 min**.** Treatment using EO water on tilapia specimen inoculated with *Vibrio parahaemolyticus* revealed a reduction of 1.5 log CFU cm^{-2} after 5 min treatment and achieved 2.6 log CFU cm^{-2} reduction after 10 min (Huang *et al*., 2006). They also reported an effective disinfection of the retailer platform using the EO water.

Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and a well-designed HACCP-programme are preventive processes for post-harvest contamination (FAO/WHO, 1997).

Bivalve shellfish are filter feeders, and become contaminated by filtering small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Dunphy, 2006). The old cooking process, which may not be sufficient to ensure consumer's safety, causes the contamination of bivalves to be hazardous. These conditions make them vital vectors of foodborne disease (Lees, 2000). To control the disease risk related with bivalves, therefore, necessitates Hazard

Analysis by Critical Control Point (HACCP) measures along-side with water environment quality management of growing and harvesting areas and post-harvest product processing which might involve depuration and/or heat treatment where suitable (WHO, 2010).

2.2. Aims of this study

The aims of this Chapter were to:

Isolate, identify and characterize *Listeria monocytogenes* from temperate seafood.

To further isolate, identify and characterize some potential pathogens and spoilage microorganisms from the seafood

2.3. Materials and methods

2.3.1. Sample collection

From March to April 2010, seafood samples were obtained from Edinburgh and Stirling. Fish fillets chosen from within the middle of stacks of fillets in chilled display cabinets within fishmongers were collected by use of disposable plastic gloves and packed in sealed aluminium foil packs. Enquiries of the fishmongers confirmed that in all cases the fish had been landed from fishing boats within the previous 24 h, although it was unknown how long the fish had been on the boats prior to landing at the ports. Filleting of the whole fish occurred in the shops within the same day as purchase, and the fillets were immediately displayed in the refrigerated cabinets. Smoked haddock fillets were obtained by the fishmongers from a commercial smoke house in Leith, Edinburgh. The smoked salmon was pre-packed, and was sourced from farmed Atlantic salmon that had been smoked in the Highlands. There was a lack of clarity about the origin of bags of live mussels and oysters, which were obtained by the fishmongers from wholesalers; the general comment was that the animals were sourced from Scotland. There was not any stated shelf life for any of the fresh fish or shellfish purchases.

The dates of collection and samples from Edinburgh were as follows;

March 1, 2010 - cold smoked salmon and fresh salmon;

March 5 2010 - fresh mussels, cold smoked salmon and fresh salmon;

March 22, 2010 - fresh mussels and oysters;

April 15, 2010 - fresh and smoked haddock were collected.

Samples from fishmongers in Stirling were packed in polyethylene wraps, and stated to be fresh with a 24 h use-by date. The samples were collected, as follows:

March 15, 2010 – fresh mussels, cold smoked salmon and fresh salmon

April 12 2010 - fresh mussels, cold smoked salmon and fresh salmon

The samples from Edinburgh were transported to the laboratory within one hour of collection, and were maintained in a cold box at 4ºC. The samples from Stirling were transported at room temperature, and were processed microbiologically within 20 min of collection. In total, 4 samples of cold smoked salmon, 4 samples of fresh salmon, 4 bags of mussels, 1 sample of cold smoked haddock, 1 sample of fresh haddock and 1 bag of oysters were examined.

2.3.2. Experimental samples and bacterial cultures

The samples were analysed for the presence of *Vibrio* spp., *Enterobacteriaceae* representatives, *Listeria monocytogenes* and total aerobic heterotrophic counts.

2.3.3. Vibrios

Ten grams of each of the seafood samples (fresh and smoked Atlantic salmon, fresh and smoked haddock, fresh oyster and mussels) were aseptically placed in sterile stomacher bags (Seward; Medical, London, UK) with 90 ml volumes of 30% (w/v) alkaline peptone water (APW; Oxoid Ltd, Cheshire, England) and homogenised using Stomacher Lab-Blender-80 (Seward; Laboratory, London, UK) for 2 min. Serial dilutions were prepared to 10^{-6} in 30% APW, and 0.1 ml of each dilution were spread over the surface of triplicate plates of thiosulphate citrate bile salts sucrose agar (TCBS; Oxoid) with incubation at 20 °C and 30 °C for 24 h (Normanno *et al.*, 2006). Representative colonies were purified as described by Al-Harbi and Uddin (2005b) and pure cultures stored as described above. The presumptive *Vibrio* species from different samples growing on TCBS plates that were purified were tested for their sensitivity to vibriostatic agent (0/129) (Oxoid). In parallel commercial API 20E (bio-Merieux,

Marcy l'Etoile, France) method was used. Further characterisation and identification were carried out based on Holt (1994), Cowan and Steel (2003) and Buller (2004).

2.3.4. *Enterobacteriaceae* representatives

Ten grams of each of the seafood samples (fresh and smoked Atlantic salmon, fresh and smoked haddock, fresh oyster and mussels) were aseptically placed in sterile stomacher bags (Seward) with 90 ml of 0.5% (w/v) peptone water (PW; Oxoid) and homogenised, as above. Serial dilutions were prepared to 10^{-6} in 0.5% APW, before 0.1 ml amounts were spread over the surface of triplicate plates of eosin methylene blue agar plates (EMB; Oxoid) [for the recovery of *Enterobacteriaceae* representatives] with incubation at 37 °C for 24 h (Ahmad *et al*., 2012). Representative colonies were purified as described by Al-Harbi and Uddin (2005b) and pure cultures stored and further characterised as described above.

2.3.5. Aerobic heterotrophs

Ten grams of each seafood samples (fresh and smoked Atlantic salmon, fresh and smoked haddock, fresh oysters and mussels) were aseptically placed in sterile stomacher bags (Seward) with 90 ml of sterile 0.85% (w/v) saline and homogenised, as described above. Serial dilutions were prepared to 10^{-6} in sterile 0.85% (w/v) saline, and 0.1 ml amounts were spread over the surface of triplicate plates of TSA plates [for the recovery of aerobic heterotrophs] with incubation at 20 °C and 30 °C for 48 h (Sallam, 2007a). Representative colonies were purified (Al-Harbi and Uddin, 2005b), and pure cultures stored and further characterised as described before.

2.3.6. *Listeria* spp.

Ten grams of each of the seafood samples (fresh and smoked Atlantic salmon, fresh and smoked haddock, fresh oyster and mussels) were aseptically placed in sterile stomacher bags (Seward) with 90 ml volumes of *Listeria* primary selective enrichment broth base CM0863 supplemented with SR0142E (UVM I) (Oxoid), homogenised as described above, with incubation at 30 °C for 24 h. One hundred µl volumes of (UVM I) homogenate were transferred to 10 ml volumes of *Listeria* secondary selective enrichment broth base CM0863 supplemented with SR0143E (UVM II) (Oxoid) and incubated at 30 °C for 24 h. Serial dilutions were prepared to 10^{-6} in (UVM II), and 0.1 ml amounts were spread over the surface of triplicate plates of polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar base CM0877 supplemented with SRO150E (PALCAM; Oxoid) [for the recovery of *Listeria monocytogenes*] with incubation at 20 °C and 30 °C for 48 h (McClain and Lee, 1988). Representative colonies were purified (Al-Harbi and Uddin, 2005b) and pure cultures stored and further characterised as described previously.

2.4. Isolation of bacterial cultures

Three to five representatives of each colony type were streaked and re-streaked on tryptone soya agar (TSA; Oxoid) plates supplemented with 1% (w/v) sodium chloride [= TNA] with incubation at 30 °C for 48 h until pure cultures were obtained (Al-Harbi and Uddin, 2005b). Pure bacterial cultures were grown routinely on TNA with incubation at 30 ^{o}C for 48 h. *Carnobacterium maltaromaticum* isolates were grown routinely on de Man Rogosa and Sharpe agar (MRS; Oxoid) at 30 °C for 48 h. Stock cultures were stored in tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) sodium chloride [= TNB] and 20% (v/v) glycerol at -70 °C (Kim *et al*., 2007).

2.5. Identification of bacteria

The cultures were examined for micro-morphology and Gram staining reaction. Biochemical tests were carried out using the methods described by Collins *et al*. (1987), Baya *et al*. (1992a), Gerner Smidt and Frederiksen (1993), Kampfer *et al*. (1993), Holt (1994), Cowan and Steel (2003), Buller (2004), Vogel *et al*. (2005), Bowman (2006), Hammes and Hertel (2006), Kocur *et al*. (2006), Stackebrandt and Jonnes (2006), Austin and Austin (2007), Vaneechoutte *et al*. (2011) and USDA, FSIS, OPHS (2013) for identification to genus and where possible to species level.

2.6. Characterisation of the bacterial isolates

The bacterial cultures were identified using a range of conventional phenotypic tests, by commercial rapid identification systems, and by 16S rDNA gene sequencing. The conventional phenotypic tests were, as follows:

2.6.1. Micro-morphology

Overnight cultures grown for 24 h on TNA were examined for micromorphology using Gram-stained smears (Hucker and Conn, 1923), at a magnification of x 1000 on an Olympus BH-2 light microscope (Japan). The data recorded included cell shape, cell arrangement, presence of endospores and Gram staining reaction.

2.6.2. Colonial morphology

Colony colour, size, shape and texture were recorded, after examining 1-2 day old cultures on TNA.

2.6.3. Catalase production

The bacterial culture was spotted on a drop of 3% (v/v) hydrogen peroxide (Sigma-Aldrich; St Louis, USA) on a glass microscope slide. The development of effervescence within 60 sec was indicative of a positive response.

2.6.4. Oxidative-fermentative test

Oxidative-fermentative (OF) basal medium (Difco; Oxford, England) was supplemented with 1% (w/v) glucose, and used to assess the oxidative or fermentative metabolism of glucose (after Hugh and Leifson, 1953). After incubation for up to 7 days at 20 °C, the presence of a yellow colour (= acid production) in the tube with liquid paraffin was indicative of fermentative metabolism. Conversely, acid production in the open tube only was interpreted as evidence of an oxidative metabolism.

2.6.5. Oxidase production

The method of Kovács (1956) was used. A flamed inoculating wire was used to pick up a bacterial colony, removed from 24 h old culture from TNA plates and streaked onto a piece of Whatman filter paper, soaked with 1% (w/v) oxidase reagent (N, N, N, N-tetramethyl-*p*-phenylenediamine dihydrochloride; (Sigma-Aldrich). A positive reaction was recorded by the development of a purple/blue colour within 30 sec.

2.6.6. Determination of motility

Overnight broth cultures were used to prepare hanging drops, which were examined at x 400 on an Olympus BH-2 light microscope.

2.6.7. Lecithinase and lipase activity

Lecithinase and lipase activities were examined as described (Liu *et al*., 1996). Thus, 48 h cultures were inoculated on to TNA supplemented with 1% (v/v) egg yolk emulsion (Oxoid) or 1% (w/v) Tween 80 (Remel; Lenexa, USA) for the determination of lecithinase and lipase activity, respectively, with incubation at room temperature for 7 days. The development of opalescence around the bacterial growth was indicative of a positive reaction.

2.6.8. Determination of haemolytic activity

Haemolytic activity against sheep defribrinated sheep blood in Alseviers solution (Oxoid) was examined. Thus, blood agar plates were prepared by mixing 25 ml volumes of blood with 500 ml of molten cooled (to 45 °C) blood agar base (Oxoid) supplemented with 1% (w/v) sodium chloride. Forty-eight hour cultures were inoculated onto the plates, and the presence of zones of clearing or opalescence around the bacterial growth following incubation at room temperature for 4 days was recorded as evidence of haemolysis.

2.6.9. Gelatinase activity

Gelatinase activity was recorded after Loghothetis and Austin (1996). Gelatin agar was prepared using the method of Smith and Goodner (1958). Briefly, 3% sodium chloride was added to the recipe and the medium was heated to dissolve the gelatin (but not to boiling). Once the gelatin was dissolved, the agar was added and heated to dissolve (with brief boiling as necessary), then autoclaved at 121°C for 15 min. Thus, 48 h cultures were inoculated onto the gelatin agar plates. After incubation at room temperature for 7 days, the presence of a zone of clearing around the colonies was recorded as positive. The results were read after adding saturated ammonium sulphate to precipitate residual protein.

2.6.10. Elastase activity

The ability to produce elastase was evaluated by using a bilayer of elastin agar (Hasan *et al*., 1992). Initially, 10 ml volumes of sterile (121°C/15 min) Columbia agar base (CAB; Oxoid) were poured into sterile petri dishes, and after setting overlayered with 10 ml of CAB supplemented with 1% (w/v) sodium chloride and 0.3% (w/v) elastin (Sigma-Aldrich). Forty-eight hour cultures of bacterial cultures were seeded onto the

medium, with incubation for 28 days at room temperature. The degradation of elastin was indicated by the development of zones of clearing around the bacterial growth.

2.6.11. Coagulation test

Test for coagulase production was carried out according to Cowan (1938). Five hundred microlitres of undiluted plasma was mixed with an equal volume of an 18-42 h broth culture and incubated at 37 °C for 4 h. Tubes were examined after 1 h and 4 h for coagulation. Negative tubes were left at room temperature overnight and then re-examined.

2.6.12. DNase test

This was carried out according to Jeffries *et al*. (1957). Cultures were inoculated as streaks on the surface of replicate DNase agar (Oxoid) plates. Plates were incubated at 25, 30 and 37 °C. After incubation for 36 h, plates were flooded with 1 N-HCl; a positive result showed a clear zone around the growth and surrounding medium, while a negative result of the test is opaqueness in the medium.

2.7. Rapid identification systems

Cultures were examined for reactions in the API 20E (for Gram-negative isolates) and API 50CH (for Gram-positive bacterial isolates) rapid identification systems (Bio-Mériéux, France). The manufacturer's methods were used with these identification systems, except that incubation was done at 20°C, 30°C and 37°C for 48 h, i.e. the temperature at which the organisms were isolated. A comparison of the results was made with the Bio-Mériéux data base.

2.8. MICROBACT™ Listeria 12L identification system

The system was used for the identification of *Listeria* spp. isolated from clinical samples, food and food-related material. The system is a standardised micro-substrate system designed to simulate conventional biochemical substrates useful for the differentiation of *Listeria* spp. Each identification strip consists of 12 tests (11 sugar utilization tests plus a rapid haemolysis test), and incubation is at (35°C +/- 2°C). The reactions occurring during the incubation period are demonstrated through either a colour change in the sugar utilization test or in the lysis of the sheep red blood cells in the haemolysis test. The Microbact™ Listeria 12L was used as an 18-24 h incubation test when inoculated from single colonies. Thus, a single colony from an 18-24 h culture was picked and emulsified in suspending medium. A thorough mixing was done to prepare a homogenous suspension. Then using a sterile Pasteur pipette, 100 µl of the bacterial suspension was placed into each well. One drop of haemolysin reagent was dropped in well number 12. To check for purity, one drop of the inoculum was inoculated onto a TNA plate with incubation at 35°C for 24 h. After incubation, the reactions were read visually and interpreted against the tables provided in the product insert. Purity was checked by examination of the inoculated the TNA plates.

2.9. Trimethylamine oxide (TMAO) test

The ability to reduce trimethylamine oxide (TMAO) to TMA and produce hydrogen sulphide (H_2S) is regarded as prominent characteristics of fish spoilage bacteria. Bacteria are able to produce, H_2S when decomposing thiosulphate and/or cysteine to form black colonies on trimethylamine oxide-medium due to precipitation of FeS. Trimethylamine oxide-medium was used when bacterial strains were tested for their ability to reduce TMAO and produce H_2S (Gram *et al*., 1987). The medium was

inoculated with bacterial isolates and covered with a thin layer of sterile paraffin oil. The tubes were incubated at 25°C for 3 days. The redox indicator in the medium changed from red to yellow when TMAO was reduced, and a black precipitate of FeS formed when H_2S is produced from thiosulphate and cysteine.

2.10. Modified *Pseudomonas* selective medium (CFC)

Differentiation between *Pseudomonas* spp. and *Enterobacteriaceae* was determined using a modification of the *Pseudomonas* selective medium, Cephaloridine-Fucidin-Centrimide (CFC), of Stanbridge and Board (1994). CFC was made according to the manufacturer's instructions but with the addition of 1% (w/v) L-arginine hydrochloride (Sigma-Aldrich) and 0.002% (w/v) phenol red (BDH; Bristol, England) before autoclaving. The *Pseudomonas* C-F-C supplement (SR 103E supplement, Oxoid) was added prior to pouring into petri dishes. The organisms were inoculated onto the medium and incubated at 25°C for 48 h. Pink colonies were scored as *Pseudomonas* spp. whereas yellow colonies were presumptive *Enterobacteriaceae* representatives. *Pseudomonas* produces ammonia from the arginine (after Stanbridge and Board 1994).

2.11. Identification of isolates by sequencing of bacterial DNA

2.11.1. Preparation of biomass

The bacterial cultures were grown in TNB for 24 h at 25°C, and harvested by centrifugation at 3000 rpm for 15 min at 4°C. Supernatants were removed without disturbing the cell pellets. The cell pellets were resuspended in 1 ml volumes of STE buffer (0.1M NaCl, 10mM Tris pH 8, 1mM EDTA), and transferred to 1.5 ml capacity Eppendorf tubes with centrifuging at 13,000 rpm for 1 min. The supernatants were removed, and the pellets were resuspended in 100 µl of TE buffer (10 mM Tris pH 8, 1 mM EDTA) and transferred to 0.5 ml volume Eppendorf tubes.

2.11.2. Extraction of DNA

The DNA was extracted from the cell pellets using the heating laboratory method. The cell pellets were heated at 95°C for 10 min (heated block; Eppendorf AG, 22331, Hambury, Germany). The pellets were cooled in ice and centrifuged at 13000 rpm for 1 min to remove cellular debris in the pellets; the DNA was contained in the supernatant. The DNA content was measured using Nanodrop, and then stored as aliquots at -20°C until required.

2.11.3. PCR components

Two different *Taq* polymerases were used, Hot Start polymerase and Abgene *Taq* polymerase. DNA templates were amplified by the polymerase chain reaction (PCR) on a Biometra® T-Gradient thermoblock (Germany), using universal primer amplifying 16S rDNA fragments, 20F (5'-AGAGTTGATCATGGCTCAG-3') and 1500R (5'-GGTTCACTTGTTACGACTT-3') (Weisburg *et al*., 1991). Four replicate PCR reactions were prepared for each template. The PCR reaction was prepared in a 0.2 ml polypropylene tube with 1 µl of template DNA, 1.50 µl Klear*Taq* Buffer (10X), 0.60 µl dNTPs (containing 5mM each of dATP, dGTP, dCTP and dTTP; AB0315/A, Thermoscientific), 0.60 µl of each oligonucleotide primer (from a 10 µM solution), 0.45 µl of (50 mM) $MgCl_2$, 0.15 µl of (5U μl^{-1}) Klear*Taq* enzyme, and 10.10 µl of nuclease-free dH_2O. The reaction mixture was vortexed briefly and flash spun.

2.11.4 Thermocycler operation

The cycle sequencing reactions were carried out for the spoilage organisms by initial preheating at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, 72 °C for 5 min and 10 °C for 1 sec. For *L. monocytogenes* by initial preheating at 95 °C for 15 min, followed

by 32 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, 72 °C for 5min and 10 °C for 1 sec. For *C. maltaromaticum* (LAB) using the Abgene *Taq* polymerase and buffer the 95 °C for 2 min, followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, 72 °C for 5 min and 10 °C for 1 sec. An aliquot of the PCR was visualized on an agarose gel to ensure that the applicon was a single band of the expected size. A quadruplet reaction were pulled and purified. PCR products were purified using a PCR purification kit (Qiagen; Hilden, Germany). Cycle sequenced templates were purified by addition of 250 µl of binding buffer (PB) to 55 µl PCR template. Samples were centrifuged at 17600 g for 1 min to bind DNA, and the flow through was removed. Seven hundred and fifty µl of wash buffer (PE) were added to the samples and were centrifuged at 17600 g for 1 min. An additional centrifugation of 17600 g for 1 min was run to remove the residual ethanol from the wash buffer. The DNA were eluted using 20 µl of 60 °C double deionized water was allowed to stand for 1min and finally centrifuged at 17600 g for 1 min. The sequencing was out sourced to a commercial supplier and undertaken on ABI 3100 DNA. Sequencing of the DNA using ABI 3100 DNA sequencer was carried out by (Genetics Core Services, Division of Medical Sciences, Human Genetics, Level 6, Ninewells Hospital and Medical School, University of Dundee). The returned trace files were assembled using Seq Man I (DNA star Inc.) and BlastN comparisons made against the non redundant nucleotide collection data base Genbank.

2.12. Results

2.12.1. Isolation of microorganisms from seafood

Fresh and smoked salmon, fresh and smoked haddock, fresh mussels and oyster fillets collected from local shops in Central Scotland were used as sources of microorganisms as shown in Figures 2.1.

A **B**

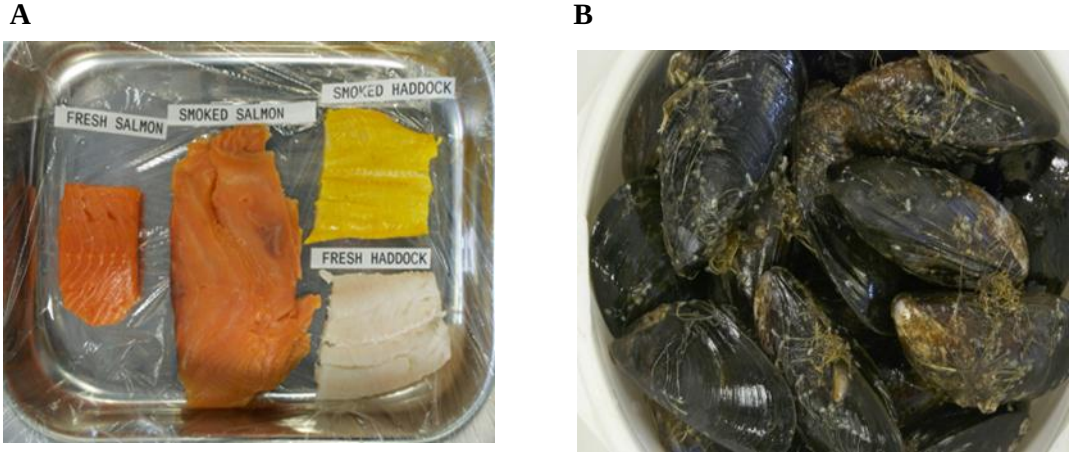


Figure 2. 1. Fresh/smoked haddock and Atlantic salmon as obtained from fishmongers (panel A); fresh Scottish mussels (panel B).

The mean presumptive *Vibrio* counts at 20 °C and 30 °C, the mean *Enterobacteriaceae* counts at 37 °C, mean aerobic heterotrophic counts (AHC) at 20 °C and 30 °C, and the mean *L. monocytogenes* counts at 20 °C and 30 °C were determined (Table 2.1, 2.2, 2.3 and 2.4., respectively). During the period of study, the mean presumptive *Vibrio* counts ranged from 2.8 x 10^3 to 2.1 x 10^6 CFU g^{-1}, the mean Enterobacteriaceae counts from 5.0 x10^2 to 2.2 x 10^6 CFU g^{-1}, mean aerobic heterotrophic counts from 1.6 x 10^3 to 5.8 x 10^7 CFU g^{-1} and the mean *L. monocytogenes* counts from 1.0 x 10^4 to 5.0 x 10^4 CFU g^{-1} in the seafood samples.

Table 2. 1. Presumptive *Vibrio* counts of the seafood samples

Date of seafood collection	seafood samples	Colony count CFU g^{-1} on TCBS at 30 °C	Colony count CFU g^{-1} on TCBS at 20 °C
01 March 2010	smoked salmon	0	0
	fresh salmon	0	1.2×10^5
05 March 2010	mussels	0	3.2×10^5
	smoked salmon	0	0
	fresh salmon	0	0
15 March	mussels	0	0
	smoked salmon	0	0
	fresh haddock	0	0
22 March	mussels	0	2.1×10^6
	oysters	0	1.2×10^6
12 April 2010	mussels	2.8×10^4	1.9×10^6
	smoked salmon	0	0
	fresh salmon	0	0
15 April 2010	smoked haddock	2.8×10^3	0
	fresh haddock	0	0

Table 2. 2. Total *Enterobacteriaceae* numbers in the seafood

Date of seafood collection	seafood samples	Colony count CFU g^{-1} on EMBA at 37 °C
01 March 2010	smoked salmon	0
	fresh salmon	2.2×10^6
05 March 2010	Mussels	0
	smoked salmon	0
	fresh salmon	0
15 March 2010	Mussels	0
	smoked salmon	0
	fresh salmon	0
22 March 2010	Mussels	0
	Oysters	0
12 April 2010	Mussels	0
	smoked salmon	0
	fresh salmon	0
15 April 2010	smoked haddock	2.0×10^6
	fresh haddock	5.0×10^2

Table 2. 3. Total aerobic heterotrophic counts of the seafood

Dates of seafood collection	seafood samples	Colony count CFU g^{-1} on TSA at 30 °C	Colony count CFU g^{-1} on TSA at 20 °C
01 March 2010	smoked salmon	0	0
	fresh salmon	5.8×10^7	3.8×10^7
05 March 2010	Mussels	2.6×10^5	6.1×10^5
	smoked salmon	2.6×10^5	1.1×10^5
	fresh salmon	5.8×10^5	3.2×10^6
15 March 2010	Mussels	3.0×10^3	6.5×10^5
	smoked salmon	0	0
	fresh salmon	0	0
22 March 2010	Mussels	1.1×10^5	1.1×10^7
	Oysters	1.1×10^5	1.1×10^6
12 April 2010	mussels	9.2×10^4	6.2×10^6
	smoked salmon	1.6×10^3	0
	fresh salmon	1.3×10^5	9.7×10^5
15 April 2010	smoked haddock	1.1×10^6	2.1×10^6
	fresh haddock	1.8×10^6	2.0×10^6

Table 2. 4. Total *Listeria monocytogenes* counts of the seafood samples

Date of seafood collection	seafood samples	Colony count CFU g^{-1} on PALCAM at 30 °C	Colony count CFU g^{-1} on PALCAM at 20 °C
01 March 2010	smoked salmon	0	0
	fresh salmon	0	0
05 March 2010	mussels	0	0
	smoked salmon	0	0
	fresh salmon	0	0
15 March 2010	mussels	0	0
	smoked salmon	0	0
	fresh salmon	0	2.5×10^6
22 March 2010	mussels	1.0×10^4	5.0×10^4
	oysters	0	0
12 April 2010	mussels	0	0
	smoked salmon	0	0
	fresh salmon	0	0
15 April 2010	smoked haddock	0	0
	fresh haddock	1.7×10^5	1.6×10^4

2.12.2. The distribution, frequency, number and percentage of bacterial isolates from the seafood

From Table 2.5, it may be observed that fresh haddock was the most contaminated species in terms of the diversity of bacteria, followed by fresh salmon and smoked haddock. In total, 20 genera of bacteria were recognised including *Acinetobacter, Aerococcus, Aeromonas, Bacillus, Brochothrix, Carnobacterium, Citrobacter,*

Corynebacterium, Enterobacter, Escherichia coli, Listeria, Moraxella, Micrococcus, Pseudomonas, pseudomonad, *Psychrobacter, Serratia, Shewanella, Staphylococcus* and *Vibrio*. The proportions of these taxa have been included in Table 2.5. Conversely, the least dominant isolates were *Bacillus, Micrococcus, Pseudomonas, Psychrobacter, Serratia* and *Staphylococcus*.

On TSA, *Acinetobacter* spp. cultured exhibited cream colonies, that were ≤ 1-2 mm in diameter, round, smooth edged, raised and creamy. Colonies comprised of non-sporeforming, Gram-negative, cocobacillary (short rods) occurring in chains. Cultures were oxidase negative and catalase positive. They were non-motile. Gelatin was produced by one of the isolates and one of the isolates utilized citrate. Ortho-nitrophenyl-galactosidase, arginine dihydrolase, lysine decarboxylase were produced, but not indole, H_2S, tryptophan deaminase and urease. The Voges-Proskauer reaction was positive. Tween 80 was not hydrolysed and haemolysin was not produced (Appendix IV Table 1).

Aerococcus spp. cultured on TSA, showed intense yellow and light yellow coloured, 1-2 mm in diameter, round, smooth edged, raised, creamy colonies. These were Gram-positive, arranged in tetrads, pairs and clusters, non-sporeforming cocci. Cultures are non-motile, (weakly fermentative), catalase production was positive and strong, oxidase production was negative. Arginine dihydrolase, indole, tryptophan deaminase and H_2S were not produced. Citrate was utilized, one isolate produced gelatin, while one isolate produced urease. Two of the isolates weakly produced acid from sucrose and glucose. One of the isolate degraded gelatin and Tween 80 (Appendix IV Table 2).

On TSA, *Aeromonas* spp. demonstrated cream, orange and creamish brown coloured colonies that were ≤ 1-4 mm in diameter, round, smooth edged, raised, flat and sticky. Colonies consist of non-sporeforming Gram-negative, (fermentative/alkaline) rods

occurring in singles, pairs and clusters. Cultures were catalase and oxidase positive. They are motile and non-motile rods. Arginine dihydrolase was produced and indole, lysine decarboxylase and ortho-nitrophenyl galactosidase were produced with variable results. The Voges Proskauer reaction was positive and blood β and α haemolysis were produced by some isolates. Gelatin, lecithin, Tween 80 and elastin tests produced variable results and the bacteria were resistance to the vibriostatic agent O/129. Urease is not degraded and acid production from glucose, sucrose, arabinose, mannitol, amygdalin and sorbitol was variable (Appendix IV Table 3 a, b and c).

Bacillus spp. cultured on TSA exhibited white coloured, 3-4 mm in diameter, round, rough edged, raised, dry colonies that Gram-positive rods, occurring in chains and non-motile, with oval endospores. The Voges-Proskauer reaction was positive, indole was not produced and the catalase test was positive. Acid was produced from glucose, mannitol, inositol, sorbitol, rhamnose and amygdalin and blood (haemolysis), gelatin and lecithin were not degraded (Appendix IV Table 4).

Brochothrix spp. cultured on TSA showed white coloured, 1-3 mm in diameter, round, smooth/rough edged, raised creamy and dry colonies. Colonies comprised of Gram-positive rods, non-sporeforming, nonmotile, occurring in short chains and thread-like chains. The catalase test was positive and oxidase test was negative. The Voges-Proskauer tests are positive, H_2S and indole were not produced, gelatin was not liquefied, urease was not produced and citrate was not utilized. Arginine was not hydrolyzed. Acid was produced from amygdalin, inositol, mannitol and sucrose. Tween 80 was not hydrolyzed, blood (haemolysin) not degraded, one of the isolates degraded gelatin (Appendix IV Table 5).

On TSA, *Carnobacterium* spp. demonstrated white colonies that were ≤ 1 mm in diameter, round, smooth edged, raised and creamy colonies. Colonies comprised of Gram-positive, non-sporeforming and non-motile rods, occurring in singles, pairs, chains and clusters. Catalase and oxidase tests are negative. They hydrolyzed arginine dihydrolase and ortho-nitrophenyl-galactosidase. The Voges-Proskauer reaction is positive. Gelatin, Tween 80, lipase, elastin and blood (haemolysin) were not degraded. Acid was produced from gluconate, amygdalin, D-turanose, inulin, melezitose, α-methyl-D-mannopyranoside, α-methyl-D-Glucopyranoside, D-mannitol, amidon, but not from D-xylose, D-tagatose (Appendix IV Table 6 a and b).

Citrobacter spp. cultured on EMBA, characteristically exhibited pink coloured, 2-4 mm in diameter, round, smooth edged, raised and creamy colonies. Colonies comprised of Gram-negative, non-sporeforming and fermentative rods, occurring in single, pairs and clusters. Cultures revealed motility at 20 °C, which produced catalase, arginine dihydrolase, but not indole, lysine or ornithine decarboxylase or oxidase. The Voges Proskauer reaction was negative. Ortho-nitrophenyl-galactosidase was produced by one of the strain. Gelatinase was not produced. One of the strains hydrolysed urea. Acid is produced from glucose by both strains. One of the strains produced acid from sucrose, arabinose, mannitol, inositol, sorbitol and amygdalin. Lecithinase and Tween 80 were degraded by one of the strains (Appendix IV Table 7).

Corynebacterium spp. cultured on TSA, demonstrated white and cream coloured colonies that were ≤ 1-2 mm in diameter, round, irregular edged and raised. Colonies were Gram-positive, flagella like bacillus (club shaped), non-sporeforming rods. Cultures revealed catalase production. Isolates are non-motile and non-fermentative. Indole, H_2S, urease, oxidase, ortho-nitrophenyl-galactosidase, lysine or ornithine decarboxylases were not produced. The Voges-Proskauer reaction was positive, one

isolate produced arginine dihydrolase. Acid was produced from glucose, sucrose and amygdalin by one of the isolates. Lecithin, gelatin and blood (haemolysis) were not degraded (Appendix IV Table 8).

Typically on EMBA *Enterobacter* spp. characteristically showed pink coloured colonies that were 2-3 mm in diameter, round, smooth edged, raised and creamy. Colonies comprised of Gram-negative, fermentative and non-sporeforming rods occurring singly, in pairs and clusters. Catalase test was positive, motility at 20 °C, indole was not produced, oxidase test was negative, gelatin was not hydrolyzed, ornithine-decarboxylase, arginine dihydrolase, ortho-nitrophenyl-galactosidase and lysine decarboxylase were produced, urease was produced by one of the strains. Acid was produced from glucose, sucrose, arabinose, mannitol, inositol, rhamnose, melibiose, amygdalin and sorbitol. H_2S is not produced. The Voges-Proskauer reaction is positive. Lecithin and Tween 80 were degraded by one of the strains (Appendix IV Table 9).

Escherichia coli cultured on EMBA, characteristically exhibited green metallic sheen coloured colonies, that were 1-4 mm diameter, round, smooth edged, raised and creamy. Colonies comprised of Gram-negative, fermentative, non-sporeforming and pleomorphic rods, occurring in singles and pairs. Cultures revealed catalase and indole production, motility at 20 °C. Citrate, ortho-nitrophenyl-galactosidase, arginine dihydrolase, ornithine decarboxylase production were (variable among strains). Lysine decarboxylase, H_2S, the Voges-Proskauer reaction, gelatinase and oxidase are not produced. Acid production from glucose, sucrose, arabinose, mannitol, inositol, sorbitol, rhamnose, melibiose and amygdalin was (variable among strains). One of the strains degraded lecithin and Tween 80, another strain revealed α-haemolysis on sheep blood (Appendix IV Table 10 a and b).

On TSA, *Moraxella* spp. demonstrated cream and orange coloured colonies, that were 1-2 mm in diameter, round, raised, smooth and creamy. Colonies comprised of Gram-negative, non-sporeforming, non-motile coccobacilli (short) rods occurring in singles, pairs and clusters. Catalase and oxidase tests were positive. The Voges-Proskauer reaction was positive. Gelatinase, H_2S, urease, ortho-nitrophenyl-galactosidase, arginine dihydrolase and lysine decarboxylase were not produced. Citrate was not utilized, indole was not produced. One of the isolates produced tryptophan deaminase, while another produced ornithine decarboxylase. Acid was not produced from any of the sugars. Three of the isolates degraded Tween 80 and two isolates degraded lecithin (Appendix IV Table 11 a and b).

Micrococcus spp. cultured on TSA showed cream-white coloured that were 1-2 mm diameter, round, rough edged, raised and dry colony. Colonies were Gram-positive, non-sporeforming and non-motile coccus occurring in tetrads and clusters. They were catalase and oxidase positive. The Voges-Proskauer reaction was positive. The culture was non-pigmented, not able to hydrolyze arginine dihydrolase, lysine and ornithine decarboxylase. Indole, H_2S and gelatin were not produced. Acids were not produced from sugars (Appendix IV Table 12).

Pseudomonas spp. cultured on TSA and TCBS agar demonstrated cream and orange, and green coloured colonies that were ≤ 1-4 mm in diameter, round, smooth and rough edged, raised, flat, dry, creamy and sticky. Colonies comprised of Gram-negative, non-sporeforming, oxidative, alkaline, motile rods occurring in singles, pairs and chains. Catalase and oxidase tests were positive. The Voges-Proskauer reaction was positive. Citrate was utilized. Arginine dihydrolase was produced by two of the strains and urease was produced by one of the strains. Acid was produced from glucose, arabinose,

mannitol, melibiose. Gelatine, lecithin, Tween 80, elastin and blood (β-haemolysis) were degraded (Appendix IV Table 13 a, b and c).

Psychrobacter spp. cultured on TSA, exhibited cream coloured, 1 mm in diameter, rough colonies with waxy consistency. Colonies comprised of Gram-negative, non-sporeforming, nonmotile, coccobacilli rods the cell though Gram-negative retained the crystal violet colour and so initially identified as *Micrococcus*. Catalase and oxidase test were positive. Tryptophan deaminase was produced and the Voges-Proskauer reaction was positive. All the other biochemical tests gave no reaction which confirms the report of Denner *et al*. (2001) that generally, *Psychrobacter* is relatively biochemically inert and will be unreactive to most of the popularly used commercial rapid tests (e.g. API 20E and API 20NE test strips, Vitek-Biomérieux). Lipase was not degraded but alpha haemolysin was produced (Appendix IV Table 14).

Serratia spp. typically cultured on EMBA, demonstrated pink coloured colonies that were 2-3 mm in diameter, round, smooth edged, raised and creamy. These were of Gram-negative, non-sporeforming, fermentative, rods occurring in singles and pairs. Cultures showed motility at 20 °C and produced catalase. The Voges Proskauer reaction, gelatinase, ortho-nitrophenyl- galactosidase, arginine dihydrolase, lysine and ornithine decarboxylase were produced, citrate was utilised. Indole, oxidase, tryptophan deaminase, H_2S and urease were not produced. Acid was produced from sucrose, arabinose, mannitol, inositol, sorbitol, amygdalin, but not from glucose, rhamnose and melibiose. Lecithin and Tween 80 were degraded (Appendix IV Table 15).

Characteristically *Shewanella* spp. cultured on TSA, showed orange coloured colonies that were ≤ 1-4mm in diameter, round, smooth edged, raised and creamy. Colonies comprised of Gram-negative, non-sporeforming rods occurring in singles, pairs and

clusters. Cultures revealed H_2S producing, motile rods with positive oxidase and catalase reactions. The strains reduced TMAO to TMA and produce H_2S from thiosulphate and cysteine. Gelatin was produced and citrate was utilized by all the strains. The isolates revealed variable results for ornithine and lysine decarboxylase, arginine dihydrolase and the Voges Proskauer reaction. Two strains showed acid production from glucose, sucrose, arabinose, melibiose, amygdalin and mannitol, while other did not metabolise any sugar. One of the strains isolated from fresh haddock was able to decarboxylate tryptophan. Indole production was negative. All the strains degraded lecithin, four of the strains degraded lipase, all strains degraded gelatin except one, two strains degraded elastin, while three were beta haemolytic all isolates grew at 4 °C, while only two isolates grew at 37 °C (Appendix IV Table 16 a, b and c).

Staphylococcus spp. cultured on TSA, revealed white coloured colonies that were 1-2 mm in diameter, round, smooth edged, raised and creamy. Colonies comprised of Gram-positive (fermentative), non-sporeforming cocci, in clusters and chains. Catalase was produced, but not oxidase. The Voges Proskauer reaction was positive; indole was not produced. Urease and H_2S were not produced and citrate was not utilized. Arginine dihydrolase and ortho-nitrophenyl-galactosidase were produced. Acid was produced from glucose, sucrose and mannitol. Coagulase and DNase were not produced. Blood (β-haemolysis) and Tween 80 were not degraded (Appendix IV Table 17).

Distinctively on TCBS agar, *Vibrio* spp. showed green/yellow colonies that were 1-2 mm in diameter, round, smooth edged, flat and creamy. Colonies were Gram-negative, motile, non-sporeforming, fermentative curved/straight rods occurring in singles, pairs and clusters. Cultures revealed catalase and oxidase positive (for oxidase producers), oxidase negative (for non-oxidase producers). Indole and gelatinase were produced by oxidase producers. The Voges-Proskauer reaction was negative for oxidase producers

and positive for non-oxidase producers. Tryptophan deaminase was degraded by non-oxidase producers. Citrate was utilized by all isolates. Arginine dihydrolase was produced. Acid was produced from glucose and sucrose. Acid was produced from mannitol by oxidase producers. The oxidase producers were sensitive to O/129; lecithin, Tween 80 and blood (β-haemolysin) were degraded. The non-oxidase producers were both sensitive and resistant to O/129 and blood (α-haemolysin) was degraded (Appendix IV Table 18).

Typically on PALCAM agar, *Listeria* colonies demonstrated black haloes with greenish surroundings that were ≤ 1 mm in diameter; they were round, smooth edged, flat and creamy. Colonies comprised non-sporeforming Gram-positive, tiny rods occurring singly, and in pairs and clusters. Cultures were catalase positive, but oxidase negative. Beta haemolysis was recorded on sheep blood agar. Hydrolysis of aesculin was positive. Acid was produced from xylose, arabitol, ribose, trehalose, M-D- gluconate and M-D-mannose (Appendix IV Table 19).

Table 2. 5. Number and percentage of bacterial isolates in the seafood

Bacterial Isolate	No. (%)	Seafood species/ No. (%)					
		Fresh salmon	Smoked salmon	Fresh haddock	Smoked haddock	Mussels	Oysters
Acinetobacter	2 (3.2)	0 (0.0)	0 (0.0)	1 (6.3)	1 (7.7)	0 (0.0)	0 (0.0)
Aerococcus	3 (4.8)	1 (7.7)	0 (0.0)	1 (6.3)	0 (0.0)	1 (12.5)	0.(0.0)
Aeromonas	9 (14.5)	1 (7.7)	1 (12.5)	3 (18.8)	3 (23.1)	1 (12.5)	0.(0.0)
Bacillus	1 (1.6)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
Brochothrix	2 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	1 (25.0)
Carnobacterium	3 (4.8)	1 (7.7)	2 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Corynebacterium	3 (4.8)	2 (15.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)
Moraxella	5 (8.1)	0 (0.0)	1 (12.5)	0 (0.0)	3 (23.1)	1 (12.5)	0 (0.0)
Micrococcus	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)
Pseudomonas	5 (8.1)	1 (7.7)	1 (12.5)	1 (6.3)	0 (0.0)	1 (12.5)	1 (25.0)
pseudomonads	1 (1.6)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Psychrobacter	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)
Shewanella	7 (11.3)	2 (15.4)	0 (0.0)	4 (25.0)	1 (7.3)	0 (0.0)	0 (0.0)
Staphylococcus	1 (1.6)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Citrobacter	2 (3.2)	1 (7.7)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)
Enterobacter	2 (3.2)	1 (7.7)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
Escherichia coli	6 (9.6)	2 (15.4)	1 (12.5)	2 (12.5)	1 (7.7)	0 (0.0)	0 (0.0)
Serratia	1 (1.6)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
Vibrio	5 (8.1)	0 (0.0)	1 (12.5)	1 (6.3)	0 (0.0)	2 (25.0)	1 (25.0)
Listeria	2 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (25.0)	0 (0.0)
Total	62(100.0)	13(21.0)	8 (12.9)	16(25.8)	13(21.0)	8(13.0)	4(6.5)

All total heterotrophs were isolated using TSA, *Enterobacteriaceae* representatives were isolated using EMBA, presumptive vibrios were isolated using TCBS and *Listeria* spp. isolated using PALCAM agar.

Bacterial codes are in Appendix III.

