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Biodiversity and dynamics of the bacterial community of packaged king scallop (*Pecten maximus*) meat during cold storage

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Abstract:

The microbial biodiversity and dynamics of king scallops meat and coral during cold storage (cold chain rupture: 1/3 storage time at 4 °C followed by 2/3 at 8 °C), was assessed by combining culture-dependant and -independent methods. Products were packaged as follows: aerobic, vacuum packed and 3 different CO_2/N_2 modified atmospheres and the impact of these conditions on the microbial communities was assessed. Results indicated that under air (current packaging condition), the dominant species corresponded to *Brochothrix thermosphacta*, *Pseudomonas* spp. and *Shewanella* spp. These species have regularly been associated in the literature with food (especially seafood), and product spoilage. *Moellerella wisconsensis* was the only species detected on VRBG medium, however, its impact on the food product is unclear. Packaging conditions influenced the ecosystem equilibrium and biodiversity. Except for day 8, the lowest counts for all studied flora were observed for modified atmosphere packaging (MAP) containing >80% CO_2. Moreover, in these conditions, higher biodiversity by Temporal Temperature Gradient Gel Electrophoresis (TTGE) and the non-detection of specific flora (i.e. *Pseudoalteromonas haloplanktis*) were observed. At day 8, scallops packaged using these conditions were still acceptable from a sensorial point of view (odour), although the initial load of the king scallop was high (total psychrotrophic flora reached 5.5 log CFU/g).

Highlights

► High microbial biodiversity associated with king scallops during storage (at least 26 species). ► Impact of MAP on flora equilibrium especially at high CO_2 concentrations. ► At day 8, scallops packaged with high CO_2 concentrations were still acceptable from a sensorial point of view.

Keywords: King scallop ; Microbial biodiversity ; Modified atmosphere packaging (MAP) ; TTGE

1. Introduction

The king scallop *Pecten maximus* is a valuable bivalve mollusc as it is a highly appreciated food product, especially in France ("coquilles Saint-Jacques"). France produces around 25 000 tons of king scallops per year (FAO, 2006), a significant portion originate from the Normandy region. A large quantity of scallops is also imported to France and mainly originates from Scotland and North America (*Pecten maximus* and *Placopecten magellanicus*, respectively). In the context of new food consumption habits, in particular, ready-to-eat foods, a larger proportion of this bivalve mollusc production is sold shelled. King scallop meat is sold either frozen or fresh. As for other seafood products, shelf-life during cold storage is limited; thus, the study of alternative packaging or storage conditions is important.

Like other seafood products, scallop quality during packaging and storage is associated with biochemical and sensorial changes that are mainly affected by storage temperature (Ehira and Uchiyama, 1987; Kawashima and Yamanaka, 1992) and the development of microbial flora that can lead to spoilage (Ocaño-Higuera et al., 2006). Several methods have been used to evaluate the freshness of fish and shellfish. These methods are based on measuring chemical, physical and microbiological changes (Luong et al., 1991; Ohashi, 1991; Olafsdottir et al., 1997). The main indices used in relation to fish spoilage (Botta, 1995; Hebbard et al., 1982) correspond to trimethylamine nitrogen (TMA) and total volatile basic nitrogen (TVBN), as well as sensory changes; however, there is very limited data based on the changes observed during mollusc's spoilage and especially king scallops. Indeed, studies have rather been carried out on alterations of closely related species including sea scallops (*Placopecten magellanicus*) (Hilts and Dyer, 1970), queen scallops (*Chlamys opercularis*) (Thomson et al., 1974), yesso scallops (*Patinopecten yessoensis*) (Kawashima and Yamanaka, 1992) and lion-paw scallops (*Nodipecten subnodosus*) (Ocaño-Higuera et al., 2006) or have been based on the characterization of bacterial communities during the early

life stages of scallops that may have an impact on the survival of bivalve larvae (Lane et al., 1985; Nicolas et al., 1996; Sandaa et al., 2003; Torkildesen et al., 2005).

Studies based on the impact of packaging conditions on bivalve mollusc spoilage during cold storage are rare. Ruiz-Capillas et al. (2001) compared the biochemical and sensory changes of frozen king scallops that were thawed and stored at 4° C in melting ice to samples wrapped in aluminium foil and cling film. Only few differences were observed between aluminium foil and cling film while the levels TMA and TVBN were low due to the leaching effect of the ice meltwater. Kimura et al. (2000) showed that storage at 5°C of scallop adductor muscle packaged under air or various O_2 and CO_2 atmospheres ranging from 20 to 40% had an impact on the bacteria found in the samples (inhibition from 20% CO_2) and that 100% O_2 allowed to prolong the shelf life by nearly two days in comparison to aerobic conditions.

Finally, the microflora associated with king scallops and their respective spoilage potential as well as the impact of packaging on the microbial diversity is not well documented. Thomson et al. (1974) indicated that the spoilage flora of the queen scallop (*Chlamys opercularis*) was similar to spoilage flora found in fish. While Llanos et al. (2002) showed that in the Peruvian scallop (*Argopecten purpuratus*), the initial flora was associated with culture conditions, farming localization and the feed used. Moreover, this flora would have a direct impact on spoilage. Bremner and Statham (1983) showed that *Vibrio* spp. were the dominant flora and persisted in vacuum packed *Pecten alba* scallops.

This study aimed at evaluating the microbial diversity of French king scallops meat and coral during cold storage and the impact of various packaging conditions on the bacterial ecosystem. The different modified atmosphere conditions were selected to test for their potential inhibiting properties towards spoilage bacteria at temperatures encountered by the consumer during food storage. Indeed, practical industrial studies tend to suggest that TVBN

content is lower in CO_2 rich environments for some seafood products. Both microbiological and molecular tools were used to follow and identify the bacterial communities during cold storage. To our knowledge, this is the first study on the bacterial community of king scallops using microbial and molecular tools.

2. Materials and Methods

2.1 Sample preparation and bacterial numerations

King scallops (*Pecten maximus*) harvested in the Basse-Normandie region were obtained just after shelling (meat and coral) on ice. They were then placed into trays (6 King scallop meats with coral per tray) and packaged under different conditions using a Multivac Galaxy TS 355 semi-automatic tray sealer (Multivac, France) with 150µm thick polyethylene film. A total of 5 different modified atmosphere packaging conditions were tested for each sampling date (day D1, D4, D6 and D8) and corresponded to air conditions, vacuum packed and three different modified atmospheres (50% CO_2 / 50% N_2, 80% CO_2 / 20% N_2, 100% CO_2). A 3:1 ratio of modified atmosphere to scallops was obtained in the sealed trays. The different modified atmosphere conditions were selected to test for their potential inhibiting properties towards spoilage bacteria at temperatures encountered by the consumer during food storage.

