International Journal of Food Microbiology 151 (2011) 164–170

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterisation of the microbiota of Atlantic mackerel (*Scomber scombrus*)

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ARTICLE INFO

Article history:
Received 21 April 2011
Received in revised form 18 July 2011
Accepted 19 August 2011
Available online 26 August 2011

Keywords:
Atlantic mackerel
Microbiota
API®
PCR-DGGE
Handling effect

ABSTRACT

In this study the microbiota of Atlantic mackerel (*Scomber scombrus*) collected by a commercial purse seiner was examined. Fish were collected directly from the purse seine and from the Refrigerated Sea Water (RSW) transport tank after loading. The culturable microbiota and Specific Spoilage Bacteria (SSB) were quantified on Iron Agar Lyngby (IAL) and identified using commercially available Biochemical API® kits on pure cultured isolates. These kits showed to be sub-optimal in characterising the isolates, since only half of the strains were identified. The same isolates were also identified by a nucleic acid based PCR-DGGE approach, and only half of the sequences gave the same results as the API®. Characterisation by PCR-DGGE was also performed on bacterial DNA from IAL plates (bulk cell samples) and on samples where the bacterial DNA was extracted directly from fish material without any cultivation (direct DNA samples). The microbiota of Atlantic mackerel was dominated by members of the Gram-negative genera as *Psychrobacter* sp., *Proteus* sp., *Photobacterium* sp., *Vibrio* sp., *Shewanella* sp., *Synechococcus* sp., *Oceanisphaerae* sp., *Bizonia* sp., *Pseudoalteromonas* sp., and members of Flavobacteriaceae. Gram-positive bacteria in the genera *Vagococcus* sp., *Bacillus* sp., *Mycobacterium* sp., *Staphylococcus* sp., *Mycoplasma* sp. and *Clostridia* sp. were also found. Examination by PCR-DGGE and sequencing of the bulk cell pellet after cultivation on IAL, gave a higher number of taxa as compared to extraction and examination of bacterial DNA from fish materials without prior cultivation. This shows the benefit of combining both culture dependent and culture independent methods, when studying the microbiota of marine fish. Several *Vibrio* spp. were found only in gut samples collected from the purse seine, but in all samples including the skin and the gills collected from the RSW tank, indicating microbial contamination by faecal bacteria from the fish under these transport conditions.

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1. Introduction

Since the sustainable harvest of fish stocks has an upper limit, increased food production has to come from a better and knowledge-based maintenance of the fish quality through the production line. The global post harvest loss of fish is estimated to account for at least 10% of captured and cultured fish, where microorganisms are important contributors to quality reduction.

When studying the microbiota of any habitat, cultivation dependent methods have shown to have limitations as a substantial proportion of the organisms will not be able to grow on the media provided (Vartoukian et al., 2010; Broekaert et al., 2011). The PCR-DGGE method is culture independent, and is well recognised worldwide for bacterial dynamics and ecology studies. Numerous habitats have been examined e.g. soil (Edenborn and Sexstone, 2007), lakes (Øvreås et al., 1997; Wu et al., 2009), seawaters and petroleum reservoirs (Kaster et al., 2009). Several foods have also been examined, such as blue-veined cheese (Flórez and Mayo, 2006) and fermented sausages (Cocolin et al., 2001) to follow changes during preservation, as well as fish (Hovda et al., 2007b,c) and meat (Russo et al., 2006) with focus on spoilage organisms.

Previous studies of the fish microbiota combining methods including cultivation and DNA analysis have been conducted on some species from different parts of the world, but none of these studies have examined Atlantic mackerel. Cahill (1990) and Austin (2006) have both published reviews on fish microbiology, gathering results from several studies. Most of these studies were conducted on wild or farmed fish species of both fresh and marine waters as tilapia, salmonids and flatfish. According to these authors, the bacterial biota of marine fish seems to be dominated by Gram negative rod formed taxa e.g. *Photobacterium* sp., *Vibrio* sp., *Pseudomonas* sp., *Acinetobacter* sp, and *Carnobacterium* sp., and some Gram-positive bacteria as lactic acid bacteria, *Bacillus* sp. and *Staphylococcus* sp. Several authors have conducted examinations on processed fish products and the effect of preservation methods on the general microbial community (Rudi et al., 2004; Hovda et al., 2007a,b,c) or the Specific Spoilage Bacteria (SSB) e.g. *Shewanella putrefaciens* and *Photobacterium phosphoreum* (Gram and Huss, 1996; Gram and Dalgaard, 2002; Broekaert et al., 2011). Wilson et al. (2008) examined the microbiota at the epidermal mucosa of wild Atlantic cod (*Gadus morhua*) collected onboard a fishing vessel, and described a diverse consortium of bacteria where the γ-proteobacteria and *Cytophaga*–

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doi:10.1016/j.ijfoodmicro.2011.08.016

Flavobacter–Bacteroides species dominated. Even though several publications as exemplified above deal with the microbiota of fish, not much have been published on Atlantic pelagic fish.