2.13. Molecular-based Identification of the isolates

In all instances, agarose gel electrophoresis confirmed that a single amplicon of approximate size of 1500 bp was amplified.

2.13.1. 16S rDNA sequencing

Two sequences were generated per amplicon, one primer via the "forward" PCR primer, and the second by the "reverse" PCR primer. High quality reads were produced for all templates (read length c.900b). Seq Man I (DNA Star Inc.) was used to contig each pair of sequences. Finally, the primer sequences were removed and the remaining template specific sequences blasted (Blasted N) against the Genbank database (November, 2011).

16S rDNA sequencing was used to identify the isolates by comparing the data in the BLAST search engine (http://www.ncbi.nlm.nih.gov:80/blast/Blast.cgi), which is a database program, at the National Library of Medicine, Bethesda, MD, USA. The sequences of FSA 20 °C produced 99% significant alignments, identical with *Sh. baltica* OS185 complete genome. SHB 20 °C had 99% identity to *Aeromonas* sp. HB-6. Furthermore, ZFSB 30 °C sequences resulted in 99% significant alignments with *Sh. baltica* complete. The sequences of ZFHB 20 °C resulted in 99% identity to *Sh. baltica* OS678 complete genome. The sequences of ZFHG 37 °C produced 99% identity to *Serratia* sp. I-113-31. ZFHTA 30 °C sequences had 99% similarity to *A. salmonicida* subsp. *achromogenes*. The sequences of SHTA 30 °C produced 100% identity with *A. hydrophila* strain HX201006-3. The sequences of MP_1C 30 °C resulted in 99% identity to *L. monocytogenes* ATCC 19114. The sequences of SSC 30 °C produced 99% identity to *Staphylococcus haemolyticus* gene. FS_2C 30 °C sequences produced 99% similarity to *C. maltaromaticum* strain MMF-32. Furthermore the sequences of ZSSC 30 °C resulted in 99% identity to *C. maltaromaticum* strain MMF-32. The sequences of ZSHK

30 °C resulted in 99% homology with *Psychrobacter* sp. OTUC8. The sequences of ZSSC 20 °C produced 99% identity with to *C. maltaromaticum* strain KOPRI 25789. The sequences of OCD 30 °C and SHC 20 °C produced 99% homology with *Brochothrix* sp. clone OTUN6 (Table 2.6.).

Table 2.6. Results of 16S rDNA

Sample number	Strain identified	Query coverage	Maximum identification	E. Value
N1-FSA 20 °C	*Shewanella baltica* OS185 complete genome	99 %	99 %	0.0
N2-SHB 20 °C	*Aeromonas* sp. HB-6	98%	99 %	0.0
N3-ZFSB 30 °C	*Shewanella baltica*	100%	99 %	0.0
N4-ZFHB 20 °C	*Shewanella baltica* OS678 complete genome	100%	99 %	0.0
N5-ZFHG 37 °C	*Serratia* sp. I-113-31	100%	99 %	0.0
N6-ZFHTA 30 °C	*Aeromonas salmonicida* subsp. *achromogenes*	100%	99 %	0.0
N7-SHTA 30 °C	*Aeromonas hydrophila* strain HX201006-3	100%	99 %	0.0
N8-MP_1C 30 °C	*Listeria monocytogenes* ATCC 19114	100%	99 %	0.0
N9-SSC 30 °C	*Staphylococcus haemolyticus*	100%	99 %	0.0
N10- FS_2C 30 °C	*Carnobacterium maltaromaticum* strain MMF-32	100%	99 %	0.0
N11-ZSSC 30 °C	*Carnobacterium maltaromaticum* strain MMF-32	100%	99 %	0.0
N12-ZSHK 30 °C	*Psychrobacter* sp. OTUC8	100%	99 %	0.0
N13-ZSSC 20 °C	*Carnobacterium maltaromaticum* strain KOPRI 25789	99 %	99 %	0.0
N14-OCD 30 °C	*Brochothrix* sp. OTUN6	99 %	99 %	0.0
N15-SHC 20 °C	*Brochothrix* sp. clone OTUN6	99 %	99 %	0.0

2.14. Identification of the isolates by API 20E

The isolates were identified by API 20E as follows:

FSA 20 °C was equated with *Sh. baltica*, SHB 20 °C was identified as *Sh. putrefaciens*, ZFSB 30 °C was characterized as *Sh. putrefaciens*, ZFHB 20 °C was equated with *Sh. putrefaciens*, ZFHG 37 °C was identified as *Serratia liquefaciens*, ZFHTA 30 °C was characterized as *A. salmonicida* subsp. *salmonicida*, SHTA 30 °C was equated with *A. hydrophila*, SSC 30 °C was identified as *Staphylococcus haemolyticus*, ZSHK 30 °C was characterized as *Pyschrobacter* spp. The identification of FS_2C 30 °C, ZSSC 30 °C and ZSSC 20 °C using API 50CH indicated the presence of *C. maltaromaticum*. OCD 30 °C and SHC 20 °C were identified as *Brochothrix* spp. and *Brochothrix thermosphacta*, respectively. Furthermore, identification of MP_1C 30 °C using MICROBACT™ 12L showed the presence of *L. monocytogenes*.

By sequencing of the 16S-rDNA, FSA 20 °C was identified as: *Sh. baltica* OS185 complete genome (score = 99 %), SHB 20 °C was determined to be *Aeromonas* sp. HB-6 (Score = 99 %), ZFSB 30 °C was equated with *Sh. baltica* (score = 99 %), ZFHB 20 °C was identified as *Sh. baltica* OS678 complete genome (score = 99 %), ZFHG 37 °C was determined to be *Serratia* sp. I-113-31 (score = 99 %), ZFHTA 30 °C was equated with *A. salmonicida* subsp. *achromogenes* strain (score = 99 %); SHTA 30 °C was identified as *A. hydrophila* strain HX201006-3 (score = 100 %), MP_1C 30 °C was determined to be *L. monocytogenes* strain ATCC 19114 (score = 99%). SSC 30 °C was regarded as *Staphylococcus haemolyticus* gene (score = 99%), FS_2C 30 °C and ZSSC 30 °C were identified as *C. maltaromaticum* strains MMF-32 respectively (score = 99%), ZSSC 20 °C was equated with *C. maltaromaticum* strain KOPRI 25789 (score = 99%), ZSHK 30 °C was determined to be *Psychrobacter* spp. OTUC8 (score = 99%),

OCD 30 °C and SHC 20 °C were determined as *Brochothrix* spp. clone OTUN6, respectively (score = 99%) (Table 2.7, 2.8, 2.9, 2.10 and 2.11).

Table 2.7. Identification of bacterial isolates

Bacterial code	API 20E identification	API 50CH identification	16S-rDNA identification (BLAST)
ZFHTI 20 °C	*Acinetobacter lwoffi*	-	-
ZSHTI 20 °C	*Acinetobacter johnsonii*	-	-
MPA 30 °C	*Aerococcus viridans* subsp. *homari*	-	-
FHP 30 °C	*Aerococcus viridans* subsp. *homari*	-	-
FSPA 20 °C	*Aerococcus viridans* subsp. *homari*	-	-
SHB 20 °C	*Shewanella putrefaciens*	-	*Aeromonas* spp. HB-6
ZSHB 20 °C	*Aeromonas salmonicida* subsp. *salmonicida*	-	-
SHTA 30 °C	*Aeromonas hydrophila*	-	*Aeromonas hydrophila* strain HX201006-3
ZFHD 30 °C	*Aeromonas hydrophila*	-	-
ZFHTA 30 °C	*Aeromonas salmonicida* subsp. *salmonicida*	-	*Aeromonas salmonicida* subsp *achromogenes*
ZFHTA 20 °C	*Aeromonas hydrophila*	-	-
MT_1A 30 °C	*Aeromonas hydrophila*	-	-
FSC 30 °C	*Aeromonas salmonicida* subsp. *salmonicida*	-	-
SSB 30 °C	*Aeromonas salmonicida* subsp. *salmonicida*	-	-

Table 2.8. Identification of bacterial isolates (contiuned)

Bacterial code	API-20E identification	API-50CH identification	16S-rDNA identification (BLAST)
FHC 20 °C	*Bacillus mycoides*	-	-
SHC 20 °C	*Brochothrix thermosphacta*	-	*Brochothrix* spp. clone OTUN6
OCD 30 °C	*Brochothrix* spp	-	*Brochothrix* spp. OTUN6
ZSSC 20 °C	-	*Carnobacterium maltaromaticum*	*Carnobacterium maltaromaticum* KOPRI 25789
ZSSC 30 °C	-	*Carnobacterium maltaromaticum*	*Carnobacterium maltaromaticum* MMF-32
FS_2C 30 °C	-	*Carnobacterium maltaromaticum*	*Carnobacterium maltaromaticum* MMF-32
FSG 37 °C	*Citrobacter freundii*	-	-
SHG 37 °C	*Citrobacter freundii*	-	-
FSC 20 °C	*Corynebacterium amycolatum*	-	-
OJ 20 °C	*Corynebacterium amycolatum*	-	-
ZFSK 30 °C	*Corynebacterium amycolatum*	-	-
FHG 37 °C	*Enterobacter cloacae*	-	-
ZFSG 37 °C	*Enterobacter cloacae*	-	-
FSH 37 °C	*Escherichia coli*	-	-
FHH 37 °C	*Escherichia coli*	-	-

Table 2.9. Identification of bacterial isolates (continued)

Bacterial code	API 20E Identification	API 50CH Identification	16S rDNA Identification (BLAST)
ZFHH 37 °C	*Escherichia coli*	-	-
ZFSH 37 °C	*Escherichia coli*	-	-
SHH 37 °C	*Escherichia coli*	-	-
SSB 20 °C	*Escherichia coli*	-	-
MBT 30 °C	*Moraxella* spp.	-	-
ZSHK 20 °C	*Moraxella osloensis*	-	-
ZSHB 30 °C	*Moraxella* spp.	-	-
ZSSB 20 °C	*Moraxella osloensis*	-	-
ZSHC 20 °C	*Moraxella osloensis*	-	-
SH2CT 30 °C	*Micrococcus lylae*	-	-
SSD 30 °C	*Pseudomonas pseudoalcaligenes*	-	-
MUB 20 °C	*Pseudomonas pseudoalcaligenes*	-	-
FSD 30 °C	*Pseudomonas aeruginosa*	-	-
ZFHTI 30 °C	*Pseudomonas aeruginosa*	-	-
OB 20 °C	*Pseudomonas alcaligenes*	-	-
FSB 30 °C	Pseudomonad	-	-
ZSHK 30 °C	*Micrococcus* spp.	-	*Psychrobacter* spp. OTUC8
ZFHG 37 °C	*Serratia liquefaciens*	-	*Serratia* spp.I-113-31

Table 2.10. Identification of bacterial isolates (continued)

Bacteria code	API 20E identification	API 50CH identification	16S-rDNA identification (BLAST)
FSA 20 °C	*Shewanella baltica*	-	*Shewanella baltica* OS185
SHP 30 °C	*Shewanella baltica*	-	-
ZFSB 30 °C	*Shewanella putrefaciens*	-	*Shewanella baltica*
FHB 20 °C	*Shewanella baltica*	-	-
ZFHB 20 °C	*Shewanella putrefaciens*	-	*Shewanella baltica* OS678
FHB 30 °C	*Shewanella baltica*	-	-
ZFHB 30 °C	*Shewanella baltica*	-	-
SSC 30 °C	*Staphylococcus haemolyticus*	-	*Staphylococcus haemolyticus* gene
MUTI 20 °C	*Vibrio parahaemolyticus*	-	-
OTA 20 °C	*Vibrio fluvialis*	-	-
MTI 20 °C	*Vibrio parahaemolyticus*	-	-
FHTI 30 °C	*Vibrio metschnikovii*	-	-
SSA 30 °C	*Vibrio metschnikovii*	-	-

Table 2.11. Identification of *Listeria monocytogenes*

Bacteria code	MICROBACT™ 12L identification	16S-rDNA identification (BLAST)
MP_1C 30 °C	*Listeria monocytogenes* NCTC 11994	*Listeria monocytogenes* ATCC 19114
MP_1C 20 °C	*Listeria monocytogenes* NCTC 11994	-

2.15. Detection of specific spoilage bacteria

Table 2.12 shows the prominent characteristics of fish spoilage bacteria, by their ability to reduce trimethylamine oxide (TMAO) red to trimethylamine (TMA) yellow, to produce H_2S when decomposing thiosulphate and or cysteine to form black colonies due to preciptation of FeS. *Aeromonas* spp., *Citrobacter freundii*, *Enterobacter cloacae* and *Shewanella* spp. were early producers of H_2S. In the present study, the prominent characteristics of fish spoilage isolates were the ability to reduce trimethylamine oxide (TMAO) to trimethylamine and to produce H_2S. The production of TMA was evidenced by the redox indicator in the medium being changed from red to yellow, and a black precipitate of FeS was formed as H_2S was produced from thiosulphate and/or cysteine. The *Sh. baltica* OS185 and *Aeromonas* spp. HB-6 were the strongest TMA producers as they were able to reduce TMAO to trimethylamine and to produce H_2S within two days of incubation. Other isolates of *Sh. baltica*, *Sh. putrefaciens*, *A. hydrophila* HZ201006-3, *A. salmonicida* subsp. *achromogenes*, *A. hydrophila* strain, *C. freundii*, *E. cloacae* were also strong producers of TMA and H_2S within three days of incubation. One strain each of *Sh. baltica, Sh. putrefaciens, V. metschnikovii, E. coli*, pseudomonad and *Serratia* spp. I-113-31 were late H_2S producers (Table 2.12).

Table 2.12. Detection of specific spoilage bacteria using trimethylamine oxide medium (TMAO)

Bacterial isolate	Bacterial code	Red to yellow	H_2S (Black)
Aeromonas sp. HB-6	SHB 20 °C	+	+
Aeromonas hydrophila HX201006-3	SHTA 30 °C	+	+
Aeromonas salmonicida subsp. *achromogenes*	ZFHTA 30 °C	+	+
Vibrio metschnikovii	FHTI 30 °C	+	(+)
Aeromonas hydrophila	ZFHTA 20 °C	+	(+)
Citrobacter freundii	SHG 37 °C	+	+
Enterobacter cloacae	ZFSG 37 °C	+	+
Escherichia coli	ZFSH 37 °C	+	(+)
Pseudomonad	FSB 30 °C	+	(+)
Serratia sp. I-113-31	ZFHG 37 °C	+	(+)
Shewanella baltica OS678	ZFHB 20 °C	+	(+)
Shewanella baltica OS185	FSA 20 °C	+	+
Shewanella baltica	ZFSB 30 °C	+	(+)
Shewanella baltica	FHB 30 °C	+	+
Shewanella putrefaciens	ZFHB 30 °C	+	+

(+) parenthesis represents late producers of H_2S

2.16. Virulent characteristics of spoilage bacteria

Table 2.13 shows the virulent characteristics of spoilage bacteria, revealing *Aeromonas* spp. and *Shewanella* spp. as high producers of lecithinase, lipase, haemolysin, gelatinase and elastinase. The proteolytic activity determined on gelatin agar for all the spoilage microorganisms, revealed that 6/15 of the spoilage microorganisms showed

activity. Determination of the lipolytic activity showed that 7/15 of the isolates could hydrolyze Tween 80. Lecithinase activity assayed on egg-yolk agar plate showed that 6/15 of the spoilage microorganisms were able to degrade lecithin. Blood (β and α haemolysin) activity on sheep blood agar and elastinase activity on elastin agar by the spoilage isolates showed activity of 6/15 isolates, respectively. *Shewanella* spp. and *Aeromonas* spp. showed the highest activity in all the substrates used, although one *Aeromonas* spp. was gelatinase positive. Pseudomonads were only able to hydrolyse gelatin and elastin, while *Serratia* spp. hydrolysed lecithin and lipase (Table 2.13).

The ability of the microorganisms to degrade haemolysin (β- haemolysis) reveals that haemolysin causes a cytolytic effect creating pores in host membranes resulting in cell lysis (Ellis *et al*., 1988). Haemolysin is an exotoxin that acts destructively on the blood cell membrane and leads to cell rupture. Haemolysis, which results from the lysis of erythrocyte membranes with release of haemoglobin, consists of β-haemolysis, i.e. complete degradation, and α-haemolysis i.e. the incomplete degradation of haemoglobin (Zhang and Austin, 2005). Proteases (gelatin) are associated in the growth and spread of the bacterium and contribute to the development of the disease by defeating host host defenses and providing nutrients for the host (Sakai, 1985ab; Janda and Abbot, 2010). Lipases have hydrolytic effect on the lipids of the membrane of the host cells, causes intestinal damage, invasiveness and establishment of infections (Lee and Ellis, 1990; Timpe *et al*., 2003). Merino *et al*. (1999) in their study reported that phospholipases associated with fish disease are the lecithinases C and A1. Furthermore, that phospholipases act as both haemolysin and glycerolphospholipid: cholesterol acyltransferase (glycerolphospholipid: cholesterol acyltransferase, GCAT) is present in all *Aeromonas* species, and its role in fish virulence may be due to a combination with other factors (Chacón *et al*., 2002; Scoaris, *et al*., 2008; Figueras *et al*., 2011b).

Table 2.13. Virulent characteristics of spoilage microorganisms

Spoilage Microorganisms	Lecithinase	Lipase	Haemolysin	Gelatinase	Elastinase
Aeromonas sp. HB-6	+	+	B	+	+
Aeromonas hydrophila HX201006-3	-	+	B	-	+
Aeromonas salmonicida subsp. *achromogenes*	-	+	B	-	+
Vibrio metschnikovii	-	-	A	-	-
Aeromonas hydrophila	+	+	-	-	-
Citrobacter freundii	-	-	-	-	-
Enterobacter cloacae	-	-	-	-	-
Escherichia coli	-	-	-	-	-
Pseudomonad	-	-	-	+	+
Serratia sp. I-113-31	+	+	-	-	-
Shewanella baltica OS678 ZFHB 20 °C	-	-	-	-	-
Shewanella baltica OS185 FSA 20 °C	+	+	B	+	+
Shewanella baltica ZFSB 30 °C	+	+	B	+	+
Shewanella baltica FHB 30 °C	-	-	-	+	-
Shewanella putrefaciens ZFHB 30 °C	+	-	-	+	-

Vibrio parahaemolyticus and *Escherichia coli* isolated from the seafood as shown in Figure 2.2.

A **B**

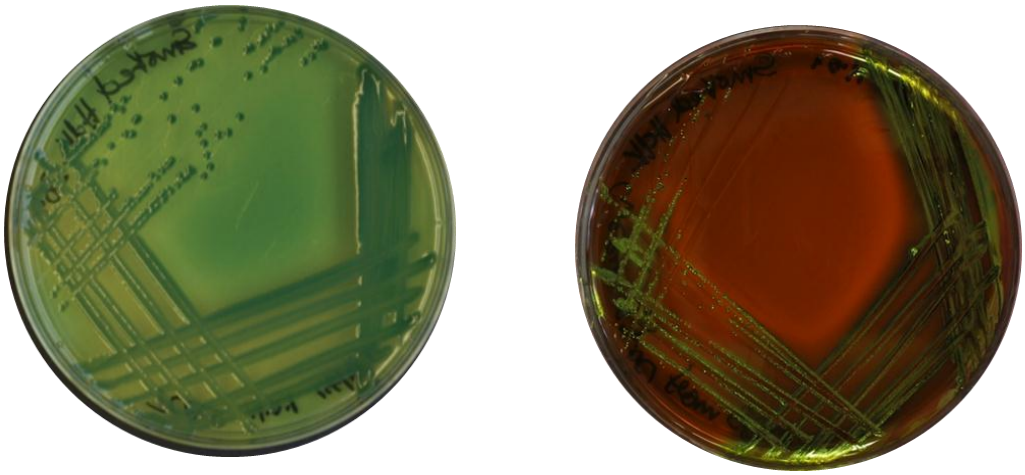


Figure 2.2. *Vibrio parahaemolyticus* on TCBS agar plate (panel A); *Escherichia coli* on EMBA plate (panel B).

2.17. Discussion

The presumptive *Vibrio* counts revealed counts ranged from 2.8 x 10^3 to 2.1 x 10^6 CFU g^{-1} (Table 2.1). However, the majority of the colonies on TCBS were equated with a wide range of taxa, including *Acinetobacter lwoffii, Acinetobacter johnsonii, Aeromonas hydrophila Aeromonas salmonicida* subsp. *achromogenes*, *Pseudomonas aeruginosa*, as well as representatives of *Vibrio parahaemolyticus*, *Vibrio metschnikovii* and *Vibrio fluvialis.* Of relevance, Farmer *et al*. (2003) reported the growth of *Pseudomonas* spp. and *Aeromonas* spp. on TCBS agar.

The *Enterobacteriaceae* counts from the seafood samples in this study (Table 2.2) were above the acceptable limits of 10 CFU g^{-1} as recommended by the International Commission on Microbiological Specifications of Foods (1986) for fresh, frozen and cold smoked fish. Thus, the data are in agreement with the results of Ghanem *et al*. (2014), who reported that the *Enterobacteriaceae* counts from marine fish fillets varied from 1.6 x 10^2 to 6.3 x 10^4 CFU g^{-1}, 2.1 x 10^2 to 4.3 x 10^4 CFU g^{-1}, 3.1 x 10^2 to 4.3 x 10^4 CFU g^{-1} and 2.3 x 10^2 to 1.1 x 10^5 CFU g^{-1} for *Epinephelus alexandrinus*, *Dicentrachus labrax*, stingray and *Scomberomorus commerson*, respectively.

The mean aerobic heterotrophic counts recovered from fresh and cold smoked salmon, fresh haddock and cold smoked haddock, mussels and oysters collected from outlets in Central Scotland were shown in Table 2.3. Bacteria numbers recovered from cold smoked salmon were within the acceptable limits 10^5 CFU g^{-1} as recommended by the International Commission on Microbiological Specifications of Foods (1986). Similarly, Tomé *et al*. (2006) revealed that the total viable count of cold-smoked salmon from two Scottish and two Spanish producers ranged between 10^2 and 10^4 CFU g^{-1}. In agreement, a lower range of bacterial counts were obtained by Al Ghabshi (2012) and Goja (2013). Their results revealed counts of 1.54 x 10^4 CFU g^{-1} in fresh fish and

2.8 x 10^3 to 9.8 x 10^4 CFU g^{-1} from the skin from fish. Similarly, the aerobic heterotrophic counts for mussels and oysters, ranged from 3.0 x 10^3 to 2.6 x 10^5 CFU g^{-1} at 30 °C, which were within the guidelines published by the Centre for Food Safety and Applied Nutrition (CFSAN, 2003).

The high bacteria numbers recovered from fresh salmon, fresh haddock and cold smoked haddock, (mussels and oysters at 20 °C) (Table 2.3) were above the acceptable limits according to the International Commission on Microbiological Specifications of Foods (1986). Similarly, Kapute *et al*. (2012), Adebayo-Tayo *et al*. (2012), and Noor *et al*. (2013) obtained high bacterial counts from a range of fish. These workers reported 9.5 x 10^8 CFU g^{-1} in Lake Malawi tilapia (*Chambo*), 1.0 x 10^4 to 1.1 x 10^6 CFU g^{-1} in fresh catfish (*Arius hendelotic*) Awka-Ibom State Nigeria and 1.1 x 10^6 to 1.7 x 10^9 CFU g^{-1} in surmai fish (*Scomberomorus guttatus*) from Dhaka city, Bangladesh, respectively. However, the method of sampling and transporting at 15 °C, may well have contributed to the high bacteria counts revealed in this study. Popovic *et al*. (2010) reported high bacterial counts on fish and fishery products involving a study of poor handling and sanitary conditions.

Some countries, including the USA, have zero tolerance for the presence of *L. monocytogenes* in food, specifically stating that the organism must not be detected in 25 g quantities of food (Huss *et al*., 2003). The European Commission (Regulation (EC) No 2073/2005) has set a limit of < 10^2 CFU g^{-1} for ready-to-eat foods available to the public in retail markets (Gelbíčova and Karpíšková, 2009). Therefore, the numbers recorded in this study (Table 2.4) exceeded the limits for both Europe and the USA. The actual *L. monocytogenes* counts recovered from mussels, ranged from 5.0 x 10^4 to 1.0 x 10^4 CFU g^{-1}. Other colonies grew on PALCAM agar plates as shown in Table 2.4. These colonies were yellow in colour and were equated with *Aerococcus viridans*

subsp. *homari* and *Shewanella baltica*. Also, Van Netten *et al*. (1989) demonstrated the growth of staphylococci and enterococci as yellow colonies on PALCAM agar, being attributed to the effect of mannitol-positive activity.

The range of organisms isolated from seafood has similarities with other work, e.g. Austin (2006) and Amoah *et al*. (2011). In particular, *Acinetobacter* has been reported on *Pangasius* fillet collected at the filleting, trimming and freezing steps (Thi *et al*., 2013). Various workers have reported the presence of *Acinetobacter* species in the gastrointestinal tract, gills, and on the surface of the flesh of farm raised fresh water fish (Austin, 2002; Hatha, 2002). Guardabassi *et al*. (1999) demonstrated the isolation of *Ac. johnsonii* and *Ac. lwoffii* from various aquatic environments including sewage, unpolluted streams, fish ponds, fish farm outlets, trout intestinal contents and frozen shrimps.

The genus *Aeromonas* belongs to the class *Gammaproteobacteria*, order *Aeromonadales* and family *Aeromonadaceae* (Martin-Carnahan and Joseph, 2005). *Aeromonas* spp. have been isolated previously from seafood samples (Palumbo *et al*., 1985; Joseph *et al*., 2013). Tsai and Chen (1996) and Vivekanandhan *et al*. (2005) reported a higher incidence of *A. hydrophila* in fishes compared to prawns, which agrees with the result of the present study having more *A. hydrophila* isolated from fish samples than from mussels. However, *A. hydrophila* are mesophilic (optimum temperature of 35-37 °C), non-pigmented, motile strains mainly associated with human clinical infections (Beaz-Hidalgo and Figueras, 2012). They cause major epidemic outbreaks (Figueras *et al*., 2011b; Beaz-Hidalgo and Figueras, 2012). *Aeromonas* species has been known as potential or emerging foodborne pathogens for more than 20 years and *A. hydrophila* has been occasionally associated with foodborne disease, the clinical manifestations being either extraintestinal (sepsis, meningitis, peritonitis,

endocarditis, pneumonia, ocular and urinary tract infections, septic arthritis, osteomyelitis and soft tissue infections) or gastroenteritis (Isonhood and Drake, 2002). The present work showed that isolates were detected at 20-30 °C and one of the isolates was nonmotile.

The presence of organisms identified putatively as *A. salmonicida* is interesting insofar as this is a fish pathogen (Austin and Austin, 2007). *A. salmonicida* is the causative agent of furunculosis, a disease that affects many species of fish and is an important cause of economic losses in the aquaculture of salmonids, eels, rainbow trout and trout (Austin and Adams, 1996; Sørum *et al*., 2000). These species includes psychrotrophic (optimum growth at 22-28 °C) nonmotile bacteria as well as mesophilic bacteria, pigmented and are principal fish pathogen (Janda and Aboot, 1996; Beaz-Hidalgo and Figueras, 2012). All *A. salmonicida* strains studied in this work were isolated at 20-30 °C and they were nonmotile. The isolates used in this work were obtained from obviously healthy fresh water fish and mussels sold in retail shops in Scotland. According to Wiklund and Dalsgaard (1998) fish habour the pathogen at the point of capture or become infected during transportation, and Ottaviani *et al*. (2011) reported that the wide-spread distribution of *Aeromonas* species in aquatic environments shows that their interactions with fish are continual and unavoidable enabling their opportunistic pathogenicity. *Aeromonas* spp. has been reported as one of the main spoilage flora fresh fish and seafood products stored aerobically at refrigeration temperature (Franzetti *et al*., 2003; Cardinal *et al*., 2004). The report of this work revealed *Aeromonas* spp. as being able to reduce TMAO to TMA. The later contributes to the characteristic ammonia-like and ‘fishy’ off-flavours (Gram and Dalsgaard, 2002). Certainly, *Aeromonas* spp. secrete toxins and enzymes that can affect host cells and can be important virulence factors. In particular, arylamidases, esterases, amylases, elastase,

deoxyribonuclease, chitinase, peptidases, haemolysin, proteases and lipases have been documented (Austin and Austin, 2007; Beaz-Hidalgo and Figueras, 2013; Dallaire-Dufresne *et al*., 2014).

Aerobic spore-forming bacteria belonging to the genus *Bacillus* and closely related genera such as *Paenibacillus* or *Brevibacillus* are ubiquitous in the environment and the widespread occurrence of sporulated bacteria is almost unavoidable in some raw food products (Coton *et al*., 2011). The presence of *Bacillus* is not surprising insofar as the genus has been previously associated with fish. Coton *et al*. (2011) isolated *Bacillus* spp. from surimi seafood products. Akinyemi and Ajisafe (2011) demonstrated the presence of *Bacillus* spp. from the skin, bucal cavity and gills of *Chrysichthys nigrodigitatu.* Rahmati and Labbe (2008) reported *B. cereus* from fresh and processed retail seafood samples. Overall, *Bacillus* spp. is regarded as ubiquitous in aquatic ecosystems and can be introduced into food during processing (Fernández-No *et al*., 2011). Some species have been involved in food spoilage such as *B. licheniformis* (ex. slime production in kamaboko; (Mori *et al*., 1973), *B. subtilis* and *B. pumilus* (Pepe *et al*., 2003) and in food intoxication cases in particular *B. cereus* (Ehling-Schulz *et al*., 2004; Dierick *et al*., 2005), likewise *B. licheniformis* (Salkinoja-Salonen *et al*., 1999), *B. subtilis* (Kramer *et al*., 1982) and *B. pumilus* (Lund, 1990).

Brochothrix has been recovered previously from fish and *Brochothrix thermosphacta* was isolated at all stages of in the production of finfish from the Gulf of Mexico, although the proportion of the total microflora rarely exceeded 5% (Nickelson *et al*., 1980). Moreover, examination of vacuum-packed cold-smoked salmon and trout has revealed a low number of *Brochothrix* spp. (Gonzalez-Rodriguez *et al*., 2002). *B. thermosphacta* was recovered from fresh fish (Olofsson *et al*., 2006), and it is recognised that the organism has an important role in the spoilage of fish (Pin *et al*.,

2002) and shrimp (Fall *et al*., 2010). Specifically, *B. thermosphacta* is responsible for off-flavours, discolouration and gas production (Braun and Sutherland, 2004), and a pungent 'cheesy' odour (McClure *et al*., 1993).

Kim and Austin (2006) isolated carnobacteria from the digestive tract content of rainbow trout (*Oncorhynchus mykiss*, Walbaum). Dalsgaard *et al*. (2003) reported the presence of carnobacteria in chilled fresh and lightly preserved seafood. Also, *Carnobacterium* spp. were isolated from seafood (cod, halibut, salmon, shrimps and roe products) (Laursen *et al*., 2005). The growth and/or presence of high numbers of *C. maltaromaticum* have been connected with spoilage of, frozen/thawed and modified atmosphere packed fish (Emborg *et al*., 2002; Mejlholm *et al*., 2004), and various lightly preserved seafoods (Jørgensen Dalsgaard *et al*., 2000b; Dalsgaard *et al*., 2003). *C. maltaromaticum* strains may be used as protective cultures in biopreserved meat and seafood (Laursen *et al*., 2005).

Enteric bacteria were isolated from fresh and smoked salmon, fresh and smoked haddock in this study. Certainly, *Citrobacter* and *Enterobacter* have been recovered previously from cultured freshwater fish (Hassan *et al*., 2012). In addition, Ghanem *et al*. (2014) recovered *Enterobacter cloacae* from *Dicentrarchus labrax* and stingray fish fillets whereas Tavakoli *et al.* (2012) and Gupta *et al*. (2013) isolated *E coli* from fresh fish and ready-to-eat fish products. *Serratia,* notably *Serratia liquefaciens* has been isolated as a predominant Enterobacteriaceae representative in fish processing (Gudbjörnsdóttir *et al*., 2005). To some extent, the presence of enteric bacteria in fish products may represent cross contamination during handling (Gonzalez-Rodriguez, *et al.*, 2001).

Addis *et al*. (2010) demonstrated the presence of *Moraxella* in the tissues of gilthead sea bream (*Sparus aurata*, L.). In addition, Austin and Austin (2007) revealed the presence of *Moraxella* among fish tissues.

Pseudomonas spp. have been regularly associated with fish tissues. Eissa *et al*. (2010) isolated *Pseudomonas* from gills, liver, kidneys and spleen, whereas Oladipo and Bankole (2013) demonstrated the presence of the organisms in fresh and dried *Clarias gariepinus* and *Oreochromis niloticus*. Kapute *et al.* (2013) reported the presence of *Pseudomonas* in high numbers from tilapia obtained from Lake Malawi after 16 days storage. The relevance is that *Pseudomonas* may exert an important role in spoilage (Mahalaxmi *et al*., 2013). It has been stated that the presence of *Pseudomonas* represents a health hazard when populations exceeds 10^6-10^7 CFU g^{-1} of product (Mena and Gerba, 2009; Craun *et al*., 2010).

Psychrobacter are common especially on salted fish, chilled fish, some shell fish and fermented seafood (Gonzalez *et al*., 2000b; Bagge-Ravn *et al*., 2003; Bjorkevoll *et al*., 2003; Yoon *et al*., 2003) therefore their presence in this study is not surprising. *Psychrobacter* may be involved in spoilage resulting in a musty off-odour, usually after the fish has been stored in the cold for 7-10 days (Bjorkevoll *et al*., 2003).

Shewanella has been recovered from oysters **(**Richards *et al*., 2008). Moreover, *Sh. baltica* has been isolated from ice-stored Danish marine fish (Gram *et al*., 1987). Vogel *et al.* (2005) demonstrated the presence of *Shewanella* spp. on newly caught fish. Using 16S rDNA sequencing, they revealed that *Sh. baltica* was the main species, which is in agreement with the present study. Yet, *Shewanella putrefaciens* is regarded a significant spoilage organism of iced fish and fish products, and is often isolated from poultry meat and beef products (Stenström and Molin, 1990; Rudi *et al*., 2004). They respire

anaerobically using several electron acceptors, and most strains reduce trimethyl-amine-N-oxide (TMAO) (Brettar *et al*., 2002).

Ananchaipattana *et al*. (2012) reported the isolation of *Staphylococcus lentus*, *S. sciuri* and *S. xylosus* from fish and seafood samples from Thailand. *S. aureus* was isolated from fresh and smoked silver carp, and smoked shad from Iran (Tavakoli *et al*., 2012). According to Himelbloom and Crapo (1998), smoked and dried king salmon processed by Alaska Natives contain coagulase-negative *Staphylococcus* spp. This is in agreement with the report of the FDA (2001) that considered that most of the staphylococci from fish are coagulase-negative, namely, *S. epidermidis, S. xylosus, S. lentus, S. capitis, S. lugdunensis, S. hominis, S. warneri, S. cohnii and S. chromogenes.* Their presence in fish may suggest post-harvest contamination due to poor hygiene (Austin and Austin, 2007).

The recovery of *Listeria* in bivalves is not surprising in view of previous work (Vinothkumar, *et al*., 2013; González *et al*., 2013). (Pinto *et al*. (2006) described the incidence of listeria in live bivalve molluscs from the North of Portugal. Similarly, *L. monocytogenes* was reported in farmed mussels harvested from approved production sites in the North Aegean Sea (Soultos *et al*., 2014). The significance of finding *L. monocytogenes* is that there is a risk to human health, i.e. as listeriosis (Farber and Peterkin, 2000). This may be serious in high risk groups, such as the elderly, individuals with lowered immunity, pregnant women and new born infants (Gelbíčová and Karpíšková, 2009).

Vibrios, notably *V. parahaemolyticus,* are common in seafood (e.g. Adeleye *et al*., 2010; Adebayo-Tayo *et al*., 2011). Adeleye *et al*. (2010) showed that *V. parahaemolyticus* were detected in seafood (11.4% of samples) collected in Nigeria. In comparison, Bauer *et al*. (2006) detected *V. parahaemolyticus* in 10.3% of blue mussels

collected in Norway from July 2002 to September 2004 from 102 production sites. It is argued that filter feeders, notably mussels and oysters, are more liable to bacterial contamination because they filter micro-organisms of which vibrios are commonplace in the coastal environment (Popovic *et al*., 2010; Merwad *et al*., 2011). Populations of *V. parahaemolyticus* of 10^4 CFU g^{-1} are regarded as potentially hazardous to human health (Aberoumand, 2010b). *V. parahaemolyticus* is causing sporadic foodborne infections and outbreaks worldwide, with gastroenteritis being the most common clinical manifestation (Papadopoulou *et al*., 2007). Thus, the vibrio populations recovered in this study are of concern, especially as the estimated numbers *of V. parahaemolyticus* in mussels were 2.1 x 10^6 CFU g^{-1}.

Furthermore, the recovery of putative *V. metschnikovii* is of concern because of its association with disease (Linde *et al*., 2004; Wallet *et al*., 2005).

Vibrio spp. produce a range of putative virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and haemagglutinins (Zhang and Austin, 2005). The results of this study revealed that *Vibrio* spp. from mussels and oyster produced β-haemolysis, whereas those isolated from smoked salmon and fresh haddock demonstrated α-haemolysis. The virulence of *V. parahaemolyticus* is commonly connected with the expression of thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), which are encoded by *tdh* and *trh* genes, respectively (Nishibuchi and Kaper, 1995). Consequently, *tdh* gene, marked by a β -type haemolysis on Wagatsuma agar (Nishibuchi and Kaper, 1995), and *trh* gene, correlate to a positive urease tests (Okuda *et al*., 1997), can serve as markers for pathogenic strains. In this respect, urease activity was recorded in this study. Furthermore, the β -haemolytic vibrios recovered in this study produced phospholipase

in comparison with the observations of Zhang and Austin (2005), who discussed is a correlation between haemolysis and enzymatic activities notably phospholipase.

In the present study, the prominent characteristics of fish spoilage isolates were the ability to reduce trimethylamine oxide (TMAO) to trimethylamine and to produce H_2S. In agreement with the present study, Serio *et al*. (2014) demonstrated strong production of TMA and H_2S by *Shewanella* spp. However, some of the *Shewanella* in the previous study did not produce both compounds, whereas this study showed late producers of H_2S. Furthermore, Serio *et al*. (2014) reported the moderate production of these compounds by some *Serratia* spp. and *Aeromonas* spp., which is in agreement with the result of this study. Similarly, Gram and Dalsgaard (2002) and Gram (2009) reported that *Aeromonas* spp., psychrotolerant *Enterobacteriaceae* representatives, *Ph. phosphoreum*, *Sh. putrefaciens*-like organisms and *Vibrio* spp. could reduce TMAO to TMA, which is in agreement with the results of this study. Pseudomonads have been associated with the production of dimethyldisulphide $(CH_3)_2S_2$ and sulphhydryl off-odours (Gram, 2009) but not production of TMA and H_2S, whereas in this study a pseudomonad from fresh salmon produced TMA and H_2S. Indeed, all of the spoilage microorganisms recovered in this study produced H_2S from cysteine and sodium thiosulphate together within three days, with the late producers requiring 4-5 days.

In conclusion, the results from the current study suggest that potential pathogenic and spoilage microorganisms are present in seafood. Seafood safety, which differs according to products, is predisposed to a number of factors such as fish origin, product characteristics, handling and processing practices and preparation before consumption (Huss *et al*., 2000). Adequate cooking of the seafood samples will inactivate the microorganisms, improper handling and cross contamination or raw seafood eating

habits, might pose a health hazard, especially to susceptible populations such as the immunosuppressed, pregnant women, children and elderly people especially as regards *L. monocytogenes* (Papadopoulou *et al*., 2007). Hazard Analysis and Critical Control Points (HACCP) should be used in all stages of food production and preparation processes including packaging and distribution to identify potential food safety hazards.

Chapter 3

Detection, characterization and partial purification of bacteriocin-like substances produced by *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789.

3.1. Introduction

3.1.1. Use of lactic acid bacteria and their bacteriocins

Lactic acid bacteria (LABs) are widely used for the production of fermented food products, their role being to improve the flavour, texture and shelf life. The following genera are regarded as belonging to the LAB group: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Ghanbari *et al*., 2010). The production of diverse antibacterial compounds, such as organic acids, diacetyl, hydrogen peroxide and proteinaceous molecules, known as bacteriocins, leads to the inhibition of food spoilage and pathogenic bacteria (O'Sullivan *et al*., 2002; Rajaram *et al*., 2010). Bacteriocins are produced by LABs **(**Zacharof and Lovitt, 2012). Bacteriocins are antimicrobial peptides synthesized by ribosomes of bacteria, which have the characteristics of inhibiting other bacteria (Maria and Janakiraman, 2012; Zacharof and Lovitt, 2012). Matamoros *et al*. (2009) demonstrated that the cell-free culture supernatant of *Carnobacterium alterfunditum* (EU2257) revealed activity when spotted against *Lactobacillus farciminis, Brochothrix thermosphacta, Shewanella putrefaciens, L. monocyogenes, Staphylococcus xylosus, Pseudomonas* spp., *Serratia liquefaciens* and *Staphylococcus aureus*. Many bacteriocins have shown strong activity against food spoilage and pathogenic bacteria, such as *Bacillus, Listeria, Clostridium,*

methicillin-resistant *Staphylococcus aureus* and vancomycin resistant enterococci, often at concentrations much lower than conventional antibiotics (Gillor *et al*., 2008; Bierbaum and Sahl, 2009). Several workers have reported *in vitro* efficiency of various bacteriocins in reducing the numbers of viable cells of *L. monocytogenes* and other potential pathogens in various food products (Nilsen *et al*., 1990; Richard *et al*., 2003; Nilsson, *et al*., 2004; Tahiri *et al*., 2009). Nisin, produced by *Lactococcus lactis* has been available and widely used since 1959 to prevent spoilage of many foods (Fowler and Gasson, 1991; De Vuyst and Vandamme, 1994a; Jack *et al*., 1995). Nevertheless, the antimicrobial activity of nisin only under acidic conditions has limited its use (Chung *et al*., 1989; Liu and Hansen, 1990). Pediocins produced by *Pediococcus acidilactici* (Henderson *et al*., 1992b) and *Pediococcus cerevisiae* (Langeveld *et al*., 1993) are cited in patents for applications in foods with a particular focus on anti-listerial activity. Various studies have shown that non-bacteriocinogenic lactic acid bacteria have the ability to control the growth of *L. monocytogenes* in food products (Buchanan and Bagi, 1997; Nilsson *et al*., 1999; Nilsson *et al*., 2004; Tahiri *et al*., 2009) although the definite mode of action of inhibition is unknown.

With the expansion of demand for seafood products, there have been considerable challenges related to preservation/spoilage (Devlieghere *et al*., 2004). LABs are generally regarded as safe (GRAS) for use in foods. Bacteriocins produced by LABs have been planned for future use in the biopreservation of food products by preventing spoilage microorganisms or pathogens (Cleveland *et al*., 2001). In addition, nisin produced by *Lactococcus lactis* is the only bacteriocin with GRAS grade for use in some foods (Rattanachaikunsopon and Phu mkhachorn, 2010), nevertheless pediocin-like bacteriocins may be supplemented to foods when produced by organisms used to

manufacture GRAS ingredients such as cultured milk or cultured whey that are accepted as food ingredients (Deegan *et al*., 2006).The ability of these compounds to change the quality and shelf life of fish products have been demonstrated by various groups (e.g. Katla, *et al*., 2001; Nilsson *et al*., 2004; Brillet *et al*., 2005; Calo-Mata *et al*., 2008). Some of the studies involve use of combinations of bacteriocins and other technologies (Zuckerman and Bem Avraham, 2002; Al-Holy *et al*., 2004; Elotmani and Assobhei, 2004).