A cold chain rupture, 1/3 storage time at 4°C and 2/3 at 8°C, was applied to each sample to mimic conditions encountered by the consumer during cold storage according to the guidelines given for best before date determinations (Norme NF V01-003). Microbial sampling was performed in triplicate. For each sampling date and packaging condition, the 6 King scallop meats per tray were separated into 3 samples (2 meats and coral) to obtain 3 sample repetitions. Then, for each repetition, 2.5 g of each meat and 2.5 g of each coral,

121 corresponding to 10 g in total per repetition, were placed in a sterile stomacher bag and
122 homogenized for 2 min in 90 ml tryptone salt buffer using a stomacher (AES laboratories,
123 France). In order to numerate total aerobic flora, homogenates were serially diluted and plated
124 on modified Long and Hammer's medium (van Spreekens, 1974), then, incubated for 5 days
125 at 15°C. For enterobacteria, serial dilutions were plated onto Violet Red Bile Glucose
126 (VRBG) agar (AES, France) and incubated for 48h at 30°C. *Pseudomonas* spp., were plated
127 and enumerated on Cephalosporine Fucidine Cetrimide (CFC) medium (AES, France), lactic
128 acid bacteria (LAB) on De Man Rogosa Sharpe (MRS, pH 6.4) (AES, France), *Brochothrix*
129 *thermosphacta* colonies were enumerated on Streptomycin Thallous Acetate Actidione
130 (STAA) agar (Oxoid, France) and H_2S producing bacteria on Iron Agar (IA) (tryptone 20 g/L,
131 NaCl 5 g/L, beef extract 3 g/L, yeast extract 3 g/L, ferric citrate 0.3 g/L, sodium thiosulfate
132 0.3 g/L and agar 12 g/L). CFC, MRS, STAA and IA plates were incubated at 25°C for 48 h, 5
133 days, 48 h and 72 h, respectively. For each Petri dish containing $n < 300$ and at each sampling
134 date, the square root of the total number of colonies were selected according to
135 morphological, microscopic and biochemical aspects (sampling was performed according to
136 morphotypes, Gram-staining, catalase and oxidase testing to ensure proper species
137 representation). Overall, 311 isolates were selected for further study using molecular tools. In
138 parallel, the king scallop homogenates used for microbiological analyses were filtered on
139 Minisart 5 µm filters (Sartorius, France) and kept frozen at -20°C for latter use in Temporal
140 Temperature Gel Electrophoresis (TTGE) analyses. Indeed, previous studies performed by
141 our laboratory have shown that there is no impact on TTGE results after freezing samples at -
142 20°C (data not shown).
143
144 *2.2. Culture conditions*

All representative strains were cultivated in tryptic soy broth (TSB, AES,) supplemented with 2.5g/l yeast extract (TSBYE) except LAB which were cultivated in MRS broth (AES, France). All isolates, except LAB, were incubated at 25°C for 24 h under agitation. LAB were incubated at 30°C for 24-48 h. Strains were finally conserved in cryotubes with 30% v/v glycerol at -80°C.

2.3 Isolate M13-PCR grouping and identification

2.3.1 Preparation of template DNA

DNA was extracted from bacterial cultures grown to stationary phase using 1 ml of culture with the NucleoSpin Tissue Kit (Macherey Nagel, France) according to the manufacturer's instructions for bacteria. Purified DNA samples were stored at -20°C.

2.3.2. PCR amplification

For strain grouping, genetic profiles were generated for each isolate as described by Guinebretière and Nguyen-The (2003). Briefly, 1 µl purified DNA (~50 ng) was used for each reaction in the presence of 2.0 µM M13 primer (5'-GAGGGTGGCGGCTCT-3'), 400 µM dNTP in the presence of 1.25U *Taq* polymerase (5 PRIME, Germany). Amplification conditions were as follows: 95°C 5 min, 45 cycles of 95°C 1 min, 36°C 1 min, 72°C 4 min.

For strain identification, amplification and sequencing of a 1533bp 16S rRNA gene fragment was carried out using the universal primers BSF8 and BSR1541 (Edwards et al., 1989) in the presence of ~50 ng DNA, 0.2 µM each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 1U *Taq* polymerase (Invitrogen, France). Amplification conditions were 94°C for 5 min, 30 cycles of 94°C for 45 sec, 59.5°C for 45 sec, 72°C for 2 min with a final extension at 72°C for 5 min. All PCR were performed in a Master Gradient thermocycler (Eppendorf, France).

2.3.3. Gel electrophoresis

Aliquots (9 µl for M13 PCR products and 18 µl for 16S rDNA fragments) of each PCR sample were analyzed using 1.2% (M13-RAPD experiments) or 0.8% (16S rDNA experiments) (wt/vol) agarose gels (Invitrogen, France) in 1X TBE buffer at 130 V for 50 min then visualized with ethidium bromide staining using a GelDoc2000 (BioRad, France).

2.3.4. Genetic profile clustering and sequence analysis

M13-PCR genetic banding patterns were analyzed using Quantity One software (BioRad, France) and BioNumerics fingerprinting software version 5.1 (Applied Maths, Belgium). For sequencing, 16S rDNA amplicons (1533bp) were purified using the GenElute PCR purification kit (Sigma, France) and sent to Eurofins MWG Operon for sequencing (Abersberg, Germany). Alignments were performed using the ClustalX program or Bionumerics software and sequence similarity was determined using BLAST (Altschul et al. 1990) in the GenBank database.

2.4 TTGE gel electrophoresis

2.4.1. Total bacterial DNA extraction.

Filtrates obtained from each sampling homogenate were thawed overnight at 4°C, and then 10 ml of homogenised filtrate were filtered on a Nucleospin Plant column (Macherey-Nagel, France) by centrifugation at 8500 rpm for 10 min. The pellet was resuspended in 400 µl of lysis solution (Tris-HCL20 mM, pH 8.0, EDTA 2 mM, Triton X-100 1.2 %, lysozyme 20 mg/ml, mutanolysin 11.6 U), then transferred in 2 ml microtubes and incubated at 37°C for 1 h. This enzymatic lysis was followed by a mechanical lysis performed by addition of 0.3 g of glass beads (150-200 µm diameters) and agitation for 2 cycles of 2 minutes at 30 Hz in a MM 200 mixer mill (Retsh, Germany). Proteins were digested by proteinase K (20 mg/ml) in

200 µl of DNeasy blood and tissue kit AL buffer (Qiagen, France) and incubation performed at 56°C for 30 min. Glass beads were pelleted by centrifugation at 10 000 rpm for 3 min and the supernatant was then transferred in a 2 ml microtube for DNA precipitation by addition of 200 µl of cold absolute ethanol at –20 °C. Total DNA was finally purified using the DNeasy blood and tissue kit (Qiagen, France) according to the manufacturer's instructions.

2.4.2. PCR amplification.

The 16S rRNA gene V3 region (194 bp) was amplified using the following primers: V3P2 (5'-ATTACCGCGGCTGCTGG-3') and V3P3 with GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAGGGGGGCCTACGGGAGGCAG CAG-3') (Jaffrès et al., 2009). Amplification was performed in 50 µl final volume with 40 µl of PCR mix containing in final concentration: dNTP 800 µM (Interchim, France), 1 X *Taq* buffer (Tris-HCL 10 mM [pH 9.0], KCL 50 mM, $MgCl_2$ 1.5 mM, Triton X-100 0.1% and BSA 0.2 mg/ml), 2.5 U of *Taq* DNA polymerase (MP Biomedicals, France), 1 µM of each primer and 10 µl of extracted total DNA. PCR amplification was performed in a PTC-100 thermocycler (MJ Research Inc, USA) with the following conditions: 94°C for 5 min, 40 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 1 min. A final extension at 72°C for 30 min was performed. The quality and size of the amplicons were evaluated on agarose gel 1% (MP Biomedicals, France) with a 100 bp ladder (Fermentas Life Science, France).