The objective for this study was to describe the microbiota of Atlantic mackerel (*Scomber scombrus*) by the combined application of conventional microbiological methods that are culture dependent, and culture independent molecular methods based on the bacterial 16S rRNA gene found associated with fish. In order to examine possible effects on the microbiota of the catching and storage conditions onboard the fishing vessel, samples from gills, skin and the gut contents were collected from fish withdrawn from the purse seine and the RSW tank. To increase the relevance of the findings, all samples were collected during a commercial catch situation.

2. Materials and methods

2.1. Sample collection

Atlantic mackerel (*S. scombrus*) were caught by a purse seiner under authentic commercial conditions in the Norwegian Sea (61°15′N/02°03′E) in October, 2009. After the mackerel had been encircled by the purse seine the fish were transferred to an onboard storage tank containing Refrigerated Sea Water (RSW) at a temperature of −1.5 °C, by a high velocity pumping device. Four fish were collected directly from the purse seine before the pumping to the RSW tank, and four fish were collected from the RSW tank 12 h after capture. All fish were collected with a sterile bucket with a rope and further treatments were done under sterile conditions. From each fish two sets of 1.0 g samples of gills, skin and gut contents, were collected. Gill filaments were cut from the gill arches (3.–7. visceral arch) by sterile scissors, whereas 10 cm^2 of skin was collected by sterile scalpel and tweezers. The gut contents were collected by squeezing the content from the fore gut towards and out the hind gut with sterile tweezers.

2.2. Conventional cultivation and phenotypic characterisation

For quantitative analysis one sample set from each material were homogenised with 9.0 ml sterile peptone water by mixing with a hand held mixer (Polytron PT 1200 Cl) immediately after sample withdrawal. The mixer was thoroughly cleaned in tap water, 70% ethanol and finally in sterile water. An additional tenfold dilution was made with 1 ml aliquots and sterile peptone water. Appropriate dilutions were spread on the surface of Iron Agar Lyngby (IAL) (Oxoid CM0964, Basingstoke, United Kingdom) added 1% Sodium Chloride (Merck KGaA 1.06404.0500, Darmstadt, Germany) and 0.04% L-Cysteine (Sigma-Aldrich C7352-100 G, St. Louis, USA), as recommended by Gram (1992) for cultivation of bacteria from fish in temperate waters. All inoculated IAL plates were incubated for 72 ± 6 h at 20.0 ± 1.0 °C under aerobic conditions. To include any anaerobic bacteria that may be present in the fish gut (Cahill, 1990), a dilution series from the gut content samples were spread on IAL plates and incubated anaerobically in an anaerobic jar (Oxoid, Basingstoke, United Kingdom) in accordance with the recommendations provided by the producer. All colonies were counted to express the number of culturable bacteria (plate counts) present in the samples, whereas black colonies were enumerated as H_2S producing Specific Spoilage Bacteria (SSB). Differently appearing colonies were isolated and cultured, including 11 colonies from gills, 12 from skin and 15 from gut samples from fish collected from the purse seine. From fish collected in the RSW tank, 13 colonies from gills, 12 from skin and 16 from gut samples were isolated. Pure culture isolates were obtained after three times transfer to IAL plates and incubation for 72 ± 6 h at 20.0 ± 1 °C. Pure isolates were stored in the Microbank™ system (Pro-Lab Diagnostics, Richmond Hill, Canada) at −80 °C until molecular analysis and are further referred to as “pure culture samples”.

Isolates were analysed for cell morphology by microscopy (Olympus BX40), Gram reaction (Buck, 1982), oxidase reaction (Becton, Dickinson and Company, ref 231746, BBL™ DRYSLIDE™) and catalase production. Isolates were assessed for haemolytic activity on TSS blood agar (Biomerieux 50300, Marcy l'Etoile, France). Based on these preliminary tests, each isolate was examined by appropriate variants of the commercially available biochemical identification kit API® (Biomerieux, Marcy l'Etoile, France). The Gram-negative and oxidase negative isolates were tested on API® 20 E, whereas the oxidase positives were tested on API® 20 NE. The Gram-positive and catalase positive isolates were tested on API® 50CHB. Because of the temperature requirements of marine psychrotrophic bacteria, the API® system was incubated at 20 °C for 72 h. If growth were not satisfactory after 72 h, the API® strips were incubated again for a maximum of 120 h. The extended incubation time was to countermeasure the lowered temperature. All isolates were identified using the database provided by the manufacturer.