Elotmani and Assobhei (2004) demonstrated the antibacterial effects of nisin and lactoperoxidase system (LP system) alone or combined using the agar diffusion method against bacterial strains isolated from sardines (*Sardina pilchardus*). Their results revealed the inhibition of only Gram-positive bacteria by nisin, whereas all the bacteria strains were inhibited by LP system. Furthermore, a significantly more effective result was realised when nisin and LP system were combined at 100 IU $ml^{-1}$and 10 mg l^{-1} respectively, inhibiting allstrains, except *Aeromonas salmonicida* subsp. *salmonicida* and *Vibrio alginolyticus*.

Nykanen *et al*. (2000) revealed the reduction in the count of *L. monocytogenes* from 3.3 to 1.8 log_{10} during 16 days of storage at 8 °C, by injecting vacuum packed-cold smoked rainbow trout with nisin and sodium lactate combined.

Bacteriocins are generally divided according to their chemical and genetic characteristics (Ennahar *et al*., 2000; Garneau *et al*., 2002; Cotter *et al*., 2005). Class I bacteriocins (= lantibiotics), including nisin, are characterized by the presence of post-translationally modified amino acid residues, such as lanthionine (Asaduzzaman and Sonomoto, 2009). Class II bacteriocins include no lanthionine and are further divided into four subgroups. Class IIa bacteriocins, i.e. pediocin-like bacteriocins, are low-molecular-weight, heat-stable peptides comprising the N-terminal region motif

YGNGVXC, referred to as the pediocin box, and are highly active against *L. monocytogenes* (Richard *et al*., 2003; Drider *et al*., 2006). Class IIb bacteriocins, such as lactococcin Q, comprise two-peptide bacteriocins, whose entire antimicrobial activities demand the presence of both peptides in equal amounts (Zendo *et al*., 2006). Class IIc bacteriocins, such as enterocin AS-48 (Galvez *et al*., 1986) and lactocyclicin Q (Richard *et al*., 2003; Sawa *et al*., 2009), are circular, large, heat labile protein bacteriocins. Class IId bacteriocins include other class II bacteriocins, such as lacticin Q (Fujita *et al*., 2007).

3.1.2 Presence of *Carnobacterium* species in seafood products

Carnobacteria are LABs that are found in various environments, such as fish and meat (Ringø and Olsen, 1999; Ringø and Holzapfel, 2000; Gonzalez *et al*., 2000a; Leisner *et al*., 2007). High concentrations of *Carnobacterium divergens* and *Carnobacterium maltaromaticum* are common in halibut, rainbow trout, salmon and surubim (*Pseudoplatystoma coruscans*), being detected in vacuum-packed cold-smoked or sugar-salted ('gravad') seafood categorized by salt content of 3-7% NaCl and a pH of 5.8-6.5 (Jørgensen *et al*., 2000a, b; Lyhs *et al*., 2002; Alves *et al*., 2005; Emborg and Dalsgaard, 2006). Carnobacteria are commonly found in chilled fresh and lightly preserved seafood. The presence of *C. divergens* and *C. maltaromaticum* has been demonstrated for modified atmosphere-packed (MAP) coalfish, cod, pollack, rainbow trout, salmon, shrimp and surubim (Franzetti *et al*., 2003; Rudi *et al*., 2004; Emborg *et al*., 2002, 2005). The ability of *Carnobacterium* spp. to grow and produce bacteriocins with high anti-listerial activity at low and high sodium concentration has focused the attention of food scientists (Buchanan and Bagi, 1997).

3.1.3. Mode of action of Class IIa bacteriocins

The class IIa bacteriocins show anti-listerial activity and kill target cells by permeabilizing the cell membrane, this leads to the disturbance of the proton motive force (Herranz *et al*., 2001). Class IIa bacteriocins exhibit cationic, and partly amphiphilic and /or hydrophobic properties. The primary structures of the peptide chains reveals the N-terminal region which is relatively hydrophilic and conserved, whereas the C-terminal region is hydrophobic and diverse (Nissen-Meyer *et al*., 2009). The Gram-positive bacterial envelop comprises peptidoglycan layers, S-layers, the phospholipidic membrane and proteins and flagellae, forming a common boundary between the bacteria and its external environment (Moll *et al*., 1999). The anionic cell surface polymers such as teichoic acid and lipoteichoic acid play a role in the initial interaction with cationic bacteriocins (Jack *et al*., 1995; Kazazic *et al*., 2002). The amphiphilic/hydrophobic property allows the addition of a hydrophobic C-terminal domain into the hydrophobic core of target membranes and makes permeability possible. Consequently, there are interactions between lipids and/or proteins, such as membrane-associated mannose phosphotransferase system permease, which causes bacteria sensitivity to class IIa bacteriocin when present (Héchard *et al*., 2001).

There is between 37 to 48 residues of the all the pediocin-like antimicrobial peptides (AMPs) and the conserved Y-G-*N*-G-V/L ‘pediocin box’ motif and two cysteine residues joined by a disulphide bridge are components of the N- terminal region. The N- terminal half have like primary structures and the C-terminal half are rather diverse they are assembled into three subgroups giving to sequence similarities and differences in this half of the peptides. Despite wide sequence similarities, these AMPs vary distinctly in their target-cell specificity, and results gotten with hybrid AMPs show that

the membrane-penetrating hairpin-like *C*-terminal domain is the main specificity determinant (Fimland *et al*., 2005).

Notwithstanding their effective uses, one important limitation is that the majority of bacteriocins produced by Gram-positive bacteria are not active against Gram-negative bacterial pathogens (Cotter *et al*., 2005; Deegan *et al*., 2006; Gillor *et al*., 2008).

3.1.4. Bacteriocins produced by *Carnobacterium* spp.

Various bacteriocins produced by *Carnobacterium* spp. have been isolated and characterized, including

- carnobacteriocin Cbn BM1 and Cbn B2 which is produced by *C. maltaromaticum* CP5 that was isolated from a French mould ripened cheese (Herbin *et al*., 1997; Jacquet *et al*., 2012).
- divergicin M35, produced by *C. divergens* M35 which was isolated from frozen smoked mussels (Tahiri *et al*., 2004),
- carnocyclin A, a novel circular bacteriocin produced by *C. maltaromaticum* UAL307 that was isolated from fresh pork (Martin-Visscher *et al*., 2008).
- carnocyclin CclA, carnobacteriocin Cbn BM1 and piscicolin 126 PisA by *C. maltaromaticum* UAL307 (Martin-Visscher *et al*., 2011).
- carnobacteriocin B2 produced by *C. piscicola* strain A9b was isolated from vacuum packed cold-smoked salmon (Paludan-Müller *et al*., 1998).
- divergicin A was produced by *C. divergens* NCIMB 702855 (Worobo *et al*., 1995). Divercin V41 was recovered from *C. divergens* 41 that was isolated from fish viscera (Métivier *et al*., 1998).
- piscicocin CS526 was produced by *C. piscicola* CS526, which was isolated from frozen surimi (Yamazaki *et al*., 2003).

Overall, not many bacteriocin producing strains and their bacteriocins have been recovered, identified and characterized from fish and seafood products. However, the gaining of these strains familiar to the seafood environment would be useful for biopreservation (Tahiri *et al*., 2004; Pinto *et al*., 2009; Chahad *et al*., 2012).

3.2. Aims of this study

The aims of the present Chapter were to detect, characterize and partially purify bacteriocins produced by *C. maltaromaticum* MMF-32 and *C. maltaromaticum* KOPRI 25789, isolated from smoked salmon, and to determine their inhibitory spectra against food-borne pathogens and food spoilage organisms.

3.3. Materials and methods

3.3.1. Statistical analysis

Statistical analysis involved use of IBM SPSS. A Kruskal-Wallis test was run to determine if there were differences in different bacteriocin samples and the treatments given to the groups. Pairwise comparisons were performed using Dunn's (1964) procedure with Bonferroni correction for multiple comparisons. Statistical significant level was fixed to $p < 0.05$.

3.3.2. Determination of the antibacterial activity of cell free supernatant from lactic acid bacteria (LAB) using bacteria indicator strains

The *in vitro* antibacterial capacity of cell free supernatant (CFS) from the two LAB cultures; *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 were determined for inhibition of the growth of *Shewanella baltica* OS185, *Aeromonas* sp. HB-6, *Shewanella baltica*, *Shewanella baltica* OS678, *Serratia* sp. I-113-31, *Aeromonas salmonicida* subsp. *achromogenes*, *Aeromonas hydrophila* HX201006-3 and *Listeria monocytogenes* ATCC 19114.

3.3.3. Extraction of LAB cell free supernatant

Carnobacterium maltaromaticum MMF-32 and KOPRI 25789 were grown in 500 ml volumes of MRS broth at 30 °C for 48 h. The optical density (OD) was 0.6. The bacterial cells were removed by centrifugation (10,000 x g for 10 min at 4 °C), and cell free supernatants were passed through 0.45 µm pore size filters (Sartorius; Stedim Biotech, GmbH Goettingen, Germany). To rule out the possibility that the inhibition might have been caused by acidification of the media induced by LAB metabolism, cell free supernatants were adjusted with 2 N NaOH to pH 6.5 ± 0.2 and filtered by passage

through 0.45 µm of pore size filters. Tenfold serial dilutions of cell-free supernatants were made using TNB to two successive dilutions.

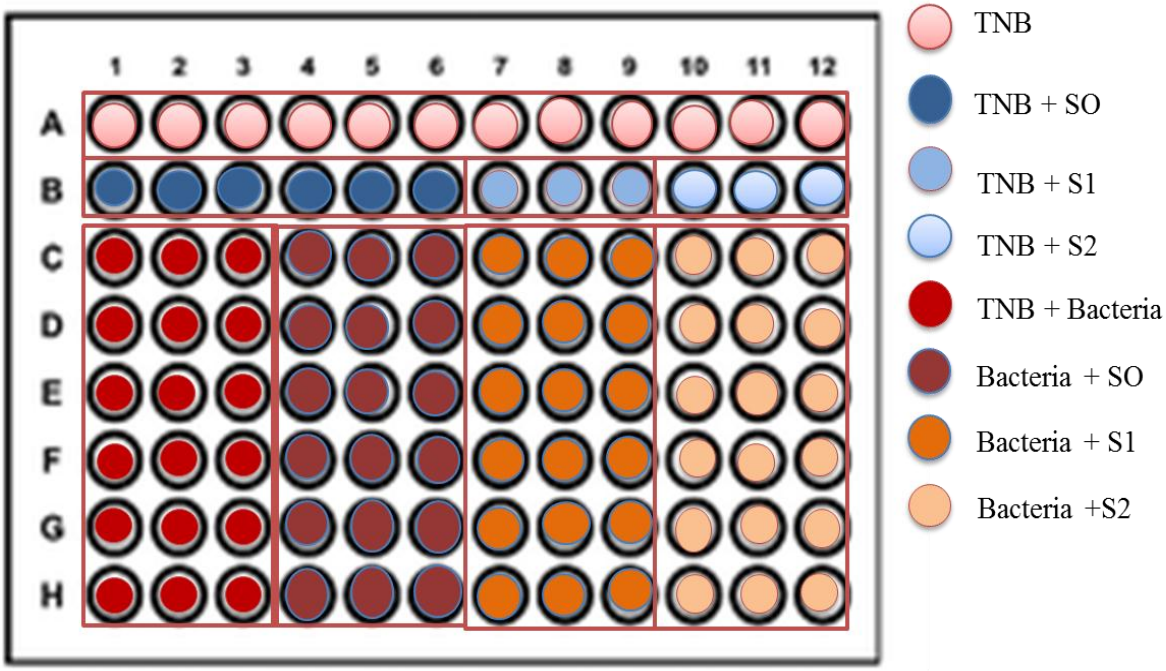
3.3.4. Preparation of bacterial indicator

Bacterial strains used as indicator were *Shewanella baltica* OS185, *Aeromonas* sp. HB-6, *Shewanella baltica*, *Shewanella baltica* OS678, *Serratia* sp. I-113-31, *Aeromonas salmonicida* subsp. *achromogene*s, *Aeromonas hydrophila* HX201006-3 and *Listeria monocytogenes* ATCC 19114. These bacterial indicators were incubated overnight at 30 °C in TNB. The overnight bacterial cultures of the indicator strains were centrifuged at 2000 g for 10 min at 4 °C, and the cell free supernatants were removed. The bacterial cells were washed twice in 0.85% (w/v) sterile saline, and serial dilutions of the washed bacterial cells were prepared in saline to realize10^5 CFU ml^{-1} as determined by Nikoskelaien *et al*. (2001).

3.3.5. Broth bioassays

Broth assays were performed as follows: 100 µl volumes of the serially diluted cell free supernatants from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 were pipetted into triplicate wells in 96-well microtitre plate together with 100 µl volumes of indicator bacteria containing 10^5 CFU ml^{-1}. Incubation of the microtitre plates was at 30 °C for 40 h and the presence of turbidity in the wells, was considered as indicative of the absence of inhibition. Hundred microlitres of positive and negative controls were also tested. Growth inhibition was measured spectrophotometrically at 600 nm and 610 nm every 8 h for 40 h with a microtitre plate reader (Fisher Scientific; BioTek, USA). Cell free supernatant extracts from the nisin-producer *Lactococcus lactis* subsp. *lactis* NCIMB 8586 were used against indicator bacteria as comparable controls.

The plate layout for the broth assay (Fig. 3.1.) is explained as follows; TNB was used as negative controls for the undiluted (TNB + SO) and diluted supernatants (TNB + S1 (1:10 dilution) and TNB + S2 (1:100 dilution)) to ensure that the supernatants were sterile. TNB + bacteria was used to assess the growth curve of the indicator bacteria. Bacteria + SO, bacteria + S1 and bacteria + S2, were the indicator bacteria with different dilutions of the cell-free supernatants of the bacteriocin producing strain, to detect their inhibitory effects.



TNB = Tryptone soya broth (TSB; Oxoid) supplemented with 1% sodium chloride [=TNB]
SO = Undiluted supernatant; S1 = First diluted supernatant; S2 = Second diluted supernatant
Bacteria = Any of the indicator strains

Figure 3.1. Diagram of the 96-well microtitre plates and the arrangement of samples on them.

3.4. Characterization of antimicrobial compounds

The tests were carried out with cell free supernatant extracts from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h.

3.4.1. Effect of thermal treatment on bacteriocin activity

The effect of temperature on cell free supernatant extracts was determined as follows: the cell free supernatant extracts in sterile Bijoux bottles were placed in a water bath (Grant Instruments; Cambridge, Barrington, England), and a thermometer was inserted into one of the Bijoux bottles in the water bath to enable reading the temperature of the supernatant directly. The cell free supernatant was heated to 56 °C using a water bath

for 30 min. A stop watch was used to take the reading for 30 min when the temperature was steady at 56 °C.

For heating at 100 °C for 10 min, sterile 1.5 ml Eppendorf tubes containing the cell free supernatants were heated on a heating block (Techne; Cambridge, England). The temperature of the cell free supernatant was increased to 100 °C by inserting a thermometer directly into one of the tubes containing the cell free supernatant, and the tubes were placed in the hot block. The supernatant was heated to 100 °C after 10 min. A stop watch was used to take the reading for 10 min when the temperature was steady at 100 °C.

3.4.2. Effect of pH range on bacteriocin activity

To check pH stability of the bacteriocins, the supernatant extracts from the two lactic acid bacteria were adjusted to pH 3.5, 4.9 and 6.5 by adding appropriate volumes of 2 N HCl or 2 N NaOH. The pH value of 4.9 was chosen because during the extraction of the supernatant the pH values ranges from 4.9 to 5.15. pH 3.5 was chosen to observe its effect on the indicator strains, and the supernatant was neutralized to pH 6.5 to check the effect of the neutralized supernatant on the indicator strains. Then, the samples were filtered through 0.45 µm filters (Sartorius), and antimicrobial activity was determined as described above. Negative controls aimed at making clear the possible role of acid pH values in the inhibition of *L. monocytogenes,* were prepared testing portions of non-inoculated MRS broth whose pH values were adjusted to 3.5, 4.9 and 6.5 (Ponce *et al*., 2008).

3.4.3a. Sensitivity of cell-free supernatants to proteases

The sensitivity of neutralized (i.e. cell-free supernatants adjusted to pH 6.5 using 2N NaOH) cell-free supernatants extracts to proteolytic digestion was investigated by the

addition of proteinase K, trypsin, α-chymotrypsin (Sigma-Aldrich) and lysozyme (Fluka) at a final concentration of 1 mg ml^{-1} (Ammor *et al*., 2005). Samples with and without proteases were incubated aerobically for 3 h at 30 °C, and the residual activity was determined as described above. The presence of turbidity in the presence of proteases indicates absence of inhibition confirming the proteinaceous nature of the antimicrobial substances (Lewus *et al*., 1991).

3.4.3b. Sensitivity of bacteriocin-producing strains to bovine liver catalase

In order to determine whether antimicrobial activity might be derived from the production of hydrogen peroxide by *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789, 300 IU ml^{-1} of bovine liver catalase (Sigma-Aldrich) was added to 1 ml portions of the cell-free supernatant extracts of the LAB exhibiting antimicrobial activity, and the mixtures were incubated at 37 °C for 24 h before the treated extracts were subjected to broth assay, as described above. The untreated cell-free supernatants were used as controls, and were tested in parallel.

3.4.3c. Screening for enzyme activities in LAB extracellular extracts.

(i) Gelatinase activity was recorded after (Loghothetis and Austin, 1996). Gelatin agar was prepared using the method of (Smith and Goodner, 1958). The medium was heated to dissolve the gelatin (without boiling). Once the gelatin was dissolved, the agar was added and heated to dissolve (a brief boiling was carried out as necessary), then autoclaved at 121 °C for 15 min. Twenty µl portions of each undiluted supernatant from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h in MRS broth without shaking – were placed in 6 mm diameter wells which were cut in the agar plates. After incubation at room temperature and at 4°C in the

refrigerator for 2 days, the plate was flooded with saturated ammonium sulphate; the presence of zones of clearing around the wells indicating enzyme activity.

(ii) Casein activity modified from Hastings (1903). A 10% aqueous solution of skimmed milk (Marvel®) Premier International Foods (UK) Ltd, powder was used. The skimmed milk was autoclaved at 121 °C for 5 min. Fifty ml of the skimmed milk was added to 100 ml of melted and cooled to ~50°C TSA, mixed and poured unto sterile petri dishes. Twenty µl portions of each undiluted supernatant from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h in MRS broth without shaking were placed in 6 mm wells which were cut in the agar plates. After incubation at room temperature and at 4°C in the refrigerator for 2 days, the presence of a zone of clearing around the well indicates enzyme activity.

(iii) Starch hydrolysis: TSA was prepared and 0.2% soluble starch was added before boiling, followed by autoclaving at 115 °C for 10 min. Twenty µl portions of each undiluted supernatant from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h in MRS broth without shaking were placed in 6 mm wells which were cut into the agar plates. After incubation at room temperature and at 4°C in the refrigerator for 2 days, the plates were flooded with iodine solution. A positive result was indicated by a colourless area around the well. The medium became blue where starch was not hydrolysed.

(iv) Haemolytic activity against defibrinated sheep blood in Alseviers solution (Oxoid) was examined. Thus, 25 ml of 5% sheep blood was added to 500 ml of melted cooled (45°C) TSA, mixed gently and poured unto sterile petri dishes. Twenty µl portions of each undiluted supernatant from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 strains grown at 30 °C for 48 h in MRS broth without shaking were

placed in 6 mm wells with incubation at room temperature and at 4°C for 2 days. The presence of zones of clearing or opalescence around the well following was recorded as evidence of haemolysis.

3.5. Preparation of concentrated bacteriocin from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789

The crude preparations of bacteriocins used were the supernatant fractions obtained after centrifugation (10,000 x g, 10 min, 4 °C) of cultures of *C. maltaromaticum* strains MMF-32 and KOPRI 25789 inoculated in MRS broth incubated at 30 °C for 48 h. Concentrated preparations of bacteriocins were prepared from crude preparations by precipitation with solid $(NH_4)_2SO4$ to a final concentration of 516 g l^{-1} at 4 °C for 1 h with stirring. The precipitate was collected by centrifugation at 20,000 x g for 30 min at 4 °C, redissolved in 50 mM potassium phosphate buffer (pH 5.8), and filter sterilized (pore size, 0.45 µm; type Minisart NML; Sartorius) before its spectrum of activity was determined (Jack *et al*., 1996).

3.6. Quantification of total protein with BCA protein assay kit

The Thermo Scientific Pierce bicinchoninic acid (BCA; Thermo Scientific, Rockford USA) Protein Assay is a detergent-compatible formulation based on BCA for the calorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with highly sensitive and selective colorimeteric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing BCA (Smith *et al*., 1985). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg ml^{-1}). Accordingly, protein concentration of the cell free supernatants,

concentrated concentrated bacteriocin and semi purified from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 were generally determined and reported with reference to standards of a common protein such as bovine serum albumen (BSA; Thermo Scientific, Rockford USA). A series of dilutions of known concentration were prepared from BSA and assayed alongside the unknowns that is the cell free supernatants, concentrated concentrated bacteriocin and semi purified from *Carnobacterium maltaromaticum* MMF-32 and KOPRI 25789 before concentration of each unknown is determined based on the standard curve.

3.7. Purification of bacteriocin

Culture supernatants were obtained by centrifugation (10,000 x g, 10 min, 4 °C) of *C. maltaromaticum* strains MMF-32 and KOPRI 25789 inoculated onto MRS broth incubated at 30 °C for 48 h. Concentrated preparations of bacteriocins were prepared from crude preparations by precipitation with solid $(NH_4)_2SO_4$ to a final concentration of 516 g l^{-1} at 4 °C with stirring for 1 h. The precipitate was collected by centrifugation at 20,000 x g for 30 min at 4 °C, redissolved in 50 mM potassium phosphate buffer (pH 5.8), and filter sterilized (pore size, 0.45 µm; type Minisart NML; Sartorius) (Jack *et al*., 1996). The concentrated ammonium sulphate precipitate was equilibrated to the composition of binding buffer (50 mM sodium phosphate and 1.0 M ammonium sulphate) by adjusting to pH 7.0 and filtered through 0.45 µm filter. The column was equilibrated (to rehydrate the column) with binding buffer (50 mM sodium phosphate and 1.0 M ammonium sulphate, pH 7.0) and the elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1}. Sixty-five ml of concentrated ammonium sulphate precipitate was applied to hydrophobic interaction chromatography (HIC) column Hi Trap™ Octyl sepharose FF column (1 ml) (AKTA Prime, Pharmacia Biotec AB, Uppsala, Sweden), then eluted with a linear increasing gradient using

elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1} (Oh *et al*., 2000). The absorbance was monitored at 280 nm and bacteriocin activity of each fraction was determined by microtitre broth bioassay. Protein content was estimated by the BCA Protein Assay Kit. The spectrum of activity was determined against *L. monocytogenes* ATCC 19114.

The semi-purified bacteriocin samples were further purified by hydrophobic interaction Chromatography using C18 Cartridge. The bacteriocin samples were dissolved in a total of 600 µl of 0.1% trifluoro-acetic acid (TFA) and peptide/proteins were fractionated using Sep Pack Light C18 cartridge (Waters). The cartridge was first washed with 1 ml methanol, then 70% acetonitrile containing 0.1% TFA. Cartridges were equilibrated with 2 ml 0.1% TFA and samples were applied on the C18 cartridge, proteins/peptides were desalted using 1 ml 0.1% TFA and then eluted sequentially from the C18 matrix using 500 µl of 10%, 20%, 40%, 60%, 80% and 100% acetonitrile. Fractions were dried using a speed vac and tested for bacteriocin activity.

To detect the molecular weight of the purified bacteriocin samples (using SDS-PAGE) they were further desalted. Samples were suspended in 500 µl 0.1% TFA and desalted using Sep Pack Light C18 cartridge (Waters). The cartridge was first washed with 1 ml methanol, then 70% acetonitrile containing 0.1% TFA. Cartridges were equilibrated with 2 ml 0.1% TFA and samples were applied on the C18 cartridge. Salt was removed by washing with 2 ml 0.1% TFA and peptides and proteins retained on the cartridge were eluted with 70% acetonitrile (500 µl twice) then dried using speed vac.

3.8. SDS-PAGE of semi-purified bacteriocin

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (Laemmli, 1970) was used to detect the molecular weights of the bacteriocins produced by *C. maltaromaticum* strains MMF-32 and KOPRI 25789 through all the stages of the purification: the

supernatants, ammonium sulphate precipitated supernatants and the semi-purified peptides. Concentrations for supernatants and ammonium sulphate precipitated supernatants were 1.0 µg μl^{-1}, giving final concentrations of 15 µg respectively. The concentration of semi-purified peptides was 0.5 µg μl^{-1} giving a final of concentration of 7.5 µg. Samples of supernatants, ammonium sulphate precipitated supernatants and purified peptides were heated with SDS-PAGE sample buffer. Heated samples were separated on 15% SDS-polyacrylamide gels (Next gel, Amresco, USA). Fifteen µl of each sample was loaded on each lane of the gel and 5 µl volumes of the molecular weight marker, the gel was run at 100 volts for 15 min and the voltage increased to 150 for 1 h 45 min until the tracking dye reaches bottom. The gels were fixed in 50% (v/v) ethanol-10% glacial acetic acid overnight and silver-stained (ProteoSilver™ Silver Stain Kit, Sigma-Aldrich) (Rabilloud *et al*., 1994).

3.9. SDS-PAGE of purified bacteriocin

Further desalted samples were suspended in 10 MilliQ H_2O, 10 µl of NuPAGE LDS sample buffer (Novex, life technologies) and 5 µl 50 milli Moles Dithiothreitol (DTT). Samples were loaded on NuPAGE 10% Bis-Tris gel and proteins were separated on the gel using MES SDS running buffer (20X) (Novex, life technologies) at 200 V for 40 min. Pre-stained protein standard electrophoresis size markers (Novex®) were used for estimation of bacteriocin molecular weight. Gels were washed briefly with MilliQ water and proteins were fixed on the gel using 50% ethanol 12% acetic acid 1 h. Gels are washed 3 times with 50% ethanol for 20 min. The gel was aseptically placed in a sterile petri dish rinsed abundantly with sterile water and overlaid with approximately 55 ml of soft agar containing 5 ml of an overnight culture of *L. monocytogenes* ATCC 19114 containing approximately 1 x 10^6 CFU ml^{-1}. The plate was incubated at 30 ^oC for 18 h and examined for zone of inhibition (Bhunia and Johnson, 1992).

3.10. Results

3.10.1. Identification and screening of bacteriocinogenic LABs

In this study *C. maltaromaticum* MMF-32 and KOPRI 25789 were isolated from smoked salmon. The strains were identified using 16S rDNA-targeted PCR, and demonstrated homology levels of 99%, (Weisburg *et al*., 1991), and by phenotypic characteristics using the API-50CH system based on Collins *et al*. (1987) and Hammes and Hertel (2006). The broth assay method (3.8.) using the 96-well microtitre plates were used to study the antimicrobial activity of the 2 LAB cultures.

Undiluted cell-free supernatant (3.3.3.), ammonium sulphate precipitated supernatant (3.5.) and semi-purified bacteriocin (3.7.) were shown to exhibit inhibitory activity against *Shewanella baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Shewanella baltica*, *Shewanella baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes*.

Optical density measurements using microplate readers are used to determine the inhibitory effect of antimicrobials following the method of Vijayakimar and Muriana (2015).

The following data is used to represent inhibition on graph; strong inhibition represents OD between 0.000 to 0.200 at 40 h of incubation; fairly strong inhibition represents OD between 0.200 to 0.400 at 32 h; weak inhibition represents OD between 0.400 to 0.600 at 24 h of incubation; fairly weak inhibition represents OD between 0.600 to 0.800 at 16 h of incubation; very weak inhibition represents OD between 0.800 to 1.000 at 8 h of incubation.

The inhibitory effect of cell-free supernatants from *C. maltaromaticum* KOPRI 25789 and MMF-32 against *Shewanella baltica* OS185 were shown in (Fig. 3.2.). The

inhibitory assay revealed very weak inhibition of *Shewanella baltica* OS185 by the supernatants of KOPRI 25789 and MMF-32 during the 0-8 h incubation, respectively. The 0-8 h incubation period showed bacteriostasis.

C. maltaromaticum KOPRI 25789 and MMF-32 cell-free supernatants had no effect on the activity of *L. monocytogenes* ATCC 19114 after 8 h of incubation, respectively (Fig. 3. 3.).

The addition of the cell-free supernatants of both LAB cultures to *Aeromonas* HB-6 (Fig 3.4.) revealed strong inhibition for 40 h. Fairly strong inhibition was observed with *A. hydrophila* HX201006-3 (Fig. 3.5.) from 0-40 h with the addition of the cell-free supernatants of both LAB cultures.

Growth inhibition of *Sh. baltica* and *Sh. baltica* OS678 by the addition of the cell-free supernatants of both LAB cultures showed strong inhibition (Figs. 3.6 and 3.7). Addition of *C. maltaromaticum* KOPRI 25789 and MMF-32 to *Serratia* I-113-31 revealed weak inhibition during the 40 h of incubation (Fig. 3.8.).

Very weak inhibitory effect of cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 against *A. salmonicida* subsp. *achromogenes* was observed from 0-8 h of incubation (Fig. 3.9). The inhibitory effects produced by organic acids were eliminated by adjusting the supernatants to pH 6.5 using 2 N NaOH. The effect of hydrogen peroxide was removed by using bovine liver catalase.

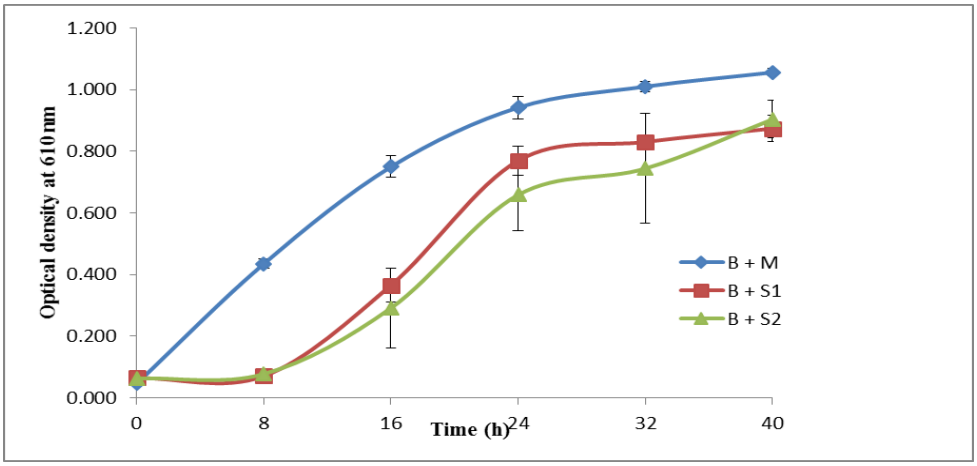


Figure 3.2. Growth inhibition test of *Sh. baltica* OS185 by addition of cell-free supernatant of 2 LABs at 30 °C for 40 h. (B + M) *Sh. baltica* OS185 with media; (B + S1) *Sh. baltica* OS185 with supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *Sh. baltica* OS185 with supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

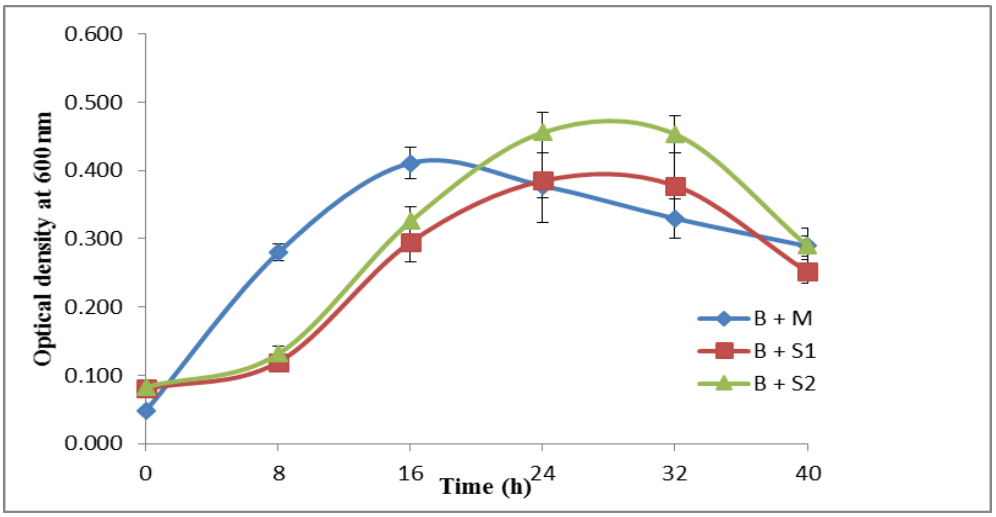


Figure 3.3. Growth inhibition test of *L. monocytogenes* ATCC19114 by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B + M) *L. monocytogenes* ATCC19114 with media; (B + S1) *L. monocytogenes* ATCC19114 with supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *L. monocytogenes* ATCC19114 with supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

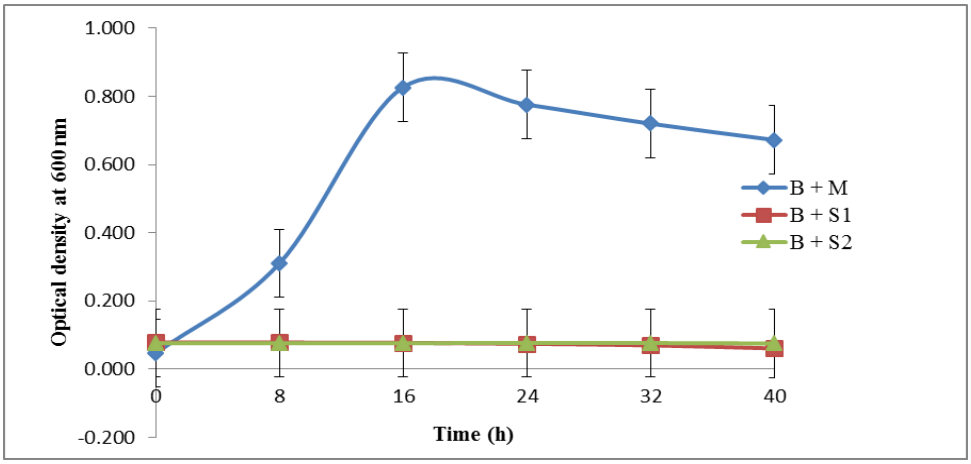


Figure 3.4. Growth inhibition test of *Aeromonas* HB-6 by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B + M) *Aeromonas* HB-6 with media; (B + S1) *Aeromonas* HB-6 with supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *Aeromonas* sp. HB-6 with supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

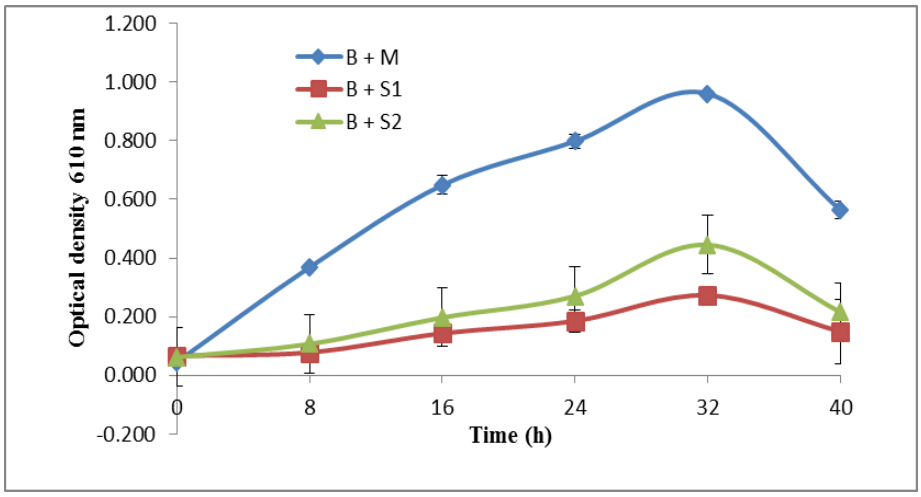


Figure 3.5. Growth inhibition test of *A. hydrophila* HX201006-3 by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B + M) *A. hydrophila* HX201006-3 with media; (B + S1) *A. hydrophila* HX201006-3 with cell-free supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *A. hydrophila* HX201006-3 with cell-free supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

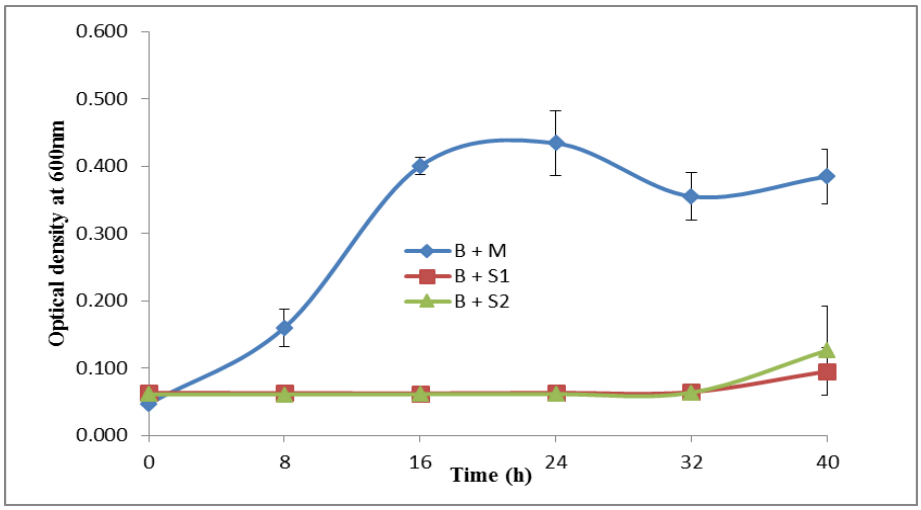


Figure 3.6. Growth inhibition test of *Sh. baltica* by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B+M) *Sh. baltica* with media; (B + S1) *Sh. baltica* with cell-free supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *Sh. baltica* with cell-free supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

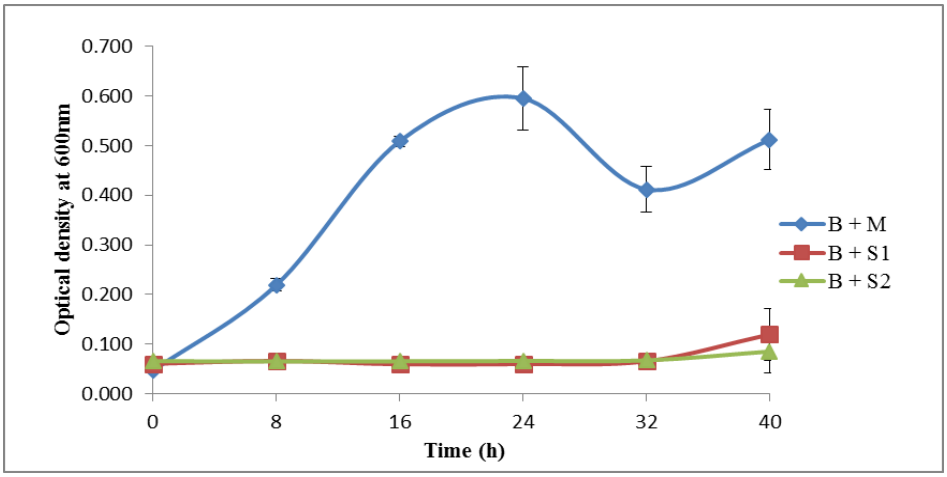


Figure 3.7. Growth inhibition test of *Sh. baltica* OS678 by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B+M) *Sh. baltica* OS678 with media; (B + S1) *Sh. baltica* OS678 with cell-free supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *Sh. baltica* OS678 with cell-free supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

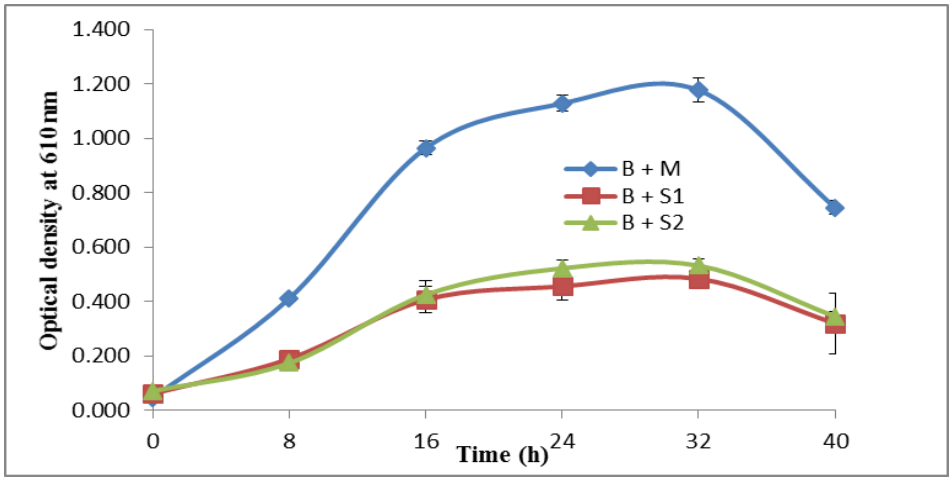


Figure 3.8. Growth inhibition test of *Serratia* sp. I-113-31 by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B + M) *Serratia* sp. I-113-31 with media; (B + S1) *Serratia* sp. I-113-31 with cell-free supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *Serratia* sp. I-113-31 with cell-free supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

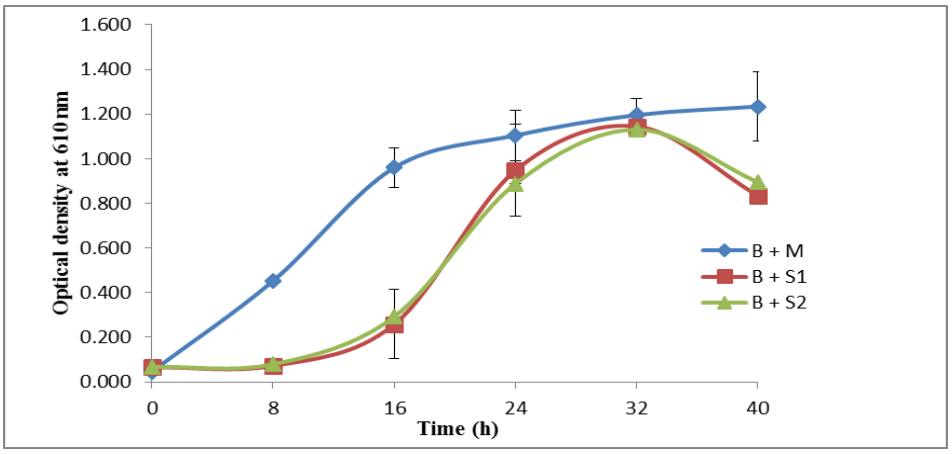


Figure 3.9. Growth inhibition test of *A. salmonicida* subsp. achromogenes by addition of cell-free supernatant of 2 LAB at 30 °C for 40 h. (B+M) *A. salmonicida* subsp. *achromogenes* with media; (B + S1) *A. salmonicida* subsp. *achromogenes* with cell-free supernatant of *C. maltaromaticum* KOPRI 25789 and (B + S2) *A. salmonicida* subsp. *achromogenes* with cell-free supernatant of *C. maltaromaticum* MMF-32. The values presented are the mean of six independent experiments. Points = Means ± SE.