2.4.3 TTGE analysis

Amplified fragments were separated using the DCode Universal Mutation Detection System (BioRad, France). Polyacrylamide gels (16 cm x 16 cm, 1 mm thickness) consisted of two layers including resolving and stacking gels. Resolving gels were prepared with 9.5 % (w/v) of an acrylamide solution (acrylamide-bisacrylamide, 37.5 :1) and urea 8 M (final

concentration) in 1.25 X Tris base Acetic acid EDTA (TAE) buffer obtained from a 50 X TAE buffer (Tris base 2 M, glacial acetic acid 1 M, EDTA 50 mM). Stacking gels were prepared without urea with 16% (w/v) of acrylamide solution (acrylamide-bisacrylamide, 37.5:1) in 1.25 X TAE buffer. Acrylamide gel polymerisation was initiated by addition of ammonium persulfate (10%) and N, N, N', N'-tetramethylethylenediamine (TEMED) just before pouring into the vertical glass plate sandwich. After polymerisation, the gels were stored overnight at 4°C. Electrophoresis was run in 1.25X TAE buffer. Before loading the PCR products, the wells were rinsed with 1.25X TAE buffer. Thirty µl of amplified fragments with 1X of loading buffer (1:1) were then loaded in the wells. Electrophoresis were performed at 50 V for 12h30 with a temperature gradient ranging from 65°C to 70°C (0.4°C/h ramp) under stirring with a magnetic stirrer to mix the buffer and improve the temperature gradient homogeneity. After electrophoresis, gels were rinsed for 20 min in MilliQ water (Millipore, France); stained for 30 min in 300 ml of 3X GelRed staining solution (Fluoprobes, Interchim, France) in water containing NaCl 0.1 M and then rinsed in MilliQ water. Finally, gels were photographed by UV illumination using an ImageMaster VDS-CL imaging system (Amersham Pharmacia Biotech, France).

TTGE gels were standardized by using a V3 identification ladder made up of ten reference species resulting in ten bands well spread over the track length. TTGE banding patterns were analysed using BioNumerics fingerprinting software version 5.1 (Applied Maths, Belgium).

2.5 Total volatile basic nitrogen (TVBN) and trimethylamine (TMA) content

TVBN and TMA content were determined in this study using the Conway method as an index of freshness quality for the scallop samples at each sampling date (Conway and Byrne, 1936). Briefly, 100 g sample were homgenized then TVBN were extracted with

trichloroacetic acid (TCA). Mixtures were filtered using Whatman filter paper and the filtrate was used for analysis. For TMA determination, a 40% formaldehyde solution was added to the solution to fix the ammonia present in the sample. TVBN and TMA were released after addition of a 112% potassium carbonate solution and diffused into a 1% boric acid solution. Samples were then titrated by 0.01 N HCl until a rose colour appeared and TVBN and TMA contents were determined. TVBN was expressed as the quantity of NH_3 in the homogenised product using the following formula: [V in ml hydrochloric acid X 0.17 (mass in g homogenised product + mass in g water + mass in g 20% TCA) X 100] / [1 X TCA density X ml]. TMA was expressed as mg nitrogen per 100g product using the formula: [V in ml hydrochloric acid X 0.14 (mass in g homogenised product + mass in g water + mass in g 20% TCA) X 100] / [1 X TCA density X ml].

2.5 Sensory Evaluation

Sensory evaluation was carried out on both raw and cooked scallop samples at four different sampling dates (D1, D4, D6, D8) with a trained panel of 6-8 judges. To do so, different factors were considered and included visual aspect, odour and texture/firmness for both raw and cooked scallops and corals. A list of descriptors was made and grades were given based on 4 major sensorial criteria (odour, scallop aroma, coral aroma, and presence of spots) in order to judge scallop quality and freshness. For cooked scallops, samples were placed for 1 minute in a microwave at 750 W. Before each session, samples were placed in airtight containers prior to disposing them onto tasting plates.

3. Results

3.1 Culture-dependent community dynamics

Microbiological analyses targeting various bacterial floras (total psychrotrophic flora, *B. thermosphacta*, pseudomonads, enterobacteria, lactic acid bacteria and H_2S-producing bacteria) were performed at each sampling day (D1, D4, D6 and D8) and for each packaging condition (aerobic, vacuum, 50%CO_2 /50%N_2, 80%CO_2 /20%N_2 and 100% CO_2) by triplicate analyses (Figure 1).

The results obtained on Long & Hammer medium indicated that total psychrotrophic flora counts were high during the entire storage period and under all packaging conditions. Counts ranged from 5.5 log CFU/g at day D1 and almost reached 8 log CFU/g at day D8. Noteworthy, a difference of about 1 log was observed at day D4 between aerobic and vacuum packed conditions (~ 7 logs CFU/g) versus the 3 different CO_2 rich modified atmosphere conditions (~ 6 logs CFU/g) (Figure 1A).

For VRBG medium targeting enterobacteria (Figure 1B), counts were the lowest with 1 log CFU/g at day D1 and a maximum of about 4.5 log CFU/g at day D8 under aerobic conditions. While the largest differences were observed at day D6, with vacuum packaging allowing for faster growth and 80% CO_2 allowing for the slowest growth, at day D8 the maximum counts observed were relatively close to each other (ranging from 3.9 to 4.5 log CFU/g).

Concerning STAA medium numerations, results indicated that the packaging conditions had a limited but statistically significant effect. Indeed, although growth curves were parallel, they showed, at day D8, that aerobic packaging conditions allowed for the highest growth (6.9 log CFU/g) followed by vacuum packed samples and 50% CO_2 /50% N_2 modified atmosphere conditions (~ 6.5 log CFU/g). Packaging conditions with more than 80% CO_2 presented the lowest counts (≤ 6 log CFU/g) (Figure 1C).

For lactic acid bacteria numerated on MRS medium (Figure 1D), growth curves were parallel except for 50% CO_2/50% N_2 that reached a plateau at day 6 (~ 7.0 log CFU/g). At day

D8, the highest growth was observed for air packaged scallops while slowest growth rates were observed for modified atmosphere conditions with over 80% CO_2 (~ 6.0 log CFU/g).

Concerning CFC medium targeting pseudomonads, the obtained results showed the highest differences (Figure 1E). While packaging under aerobic conditions allowed for the growth of *Pseudomonas* spp. up to 6.5 log CFU/g ; the other packaging conditions had an impact on this flora with almost no growth observed for vacuum packed samples or in the presence of CO_2.

Finally, H_2S-producing bacteria were numerated on IA medium and appeared with a black halo. At day D8, significant differences were found as highest counts (~ 6.2 log CFU/g) were observed for the air packaged scallops while lowest counts corresponded to all the other conditions (< 5.0 log CFU/g)(Figure 1F). However, at day D6, modified atmosphere conditions with 100 % CO2 showed the lowest counts.

Overall, highest microbial flora counts were observed for air and in some cases vacuum packed and 50% CO_2 / 50% N_2 packaged scallops during conservation; these results were in agreement with the highest levels of TVBN and TMA detected under these packaging conditions (Table 2). Moreover, sensory evaluation data also showed that air packaged, vacuum packed and 50% CO_2 / 50% N_2 modified atmosphere samples were significantly different and considered unacceptable after day D6 while those for 80% CO_2 / 20% N_2 and 100% CO_2 modified atmosphere samples were inferior to the sensory evaluation acceptability limits (data not shown) These results allowed us to determine TVBN and TMA sensorial evaluation acceptability limits of <30 mg N / 100 g and <10 mg N / 100 g for *Pecten maximus* scallops, respectively.

3.2. M13-PCR typing and inter- and intra-species biodiversity

Genetic diversity of 311 representative isolates originating from the various media was assessed by obtaining genetic profiles using the coliphage M13 sequence-based PCR (M13-PCR) method (Henderson et al. 1994; Guinebretière and Nguyen-The 2003). The profiles generated for the 311 strains were then integrated and analyzed in a Bionumerics database (Figure 2 presents an example of profiles generated for isolates at day 8 from the various media). The repeatability and reproducibility of all experiments was also evaluated by using 2 control strains (laboratory control strains) in all M13-PCR experiments. Overall, a high level of M13-PCR genetic profile diversity was observed with at least 104 different profile types, of which 57 contained from 2 to 20 isolates (data not shown).