The bacterial population from IAL plates were gathered and washed in Phosphate Buffered Saline (one tablet Phosphate Buffered Saline, Sigma-Aldrich P4417-100TAB, St. Louis, USA, dissolved in 200 ml MilliQ H_2O) and frozen at −20 °C. These samples are referred to by the term “bulk cell samples” as previously applied by (Ercolini et al., 2001; Ercolini, 2004).

2.3. Extracting bacterial DNA from fish material

One sample set of gill, skin and gut contents were diluted in a proportion of 1:10, followed by homogenisation using a handheld mixer for 2 min. The mixer was cleaned in between samples by tap water, 70% ethanol and finally sterile water. The homogenates were then centrifuged to sediment the solid fish materials whilst the bacteria that were present would remain in the supernatants, in accordance to the principals described by Rudi et al. (2004). These samples are referred to as “direct DNA samples”.

2.4. DNA purification

DNA purification was performed on “bulk cell samples”, “pure culture samples”, “direct DNA samples” and reference strains (*S. putrefaciens* (CCUG 13452) and *P. phosphoreum* (CCUG 16288)) by using the commercially available DNeasy® Blood and Tissue Kit (Qiagen 69504, Hilden, Germany) according to the supplied protocol. All samples were pre-treated with lysozyme buffer for 30 min at 37 °C, to ensure that the DNA from the Gram-positive bacteria was available for purification. The DNA outcome and the purity of the samples were measured by the NanoDrop™ 1000 spectrophotometer (Thermo Scientific).

2.5. PCR protocol

The hyper variable V3 region of the 16 rRNA gene was the target region for amplification for all samples. Universal primers for the domain Bacteria were used including the forward primer BA338f (5′ACT CCT ACG GGA GGC AGC AG 3′) that contain a 40 base pairs long GC-clamp at the 5′ end and the reverse primer UN518r (5′ATT ACC GCG GCT GCT GG 3′) (Øvreås et al., 1997) resulting in fragments of approximately 200 bp. Aliquots of 100 ng DNA were used as templates, and a PCR reaction was performed on 50 µl containing the final concentration of 0.2 µM of each primer (MWG Biothech AS 11-1032, Ebersberg, Germany), 0.2 mM of each dNTP (Roche 1969064, Oslo, Norway), 1 U of Platinum® *Taq* Polymerase High Fidelity, 1 × High Fidelity Buffer and 2 mM $MgSO_4$ (Invitrogen 11304-011, Parsley, United Kingdom). Each sample was run on a GeneAmp® PCR System 9700 (Applied Biosystems, Life Technologies™, California, USA) for 2 min at 94 °C, and further 30 cycles for 30 s at 94 °C, 30 s at 54 °C and 1 min at 68 °C. The reaction was terminated by a 7 min long extension step at 68 °C. The PCR product length were verified by a

1.5% analytic agarose gel (Invitrogen 16500-100, Parsley, United Kingdom) stained by GelRed (Biotium 41003-0.5ml, Hayward, USA) and visualised in UV illuminator (Bio-Rad Laboratories GelDoc 2000, Oslo, Norway).

2.6. DGGE analysis

The PCR products were analysed by the vertical dual heater system DGGE-TV400 unit (Scie-Plas, Cambridge, United Kingdom) and Denaturing Gradient Gel Electrophoresis (DGGE) were performed on 10 µl templates of PCR products loaded in a 1.0 mm thick 8% polyacrylamide gel with a denaturing gradient from 30 to 55%. The 100% denaturant solution stock contained 40% deionised formamide (Sigma-Aldreich F9037-100ml, St. Louis, USA) and 7M Urea (Bio-Rad Laboratories 161-0730, Oslo, Norway). Electrophoresis was performed in 0.5×TAE (40 mM Tris-Acetate and 1 mM EDTA pH 8.3) for 10 min at 20 V to focus samples, and further for 18 h at 70 V. The gel was stained by Sybr®Gold (Invitrogen S-11494, Parsley, United Kingdom) in the dark for 1 h before visualisation of the fragments under UV illumination. The number of bands in each sample was counted as a measurement of band diversity.