These indicator strains, which are all Gram-negative bacteria except *Listeria*, were inhibited at various time intervals by cell-free supernatants of *Lactococcus lactis* subsp. *lactis* NCIMB 8586, used in the bioassay as a comparable control, during the 40 h incubation as shown below.

Figure 3.10 revealed a weak inhibition of *Sh. baltica* OS185 by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. The inhibitory effect of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 against *L. monocytogenes* revealed very weak inhibition (Fig.3.11). Addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 to *Aeromonas* HB-6 showed fairly strong inhibition (Fig. 3.12). The inhibitory effect of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 against showed *A. hydrophila* HX201006-3 revealed fairly strong inhibition (Fig. 3.13). Figure 3.14 and 3.15 showed fairly strong inhibition of *Sh. baltica* and *Sh. baltica* OS678 by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 to *Serratia* spp. I-113-31 revealed fairly weak inhibition (Fig. 3.16). The inhibitory effect of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 against *A. salmonicida* subsp. *achromogenes* showed very weak inhibition (Fig. 3.17).

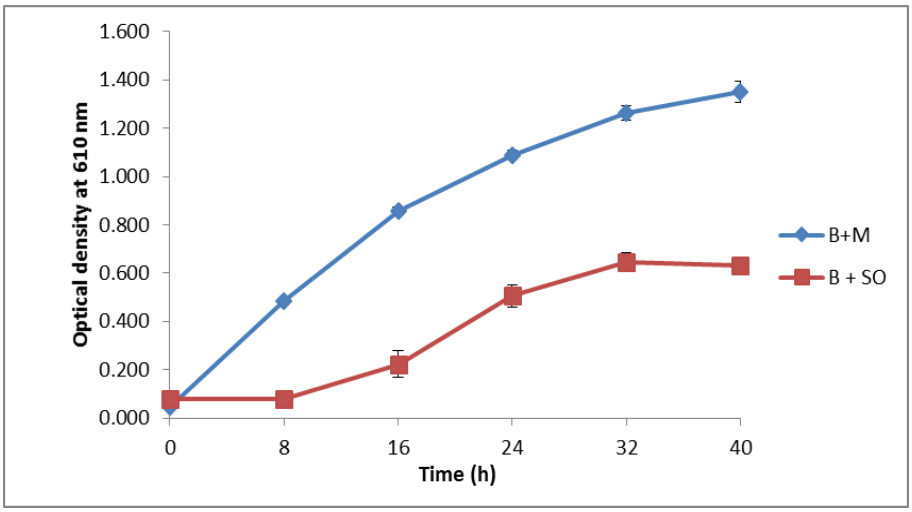


Figure 3.10 Growth inhibition test of *Sh. baltica* OS185 by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B + M) *Sh. baltica* OS185 with media and (B + SO) *Sh. baltica* OS185 with cell-free supernatant of of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

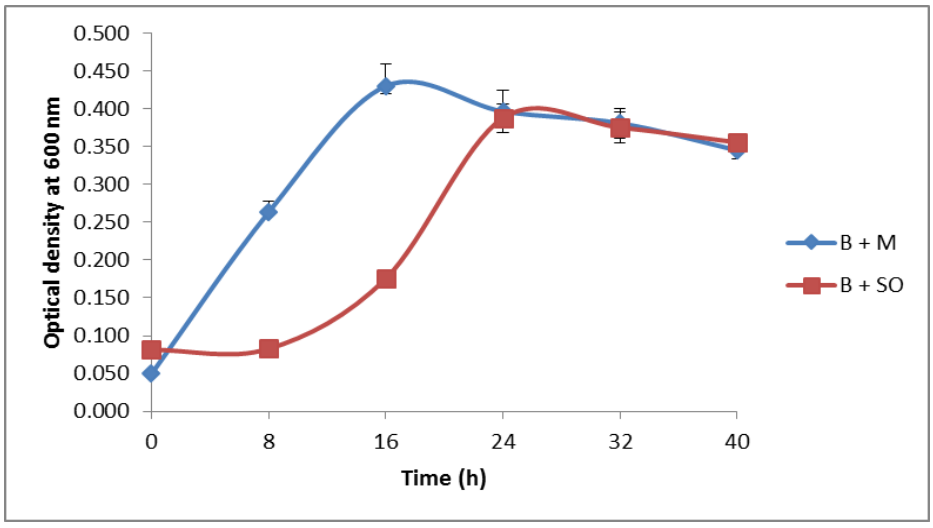


Figure 3.11 Growth inhibition test of *L. monocytogenes* ATCC 19114 by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B + M) *L. monocytogenes* ATCC 19114 with media and (B + SO) *L. monocytogenes* ATCC 19114 with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

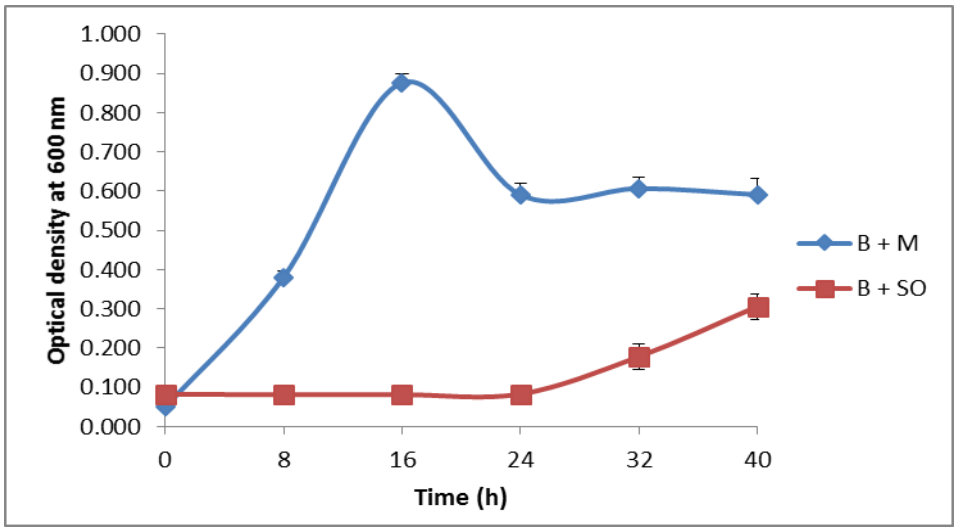


Figure 3.12 Growth inhibition test of *Aeromonas* HB-6, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B + M) *Aeromonas* HB-6 with media and (B + SO) *Aeromonas* HB-6 with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

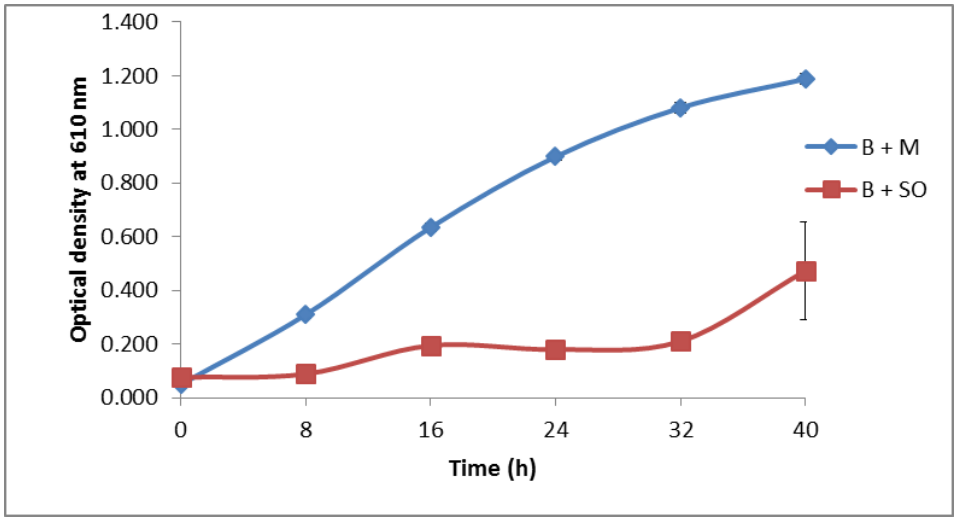


Figure 3.13 Growth inhibition test of *Aeromonas hydrophila* HX201006-3, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B + M) *Aeromonas hydrophila* HX201006-3 with media and (B + SO) *Aeromonas hydrophila* HX201006-3 cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

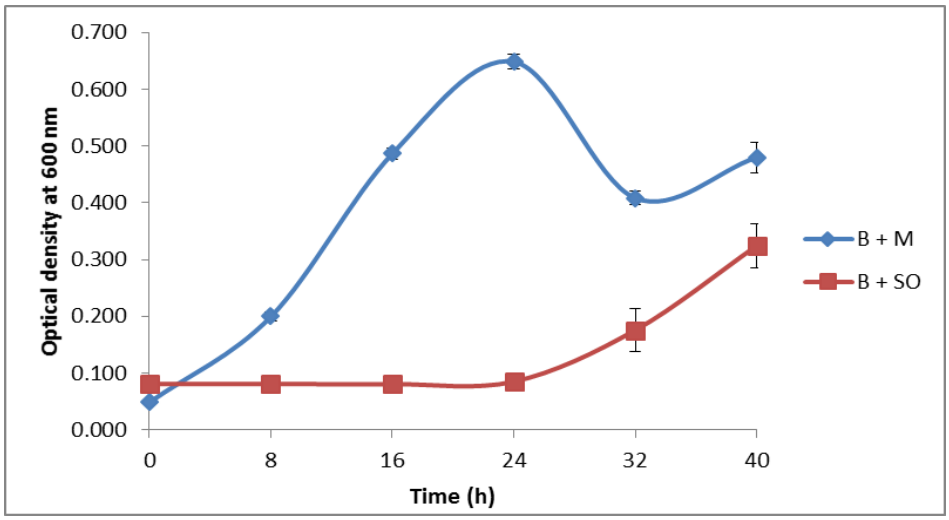


Figure 3.14 Growth inhibition test of *Sh. baltica*, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B +O) *Sh. baltica* with media and *Sh. baltica* with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

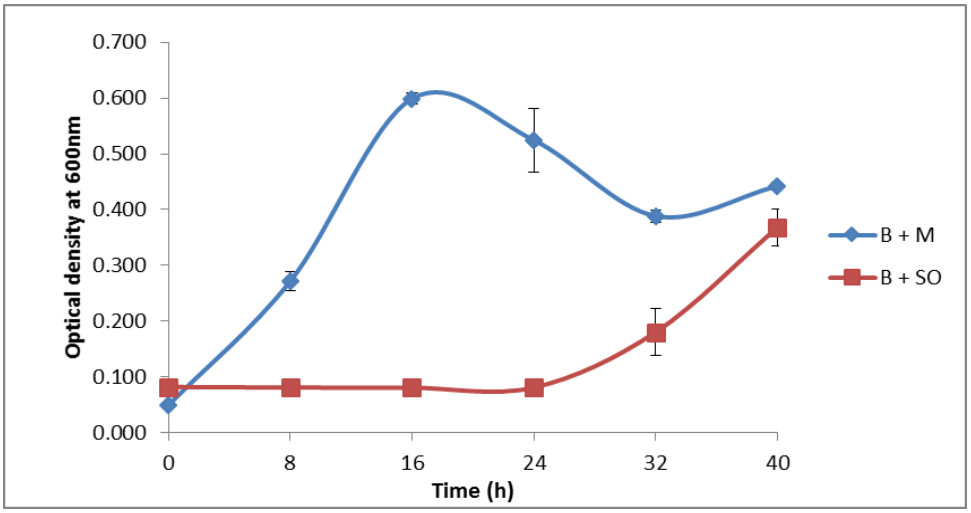


Figure 3.15 Growth inhibition test of *Sh. baltica* OS678, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B +O) *Sh. baltica* OS678 with media and (B +SO) *Sh. baltica* OS678 with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

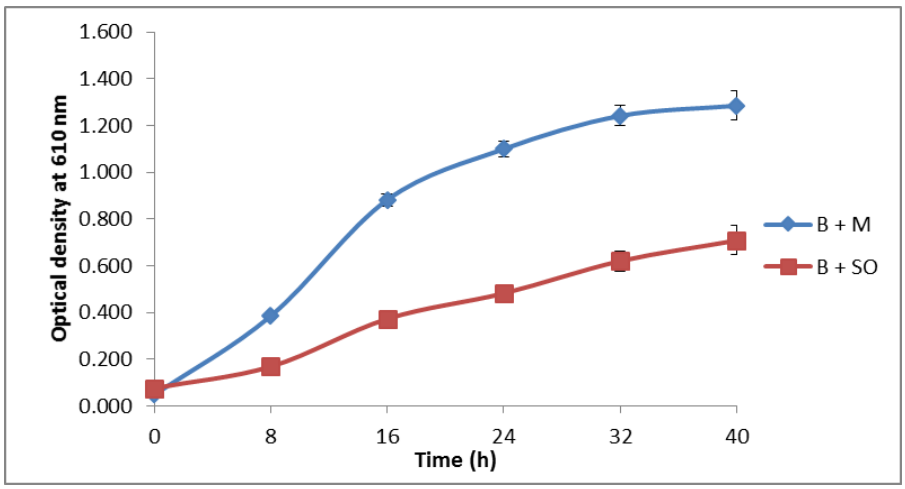


Figure 3.16 Growth inhibition test of *Serratia* sp. I-113-31, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B +O) *Serratia* sp. I-113-31with media and (B +SO) *Serratia* sp. I-113-31with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

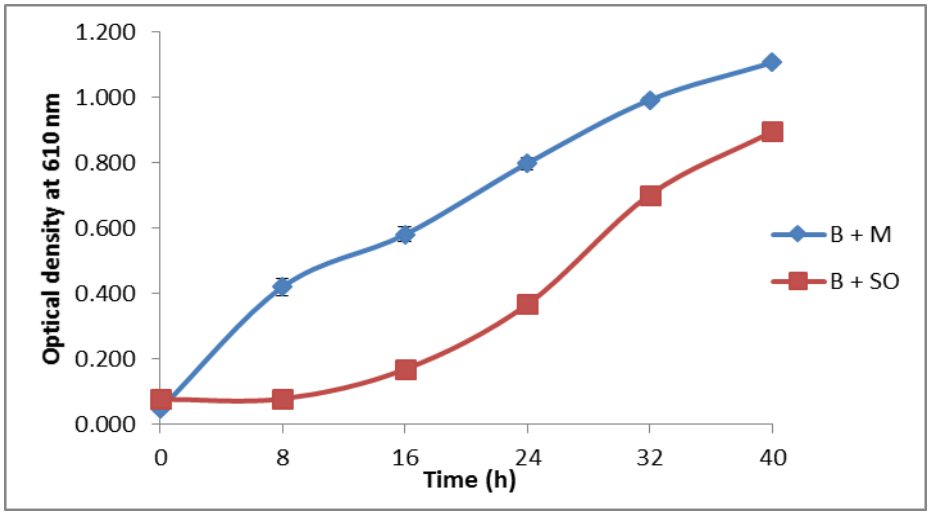


Figure 3.17 Growth inhibition test of *Aeromonas salmonicida* subsp. *achromogenes*, by addition of cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586 at 30 ºC for 40 h. (B +O) *Aeromonas salmonicida* subsp. *achromogenes* with media and (B +SO) *Aeromonas salmonicida* subsp. *achromogenes* with cell-free supernatant of *Lactococcus lactis* subsp. *lactis* NCIMB 8586. Points = Means ± SE.

The use of tabulated data gives a more accurate representation of data than figures (Editorial Policy of Aquaculture).

The antimicrobial effects of the ammonium sulphate precipitated supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 against the indicator microorganisms at 40 h were explained in Table 3.1.

A fairly strong growth inhibition against *Shewanella baltica* OS185, *Listeria monocytogenes* ATCC 19114, *Aeromonas* HB-6 and *Shewanella baltica* were observed, by the addition of ammonium sulphate precipitated supernatants of KOPRI 25789 and MMF-32.

The addition of ammonium sulphate precipitated supernatants of KOPRI 25789 and MMF-32 revealed a strong growth inhibition against *Shewanella baltica* OS678.

Weak growth inhibition against *Aeromonas hydrophila* HX201006-3, *Serratia* spp. I-113-31 and *Aeromonas salmonicida* subsp. *achromogenes* were revealed by the addition of ammonium sulphate precipitated supernatants of KOPRI 25789 and MMF-32.

Table 3.1. Growth inhibition of ammonium sulphate precipitated supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 against the indicator strains using broth assay method.

Indicator strains	*C. maltaromaticum* KOPRI 25789	*C. maltaromaticum* MMF-32
Shewanella baltica OS185	++++	++++
Listeria monocytogenes ATCC 19114	++++	++++
Aeromonas HB-6	++++	++++
Aeromonas hydrophila HX201006-3	+++	+++
Shewanella baltica	++++	++++
Shewanella baltica OS678	+++++	+++++
Serratia spp. I-113-31	**+++**	+++
Aeromonas salmonicida subsp. *achromogenes*	+++	+++

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of three independent experiments.

Very weak inhibition was observed against *L. monocytogenes* ATCC 19114 treated with semi-purified bacteriocin of *C. maltaromaticum* MMF-32 (Fig. 3.18).

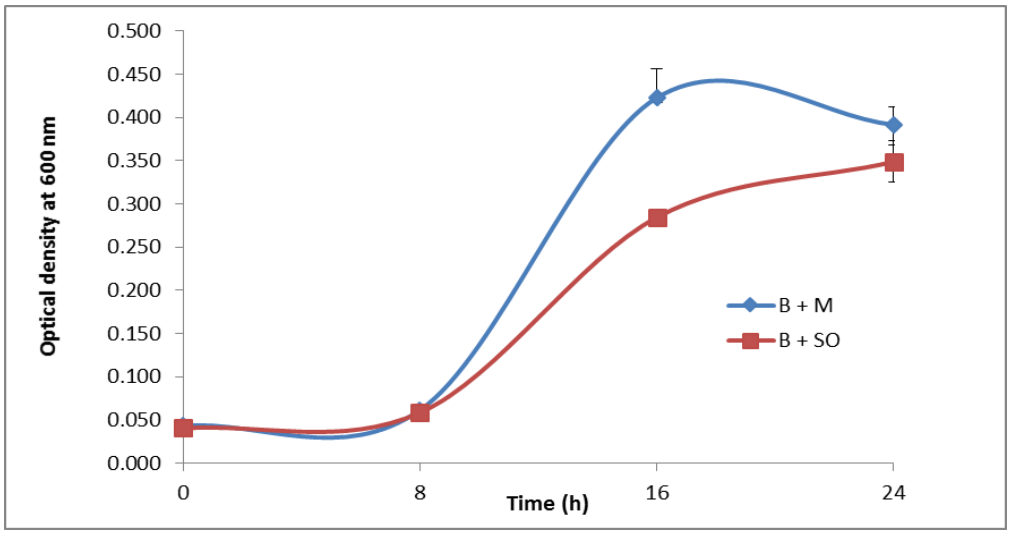


Figure 3.18. Growth inhibition test of *L. monocytogenes* ATCC 19114 by semi-purified bacteriocin from ammonium sulphate precipitated culture supernatant of *C. maltaromaticum* MMF-32. B +M represent *L. monocytogenes* ATCC 19114 and media, SO +B represent bacteriocin with media and *L. monocytogenes* ATCC 19114. The values presented are the mean of three independent experiments. Points = Means ± SE.

3.11. Characterization of the antimicrobial compounds

3.11.1. Effect of temperature treatments

The thermal resistance of the bacteriocins produced by strains KOPRI 25789 and MMF-32 was determined. The treatment of the extracellular extracts of the two bacteriocin-producing LAB strains at 56 °C for 30 min and 100 °C for 10 min led to antimicrobial activity against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* sp. I-113-31 and *A. salmonicida* subsp. *achromogenes* (Table 3.2.).

Inhibitory activities of KOPRI 25789 and MMF-32 were not completely destroyed by the heat treatment, i.e. 56 °C for 30 min (fairly weak inhibition) and 100 °C for 10 min (very weak inhibition), against *Sh. baltica* OS185. Thus, significant differences were observed between heated samples at 8 h and unheated samples at 40 h of incubation at 56 °C for 30 min (p = 0.034 and p = 0.029), respectively, for the 2 LAB. Similarly, significant differences were observed between heated samples at 8 h and unheated samples at 40 h of incubation at 100 °C for 10 min (p = 0.004 and p = 0.038) respectively, for the 2 LAB. Residual activity of 40% and 20% was retained respectively, at 56 °C for 30 min and 100 °C for 10 min by the supernatants of the 2 LAB after heat treatment.

The antibacterial activities of KOPRI 25789 and MMF-32 were not completely lost during heat incubation at 56 °C for 30 min (very weak inhibition) and 100 °C for 10 min (very weak inhibition) against *L. monocytogenes* ATCC 19114. Significant differences were observed between heated samples at 8 h and unheated samples at 16 h of incubation at 56 °C for 30 min (p = 0.017 and p = 0.004) respectively, for the 2 LAB. In contrast, significant differences were observed between heated samples at 0 h and unheated samples at 16 h of incubation at 100 °C for 10 min (p = 0.12 and p = 0.017) respectively, for the 2 LABs. Some 20% of the residual activity of the supernatants was retained from the 2 LABs after both heat treatments.

KOPRI 25789 and MMF-32 retained their antagonistic activities when heat treatment was applied, i.e. 56 °C for 30 min (fairly strong and strong inhibition, respectively) and 100 °C for 10 min (fairly weak and strong inhibition, respectively) against *Aeromonas* HB-6. Significant differences were observed between unheated samples at 8 h and heated samples at 32 h of incubation for strain KOPRI 25789 (p = 0.003). However, significant differences were not observed between the treated samples of strain MMF-

32 for 56 °C for 30 min. Heating at 100 °C for 10 min revealed a significant difference between the heated sample at 8 h and unheated material at 40 h for strain KOPRI 25789 (p = 0.032) and a significant difference of (p = 0.014) was observed between unheated at 16 h and unheated at 40 h for strain MMF-32. The supernatants from the two LABs retained 80% and100% residual activities respectively, after treatment at 56 °C for 30 min, while 40% and 100% activities were recorded respectively, at 100 °C for 10 min.

Antagonistic activity of KOPRI 25789 supernatant was not completely lost when heat treatment was applied at 56 °C for 30 min (very weak inhibition) against *A. hydrophila* HX201006-3, a significance difference of (p = 0.005) revealed a total activity between the heated sample at 0 h and unheated material at 32 h; a residual activity of 20% was retained. Heat treatment at 56 °C for 30 min applied to the supernatant of strain MMF-32 against *A. hydrophila* HX201006-3 revealed that total activity was not completely lost (fairly weak inhibition). A significant difference of (p = 0.008) was observed in the unheated sample at 0 h and heated material at 40 h of incubation; a residual activity of 40% was retained. Heating of KOPRI 25789 supernatant at 100 °C for 10 min showed an activity loss after 8 h of incubation (very weak inhibition). A significance difference (p = 0.007) was detected between the unheated sample at 0 h and unheated material at 24 h of incubation. Under the same heat treatment regime, the supernatant of MMF-32, revealed a significant difference of (p = 0.015) between heated sample at 8 h and unheated at 32 h (very weak inhibition). Residual activities of 20% were retained, respectively of 2 LAB after heat treatment at 56 °C for 30 min and 100 °C for 10 min.

The antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants were retained after heat treatment at 56 °C for 30 min against *Sh. baltica* (fairly strong and strong inhibition, respectively). A significant difference (p = 0.030) was observed only for KOPRI 25789 between unheated material at 0 h and the heated supernatant at 40 h

incubation. Residual activities of 80% and100% were retained by the 2 LABs respectively. Heat treatment of KOPRI 25789 and MMF-32 cell-free supernatants at 100 °C for 10 min against *Sh. baltica* revealed loss of activity at 16 and 32 h respectively by the 2 LABs (very weak and fairly strong inhibition). A significant difference ($p = 0.047$) for KOPRI 25789 was observed between the heated sample at 40 h and unheated material at 32 h, and for MMF-32 a significant difference ($p = 0.0005$) was detected between unheated supernatant at 0 and at 40 h of incubation. The residual activities retained by the 2 LABs were 20% and 80% respectively.

Heat treatment at 56 °C for 30 min for KOPRI 25789 and MMF-32 cell-free supernatants against *Sh. baltica* OS678 revealed that their antagonistic activities were retained. Complete residual activity was retained with KOPRI 25789 and MMF-32, respectively (strong inhibition). A significant difference ($p = 0.001$) was observed between the unheated sample at 16 h and heated sample at 40 h incubation only for KOPRI 25789; residual activity of 100% was retained. Antagonistic activity of KOPRI 25789 cell-free supernatant with heat treatment of 100 °C at 10 min against *Sh. baltica* OS678 revealed very weak inhibition at 40 h of incubation, with a significant difference ($p = 0.0005$) between the unheated material at 0 h and heated supernatant at 40 h, with a residual activity of 20% retained. Antagonistic activity of MMF-32 cell-free supernatant was retained with heat treatment of 100 °C at 10 min against *Sh. baltica* OS678 (fairly strong inhibition); a significant difference ($p = 0.002$) was observed between the unheated sample at 0 h and heated material at 40 h of incubation. Residual activity of 80% was retained.

Antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants with heat treatments at 56 °C for 30 min and 100 °C at 10 min against *Serratia* I-113-31 were retained (weak inhibition). Significant differences ($p = 0.012$ and $p = 0.008$) and ($p =$

0.035 and p = 0.006) were observed for both heat treatments, respectively. A residual activity of 60% was retained.

Antagonistic activities of KOPRI 25789 and MMF-32 cell-free supernatants with treatments at 56 °C at 30 min and 100 °C at 10 min against *A. salmonicida* subsp. *achromogenes* were not completely lost (very weak inhibition). Heat treatment at 56 °C for 30 min revealed a loss of antagonistic activity of KOPRI 25789 cell-free supernatants at 8 h of incubation, with a significant difference (p = 0.006) observed between unheated sample at 8 h and heated material at 24 h of incubation. With the latter, heat treatment applied to MMF-32 cell-free supernatants resulted in antagonistic activity being lost at 8 h of incubation, with a significant difference of p = 0.010 shown between the unheated sample at 0 and heated preparation at 24 h of incubation. Residual activity retained was 20%, respectively. Loss of antagonistic activity was detected at 8 h of incubation when heat treatment of 100 °C at 10 min was applied to KOPRI 25789 cell-free supernatants; a significant difference (p = 0.002) was observed between unheated sample at 0 h and heated material at 24 h of incubation. MMF-32 cell-free supernatants lost activity at 8 h of incubation with the latter heat treatment; a significant difference (p = 0.002) was detected between the unheated sample at 8 h and the heated preparation at 32 h of incubation. The residual activities retained were 20%, respectively.

Table 3.2. Effect of thermal treatments on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and MMF-32 against indicator strains using broth assay method.

Indicator strains	Time of thermal treatment	Temperature (°C)	KOPRI 25789	Thermal	MMF-32	Thermal
			Untreated[a]	Treated	Untreated[a]	Treated
Sh. baltica OS185	30 min	56	++	++	++	++
	10 min	100	+	+	+	+
L. monocytogenes ATCC19114	30 min	56	+	+	+	+
	10 min	100	+	+	+	+
Aeromonas sp. HB-6	30 min	56	++++	++++	++++	+++++
	10 min	100	++	++	+++	+++++
A. hydrophila HX201006-3	30 min	56	+	+	+	++
	10 min	100	+	+	+	+
Sh. baltica	30 min	56	+	++++	++++	+++++
	10 min	100	+	+	+	++++
Sh. baltica OS678	30 min	56	+++++	+++++	+++++	+++++
	10 min	100	+	+	++++	++++
Serratia I-113-31	30 min	56	+++	+++	+++	+++
	10 min	100	+++	+++	+++	+++
A. salmonicida subsp. *achromogenes*	30 min	56	+	+	+	+
	10 min	100	+	+	+	+

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consists of freshly prepared cell-free supernatants without thermal treatment.

3.11.2. Effect of pH treatment

Antagonistic activity of the cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 treated at pH 3.5 and 4.9 against indicator organisms have been detailed in Table 3.3. Cell-free supernatants of KOPRI 25789 and MMF-32 treated to pH 3.5 and 4.9 retained their antagonistic activity against *Sh. baltica* OS185 for 40 h of incubation; significant differences ($p = 0.014$, $p = 0.018$ and $p = 0.023$) were observed between treated samples at 0 h and untreated material at 40 h respectively (strong inhibition). Residual activity retained was 100%. No significant difference was observed with the cell-free supernatant of MMF-32 treated at pH 4.9.

As a second example, antagonistic activities of cell-free supernatants of strains KOPRI 25789 and MMF-32 were retained when treated at pH 3.5 and 4.9 against *L. monocytogenes* ATCC 19114 (strong inhibition), with significant difference ($p = 0.043$) detected between treated samples at 8 h and untreated material at 24 h for KOPRI 25789 cell-free supernatants treated to pH 3.5. A significant difference ($p = 0.033$) was observed between treated samples at 0 h and untreated material at 16 h for MMF-32 cell-free supernatants treated to pH 3.5. Cell-free supernatants of KOPRI 25789 and MMF-32 treated to pH 4.9 showed significant differences ($p = 0.020$ and $p = 0.042$) between treated samples at 8 h and untreated preparations at 24 h, respectively. The residual activity retained was 100%.

Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated to pH 3.5 retained their antagonistic activities against *Aeromonas* HB-6 (strong inhibition), showing significant differences of ($p = 0.028$ and $p = 0.029$) between treated samples at 0 h and untreated at 32 h, respectively. Antagonistic activities of cell-free supernatants of strains KOPRI 25789 and MMF-32 treated to pH 4.9 retained activities against

Aeromonas HB-6, significant difference of (p = 0.023) was only observed with strain KOPRI 25789 cell-free supernatant. Residual activity retained was 100%.

Strains KOPRI 25789 and MMF-32 cell-free supernatants treated to pH 3.5 and pH 4.9 retained their antagonistic activities against *A. hydrophila* HX201006-3 (strong inhibition), significant differences of (p = 0.010 and p = 0.007) between treated samples at 0 h and untreated samples at 40 h of pH 3.5 treated samples respectively. Significant difference of (p = 0.036) was only observed between treated samples at 8 h and untreated samples at 40 h for MMF-32 cell-free supernatants treated to pH 4.9. Residual activity retained was 100%.

Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated to pH 3.5 and 4.9 retained their antagonistic activities against *Sh. baltica* (strong inhibition), significant difference of (p = 0.026) was observed between treated sample at 8 h and untreated sample at 24 h and significant difference of (p = 0.026) was observed between treated sample at 0 h and untreated sample at 32 h, respectively of pH 3.5 treated samples. Only cell-free supernatants of MMF-32 samples treated to pH 4.9 showed a significant difference of (p = 0.012). Residual activity retained was 100%.

Antagonistic activities of cell-free supernatants of strains KOPRI 25789 and MMF-32 were retained when treated to pH 3.5 and 4.9 against *Sh. baltica* OS678 (strong inhibition), significant difference of (p = 0.032) was detected between treated samples at 0 h and untreated samples at 32 h for KOPRI 25789 cell-free supernatants and also significant difference of (p = 0.021) was detected between treated samples at 0 h and untreated samples at 40 h for MMF-32 cell-free supernatants of pH 3.5 treated samples. Significant differences were not observed in pH 4.9 treated samples. Residual activity retained was 100%.

Strains KOPRI 25789 and MMF-32 cell-free supernatants treated to pH 3.5 and 4.9 against *Serratia* I-113-31 retained the antagonistic activities (strong inhibition). Significant difference of (p = 0.025) was observed between treated samples at 0 h and untreated samples at 32 h only in MMF-32 cell-free supernatants treated to pH 3.5. Only strain MMF-32 cell-free supernatants treated to pH 4.9 showed a significant difference of (p = 0.018) between treated samples at 0 h and untreated samples at 40 h. Residual activity retained was 100%.

Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated to pH 3.5 and 4.9 retained their antagonistic activities against *A. salmonicida* subsp. *achromogenes*. Significant differences of (p = 0.010 and p = 0.009) were only detected in pH 3.5 treated samples between treated samples at 0 h and untreated samples at 24 h, respectively. Residual activity retained was 100%.

Table 3.3. Effect of pH on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against indicator strains using broth assay method.

Indicator strain	pH treatment	KOPRI 25789		MMF-32 Untreated	
		Untreated	Treated		Treated
Sh. baltica OS185	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
L. monocytogenes ATCC19114	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
Aeromonas HB-6	3.5	+++	+++++	++	+++++
	4.9	++	+++++	+	+++++
A. hydrophila HX201006-3	3.5	++	+++++	+	+++++
	4.9	++	+++++	+	+++++
Sh. baltica	3.5	+++++	+++++	+++	+++++
	4.9	++++	+++++	+++	+++++
Sh. baltica OS678	3.5	++	+++++	++	+++++
	4.9	++	+++++	++	+++++
Serratia I-113-31	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++
A. salmonicida subsp. *achromogenes*	3.5	+	+++++	+	+++++
	4.9	+	+++++	+	+++++

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consist of freshly prepared cell-free supernatants without thermal treatment.

3.11.3. Enzymatic activity directed towards putative bacteriocins

Cell-free supernatants of KOPRI 25789 and MMF-32 exhibited no reduction, partially reduced, reduced, weakly reduced and very weakly reduced antimicrobial activity due to the action of proteases (Table 3.4). The residual antagonistic activity of the LAB supernatants after protease treatment was a function of the indicator microorganisms used and did not follow a particular pattern. For example, the cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin showed very weak and fairly weak activity against *Sh. baltica* OS185, respectively. A significant difference ($p = 0.014$) between the untreated samples at 0 h and treated material at 40 h, for KOPRI 25789. No significant difference was observed with MMF-32 cell-free supernatant. Treatment of cell-free supernatants with lysozyme against *Sh. baltica* OS185 showed very weak reduced activity, with significant differences ($p = 0.014$ and $p = 0.024$) between the untreated samples at 0 h and treated preparations at 32 h of incubation, respectively. Antagonistic activities of the cell-free supernatants was very weak reduced when treated with proteinase K against *Sh. baltica* OS185. Significant differences were not detected between treated and untreated samples. A very weak reduced antagonistic activities of cell-free supernatants treated with trypsin against *Sh. baltica* OS185 was observed, with significant differences ($p = 0.021$ and $p = 0.030$) between the untreated samples at 0 h and treated material 32 h of incubation, respectively.

The antimicrobial activity exhibited by cell-free supernatants of KOPRI 25789 and MMF-32 treated with α-chymotrypsin against *L. monocytogenes* ATCC 19114 was very weakly reduced. Thus, significant differences ($p = 0.040$ and $p = 0.008$) were detected between treated samples at 0 h and untreated samples at 24 h, respectively. Treatment of the cell-free supernatants with lysozyme showed strong antimicrobial activity against *L. monocytogenes* ATCC 19114 for KOPRI 25789 and MMF-32 cell-free supernatants.

A significant difference (p = 0.012) was observed between treated samples at 0 h and untreated material at 24 h for MMF-32 cell-free supernatants. No significant difference was observed between the treatments for cell-free supernatants of KOPRI 25789. Cell-free supernatants of KOPRI 25789 and MMF-32 showed a significant very weakly reduced antimicrobial activity against *L. monocytogenes* ATCC 19114, when treated with proteinase K. Significant differences (p = 0.009 and p = 0.026) were observed between untreated at 0 h and treated material at 16 h, respectively. Similarly use of trypsin led to a significant very weakly reduced antimicrobial activity against *L. monocytogenes* ATCC 19114 (p = 0.019 and p = 0.020) between untreated material at 0 h and treated preparation at 16 h.

No significant reduction in the antimicrobial activity of the cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin and proteinase K against *Aeromonas* HB-6 was observed (strong activity). Cell-free supernatants of strain KOPRI 25789 treated with lysozyme led to a significant reduction of (p = 0.034) between untreated samples at 0 h and treated at 40 h (weak reduction). Cell-free supernatants of strain KOPRI 25789 treated with trypsin against *Aeromonas* HB-6 had significant reductions of (and p = 0.029) between untreated samples at 0 h and treated at 40 h, respectively. The cell-free supernatants treated with lysozyme and trypsin revealed weakly reduced activity. Cell-free supernatants of strain MMF-32 treated with lysozyme and trypsin did not show any significant difference.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin, against *A. hydrophila* HX201006-3 revealed significant differences of (p = 0.017) between untreated samples at 0 h and treated samples at 40 h (very weak and fairly weak activity), respectively. Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with lysozyme, showed significant differences of (p = 0.026 and p =

0.046) between untreated samples at 0 h and treated samples at 40 h (very weak activity), respectively. Proteinase K treated with cell-free supernatants of strains KOPRI 25789 and MMF-32 revealed significant differences of (p = 0.027 and p = 0.037) between untreated samples at 0 h and treated samples at 40 h (very weak activity), respectively. Trypsin treated cell-free supernatants showed significant differences of (p = 0.010 and p = 0.016) between untreated samples at 0 h and treated samples at 40 h (very weak activity), respectively. The cell-free supernatants treated with all the enzymes except lysozyme treated MMF-32 supernatants all had weakly reduced antimicrobial activities.

Cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin and proteinase K against *Sh. baltica* showed no significant difference in the reduction of antimicrobial activity (strong activity). Trypsin treated cell-free supernatants of strains KOPRI 25789 and MMF-32, revealed fairly strong activity, there was no significant difference in the reduction of antimicrobial activity. However, cell-free supernatant of strain KOPRI 25789 treated with lysozyme against *Sh. baltica* had significant differences of (p = 0.042) between untreated samples at 0 h and treated samples at 32 h of incubation (fairly strong) whereas cell-free supernatant of strain of MMF-32 showed a significant difference of (p = 0.018) between untreated samples at 0 h and treated samples at 32 h of incubation (fairly strong).

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin against *Sh. baltica* OS678 resulted in significant differences of (p = 0.034 and p = 0.047) between treated samples at 40 h and untreated samples at 0 h, respectively. There was no reduction in antimicrobial activity of the cell-free supernatants. Treatment of cell-free supernatant of KOPRI 25789 with lysozyme against *Sh. baltica* OS678 showed a significant difference of (p = 0.049) between

treated samples at 0 h and untreated samples at 40 h of incubation. The cell-free supernatants of strains KOPRI 25789 and MMF-32 revealed very weak activity. No significant difference was observed in the cell-free supernatants of MMF-32 treated with lysozyme against *Sh. baltica* OS678. No significant difference was detected in the antimicrobial activity of KOPRI 25789 when the cell-free supernatants were treated with proteinase K against *Sh. baltica* OS678 (strong activity). Nevertheless, a significant difference of (p = 0.044) in the antimicrobial activity of cell-free supernatants of MMF-32 was observed between treated samples at 40 h and untreated sample at 0 h when treated with proteinase K against *Sh. baltica* OS678 (strong activity). Cell-free supernatant of strain KOPRI 25789 treated with trypsin against *Sh. baltica* OS678, revealed significant differences of (p = 0.010) between untreated samples at 0 h and treated samples at 40 h, whereas cell-free supernatant of strain MMF-32 revealed significant differences of (p = 0.039) between untreated samples at 0 h and treated samples at 40 h. Weak activities were observed in both cell-free supernatants.

The cell-free supernatants of strains KOPRI 25789 and MMF-32 treated with α-chymotrypsin, showed significant differences of (p = 0.043 and p = 0.016) between untreated samples at 0 h and treated samples at 32 h of incubation respectively. Lysozyme treated cell-free supernatants led to significant differences of (p = 0.022 and p = 0.035) between untreated samples at 0 h and treated samples at 32 h of incubation respectively. Cell-free supernatants treated with proteinase K revealed significant differences of (p = 0.022 and p = 0.014) between untreated samples at 0 h and treated samples at 32 h of incubation respectively. Trypsin treated cell-free supernatant against *Serratia* I-113-31 led to significant differences (p = 0.028 and p = 0.024) between

untreated samples at 0 h and treated samples at 32 h of incubation respectively. All treated cell-free supernatants revealed weak reduced antimicrobial activity.

The cell-free supernatant of strains KOPRI 25789 treated with α-chymotrypsin *A. salmonicida* subsp. *achromogenes* revealed significant differences of (p = 0.036) between treated samples at 0 h and untreated samples at 40 h (very weak activity), while cell-free supernatant of MMF-32 treated with α-chymotrypsin against *A. salmonicida* subsp. *achromogenes* revealed significant differences of (p = 0.008) between treated samples at 0 h and untreated samples at 40, respectively. Cell-free supernatant of LAB treated with lysozyme against *A. salmonicida* subsp. *achromogenes* had significant differences of (p = 0.043) between treated sample at 0 h and untreated at 32 h for KOPRI 25789, whereas a significant reduction of (p = 0.016) in the antagonistic activity MMF-32 was observed between untreated sample at 0 h and treated sample at 40 h very weak activity). Proteinase K treated cell-free supernatants of LAB against *A. salmonicida* subsp. *achromogenes* had a significant difference of (p = 0.015) between untreated sample at 0 h and treated sample at 32 h for KOPRI 25789, a significant difference of (p = 0.016) between treated samples at 0 h and untreated samples at 40 h was observed for MMF-32. For trypsin treated cell-free supernatants significant difference of (p = 0.022) between untreated samples at 0 h and treated samples at 24 h was observed only in MMF-32. Cell-free supernatants treated with α-chymotrypsin, lysozyme, proteinase K and trypsin against *A. salmonicida* subsp. *achromogenes* revealed very weak activity.

Table 3.4. Effect of proteases on cell-free supernatant activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against indicator strains using broth assay.

Indicator strain	Enzyme treatment	KOPRI 25789 Untreated	Treated	MMF-32 Untreated	Treated
Sh. baltica OS185	α-Chymotrypsin	+	+	+	++
	Lysozyme	+	+	+	+
	Protease K	+	++	+	++
	Trypsin	+	+	+	+
L. monocytogenes ATCC19114	α-Chymotrypsin	+	+	+	+
	Lysozyme	+	+++++	+	+++++
	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+
Aeromonas HB-6	α-Chymotrypsin	+++++	+++++	+++++	++++
	Lysozyme	+++	+++	+++	+++
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	+++	+++	+++	+++
A.hydrophila HX201006-3	α-Chymotrypsin	+	+	+	++
	Lysozyme	+	+	+	+

A.hydrophila HX201006-3	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+
Sh. baltica	α-Chymotrypsin	+++++	+++++	+++++	+++++
	Lysozyme	++++	++++	++++	++++
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	++++	++++	++++	++++
Sh. baltica OS678	α-Chymotrypsin	+++++	+++++	+++++	+++++
	Lysozyme	+	+	+	+
	Proteinase K	+++++	+++++	+++++	+++++
	Trypsin	+++	+++	+++	+++
Serratia I-113-31	α-Chymotrypsin	+++	+++	+++	++++
	Lysozyme	+++	+++	++	+++
	Proteinase K	++	+++	++	+++
	Trypsin	++	+++	++	+++
A. salmonicida subsp. *achromogenes*	α-Chymotrypsin	+	+	+	+
	Lysozyme	+	+	+	+
	Proteinase K	+	+	+	+
	Trypsin	+	+	+	+

+++++ represents strong inhibition at OD between 0.000 to 0.200 at 40 h of incubation; ++++ represents fairly strong inhibition at OD between 0.200 to 0.400 at 32 h; +++ represents weak inhibition at OD between 0.400 to 0.600 at 24 h of incubation; ++ represents fairly weak inhibition at OD between 0.600 to 0.800 at 16 h; + represents very weak inhibition at OD between 0.800 to 1.000 at 8 h of incubation. The values presented are the mean of six independent experiments. Control samples consist of freshly prepared cell-free supernatants without thermal treatment.