Strain identifications at the species level were then carried out, *via* sequencing of a 1533 bp 16S rRNA gene fragment, on 58 representative isolates (isolates within dominant M13 profile type clusters or unique isolates). Overall, high species diversity was observed with at least 26 different species (Table 1). The non-selective Long & Hammer medium showed the presence of various genera and species including numerous Gram-positive and Gram-negative bacteria. Overall, on this medium, the dominant genera corresponded to *Pseudomonas, Brochothrix* and *Shewanella* (20.2%, 15.9% and 14.9% of all LH isolates, respectively). A certain level of species biodiversity was also observed on CFC medium; dominant species corresponded to pseudomonads (46.9% of all CFC isolates) (*Pseudomonas* sp., *P. fluorescens*, *P. jensenii*, *P. syringae* and *P. fragi*) followed by *Shewanella* spp. (17.2% of all CFC isolates) (*S. baltica S. putrefaciens* and *S. frigidimarina*) and to a lesser extent *Pseudoalteromonas haloplanktis* and *Morganella morganii.* For Iron Agar, *Shewanella* spp. (in particular, *S. baltica* and *S. putrefaciens*) were dominant represented 61.7% of all IA isolates. On MRS medium, *Carnobacterium maltaromaticum* and *Actinomyces radicidentis* were observed (data not shown). *Moellerella wisconsensis* was the only species identified on VRBG medium; while bacteria growing on the STAA medium almost only corresponded to

B. thermosphacta. Low intra-species diversity was observed using these tools for strains belonging to the *Moellerella wisconsensis* and *S. putrefaciens* species (only 1 or 2 genetic profile clusters for each species). On the contrary, *B. thermosphacta* and *S. baltica* presented high infraspecific diversity and were distributed on the generated dendrogram in 5 different groups for both species (data not shown).

3.3 Incidence of storage duration and packaging conditions

Bacterial counts combined with species identification data allowed to study the impact of the storage period and packaging conditions on king scallop microbial ecosystem equilibrium. Concerning the storage duration (all packaging conditions considered), some variations of the represented species from day D1 to day D8 were observed except in the case of *Pseudomonas* spp. that were dominant at day 1 and remained stable during the following days (except under air). In particular, *B. thermosphacta* showed highest prevalence in all conditions followed by *Shewanella* spp. and *Moellerella wisconsensis* (except at day D1) (Table 3).

Regarding the impact of packaging conditions on bacterial genus distribution (all storage days considered), the analysis indicated that the dominant genera *Pseudomonas, Brochothrix*, *Shewanella* and to a lesser extent *Staphylcoccus* spp. were present in every packaging condition although some differences in the respective ratios were observed (Table 3). Mainly, bacteria growing on CFC and IA (in particular, *Pseudomonas* and *Shewanella* spp.) were affected by all conditions eliminating oxygen from the packaging atmosphere. *B. thermosphacta* and *C. maltaromaticum* were slightly affected by the presence of CO_2 with an approximately 1 log unit reduction in "100% CO_2" MAP as observed by STAA and MRS media counts, respectively, while *Moellerella wisconsensis* showed lower differences. The obtained result also indicated that the packaging conditions could have a deeper impact on

specific microorganisms. For example, the *Pseudoalteromonas* genus, represented in this study by the *Pseudoalteromonas haloplanktis* species, was not found in "80% CO_2/20% N_2" and "100% CO_2" MAP conditions. This result suggested that this species is sensitive to high CO_2 concentrations rather than to anaerobic conditions as it was detected in the "vacuum packed" and "50% CO_2/50% N_2" packaging conditions.

3.4 Culture-independent community dynamics

In parallel, the TTGE molecular method was used in order to contribute to the study of the biodiversity as well as the dynamics of the king scallop meat ecosystem during storage. The obtained TTGE profiles indicated that the repeats were highly similar (data not shown); therefore, only one representative TTGE profile was selected for further analysis. The corresponding 17 selected profiles were analyzed and compared using the Bionumerics software which generated a proximity dendrogram (Figure 3).

After day D6, the bacterial flora seemed to be stable in each packaging condition and patterns at day D6 and day D8 were similar. These patterns have been clustered according to the different packaging atmospheres showing their respective effect on the microbiota composition. The profiles obtained at day D4, although close to those from day D6 and day D8, did not group as closely suggesting that at this sampling date the flora ecosystem was still evolving.

The effect of each packaging condition on the evolution of the TTGE profiles during the storage period was also analyzed. For the king scallop meats packaged under aerobic or vacuum packed conditions, the profiles obtained between days D4 and D8 were similar (except for day D6 under vacuum packaging). The largest modification of the microbial ecosystem composition seemed to appear between the first and the fourth day.

For the products packaged in the presence of CO_2, the profiles evolved between days D1, D4 and D6. From day D6, the ecosystems seemed to be stabilized. Moreover, on these products, a larger number of bands could reflect a higher diversity of the king scallop microbiota in these samples, however, some species may present multiple bands.

By comparing the profiles obtained from the different products to the profiles obtained from pure strains representative of the biodiversity as observed by M13-PCR typing, band assignment was performed although some pure strains exhibited several bands in their TTGE profiles (data not shown). The band associated with the dominating genus on culture media (i.e. *Pseudomonas*) was not found in the product profiles and sporadically present for *Brochothrix* and *Shewanella putrefaciens*.

4. Discussion

The first part of this work consisted in studying the main groups of flora associated with king scallops and their dynamics during cold storage under different modified atmosphere packaging. These different conditions were selected to determine their impact on the microbial communities and their potential inhibiting properties towards spoilage bacteria at temperatures encountered by the consumer during cold food storage. Indeed, practical industrial studies tend to suggest that TVBN content is lower in CO_2 rich environments for some seafood products although it can evolve with microbial development.

The first observation was that for every flora considered (except *Enterobacteriaceae*), the initial counts were relatively high. Indeed, at day D1, depending on the considered medium the counts ranged from 3.04 log CFU/g for H_2S-producing bacteria to 5.5 log CFU/g for the total psychrotrophic flora while enterobacteria were at the detection limit with 1 log CFU/g. This could be explained by two facts, first *Pecten maximus,* like other bivalve molluscs, are filtrating water to feed on plankton; in this context they might concentrate

418 microorganisms; secondly, to obtain the meat and coral, the rest of the scallop, including the
419 mantel and the digestive gland, are removed manually which can contribute to the
420 contamination of the eatable parts. Concerning the dynamics of the various populations, the
421 most obvious effect of the tested packaging conditions was the impact of anaerobic
422 conditions, either through vacuum packaging or CO_2/N_2 mixtures on pseudomonads. In these
423 conditions, minimum growth potentials (<log 0.5 CFU/g) were observed at day D8 while a
424 growth potential of 1.6 log CFU/g was observed under aerobic conditions. The impact of O_2
425 depletion, through vacuum or modified atmosphere packaging, on *Pseudomonas* was not a
426 surprise as most members of this flora are strictly aerobic. The inhibition of *Pseudomonas* in
427 anaerobic packaging conditions has been reported on different food products (Crowley et al.,
428 2010; Pantazi et al., 2008; Ravi Sankar et al., 2008). Except for day D8, the lowest counts for
429 every studied flora as well as lowest TVBN and TMA contents were observed for the
430 modified atmosphere packaging (MAP) with >80% CO_2. These results were clearly
431 confirmed by sensory evaluation criteria as only >80% CO2 modified atmosphere samples
432 were acceptable after day D8 (data not shown). Both TVBN and TMA content limits were
433 determined based on all of the results in this study and were considered to be <30 mg N / 100
434 g and <10 mg N / 100 g, respectively. No European regulation exists for TVBN and TMA
435 contents in *Pecten maximus* scallops, however, EC regulation No 2074/2005 fixed TVBN
436 values at 25-35 mg N / 100g muscle according to the fish species. Based on the results of this
437 study, the determined TVBN and TMA limits are therefore in accordance with those for fish
438 species.