2.7. Sequencing of DGGE fragments

From "bulk cell samples" and "direct DNA samples", unique bands that appeared in the gel under UV light were excised using a sterile scalpel and transferred into tubes containing 30 µl of MilliQ H_2O. Similar positioned bands in different samples were registered. From "pure isolate samples", one strong band from each isolate was excised as described above. All excised fragments eluted into the solution during incubation for 30 min at 37 °C. From the eluate, 5 µl of template was used in a re-amplification before the bands were re-run on DGGE under identical conditions as earlier described. This was done to ensure the purity of the fragments, and was repeated until each band migrated as a single band to the original position. Pure DNA fragments was re-amplified by PCR and purified for gel residues and other undesirables by the commercially available E.Z.N.A.™ Cycle-Pure Kit (Omega Bio-Tek, Inc., Georgia, USA) according to the supplied protocol from the producer. Sequencing was performed by the University of Bergen Sequencing Facility (SeqLab, Bergen, Norway). All fragments were prepared by the Big Dye® Terminator v3.1 Cycle Sequencing Kit according to protocol, in advance of sequencing by an ABI 3730 xl DNA Analyzer (Applied Biosystems, Warrington, UK). Both forward and reverse fragments were analysed using the same primers as for PCR. Obtained sequences were searched for in the GenBank library BLAST to find the closest relative for the partial 16S rRNA gene (Altschul et al., 1990). Sequence match above 97% similarity was recognised as the same (Venter et al., 2004).

3. Results and discussion

Samples from gills, skin and gut contents were collected from Atlantic mackerel harvested directly from the purse seine and additionally from the RSW tank to characterise the bacterial biota, as well as to describe possible changes in the biota during these two initial steps in the production chain. Both the conventional cultivation based microbiological analyses and the molecular PCR-DGGE analysis were performed. Bacterial DNA was extracted directly from fish materials without a prior cultivation step, and from bulk cells and bacterial isolates after cultivation on IAL.

3.1. Conventional quantification

The suitability of IAL as the agar for cultivation of the microbiota and the SSB population on marine fish from cold waters have previously been recommended by several authors (Gram et al., 1987; Gram, 1992; Hovda et al., 2007b). The average plate counts and the count of SSB for each material and sampling site are shown in Table 1.

An increase in plate counts of gills and skin samples was observed when comparing samples collected from the purse seine to those collected from the RSW tank. The microbial numbers in gut content samples were rather constant. If some of the gut contents were squeezed out during pumping, the average CFU/g of gut contents would not be affected as long as some gut contents were left for sampling, however, mixing of the gut contents with the RSW storage water would affect the CFU/g of gills and skin. The increased number of CFU/g in samples of gills and skin in samples from the RSW tanks could indicate that these tissues were contaminated by gut material. Samples collected from skin material gave the lowest plate count. Similarly to what was found for the general plate counts, there was an increase in the SSB when comparing samples collected from the purse seine to those collected from the RSW tank. This observation also supports the thesis that the transportation step from the purse seine to the RSW tank may cause contamination by the faeces of the fish. The absence of SSB in the anaerobically incubated gut content samples collected from the RSW tank compared to the high numbers from those collected from the purse seine is quite significant. This result indicates that the bacterial biota of the gut changes from the proximal towards the distal part of the gut, and that the outer part is highly populated by SSB. *S. putrefaciens* was found in all samples from both the purse seine and from the RSW tank, as examined by the DGGE analysis. *S. putrefaciens* is a facultative anaerobic bacterium able to produce H_2S and identified as an important contributor to spoilage of chilled fish shown (Ringø et al., 1984; Semple and Westlake, 1987; Gram, 1992).

Some studies have shown that the correlations between the plate counts of fish and fish spoilage are insignificant since the non-SSB, i.e. white colonies on IAL plates, contributes with little or not to spoilage. On the other hand, the number of black colonies on IAL correlates closely to the degree of fish spoilage, as most SSB associated with chilled fish are found to produce H_2S (Gram et al., 1987; Jørgensen and Huss, 1989; Tryfinopoulou et al., 2007). Broekaert et al. (2011) found, however, that one of the known SSB in fish, *Brochothrix thermospacta*, was unable to grow on IAL. The detection of SSB in samples collected from fish in the purse seine suggests that they are a part of the indigenous bacterial biota of the fish.