3.12. Purification of bacteriocins produced by *C. maltaromaticum* MMF-32 and KOPRI 25789

The bacteriocins produced by *C. maltaromaticum* MMF-32 and KOPRI 25789 were semi-purified from the cell-free supernatant fractions of cultures by ammonium sulphate precipitation and hydrophobic interaction chromatography (HIC) using a Hi Trap™ Octyl sepharose FF column. The purification steps and the recovery values of the bacteriocins are summarized in Table 3.5. The bacteriocins were recovered following the 50% saturation of the cell- free supernatants culture broths with ammonium sulphate with an increase to specific activity of 14.26 and 16.10 AU mg^{-1} protein, respectively. The purification protocol of binding with (50 mM sodium phosphate and 1.0 M ammonium sulphate) and eluting with (50 mM sodium phosphate) giving rise to semi-purified fractions. The percentage recovery at this stage of purification was 10.78% and 17.64% respectively. The specific activity decreased to 11.24 and 8.73 AU mg^{-1}, respectively. The inhibitory spectrum of the semi-purified bacteriocins against *L. monocytogenes* ATCC19114 using broth assay method (data shown in Figure 3.18 revealed inhibition, but *L. monocytogenes* ATCC 19114 did not grow as in control. The SDS-PAGE results of the cell free supernatant, ammonium sulphate precipitated supernatant and semi-purified bacteriocin carried out were not successful, so the results from this experiment were discarded. The purified fractions that were eluted following hydrophobic interaction chromatography using C_{18} hydrophobic chromatography column were not inhibitory against *L. monocytogenes* ATCC 19114.

Table 3.5. Purification stages of bacteriocin produced by *C. maltaromaticum* MMF-32 and KOPRI 25789

Organisms	Purification stages	Volume (ml)	Activity (Au ml^{-1})	Total activity (AV)[a]	Protein (mg ml^{-1}) [b]	Specific activity[c]	Purification factor[d]	Recovery (%)[e]
C. maltaromaticum MMF-32	Culture supernatant	500	80	40000	12.98	6.16	1	100
	Ammonium sulphate precipitation	80	80	6400	5.61	14.26	2.32	43.22
	Semi-purified protein	1	20	20	2.29	8.73	1.42	17.64
C. maltaromaticum KOPRI 25789	Culture supernatant	500	80	40000	16.51	4.85	1	100
	Ammonium sulphate precipitation	60	80	4800	4.97	16.10	3.31	30.10
	Semi-purified protein	1	20	20	1.78	11.24	2.32	10.78

[a]Total activity was determined by the multiplication of volume by activity

[b]Protein concentration was determined by the Smith method

[c]Specific activity is the activity units divided by the protein concentration (AU/mg)

[d]Purification factor is the increase in the initial specific activity

[e]Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration

NOTE for semi-purified protein undiluted stock was used because of limited volume

AU ml^{-1} = 2^n x (1000/100), n = 3 for culture supernatant and ammonium sulphate precipitation, n = 1 for semi-purified protein.

3.13. Discussion

Bacteriocin-producing *Carnobacterium* strains were isolated from smoked salmon and were identified using a 16S rDNA-targeted PCR method (Weisburg *et al*., 1991) and by morphological, cultural and biochemical characteristics using API-50CH, based on the methods of Collins *et al*. (1987) and Hammes and Hertel (2006). *C. maltaromaticum* often abounds as a member of the microflora of chilled vacuum or modified atmosphere-packed meat and seafood. Moreover, several studies have been dedicated to the application of carnobacteria for biopreservation of foods (Ahn and Stiles, 1990; Coventry *et al*., 1997) including various sea foods (Lakshmanan and Dalsgaard, 2004; Rudi *et al*., 2004; Emborg *et al*., 2005).

The bacteriocin like substances (BLIS) from *C. maltaromaticum* KOPRI 25789 and MMF-32 are interesting antimicrobial compounds because they exhibit broad spectrum inhibitory activity against Gram-positive and Gram-negative bacteria (Figs 3.2.-3.9.). Thus, the bacteriocins were tested for antibacterial activity against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes*. The highest inhibitory activity was exhibited against *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678 and *Serratia* sp. I-113-31, whereas the least activity was exhibited against *A. salmonicida* subsp. *achromogenes*, *Sh. baltica* OS185 and *L. monocytogenes* ATCC 19114. The antimicrobial substances researched in this work are referred to as BLIS as they have not been isolated and their amino acid sequences have not been characterized.

The antibacterial activity of LAB may be explained by organic acid production following a pH reduction or the production of hydrogen peroxide. Bacteriocins or bacteriocin-like producing compounds may also be produced (Gonźalez *et al*., 2007).

For this reason, antimicrobial activity was checked after treatment to remove inhibition from organic acids and hydrogen peroxide. The results of the present study show the presence of bacteriocin in the LAB. Studies have shown bacteriocins to be inhibitory against several other bacteria (Yang *et al*., 2012; Rajaram *et al.*, 2010; Maria and Janakiraman, 2012). Some carnobacterial bacteriocin producing strains have been reported to be active against *Lactobacillus farciminis*, *Brochothrix thermosphacta*, *Shewanella putrefaciens*, *L. monocyogenes*, *Staphylococcus xylosus*, *Pseudomonas* spp., *Serratia liquefaciens* and *Staphylococcus aureus* (Matamoros *et al*., 2009). These compounds may serve as natural substitutes for chemical food preservatives to enhance shelf-life of food, (Cleveland *et al*., 2001).

Furthermore, the results revealed a high level of antibacterial activity of *C. maltaromticum* KOPRI 25789 and MMF-32 against Gram-negative bacteria (Figs 3.4. – 3.8.), which is unusual, and has been reported for only a few LAB bacteriocins (Todorov and Dicks, 2005; De Kwaadsteniet *et al.,* 2005). For example, Gao *et al*. (2010) demonstrated the activity of sakacin C2 produced by *Lactobacillus sakei* C2 against *E. coli* ATCC 25922, *Salmonella enterica* serovar Typhimuriu*m* CMCC 47729 and *Shigella flexineri* CMCC 51606. Furthermore, sakacin LSJ618 inhibited *E. coli* ECX4 and *Proteus* sp. (Jiang *et al*., 2012). Sakacin has been used as an example of bacteriocin that inhibited Gram-negative bacteria. In contrast, Yamazaki *et al*. (2005) demonstrated that the antagonistic effect of cell-free supernatant from *C. piscicola* 526 did not inhibit *E. coli* IFO15034, *Salmonella enterica* serovar Enteritidis RIMD1933001, *Ps. fluorescens* JCM5963m and *A. hydrophila* IFO3820.

In addition, the results from this study showed a reduced level of activity against *L. monocytogenes* ATCC 19114 by the undiluted cell-free supernatants of *C. maltaromaticum* KOPRI 25789 and MMF-32 (Fig 3.3.). Higher dilutions (i.e 1:10 and

1:100) of the cell-free supernatants were not effective, and therefore as determined by Himelbloom (2001) using 'critical dilution' 1:2 should be employed. The work is consistent with the results of Matamoros *et al*. (2009), who demonstrated that their LAB strains revealed only weak inhibition of *L. monocytogenes*. However, it should be mentioned that several studies have confirmed the ability of LAB strains, isolated from various food products, to inhibit *L. monocytogenes* (Weiss and Hammes, 2006; Afzal *et al*., 2013; Barbosa *et al*., 2014; Wang and Wang, 2014). *Listeria* spp. comprises part of the inhibition range of carnobacterial class IIa bacteriocins, and the inhibitory activity is the result of pore formation, dispersion of membrane potential, and leakage of internal low molecular weight substances (Suzuki *et al*., 2005; Drider *et al*., 2006). Resistance of *L. monocytogenes* to divergicin M35 a bacteriocin produced by *C. divergens* M35 was likely due to change in structure of the cell wall fatty acid composition (Naghmouchi *et al*., 2006). According to Duffes *et al*. (2000), resistance of *L. monocytogenes* strains to class IIa divercin V41 also revealed considerable differences in protein expression as compared with the wild type. This means that for the resistant strain of *L. monocytogenes* the molecular mass of the protein ranges from 25 – 65 kDa, while the sensitive type had a protein molecular mass ranging from <20 – 35 kDa. Thus changes in cell envelope fatty acid composition (Vadyvaloo *et al*., 2004; Naghmouchi *et al*., 2007), D-alanine content of teichoic acids i.e. α-amino acid that occurs in fortified (teichoic acids) bacterial cell wall (Vadyvaloo *et al*., 2004), alterations in cell surface charge (Vadyvaloo *et al*., 2004) or changes in the major cell membrane components (Calvez *et al*., 2007; Tessema *et al*., 2009) were reported to be involved in the mechanisms of resistance to class IIa bacteriocins.

Todorov and Dicks (2004) reported that bacteriocin production was strongly dependent on temperature, pH and nutrient source. Different physicochemical factors seemed to

affect bacteriocin production as well as its activity (Rajaram *et al*., 2010). Nevertheless, it is important to note that in the present study the thermal effect of these BLIS on indicator strains revealed strong activity (represents OD between 0.000 to 0.200 nm), fairly strong activity (represents OD between 0.200 to 0.400 nm), weak activity (represents OD between 0.400 to 0.600 nm), fairly weak (represents OD between 0.600 to 0.800) at 16 h and very weak activity (represents OD between 0.800 to 1.000) (Table 3.2.). The BLIS under the thermal regimes of (56 °C for 30 min and 100 °C for 10 min) against *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *A. hydrophila* HX201006-3 and *A. salmonicida* subsp. *achromogenes* showed fairly weak and very weak activity. The thermal regimes of the BLIS against *Aeromonas* sp. HB-6, *Sh. baltica* and *Sh. baltica* OS678, revealed strong activity, fairly strong activity and weak activity, respectively, whereas for *Serratia* sp. I-113-31 it showed fairly strong activity. Thus the inhibitory activity of both cell-free supernatant under both heat regimes treated against *Aeromonas* sp. HB-6, *Sh. baltica*, *Sh. baltica* OS678 and *Serratia* sp. I-113-31 were stable.

Lee *et al*. (1999) reported the stability of partially purified bacteriocin by *L. lactis* subsp. *lactis* H-559 at 100 °C for 10 min. According to De Vuyst and Vandamme (1994b), the thermal stability may be caused by the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkages, and high glycine content. Piscicolin 126 produced by *C. piscicola* JG was stable at 100 °C over a wide range of pH (Jack *et al*., 1996). As reported in the present study (Table 3.2.), Kim and Austin (2008) demonstrated that both treated (at 100 °C for 30 min) and nontreated supernatants of B26 and B33 of carnobacteria isolates revealed stability against various bacteria. The inhibitory activity of cell-free supernatant of *C. piscicola* CS526 was completely stable after 30 min at 100°C (Yamazaki *et al*., 2005). Thermal

treated and untreated supernatants exhibited reduced or stable activity against indicator microorganisms, showing that it is proteinaceous in nature and that it denatures at certain temperature (Murtaza *et al*., 2012).

The activity of BLIS in this study was dependent on the producer strain and indicator microorganisms. The results showed that antibacterial activities of KOPRI 25789 and MMF-32 were lost at 8 h of incubation against *L. monocytogenes* ATCC 19114 when the heat regimes of 56 °C for 30 min and 100 °C for 10 min were applied. Similarly, the antibacterial activity of BLIS substances were lost at 8 h of incubation against *A. salmonicida* subsp. *achromogenes* when heat treatment of 56 °C for 30 min was applied. Conclusively, there was no antibacterial effect on *L. monocytogenes* ATCC 19114 and *A. salmonicida* subsp. *achromogenes* by the 2 LAB with the application of the heat regimes. The thermal stability at 56 °C for 30 min and 100 °C for 10 min (Table 3.2.) of BLIS produced by the bacteriocin-producing LAB isolates described here may suggest possible use as biopreservatives in combination with heat processing in order to preserve food products, in procedures like pasteurization, drying, refrigeration and freezing.

pH can enhance the antimicrobial activity of LAB, it must be an increase, decrease or within certain range. pH of media affected the antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 against for *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* I-113-31 and *A. salmonicida* subsp. *achromogenes* (Table 3.3.). The maximum antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 was increased significantly at pH 3.5 and 4.9. A reduced activity was observed at pH 6.5 used in analysing the inhibitory effect of the cell-free supernatants against the indicator microorganisms. Thus, the

antimicrobial activity increased significantly with decrease in pH. Residual activity of 100% was observed for all samples treated to pH 3.5 and 4.9. Dalié *et al*. (2010) hypothesized that organic acids act on the cytoplasmic membrane by neutralizing its electrochemical potential and increasing its permeability, thus leading to bacteriostasis and eventual death of the susceptible bacteria. Further to the effect of bacteriocin produced by these strains, the potent activity could be attributed to the production of lactic acid at lower pH value (Saranya and Hemashenpagam, 2011) or plausibly because of the better penetration of organic acids produced by LAB into the microbial cell wall at pH around 4.9 (Muyncka *et al*., 2004). Reduced activity at higher pH is a mark of alkali lysis of bacteriocin at high pH (Mandal *et al*., 2008). The activity of cell-free supernatant of *C. piscicola* CS526 was completely stable at pH 2 to 8 but reduced at values pH of 9 to 11, antimicrobial activity of 67, 44 and 30% was observed respectively, of the cell-free supernatant (Yamazaki *et al*., 2005).

Bacteriocins produced by lactic acid bacteria are endowed with general stability at acidic or neutral pH, showing that these substances are well modified to the environmental conditions produced by the bacteria (Vignolo *et al*., 1995). The maximum antimicrobial activity of *L. lactis* subsp. *lactis* TI-4 was observed at pH 5 (Sadiq *et al*., 2014). Maria and Janakiraman (2012) demonstrated the stability at broader pH value between 4.0 and 9.0 of bacteriocin from *L. acidophilus* NCIM5426. *L. brevis* OG1 exhibited the highest antibacterial activity in an acidic pH range of 2 to 6, while inactivation occurred at pH 8 to 12 (Ogunbanwo *et al*., 2003). Our result showed that *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 inhibited the growth of *L. monocytogenes* ATCC 19114 and *A. salmonicida* subsp. *achromogenes* at the pH range of 3.5 and 4.9 in contrast to the result at pH 6.5. These results indicate that the BLIS described in this study might be possibly used for application in both low and

medium–acid fermented food product having final pH with in such ranges and may well be used in fermented foods.

Bacteriocins may be degraded by some proteolytic enzymes resulting in a loss in their antimicrobial activity (Yang *et al*., 2012). In this study, *Sh. baltica* OS185, *L. monocytogenes* ATCC 19114, *Aeromonas* HB-6, *A. hydrophila* HX201006-3, *Sh. baltica*, *Sh. baltica* OS678, *Serratia* sp. I-113-31 and *A. salmonicida* subsp. *achromogenes* were used as indicators and the BLIS produced by *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 had various inhibitory effects as a result of treatment with α-chymotrypsin, lysozyme, proteinase K and trypsin (Table 3.4.).

Antimicrobial activity was reduced against *L. monocytogenes* ATCC 19114 with cell-free supernatant treated with α-chymotrypsin, protease K and trypsin residual activity of 20% was retained respectively. In contrast, complete activity was lost against *L. monocytogenes* when cell-free supernatant from *C. piscicola* CS526 treated with α-chymotrypsin and protease K, whereas trypsin partially inhibited with a residual activity of 67% (Yamazaki *et al*., 2005). Antimicrobial activity was not lost against *L. monocytogenes* ATCC 19114 with cell-free supernatants treated with lysozyme, residual activity of 100% was recorded.

Yang *et al.* (2012) demonstrated the use of *Listeria innocua* and *Lactbacillus sakei* as indicators, and the BLIS produced by different LAB strains had various inhibitory effects following use the treatment with proteolytic enzymes. The BLIS in the cell-free supernatants was sensitive to amylase (starch hydrolysis) and caseinase (skimmed milk). Sensitivity of the BLIS to amylase and caseinase suggests that carbohydrates and proteins are bound to the peptide (Korenblum *et al*., 2005). Khalil *et al*. (2009)

demonstrated the sensitivity of *B. megaterium* bacteriocin to amylase. The presence of amylase partially inhibited the bacteriocin activity of *Lactococcus lactis* isolated from Hukuti Maas, an indigenous fermented fish product (Kumar *et al*., 2012). With regard to the antibacterial activity exhibited by proteolytic enzyme treatments and the fact that the antimicrobial activities of the BLIS were not affected by catalase, I presume that antibacterial activities could be mainly due to proteinaceous compounds. The failure of the proteolytic enzymes to modify the antimicrobial activity of the bacteriocin is not unusual. Cherif *et al*. (2001) demonstrated the use of pepsin, papain, trypsin, chymotrypsin, protease K, lysozyme, catalase, DNase and RNase to treat thuricin 7, a bacteriocin produced *Bacillus thuringiensis* BNG 1.7 and they observed that the inhibitory activity was only susceptible to protease K. Thus, the observed resistance to proteolytic enzymes might be due to the presence of unusual amino acids in the bacteriocin structure, or cyclic N- and C-terminal blocked peptides (Khalil *et al*., 2009). The resistance of cyclic peptide to hydrolysis by proteases may be mainly due to their cyclic structure rendering them relatively inflexible, which may make cleavage sites inaccessible due to steric hindrance (Eckart, 1994).

An increased recovery in activity was observed in ammonium sulphate precipitated samples, which was followed by semi-purified and then supernatants. In this research, we endeavoured to purify the inhibitory substances using the octyl sepharose FF column hydrophobic chromatography. The elute of KOPRI 25789 and MMF-32 was precipitated in 70% (v/v) acetonitrile containing 0.1% trifluoro-acetic acid using C_{18} hydrophobic chromatography column, showed no inhibitory activity against *L. monocytogenes* ATCC 19114.

In conclusion, the antimicrobial activity of *C. maltaromaticum* KOPRI 25789 and *C. maltaromaticum* MMF-32 were similar. Their stability at 100°C for 10 min and 56°C

for 30 min and pH ranges of (3.5, 4.9 and 6.5), and susceptibility to enzyme treatments confirms their potential application as biopreservatives for food products subjected to pasteurization, cook-chilling, sterilization, fermentation and other heat processing treatments.

Chapter 4. Application of bacteriocins produced by *Carnobacterium maltaromaticium* MMF-32 for inhibition of *Listeria monocytogenes* ATCC 19114 in cold-smoked haddock.

4.1. Introduction

4.1.1. Increasing demand for safe seafood

The food industry has been faced with major concerns of contamination by pathogens, which are regular causes of diseases (Parada *et al*., 2007). The bacterial pathogens that account for many of these cases of disease include *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* (Buzby *et al*., 1996).

It is recognised that microbiological and enzymatic reaction rates increase directly at room temperature and in the presence of oxygen. Microbial growth builds up over time and causes spoilage that lead to economic losses and adverse human health. For this reason after harvesting, fish should be preserved properly (Briones-Labarca *et al*., 2012). In the United Kingdom the common causes of post-harvest fish losses are:

1) Insufficient preservation practices, such as poor storage amenities resulting in spoilage and insect infestation.

2) Poor handling methods during fishing resulting in fish falling from the nets, over handling resulting in bruising, fish being left for a long periods in nets leading to spoilage, and lack of chilling facilities on board with the exposure of fish to high ambient temperature.

3) Unpredictable transportation resulting to mechanical damage of fish.

4) Insufficient preservation methods as a result of poor storage amenities leading to spoilage.

5) Adverse weather conditions during processing making drying difficult and risk of invasion by insects.

6) Fish supply being greater than demand (Ward and Jeffries, 2000).

The development of effective processing treatments to extend the shelflife of fresh fish products is important (Campos *et al*., 2012). Moreover, the consumer demand for high quality and minimally processed seafood has recently drawn great attention to this issue (Calo-Mata *et al*., 2008; Alzamora *et al*., 2012). Lightly preserved fish products (LPFP), which are uncooked or mildly cooked, with low levels of preservatives (NaCl < 6% WP, pH >5), include cold-smoked salmon (CSS), fish carpaccio, and slightly cooked shrimp. LPFP are usually produced from fresh seafood and further processing increases the risk of cross contamination (Leroi *et al*., 2006; Pilet and Leroi, 2011). The use of effective chemical preservatives or the application of more drastic physical treatments, such as heating, refrigeration, high hydrostatic pressure (HHP), ionising radiation, pulsed-light, ozone or ultrasound, are approaches used to reduce the risk of outbreaks of food poisoning (Cortesi *et al*., 2009; Alzamora *et al*., 2012). Unfortunately, these treatments are not always sufficient to destroy microorganisms, and in some cases psychrotolerant pathogenic and spoilage bacteria may develop during the shelflife of LPFP, i.e. usually up to 10 days. As some of these products are eaten raw, the reduction and inhibition of microorganisms is regarded as important for food quality and safety. However, it is realised that not all microorganisms present in food are pathogenic. Some may be responsible for organoleptic changes such as off-odours and change in taste, texture and visual defects. Inhibiting the growth of these

microorganisms is also a challenge in need of solutions (Leroi *et al*., 2006; Pilet and Leroi, 2011). New technologies and methodologies are being developed to deal with fish preservation and shelflife extension (Alpas, and Akhan, 2012).

In order to reconcile consumer demands with important safety standards, traditional means of regulating microbial spoilage and safety hazards in foods are combined with novel technologies that include biological antimicrobial systems, such as the use of LABs and/or their bacteriocins, such as *Carnobacterium maltaromaticum* CS526 and its bacteriocin piscicocin CS526. The use of these microbial solutions, either alone or in combination with mild physicochemical treatments, may be effective for extending shelf-life and food safety through the inhibition of spoilage and pathogenic bacteria without changing the nutritional quality of raw materials and food products (Guzel-Zeydim and Ekinci, 2007).

4.1.2. *Listeria monocytogenes* in fish

Listeria monocytogenes is a Gram-positive, rod-shaped, food-borne pathogen that exists in a wide range of seafood and lightly preserved fish products (Ben Embarek, 1994; Rorvik *et al*., 2000; Kuzmanović, *et al*., 2011). The ability to grow at low temperatures obviously presents a major challenge to food safety with regard to *L. monocytogenes* as this pathogen persists in food processing environments and proliferates during chilled food storage (Hof *et al*., 1994; Nufer *et al*., 2007). *L. monocytogenes* can multiply in a wide pH (4.4 to 9.4) and temperature (-0.4 °C to 45 °C) range, as well as low water activity (a_w 0.92) (the unbound water to the food molecule). At 4°C, the reproduction time for the organism is 12 to 36 hours. Moreover, the organism is halotolerant and survives for up to 100 days at 30.5% salt concentration at 4°C. *Listeria* thrives best in thermally untreated food, that has spent a long time in storage, especially food that has

been produced in non-hygienic factories as well as cooked, cooled "ready to eat" (RTE) meals (Kuzmanović *et al*., 2011). Any growth of *L. monocytogenes* in lightly preserved fish products has been considered dangerous, and it is recognised that chemical treatments do not inhibit growth (Huss, 1997).

FSA (2008) reported the presence of *L. monocytogenes* in 302 ready-to-eat and cold smoked fish out of 3, 226 sampled in over 1000 retail outlets within the UK. Of the 302 samples, the weighted prevalence was 8.3%. The occurrence of *L. monocytogenes* in raw fish fillets and smoked-fish has been demonstrated in many recent studies. Thus, in smoked-fish, the incidence of contamination by *L. monocytogenes* was *ca*. 30%, with populations of <100 CFU g^{-1} (Uyttendaele *et al*., 2009). Pao *et al*. (2008) detected *L. monocytogenes* in raw fillets of catfish (23.5% of samples), trout (5.7% of samples), tilapia (10.3% of samples), and salmon (10.6% of samples). Basti *et al*. (2006) detected populations of *L. monocytogenes* at > 10^2 CFU g^{-1} in 2.6 % of fresh fish, 5.1% of smoked fish and 10% of salted-fish bought from fish farms, whereas 20% of smoked fish from a fish market revealed the organism.

Furthermore, *L. monocytogenes* contamination is one of the leading causes of recalls in industrially processed foods due to microbiological safety concerns (Nufer *et al*., 2007). As this organism is capable of growth at refrigeration temperatures, the zero-tolerance ruling issued by the U.S. Food and Drug Administration and in the UK for *L. monocytogenes* in ready-to-eat foods presents serious challenges to the food industry. Cold-smoked fish products are of major concern due to the lack of heat inactivation during processing, and consumption without any cooking step (Nufer *et al*., 2007; Gelbíčová and Karpíšková, 2009). Many preservation methods have been explored with the aim of reducing the incidence of *L. monocytogenes* in smoked fish. It is realised that salting selects Gram-negative, halophilic anaerobic bacteria, and a water activity of 0.95

does not inhibit *L. monocytogenes* development (Cheroute-Vialette *et al*., 1998). Moreover, refrigeration and vacuum storage do not guarantee inhibition of *L. monocytogenes* growth (Dorsa *et al*., 1993).

4.1.3. Biopreservation

Biotechnology in the food industry aims at the selection, production and improvement of useful microorganisms and their products, as well as in application to improving food quality (Parada *et al*., 2007). The inoculation of food with microorganisms or their metabolites, which have been selected for their antibacterial properties, may be an effective way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of the food product. This is defined as biopreservation (Galvez *et al*., 2010; Garcia *et al*., 2010). Additionally, the use of bacteriocin-producing cultures has an advantage of overcoming the decomposition and binding of food particles when used as additives (Rodgers, 2001). The antagonistic properties of LABs together with their history of use in traditional fermented food products makes them very attractive as biopreservatives. Moreover, during storage, they naturally dominate the microbiota of many foods. Some LABs detected from seafood have been shown to have strong inhibitory activity against spoilage and pathogenic microorganisms, including *Listeria*, *Clostridium*, *Staphylococcus* and *Bacillus* spp. (Brillet *et al*., 2005; Pinto *et al*., 2009; Leroi *et al*., 2010). The preservative effect of LABs is often due to the ability to produce inhibitory compounds, including hydrogen peroxide, ethanol, organic acids (lactic and acetic acid), carbon dioxide, bacteriocins or antibiotic-like substances (De Vuyst and Vandamme, 1994b; Collins *et al*., 2010). The ability of some LABs to grow at refrigeration temperatures and to endure the modified atmosphere obtained in packaging, low pH, high salt-concentrations, and the presence of additives, such as

lactic acid, acetic acid and ethanol, makes their use possible to inhibit the growth of undesired spoilage and pathogenic bacteria, with the subsequent benefits in terms of food safety (Calo-Mata *et al*., 2008; Collins *et al*., 2010; Leroi *et al*., 2010). As a food additive, *Lactococcus lactis* producing nisin has been granted the generally regarded as safe (GRAS) grade for use in some foods (Rattanachaikunsopon and Phumkhachorn, 2010), notably fermented products, but not yet in cold-smoked fish products.

4.1.4. The use of LABs in the control of pathogenic bacteria.

Carnobacterium spp. have been studied for their role as a component of the protective flora in cold smoked salmon (CSS), because of the ability to grow in foods with low carbohydrate content, e.g. fish products (Leroi *et al*., 1998; Drider *et al*., 2006). Fortunately, there is not any evidence that carnobacteria have any effect on the sensorial properties of cold-smoked salmon (Stohr *et al*., 2001) in contrast to other bacteriocin-producing LABs (Stiles, 1996). It is apparent that anti-listerial bacteriocins have been developed from several *Carnobacterium* strains (Drider *et al*., 2006). In particular, workers have demonstrated the bio-preservative power of bacteriocin-producing LABs against *L. monocytogenes* in cold-smoked salmon (Nilsson *et al*., 1999; Yamazaki *et al*., 2003; Tahiri *et al*., 2009). Another strategy for applying bacteriocins for biopreservation of food is the use of ammonium sulphate precipitated supernatant containing bacteriocin (Nilsson *et al*., 2004).

The biopreservative role of bacteriocins and/or bacteriocin producing strains in the food industry is practical if the limiting factors could be overcome (Nilsson *et al*., 2004). Workers have shown that nonbacteriocinogenic LABs have acid independent antibacterial properties (Degan *et al*., 1992; Leroi *et al*., 1996; Buchanan and Bagi, 1997; Nilsson *et al*., 1999). Moreover, Nilsson *et al*. (2004) demonstrated that the *C.*

piscicola A9b bac⁻ nonbacteriocin producing strain inhibited *L. monocytogenes* and that a significant nonbacteriocin-dependent inhibition was functioning.

Previous work has been done on shelf-life extension of haddock stored on ice at 0 °C (Banja, 2002). Thus, Olafsdottir *et al*. (2006) demonstrated the influence of storage temperature on microbial spoilage characteristics of haddock fillets evaluated by multivariate quality prediction. Shelf-life extension and safety concerns about haddock under high hydrostatic pressure have been demonstrated by Alpas and Akhan (2012). However, work has so far not been performed on the bio-preservation of cold-smoked haddock using nonbacteriocin producing strains, bacteriocin producing strains and bacteriocins.

4.2. Aims of this study

The aims of this Chapter were to:

Evaluate the inhibitory effects of *C. maltaromaticum* MMF-32, a bacteriocin producing strain, and a nonbacteriocin-producing mutant of *C. piscicola* A9b bac⁻ on the growth of *L. monocytogenes* ATCC 19114 in cold-smoked haddock.

To further determine the anti-listerial effect of *C. maltaromaticum* MMF-32 bacteriocins on cold-smoked haddock using:

1. *C. maltaromaticum* MMF-32 supernatant
2. *C. maltaromaticum* MMF-32 ammonium sulphate precipitated supernatant
3. *C. maltaromaticum* MMF-32 semi purified ammonium sulphate precipitated supernatant

4.3. Materials and methods

4.3.1. Statistical analysis

Statistical analysis involved use of MINI TAB. Significant differences among treatment means for each parameter measured over the 10 days of storage of smoked haddock were tested by analysis of variance using the general linear model and comparisons with controls using Dunnet's test. Pair-wise comparisons were analyzed using Tukey's test for statistical significant differences, with a P value of < 0.005 considered significant.

4.3.2. Bacterial strains and culture media

C. maltaromaticum MMF-32 was isolated in this study from a sample of cold-smoked salmon. *C. piscicola* A9b bac$^-$ (which does not produce bacteriocin) was obtained from Professor Lone Gram. *L. monocytogenes* ATCC 19114 was obtained from the American Type Culture Collection. All strains were maintained as 20% glycerol stock at -70 °C in TSB supplemented with 1% (w/v) sodium chloride. *C. maltaromaticum* MMF-32 was grown aerobically at 30 °C in de Man Rogosa and Sharpe (MRS) broth. *C. piscicola* A9b bac$^-$ and *L. monocytogenes* ATCC 19114 were grown in TNB at 30 °C.

4.3.3. Production of crude, concentrated ammonium sulphate precipitated bacteriocin and semi-purified bacteriocin of *C. maltaromaticum* MMF-32.

The crude preparations of bacteriocins were the supernatant fractions obtained after centrifugation (10,000 x *g*, 10 min at 4 °C) of 48 h culture of *C. maltaromaticum* MMF-32 grown in MRS at 30 °C.

Concentrated preparations of bacteriocins were prepared from crude preparations by precipitation with solid $(NH_4)_2SO4$ to a final concentration of 516 g l^{-1} at 4 °C with stirring. The precipitate was collected by centrifugation at 20,000 x *g* for 30 min at 4 °C, redissolved in 50 mM potassium phosphate buffer (pH 5.8) or distilled water, and filter sterilized (pore size, 0.45 µm; type Minisart NML; Sartorius) before its spectrum of activity was determined. The crude and concentrated ammonium sulphate precipitated bacteriocins were stored at -70 °C until use.

Semi-purified bacteriocin of *C. maltaromaticum* MMF-32 was obtained from concentrated preparations of bacteriocins using hydrophobic interaction chromatography. The concentrated ammonium sulphate precipitate was equilibrated to the composition of binding buffer (50 mM sodium phosphate and 1.0 M ammonium sulphate) by adjusting to pH 7.0 and filtered through 0.45 µm filters. The column was equilibrated with binding buffer (to rehydrate the column) (50 mM sodium phosphate and 1.0 M ammonium sulphate, pH 7.0) and the elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1}. Sixty five ml of concentrated ammonium sulphate precipitate was applied to hydrophobic interaction chromatography (HIC) column Hi Trap™ Octyl sepharose FF column (1 ml) (AKTA Prime, Pharmacia), then eluted with elution buffer (50 mM sodium phosphate, pH 7.0) at a flow rate of 1 ml min^{-1} (Lia *et al*., 2004). The absorbance was monitored at 280 nm, and bacteriocin activity of each fraction was determined by microtitre broth bioassay. The protein contents of the crude, concentrated ammonium sulphate precipitated bacteriocin and purified bacteriocin of *C. maltaromaticum* MMF-32 were estimated with the Pierce BCA Protein Assay Kit and bovin albumin as standard (Thermo Scientific), using the method of Smith *et al*. (1985). Specific activity was noted in arbitrary activity units (AU) per gram of protein.

4.3.4. Inhibition of *L. monocytogenes* in cold-smoked haddock

L. monocytogenes ATCC 19114 and *C. piscicola* A9b bac⁻ were subcultured three times in their respective culture media at 24 h intervals, whereas *C. maltaromaticum* MMF-32 was subcultured at 48 h intervals (the differences in subculture times is due to their different growth rates). Cells were harvested by centrifugation, washed three times with sterile PBS (0.01 M phosphate, pH 7.2) and finally resuspended in PBS to obtain cell concentrations of approximately 2.2 x 10^5 CFU ml^{-1} for *L. monocytogenes* ATCC 19114, 4.0 x 10^8 CFU ml^{-1} for *C. piscicola* A9b bac⁻, and 4.4 x 10^8 CFU ml^{-1} for *C. maltaromaticum* MMF-32.

Cold-smoked haddock fillets (undyed) were purchased from a retail shop in Scotland, cut into thin slices of 10 g ± 1.0 g weight and treated first by inoculating with 100 µl of 4.0 x 10^6 CFU g^{-1} fish of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac⁻. Fish slices were kept in a laminar–flow biological safety cabinet for approximately 10 min in order to dry off excess liquid, after which 100 µl of *L. monocytogenes* ATCC 19114 suspensions containing 2.2 x 10^5 CFU ml^{-1} (confirmed by plate drop count) were inoculated onto each sample to give a final inoculum of 2.2 x 10^3 CFU g^{-1} of fish. Following treatment, samples were individually packed in sterile petri dishes and kept at 4 °C for 10 days; this was the first application test to determine the inhibitory effect of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac⁻on cold-smoked haddock fillet before using the bacteriocins. For organoleptic evaluation, samples were prepared as above, with the exception that *L. monocytogenes* ATCC 19114 was not added (treatments E1 to F1).

The treatments are summarized in Table 4.1. A1 corrresponds to the addition of *L. monocytogenes* ATCC 19114 alone in cold-smoked haddock; B1 corresponds to the co-culture of *L. monocytogenes* ATCC 19114 with *C. maltaromaticum* MMF-32 in cold-

smoked haddock; C1 is the co-culture of *L. monocytogenes* ATCC 19114 with *C. piscicola* A9b bac⁻ in cold-smoked haddock; D1 is the control-uninoculated cold-smoked haddock; E1 corresponds to the cold-smoked haddock inoculated with *C. maltaromaticum* MMF-32 only and F1 corresponds to cold-smoked haddock inoculated with *C. piscicola* A9b bac⁻.

Table 4. 1. Experimental treatments of cold-smoked haddock with *Carnobacterium* spp.

Treatment code	Agent added		
	L. monocytogenes ATCC 19114	*C. maltaromaticum* MMF-32	*C. piscicola* A9b bac⁻
A1	+	-	-
B1	+	+	-
C1	+	-	+
D1 (control)	-	-	-
E1	-	+	-
F1	-	-	+

L. monocytogenes ATCC 19114 was added at 2.2 x 10^3 CFU g^{-1}

C. maltaromaticum MMF-32 culture was added at 4.0 x 10^6 CFU g^{-1}

C. piscicola A9b bac⁻ was added at 4.0 x 10^6 CFU g^{-1}

Control sample for Table 4.1. is D1

Following the first application test, each haddock slice surface was inoculated with 36 µg of semi-purified peptide solution (2.7 x 10^5 AU g l^{-1} protein) per g of fish or 0.5 ml of concentrated ammonium sulphate precipitated supernatant (1.7 x 10^4 AU g l^{-1}) or MRS (6.72 x 10^3 AU g l^{-1}) culture supernatant.

Fish slices were kept in a laminar–flow biological safety cabinet for approximately 10 min in order to dry off excess liquid, after which 100 µl of *L. monocytogenes* ATCC 19114 suspensions containing 4.0 x 10^5 CFU ml^{-1} were inoculated onto each sample to

give a final inoculum of 4.0 x 10^3 CFU g^{-1} of fish. For organoleptic evaluation, samples were prepared as above, with the exception that *L. monocytogenes* ATCC 19114 was not added (treatments F to H). Following treatment, the samples were individually packed in sterile petri dishes and kept at 4 °C for 10 days. The treatments applied are summarized in Table 4. 2. A corresponds to the addition of *L. monocytogenes* ATCC 19114 alone in cold-smoked haddock; B corresponds to the co-culture of *L. monocytogenes* ATCC 19114 with MRS supernatant in cold-smoked haddock; C is the co-culture of *L. monocytogenes* ATCC 19114 with supernatant concentrated with $(NH_4)_2SO_4$ in cold-smoked haddock; D is the co-culture of *L. monocytogenes* ATCC 19114 with semi-purified bacteriocin in cold-smoked haddock; E corresponds to the control-uninoculated cold-smoked haddock; F corresponds to the cold-smoked haddock inoculated with MRS supernatant only; G is the cold-smoked haddock inoculated with supernatant concentrated with $(NH_4)_2SO_4$ only, and H is the cold-smoked haddock inoculated with semi-purified bacteriocin only.

Table 4. 2. Experimental treatments of cold-smoked haddock with bacteriocins

Treatment code	Agent added			
	L. monocytogenes ATCC 19114[a]	MRS supernatant[b]	Supernatant conc. with $(NH_4)_2SO_4$[c]	Semi-purified[d]
A	+	-	-	-
B	+	+	-	-
C	+	-	+	-
D	+	-	-	+
E (control)	-	-	-	-
F	-	+	-	-
G	-	-	+	-
H	-	-	-	+

[a] *L. monocytogenes* ATCC 19114 was added at 4.0×10^3 CFU g^{-1}.

[b] Supernatant of *C. maltaromaticum* MMF-32 culture in MRS broth added at 6.2×10^3 AU g l^{-1}.

[c] Supernatant of *C. maltaromaticum* MMF-32 culture in MRS broth, concentrated with $(NH_4)_2SO_4$ (1.7×10^4 AU g l^{-1}).

[d] Semi-purified concentrated $(NH_4)_2SO_4$ supernatant added at 2.7×10^5 AU g l^{-1} of haddock (36 μg g l^{-1} of haddock).

Control sample for Table 4.2. is E

The experiments were performed twice, and samples were taken in duplicate at day 1 and periodically during the 10 days of storage for microbiological analyses and pH measurement, respectively. Texture, colour and organoleptic analyses were done at intervals of 3 days during the 10 days of storage (i.e. all the organoleptic studies for both experiments).

4.3.5. Microbiological analysis

Ten grams of each fish slice were aseptically placed into a sterile stomacher bag (Seward) and homogenised for 3 min in 90 ml volumes of *Listeria* primary selective enrichment broth base CM0863 supplemented with SR0142E (UVM I; Oxoid) using a Lab Blender 400 Stomacher (Seward). The homogenate was incubated at 30 °C for 24 h. One hundred µl volumes of (UVM I) homogenate were transferred to 10 ml volumes of *Listeria* secondary selective enrichment broth base CM0863 supplemented with SR0143E (UVM II; Oxoid) and incubated at 30 °C for 24 h. Serial dilutions were prepared to 10^{-8} in UVM II, and 0.1 ml amounts were spread over the surface of triplicate plates of PALCAM agar base CM0877 supplemented with SRO150E (PALCAM; Oxoid) [for the recovery of *L. monocytogenes*] plates with incubation aerobically at 30 °C for 48 h. The total number of LABs were determined on All Purpose Tween agar (APT) (VWR) supplemented with $NaNO_2$ (0.6%), polymyxin B-sulphate (0.003 g l^{-1}) and actidione cycloheximide (0.01 g l^{-1}) all obtained from Sigma-Aldrich to form nitrite actidione polymyxin (NAP) agar (Truelstrup Hansen *et al*., 1995). Control samples were tested alongside others. Total viable bacterial counts were determined using TNA with incubation aerobically at 30 °C for 48 h. *Carnobacterium* spp. were selectively enumerated on cresol red thallous acetate sucrose agar (CTAS) as proposed by Wasney *et al*. (2001), with some modification. Thus, the inoculated plates were incubated at 25 °C for 48 h for LAB, and followed by 48 h at 15 °C instead of 48 h at 8°C, as proposed by Wasney *et al*. (2001).

4.3.6. Total volatile base nitrogen (TVBN)

The steam distillation method of Malle and Poumeyrol (1989) was used. Thus, 200 ml of 7.5% aqueous trichloroacetic acid solution was added to 100 g of fish muscle in a

metal beaker and homogenized in a Waring blender before the mixture was filtered through Whatman No 3 filter paper. The extract was stored in a cooler for one week. Using a Kjeldahl-type distillator (Struer TVN), steam distillation was performed by transferring 25 ml of filtrate into a distillation flask followed by 6 ml of 10% NaOH. Ten millilitre of 4% boric acid (containing 0.04 ml of methyl red and bromocresol green indicator) was pipetted into an Erlenmeyer flask and placed under the condenser for the titration of ammonia. Distillation was started and continued until a final volume of 50 ml was obtained in the beaker (40 ml of distillate). The boric acid solution turned green when alkalinized by the distilled TVB-N, which was titrated with aqueous 0.025 N sulphuric acid solutions using a 0.05 ml graduated burette. Complete neutralization was obtained when the colour turned grey/pink on the addition of a further drop of sulphuric acid.

4.3.7. Biogenic amine measurement

Approximately 50 g of fish sample was weighed into a glass container and homogenized for 45 sec in *ca*. 100 ml 10% TCA. The extract was filtered through Whatman 542 filter paper under vacuum and made up to 100 ml in a volumetric flask, mixed thoroughly, and then a small amount was filtered through a 0.45 µm filter. Derivatization was determined by adding 0.25 ml of sample/standard to 0.5 ml of o-phthaldialdehyde (OPA) reagent in a test tube with a screwed cap. The solution was kept in dark for exactly 3.5 min. Then 2 ml of ethyl acetate was added and vortexed for 1 min and kept until phase separation was completed. A volume of 1.5 ml from the top phase was pipetted into a vial and kept for 3.5 min. After the addition of ethyl acetate, the sample/standard was injected for analysis. Approximately 100 mg of each standard was weighed and made up to 100 ml with 10% TCA in volumetric flasks. A stock solution was made by mixing the four amines (histamine, tyramine, putrescine and

cadaverine) in volumes, which were about 10 mg of the amines 100 ml^{-1} of 10% TCA, the amount is for each of the amines. Suitable dilutions were then made for standard curve preparation. Quantification of samples was by area measurement determined from a standard area versus concentration plot (Gouygou *et al*., 1987, 1989; Corbin *et al*., 1989; Cichy *et al*., 1993; Taibi and Schiavo, 1993; Kirschbaum and Luckas, 1994; Marcé *et al.*, 1995; Malle and Vallé, 1996).