439 The microbial diversity of the king scallop ecosystem was assessed by M13-PCR
440 grouping followed by identification of representative isolates by 16S rRNA gene sequencing.
441 This approach allowed to show a large biodiversity with at least 26 species identified. Under
442 air, the dominant species corresponded to *B. thermosphacta*, *Pseudomonas* spp. and

443 *Shewanella* spp.. These species have been regularly associated with food, and especially
444 seafood products (Fonnesbech Vogel et al., 2005; Hovda et al., 2007; Laursen et al., 2006).
445 *Pseudomonas* was also the predominant genus along with *Moraxella* and *Vibrio* in the scallop
446 *Argopecten purpuratus* cultured in Chile (Riquelme et al., 1995). The species *Moellerella*
447 *wisconsensis* also seemed to be well associated with the king scallop ecosystem as it was the
448 only species identified on VRBG. This species, first described from human stools (Hickman-
449 Brenner et al., 1984), has been rarely isolated from food products and its natural reservoir is
450 still unclear (Stock et al., 2003). Interestingly, Matyar (2007) indicated that this species was
451 one of the dominant Gram-negative bacterial species (18%) in Turkish sea bass; while,
452 Skrodenytė-Arbačiauskienė et al. (2008) identified it in the intestinal flora of freshwater
453 salmon and sea trout. Finally, it was also associated with sea mammals and marine birds
454 (Bogomolni et al., 2008) as well as the sponge *Halichondria rugosa* (Li, 2009). As a whole,
455 these facts suggest that the marine environment could constitute one of *Moellerella*
456 *wisconsensis* main natural reservoirs.

457 The analysis of the obtained biodiversity data by both culture-dependent and culture-
458 independent methods allowed to evaluate the impact of the modified atmosphere packaging
459 conditions during storage with a cold chain rupture on the king scallop ecosystem. The results
460 indicated an impact of the packaging conditions on the ecosystem's equilibrium during
461 storage as well as on TVBN and TMA levels. In particular, the MAP with elevated CO_2 levels
462 (80 and 100% CO_2) allowed for the reduction of the different spoilage bacteria and the non-
463 detection of specific flora (i.e. *P. haloplanktis*). The use of MAP, specifically elevated CO_2
464 levels, has been shown to inhibit normal spoilage bacteria, such as *Pseudomonas*, *Aeromonas*
465 and *Shewanella* spp. in fish and to extend shelf life (Stammen et al., 1990). For scallops, the
466 use of CO_2 was shown to extend scallop shelf life (Bremner and Statham, 1987). More
467 recently, Bremer and Fletcher (1999) compared the impact of "100% CO_2" MAP and aerobic

conditions on *Pecten alba* scallop meat. Under aerobic conditions, the bacterial development was exponential from day 3 and reached 8 log CFU/g at day 10; while, under "100% CO_2" MAP, bacteria only developed from day 7 and reached 6.7 log CFU/g at day 28. Moreover, at the end of shelf life a large presence of Gram-positive bacteria corresponding to lactobacilli was observed.

In parallel, a culture-independent method (TTGE) was performed. TTGE and denaturing gradient gel electrophoresis (DGGE) have been previously used to study microbial dynamics of numerous foods including during food fermentations (Ercolini, 2004; Cocolin et al., 2004; Ogier et al., 2004) and more recently rainbow trout fish gut microbiota (Navarette et al., 2012). The obtained patterns have been clustered according to the different packaging atmospheres showing their respective effect on the microbiota composition. Moreover, the TTGE profiles obtained for the high CO_2 level packaging seemed to indicate an increase of the bacterial biodiversity. The dominance of the *Shewanella* spp. and *B. thermosphacta* was confirmed while the profiles corresponding to *Pseudomonas* spp. and *C. maltaromaticum* were not found. On the contrary, two genera, *Psychrobacter* and *Vibrio*, non-dominant on the culture media, clearly displayed their respective patterns in the product profiles. These findings emphasize that both methods (culture-dependent and –independent) provide complementary insights into the microbial ecosystem composition of the packaged king scallop meat during storage.

The sensory parameters (appearance and smell of raw king scallops) assessed by an internal panel of six experts and the biochemical spoilage indicator levels (TMA and TVBN obtained by the Conway's Microdiffusion Units - Conway and Byrne, 1936) also indicated that there was an impact by the packaging conditions on these criteria. For example, the scallops packaged with 100% CO_2 presented TMA and TVBN levels representing almost half of the ones observed under aerobic conditions (TMA 30.9 mg/100g and TVBN 78.2 mg/100g

493 under aerobic conditions vs. TMA 16.3 mg/100g and TVBN 37.4 mg/100g under 100% CO_2)
494 and were still acceptable from an odour point of view while the declared shelf life of king
495 scallops is 6 days in cold storage. At day D8, all the products were considered spoiled and the
496 results suggested that *B. thermosphacta*, and to a lesser extent *Shewanella* species, were
497 associated with king scallop spoilage. Pseudomonads could also contribute to spoilage;
498 however, their involvement is less clear as the vacuum packaged scallops as their growth was
499 almost always inhibited (89% of pseudomonads were identified in the other packaging
500 conditions) were already unacceptable from an organoleptic point of view (aspect, texture and
501 smell) at day D4 (no link with TMA and TVBN levels was observed). Interestingly, the
502 scallops packaged at high CO_2 levels (80 and 100%), although unacceptable at day D8 from
503 visual and texture points of view, were acceptable from an odour point of view (data not
504 shown). The potential of packaging conditions for shelf life extension is directly linked to the
505 initial bacterial load. In this study, we showed that the initial microbial load was high (total
506 psychotrophic flora of 5.5 log CFU/g) and therefore, efforts should be done to reduce the
507 initial load to make the most of MAP effects.

508 Little data is available in the literature on the flora associated with king scallop
509 (*Pecten maximus*). The results obtained during this study, combining culture-dependent and -
510 independent methods, allowed comprehending the complex biodiversity and dynamics of the
511 bacterial flora during cold storage under various packaging conditions.

512

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654 medium for the enumeration of more fastidious bacteria from fresh fishery products.
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656 Table 1. Species biodiversity as observed by the identification of 58 representative isolates.