3.2. Phenotypic identification of "pure culture samples"

A total of 79 pure cultures recovered from IAL were phenotypically described by microscopy, Gram reaction, oxidase reaction, catalase production, haemolytic activity on blood agar and biochemical reactions in appropriate kit API®. Amongst these strains, 69 isolates were found to be Gram-negative rod formed bacteria. The rest four Gram-negative isolates were cocci- or spiral shaped, whilst the six Gram-positive isolates were mainly rod formed bacteria. These observations correspond to previous studies where Gram-negative rod shaped bacteria are found to dominate the biota (Cahill, 1990; Austin, 2006; Broekaert et al., 2011).

Table 1
The average of CFU/g presented in log ± SD for plate counts and SSB in samples collected from gills, skin and gut contents from fish withdrawn from the purse seine and the RSW tank. n = 4 for each sampling site. All samples were incubated for 72 h at 20 °C under aerobic conditions. An additional sample set of the gut contents was incubated anaerobically.

		Gills	Skin	Gut contents	Gut contents anaerobe
Plate counts	Purse seine	3.7 ± 0.6	2.4 ± 0.4	6.2 ± 0.6	6.4 ± 0.7
	RSW tank	4.8 ± 0.2	3.3 ± 1.3	6.2 ± 0.4	7.0 ± 0.5
SSB	Purse seine	1.9 ± 0.5	2.6 ± 0.1	3.9 ± 0.6	4.2 ± 0.8
	RSW tank	3.6 ± 0.2	3.0 ± 1.2	4.3 ± 0.5	1

Table 2
The closest relative for sequences obtained from "bulk cell samples" and "direct DNA samples" in BLAST GenBank. Similarity (%) and accession no.

Band no.	Closest relative in BLAST	Similarity (%)	Accession no.
1	*Psychrobacter immobilis*	100	AJ309942
2	*Shewanella* sp.	97	AY050528
3	*Psychrobacter* sp.	96	AY573040
4	*Shewanella* sp.	98	AY050528
5	*Oceanisphaera* sp.	100	FN377705
6	*Shewanella putrefaciens*	100	HM10335
7	*Psychrobacter* sp.	100	GQ370385
8	*Psychrobacter* sp.	100	GQ370385
9	*Vagococcus carniphilus*	100	NR025689
10	*Psychrobacter* sp.	100	GU932625
11	*Proteus vulgaris*	100	FJ799903
12	*Proteus vulgaris*	98	GU361619
13	*Proteus* sp.	95	GQ383895
14	*Psychrobacter* sp.	100	GU574735
15	Uncultured *Photobacterium* sp.	100	AM933561
16	Uncultured *Vibrio* sp.	100	FN646711
17	*Mycobacterium* sp.	97	HM022198
18	Uncultured bacterium clone	100	HM345507
19	*Psychrobacter immobilis*	100	AJ309942
20	*Shewanella* sp.	97	AY050528
21	*Psychrobacter* sp.	96	AY573040
22	*Psychrobacter* sp.	97	GQ200528
23	*Vagococcus* sp.	100	FJ514032
24	*Shewanella* sp.	98	AY050528
25	*Shewanella* sp.	98	AY050528
26	*Shewanella putrefaciens*	100	HM103350
27	*Vagococcus carniphilus*	100	NR025689
28	*Vagococcus carniphilus*	100	NR025689
29	*Shewanella putrefaciens*	100	HM103350
30	*Vibrio* sp.	98	GU826597
31	*Vibrio* sp.	98	GU826597
32	*Vibrio* sp.	98	GU826597
33	*Mycoplasma iguanae*	97	EU859973
34	Uncultured γ-proteobacterium	97	EU861205
35	*Mycoplasma iguanae*	97	EU859973
36	Uncultured γ-proteobacterium	97	EU861205
37	*Staphylococcus sciuri*	100	AM062696
38	*Staphylococcus sciuri*	100	AM062696
39	*Shewanella putrefaciens*	100	HM103350
40	*Synechococcus* sp.	100	FJ763789
41	Uncultured Clostridiales bacterium	100	HM074643
42	Uncultured teleost isolate 18S rRNA gene	100	EU004795
43	*Staphylococcus sciuri*	100	AM062696
44	*Shewanella putrefaciens*	100	HM103350
45	*Synechococcus* sp.	100	FJ763789

About half of the isolates assessed by the API® 20 E and API® 20 NE kits could be identified, whereas none of the Gram-positive isolates tested by the API® 50 CHB could be identified by these systems. Since most of the isolates were not able to grow above 20 °C, the API® kits were incubated at this temperature, but with an extended incubation time. Some isolates were incubated for as long as seven days without achieving proper growth, resulting in a non identifiable profile. Several authors have reported studies were the API® kits are not found optimal for identifying temperate marine bacteria (Hansen and Sørheim, 1991; Hovda et al., 2007a), however these systems are well established and provides a methodological tool that is generally recognised for bacterial determination, especially for pathogens of human health concern.