4.3.8. Sensorial analysis

Postgraduate students from the Institute of Aquaculture at the University of Stirling, Scotland, were asked to evaluate the acceptability of samples from duplicate trials of treatment D1 to F1 and E to H to compare them to the control (treatment D1 and E) with respect to colour, odour and texture. Scales for colour, odour and texture features were presented to the panelists. The sample was considered discoloured if the colour was pale. Odour was classified as 10-8 for 'smoke aroma', 7.8-4 for absence of smoke and 3.9-below as spoilage. Texture was scored qualitatively as either 'firm' (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock - this was to enable proper observation of changes in the organoleptic parameters.

4.4. Results

4.4.1. Broth assay: inhibition of *L. monocytogenes* ATCC 19114 by *C. maltaromaticum* MMF-32 culture supernatant, ammonium sulphate precipitated culture supernatant and semi-purified bacteriocin.

The broth assay tests showed the inhibitory effects of supernatants from cultures of *C. maltaromaticum* MMF-32, ammonium sulphate precipitated supernatant and semi-purified bacteriocin on *L. monocytogenes* (Fig. 4.1.). The addition of *C. maltaromaticum* MMF-32 cell-free supernatant inhibited the growth of *L. monocytogenes* ATCC 19114 at 8 h, but thereafter increased growth of *L. monocytogenes* was obvserved (Fig. 4.1. A). A significant growth inhibition in the growth of *L. monocytogenes* ATCC 19114 was observed by ammonium sulphate precipitated supernatants of MMF-32 over 40 h of incubation (Fig. 4.1. B). Inhibition was observed against *L. monocytogenes* ATCC 19114 following treatment with semi-purified bacteriocin of *C. maltaromaticum* MMF-32, only at 16 h post incubation (Fig. 4.1. C).

4.4.2. Inhibition in the growth of *L. monocytogenes* ATCC 19114 on cold-smoked haddock following incubation with *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac$^-$ during storage at 4 °C for 10 days.

In this experiment there were 4 treatments (Table 4.1). These were cold smoked haddock with A1. *L. monocytogenes* ATCC 19114 added, B1. *L. monocytogenes* ATCC 19114 and *C. maltaromaticum* MMF-32 added, C1. *L. monocytogenes* ATCC 19114 and non-bacteriocin producing *C. piscicola* A9bac$^-$ added and D1. Untreated control of cold smoked haddock did not have anything added to them. Initial counts of *C. maltaromaticum* MMF-32 and *C. piscicola* A9bac$^-$ added were 4.0 x 10^6 CFU g^{-1}.

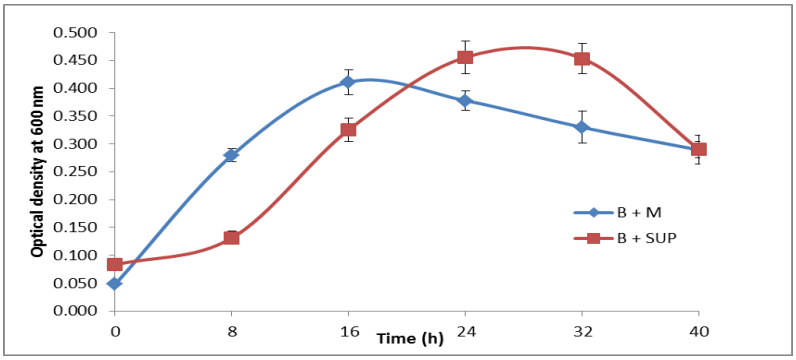
However, at the first sampling time at 24 hours post inoculation, the count of *Carnobacterium* was higher in the treatments to which no *Carnobacterium* had been added (A1 and D1) than in the treatment to which it had been added. This suggested that either there had been a problem with the inoculation or subsequent enumeration procedures, therefore the results from this experiment were discarded.

The *Carnobacterium* counts revealed that these were not significantly different, nor were there any significant differences between the treatment on day 3, 7 or 10. Although the control (D1) was significantly higher than the treatment on day 10 (Table 4.3).

Table 4.3. *Carnobacterium* counts on the days of inoculation

Days of inoculation	A1. *L. monocytogenes* ATCC 19114 added	B1. *L. monocytogenes* ATCC 19114 and ***C.*** *maltromaticum* MMF-32 added	C1. *L. monocytogenes* ATCC 19114 and non-bacteriocin producing *C. piscicola* A9bac$^-$ added	D1. Untreated control of cold smoked haddock with nothing added.
1	1.75×10^{9}	8.38×10^{8}	4.63×10^{8}	2.13×10^{9}
3	5.75×10^{9}	1.25×10^{10}	1.38×10^{10}	1.25×10^{8}
7	2.37×10^{8}	2.25×10^{8}	2.63×10^{8}	1.25×10^{7}
10	1.23×10^{8}	1.93×10^{8}	2.00×10^{8}	7.68×10^{8}

A



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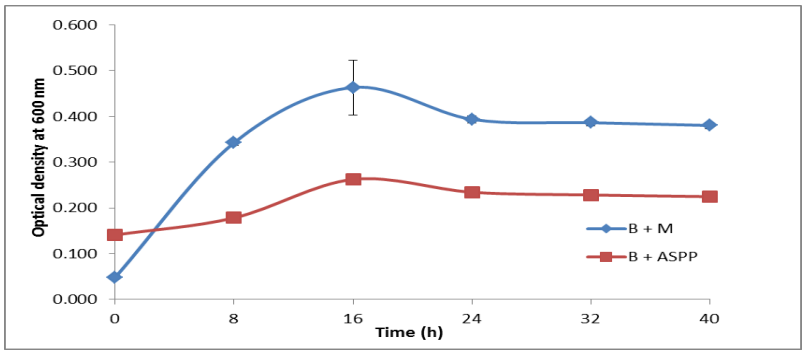

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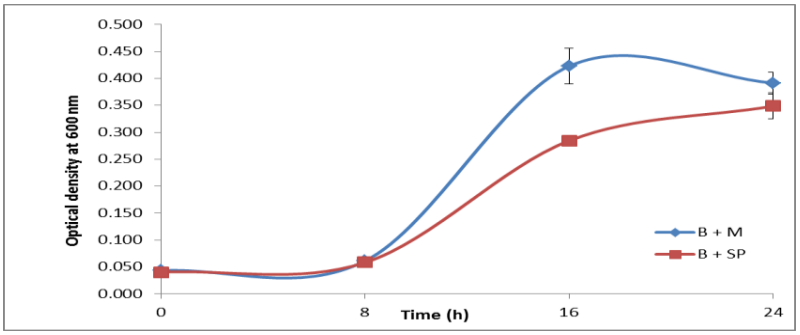

Figures 4.1. Broth assay using 96-well microtitre plates showing inhibition of *L. monocytogenes* ATCC 19114 by (A) *C. maltaromaticum* MMF-32 culture supernatant (B) ammonium sulphate precipitated culture supernatant of *C. maltaromaticum* MMF-32 (C) semi-purified bacteriocin from ammonium sulphate precipitated culture supernatant of *C. maltaromaticum* MMF-32. B + M represents *L. monocytogenes* ATCC 19114 and media; B + SUP represents *L. monocytogenes* ATCC 19114 with media and supernatant bacteriocin; B + ASPP represents *L. monocytogenes* ATCC 19114 with media and ammonium sulphate precopitated supernatant; B + SP represents semi-purified bacteriocin. Points = Means ± SE.

4.4.3. Inhibition in the growth of *L. monocytogenes* ATCC 19114 on cold-smoked haddock following incubation with MRS crude supernatant, ASP supernatant and semi-purified bacteriocin during storage at 4 °C for 10 days

Fig. 4.2 shows the growth pattern of *L. monocytogenes* ATCC 19114 viable counts during storage of smoked haddock using different bacteriocin applications *i.e.* MRS crude supernatant (6.72 x 10^3 AU g l^{-1}), ASP supernatant (0.5 ml; 1.7 x 10^4 AU g l^{-1}) and semi-purified bacteriocin (2.7 x 10^5 AU g l^{-1}; 36 µg l^{-1}) (Table 4.2.). *L. monocytogenes* ATCC 19114 alone (treatment A) grew rapidly in smoked haddock in the first 24 h, with counts increasing from 4.0 x 10^3 CFU g^{-1} initial inoculum (section 4.3.4.) to 5.3 x 10^8 on CFU g^{-1} day 1, and then similar levels were maintained until day 10 (4.7 x 10^8 CFU g^{-1}) during storage (Fig. 4.2). In the control samples (i.e. haddock only), *L. monocytogenes* ATCC 19114 was not observed, confirming that *L. monocytogenes* was introduced by the treatment (data not shown). A statistical significant reduction in *L. monocytogenes* ATCC 19114 counts was only observed by the application of semi-purified bacteriocin on day 7, resulting in a count of 8.59 log CFU g^{-1} (= 3.9 x 10^8 CFU g^{-1}) when compared with the positive control having count of 8.71 log CFU g^{-1} (=5.1 x 10^8) (treatment A). Neither the application of crude MRS supernatant nor ammonium sulphate precipitated supernatant (treatments B and C, section 4.3.3.) resulted in inhibition in the growth of *L. monocytogenes* ATCC 19114 in the smoked haddock). The application of ammonium sulphate precipitated supernatant (treatment C) showed a statistical significant increase of 8.78 log CFU g^{-1} (= 6.3 x 10^8) of *L. monocytogenes* ATCC 19114 on day 7. The increase in count of *L. monocytogenes* ATCC 19114 in the ammonium sulphate precipitated supernatant may be due to variation in samples.

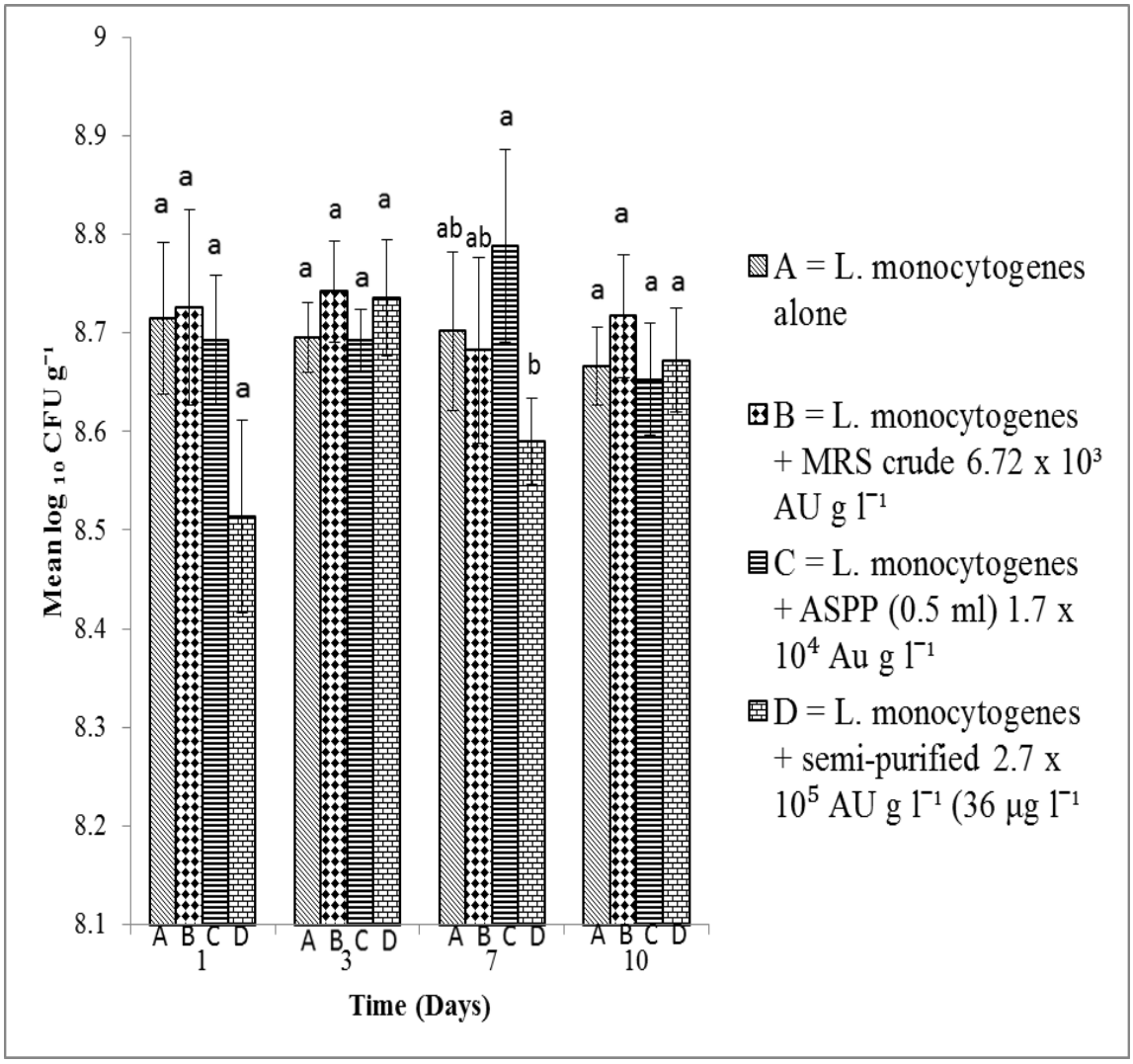

Figure 4.2. Total of viable counts of *L. monocytogenes* ATCC 19114 added to cold-smoked haddock stored at 4 °C for 10 days. (▨) *L. monocytogenes* (treatment A); (▦) *L. monocytogenes* with MRS supernatant (treatment B); (▤) *L. monocytogenes* with ammonium sulphate precipitated supernatant (treatment C) and (▥) *L. monocytogenes* with semi-purified bacteriocin (treatment D). The error bars indicate standard deviations of repeated treatments. Means ± STD with different low case within the same group of bars are significantly different (P < 0.005).

4.4.4. Total bacteria count on cold smoked haddock

This was carried out to determine the total bacteria count in the cold-smoked haddock and to determine any inhibition by the bacteriocins. Control samples gave counts from 2.0 x 10^7 to 1.9 x 10^8 log CFU g^{-1} during the storage peroid. The total bacterial counts of the treated samples were high, ranging from 3.7 x 10^8 to 7.0 x 10^8 CFU g^{-1} over the 10 day storage period. Significant differences ($p \leq 0.05$) were observed between the treated samples (treatment A, B, C and D) and control samples (treatment E, untreated fish) during the storage period except for day 1 (Table 4.4). However the appropriate controls (without *L. monocytogenes*) were not included and therefore the effect of the bacteriocins on total bacteria count could not be extrapolated.

Table 4.4. Total bacteria count on cold smoked haddock

Days of inoculation	A. *L. monocytogenes* ATCC 19114 alone	B. *L. monocytogenes* ATCC 19114 and MRS supernatant	C. *L. monocytogenes* ATCC 19114 and $(NH_4)_2SO_4$	D. *L. monocytogenes* ATCC 19114 and semi – purified	E. Control Untreated smoked haddock with nothing added
1	4. 7 x 10^8	5.6 x 10^8	4.5 x 10^8	5.8 x 10^8	1.9 x 10^8
3	5.7 x 10^8	7.0 x 10^8	5.5 x 10^8	5.1 x 10^8	1.3 x 10^8
7	4.9 x 10^8	5.6 x 10^8	4.9 x10^8	3.7 x 10^8	2.0 x 10^7
10	5.0 x 10^8	5.2 x 10^8	5.2 x 10^8	4.7 x 10^8	1.1 x 10^8

4.4.5. Biogenic amine production

MRS supernatant had the highest cadavarine content of 297.5 mg N 100 g^{-1}, at day 10 (treatment F) (Fig. 4.3). The uninoculated flesh had a lower cadavarine content of 83.5 mg N 100 g^{-1} (treatment E) whereas the cold-smoked haddock inoculated with ammonium sulphate precipitated supernatant and purified bacteriocin had ~60 mg N 100 g^{-1} (treatment G and H) of cadavarine on day 10. Putrescine content was detected only in MRS supernatant, having 61 mg N 100 g^{-1}, at day 10. Cadavarine and putrescine were not detected at day 0 from the bacteriocin inoculated and uninoculated flesh. Furthermore histamine was not detected from bacteriocin inoculated and uninoculated flesh on day 0 and 10 (data not shown). High levels of putrescine and cadavarine have been identified as potentiators of histamine or tyramine toxicity, but no recommendation about levels have been suggested (Park *et al*., 2010).

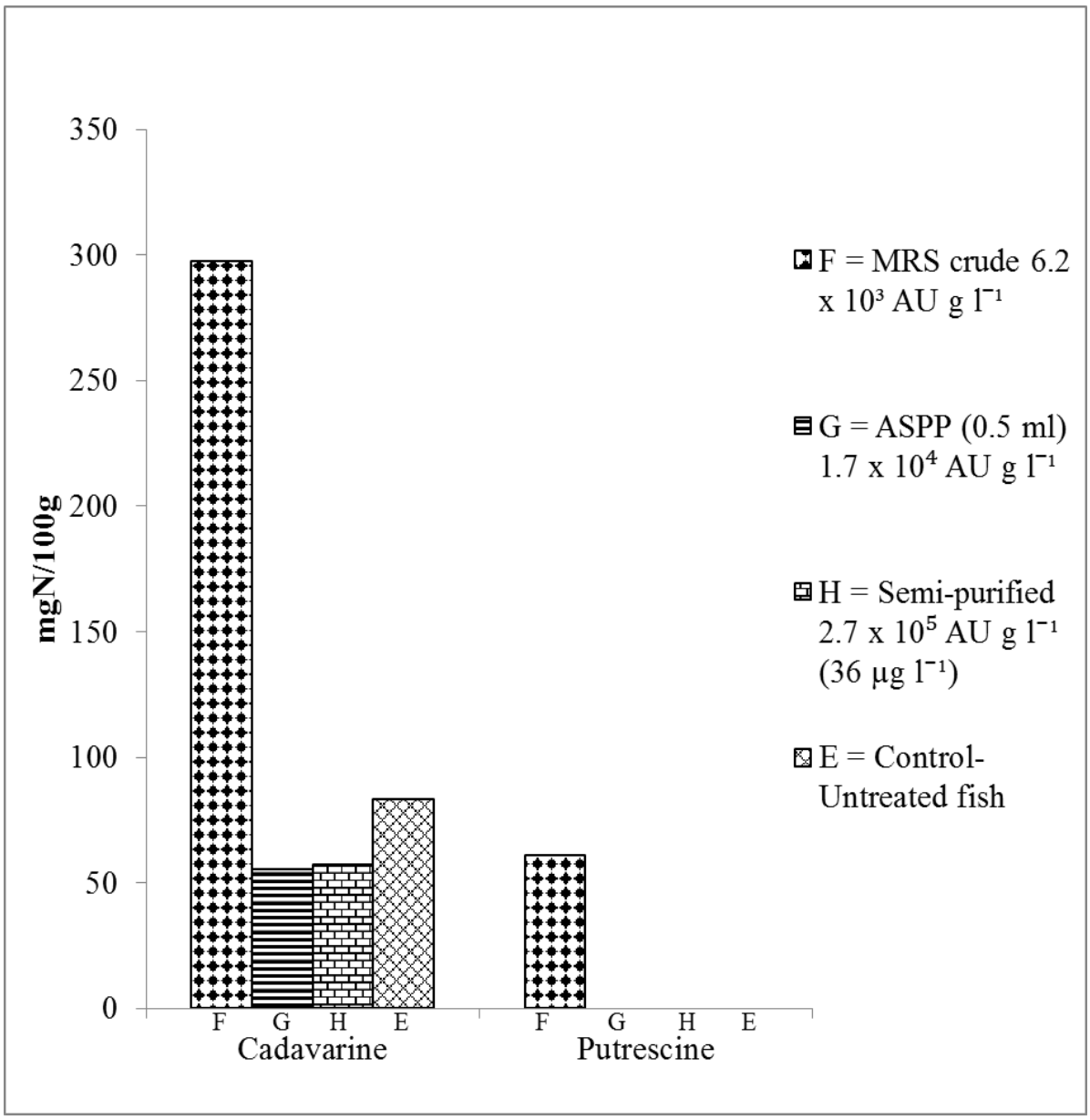

Figure 4.3. Biogenic amine concentration at day 10 in cold-smoked haddock during (storage at 4 °C). ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish).

4.4.6. Total volatile base nitrogen production

For all treatments excluding (treatment G), the volatile base nitrogen increased from 30 – 73 to 104 – 253 mg N 100 g^{-1} after 10 days of storage at 4 °C. Treatment G was exceptionally high values from 263-405 mg N 100 g^{-1} during the storage period. Significant differences were observed within treatments (Fig. 4.4.). The TVBN levels for all the treatments were above the acceptable limit for fresh fish appreciation.

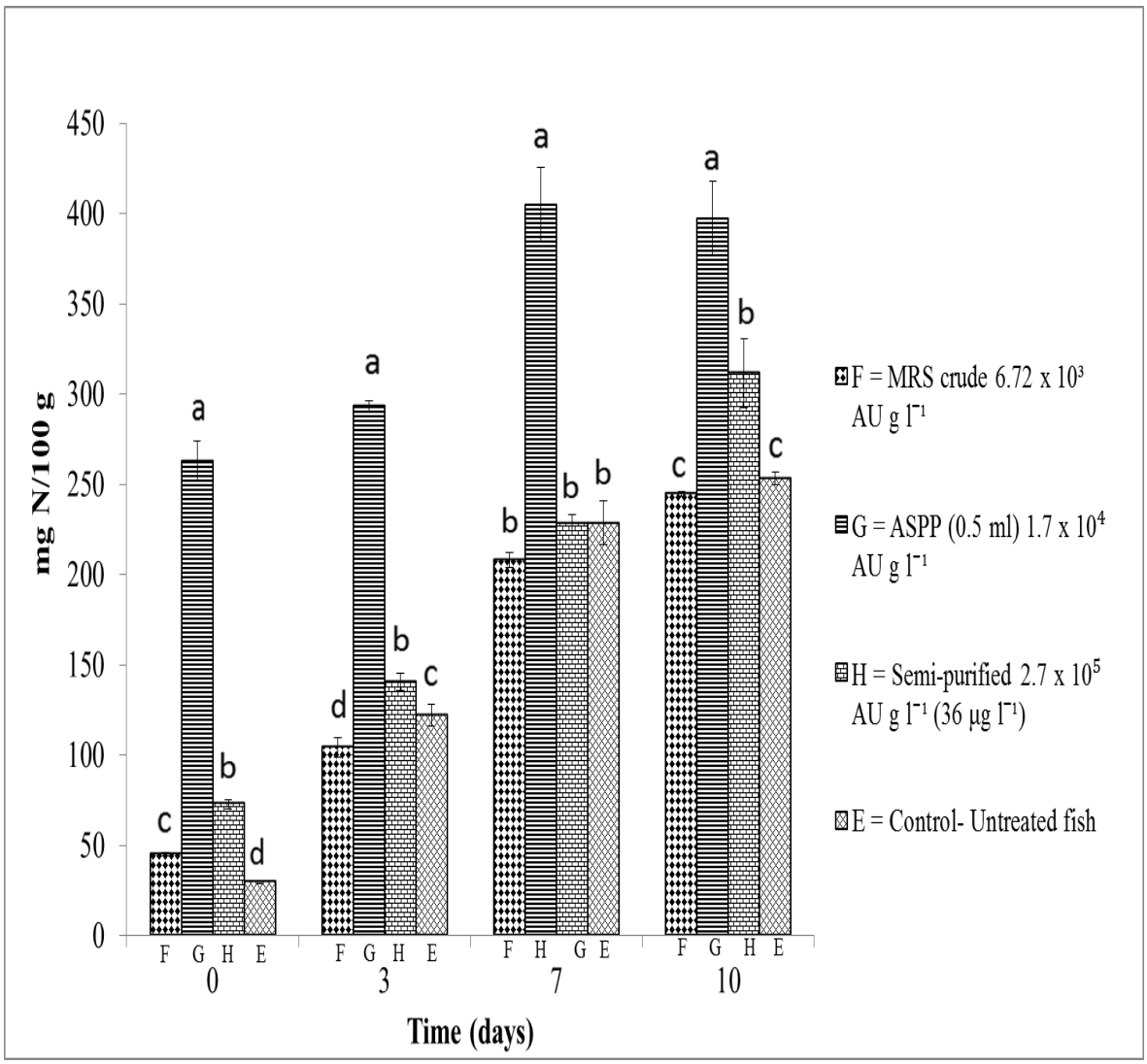

Figure 4.4. Total volatile base nitrogen production in cold-smoked haddock during storage at 4 °C for 10 days. ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish). The error bars indicate standard deviations of repeated treatments. Means that do not share a letter within the same group are significantly different (P < 0.005).

4.4.7. pH readings for *Carnobacterium* spp. and bacteriocin treated samples

There was no significant change revealed in the pH readings for the ten days of storage for any treatment conditions for *Carnobacterium* spp. and bacteriocin treated samples including the controls (Figs. 4.5. and 4.6.).

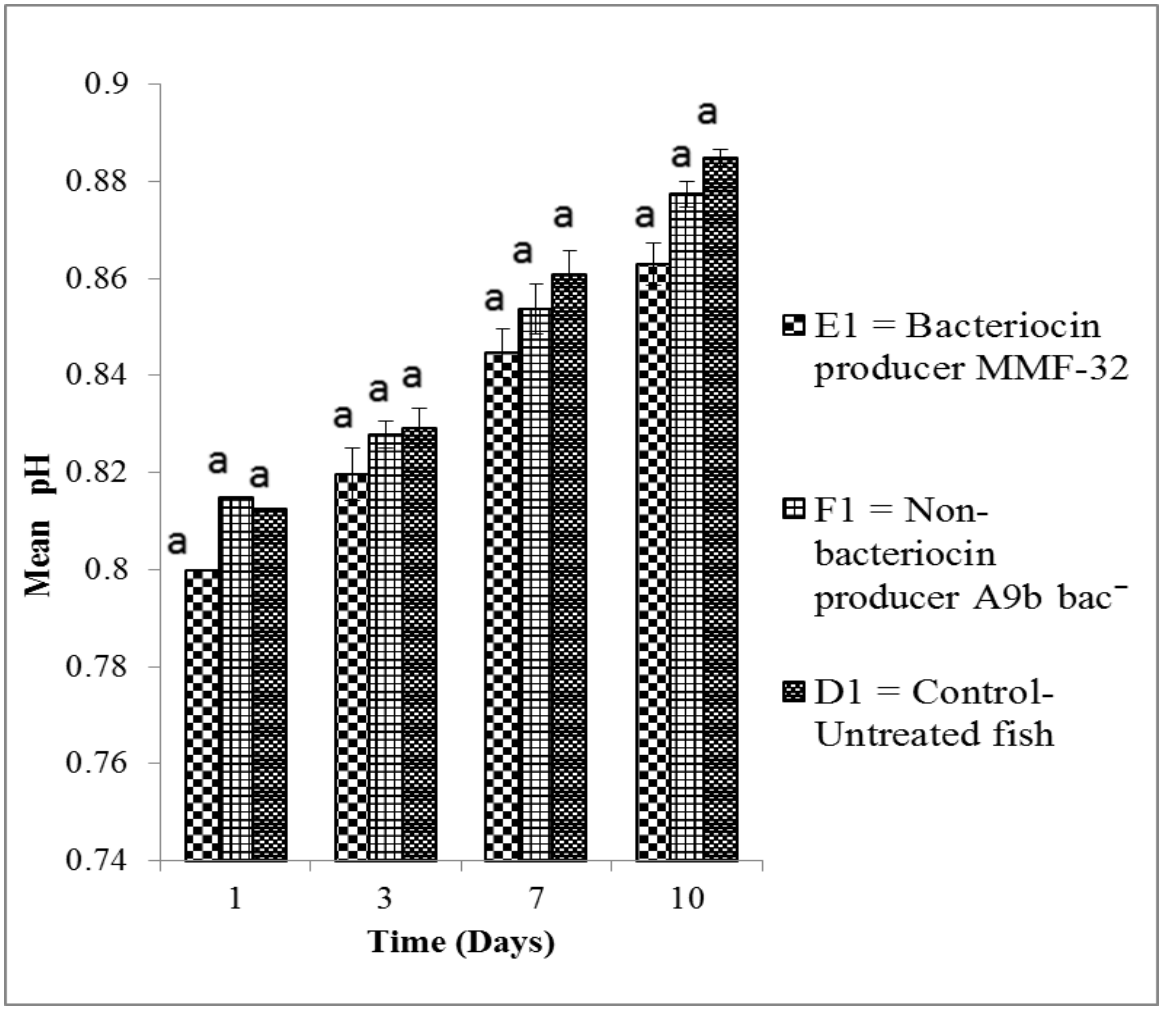

Figure 4.5. pH readings of *Carnobacterium* spp. treatment added to cold-smoked haddock stored at 4 °C for 10 days. ( ) *C. maltaromatic*um MMF-32 (treatment E1) and ( ) *C. piscicola* A9b bac⁻ (treatment F1); ( ) Control (treatment D1, untreated fish). The error bars indicates standard deviations of repeated treatments.

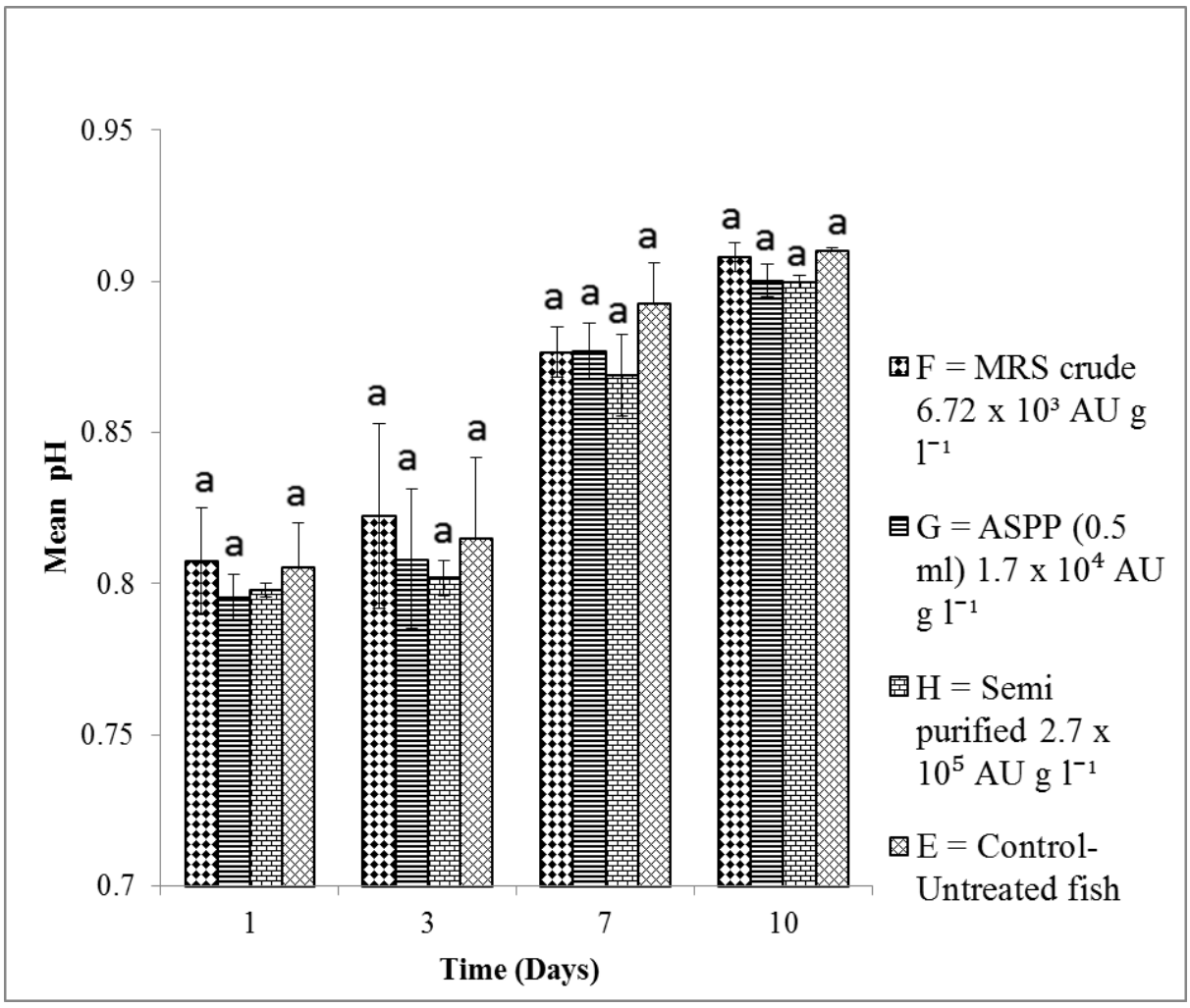

Figure 4.6. pH readings of all bacteriocin treatment added to cold-smoked haddock stored at 4 °C for 10 days. ( ) cold-smoked haddock with MRS supernatant (treatment F); ( ) cold-smoked haddock with ammonium sulphate precipitated supernatant (treatment G); ( ) cold-smoked haddock with semi-purified bacteriocin (treatment H) and ( ) control (treatment E, untreated fish). The error bars indicate standard deviations of repeated treatments.

The initial pH of smoked haddock was between 6.1 and 6.7 and did not get below 6.1 (Table 4.5. and 4.6.). On day 10 of storage, the pH readings of *Carnobacterium* spp. treated samples and bacteriocin applications varied between 7.22 to 7.78 and 7.84 to

8.19 respectively. Control is treatment D1 (untreated fish) for Table 4.5. Control treatment E (untreated fish) for Table 4.6.

Table 4.5. pH readings for *Carnobacterium* spp. treatment

Days	*C. maltaromaticum* MMF-32 Treatment E1	*C. piscicola* A9b bac$^-$ Treatment F1	Control Treatment D1, untreated fish
1	6.37	6.56	6.44
	6.38	6.47	6.50
	6.23	6.55	6.58
	6.25	6.54	6.46
3	6.63	6.76	6.71
	6.38	6.72	6.66
	6.23	6.81	6.81
	6.25	6.62	6.81
7	7.09	7.08	7.24
	7.00	7.17	7.24
	6.94	7.14	7.30
	6.94	7.17	7.25
10	7.36	7.71	7.77
	7.22	7.69	7.78
	7.28	7.25	7.63
	7.31	7.52	7.51

Table 4.6 . pH readings for bacteriocin treatment

Days	MRS supernatant Treatment F	Ammonium sulphate precipitated Treatment G	Semi-purified Peptide Treatment H	Control Treatment E untreated fish
1	6.19	6.14	6.27	6.19
	6.21	6.16	6.31	6.23
	6.58	6.34	6.24	6.53
	6.71	6.34	6.30	6.62
3	6.26	6.14	6.24	6.17
	6.24	6.14	6.29	6.21
	7.03	6.71	6.41	6.89
	7.09	6.75	6.41	6.88
7	7.62	7.42	7.59	7.94
	7.67	7.39	7.60	7.99
	7.35	7.74	7.15	7.47
	7.47	7.58	7.26	7.85
10	8.10	7.89	7.90	8.13
	8.19	7.84	7.92	8.15
	8.09	8.03	7.96	8.10
	7.98	8.04	7.98	8.14

4.4.8. Organoleptic parameters

C. maltaromaticum MMF-32 treated samples (treatment E1) were firm in texture throughout the storage period; *C. piscicola* A9b bac$^-$ treated samples (treatment F1) showed firmness from day 0 to 7; the control samples (treatment D1, untreated fish) were firm from day 0 to 3 (Table 4.7.). *Carnobacteriocin* spp. treated samples (treatment E1 and F1) retained the light pink colour during the first 7 days of storage,

whereas the control sample (treatment D1) retained a light pink colour from day 0 to 3 (Table 4.7.). The *C. maltaromaticum* MMF-32 treated samples (treatment E1) retained the smoky odour for the first 7 days of storage, but an absence of the smoky odour was recorded on day 10. *C. piscicola* A9b bac^{-} and control treated samples (treatment F1 and D1) had the smoky odour from day 0 to 3; on day 7 an absence of the smoky odour was noted (Table 4.7.).

Table 4. 7. Organoleptic parameters of *Carnobacterium* spp. treated samples

Treatment	Days	Colour	Odour	Texture
C. maltaromaticum MMF- 32	0	Light pink	9.80	Firm
	3	Light pink	9.20	Firm
	7	Light pink	8.25	Firm
	10	Pale pink	7.20	Firm
C. piscicola A9b bac^{-}	0	Light pink	9.55	Firm
	3	Light pink	8.88	Firm
	7	Light pink	7.45	Firm
	10	Pale pink	3.95	Slightly firm
Control – untreated fish	0	Light pink	9.40	Firm
	3	Light pink	8.32	Firm
	7	Pale pink	4.40	Slightly firm
	10	Pale pink	3.6	Pasty

For odour:

10 - 9 represents smoky odour

8.9 - 8 represents smoky odour

7.8 - 4 represents absence of smoky odour

3.9 – and below represents spoilage

Texture was scored qualitatively as either ‘firm’ (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock, this is to enable proper observation of changes in the organoleptic parameters.

All the bacteriocin treated samples did not reveal any difference in firmness at day 0 (Table 4.8.). The MRS supernatant (treatment F) did not affect flesh firmness. The semi-purified bacteriocin (treatment H) retained firmness for 7 days. However, the ammonium sulphate precipitated supernatant (treatment G) and control (treatment E) decreased in firmness after day 3. The semi purified bacteriocin (treatment H) samples retained a light pink colour until day 7, unlike the control (treatment E, untreated fish) which was pale pink in colour on day 7. The MRS supernatant (treatment F), ammonium sulphate precipitated supernatant (treatment G) lost their light pink colour after day 3 (Table 4.8.). A change in the smoky odour was observed on day 10 in semi purified bacteriocin sample (treatment H). However, the samples treated with MRS supernatant (treatment F) had a spoilage odour from day 7 of storage; the ammonium sulphate precipitated supernatant and control samples (treatment G and E) had a spoilage odour on day 10 (Table 4.8.).

Table 4.8. Organoleptic parameters of bacteriocin treated samples

Treatment	Day	Colour	Odour	Texture	TVBN
MRS supernatant	0	Light pink	9.70	Firm	45 ± 0.8
	3	Light pink	7.50	Slightly firm	104 ± 5.4
	7	Pale pink	4.00	Pasty	208 ± 4.2
	10	Pale pink	3.00	Pasty	245 ± 1.3
Ammonium sulphate precipitated	0	Light pink	9.59	Firm	263 ± 11.3
	3	Light pink	8.14	Firm	293 ± 3.3
	7	Pale pink	6.50	Slightly firm	405 ± 20.7
	10	Pale pink	3.80	Pasty	397 ± 20.7
Semi-purified peptide	0	Light pink	9.50	Firm	73 ± 2.5
	3	Light pink	8.40	Firm	141± 4.5
	7	Light pink	7.58	Firm	229 ± 12.2
	10	Pale pink	6.86	Slightly firm	312 ± 19.0
Control–untreated fish	0	Light pink	9.40	Firm	30 ± 0.8
	3	Light pink	8.60	Firm	122 ± 6.0
	7	Pale pink	4.88	Slightly firm	229 ± 12.2
	10	Pale pink	3.56	Pasty	253 ± 3.5

For odour:

10 - 9 represents smoky odour

8.9 - 8 represents smoky odour

7.8 - 4 represents absence of smoky odour;

3.9 - below represents spoilage

Texture was scored qualitatively as either ‘firm’ (normal texture), slightly firm and pasty.

The cold-smoked haddock used for the present analysis was undyed cold-smoked haddock, this is to enable proper observation of changes in the organoleptic parameters.

A B

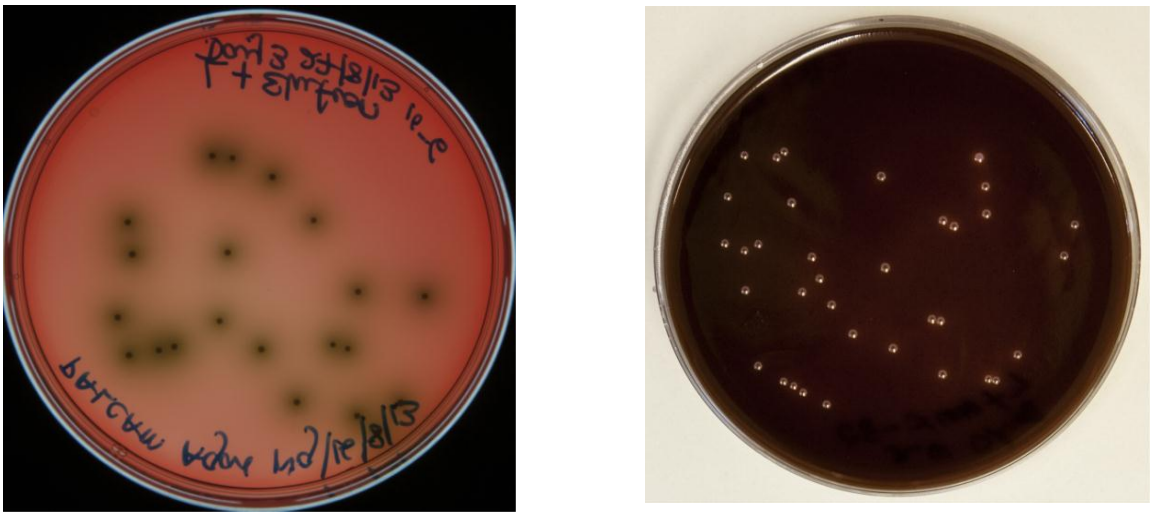

Figure 4.7. Recovery of *Listeria monocytogenes* ATCC19114 from cold-smoked haddock on polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar (Panel A) and recovery of *Carnobacterium* spp. from cold-smoked haddock on cresol red thallium acetate sucrose inulin (CTSI) agar (Panel B).

4.5. Discussion

Carnobacteria are commonly found in chilled fresh and lightly preserved seafood. The presence of *C. divergens* and *C. maltaromaticum* has been demonstrated for modified atmosphere-packed (MAP) coalfish, cod, pollack, rainbow trout, salmon, shrimp and surubim (Franzetti *et al*., 2003; Rudi *et al*., 2004; Emborg *et al*., 2002, 2005). The ability of *Carnobacterium* spp. to grow and produce bacteriocins with high anti-listerial activity at low and high sodium concentration has focused the attention of food scientists (Buchanan and Bagi, 1997). In this study, the broth assay demonstrated a reduction in the growth of *L. monocytogenes* ATCC 19114 (Figs. 4.1 A, B and C). The culture was sensitive to *C. maltaromaticum* strain MMF-32 supernatant, ammonium sulphate precipitated supernatant and semi-purified bacteriocin. *C. maltaromaticum* MMF-32 cell-free supernatant and ammonium sulphate precipitated supernatant inhibited the growth of *L. monocytogenes* ATCC 19114 until 8 h and 40 h incubation, respectively (Figs. 4.1 A and B). Addition of the semi-purified bacteriocicin only showed inhibition in *L. monocytogenes* ATCC 19114 growth at 16 h. Although OD and not CFU was used to quantify this result, this method was also used by Vijayakimar and Muriana (2015) to determine the inhibitory effect of bacteriocin preparations of *Lactobacillus curvatus* FS47, *Lb. curvatus* Beef3 and *Pediococcus acidilactici* against wild-type *L. monocytogenes*. Campos *et al*. (2006) demonstrated inhibition of *L. monocytogenes* and or *Staphylococcus aureus* by all tested LAB strains cell-free supernatant using OD measurements. Pinto *et al.* (2009) reported the inhibition of *L. monocytogenes* by ammonium sulphate precipitated supernatant, also using OD measurements.