657 (UA: air, UV: vacuum, Ga: 50% CO_2 / 50% N_2, Gb: 80% CO_2 / 20% $N_2$and Gc: 100% CO_2)

Strain code (accession n°)	Closest relative	% identity[1]	Genbank accession number	Other strain codes associated to this identification
D6-MRS-Ga-3	*Actinomyces radicidentis*	98.9%	AJ251986	
D8-VRBG-Ga-3	*Aeromonas molluscorum*	99.4%	AY987772	
D8-VRBG-Ga-1	*Aeromonas* sp.	94.2%	GQ266405	
D1-LH-10	*Arthrobacter bergerei*	100%	AJ609633	
D4-LH-UV-2	*Brevibacterium antiquum*	98.5%	AY243344	
D4-LH-UV-6	*Brevibacterium* sp.	98.4%	FJ652620	
D4-LH-A-3	*Brochothrix thermosphacta*	99.2%	M58798	D4-STAA-UA-2; D6-STAA-UA-2; D8-STAA-UA-2; D8-STAA-UV-2; D8-STAA-Ga-1
D6-VRBG-UV-2	*Moellerella wisconsensis*	98.5%	AM040754	D8-VRBG-UA-2; D8-VRBG-UV-1; D8-VRBG-UV-3
D8-CFC-UV-4	*Morganella morganii*	98.5%	DQ513315	D8-CFC-UA-4 ; D8-IA-Gb-3
D8-LH-Ga-5	*Photobacterium iliopiscarium*	99.4%	AY849429	
D4-CFC-UA-5	*Pseudoalteromonas haloplanktis*	99.9%	CR954246	D6-CFC-Ga-4
D4-CFC-Ga-4	*Pseudomonas fluorescens*	99.9%	DQ084460	D4-LH-Gc-6; D8-CFC-UA-3; D8-CFC-Gc-3; D4-VRBG-Gc-1
D8-CFC-UA-1	*Pseudomonas fragi*	99.8%	AF094733	
D1-LH-9	*Pseudomonas jessenii*	99.2%	AM933510	
D6-LH-Gc-5	*Pseudomonas* sp.	99.8%	EU681009	D1-CFC-5; D4-CFC-Gc-3
D6-CFC-UV-4	*Pseudomonas syringae*	99.9%	AJ576247	
D1-LH-3	*Psychrobacter* sp.	99.8%	AB365059	D6-LH-Ga-3; D8-LH-UA-1
D4-CFC-V-2	*Shewanella baltica*	99.3%	CP001252	D8-LH-UA-5; D4-IA-UA-2; D4-IA-UV-2; D6-IA-Ga-1 ; D8-IA-UA-2 ; D8-IA-Gc-2
D8-LH-Gb-4	*Shewanella frigidimarina*	99.7%	AJ300833	
D8-LH-UA-7	*Shewanella putrefaciens*	100%	AB205575	D4-IA-Gc-1; D8-IA-UA-1 ; D8-IA-UV-3
D4-LH-Gb-1	*Staphylococcus cohnii*	99.9%	AJ717378	
D8-LH-Ga-9	*Staphylococcus pasteuri*	99.9%	FJ435675	
D8-LH-UV-5	*Staphylococcus* sp.	99.9%	EU195954	
D8-STAA-UV-3	*Vagococcus salmoninarum*	99.9%	Y18097	
D8-CFC-Gb-1	*Vibrio* sp.	98.4%	DQ097524	
D8-LH-UA-6	*Vibrio logei*	99.9%	AY292932	D8-LH-UV-3

658 [1] Identical nucleotides percentage in the sequence obtained from the 16S rRNA gene and the sequence found in

659 Genbank.

660 Table 2. Evolution of TVBN and TMA content in scallops under different packaging

661 conditions. Results are presented in mg N / 100 g scallop meat.

	TVBN mg N / 100 g					TMA mg N / 100 g				
Time (days)	**air**	**VP**	**50% CO2**	**80% CO2**	**100% CO2**	**air**	**VP**	**50% CO2**	**80% CO2**	**100% CO2**
1	3.7	3.7	3.7	3.7	3.7	3	3	3	3	3
4	10	10.2	8.1	8.4	7.32	1.1	1.8	3	3	3
6	42.2	35.9	24.3	27.7	21.7	12.2	10.6	8.6	9	8.6
8	78.2	67.9	48.8	49.9	37.4	30.9	22.5	19	21.1	16.3

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681 Table 3. Species encountered in scallops under different packaging conditions during cold
682 storage. Results are presented as the number of isolates identified to a given species at each
683 sampling date and according to packaging conditions. For each medium, the square root of the
684 total enumerated bacteria were randomly analyzed.

685

Species	Day 1	Day 4	Day 6	Day 8	Packaging conditions*
Actinomyces radicidentis	0	2	3	3	UV, GA, GB, GC
Aeromonas molluscorum	0	0	0	1	GA
Aeromonas sp.	0	0	0	1	GA
Arthrobacter bergerei	3	1	2	0	GA, GC
Brevibacterium antiquum	0	1	0	0	UV
Brevibacterium sp.	0	3	1	0	UA, UV, GC
Brochothrix thermosphacta	2	19	13	19	UA, UV, GA, GB, GC
Carnobacterium maltaromaticum	0	0	4	3	GB, GC
Moellerella sp.	0	0	5	0	UA, UV, GA, GB
Moellerella wisconsensis	0	8	2	8	UA, UV, GA, GB, GC
Morganella morganii	3	2	5	8	UA, UV, GA, GB, GC
Photobacterium iliopiscarium	0	0	0	1	GA
Pseudoalteromonas haloplanktis	2	3	6	1	UA, UV, GA
Pseudomonas fluorescens	4	7	2	7	UA, UV, GA, GB, GC
Pseudomonas fragi	0	1	3	2	UA, UV, GC
Pseudomonas jessenii	2	2	1	0	GA, GC
Pseudomonas sp.	4	15	4	4	UA, UV, GA, GB, GC
Pseudomonas syringae	0	1	2	0	UV, GB
Psychrobacter sp.	2	7	3	1	UA, UV, GA, GC
Shewanella baltica	0	5	8	14	UA, UV, GA, GB, GC
Shewanella frigidimarina	0	0	0	1	GB
Shewanella putrefaciens	5	6	2	8	UA, UV, GA, GC
Staphylococcus cohnii	0	1	0	0	GB
Staphylococcus pasteuri	0	0	0	1	GA
Staphylococcus sp.	1	1	5	3	UA, UV, GA, GB, GC
Unidentified	4	11	15	2	UA, UV, GA, GB, GC
Vagococcus salmoninarum	0	0	0	1	UV
Vibrio sp.	0	0	0	1	GB
Vibrio logei	0	2	2	3	UA, UV, GA, GB, GC

686 * UA: air, UV: vacuum, Ga: 50% CO_2 / 50% N_2, Gb: 80% CO_2 / 20% N_2 and Gc: 100% CO_2

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689 **Figure legends**

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691 Figure 1. Numeration of bacterial flora associated with king scallop during storage (cold chain
692 rupture 1/3 storage time at 4°C and 2/3 at 8°C) under different packaging conditions (air:■,
693 vacuum:□ , 50% CO_2 / 50% N_2:▩ , 80% CO_2 / 20% N_2 :◫ and 100% CO_2:▤) . The following
694 flora were numerated: total aerobic flora (A), enterobacteria (B), *Brochothrix thermosphacta*
695 (C), lactic acid bacteria (D), pseudomonads (E) and H_2S^+ bacteria (F).

696

697 Figure 2. Genetic profiles generated by M13-PCR for strains isolated at D8 in the different
698 packaging conditions. The tree was constructed using the Pearson correlation and UPGMA.
699 (UA: air, UV: vacuum, Ga: 50% CO_2 / 50% N_2, Gb: 80% CO_2 / 20% N_2 and Gc: 100% CO_2)

700

701 Figure 3. Proximity dendrogram of the TTGE profiles obtained during storage (D1, 4, 6 and
702 8) in the different packaging conditions using the Dice coefficient and UPGMA.