In addition to the API® identification, bacterial DNA from each pure culture were also analysed by PCR-DGGE and sequencing. Most of the 33 cultures were identified by the API® and BLAST as belonging to the same phylum and subphylum. Amongst these, 15 isolates were identified with similar results and identified to either genus or species level. However, five of the isolates that were identified by API® as *Empedobacter brevis* were identified by BLAST as *Pseudoalteromonas* sp. One isolate that was identified as *Proteus vulgaris* by API®, was identified by the sequence as either *Bizionia* sp. or *Gelidibacter* sp., both found in the phylum Bacteroidetes.

3.3. Bacterial profile by PCR-DGGE

All DNA samples were amplified by universal primers targeting the hyper variable V3 region of the 16S rRNA gene (Hovda et al.,

Table 3
The closest relative for sequences obtained from "pure culture samples" in BLAST GenBank. Similarity (%) and accession no.

Band no.	Closest relative in BLAST	Similarity%	Accession no.
1	*Bizionia paragorgiae*	99	HQ538734
2	*Proteus vulgaris*	98	FJ799903
3	*Proteus vulgaris*	100	FJ799903
4	*Proteus vulgaris*	100	AY880201
5	*Shewanella* sp.	98	GU371694
7	*Shewanella* sp.	99	GU371694
8	*Proteus vulgaris*	98	FJ799903
9	*Proteus vulgaris*	98	FJ799903
10	Flavobacteriaceae bacterium	97	FJ348469
11	*Proteus vulgaris*	98	FJ799903
12	*Pseudoalteromonas tetraodonis*	100	DQ520896
13	*Pseudoalteromonas* sp.	99	EU137104
14	*Proteus vulgaris*	98	HQ640434
15	*Photobacterium phosphoreum*	98	AY780009
16	*Bizionia* sp.	99	DQ873781
17	*Bizionia* sp.	97	HQ727230
18	*Proteus vulgaris*	98	FJ799903
19	*Vibrio splendidus*	98	AJ515225
20	*Vibrio splendidus*	98	HQ694833
21	*Proteus vulgaris*	98	FJ799903
22	*Proteus vulgaris*	100	FJ799903
23	*Proteus vulgaris*	99	AY880201
24	*Proteus vulgaris*	98	FJ799903
25	*Proteus vulgaris*	99	AY880201
26	*Proteus vulgaris*	99	HQ640434
27	*Vibrio splendidus*	97	AJ515225
28	*Proteus vulgaris*	98	FJ799903
29	*Vibrio* sp.	98	FN645429
30	*Proteus vulgaris*	100	FJ799903
31	*Vibrio splendidus*	98	AJ515225
32	*Pseudoalteromonas tetraodonis*	99	EU330363
33	*Pseudoalteromonas* sp.	97	HQ882794
34	*Pseudoalteromonas tetraodonis*	100	EU330363
35	*Shewanella* sp.	100	AB543434
36	*Pseudoalteromonas* sp.	96	HQ882794
37	*Vibrio kanaloae*	97	HM584064
38	*Psychrobacter marincola*	99	EU652050
39	*Shewanella* sp.	98	DQ492736
40	*Proteus vulgaris*	99	FJ799903
41	*Psychrobacter* sp.	98	HQ836467
42	*Vibrio kanaloae*	98	HM584064
43	*Vibrio kanaloae*	97	HM584064
44	*Pseudoalteromonas tetraodonis*	98	DQ520896
45	*Shewanella* sp.	99	GU584178
46	*Shewanella* sp.	99	DQ060405
47	*Psychrobacter cibarius*	100	HQ98586
48	*Psychrobacter* sp.	99	HQ693280
49	*Vibrio* sp.	98	FN645427
50	*Vibrio* sp.	98	GU225808
51	*Vibrio pomeroyi*	98	HM584085
52	*Oceanisphaera* sp.	100	HM566011
53	*Shewanella* sp.	100	AB543434
54	No match		
55	*Vibrio splendidus*	99	AJ515225
56	*Pseudoalteromonas* sp.	100	HQ882794
57	*Pseudoalteromonas* sp.	99	HQ882794
58	*Oceanisphaera* sp.	98	AB518935
59	*Pseudomonas* sp.	98	FJ161242
60	*Shewanella* sp.	99	GU371694
61	*Proteus vulgaris*	98	FJ799903
62	*Pseudomonas* sp.	98	FJ161242
63	*Psychrobacter faecalis*	99	FR749849
64	*Bacillus* sp.	100	HQ711450

