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Isolation, identification and characterization of Listeria monocytogenes, potential pathogens and spoilage microorganisms from temperate seafood.

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ABSTRACT— Seafood permits the transmission of many bacterial pathogens. *Vibrio* spp., Enterobacteriaceae representatives, aerobic heterotrophic bacteria and *Listeria monocytogenes* were isolated from commercially prepared smoked and fresh Atlantic salmon, smoked and fresh haddock, live mussels and oysters collected from local shops in Central Scotland using selective media and tryptone soya agar (TSA). Isolates were identified using API 20E, API-50CH and16S rDNA-targeted PCR method. Vibrio spp. occurred in high densities (>10^6 CFU g^{-1}) in mussels. Enterobacteriaceae representatives were recorded at 2.2 x 10^6 CFU g^{-1} and 2.0 x 10^6 CFU g^{-1} in fresh salmon and smoked haddock, respectively. Total 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. The spoilage organisms revealed the ability to produce hydrolytic enzymes.

Key words: Enterobacteriaceae, fiiter feedres, fish spoilage bacteria, *Listeria monocytogenes*, *Vibrio* spp.

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1 INTRODUCTION

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 [1]; Hellberg *et al*., 2012 [2]. They are regarded as important part of a healthy diet because of the presence of high quality protein and essential nutrients, namely omega-3 fatty acids. All fishery products may be low in saturated fat (Okonko *et al*., 2009 [3]. Fish consumption is recognised to reduce the risks of coronary heart diseases (Din *et al*., 2004 [1]. 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 occurrences (Ayers *et al*., 2008 [4].

Contamination of fish tissues with microorganisms may reflect the presence of pollutants in the aquatic environment (Adeyemo, 2003 [5]. 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[6]. 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 (Popovic *et al*., 2010 [7].

The presence of putative pathogenic microorganisms such as *V. parahaemolyticus*, *Aeromonas* spp., *Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens* and *Edwardsiella tarda* has been revealed in fresh marine fish, *i.e*. conger, swordfish, sole, grouper, and whiting (Herrera *et al*., 2006 [8]. In some situations, the contamination of seafood by pathogenic bacteria, such as *E. coli, Salmonella* and *Shigella,* is a result of the presence of animal or human faeces being introduced into water bodies (Goja, 2013 [6]. 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 [9].

Most published work on microbial contamination concentrates on limited spectra of bacteria, mostly vibrios and coliforms, this study attempts to provide a broader insight into the isolation of *Listeria monocytogenes*, potentially pathogenic and spoilage microorganisms from temperate seafood.

2 MATERIALS AND METHODS

2.1 **Collection of seafood samples**:

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. All animals were sourced from Scotland. There was not any stated shelf life for any of the fresh fish or shellfish purchases. Samples from fishmongers in Stirling were packed in polyethylene wraps, and stated to be fresh with a 24 h use-by date. The samples from Edinburgh were transported to the laboratory within one hour of collection, and were maintained in a cold box at 4^{o}C. 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.

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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2.2 Experimental samples and bacterial cultures:

The samples were analysed for the presence of *Vibrio* spp., *Enterobacteriaceae* representatives, *Listeria monocytogenes* and total aerobic heterotrophic counts. The microbiological procedures included the homogenization of 10 g of each seafood sample in 90 ml of appropriate broth. The methods specifically used were: for *Vibrio* spp. was according to Normanno *et al.* (2006 [10]; for Enterobacteriaceae representatives on Eosin methylene blue agar were according to Ahmad *et al*. (2012 [11]; for Aerobic heterotrophic counts was Sallam (2007 [12] and for *Listeria* spp. pre-enrichment on *Listeria* primary selective enrichment broth base CM0863 supplemented with SR0142E (UVM I) (Oxoid) and *Listeria* secondary selective enrichment broth base CM0863 supplemented with SR0143E (UVM II) (Oxoid) followed by cultures on PALCAM agar as recommended by McClain and Lee (1988 [13].

2.3 Isolation of bacterial cultures:

Then, representative colonies were picked, and purified by streaking and restreaking 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, 2005 [14]. *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 [15].

2.4 Identification of bacteria:

All cultures were examined for the following characteristics: micromorphology using Gram-stained smears (Hucker and Conn, 1923 [16] and motility (optical microscopy), colonial morphology using colour, size, shape and texture, catalase production 3% (v/v) hydrogen peroxide, Oxidative-fermentative (OF) test (Hugh and Leifson, 1953 [17], Oxidase production (Kovács, 1956 [18].

