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## Unveiling hákarl: A study of the microbiota of the traditional Icelandic fermented fish

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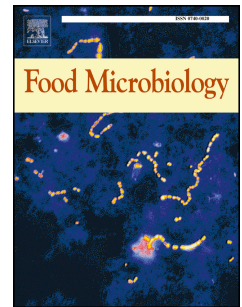
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1 **Unveiling *hákarl*: a study of the microbiota of the traditional Icelandic fermented fish**

2

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32 **Abstract**

33

34 *Hákarl* is produced by curing of the Greenland shark (*Somniosus microcephalus*) flesh, which before fermentation is  
35 toxic due to the high content of trimethylamine (TMA) or trimethylamine N-oxide (TMAO). Despite its long history of  
36 consumption, little knowledge is available on the microbial consortia involved in the fermentation of this fish. In the  
37 present study, a polyphasic approach based on both culturing and DNA-based techniques was adopted to gain insight  
38 into the microbial species present in ready-to-eat *hákarl*. To this aim, samples of ready-to-eat *hákarl* were subjected to  
39 viable counting on different selective growth media. The DNA directly extracted from the samples was further  
40 subjected to Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and 16S amplicon-  
41 based sequencing. Moreover, the presence of Shiga toxin-producing *Escherichia coli* (STEC) and *Pseudomonas*  
42 *aeruginosa* was assessed via qualitative real-time PCR assays. pH values measured in the analyzed samples ranged  
43 from between  $8.07\pm 0.06$  and  $8.76\pm 0.00$ . Viable counts revealed the presence of total mesophilic aerobes, lactic acid  
44 bacteria and Pseudomonadaceae. Regarding bacteria, PCR-DGGE analysis highlighted the dominance of close relatives  
45 of *Tissierella creatinophila*. For amplicon sequencing, the main operational taxonomic units (OTUs) shared among the  
46 data set were *Tissierella*, *Pseudomonas*, *Oceanobacillus*, *Abyssivirga* and *Lactococcus*. The presence of *Pseudomonas*  
47 in the analyzed samples supports the hypothesis of a possible role of this microorganism on the detoxification of shark  
48 meat from TMAO or TMA during fermentation. Several minor OTUs (<1%) were also detected, including  
49 *Alkalibacterium*, *Staphylococcus*, *Proteiniclasticum*, *Acinetobacter*, *Erysipelothrix*, *Anaerobacillus*, *Ochrobactrum*,  
50 *Listeria* and *Photobacterium*. Analysis of the yeast and filamentous fungi community composition by PCR-DGGE  
51 revealed the presence of close relatives of *Candida tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. zeylanoides*,  
52 *Saccharomyces cerevisiae*, *Debaryomyces*, *Torulasporea*, *Yamadazyma*, *Sporobolomyces*, *Alternaria*, *Cladosporium*  
53 *tenuissimum*, *Moristroma quercinum* and *Phoma/Epicoccum*, and some of these species probably play key roles in the  
54 development of the sensory qualities of the end product. Finally, qualitative real-time PCR assays revealed the absence  
55 of STEC and *Pseudomonas aeruginosa* in all of the analyzed samples.

56

57 **Keywords:** *Tissierella*; *Pseudomonas*; *Debaryomyces*; 16S amplicon-based sequencing; PCR-DGGE.

## 58 1. Introduction

59

60 Fermentation represents one of the most ancient techniques for food preservation. Traces of this practice can be seen as  
61 far back as 6000 B.C. in the Fertile Crescent (Franco et al. 2016). Moreover, the production of fermented foods became  
62 very popular among the Egyptian, Greek and Roman civilizations (Huang, 2016).

63 Food fermentation is mainly based on the metabolic activities of microorganisms that are either naturally present in the  
64 raw materials or artificially inoculated (Shiferaw Terefe, 2016). The most well-known processes include lactic acid  
65 fermentation, fungal fermentation, and alkaline fermentation, where pro-technological microorganisms improve the  
66 aroma, flavor, texture, and nutritional characteristics of the raw materials and inhibit spoilage and pathogenic  
67 microorganisms (Shiferaw Terefe, 2016). Moreover, species belonging to some microbial groups mostly associated  
68 with food fermentation (e.g., lactic acid bacteria) can reduce the health hazards associated with the consumption of food  
69 containing some toxic substances (Luz et al., 2018). In this context, microorganisms can be considered human beings'  
70 coevolutionary partners responsible for providing a wide variety of fermented foods with enhanced nutritional and  
71 sensory characteristics.