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704

705 **Figure 1**

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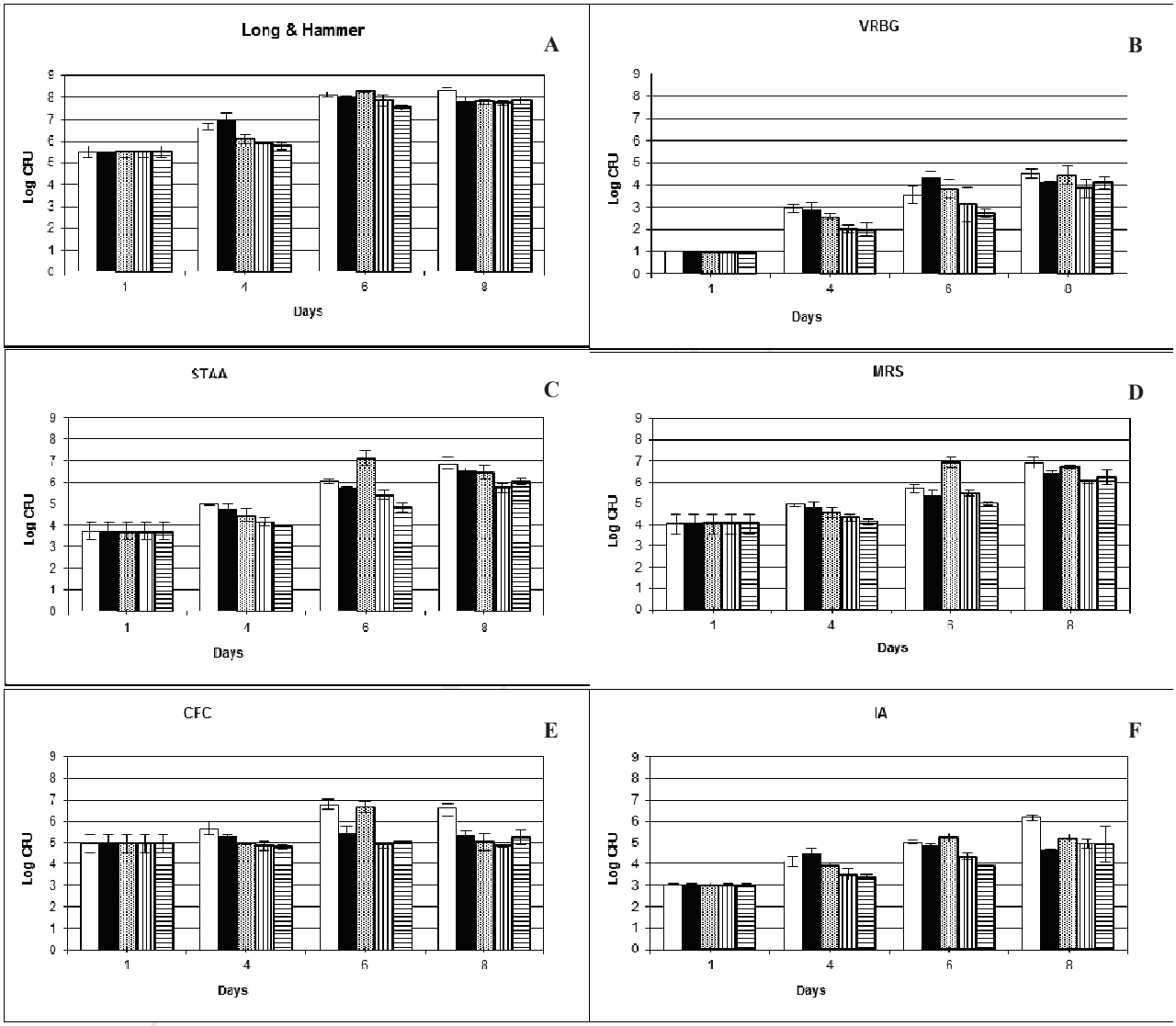
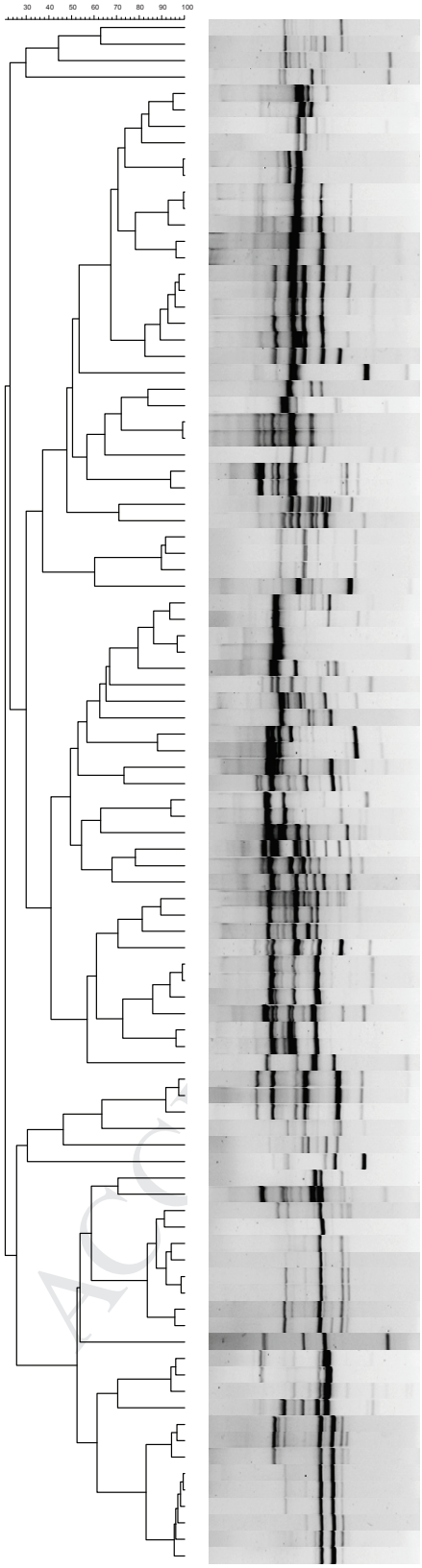