2.5 Physiological characterization:

Identification of the bacterial isolates to specie level was performed using API 20E (Gram-negative isolates), API 50CH (Gram-positive isolates) (BioMerieux® Marcy-l'Etoile, France) and MICROBACT™ Listeria 12L (Thermoscientific).

DNase activity was assessed on DNase test Agar (Oxoid) according to Jeffries *et al*., (1957 [19]. Trimethylamine oxide (TMAO) reduction and hydrogen sulphide (H_2S) production were estimated according to Gram *et al*. (1987 [20]. Lecithinase and lipase activities were examined as described (Liu *et al*., 1996 [21]. Haemolytic activity against sheep defribrinated sheep blood in Alseviers solution (Oxoid) was examined. Gelatinase activity was recorded after Loghothetis and Austin (1996 [22]. Elastase production using a bilayer of elastin agar was examined according to (Hasan *et al*., 1992 [23]. Coagulase production was carried out according to Cowan (1938 [24].

2.6 Molecular identification and characterization;

Isolates were identified by 16S rDNA amplification, sequencing and species attribution (Weisburg *et al*., 1991[25].

3 RESULTS

3.1 Isolation of microorganisms from seafood:

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 1, 2, 3 and 4., respectively). During the period of study, the mean presumptive *Vibrio* counts ranged from 2.8×10^3 to 2.1×10^6 CFU g^{-1}, the mean Enterobacteriaceae counts from 5.0×10^2 to 2.2×10^6 CFU g^{-1}, mean aerobic heterotrophic counts from 1.6×10^3 to 5.8×10^7 CFU g^{-1} and the mean *L. monocytogenes* counts from 1.0×10^4 to 5.0×10^4 CFU g^{-1} in the seafood samples.

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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Table 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. 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 3. Total aerobic heterotrophic counts of the seafood

Date 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 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

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

From Table 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, 19 genera of bacteria were recognised including *Acinetobacter, Aerococcus, Aeromonas, Bacillus, Brochothrix, Carnobacterium,*

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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Citrobacter, *Corynebacterium*, *Enterobacter*, *Escherichia coli*, *Listeria*, *Moraxella*, *Micrococcus*, *Pseudomonas*, *Psychrobacter*, *Serratia*, *Shewanella*, *Staphylococus* and *Vibrio* (Table 5). The proportions of these taxa have been included in Table 5. Data in Table 5 summarizes the prevalence of foodborne pathogens in fresh and smoked salmon, fresh and smoked haddock, fresh mussels and oyster fillets collected from local shops in Central Scotland.

Table 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.

3.3 Detection of specific spoilage bacteria:

Table 6 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. 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 6).

Table 6. 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	+	+

(+) represents late producers of H_2S

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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Table 7 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 7).

Table 7. 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	+	-	-	+	-

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 [26]. 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 [27]. Proteases (gelatin) are associated in the growth and spread of the bacterium and contribute to the development of the disease by defeating host defenses and providing nutrients for the host (Sakai, 1985 [28]; 1986 [29]; Janda and Abbot, 2010 [30]. 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 [31]; Timpe *et al*., 2003 [32]. Merino *et al*. (1999 [33] 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 [34]; Scoaris, *et al*., 2008 [35]; Figueras *et al*., 2011 [36].

DISCUSSION

Seafood permits the transmission of many bacterial pathogens (Davis *et al*., 2001 [37]; Hosseini *et al*., 2004 [38]. 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 [39]; Popovic *et al*., 2010 [7]; Adebayo-Tayo *et al*., 2011 [40]; Joh *et al*., 2013 [41]. It is likely that these bacteria may have been contaminants on fish, possibly introduced during harvesting and filleting operations (Papadopoulou *et al*., 2007 [42]; Huss *et al*., 2003 [43]; Eze *et al*., 2011[44]). 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

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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swordfish and tuna alongside non-H_2S producers, such as *Ps. fluorescens*, *Ps. fragi* and *Ac. radioresistens* (Serio *et al*., 2014 [45].

The presumptive *Vibrio* counts revealed counts ranged from 2.8 x 10^3 to 2.1 x 10^6 CFU g^{-1} (Table 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 [46] reported the growth of *Pseudomonas* spp. and *Aeromonas* spp. on TCBS agar. It is not surprising that vibrios were common in marine fish and shellfish (Popovic *et al*., 2010 [7]; Merwad *et al*., 2011[47]. Vibrios, notably *V. parahaemolyticus,* are common in seafood (e.g. Adeleye *et al*., 2010 [48]; Adebayo-Tayo *et al*., 2011[40]. Adeleye *et al*. (2010 [48] showed that *V. parahaemolyticus* were detected in seafood (11.4% of samples) collected in Nigeria. In comparison, Bauer *et al*. (2006 [49] 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 [7]; Merwad *et al*., 2011[47]. Populations of *V. parahaemolyticus* of 10^4 CFU g^{-1} are regarded as potentially hazardous to human health (Aberoumand, 2010 [50]. *V. parahaemolyticus* is causing sporadic foodborne infections and outbreaks worldwide, with gastroenteritis being the most common clinical manifestation (Papadopoulou *et al*., 2007 [42]. 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 [51]; Wallet *et al*., 2005 [52].