72 Most fermented foods available on the market are still produced in accordance with ancient traditions deeply rooted in  
73 the territory of origin. The obtained products represent an invaluable source of microbial diversity where complex  
74 microbial populations coexist in a dynamic equilibrium.

75 The most popular fermented foods are produced with raw materials from the dairy, meat or vegetable food chains. For  
76 such products, an ample scientific literature on both manufacturing technologies and the microbial communities  
77 involved during their transformation is available. Regarding fermented foods produced with raw materials from the  
78 marine environment, a lack of knowledge on the technological processes and the relevant microbial populations is  
79 highlighted (Rajauria et al., 2016). Notwithstanding, fermented marine-based products are currently consumed by  
80 several cultural groups worldwide (Rajauria et al., 2016).

81 Brilliant examples of traditional fermented fish products are represented by *surströmming* and *rakfisk*, produced in  
82 Sweden and Norway, respectively, and *hákarl*, produced in Iceland (Skåra et al., 2015). The production of such  
83 delicacies dates back to the Viking Age, when preservation of foodstuffs with salt was expensive, especially in the  
84 remote regions of northern Europe. Therefore, instead of salting, new empiric methods of preservation of caught fish  
85 were carried out by local populations, thus leading to the production of edible and safe products.

86 Among the abovementioned fermented fish, *hákarl* is produced by curing of the Greenland shark (*Somniosus*  
87 *microcephalus*). As reported by Skåra et al. (2015), the origin of the production technique of *hákarl* is still not clear,  
88 and it is unknown whether the shark was specifically caught or simply collected from specimens that drifted ashore.

89 The consumption of fresh Greenland shark is considered unsafe, although the toxic substances responsible of poisoning  
90 have not been recognized. Different authors have reported cases of poisoning from the flesh of the Greenland shark  
91 likely due to a high level of trimethylamine (TMA) (Anthoni et al., 1991; Halsted, 1962; Simidu, 1961).

92 In ancient times, *hákarl* was produced by cutting the shark into pieces that were left to ferment for weeks or months in  
93 gravel pits often close to the sea. The pits were usually covered with stones, seaweed, or turf. These structures were  
94 constantly exposed to seawater, which flooded over the fish at high tide (Skåra et al., 2015).

95 In the modern era, the fermentation of shark pieces is carried out in closed containers that allow the resulting leachate to  
96 be drained. Such a process can last from 3 to 6 weeks depending on the environmental temperature and season. After  
97 fermentation, the shark pieces are further cut and hung to dry in dedicated sheds for weeks or months, depending on the  
98 outdoor environmental conditions (Skåra et al., 2015).

99 In both the ancient or the modern processes, the metabolic activities of microorganisms occurring during shark  
100 fermentation lead to the conversion of a poisonous raw material into a safe and tasty ready-to-eat food product with a  
101 long shelf-life. The *hákarl* is characterized by a soft texture with a whitish cheese-like appearance, strong ammonia  
102 smell and fishy taste (Skåra et al., 2015).

103 Despite the long history of *hákarl* consumption, a lack of knowledge is available on the microbial consortia involved in  
104 the shark fermentation. Indeed, to our knowledge, only one study that dates back to 1984 is available in the scientific  
105 literature (Magnússon and Gudbjörnsdóttir, 1984).

106 Since many years ago, the cultivation of microorganisms on synthetic growth media was the primary way to study  
107 microbial communities in foods. The development of molecular techniques based on the use polymerase chain reaction  
108 (PCR) opened new frontiers for the study of microbial ecology in complex matrices (Garofalo et al., 2017). A variety of  
109 studies have shown that combinations of different microbiological techniques can provide sound information on the  
110 microbial composition of complex food matrices, including those subjected to fermentation. Among the most adopted  
111 and sensitive molecular methods, PCR-Denaturing Gradient Gel Electrophoresis (DGGE), real-time PCR and next-  
112 generation sequencing provide reliable data for microbiological profiling of foods.

113 Based on these concepts, a polyphasic approach based on both culture and DNA-based techniques was adopted to  
114 provide insight into the microbial species present in ready-to-eat *hákarl*.