2007b). The primer set used during this study have previously been used in similar PCR-DGGE studies (Øvreås et al., 1997). Fragments with different sequences were separated by DGGE. Samples were analysed, rerun until single fragments migrated to the original position as one band, and pure excised fragments were sequenced and subsequently identified by BLAST. Amongst the "bulk cell samples" and the "direct DNA samples" 34 and 11 unique bands were identified, respectively, whereas 62 of the "pure culture isolates" were identified. The number of identified bands from the "bulk cell samples" was higher than the comparable number from the "direct DNA samples". This was not as expected, as extracting bacterial DNA directly from the fish material would include both culturable and non-culturable bacteria present in the samples. The differences in the obtained results were most likely connected to the homogenisation of the samples material before centrifugation and collection of fish material. The skin samples were in particular difficult to homogenise and adherent bacteria might have been settled and discharged together with the fish material during centrifugation. The cultivation based approach had no centrifugation step and the effect of material bound bacteria would not be as clearly expressed. Several authors have addressed the homogenisation step of foods as giving a crucial bias possibly resulting in low DNA yield (Ercolini, 2004; Cocolin et al., 2007). In PCR-DGGE analysis of sausages, the detection limit of individual bacterial members in mixed samples was found to be over 10^4 CFU/g (Cocolin et al., 2001). This could be similar for fish products, and if so, all plate counts were close to detection limit possibly explaining the low yield of "direct DNA samples". If during this study, only molecular based methods without cultivation were performed, important information about the bacterial biota would have been excluded.

Tables 2 and 3 shows all the identified bands, their similarity and accession no. in BLAST. A total of 32 different taxa were found and Table 4. shows in which samples the different taxa were present.

The bacterial profile of Atlantic mackerel based on PCR-DGGE analysis was dominated by Gram-negative bacteria i.e. *Psychrobacter* sp., *P. immobilis*, *P. marincola*, *P. cibarius*, *P. faecalis*, *Proteus* sp., *P. vulgaris*, *Photobacterium* sp., *P. phosphoreum*, *Vibrio* sp., *V. kanaloae*, *V. splendidus*, *V. pomeroyi*, *Shewanella* sp., *S. putrefaciens*, *Oceanisphaera* sp., Flavobacteriaceae, *Bizonia* sp., *B. paragorgiae*, *Pseudoalteromonas* sp., *P. tetradonis*, *Synechococcus* sp. and γ-proteobacteria. Similar results have previously been found for marine fish species, seafood or sea waters (Gjerde, 1976; Juni and Heym, 1986; Waterbury and Rippka, 1989; Gram and Huss, 1996; Spanggaard et al., 2000; Gram and Dalgaard, 2002; Romanenko et al., 2002; Austin, 2006; Waterbury, 2006; Wilson et al., 2008; Yu et al., 2009; Srinivas et al., 2011). Additionally the bands that were identified as Gram-positive Clostridiales, *Vagococcus* sp., *Vagococcus carniphilus*, *Bacillus* sp. and *Mycobacterium* sp. have also previously been found in marine samples (Matches et al., 1974; Huss and Pedersen, 1979; González et al., 2000; Hartmans et al., 2006; Françoise, 2010). *Staphylococcus sciuri* has previously been isolated from terrestrial animals (Stepanovic et al., 2001), whereas the Gram-positive related *Mycoplasma iguana* have been isolated from the green iguana (*Iguana iguana*) (Brown et al., 2006; Razin, 2006). One band was identified as an uncultured bacterial clone (Kong et al., 2010). Some bands matched the teleost 18S rRNA gene, and previous studies have found that this

Table 4
The different obtained taxa and in which samples they were present. The results are organised into their known Gram classifications.