In order to determine if the inhibitory activity of the supernatant might arise from the production of hydrogen peroxide and lactic acid by LAB, catalase and 2NaOH were

added to the supernatant extracts to exclude hydrogen peroxide and lactic acid (Ponce *et al*., 2008).

Several studies have demonstrated the inhibitory activity of *Carnobacterium* spp. bacteriocins of class IIa against *L. monocytogenes* isolated from food (Katla *et al*., 2003; Nilsson *et al*., 2004; Brillet *et al*. 2004). In the current study, the activity of bacteriocin production in inhibiting *L. monocytogenes* ATCC 19114 was demonstrated by comparing the anti-listerial effects of *C. maltaromaticum* MMF-32 with nonbacteriocin producing *C. piscicola* A9b bac^- mutant of *C. piscicola* A9b bac^+ using cold smoked haddock stored at 4 °C for 10 days. The anti-listerial effect of *C. maltaromaticum* MMF-32 and *C. piscicola* A9b bac^- did not work on the cold smoked haddock, this is because there were few *Carnobacterium* cells in the samples that were inoculated with 4×10^6 CFU g^{-1}, than the control *i.e* untreated fish samples (treatment D1) and sample having only *L. monocytogenes* ATCC 19114 (treatment A1). This suggests there was a serious problem with some part of the protocol.

The observed non pH acidification in the present study confirms the non-acidic position of carnobacteria (Table 4.5). In the study, *C. maltaromaticum* MMF-32 cells were inoculated into the cold smoked haddock. According to Brillet *et al*. (2005) they demonstrated a non pH acidification of cold-smoked salmon blocks when inoculated with *C. divergens* V41, *C. piscicola* V1 or SF668. *C. maltaromaticum* MMF-32 did not show any spoiling capacity from the odour. This agrees with the work of Leroi *et al*., 1998; Joffraud *et al*., 2001; Stohr *et al*., 2001, that *Carnobacterium* spp. are not considered as spoilage organisms. *C. piscicola* A9b bac^- on cold-smoked salmon during the storage period showed a non pH acidification, spoilage odour was observed on day 10 along with the control samples (Tables 4.5. and 4.7.).

Firmness or hardness of flesh has been regarded as an important quality characteristic of fish product (Ando, 1999; Mitchie, 2001). Texture is considered as one of the most significant parameters when the overall quality perception of fish product is being determined (Koteng, 1992). Mørkøre and Einen (2003) defined food texture as a collective term that covers several related physical properties. Jittinandana *et al*. (2002) demonstrated that reduced moisture content, leads to increased texture firmness in smoked trout. Water content and lipid content of fish muscles determines the textural characteristics. The firmness observed during the period of storage by samples inoculated with *C. maltaromaticum* MMF-32 might be due to moisture loss thus resulting to a more tightly packed myofibrillar structure. Tahiri *et al*. (2009) demonstrated firmness in cold-smoked salmon treated with different strategies of divergicin M35 for three weeks. In the present study cold-smoked haddock samples treated with *C. piscicola* A9b bac$^-$ and control lost their firmness on days 10 and 7, respectively. Thus showing that the trend to reduce or remain constant depends on the application or treatment (Tahiri *et al*., 2009). According to Morzel *et al*. (1997) these changes in texture may be due to different increased proteolytic activities by microbial enzymes specific *C. divergens* strains or endogenous spoilage flora.

The effect of cell-free supernatant, ammonium sulphate precipitated supernatant and semi-purified bacteriocin by the producing strain for inactivation of *L. monocytogenes* ATCC 19114 in cold-smoked haddock at 4 °C revealed; rapid growth of *L. monocytogenes* ATCC 19114 on smoked haddock when inoculated alone might be due to haddock being highly perishable, its scanty connective tissue content and loose meat tissue (Samsun *et al*., 2006).

The inoculation of fish products or any type of food product with purified antimicrobial agent is liable to food preservative legislation. The addition of bacteriocins to cold-

smoked haddock to the best of our knowledge has not been demonstrated. Semi-purified bacteriocin had a bacteriostatic effect on the growth of *L. monocytogenes* ATCC 19114 inoculated in smoked haddock, as seen on Fig. 4.2 on day 1, but this was not statistically significant. There was growth on day 3, but no statistically significant difference in growth of *L. monocytogenes* ATCC 19114 ($P > 0.005$) was observed in any of the treatments, until day 7. A significant difference was observed between treatment with the semi-purified bacteriocin and ammonium sulphate precipitated supernatant on day 7 (Fig. 4.2). Delay in growth was observed by Tahiri *et al*. (2009) when using divergicin M35 from *Carnobacterium divergens* M35 to inactivate *L. monocytogenes* added to cold-smoked salmon stored at 4 °C. Katla *et al*. (2001) observed a delay also in growth when using sakacin P from *Lactobacillus sakei* on *L. monocytogenes* added to cold-smoked salmon stored at 10 °C. Duffes *et al*. (1999) demonstrated the same delay in growth when semi-purified bacteriocins from *Carnobacterium* spp. V41 was used as co-culture with *L. monocytogenes* added to cold smoked salmon stored at 8 °C. The use of MRS supernatant (treatment B) (Fig. 4.2) had no effect on growth reduction of *L. monocytogenes* ATCC 19114 added to cold smoked haddock during the storage period. In contrast, Vaz-Velho *et al*. (2005) demonstrated a reduction of *L. innocua* level greater than 3 log cycles obtained on samples treated with 5% (v/v) supernatant V41 from *C. divergens* 41 added to cold smoked salmon trout during the storage at 5 °C after 1 week. The report of Tahiri *et al*. (2009) revealed a rapid in activation of *L. monocytogenes* by divergicin M35 in culture supernatant and persistence in the salmon flesh longer (over 15 days). In the current study, the application of ammonium sulphate precipitated supernatant (treatment C) had a growth reduction of 0.1 log CFU g^{-1} of *L. monocytogenes* ATCC 19114 when added to cold smoked haddock during the storage period on day 1. A statistically significant

difference was observed on the growth of *L. monocytogenes* ATCC 19114 viable counts in co-culture with semi-purified bacteriocin on day 7 of the storage. Nilsson *et al*. (2004) demonstrated a decline of 3.5 log in viable count of listeria cells when precipitated carnobacteriocin (1024 BU ml^{-1}) from wild-type strain was co-cultured on cold-smoked salmon after day 6.

The effectiveness of using bacteriocins in food is limited by intrinsic and extrinsic factors related with a food product. These limiting factors are the inactivation of food components by proteases, lipids, microorganisms, packaging due to their molecular properties or their activity could be affected during processing (i.e. temperature and drying) (Duffes *et al*., 1999; Katla *et al*., 2001; Aasen *et al*., 2003). Consequently, Nes *et al*. (2002) reported that the actual bacteriocin activity in the environment of the bacteria is much lower than expected. Resistance-development among target bacteria is among the factors that limit use of bacteriocins (Crandall and Montville, 1998; Nilsson *et al*. 2000).

Various studies have revealed that the microbial flora of haddock comprised *Pseudomonas* spp., *Shewanella* spp., *Aeromonas* spp., *Vibrio* spp. and *Ph. phosphoreum* (Banja, 2002; Olafsdottir *et al*., 2006; Gram, 2009). In cold smoked and vacuum packed fish products, Gram and Dalsgaard (2002) reported that *Lactobacillus* species are present in the largest number and therefore are the most important microorganisms for sustained shelf life and as well as sensory characteristics. Tahiri *et al*. (2009) revealed that although they increased progressively in all samples during storage the cold-smoked salmon was still acceptable to the trained panelists until the third week of storage. The present study showed that by day 7 some of the treated samples and controls had been rejected by the sensory panelists (Table 4.8.), because of very strong amine off-odours. *Lb. curvatus* and *Lb. sakei* species often dominate the LAB

colonizing lightly preserved fish products, although not specifically identified in this study (Leroi *et al*., 1998; Truelstrup Hansen and Huss, 1998; Lyhs *et al*., 1999; Jorgensen *et al*., 2000a, b).

The total viable bacterial count analysis was carried out to determine the total aerobic bacterial count of the cold smoked haddock analysed. The cold-smoked haddock used in this study, revealed total aerobic flora from 2.0 x 10^7 to 1.0 x 10^8 CFU g^{-1} in control samples. The effect of bacteriocins on total bacterial counts could not be determined as the appropriate controls (without *L. monocytogenes*) were not included in this experiment.

With regards to safety, all treated samples produced cadavarine, MRS supernatant treated samples only produced putrescine (treatments F) (Fig 4.3.) Jorgensen *et al*. (2000a, b) reported that cadavarine and putrescine are often correlated spoilage. They reported the production of biogenic amines primarily by *Ph. phosphoreum* in vacuum-packed cold-smoked salmon where agmatine (160-220 mg kg^{-1}), cadaverine (260-470 mg kg^{-1}), histamine (100-220 mg kg^{-1}) and tyramine (50-130 mg kg^{-1}) were formed at 5 °C. None of the treatments were able to produce histamine, which is known as the main agent for scrombroid fish poisoning (Taylor, 1986). Tyramine was not produced by any of the treated samples. Tyramine may cause migraine headaches and hypertensive effects, and in some cases can act as an existing possibility for measuring histamine (Ten Brink *et al*., 1990). Cadavarine was detected in the control sample (treatment E). According to Brillet *et al*. (2005) and Russo *et al*. (2010) the most effective methods for stopping biogenic amines formation are handling and processing under sanitised and temperature control (<5 °C) conditions throughout the process. In this study there is marked spoilage odour observed in samples treated with MRS supernatant, ammonium sulphate precipitated supernatant and controls (treatments F, G and E).

Based on the fixed TVB-N limit (35 mg N 100 g^{-1}) as quoted in the EU regulations for gadoids (European Union, 1995), only the control sample was within the limit on the day zero (Fig 4.4.). During the period of storage, all treated samples exceeded the limit of 35 mg N 100 g^{-1}. TVB-N is only useful to detect advanced spoilage because values only begin to increase at later stages of storage (Oehlenschläger, 1998; Baixas-Nogueras *et al*., 2003). In contrast to the results of the present study, spoilage was observed from day 3 when increase in the TVB-N was detected. Olafsdottir *et al*. (2006) suggested that high TVB-N levels in haddock fillets at sensory rejection are related to high *Ph. phosphoreum* counts that had reached TVC levels (>log 8 g^{-1}) for most sample groups. The present study showed the TVC levels of control samples to be from (2.0 x 10^7 to 1.0 x 10^8 CFU g^{-1}). TVB-N is mainly a composition of TMA and ammonia (Banja, 2002). According to Banja (2002), haddock samples did not reveal any increase in TMA content, but TVB-N increased and was therefore considered to be due to the production of ammonia. Furthermore, the above worker reported that the *Vibrio/Photobacterium* group were found on the flesh of haddock on the last sampling day on LH medium after 4 days of incubation at 15 °C.

Changes were observed in the pH of bacteriocin treated and control samples, but were not statistically significant (Fig 4.6 and Table 4.6.). The increase of pH in treated and control samples during storage at 4 °C after day 3 may be directly related to the multiplication of psychrotrophic and mesophilic microorganisms, and connected with the autolytic reaction, which gives rise to the production of basic compounds that increase the pH. Later on the proteolytic action of spoilage bacteria also stimulated the same effect (Sant'Anna and Torres, 1998).

In conclusion, the presence of *L. monocytogenes* in cold-smoked fish cannot be completely controlled, but should be reduced to a minimum by Good Manufacturing

Practice (GMP). This study has revealed that the inoculation of cold-smoked haddock with *C. maltaromaticum* MMF-32 resulted in no changes in either firmness or sensory perception of the product. During the use of bacteriocins for inactivation of listeria cells, semi-purified bacteriocin showed a statistically significant reduction in *L. monocytogenes* ATCC 19114 growth on day 7. Although the study on anti-listerial effects of *C. maltaromaticum* MMF-32 was not successful, this organism did have a positive effect on retention of firmness and sensory perception in cold smoked haddock.

Chapter 5. General discussion and conclusions

Seafood permits the transmission of many bacterial pathogens (Davis *et al.*, 2001; Hosseini *et al*., 2004). In particular, the microorganisms potentially pathogenic to man, include *Salmonella* spp., *E. coli*, *St. aureus*, *L. monocytogenes*, *Aeromonas* spp., *V. cholerae* and *V. parahaemolyticus;* all of which have been recovered at various times from seafood, namely fresh, frozen and smoked products (fish, shellfish, crustaceans, molluscs) (Basti *et al*., 2006; Popovic *et al*., 2010; Adebayo-Tayo *et al*., 2011; Joh *et al*., 2013). It is likely that these bacteria may have been contaminants on fish, possibly introduced during harvesting and filleting operations (Papadopoulou *et al*, 2007; Huss, 2003; Eze *et al*., 2011). Moreover, this study revealed the presence of potential pathogenic and spoilage microorganisms, such as *Listeria monocytogenes* and *Aeromonas* spp., *Vibrio* spp., *Enterobacteriaceae* representatives, *Shewanella* spp. and pseudomonads. Spoilage microorganisms, including those that produce hydrogen sulphide, for example *Sh. baltica*, *Sh. putrefaciens* and *Serratia* spp., have been detected from swordfish and tuna alongside non-H_2S producers, such as *Ps. fluorescens*, *Ps. fragi* and *Ac. radioresistens* (Serio *et al*., 2014).

The initial work carried out in this study was the isolation, and characterization of *L. monocytogenes*. Various authors have isolated bacterial pathogens from seafood by using a range of conventional methods (Udgata *et al*., 2009; Eissa *et al*., 2010; Uddin and Al-Harbi, 2012; Tavakoli *et al*., 2012; Oladipo and Bankole, 2013). In this study, the homogenates of the seafood were inoculated onto/into general purpose media and selective isolation media for the recovery of specific groups of bacteria, with their identification including the use of 16SrDNA sequencing (www.ncbi.nlm.nih.gov/genbank/).

Among the organisms recovered in this study, a range of *Enterobacteriaceae* representatives were recovered. The presence of these organisms in fish is regarded as a pointer to possible sewage pollution. Also, some taxa are opportunistic pathogens of fish (Rajasekaran, 2008). It remains a possibility that biogenic amines, such as putrescine, tyramine and histamine, may be produced in fish tissues; the presence of which accounts for some human illnesses (Tsai *et al*., 2002). Certainly, the occurrence of *E. coli* may well indicate the possibility of faecal contamination. Notwithstanding, it is argued that measures should be taken to ensure that seafood is not a means of transmission of *E. coli* namely:

1) to uphold the microbiological quality of the harvesting sites;

2) taking care of post harvest capture;

3) ensuring proper hygiene conditions in the handling processes;

4) with regard to processed food, careful measures should be put in place to avoid recontamination and above all, consumption of raw or undercooked seafood should be avoided (Costa, 2013).

Shewanalla spp. were recovered in this study, but this is not surprising as Vogel *et al*. (2005) demonstrated the presence of cells on newly caught fish. It should not be overlooked that the organisms may be opportunistic human pathogens, being associated with bacteraemia and skin and soft tissue infections (Chen *et al*., 1997, Aubert *et al*., 2009). However and in agreement with this study, Serio *et al*. (2014) demonstrated *Shewanella* spp., as a spoilage microorganism by its strong production of TMA and H_2S.

The presence of staphylococci on seafood is not unusual. Indeed, Ananchaipattana *et al*. (2012) reported the isolation of *Staphyococus lentus, S. sciuri* and *S. xylosus* from fish and seafood samples (22% of the total) from Thailand. However, *Staphylococcus* is not usually regarded as an indigenous component of the microflora of fish, and may well reflect contamination. Yet, the organism may be associated with environments containing high quantities of sodium chloride (Hansen *et al*., 1995; Herrero *et al*., 2003), such as fish smokers (Ferreira *et al*., 2007). It is apparent that the detection of staphylococci in fish suggests:

(a) post harvest contamination due to poor hygiene, or

(b) disease in fish (Huss, 1988; Austin and Austin, 2007).

Basti *et al*. (2003) reported that some type of smoked fish may be associated with populations of both *L. monocytogenes* and *S. aureus*. In this connection, it was noted that *S. haemolyticus* was recovered from smoked salmon in this study.

It is not surprising that vibrios were common in marine fish and shellfish (Popovic *et al*., 2010; Merwad *et al*., 2011). Certainly, Adeleye *et al*. (2010) reported that *V. parahaemolyticus* were detected from seafood samples (11.4% of the total), which were collected from seven fishing companies and local fishermen in Nigeria. In parallel, Bauer *et al*. (2006) noted the presence of *V. parahaemolyticus* in 10.3% of blue mussels collected from July 2002 to September 2004 at 102 production sites authorized by the Norwegian Food Safety Authorities (NFSA). It was suggested by Aberoumand (2010b) that the high level (10^3-10^4 g^1) of *Vibrio* spp. in some raw seafood reflected insufficient control in the storage temperatures from the time of harvesting.

Listeria spp., especially *L. monocytogenes,* were reported in farmed mussels in the North Aegean Sea (Soultos *et al*., 2014). Of relevance, Vernocchi *et al*. (2007) noted that contamination with listeria could occur as a result of improper harvesting, handling, processing and sanitation (Bremer *et al*., 2003). It is appreciated that *L. monocytogenes* is a facultative anaerobic opportunistic intracellular bacterial pathogen, whose primary route of transmission to humans is the consumption of contaminated food (Vázquez-Boland *et al.,* 2001). The invasive form of listeriosis is observed primarily in high-risk groups, namely the elderly, individuals with lowered immunity, pregnant women and new borns (Gelbíčová and Karpíšková, 2009). The outcome of listeriosis in pregnant women is abortion. In healthy people, it has been reported that *L. monocytogenes* causes a non-invasive febrile gastroenteritis resulting from the consumption of contaminated smoked trout (Miettinen *et al*., 1999).

It is apparent that with the susceptibility of seafood to both microbiological and biochemical spoilage pathways, the increase in foodborne illness outbreaks and the consumers' demand for high-quality and minimally processed seafood (Calo-Mata *et al*., 2008; Campos *et al*., 2012; Alzamora *et al*., 2012), a great deal of emphasis has been placed on the need to develop effective processing treatments to extend the shelf life of fresh fish products. In this respect, bacteriocins appear to offer promise, having a wide antibacterial spectrum with potential applications in foods such as meat and fish products, fruits and vegetables, cereals and beverages (Ivanova *et al*., 2000; Cleveland *et al*., 2001). In spite of the fact that many bacteria are able produce bacteriocins, those produced by LABs are of particular benefit to the food industry, because these bacteria have GRAS (generally regarded as safe) status (Barefoot and Nettles, 1993; Elgado *et al*., 1997; Anastasiadou *et al*., 2008; Altuntas *et al*., 2010). LABs have been used in food production and are successful in extending shelf life of foodstuffs by simple

fermentation (Galvez *et al*., 2008). Certain LABs, including *Carnobacterium, Lactobacillus, Enterococcus* and *Lactococcus,* have been isolated in freshwater and seawater fish (Ringø and Gatesoupe, 1998; González *et al*., 2000a; Bucio *et al*., 2006; Ghanbari *et al*., 2010). These bacteriocin producing bacteria are possibly among the most promising natural food biopreservatives (Atanassaova *et al*., 2001; Leroy and De Vuyst, 2003). Using bacteriocins and/or bacteriocin producing strains is of great interest to the food industry (Montville and Winkowski, 1997).

The bacteriocinogenic strains used in this study were isolated from smoked salmon, which have been the focus of most early research. It has been reported in the literature that many bacteria isolated from smoked fish and intestinal contents are beneficial to fish processing industries because of the ability to inhibit the growth of pathogens (Brillet *et al*., 2004; Nilsson *et al*., 2004; Campos *et al*., 2006). Certainly, biopreservation is becoming popular in combined strategies for the development of lightly processed novel foods, as an alternative to conventional and more aggressive preservation techniques. The consumer demand for high-quality and minimally processed seafood has opened the way to new applications of food-grade LABs and/or their metabolites for the biopreservation of lightly processed foods (Gálvez *et al*., 2007). In the present study, bacteriocin producing *Carnobacterium* strains were isolated from smoked Atlantic salmon and identified by 16S rDNA sequencing as *C. maltaromaticum* MMF-32 and KOPRI 25789. These *Carnobacterium* strains inhibited the growth of *L. monocytogenes* and other potentially pathogenic spoilage microorganisms.

Flesh and intestinal tract samples of fish species contain large amounts of LAB producing antibacterial compounds able to inhibit the growth of pathogens (Ringø and

Gatesoupe, 1998; Campos *et al*., 2006; Itoi *et al*., 2008; Chahad *et al*., 2012; Zapata *et al*., 2013; Tulini *et al*., 2014). To date, 9 *Carnobacterium* species have been described, but only *C. divergens* and *C. maltaromaticum* are frequently isolated from the intestinal tract and lightly preserved fish products (LPFP; Emborg *et al*., 2002, 2005; Seppola *et al*., 2005; Dalsgaard *et al*., 2006; Ringø *et al*., 2006).

The antimicrobial spectra for the carnobacteria in this study revealed broad spectrum activity against pathogenic Gram-positive and Gram-negative potentially pathogenic and spoilage organisms. Several studies have shown that bacteria isolated from seafood produce antimicrobial substances inhibiting bacterial pathogens in LPFP (Brillet *et al*., 2004; Nilsson *et al*., 2004; Kim and Austin, 2007; Matamoros *et al*., 2009). Unlike meat or dairy products, seafood products are often non-fermented. Consequently the addition of bacterial cultures, even with protective effects, is novel and not yet completely accepted by seafood producers, for which the main objective is to avoid bacterial contamination by use of good hygienic practices. Nevertheless, in LPFP, the use of protective cultures is gradually being considered as an alternative to the use of chemical food additives, and is gaining interest in the seafood industry (Pilet and Leroi *et al*., 2011).

Inhibition of Gram-negative bacteria is not common, and has so far only been reported for a few LAB bacteriocins (Messi *et al*., 2001; Todorov and Dicks, 2004; De Kwaadsteniet *et al*., 2005). For example, Martin-Visscher *et al*. (2011) revealed that *C. maltaromaticum* UAL307 bacteriocins could inhibit Gram-negative bacteria if the outer membrane is weakened. From this study. It was determined that these bacteriocin-like substances against Gram-negative bacteria have potential as food preservatives. The data for the LAB isolated from smoked salmon confirm previous reports that have described a stronger effect of bacteriocins at acidic pH values (Blom *et al*., 2001;

Guerra and Pastrana, 2002). Bacteriocins produced by LABs are usually stable at acid or neutral pH, showing that these substances are well adapted to the environmental conditions (Vignolo *et al*., 1995). The results from this study suggests that the bacteriocins may be of benefit to application in both low and medium-acid fermented food products; this includes a number of fermented and ripened dairy and meat products.

Unfortunately, purification of *C. maltaromaticum* MMF-32 and KOPRI 25789 led to a loss of bioactivity. A more effective recovery of bacteriocin activity may have been realised if the method described by Atrih *et al*. (2001) for purification of plantaricin C19 produced by *Lactobacillus plantarum* C19 had been used. Atrih *et al*. (2001) exploited the influence of pH on adsorption and release of plantaricin C19 from producing cells to purify plantaricin C19 from supernatant fluids by high-performance liquid chromatography purification. A total activity of 2048000 AU was recovered from the culture supernatant of *Lb. plantarum* C19 (Atrih *et al.,* 2001), whereas in this study a total activity of 40000 AU was recovered.

The addition of bacteriocins to cold-smoked haddock is novel. Certainly, a delay in growth of *L. monocytogenes* was observed by Tahiri *et al*. (2009) when using divergicin M35 from *Carnobacterium divergens* M35 to inactivate *L. monocytogenes* which had been added to cold-smoked salmon stored at 4 °C. Also, Katla *et al*. (2001) observed a delay in growth when using sakacin P from *Lb. sakei* on *L. monocytogenes* which had been inoculated onto cold-smoked salmon stored at 10 °C. Duffes *et al*. (1999) demonstrated the same delay in growth when semi-purified bacteriocins from *Carnobacterium* spp. V41 was used as co-culture with *L. monocytogenes* added to cold smoked salmon stored at 8 °C. Furthermore, Vaz-Velho *et al*. (2005) demonstrated a reduction of *L. innocua* level greater than 3 log cycles obtained on samples treated with

5% (v/v) supernatant V41 from *C. divergens* 41, which was added to cold smoked salmon trout during storage at 5 °C for 1 week. Tahiri *et al*. (2009) revealed a rapid inactivation of *L. monocytogenes* by divergicin M35 in culture supernatant and persistence in the salmon flesh longer (over 15 days).

Nilsson *et al*. (2004) demonstrated a decline of 3.5 logs in the viable count of listeria cells when precipitated carnobacteriocin (1024 BU ml^{-1}) from a wild-type strain was co-cultured on smoked haddock after day 6. Thus, the effectiveness of bacteriocins in food is limited by intrinsic and extrinsic factors related with the food product. These limiting factors are the inactivation of food components by proteases, lipids, micro-organisms, packaging due to their molecular properties or their activity could be affected during processing (temperature, drying) (Duffes *et al*., 1999; Katla *et al*., 2001; Aasen *et al*., 2003). Consequently, Nes *et al*. (2002) reported that the actual bacteriocin activity in the environment of the bacteria is much lower than expected. Resistance-development among target bacteria is among the factors that limit use of bacteriocins (Crandall and Montville, 1998; Nilsson *et al*. 2000).

High levels of tyramine can be produced by some strains of *Carnobacterium* (Masson *et al*., 1997; Bover-Cid and Holzapfel, 1999). When compared to other carnobacteria, *C. divergens* M35 produced low amounts of tyramine approximately 72 μg g^{-1} after 21 days of storage showing its weak ability to produce biogenic amine in cold-smoked salmon (Tahiri *et al*., 2009). In a fermented synthetic medium (decarboxylase medium), a wide difference was observed in tyramine production by LAB and *Carnobacterium* strains, ranging from 20 to 5000 mg l^{-1} depending on the tested strain (Bover-Cid and Holzapfel, 1999). Brillet *et al*. (2005) observed in commercial cold-smoked salmon during vacuum storage of 28 days, *C. divergens* V41 produced low

levels of putrescine and cadaverine in control samples (10 ± 5 and 28 ± 9 μg g^{-1} respectively). However, in this study, *C. maltaromaticum* MMF-32 during 10 days storage revealed cadavarine production in the control samples as 83.5 mg N 100 g^{-1}, whereas putrescine was not detected in the control. The treated samples showed high cadavarine production ranging from 55.5 to 297.5 mg N 100 g^{-1}, while putrescine was only detected in MRS treated samples with a value of 61 mg N 100 g^{-1}. According to Brillet *et al*. (2005) and Russo *et al*. (2010), the most effective methods for stopping biogenic amines formation are handling and processing under sanitary conditions and temperature control (<5 °C) throughout the process.

The presence of TVBN gives information on the end spoilage of fish and is appropriate for validation of sensory data (Antonacopoulos and Vyncke, 1989). TVBN or biogenic amines has been regarded as chemical quality indices (Jorgensen *et al*., 2000a; Leroi *et al*., 2001). The level of TVBN in seafood is based mainly on the fish type and the processing method involved (Connell, 1995). For different groups of fish species, a TVBN levels of 25, 30 and 35 mg N 100 g^{-1} have suggested as acceptable. Seafood concentrations of TVBN at sensory product rejection differ, in processed, lightly or semi preserved. At sensory rejection of sliced vacuum packed cold smoked salmon, TVBN levels of 30-40 mg N 100 g^{-1} have detected, whereas in modified atmosphere packed, cooked and brined shrimps lower concentrations of 10-20 mg N 100 g^{-1} (Dalgaard, 2000). Certainly, the production of total volatile compounds is narrowly associated to microbiological contamination (Tahiri *et al*., 2009). Olafsdottir *et al*. (2006) suggested that high TVB-N levels in haddock fillets at sensory rejection are related to high *Ph. phosphoreum* counts that had reached TVC levels of >log 8 g^{-1} for most samples. The present study showed the TVC levels of control samples to be between 2.0 x 10^7 to 1.0 x 10^8 CFU g^{-1}.

CONCLUSIONS

The identification of *L. monocytogenes* and other potentially pathogenic and spoilage microorganisms in seafood was considered to have importance for safety. Due to the potential hazard of some bacteria, it is important that rules should be put in place as regards harvesting, transportation, handling and cooking of the seafood. The thermal and pH stability of the supernatants widens the application of BLIS as biopreservatives for minimally processed and fermented foods. It is known that carnobacteria are capable of producing bacteriocins, some of which have wide range of antibacterial activity. Thus, the above mentioned characteristics led to the possibility of using carnobacteria as potential control mechanisms for *Listeria* in food. The absence of pronounced inhibitory effects by the bacteriocin extracts applied on cold-smoked haddock in this study may be attributed to the deterioration of activity during stoage. Heating the supernatants after extraction would have inactivated any protease activity allowing storage at -70 ºC without loss of activity. This study further revealed that the inoculation of cold-smoked haddock with *C. maltaromaticum* MMF-32 resulted in no changes in either firmness or sensory perception of the product. During the use of bacteriocins for inactivation of listeria cells in this current study, semi-purified bacteriocin showed a statistically significant reduction in *L. monocytogenes* ATCC 19114 growth on day 7. Although the study on anti-listerial effects of *C. maltaromaticum* MMF-32 was not successful, this organism did have a positive effect on retention of firmness and sensory perception in cold smoked haddock.

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Appendix I. Media, Buffers and Reagents

Trimethylamine Oxide Medium (TMAO)	500 ml^{-1}
Peptone (Oxoid)	2%
Lab lemco powder (Oxoid)	0.3%
Yeast extract (Oxoid)	0.3%
Ferric citrate (Sigma)	0.03%
Sodium thiosulphate (BDH) chemicals	0.03%
Sodium chloride (BDH) chemicals	0.4%
Potassium di-hydrogen phosphate (BDH) chemicals	0.4%
Di-potassium hydrogen phosphate (BDH) chemicals	0.575%
Magnesium sulphate.H_2O	0.05%
Resazurin tablets (BDH Prolabo)	2
Bacteriological agar No.1 (Oxoid)	0.4%
L-Cysteine (Sigma)	0.04%
TMAO.$2H_2O$	0.5%
pH	6.8

Gelatin Agar	
Difco Bacto peptone	2.0g
Yeast extract (Oxoid)	0.5g
Gelatin (Oxoid)	7.5g
Bacteriological agar No.1 (Oxoid)	7.5g
Distilled water	500 ml

Cresol Red Thallium Acetate Sucrose Inulin (CTSI)

agar

Component	g l^{-1}
Peptone	10.0
Yeast extract	10.0
Sucrose	10.0
Inulin	10.0
Tween 80	1.0
Sodium citrate	5.0
Magnesium sulphate. H_2O	10.0
Di-potassuim hydrogen phosphate	2.0
Thiamine hydrochloride	0.001
Chrisin ® (nisin)	0.00125
Vancomycin	0.001
Thallium acetate	0.5
Nalidixic acid	0.04
Cresol red	0.004
pH to 9.1 with 1 N NaOH	
Bacteriological agar No.1 (Oxoid)	15.0
Distilled water (ml)	1000
Triphenyl-tetrazolium chloride (TTC)	0.01

Nitrite Actidion Polymyxin agar (NAP)

Component	g l^{-1}
All purpose Tween agar (Merck)	59.5g
Sodium Nitrite ($NaNO_2$)	0.6g
Actidione cycloheximide (Sigma)	0.01g
Polymyxin-B (Sigma)	0.003g
pH modified from	5.5 to 6.7

Oxidative-Fermentative Media

Distilled water	500 ml
O/F Basal medium	4.7g
Bacteriological agar No.1 (Oxoid)	0.5g
Glucose (Oxoid)	5.0g

Phosphate Buffered Saline

Component	g l^{-1}
Sodium di-hydrogen phosphate (NaH_2PO_4. $2H_2O$)	0.876
Di-sodium hydrogen phosphate $Na_2HPO_4.2H_2O$	2.56
Sodium chloride	8.77
pH	7.2

Buffers for Bacteriocin Purification

Binding or starting buffer	50 mM sodium phosphate + 1. M ammonium sulphate (pH 7.0)
Elution buffer	50 mM sodium phosphate (pH 7.0)

Buffers used for Electrophoresis

Sample Buffer (2X)

Tris-HCl (0.5 M, pH 6.8)	2.5 ml
Glycerol (1 M)	2.0 ml
SDS (10% w/v = 0.35 M)	4.0 ml
Dithiothreitol (DTT) 0.2 M	0.31 g
Bromophenol blue	2.0mg
Distilled water	to 10ml

Stored at -20 °C

Reservoir buffer for 1D SDS-PAGE (5 X, pH 8.3)

Tris base (123 mM)	7.5 g
Glycine (0.96 M)	36 g
SDS (17 mM)	2.5 g
Distilled water	500 ml

Store at 4 °C

Separating gel buffer (pH 8.7)

Tris base (1.5 M)	91g
SDS (0.4 %,13.8 mM)	2g
Distilled water	500ml

Stored at 4 °C

Stacking gel buffer (pH 6.8)

Tris base (0.5 M)	6.05g
SDS (0.4 %, 13.8 mM)	0.4g
Distilled water	100ml

Stored at 4 °C

Separating gel (10 %)

Separating gel buffer	5ml
Distilled water	8.35ml
Acrylamide (30 % w/v)	6.65ml
(29 % acrylamide + 1 % bis acrylamide	
N,N,N,N-tetramethyl-ethylenediamine	15µl
TEMED (1 M)	
Ammonium persulphate (10 %)	70µl

Stacking gel (4 %)

Stacking gel buffer	2.5ml
Distilled water	6.1ml
Acrylamide (30 % w/v)	1.34ml
(29 % acrylamide + 1 % bis acrylamide	
N,N,N,N-tetramethylethylenediamine	10µl
TEMED (1 M)	
Ammonium persulphate (10 %)	50µl

PCR reagents	Per Reaction
Klear *Taq* buffer (10X)	1.50µl
dNTPs (5mM each)	0.60µl
$MgCl_2$ (50mM)	0.45µl
Primer 1 (10µM)	0.60µl
Primer 2 (10µM)	0.60µl
Klear *Taq* enzymes (5U/µl)	0.15µl
Nuclease-free dH_2O	10.10µl
Total	14.00µl
DNA (at 1/100 dilution)	+
	1.00µl
	15.00µl

Appendix II. Abstracts and Presentations from the project

Izuchukwu, N. O., Adams, A., Austin, B. "Detection, purification and partial characterization of bacteriocin-like substances produced by *Carnobacterium maltaromaticum* MMF-32 and KOPR I25789". Abstract presented at International Scientific Conference on Bacteriocins and Antimicrobial Peptides, 21st – 23rd May, 2013, Kosice, Slovakia.

Izuchukwu, N. O., Adams, A., Austin, B. "Bacterial Contaminants of Scottish Seafood". Oral presentation, SGRS Postgraduate Research Conference, May 2012, University of Stirling, Scotland, UK.

Izuchukwu, N. O., Adams, A., Austin, B. "Preliminary characterization of bacteriocin-like substances from *Carnobacterium maltaromaticum strains* KOPRI 25789 and MMF-32 isolated from smoked salmon (*Salmo salar*)". Oral poster presentation at Prebiotics and Probiotics in Medicine, Veterinary Sciences and Aquaculture: the Future, 10th and 11th September, 2012, Keele University, England, UK.

Izuchukwu, N. O., Adams, A., Austin, B. "Bacterial Contaminants of the Scottish Seafood". Poster presentation, SGRS Postgraduate Research Conference, May 2011, University of Stirling, Scotland, UK.

Izuchukwu, N. O., Adams, A., Austin, B. "Environmental Impact of Pollutants on sea Food". Poster presentation PhD Conference of the Institute of Aquaculture, 28th June, 2010, University of Stirling, Scotland, UK.

Appendix III. Table 1. Bacterial codes

ZFHTI 20 °C	Fresh haddock from TCBS green colony, isolated at 20 °C, Z a general code
ZSHTI 20 °C	Smoked haddock from TCBS green colony, isolated at 20 °C, Z a general code
MPA 30 °C	Mussels from PALCAM agar, isolated at 30 °C
FHP 30 °C	Fresh haddock from PALCAM agar, isolated at 30 °C
FSPA 20 °C	Fresh salmon from PALCAM agar, isolated at 20 °C
SHB 20 °C	Smoked haddock orange colony, isolated at 20 °C
ZSHB 20 °C	Smoked haddock orange colony, isolated at 20 °C, Z a general code
SHTA 30 °C	Smoked haddock TCBS yellow colony, isolated at 30 °C
ZFHD 30 °C	Fresh haddock isolated at 30 °C, Z a general code
ZFHTA 30 °C	Fresh haddock TCBS yellow colony, isolated at 30 °C, Z a general code
MT_1A 30 °C	Mussels TCBS yellow colony, isolated at 30 °C
FSC 30 °C	Fresh salmon cream colony, isolated at 30 °C
SSB 30 °C	Smoked salmon orange colony, isolated at 30 °C
FHC 20 °C	Fresh haddock white colony, isolated at 20 °C
SHC 20 °C	Smoked haddock white colony, isolated at 20 °C
OCD 30 °C	Oyster white colony, isolated at 30 °C
ZSSC 20 °C	Smoked salmon white colony, isolated at 20 °C, Z a general code
ZSSC 30 °C	Smoked salmon white colony, isolated at 30 °C, Z a general code
FS_2C 30 °C	Fresh salmon white colony, isolated at 30 °C
FSG 37 °C	Fresh salmon pink colony, isolated at 37 °C
SHG 37 °C	Smoked haddock pink colony, isolated at 37 °C
FSC 20 °C	Fresh salmon white colony, isolated at 20 °C
OJ 20 °C	Oyster cream colony, isolated at 20 °C
FHG 37 °C	Fresh haddock pink colony, isolated at 37 °C
ZFSG 37 °C	Fresh salmon pink colony, isolated at 37 °C, Z a general code
FSH 37 °C	Fresh salmon metallic green sheen colony, isolated at 37 °C

Appendix III. Table 2. Bacterial codes (continued)

FHH 37 °C	Fresh haddock metallic green sheen colony, isolated at 37 °C
ZFHH 37 °C	Fresh haddock metallic green sheen colony, isolated at °C, Z a general code
ZFSH 37 °C	Fresh salmon metallic green sheen colony, isolated at 37 °C, Z a general code
SHH 37 °C	Smoked haddock metallic green sheen colony, isolated at 37 °C
SSB 20 °C	Smoked salmon orange colony, isolated at 20 °C
MBT 30 °C	Mussels orange colony, isolated at 30 °C
ZSHK 20 °C	Smoked haddock cream colony, isolated at 20 °C, Z a general code
ZSHB 30 °C	Smoked haddock orange colony, isolated at 30 °C, Z a general code
ZSSB 20 °C	Smoked salmon orange colony, isolated at 20 °C, Z a general code
ZSHC 20 °C	Smoked haddock cream colony, isolated at 20 °C, Z a general code
SH2CT 30 °C	Smoked haddock cream- white colony, isolated at 30 °C
SSD 30 °C	Smoked salmon cream colony, isolated at 30 °C
MUB 30 °C	Mussels orange colony, isolated at 30 °C
FSD 30 °C	Fresh salmon cream colony, isolated at 30 °C
ZFHTI 30 °C	Fresh haddock TCBS green colony, isolated at 30 °C, Z a general code
OB 20 °C	Oyster orange colony, isolated at 20 °C
FSB 30 °C	Fresh salmon orange colony, isolated at 30 °C
ZSHK 30 °C	Smoked haddock cream colony, isolated at 30 °C, Z a general code
ZFHG 37 °C	Fresh haddock metallic green sheen colony, isolated at 37 °C, Z a general code
FSA 20 °C	Fresh salmon orange colony, isolated at 30 °C
SHP 30 °C	Smoked haddock PALCAM agar, isolated at 30 °C
ZFSB 30 °C	Fresh salmon orange colony, isolated at 30 °C
FHB 20 °C	Fresh haddock orange colony, isolated at 20 °C
ZFHB 20 °C	Fresh haddock orange colony, isolated at 20 °C, Z a general code

Appendix III. Table 3. Bacterial codes (continued)

FHB 30 °C	Fresh haddock orange colony, isolated at 30 °C
ZFHB 30 °C	Fresh haddock orange colony, isolated at 30 °C, Z a general code
SSC 30 °C	Smoked salmon white colony, isolated at 30 °C
MUTI 20 °C	Mussels TCBS green colony, isolated at 20 °C
OTA 20 °C	Oyster TCBS yellow colony, isolated at 20 °C
MTI 20 °C	Mussels TCBS green colony, isolated at 20 °C
FHTI 30 °C	Fresh haddock TCBS green colony, isolated at 30 °C
SSA 30 °C	Smoked salmon yellow colony, isolated at 30 °C
MP1C 30 °C	Mussels PALCAM agar black halo with greenish surroundings, isolated at 30 °C
MP1C 20 °C	Mussels PALCAM agar black halo with greenish surroundings, isolated at 20 °C

Appendix IV Table 1. Characteristics of *Acinetobacter* species

	Culture	
Character	ZFHTI 20 °C	ZSHTI 20 °C
Morphology:		
Colony colour	cream	cream
Colony size	≤ 1 mm	1-2 mm
Colony shape	Round, smooth edge, raised	Round, smooth edge,
Colony texture	creamy	raised
Micromorphology:		creamy
Cell shape	short rod	
Cell arrangement	in chains	short rod
Gram stain	negative	in chains
Endospore	no	negative
API 20E character:		no
Catalase production	+	
Oxidase production	-	+
Motility	-	-
Oxidation/fermentation		-
reaction	-	
Tryptophan deaminase	-	-
Indole production	-	-
Voges-Proskauer reaction	+	-
Gelatinase production	-	+
H_2S production	-	+
Citrate utilization	+	-
Urease production	-	-
Ortho-nitrophenyl-		-
galactosidase	+	
Arginine dihydrolase	+	+
Lysine decarboxylase	-	+
Ornithine decarboxylase	-	-
Production of Acid from:		-
Glucose	-	
Sucrose	-	-
Arabinose	-	-
Mannitol	-	-
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Melibiose	-	-
Amygdalin	-	-
		-
Degradation of:		
Lecithin	-	
Tween 80	-	-
Gelatin	-	-
Elastin	-	-
Blood (haemolysis)	-	-
Triple sugar iron agar	-	-
		-

Based on Gerner Smidt and Frederiksen (1993), Kampfer *et al*. (1993), Cowan and Steel (2003) and Vaneechoutte *et al*. (2011).

Appendix IV Table 2. Characteristics of *Aerococcus* species

	Culture		
	MPA 30 °C	FHP 30 °C	FSPA 20 °C
Morphology:			
Colony colour	intense yellow	light yellow	intense yellow
Colony size	1-2 mm	1-2 mm	1-2 mm
Colony shape	round, smooth edge, raised,	round, smooth edge, raised	round, smooth edge, raised
Colony texture	creamy	creamy	creamy
Micromorphology:			
Cell shape	Coccus	Coccus	Coccus
Cell arrangement	tetrads, pairs, clusters	tetrads, pairs, clusters	tetrads, pairs, clusters
Gram stain	positive	positive	positive
Endospore	No	No	No
API 20E character:			
Catalase production	+	+	+
Oxidase production	-	-	-
Motility	-	-	-
Oxidation/fermentation reaction	F^+	F^+	F^+
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Gelatinase production	+	-	-
H_2S production	-	-	-
Citrate utilization	+	+	+
Urease production	-	-	+
Ortho-nitrophenyl-galactosidase	-	-	-
Arginine dihydrolase	-	-	-
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	+	+	-
Sucrose	+	+	-
Arabinose	-	-	-
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Degradation of:			
Lecithin	-	-	-

Appendix IV Table 2. (Continued)

	Culture		
Character	MPA 30 °C	FHP 30 °C	FSPA 20 °C
Tween 80	-	-	+
Gelatin	-	-	+
Elastin	-	-	-
Blood (haemolysis)	-	A	α
Triple sugar iron agar	-	-	-

Based on Holt (1994), Cowan and Steel (2003), Buller (2004).