115 To this end, samples of ready-to-eat *hákarl* were subjected to viable counting on different selective growth media. The  
116 DNA directly extracted from the samples was further subjected to PCR-DGGE and Illumina sequencing. Moreover, the  
117 presence of *Shiga* toxin-producing *E. coli* (STEC) and *Pseudomonas aeruginosa* was assessed via qualitative real-time  
118 PCR assays.

119

## 120 2. Materials and methods

121

### 122 2.1. Sampling

123

124 Ten samples of ready-to-eat *hákarl* (Figure 1) codified from H1 to H10 were analyzed, for each sample two 100 g boxes  
125 were purchased (for a total of 20 analyzed boxes). The samples were purchased via the internet from a dealer located in  
126 Iceland. In more detail, the samples were collected through different orders placed from January to May 2018. The  
127 samples were shipped by international express courier in plastic boxes at room temperature in aerobic conditions and  
128 analyzed after 24 hours from shipping. No further information on the samples was provided by the producer.

129

### 130 2.2. pH measurements

131

132 pH values of the *hákarl* samples were determined with a pH meter equipped with an HI2031 solid electrode (Hanna  
133 Instruments, Padova, Italy). For each sample, the measurements were performed in duplicate.

134

### 135 2.3. Microbial viable counts

136

137 Twenty-five grams of each *hákarl* sample were homogenized for 5 min at 260 rpm in 225 mL of sterile peptone water  
138 (bacteriological peptone 1 g L<sup>-1</sup>, Oxoid, Basingstoke, UK) using a Stomacher 400 Circulator apparatus (VWR  
139 International PBI, Milan, Italy). The obtained suspensions were diluted 10-fold and subjected to microbial counts of  
140 total mesophilic aerobes, lactic acid bacteria, Pseudomonadaceae, Enterobacteriaceae and eumycetes. Briefly, total  
141 mesophilic aerobes were counted as reported by Osimani et al. (2011); presumptive mesophilic lactobacilli and  
142 lactococci were enumerated in De Man, Rogosa and Sharpe (MRS) agar medium incubated at 30 °C for 48 h and M17  
143 agar medium incubated at 22 °C for 48 h, respectively as previously described (Aquilanti et al., 2013). The enumeration  
144 of Pseudomonadaceae was carried out using Pseudomonas Agar Base (PAB) with ceftrimide-fucidin-cephalosporin  
145 (CFC) selective supplement (VWR International, Milan, Italy), incubated at 30 °C for 24–48 h (Garofalo et al., 2017),  
146 whereas Enterobacteriaceae were counted in Violet Red Bile Glucose Agar (VRBGA) incubated at 37 °C for 24 h  
147 (Garofalo et al., 2017). Finally, the enumeration of eumycetes was carried out on Wallerstein Laboratory Nutrient  
148 (WLN) agar medium supplemented with chloramphenicol (0.1 g/L) to inhibit the growth of bacteria and incubated at 25  
149 °C for 72 h (Taccari et al., 2016).

150 A miniVIDAS apparatus (Biomérieux, Marcy l'Etoile, France) was used to assess the presence of *Listeria*  
151 *monocytogenes* through the enzyme-linked fluorescent assay (ELFA) method in accordance with the AFNOR BIO  
152 12/11-03/04 validated protocol (Aquilanti et al., 2007).

153

#### 154 2.4. DNA extraction from hákarl samples

155

156 Aliquots (1.5 mL) of each homogenate (dilution  $10^{-1}$ ) prepared as described above were centrifuged for 5 min at 16000  
157 g, and the supernatants were discarded. The cell pellets were then used for the extraction of total microbial DNA using  
158 an E.Z.N.A. soil DNA kit (Omega bio-tek, Norcross, GA, USA) following the manufacturer's instructions. A Nanodrop  
159 ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the quantity and purity of the  
160 extracted DNAs, which were then standardized to a concentration of  $25 \text{ ng } \mu\text{L}^{-1}$  for further analysis. DNA extracts  
161 obtained from the hákarl from each of the two boxes representing one sample (H1-H10) were then pooled and subjected  
162 to PCR-DGGE analyses and 16S rRNA gene amplicon target sequencing (Milanović et al., 2018).