Sequence result		Viable DNA samples								Direct DNA samples						Pure culture samples					
		Purse seine				RSW tank				Purse seine			RSW tank			Purse seine			RSW tank		
		Gills	Skin	Gut	Gut[a]	Gills	Skin	Gut	Gut[a]	Gills	Skin	Gut	Gills	Skin	Gut	Gills	Skin	Gut	Gills	Skin	Gut
Gram −	*Psychrobacter immobilis*			x		x		x	x												
	Psychrobacter sp.	x	x	x		x	x	x										x			
	Psychrobacter marincola																		x		
	Psychrobacter cibarius																			x	
	Psychrobacter faecalis															x					
	Proteus sp.	x	x	x		x															
	Proteus vulgaris	x	x	x		x										x	x	x	x		x
	Uncultured *Photobacterium* sp.	x	x	x																	
	Photobacterium phosphoreum																			x	
	Vibrio sp. W208			x	x	x	x	x	x									x			x
	Vibrio kanaloae															x	x				
	Vibrio splendidus																	x		x	x
	Vibrio pomeroyi																	x			
	Shewanella sp. SIGA172a			x	x		x	x	x							x		x	x		x
	Shewanella putrefaciens	x	x	x	x	x	x	x	x	x		x	x		x						
	Oceanisphaera sp. V1-41			x	x	x												x			x
	Uncultured γ-proteobacterium						x			x											
	Bizonia paragorgiae															x					
	Bizonia sp.																x			x	
	Psaudoalteromonas tetradonis															x	x			x	
	Psaudoalteromonas sp.															x	x				x
	Synechococcus sp.									x		x	x		x						
Gram +	Clostridiales bacterium											x									
	Bacillus sp.																x				
	Vagococcus sp. H2914				x				x												
	Vagococcus carniphilus strain	x	x	x		x	x	x	x												
	Staphylococcus sciuri									x	x	x		x	x						
	Mycoplasma iguanae						x			x											
	Mycobacterium sp. FI-09129					x															
	Uncultured bacterium clone	x	x	x		x															
	Uncultured teleost isolate 18S rRNA gene												x	x	x						

[a] Were incubated anaerobically.

universal primer set could co-amplify parts of the eukaryotic 18S rRNA gene giving rise to bands that represents either the animal in question or any consumed animal or plant material (Jensen et al., 2004; Hovda et al., 2007a,b).

Considerable attention has been paid to potential human pathogens in and on fish, including members of the geni *Vibrio*, *Mycoplasma* and *Mycobacterium* (Austin, 2006). The genus *Vibrio* includes 12 species that have so far been reported to be pathogenic to humans (West, 1989; Oliver and Kaper, 2001; Farmer and Hickman-Brenner, 2006). These bacteria are common in marine and estuarine environments, and on the surfaces of marine plants and animals (Baumann et al., 1984). In the present study several isolates were found to belong to the genus *Vibrio*, however no human pathogenic *Vibrio* was identified to species level. Several members of the genus *Mycoplasma*, class Mollicutes, are found as pathogens of human, animals and plants (Razin, 2006). The mollicute sequence found in this study matched 100% to *Mycoplasma iguanae* and were distinguished from other *Mycoplasma* members by Brown et al. (2006). Several mycobacteria are known as pathogens of animal and humans. *M. marinum*, *M. fortuitum* and *M. chelonae* are commonly associated with piscine tuberculosis, which have been reported in more than 150 fish species. These bacteria are also known to infect humans (Decostere et al., 2004; Piersimoni and Scarparo, 2009). In Atlantic mackerel we detected a *Mycobacterium* sp. not identified to the species level.

3.4. Effect of handling on fish quality

The bacterial biota in samples from gills, skin and gut contents from fish from two sites, the purse seine and the RSW tank, was examined to see possible differences in the bacterial biota before and after pumping. The increase in the number of bands in both the "bulk cell samples" and the "direct DNA samples", concerning gills and skin samples collected from the RSW tank, indicates that there was a higher bacterial diversity in the samples collected from the RSW tank. The different *Vibrio* spp., with exception of *Vibrio kanaloae*, that were only found in gut samples collected from the purse seine but in all samples collected from the RSW tank, reveal that the bacterial increase was caused by contaminating faeces from the fish itself. This also supports the theory that the contamination occurs somewhere during the handling step where the fish are pumped onboard into the RSW tank. If this is the case, the fish that were delivered to the processing plant, had already been contaminated before landing possibly reducing the fish quality and thus affecting the remaining shelf life.

4. Conclusion

This study shows that the bacterial biota was dominated by Gram-negative taxa. It did also reveal that there was a difference in the bacterial biota when comparing fish samples from the purse seine to those collected from the RSW tank. The increase in plate counts and number of SSB in samples from the RSW tank support the hypothesis that the fish is being contaminated during the transfer from the purse seine into the RSW tank. During handling the fish are pumped onboard at high velocity and the pressure and the density may cause the gut contents of the fish to get squeezed out and contaminate the fish surface. Further typing of isolated strains would give a stronger evidence for this hypothesis, however it is supported by the observation that members of the genera *Vibrio* were only found in the gut samples from the purse seine fish, but in all examined fish tissues of the RSW tank samples.

Acknowledgements

The authors want to thank the technical staff at NIFES for excellent support.

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