The *Enterobacteriaceae* counts from the seafood samples in this study (Table 2) were above the acceptable limits of 10 CFU g^{-1} as recommended by the International Commission on Microbiological Specifications of Foods (1986 [53] for fresh, frozen and cold smoked fish. Thus, the data are in agreement with the results of Ghanem *et al*. (2014 [54], 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.

Citrobacter and *Enterobacter* have been recovered previously from cultured freshwater fish (Hassan *et al*., 2012 [55]. In addition, Ghanem *et al*. (2014 [54] recovered *Enterobacter cloacae* from *Dicentrarchus labrax* and stingray fish fillets whereas Tavakoli *et al.* (2012 [56] and Gupta *et al*. (2013 [57] 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 [58].

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 [59]. 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 [60]. 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 postharvest 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 [61].

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 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 [53]. Similarly, Tomé *et al*. (2006 [62] 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 [63] and Goja (2013 [6]. 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

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
ISSN 2229-5518

Centre for Food Safety and Applied Nutrition (CFSAN, 2003 [64].

The high bacteria numbers recovered from fresh salmon, fresh haddock and cold smoked haddock, (mussels and oysters at 20 °C) (Table 3) were above the acceptable limits according to the International Commission on Microbiological Specifications of Foods (1986 [53]. Similarly, Kapute *et al*. (2012 [65], Adebayo-Tayo *et al*. (2012 [66], and Noor *et al*. (2013 [67] 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 [7] 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 [43]. 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 [68]. Therefore, the numbers recorded in this study (Table 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 4. These colonies were yellow in colour and were equated with *Aerococcus viridans* subsp. *homari* and *Shewanella baltica*. Also, Van Netten *et al*. (1989 [69] demonstrated the growth of staphylococci and enterococci as yellow colonies on PALCAM agar, being attributed to the effect of mannitol-positive activity. *Listeria* spp., especially *L. monocytogenes,* were reported in farmed mussels in the North Aegean Sea (Soultos *et al*., 2014 [70]. Of relevance, Vernocchi *et al*. (2007 [71] noted that contamination with listeria could occur as a result of improper harvesting, handling, processing and sanitation (Bremer *et al*., 2003 [72]. 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[73]. 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 [68]. 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 [74].

The range of organisms isolated from seafood has similarities with other work, e.g. Austin (2006 [75] and Amoah *et al*. (2011 [76]. In particular, *Acinetobacter* has been reported on *Pangasius* fillet collected at the filleting, trimming and freezing steps (Thi *et al*., 2013 [77]. 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 [78]; Hatha, 2002 [79]. Guardabassi *et al*. (1999 [80] 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 [81]. *Aeromonas* spp. have been isolated previously from seafood samples (Palumbo *et al*., 1985 [82]; Joseph *et al*., 2013 [83]. Tsai and Chen (1996 [60] and Vivekanandhan *et al*. (2005 [84] 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 [85]. They cause major epidemic outbreaks (Figueras *et al*., 2011 [36]; Beaz-Hidalgo and Figueras, 2012 [85]. *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 [86]. 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 [87]. *A. salmonicida* is the causative agent of furunculosis, a disease that affects many species of fish

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
ISSN 2229-5518

and is an important cause of economic losses in the aquaculture of salmonids, eels, rainbow trout and trout (Austin and Adams, 1996 [88]; Sørum *et al*., 2000 [89]. 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 [90]; Beaz-Hidalgo and Figueras, 2012 [85]. 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 [91] fish habour the pathogen at the point of capture or become infected during transportation, and Ottaviani *et al*. (2011[92] 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 [93]; Cardinal *et al*., 2004 [94]. 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 [95].

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 [87]; Beaz-Hidalgo and Figueras, 2013 [96]; Dallaire-Dufresne *et al*., 2014 [97].