163

#### 164 2.5. PCR-DGGE analysis of bacteria

165

166 The extracted DNA was first amplified by PCR in a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA,  
167 USA) using the universal prokaryotic primers 27F and 1495R described by Weisburg et al. (1991) for the amplification  
168 of 16S rRNA gene. In detail,  $2 \mu\text{L}$  (approximately 50 ng) of DNA from each sample was amplified in a  $25 \mu\text{L}$  reaction  
169 volume composed of 0.5 U of Taq DNA polymerase (Sibenzyme, Novosibirsk, Russia), 1X reaction buffer, 0.2 mM  
170 dNTPs and  $0.2 \mu\text{M}$  of each primer using the cycling program described by Osimani et al. (2015). The PCR products  
171 were checked by routine electrophoresis on 1.5% agarose (w/v) gels and then purified using the Illustra GFX PCR DNA  
172 and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's  
173 instructions.  $2 \mu\text{L}$  of the purified PCR products was used as a template for the amplification of the V3 region of the 16S  
174 rRNA gene with the 338F-518R primer pair (Alessandria et al., 2010). The forward primer, 338F, was attached with the  
175 GC clamp necessary for the following DGGE analysis as described by Ampe et al. (1999). The PCR conditions were  
176 those described by Osimani et al. (2015), except for the Taq polymerase (Sibenzyme) used in the present study.  $5 \mu\text{L}$  of  
177 PCR amplicons was loaded on a 1.5% agarose (w/v) gel with a 100 bp molecular weight marker (HyperLadder™ 100  
178 bp) to check for the expected PCR product size of 180 bp prior to the PCR-DGGE analysis. Subsequently,  $20 \mu\text{L}$  of the  
179 PCR products was loaded on a 30-60% urea-formamide (w/v) gradient DGGE gel (100% corresponds to 7 M urea and  
180 40% (w/v) formamide), and the gel was run at a constant voltage of 130 V for 4 h at  $60 \text{ }^\circ\text{C}$  in  $1\times$  TAE buffer ( $0.04 \text{ mol}$



181 L<sup>-1</sup> Tris–acetate, 0.001 mol L<sup>-1</sup> EDTA) in a DGGE Bio-Rad D-code™ apparatus (Bio-Rad Laboratories). After the  
182 DGGE run, the gel was stained with SYBR Green I Stain 1X (Lonza, Walkersville, MD, USA) in 1X TAE for 30 min,  
183 visualized under UV light and photographed with a Complete Photo XT101 system (Explera). All of the single bands  
184 visible by eye after UV light exposure were excised with gel cutting pipette tips, introduced into 50 µL of molecular  
185 biology grade water and left overnight at 4 °C to allow the elution of the DNA. 5 µL of the eluted DNA was amplified  
186 by PCR as described above but using the 338F and 518R primers without the GC clamp. The amplicons were checked  
187 by electrophoresis and sent to Genewiz (Takeley, UK) for purification and sequencing. The resulting sequences in  
188 FASTA format were compared with those previously deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>)  
189 using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), and only the sequences showing ≥ 97%  
190 similarity was unambiguously assigned into species or genus levels.

191

## 192 2.6. PCR-DGGE analysis of the yeast and filamentous fungal communities

193

194 DNA extracted as previously reported was used for the analysis of the yeast and filamentous fungal communities. An  
195 approximately 250 bp long fragment of the D1/D2 region of the 26S rRNA gene was amplified using NL1 (5'-GCC  
196 ATA TCA ATA AGC GGA GGA AAA G-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') primers (Cocolin  
197 et al., 2000). An additional GC clamp (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG  
198 G-3') was added to the NL1 forward primer. The amplification reactions and conditions were carried out as described in  
199 Palla et al. (2017). The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gels stained with  
200 20,000X REALSAFE Nucleic Acid Staining Solution (Durviz, s.l., Valencia, Spain). All gels were visualized using UV  
201 light and captured as TIFF format files using the UVI 1D v. 16.11a program for the FIRE READER V4 gel  
202 documentation system (Uvitec Cambridge, Eppendorf, Milan, Italy).