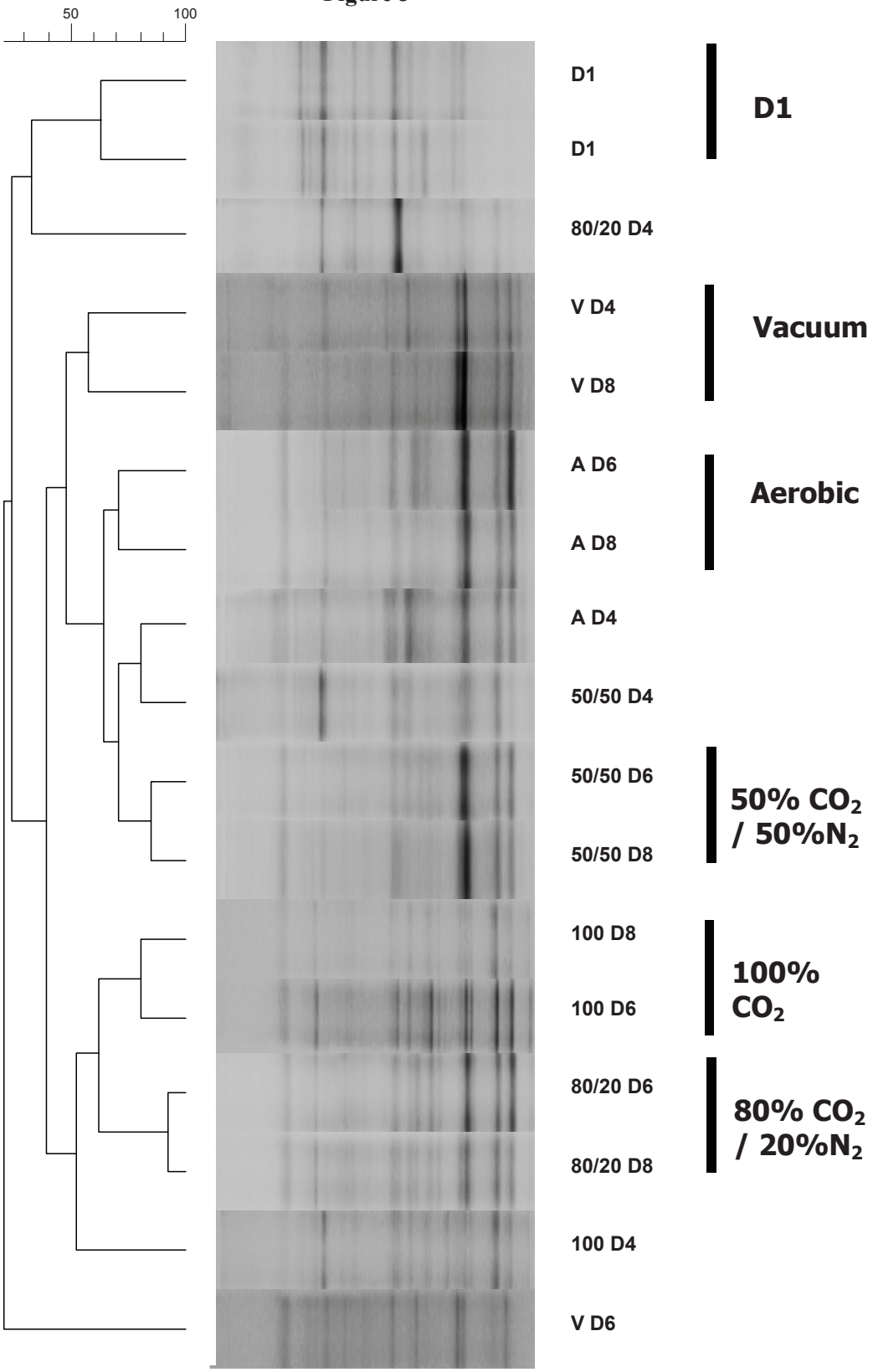
707

Figure 2



Species	Strain code
Brochothrix thermosphacta	D8-STAA-UV-2
Actinomyces radicidentis	D8-STAA-Gc-3
Actinomyces radicidentis	D8-STAA-Gb-3
Vagococcus salmoninarum	D8-STAA-UV-3
Shewanella putrefaciens	D8-LH-UA-7
Shewanella putrefaciens	D8-CFC-Ga-1
Shewanella putrefaciens	D8-LH-Gc-3
Shewanella putrefaciens	D8-CFC-UA-6
Shewanella putrefaciens.	D8-GF-UA-1
Shewanella putrefaciens.	D8-GF-UA-3
Morganella morganii	D8-CFC-Ga-2
Morganella morganii	D8-CFC-Ga-4
Morganella morganii	D8-CFC-UA-4
Moellerella wisconsensis	D8-VRBG-UA-1
Moellerella wisconsensis	D8-VRBG-UA-2
Moellerella wisconsensis	D8-VRBG-UA-3
Moellerella wisconsensis	D8-VRBG-UV-2
Moellerella wisconsensis	D8-VRBG-Gb-1
Moellerella wisconsensis	D8-VRBG-SV-3
Moellerella wisconsensis	D8-VRBG-Gb-2
Moellerella wisconsensis	D8-VRBG-SV-1
ND	D8-LH-Ga-2
ND	D8-LH-Gb-3
Shewanella baltica	D8-LH-Gc-2
Pseudomonas sp.	D8-VRBG-Ga-2
Aeromonas molluscorum	D8-VRBG-Ga-3
ND	D8-GF-UV-1
Shewanella putrefaciens	D8-GF-UV-2
Shewanella putrefaciens	D8-GF-UV-3
Pseudomonas fluorescens	D8-CFC-UV-3
Pseudomonas fluorescens	D8-VRBG-Gc-2
Carnobacterium maltaromaticum	D8-MRS-Gc-2
Carnobacterium maltaromaticum	D8-MRS-Gc-3
Carnobacterium maltaromaticum	D8-MRS-Gc-1
ND	D8-LH-Ga-6
Photobacterium iliopiscarium	D8-LH-Ga-5
Morganella morganii	D8-LH-Gb-1
Morganella morganii	D8-GF-Gb-1
Morganella morganii	D8-GF-Gb-3
Psychrobacter sp.	D8-LH-UA-1
Shewanella baltica	D8-GF-UA-2
Shewanella baltica	D8-GF-Ga-3
Shewanella frigidimarina	D8-LH-Gb-4
Pseudomonas fluorescens	D8-CFC-Gb-2
Pseudomonas fluorescens	D8-CFC-Gc-3
Pseudomonas sp.	D8-CFC-SA-5
ND	D8-CFC-Gc-1
Vibrio logei	D8-LH-UA-6
Vibrio logei	D8-LH-UV-4
Pseudomonas fluorescens	D8-CFC-UA-3
ND	D8-GF-Gb-2
Aeromonas salmonicida	D8-VRBG-Ga-1
Pseudomonas fragi	D8-LH-Gc-1
Pseudomonas sp.	D8-CFC-UA-2
Pseudomonas sp.	D8-VRBG-Gb-3
Pseudomonas fragi	D8-CFC-UA-1
Morganella morganii	D8-CFC-UV-4
Shewanella baltica	D8-GF-Gc-1
Shewanella baltica	D8-GF-Gc-2
Shewanella baltica	D8-CFC-Gc-2
Shewanella baltica	D8-LH-Ga-1
Shewanella baltica	D8-GF-Ga-1
Shewanella baltica	D8-GF-Ga-2
Shewanella baltica	D8-VRBG-Gc-3
Staphyloccocus pasteuri	D8-LH-UV-2
Staphyloccocus pasteuri	D8-LH-Ga-9
Staphyloccocus pasteuri	D8-LH-Gb-2
Staphyloccocus pasteuri	D8-LH-UV-5
Brochothrix thermosphacta	D8-LH-Ga-7
Pseudoalteromonas haloplanktis	D8-LH-UA-3
Brochothrix thermosphacta	D8-LH-UA-2
Pseudomonas fluorescens	D8-VRBG-Gc-1
Brochothrix thermosphacta	D8-LH-Gb-5
Vibrio litoralis	D8-CFC-Gb-1
Brochothrix thermosphacta	D8-LH-UV-1
Brochothrix thermosphacta	D8-STAA-Gb-2
Brochothrix thermosphacta	D8-STAA-Ga-2
Brochothrix thermosphacta	D8-STAA-Ga-3
Brochothrix thermosphacta	D8-STAA-UA-1
Brochothrix thermosphacta	D8-STAA-UV-1
Vibrio logei	D8-LH-UV-3
Shewanella baltica	D8-LH-UA-5
Shewanella baltica	D8-CFC-Ga-3
Shewanella baltica	D8-CFC-UV-1
Shewanella baltica	D8-CFC-UV-2
Brochothrix thermosphacta	D8-STAA-UA-2
Brochothrix thermosphacta	D8-STAA-UA-3
Brochothrix thermosphacta	D8-LH-Gc-5
Brochothrix thermosphacta	D8-STAA-Gc-1
Brochothrix thermosphacta	D8-STAA-Gc-2
Brochothrix thermosphacta	D8-STAA-Ga-1
Brochothrix thermosphacta	D8-STAA-Gb-1
Brochothrix thermosphacta	D8-LH-Ga-3
Brochothrix thermosphacta	D8-LH-Gc-4

Figure 3


50
100
D1
D1
80/20 D4
V D4
V D8
A D6
A D8
A D4
50/50 D4
50/50 D6
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100 D8
100 D6
80/20 D6
80/20 D8
100 D4
V D6
D1
Vacuum
Aerobic
50% CO2 / 50%N2
100% CO2
80% CO2 / 20%N2