$F^{+ =}$ weak fermentation.

Catalase production was strong.

Appendix IV Table 3a. Characteristics of *Aeromonas* species

Character	Culture		
	SHB 20 °C	SSB 30 °C	ZSHB 20 °C
Morphology:			
Colony colour	Orange	cream	creamish brown
Colony size	2-3 mm	2 mm	≤ 1 mm
Colony shape	Round, smooth edge, raised	Round, smooth edge, raised	Round, smooth edge, raised
Colony texture	creamy	creamy	creamy
Micromorphology:			
Cell shape	Rod	Rod	Rod
Cell arrangement	singles	singles	singles, pairs
Gram stain	negative	negative	negative
Endospore	No	No	No
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	-	-
Oxidation/fermentation reaction	A	F	A
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Gelatinase production	+	-	-
H_2S production	+	-	-
Citrate utilization	+	+	+
Urease production	+	-	+
Ortho-nitrophenyl-galactosidase	-	+	-
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	+	-	-
Production of Acid from:			
Glucose	-	+	-
Sucrose	-	+	-
Arabinose	-	-	-
Mannitol	-	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	+	-
Amygdalin	-	-	-
O/129 Sensitivity:			
10 µg	R	R	R
150 µg	R	R	R

Appendix IV Table 3a. (Continued)

	Culture		
Character	SHB 20 °C	SSB 30 °C	ZSHB 20 °C
Degradation of:			
Lecithin	+	-	-
Tween 80	+	-	-
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	B	-	-
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 3b. Characteristics of *Aeromonas* species

	Culture		
Character	SHTA 30 °C	ZFHD 30 °C	ZFHTA 30 °C
Morphology:			
Colony colour	creamish brown	cream	creamish brown
Colony size	1-2 mm	≤ 1 mm	3-4 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	Creamy	Creamy
Micromorphology:			
Cell shape	rod	rod	rod
Cell arrangement	chains	Pairs, chains, clusters	chains
Gram stain	Negative	Negative	negative
Endospore	No	No	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	-	+	-
Oxidation/fermentation reaction	F	F	F
Tryptophan deaminase	-	+	-
Indole production	+	-	+
Voges-Proskauer reaction	+	+	+
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	+	+	-
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	+	-	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-

Appendix IV Table 3b. (Continued)

	Culture		
Character	SHTA 30 °C	ZFHD 30 °C	ZFHTA 30 °C
Production of Acid from:			
Glucose	+	+	+
Sucrose	+	-	+
Arabinose	+	-	+
Mannitol	+	-	+
Inositol	-	-	-
Sorbitol	+	-	-
Rhamnose	-	-	-
Melibiose	-	+	-
Amygdalin	+	-	+
O/129 Sensitivity:			
10 µg	R	R	R
150 µg	R	R	R
Degradation of:			
Lecithin	-	-	-
Tween 80	+	-	+
Gelatin	-	-	-
Elastin	+	-	+
Blood (haemolysis)	B	A	B
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 3c. Characteristics of *Aeromonas* species

	Culture		
	ZFHTA 20 °C	FSC 30 °C	MT_1A 30 °C
Morphology:			
Colony colour	Cream	cream	cream
Colony size	1-2 mm	≤ 1 mm	3-4 mm
Colony shape	Round, smooth edge, flat	Round, smooth edge, raised	Round, smooth edge, flat
Colony texture	Creamy	Creamy	creamy, sticky
Micromorphology:			
Cell shape	Rod	Rod	rod
Cell arrangement	singles, pairs, clusters	singles, pairs	chains, clusters
Gram stain	Negative	Negative	negative
Endospore	No	No	No
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	-	+
Oxidation/fermentation reaction	F	F	F
Tryptophan deaminase	-	-	+

Appendix IV Table 3c. (Continued)

Character	Culture		
	ZFHTA 20 °C	FSC 30 °C	MT_1A 30 °C
Indole production	-	-	+
Voges-Proskauer reaction	+	+	+
Gelatinase production	+	-	+
H_2S production	-	-	+
Citrate utilization	-	+	+
Urease production	-	-	+
Ortho-nitrophenyl-galactosidase	+	-	-
Arginine dihydrolase	+	+	+
Lysine decarboxylase	+	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	+	+	+
Sucrose	+	-	+
Arabinose	-	+	+
Mannitol	+	-	+
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	+	-
Amygdalin	+	-	-
O/129 Sensitivity:			
10 µg	R	R	R
150 µg	R	R	R
Degradation of:			
Lecithin	+	+	+
Tween 80	+	-	-
Gelatin	-	+	-
Elastin	-	-	-
Blood (haemolysis)	A	A	β
Triple sugar iron agar (H_2S)	-	-	-

Based on Holt (1994), Cowan and Steel (2003) and Austin and Austin (2007).

R = Resistant, F = Fermentative.

Appendix IV Table 4. Characteristics of *Bacillus* species

Character	Culture FHC 20 °C
Morphology:	
Colony colour	white
Colony size	3-4 mm
Colony shape	Round, rough edge, raised
Colony texture	Dry
Micromorphology:	
Cell shape	Rod
Cell arrangement	Chains
Gram stain	positive
Endospore	yes (oval)
API 20E character:	
Catalase production	+
Oxidase production	-
Motility	-
Oxidation/fermentation reaction	A
Tryptophan deaminase	-
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	-
H_2S production	-
Citrate utilization	-
Urease production	-
Ortho-nitrophenyl-galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Production of Acid from:	
Glucose	+
Sucrose	-
Arabinose	-
Mannitol	+
Inositol	+
Sorbitol	+
Rhamnose	+
Melibiose	-
Amygdalin	+
Degradation of:	
Lecithin	-
Tween 80	-
Gelatin	-
Elastin	-
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	-

Based on Cowan and Steel (2003) and Austin and Austin (2007).

A = Alkaline

Appendix IV Table 5. *Brochothrix* species

Character	Culture	
	SHC 20 °C	OCD 30 °C
Morphology:		
Colony colour	White	white
Colony size	2-3 mm	1-2 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	dry
Micromorphology:		
Cell shape	rod	Rod
Cell arrangement	short/thread-like chains	short/thread-like chains
Gram stain	positive	Positive
Endospore	no	no
API 20E character:		
Catalase production	+	+
Oxidase production	-	-
Motility	-	-
Oxidation/fermentation reaction	A	-
Tryptophan deaminase	-	-
Indole production	-	-
Voges-Proskauer reaction	+	+
Gelatinase production	-	-
H_2S production	-	-
Citrate utilization	-	-
Urease production	-	-
Ortho-nitrophenyl-galactosidase	-	-
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Production of Acid from:		
Glucose	-	-
Sucrose	+	-
Arabinose	-	-
Mannitol	+	-
Inositol	+	-
Sorbitol	-	-
Rhamnose	-	-
Melibiose	-	-
Amygdalin	+	-
Degradation of:		
Lecithin	-	-
Tween 80	-	-
Gelatin	-	+
Elastin	-	-
Blood (haemolysis)	-	-
Triple sugar iron agar (H_2S)	-	-

Based on Cowan and Steel (2003) and Stackebrandt and Jonnes (2006).

A = Alkaline

Appendix IV Table 6a. Characteristics of *Carnobacterium* species

Character	Culture		
	ZSSC 20 °C	ZSSC 30 °C	FS_2C 30 °C
Morphology:			
Colony colour	white	white	white
Colony size	≤ 1 mm	≤ 1 mm	≤ 1 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	Creamy	Creamy
Micromorphology:			
Cell shape	Rod	Rod	rod
Cell arrangements	Clusters	singles, pairs, chains, clusters	singles, pairs, clusters, tetrads, chains
Gram stain	Positive	Positive	positive
Endospore	No	No	No
API 20E character:			
Catalase production	-	-	-
Oxidase production	-	-	-
Motility	-	-	-
Oxidation/fermentation reaction	-	-	-
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	-	-	-
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	+	+	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	-	-	-
Sucrose	-	-	-
Arabinose	-	-	
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Degradation of:			
Lecithin	-	-	-

Appendix IV Table 6a. (Continued)

Character	Culture		
	ZSSC 20 °C	ZSSC 30 °C	FS_2C 30 °C
Tween 80	-	-	-
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	-	-	-
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 6b. Characteristics of *Carnobacterium maltaromaticum* isolates by use of the API 50CH

API 50CH character	Culture		
	ZSSC 20 °C	ZSSC 30 °C	FS_2C 30 °C
Glycerol	+	+	+
Erythritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	-	-	-
D-Ribose	+	+	+
D-Xylose	-	-	-
L-Xylose	-	-	-
D-Adonitol	-	-	-
β-Methyl-D-Xylopyranoside	-	-	-
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	-	-	-
L-Rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
D-Mannitol	+	+	+
D-Sorbitol	-	-	-
α-Methyl-D-Mannopyranoside	+	+	+
α-Methyl-D-Glucopyranoside	+	+	+
N-Acetyl-Glucoamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin ferric citrate	+	+	+
Salicin	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose (bovine origin)	+	+	+
D-Melibiose	+	+	+
D- Saccharose	+	+	+

Appendix IV Table 6b. (Continued)

	Culture		
API 50CH character	ZSSC 20 °C	ZSSC 30 °C	FS_2C 30 °C
D-Trehalose	+	+	+
Inulin	+	+	+
Melezitose	+	+	+
D-Raffinose	-	-	-
Amidon	+	+	+
Glycogen	-	-	-
Xylitol	-	-	-
β-Gentiobiose	+	+	+
D-Turanose	+	+	+
D-Lyxose	-	-	-
D-Tagatose	-	-	-
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabitol	-	-	-
L-Arabitol	-	-	-
Gluconate	+	+	+
2-keto-gluconate	-	-	-
5-Keto-gluconate	-	-	-

Based on Collins *et al*. (1987) and Hammes and Hertel (2006).

Appendix IV Table 7. Characteristics of *Citrobacter* species

	Culture	
Character	FSG 37 °C	SHG 37 °C
Morphology:		
Colony colour	pink	pink
Colony size	3-4 mm	2-3 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	creamy
Micromorphology:		
Cell shape	rod	rod
Cell arrangement	pairs, clusters	single
Gram stain	negative	negative
Endospore	No	no
API 20E character:		
Catalase production	+	+
Oxidase production	-	-
Motility at 20 °C	+	+
Oxidation/fermentation reaction	F	F
Tryptophan deaminase	-	+
Indole production	-	-
Voges-Proskauer reaction	-	-
Gelatinase production	-	-
H_2S production	-	-
Citrate utilization	+	+
Urease production	+	-
Ortho-nitrophenyl-galactosidase	+	-
Arginine dihydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Production of Acid from:		
Glucose	+	+
Sucrose	+	-
Arabinose	+	-
Mannitol	+	-
Inositol	+	-
Sorbitol	+	-
Rhamnose	-	-
Melibiose	-	-
Amygdalin	+	-
Degradation of:		
Lecithin	+	-
Tween 80	+	-
Gelatin	-	-
Elastin	-	-
Blood (haemolysis)	-	-
Triple sugar iron agar	-	-

Based on Holt (1994), Cowan and Steel (2003) and Austin and Austin (2007).

F = Fermentative

Appendix IV Table 8. Characteristics of *Corynebacterium* species

Character	Culture		
	FSC 20 °C	OJ 20 °C	ZFSK 30 °C
Morphology:			
Colony colour	white	cream	white
Colony size	≤ 1 mm	≤ 1 mm	1-2 mm
Colony shape	round, irregular edge, raised	round, irregular edge, raised	round, irregular edge, raised
Colony texture	creamy	dry	dry
Micromorphology:			
Cell shape	Rod	rod	rod
Cell arrangement	flagella like bacillus	flagella like bacillus	flagella like bacillus
Gram stain	Positive	positive	positive
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	-	-	-
Motility	-	-	-
Oxidation/fermentation reaction	A	-	-
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	-	-	-
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	-	-	-
Arginine dihydrolase	+	-	-
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	+	-	-
Sucrose	+	-	-
Arabinose	-	-	-
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	+	-	-
Degradation of:			
Lecithin	-	-	-
Tween 80	-	-	-
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	-	-	-
Triple sugar iron agar (H_2S)	-	-	-

Based on Baya *et al*. (1992a), Cowan and Steel (2003) and Austin and Austin (2007). A = Alkaline

Appendix IV Table 9. Characteristics of *Enterobacter* species

	Culture	
Character	FHG 37 °C	ZFSG 37 °C
Morphology:		
Colony colour	Pink	pink
Colony size	2-3 mm	3 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised
Colony texture	creamy	creamy
Micromorphology:		
Cell shape	Rod	rod
Cell arrangement	single, pair, cluster	cluster
Gram stain	negative	negative
Endospore	No	no
API 20E character:		
Catalase production	+	+
Oxidase production	-	-
Motility at 20 °C	+	+
Oxidation/fermentation reaction	F	F
Tryptophan deaminase	-	-
Indole production	-	-
Voges-Proskauer reaction	+	+
Gelatinase production	-	-
H_2S production	-	-
Citrate utilization	+	+
Urease production	+	-
Ortho-nitrophenyl-galactosidase	+	+
Arginine dihydrolase	+	+
Lysine decarboxylase	-	+
Ornithine decarboxylase	+	+
Production of Acid from:		
Glucose	+	+
Sucrose	+	-
Arabinose	+	+
Mannitol	+	+
Inositol	+	+
Sorbitol	+	+
Rhamnose	+	-
Melibiose	+	-
Amygdalin	+	+
Degradation of:		
Lecithin	+	-
Tween 80	+	-
Gelatin	-	-
Elastin	-	-
Blood (haemolysis)	-	-
Triple sugar iron agar (H_2S)	-	-

Based on Holt (1994) and Cowan and Steel (2003). F = Fermentative

Appendix IV Table 10 a. Characteristics of *Escherichia coli*

	Culture		
	FSH 37 °C	FHH 37 °C	ZFHH 37 °C
Morphology:			
Colony colour	Green metallic sheen	Green metallic sheen	Green metallic sheen
Colony size	3-4mm	1-2 mm	2-3 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	creamy	Creamy
Micromorphology:			
Cell shape	pleomorphic rod	pleomorphic rod	pleomorphic rod
Cell arrangement	single, pair	single, pair	single, pair
Gram stain	Negative	negative	negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	-	-	-
Oxidation/fermentation reaction	F	F	F
Tryptophan deaminase	-	-	-
Indole production	+	+	+
Voges-Proskauer reaction	-	-	-
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	+	-	+
Urease production	+	-	-
Ortho-nitrophenyl-galactosidase	+	+	-
Arginine dihydrolase	-	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	+
Production of Acid from:			
Glucose	+	+	-
Sucrose	+	+	-
Arabinose	+	+	+
Mannitol	+	+	-
Inositol	+	-	-
Sorbitol	+	-	+
Rhamnose	-	+	+
Melibiose	-	-	+
Amygdalin	+	+	+
Degradation of:			
Lecithin	+	-	-
Tween 80	+	-	-
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	-	-	-
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 10 b. Characteristics of *Escherichia coli*

Character	Culture		
	ZFSH 37 °C	SSB 20 °C	SHH 37 °C
Morphology:			
Colony colour	Green metallic sheen	Green metallic sheen	Green metallic sheen
Colony size	2-3 mm	3-4 mm	2-3 mm
Colony shape	Round, smooth edge, flat	Round, smooth edge, flat	Round, smooth edge, flat
Colony texture	Creamy	creamy	Creamy
Micromorphology:			
Cell shape	pleomorphic rod	pleomorphic rod	pleomorphic rod
Cell arrangement	single, pair	single, pair	single, pair
Gram stain	Negative	negative	Negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	-	-	-
Motility at 20 °C	+	+	+
Oxidation/fermentation reaction	F	F	F
Tryptophan deaminase	-	-	-
Indole production	+	+	+
Voges-Proskauer reaction	-	-	-
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	-	-	-
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	-	+	+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	+	-	-
Production of Acid from:			
Glucose	+	-	-
Sucrose	-	-	+
Arabinose	+	-	+
Mannitol	-	-	+
Inositol	-	-	-
Sorbitol	+	-	-
Rhamnose	+	-	+
Melibiose	+	-	-
Amygdalin	+	-	+
Degradation of:			
Lecithin	-	-	-
Tween 80	-	-	-
Gelatin	-	-	
Elastin	-	-	-
Blood (haemolysis)	-	α	-
Triple sugar iron agar (H_2S)	-	-	-

Based on Holt (1994) and Cowan and Steel (2003).

Appendix IV Table 11 a. Characteristics of *Moraxella* species

	Culture		
	ZSHB 30 °C	MBT 30 °C	ZSSB 20 °C
Morphology:			
Colony colour	Orange	orange	Orange
Colony size	≤1 mm	1-2 mm	≤1 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	creamy	Creamy
Micromorphology:			
Cell shape	short rod	short rod	short rod
Cell arrangement	single, pair, cluster	single, pair, cluster	single, pair, cluster
Gram stain	Negative	negative	Negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	-	-	-
Oxidation/fermentation reaction	-	-	A
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	+	+	+
Gelatinase production	-	-	-
H_2S production	-	-	-
Citrate utilization	-	-	-
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	-	-	-
Arginine dihydrolase	-	-	-
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	+	-	-
Production of Acid from:			
Glucose	-	-	-
Sucrose	-	-	-
Arabinose	-	-	
Mannitol	-	-	
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Degradation of:			
Lecithin	+	-	-
Tween 80	-	-	+
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	-	-	-
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 11 b. Characteristics of *Moraxella* species

	Culture	
Character	ZSHK 20 °C	ZSHC 20 °C
Morphology:		
Colony colour	Cream	Cream
Colony size	1-2 mm	1 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised
Colony texture	creamy	creamy
Micromorphology:		
Cell shape	short rod	short rod
Cell arrangement	Clusters	Clusters
Gram stain	Negative	Negative
Endospore	No	no
API 20E character:		
Catalase production	+	+
Oxidase production	+	+
Motility	-	-
Oxidation/fermentation reaction	A	A
Tryptophan deaminase	+	-
Indole production	-	-
Voges-Proskauer reaction	+	+
Gelatinase production	-	-
H_2S production	-	-
Citrate utilization	-	-
Urease production	-	-
Ortho-nitrophenyl-galactosidase	-	-
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Production of Acid from:		
Glucose	-	-
Sucrose	-	-
Arabinose	-	-
Mannitol	-	-
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Melibiose	-	-
Amygdalin	-	-
Degradation of:		
Lecithin	+	-
Tween 80	+	+
Gelatin	-	-
Elastin	-	-
Blood (β-haemolysis)	-	-
Triple sugar iron agar (H_2S)	-	-

Based on Cowan and Steel (2003) and Vaneechoutte *et al*. (2011).

A = Alkaline

Appendix IV Table 12. Characteristics of *Micrococcus* species

Character	Culture SH_2CT 30 °C
Morphology:	
Colony colour	cream-white
Colony size	1-2 mm
Colony shape	round, rough edge, raised
Colony texture	Dry
Micromorphology:	
Cell shape	Coccus
Cell arrangement	tetrads and clusters
Gram stain	Positive
Endospore	no
API 20E character:	
Catalase production	+
Oxidase production	+
Motility	-
Oxidation/fermentation reaction	-
Tryptophan deaminase	-
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	-
H_2S production	-
Citrate utilization	-
Urease production	-
Ortho-nitrophenyl-galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Production of Acid from:	
Glucose	-
Sucrose	-
Arabinose	-
Mannitol	-
Inositol	-
Sorbitol	-
Rhamnose	-
Melibiose	-
Amygdalin	-
Degradation of:	
Lecithin	-
Tween 80	-
Gelatin	-
Elastin	-
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	-

Based on Cowan and Steel (2003) and Kocur *et al*. (2006).

Appendix IV Table 13 a. Characteristics of *Pseudomonas* species

Character	Culture		
	SSD 30 °C	MUB 20 °C	FSD 20 °C
Morphology:			
Colony colour	Cream	orange	cream
Colony size	≤ 1 mm	1 mm	1-2 mm
Colony shape	Round, rough edge, raised	Round, smooth edge, raised	Round, rough edge, raised
Colony texture	dry	creamy	dry
Micromophology:			
Cell shape	rod	rod	rod
Cell arrangement	Chain	single, pair	single
Gram stain	negative	negative	negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	+	+
Oxidation/fermentation reaction	A	A	O
Tryptophan deaminase	-	-	+
Indole production	-	-	-
Voges-Proskauer reaction	-	+	-
Gelatinase production	-	-	+
H_2S production	-	-	-
Citrate utilization	+	+	+
Urease production	-	+	-
Ortho-nitrophenyl-galactosidase	-	-	-
Arginine dihydrolase	-	-	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	-	+	-
Sucrose	-	-	-
Arabinose	+	-	+
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	+	-	+
Amygdalin	-	-	-
Degradation of:			
Lecithin	-	-	+
Tween 80	-	-	-
Gelatin	-	-	+
Elastin	-	-	-
Blood (haemolysis)	-	-	-
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 13 b. Characteristics of *Pseudomonas* species

	Culture	
Character	ZFHTI 30 °C	OB 30 °C
Morphology:		
Colony colour	green	orange
Colony size	1-2 mm	1-2 mm
Colony shape	round, smooth edge, flat	round, smooth edge, raised
Colony texture	creamy	creamy, sticky
Micromorphology:		
Cell shape	rod	rod
Cell arrangement	single	single, pair, chain
Gram stain	Negative	negative
Endospore	No	no
API 20E character:		
Catalase production	+	+
Oxidase production	+	+
Motility	+	+
Oxidation/fermentation reaction	O	A
Tryptophan deaminase	-	-
Indole production	-	+
Voges-Proskauer reaction	+	+
Gelatinase production	-	+
H_2S production	-	-
Citrate utilization	+	+
Urease production	-	-
Ortho-nitrophenyl-galactosidase	-	-
Arginine dihydrolase	+	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Production of Acid from:	-	-
Glucose	-	-
Sucrose	-	-
Arabinose	-	-
Mannitol	-	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Melibiose	-	-
Amygdalin	-	-
Degradation of:		
Lecithin	+	+
Tween 80	+	-
Gelatin	-	+
Elastin	-	-
Blood (haemolysis)	-	β
Triple sugar iron agar (H_2S)	-	-

Appendix IV Table 13 c. Characteristics of Pseudomonad

Character	Culture FSB 30 °C
Morphology:	
Colony colour	orange
Colony size	3-4 mm
Colony shape	Round, smooth edge, flat
Colony texture	creamy
Micromorphology:	
Cell shape	rod
Cell arrangement	single, pair
Gram stain	negative
Endospore	No
API 20E character:	
Catalase production	+
Oxidase production	+
Motility	+
Oxidation/fermentation reaction	A
Tryptophan deaminase	-
Indole production	-
Voges-Proskauer reaction	-
Gelatinase production	-
H_2S production	-
Citrate utilization	-
Urease production	-
Ortho-nitrophenyl-galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Production of Acid from:	
Glucose	-
Sucrose	-
Arabinose	-
Mannitol	-
Inositol	-
Sorbitol	-
Rhamnose	-
Melibiose	-
Amygdalin	-
Degradation of:	
Lecithin	-
Tween 80	-
Gelatin	+
Elastin	+
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	-

Based on Holt (1994) and Cowan and Steel (2003).

A = Alkaline. O = Oxidation.

Appendix IV Table 14. Characteristics of *Psychrobacter* species

	Culture
Character	ZSHK 30 °C
Morphology:	
Colony colour	cream
Colony size	1 mm
Colony shape	round, rough edge, raised
Colony texture	Buttery or waxy
Micromorphology:	
Cell shape	rod
Cell arrangement	single, pair, cluster
Gram stain	negative
Endospore	No
API 20E character:	
Catalase production	+
Oxidase production	+
Motility	-
Oxidation/fermentation reaction	-
Tryptophan deaminase	+
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	-
H_2S production	-
Citrate utilization	-
Urease production	-
Ortho-nitrophenyl-galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Production of Acid from:	
Glucose	-
Sucrose	-
Arabinose	-
Mannitol	-
Inositol	-
Sorbitol	-
Rhamnose	-
Melibiose	-
Amygdalin	-
Degradation of:	
Lecithin	-
Tween 80	-
Gelatin	-
Elastin	-
Blood (α-haemolysis)	A
Triple sugar iron agar (H_2S)	-

Based on Bowman (2006).

Appendix IV Table 15. Characteristics of *Serratia* species

Character	Culture ZFHG 37 °C
Morphology:	
Colony colour	Pink
Colony size	2-3 mm
Colony shape	round, smooth edge, raised
Colony texture	Creamy
Micromorphology:	
Cell shape	Rod
Cell arrangement	single, pair
Gram stain	Negative
Endospore	No
API 20E character:	
Catalase production	+
Oxidase production	-
Motility at 20 °C	+
Oxidation/fermentation reaction	F
Tryptophan deaminase	-
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	+
H_2S production	-
Citrate utilization	+
Urease production	-
Ortho-nitrophenyl-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Production of Acid from:	
Glucose	-
Sucrose	+
Arabinose	+
Mannitol	+
Inositol	+
Sorbitol	+
Rhamnose	-
Melibiose	-
Amygdalin	+
Degradation of:	
Lecithin	+
Tween 80	+
Gelatin	-
Elastin	-
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	-

Based on Holt (1994), Cowan and Steel (2003) and Austin and Austin (2007).

Appendix IV Table 16 a. Characteristics of *Shewanella* species

	Culture		
Character	FSA 20 °C	SHP 30 °C	ZFSB 30 °C
Morphology:			
Colony colour	Orange	cream	orange
Colony size	3-4 mm	≤ 1 mm	3-4 mm
Colony shape	Round, smooth edge, raised	Round, smooth edge, raised	Round, smooth edge, raised
Colony texture	Creamy	creamy	creamy
Micromorphology:			
Cell shape	short rod	short rod	short rod
Cell arrangement	pairs, clusters	pairs, singles, clusters	clusters
Gram stain	negative	negative	negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	+	+
Oxidation/fermentation reaction	F	F	-
Tryptophan deaminase	+	+	-
Indole production	+	-	-
Voges-Proskauer reaction	+	+	-
Gelatinase production	+	+	+
H_2S production	+	+	+
Citrate utilization	+	+	+
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	+	-	-
Arginine dihydrolase	+	+	-
Lysine decarboxylase	+	+	-
Ornithine decarboxylase	-	-	+
Production of Acid from:			
Glucose	+	+	-
Sucrose	+	-	-
Arabinose	+	+	-
Mannitol	+	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	+	-	-
Amygdalin	+	-	-
Degradation of:			
Lecithin	+	+	+
Tween 80	+	+	+
Gelatin	+	-	+
Elastin	+	-	+

Appendix IV Table 16 a. (Continued)

Character	Culture		
	FSA 20 °C	SHP 30 °C	ZFSB 30 °C
Blood (β-haemolysis)	β	β	β
Triple sugar iron agar (H_2S)	-	-	+
Production of TMA	+	+	+
Growth at 4 °C	+	+	+
Growth at 37 °C	+	-	+

Appendix IV Table 16 b. Characteristics of *Shewanella* species

Character	Culture		
	FHB 20 °C	FHB 30 °C	ZFHB 30 °C
Morphology:			
Colony colour	orange	orange	orange
Colony size	3-4 mm	2-3 mm	1-2 mm
Colony shape	round, smooth edge, raised	round, smooth edge, raised	round, smooth edge, raised
Colony texture	Creamy	creamy	creamy
Micromorphology:			
Cell shape	short rod	short rod	short rod
Cell arrangement	Singles	singles	singles, pairs
Gram stain	negative	negative	negative
Endospore	No	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	+	+
Oxidation/fermentation reaction	A	A	A
Tryptophan deaminase	-	-	-
Indole production	-	-	-
Voges-Proskauer reaction	-	-	-
Gelatinase production	+	+	+
H_2S production	+	+	+
Citrate utilization	+	+	+
Urease production	-	-	-
Ortho-nitrophenyl-galactosidase	-	-	-
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	+	-	-
Production of Acid from:			
Glucose	-	-	-
Sucrose	-	-	-

Appendix IV Table 16 b. (Continued)

	Culture		
Character	FHB 20 °C	FHB 30 °C	ZFHB 30 °C
Arabinose	-	-	-
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Degradation of:			
Lecithin	-	-	+
Tween 80	-	-	-
Gelatin	+	+	+
Elastin	-	-	-
Blood (haemolysis)	A	-	-
Triple sugar iron agar (H_2S)	+	+	+
Production of TMA	+	+	+
Growth at 4 °C	+	+	+
Growth at 37 °C	-	-	-

Appendix IV Table 16 c. Characteristics of *Shewanella* species

	Culture
Character	ZFHB 20 °C
Morphology:	
Colony colour	orange
Colony size	1-2 mm
Colony shape	round, smooth edge, raised
Colony texture	Creamy
Micromorphology:	
Cell shape	short rod
Cell arrangement	singles, pairs
Gram stain	negative
Endospore	No
API 20E character:	
Catalase production	+
Oxidase production	+
Motility	+
Oxidation/fermentation reaction	A
Tryptophan deaminase	+
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	+
H_2S production	+
Citrate utilization	-

Appendix IV Table 16 c. (Continued)

	Culture
Character	ZFHB 20 °C
Urease production	-
Ortho-nitrophenyl-galactosidase	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	+
Production of Acid from:	
Glucose	-
Sucrose	-
Arabinose	-
Mannitol	-
Inositol	-
Sorbitol	-
Rhamnose	-
Melibiose	-
Amygdalin	-
Degradation of:	
Lecithin	+
Tween 80	+
Gelatin	+
Elastin	-
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	+
Production of TMA	+
Growth at 4 °C	+
Growth at 37 °C	-

Based on Vogel *et al*. (2005).

A = Alkaline. F = Fermentation.

Appendix IV Table 17. Characteristics of *Staphylococcus* species

	Culture
Character	SSC 30 °C
Morphology:	
Colony colour	white
Colony size	1-2 mm
Colony shape	round, smooth edge, raised
Colony texture	creamy
Micromorphology:	
Cell shape	coccus
Cell arrangement	chain, cluster
Gram stain	positive
Endospore	No
API 20E character:	
Catalase production	+
Oxidase production	-
Motility	-
Oxidation/fermentation reaction	F
Tryptophan deaminase	-
Indole production	-
Voges-Proskauer reaction	+
Gelatinase production	-
H_2S production	-
Citrate utilization	-
Urease production	-
Ortho-nitrophenyl-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Production of Acid from:	
Glucose	+
Sucrose	+
Arabinose	-
Mannitol	+
Inositol	-
Sorbitol	-
Rhamnose	-
Melibiose	-
Amygdalin	-
Degradation of:	
Lecithin	-
Tween 80	-
Gelatin	-
Elastin	-
Blood (haemolysis)	-
Triple sugar iron agar (H_2S)	-
Coagulase	-
DNase	-

Based on Cowan and Steel (2003). F = Fermentative.

Appendix IV Table 18 a. Characteristics of *Vibrio* species

	Culture		
Character	MUTI 20 °C	OTA 20 °C	MTI 20 °C
Morphology:			
Colony colour	green	yellow	green
Colony size	1-2 mm	1-2 mm	1-2 mm
Colony shape	Round, smooth edge, flat	Round, smooth edge, flat	Round, smooth edge, flat
Colony texture	creamy	creamy	creamy
Micromorphology:			
Cell shape	straight rod	curved rod	straight rod
Cell arrangement	single, pair	cluster	single, pair
Gram stain	Negative	negative	negative
Endospore	no	no	no
API 20E character:			
Catalase production	+	+	+
Oxidase production	+	+	+
Motility	+	+	+
Oxidation/fermentation reaction	F	F	F
Tryptophan deaminase	-	-	-
Indole production	+	+	+
Voges-Proskauer reaction	-	-	-
Gelatinase production	+	+	+
H_2S production	-	-	-
Citrate utilization	+	+	+
Urease production	+	-	-
Ortho-nitrophenyl-galactosidase	-	+	+
Arginine dihydrolase	-	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
Production of Acid from:			
Glucose	-	-	+
Sucrose	-	+	-
Arabinose	-	-	-
Mannitol	+	+	+
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
O/129 Sensitivity:			
10 µg	S	S	S
150 µg	S	S	S
Degradation of:			
Lecithin	+	+	+
Tween 80	-	-	+

Appendix IV Table 18 a. (Continued)

	Culture		
Character	MUTI 20 °C	OTA 20 °C	MTI 20 °C
Gelatin	-	-	-
Elastin	-	-	-
Blood (haemolysis)	B	B	β
Triple sugar iron agar (H_2S)	-	-	-

Appendix IV Table 18 b. Characteristics of *Vibrio* species

	Culture	
Character	FHTI 30 °C	SSA 30 °C
Morphology:		
Colony colour	green	yellow
Colony size	1-2 mm	1-2 mm
Colony shape	Round, smooth edge, raised	Round, smooth edge, raised
Colony texture	Creamy	creamy
Micromorphology:		
Cell shape	curved rod	curved rod
Cell arrangement	chains	singles, pairs
Gram stain	Negative	negative
Endospore	No	no
API 20E character:		
Catalase production	+	+
Oxidase production	-	-
Motility	+	+
Oxidation/fermentation reaction	F	F
Tryptophan deaminase	+	+
Indole production	-	-
Voges-Proskauer reaction	+	+
Gelatinase production	-	-
H_2S production	-	-
Citrate utilization	+	+
Urease production	-	+
Ortho-nitrophenyl-galactosidase	-	-
Arginine dihydrolase	+	+
Lysine decarboxylase	+	-
Ornithine decarboxylase	-	-
Production of Acid from:		
Glucose	+	-
Sucrose	-	+
Arabinose	-	-
Mannitol	-	-
Inositol	-	-

Appendix IV Table 18 b. (Continued)

Character		Culture	
		FHTI 30 °C	SSA 30 °C
Sorbitol		-	-
Rhamnose		-	-
Melibiose		-	-
Amygdalin		-	-
O/129 Sensitivity:			
	10 µg	S	R
	150 µg	S	R
Degradation of:			
Lecithin		-	-
Tween 80		-	-
Gelatin		-	-
Elastin		-	-
Blood (α-haemolysis)		A	α
Triple sugar iron agar (H_2S)		-	-

Based on Holt (1994) Cowan and Steel (2003) and Buller (2004).

F = Fermentative.

Appendix IV Table 19. Characteristics of *Listeria monocytogenes*

Character	Reaction	Culture	
		MP1C 30 °C	MP1C 20 °C
Morphology:			
Colony colour		black halo with green surrounding	black halo with green surrounding
Colony size		≤1 mm	≤1 mm
Colony shape		round, smooth edge, flat	round, smooth edge, flat
Colony texture		Creamy	creamy
Micromorphology:			
Cell shape		tiny rod	tiny rod
Cell arrangement		pairs, singles, clusters	pairs, singles, clusters
Gram stain		positive	positive
Endospore		No	no
MICROBACT™ 12L character:			
Catalase production		+	+
Oxidase production		-	-
Motility at 20 °C		-	+
Motility at 30 °C		+	-
Esculin	Yellow	+	+
Mannitol	Purple	-	-
Xylose	Yellow	+	+
Arabitol	Yellow	+	+
Ribose	Yellow	+	+
Rhamnose	Purple	-	-
Trehalose	Yellow	+	+
Tagatose	Purple	-	-
Gluc-1-phos	Purple	-	-
M-D-Gluc	Yellow	+	+
M-D-Man	Yellow	+	+
Haemolysis	Brown	+	+

Based on USDA, FSIS, OPHS (2013).

Appendix V. Result of blast of *Carnobacterium maltaromaticum* MMF-32

Table 1. The List of top 5 significant alignments from BLAST

Result	Score (bits)	E Value
gi|254771558|gb|GQ304940.1|:2-1402 *Carnobacterium maltaromaticum* strain MMF-32 16S ribosomal RNA gene, partial sequence	2573	0.0
gi|254771554|gb|GQ304936.1|:2-1402 *Carnobacterium maltaromaticum* strain MMF-28 16S ribosomal RNA gene, partial sequence	2573	0.0
gi|254771550|gb|GQ304932.1|:2-1402 *Carnobacterium maltaromaticum* strain MMF-24 16S ribosomal RNA gene, partial sequence	2573	0.0
gi|445065276|gb|KC213911.1|:55-1456 *Carnobacterium maltaromaticum* strain Z3_S_MRS15 16S ribosomal RNA gene, partial sequence	2567	0.0
gi|445065242|gb|KC213877.1|:41-1442 *Carnobacterium maltaromaticum* strain Z2_T_MRS_39 16S ribosomal RNA gene, partial sequence	2567	0.0

gi = GenBank accession number

gb = GenBank

Score = assigned to a match between two sequences

E Value = Expectation

The example of alignments of gi|254771558|gb|GQ304940.1|:2-1402 *Carnobacterium maltaromaticum* strain MMF-32 16S ribosomal RNA gene, partial sequence (Length = 1410). Score = 2564 bits (1393), Expect = 0.0, Identities = 1397/1401 (99%), Gaps = 0/1401 (0%), Strand = Plus/Plus.

```
Query  1     GTCGACGCACGAAGTTGAAGAGCTTGCTCTTTAACCAAGTGAGTGGCGGACGGGTGAGTA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2     GTCGACGCACGAAGTTGAAGAGCTTGCTCTTTAACCAAGTGAGTGGCGGACGGGTGAGTA  61

Query  61    ACACGTGGGTAACCTGCCCATTAGAGGGGGATAACATTCGGAAACGGATGCTAATACCGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62    ACACGTGGGTAACCTGCCCATTAGAGGGGGATAACATTCGGAAACGGATGCTAATACCGC  121

Query  121   ATAGTTTCAGGRATCGCATGATTCTTGAAGGAAAGGTGGCTYCGGCTACCACTAATGGAT  180
             |||||||||| |||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  122   ATAGTTTCAGGAATCGCATGATTCTTGAAGGAAAGGTGGCTTCGGCTACCACTAATGGAT  181

Query  181   GGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACGTAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182   GGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACGTAG  241

Query  241   CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242   CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA  301

Query  301   GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302   GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT  361

Query  361   GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGGATGAGAGTAACT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362   GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGGATGAGAGTAACT  421

Query  421   GCTCATCCCCTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422   GCTCATCCCCTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG  481

Query  481   GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482   GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGT  541

Query  541   TCTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAGA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542   TCTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAGA  601

Query  601   ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   ACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGT  661

Query  661   GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662   GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG  721

Query  721   CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA  781

Query  781   GTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   GTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG  841

Query  841   AGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   AGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGC  901

Query  901   ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCAC  961

Query  961   TCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAG  1020
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  962   TCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAG  1021

Query  1021  CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGC  1080
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1022  CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGC  1081

Query  1081  CAGCATTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT  1140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1082  CAGCATTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT  1141

Query  1141  GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC  1200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1142  GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC  1201

Query  1201  AACGAGTCGCAAGGTCGCGAGGCCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTG  1260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1202  AACGAGTCGCAAGGTCGCGAGGCCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTG  1261

Query  1261  TAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAACGCCGC  1320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1262  TAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAACGCCGC  1321

Query  1321  GGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACAY  1380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1322  GGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACAC  1381

Query  1381  CCGAAGCCGGTKAGGTAACCT  1401
            |||||||||||  |||||||||
Sbjct  1382  CCGAAGCCGGTGAGGTAACCT  140
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Appendix VI. Result of blast of *Carnobacterium maltaromaticum* KOPRI 25789

Table 1. The List of top 5 significant alignments from BLAST

Result	Score (bits)	E Value
gi|325668038|gb|HQ824973.1|:7-1403 *Carnobacterium maltaromaticum* strain KOPRI 25789 16S ribosomal RNA gene, partial sequence	2566	0.0
gi|305430524|gb|HM215040.1|:40-1436 Uncultured *Carnobacterium* sp. clone BJ07179E4 16S ribosomal RNA gene, partial sequence	2566	0.0
gi|308210746|dbj|AB593337.1|:27-1423 *Carnobacterium* sp. cG53 gene for 16S ribosomal RNA, partial sequence	2566	0.0
>gi|308210743|dbj|AB593334.1|:24-1420 *Carnobacterium* sp. aG53 gene for 16S ribosomal RNA, partial sequence	2566	0.0
>gi|254771558|gb|GQ304940.1|:7-1403 *Carnobacterium maltaromaticum* strain MMF-32 16S ribosomal RNA gene, partial sequence	2566	0.0

gi = GenBank accession number

gb = GenBank

Score = assigned to a match between two sequences

E Value = Expectation

The example of alignments of gi|325668038|gb|HQ824973.1|:7-1403 *Carnobacterium maltaromaticum* strain KOPRI 25789 16S ribosomal RNA gene, partial sequence (Length = 1427). Score = 2566 bits (1389), Expect = 0.0, Identities = 1393/1397 (99%), Gaps = 0/1397 (0%), Strand (Plus/Plus).

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Query  1     CGCACGAAGTTGAAGAGCTTGCTCTTTAACCAAGTGAGTGGCGGACGGGTGAGTAACACG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7     CGCACGAAGTTGAAGAGCTTGCTCTTTAACCAAGTGAGTGGCGGACGGGTGAGTAACACG  66

Query  61    TGGGTAACCTGCCCATTAGAGGGGGATAACATTCGGAAACGGATGCTAATACCGCATAGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67    TGGGTAACCTGCCCATTAGAGGGGGATAACATTCGGAAACGGATGCTAATACCGCATAGT  126

Query  121   TTCAGGRATCGCATGATTCTTGAAGGAAAGGTGGCTYCGGCTACCACTAATGGATGGACC  180
             |||||| |||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  127   TTCAGGAATCGCATGATTCTTGAAGGAAAGGTGGCTTCGGCTACCACTAATGGATGGACC  186

Query  181   CGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACGTAGCCGAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187   CGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACGTAGCCGAC  246

Query  241   CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247   CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG  306

Query  301   CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307   CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGA  366

Query  361   AGGTTTTCGGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGGATGAGAGTAACTGCTCA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367   AGGTTTTCGGATCGTAAAACTCTGTTGTTAAAGAAGAACAAGGATGAGAGTAACTGCTCA  426

Query  421   TCCCCTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427   TCCCCTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT  486

Query  481   ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTCTTT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487   ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTCTTT  546

Query  541   AAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAGAACTTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547   AAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAGAACTTG  606

Query  601   AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607   AGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGG  666

Query  661   AACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667   AACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGG  726

Query  721   GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  727   GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT  786

Query  781   GGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  787   GGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAC  846

Query  841   GGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  847   GGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTG  906

Query  901   GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  907   GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAG  966

Query  961   AGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT  1020
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  967   AGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT  1026

Query  1021  GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCA  1080
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1027  GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCA  1086

Query  1081  TTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT  1140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1087  TTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT  1146

Query  1141  CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGA  1200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1147  CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGA  1206

Query  1201  GTCGCAAGGTCGCGAGGCCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGC  1260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1207  GTCGCAAGGTCGCGAGGCCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGC  1266

Query  1261  TGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAACGCCGCGGTGA  1320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1267  TGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAACGCCGCGGTGA  1326

Query  1321  ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACAYCCGAA  1380
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  1327  ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA  1386

Query  1381  GCCGGTKAGGTAACCTT  1397
            |||||| ||||||||||
Sbjct  1387  GCCGGTGAGGTAACCTT  1403
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