203 The amplicons were analyzed using the DCode™ Universal Mutation Detection System (Bio-Rad, Milan, Italy).  
204 Twenty µL of the PCR products in 20 µL of a 2x buffer consisting of 70% glycerol, 0.05% xylene cyanol and 0.05%  
205 bromophenol blue were loaded on an 8% polyacrylamide-bisacrilamide (37.5:1) gel with a urea-formamide denaturing  
206 gradient ranging from 20% to 80%. The gels were run at 80 V and 60 °C for 16 hours and stained for 30 min in 500 mL  
207 of 1x TAE buffer containing 50 µL of Sybr® Gold Nucleic Acid Gel Stain (Life Technologies, Milan, Italy). The  
208 profiles were visualized as previously described. The bands of interest in the DGGE profiles were cut out from the gels  
209 for sequencing. DNA was extracted by eluting for 3 days in 50 µL 10 mM TE at 4 °C. One µL of the supernatant  
210 diluted 1:100 was used to reamplify the D1/D2 regions of the DNA according to the PCR protocol described above  
211 using an NL1 primer without the GC clamp. The amplification products were then purified with the UltraClean PCR

212 CleanUp Kit (MO-BIO Laboratories, CABRU Sas, Arcore, Italy) according to the protocol of the manufacturer,  
213 quantified and 5' sequenced at the Eurofins Genomics MWG Operon (Ebersberg, Germany). Sequences were analyzed  
214 using BLAST on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The related sequences were collected and  
215 aligned using MUSCLE (Edgar, 2004a, b), and phylogenetic trees were constructed using the maximum likelihood  
216 method based on the Kimura 2-parameter model (Kimura, 1980) using Mega 6 software  
217 (<http://www.megasoftware.net/>) with 1000 bootstrap replicates (Tamura et al., 2013). The sequences were submitted to  
218 the European Nucleotide Archive under the accession numbers from LS990841 to LS990863.

219

## 220 2.7. 16S rRNA gene amplicon target sequencing

221

222 DNA directly extracted from *hákarl* samples was quantified using a QUBIT dsDNA Assay kit (Life Technologies,  
223 Milan, Italy) and standardized at 20 ng  $\mu\text{L}^{-1}$  and used a template in the PCR amplifying the V3-V4 region of the 16S  
224 rRNA gene using the primers and protocols described by Klindworth et al. (2013).

225 The PCR amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting  
226 products were tagged using the Nextera XT Index Kit (Illumina Inc. San Diego, CA) according to the manufacturer's  
227 instructions. After the second clean-up, the amplicons were quantified using a QUBIT dsDNA Assay kit and equimolar  
228 amounts of the amplicons from different samples were pooled. The pooled samples were analyzed with an Expiration  
229 workstation (Biorad, Milan, Italy) for quality analysis prior to sequencing. The sample pool was denatured with 0.2 N  
230 NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX prepared according to Illumina  
231 guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry to generate  
232 250 bp paired-end reads according to the manufacturer's instructions.

233

### 234 2.7.1. Bioinformatics analysis

235

236 After sequencing, the paired-end reads were first joined using FLASH software (Magoc and Salzberg, 2011) with  
237 default parameters. Joint reads were quality filtered (at Phred < Q20) using QIIME 1.9.0 software (Caporaso et al.,  
238 2010) and the pipeline recently described (Ferrocino et al., 2017). Briefly, USEARCH software version 8.1 (Edgar et  
239 al., 2011) was used for chimera filtering and Operational Taxonomic Units (OTUs) were clustered at a 99% similarity  
240 threshold using UCLUST algorithms (Edgar, 2010). Centroid sequences of each cluster were mapped against the  
241 Greengenes 16S rRNA gene database version 2013 for taxonomic assignment. To avoid biases due to different  
242 sequencing depths, OTU tables were rarefied at 11010 sequences. The OTU table displays the higher taxonomy

243 resolution that was reached, and the two biological replicates from each sampling point were averaged. The tables were  
244 then imported in the Gephi software (Bastian et al., 2009), and an OTU network was built.

245 All of the sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology  
246 Information (SRA accession number: SRP153795).

247

#### 248 2.7.2. Statistical analysis

249

250 Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were  
251 calculated using the *diversity* function of the *vegan* package (Dixon, 2003). Weighted and unweight UniFrac distance  
252 matrices and OTUs table were used to find differences between the samples using Anosim and Adonis statistical tests  
253 through the function *vegan* in the R environment. Pairwise Wilcoxon tests were used, as appropriate, to determine  
254 significant differences in alpha diversity or OTU abundance. The principal component analysis was plotted using the  
255 function *dudi.pca* through the *made4* R package.