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 [98]. The presence of *Bacillus* is not surprising insofar as the genus has been previously associated with fish. Coton *et al*. (2011 [98] isolated *Bacillus* spp. from surimi seafood products. Akinyemi and Ajisafe (2011 [99] demonstrated the presence of *Bacillus* spp. from the skin, bucal cavity and gills of *Chrysichthys nigrodigitatu.* Rahmati and Labbe (2008 [100] 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 [101]. Some species have been involved in food spoilage such as *B. licheniformis* (ex. slime production in kamaboko; (Mori *et al*., 1973 [102], *B. subtilis* and *B. pumilus* (Pepe *et al*., 2003 [103] and in food intoxication cases in particular *B. cereus* (Ehling-Schulz *et al*., 2004 [104]; Dierick *et al*., 2005 [105], likewise *B. licheniformis* (Salkinoja-Salonen *et al*., 1999 [106], *B. subtilis* (Kramer *et al*., 1982 [107] and *B. pumilus* (Lund, 1990 [108].

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 [109]. Moreover, examination of vacuum-packed cold-smoked salmon and trout has revealed a low number of *Brochothrix* spp. (Gonzalez-Rodriguez *et al*., 2002 [110]. *B. thermosphacta* was recovered from fresh fish (Olofsson *et al*., 2006 [111], and it is recognised that the organism has an important role in the spoilage of fish (Pin *et al*., 2002 [112] and shrimp (Fall *et al*., 2010 [113]. Specifically, *B. thermosphacta* is responsible for off-flavours, discolouration and gas production (Braun and Sutherland, 2004 [114], and a pungent 'cheesy' odour (McClure *et al*., 1993 [115].

Kim and Austin (2006 [116] isolated carnobacteria from the digestive tract content of rainbow trout (*Oncorhynchus mykiss*, Walbaum). Dalsgaard *et al*. (2003 [117] 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 [118]. 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 [119]; Mejlholm *et al*., 2004), and various lightly preserved seafoods (Jørgensen Dalsgaard *et al*., 2000 [120]; Dalsgaard *et al*., 2003 [117]. *C. maltaromaticum* strains may be used as protective cultures in biopreserved meat and seafood (Laursen *et al*., 2005 [118].

Addis *et al*. (2010 [121] demonstrated the presence of *Moraxella* in the tissues of gilthead sea bream (*Sparus aurata*, L.). In addition, Austin and Austin (2007 [87] revealed the presence of *Moraxella* among fish tissues.

Pseudomonas spp. have been regularly associated with fish tissues. Eissa *et al*. (2010 [122] isolated *Pseudomonas* from gills, liver, kidneys and spleen, whereas Oladipo and Bankole (2013 [123] demonstrated the presence of the organisms in fresh and dried *Clarias gariepinus* and *Oreochromis niloticus*. Kapute *et al.* (2013 [124] 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

International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
ISSN 2229-5518

important role in spoilage (Mahalaxmi *et al*., 2013 [125]. 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 [126]; Craun *et al*., 2010 [127].

Psychrobacter are common especially on salted fish, chilled fish, some shell fish and fermented seafood (Gonzalez *et al*., 2000 [128]; Bagge-Ravn *et al*., 2003 [129]; Bjorkevoll *et al*., 2003 [130]; Yoon *et al*., 2003 [131] 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 [130].

The presence of staphylococci on seafood is not unusual. Indeed, Ananchaipattana *et al*. (2012 [132] 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 [133]; Herrero *et al*., 2003 [134], such as fish smokers (Ferreira *et al*., 2007 [135]. It is apparent that the detection of staphylococci in fish suggests: (a) postharvest contamination due to poor hygiene, or (b) disease in fish (Huss, 1988 [136]; Austin and Austin, 2007 [87]. Basti *et al*. (2003 [39] 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.

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 [27]. 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 [137]. Consequently, *tdh* gene, marked by a β -type haemolysis on Wagatsuma agar (Nishibuchi and Kaper, 1995 [137], and *trh* gene, correlate to a positive urease tests (Okuda *et al*., 1997 [138], 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 [27], 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 (Table 6). *Shewanalla* spp. were recovered in this study, but this is not surprising as Vogel *et al*. (2005 [139] 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 [140], Aubert *et al*., 2009 [141]. However and in agreement with this study, Serio *et al*. (2014 [45] demonstrated *Shewanella* spp., as a spoilage microorganism by its strong production of TMA and H_2S.

The results of this study clearly indicate that seafood are contaminated by potential pathogenic and spoilage microorganisms. 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 [142]. It is important to handle seafood safely so to prevent increase in rapid spread of microorganisms. 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 [42]. 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.

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International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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International Journal of Scientific & Engineering Research Volume 8, Issue 5, May-2017
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