256

#### 257 2.8. Real-time PCR analyses for the detection of foodborne pathogens

258

259 Real-time PCR analyses were performed on a RotorGene Q thermal cycler (Qiagen, Hilden, Germany) exploiting  
260 TaqMan chemistry. All target probes employed were dual-labeled with 5'-FAM and a 3'-nonfluorescent quencher (as  
261 specified below). The oligonucleotides were purchased from ThermoFisher Scientific (Milan, Italy) and from LCG  
262 Biosearch Technologies (Petaluma, CA, USA). The reaction mixtures were all prepared at a final 25 µl reaction  
263 volume. Molecular-grade H<sub>2</sub>O was included in each analytical session as a negative control, as well as DNA from  
264 reference strains as positive controls. Fluorescence was measured in the green channel for the target genes, and in the  
265 yellow channel for the Internal Amplification Control.

266

##### 267 2.8.1. Detection of Shiga-toxin *E. coli* (STEC)

268

269 STEC detection was performed according to the standard ISO/TS 13136:2012 specifications, as also previously  
270 reported (Petruzzelli et al. 2013). Briefly, this method initially targets the Shiga-toxin genes *stx1* and *stx2*, followed by  
271 the *eae* adhesion factor and serogroup-specific genes (O157, O145, O103, O111, O26 and O104:H4). Amplification of  
272 2 µl of template DNA was performed using the QuantiFast Pathogen PCR+IC kit (Qiagen) in combination with the

273 previously reported primer set and 5'-FAM-3'-MGBNFQ dual-labeled probes (Osimani et al. 2018). An Internal control  
274 DNA and Internal Control Assay to be added to the reaction mix were provided with the kit.  
275 DNA from STEC strains provided by the EU Reference Laboratory for STEC - Istituto Superiore di Sanità (Rome,  
276 Italy) were included as positive controls.

277

### 278 2.8.2. Detection of *Pseudomonas aeruginosa*

279

280 *P. aeruginosa* was detected using the QuantiFast Pathogen PCR+IC kit together with a primer-probe set identifying the  
281 presence of the *ecfX* gene, which encodes an extracytoplasmic sigma factor. The probe was dual-labeled with 5'-FAM  
282 and 3'-BHQ1, and the assay mixture was prepared as described by Amagliani et al. (2013). Amplification of 2  $\mu$ l of  
283 template DNA was performed following the thermal protocol indicated by the same authors. DNA from a previously  
284 prepared boiled extract of *P. aeruginosa* ATCC 27853 was included as a positive amplification control in this assay.

285

## 286 3. Results

287

### 288 3.1. pH measurements and microbial viable counts

289

290 The pH values measured in the analyzed samples ranged from between  $8.07\pm 0.06$  and  $8.76\pm 0.00$  (Table 1). The results  
291 of the viable counts are reported in Table 1. In more detail, the mean values of the total mesophilic aerobes ranged from  
292 between  $1.00\pm 0.00$  and  $5.71\pm 0.09$  log cfu  $g^{-1}$ . Low counts of LAB on MRS at 30 °C were recorded with mean values  
293 from between  $< 1$  and  $1.60\pm 0.43$  log cfu  $g^{-1}$ . Regarding the LAB counted on M17 at 22 °C, the mean values ranged  
294 from between  $1.95\pm 0.07$  and  $4.51\pm 0.04$  log cfu  $g^{-1}$ . Pseudomonadaceae counts showed mean values from between  $< 1$   
295 and  $1.59\pm 0.16$  log cfu  $g^{-1}$ . For both the Enterobacteriaceae and Eumycetes counts, mean values  $< 1$  log cfu  $g^{-1}$  were  
296 recorded. Finally, no *Listeria monocytogenes* was detected.

297

### 298 3.2. PCR-DGGE analyses

299

#### 300 3.2.1. Bacteria

301 Regarding the bacteria, the results of PCR-DGGE analysis of the *hákarl* samples are reported in Table 2, while  
302 Supplementary Figure 1 shows the DGGE profiles obtained from the analysis of the microbial DNA directly extracted  
303 from the samples.

304 In more detail, the dominance of close relatives to *Tissierella creatinophila* was clear in all of the pooled samples with  
305 sequence identities from between 85 and 98%. Moreover, close relatives to *Anaerosalibacter* species were detected in  
306 the pooled samples H1, H2 and H10. Finally, close relatives to *Murdochiella massiliensis*, *Sporanaerobacter acetigenes*  
307 and *Pontibacillus marinus* were also found in samples H5, H6 and H10, respectively.

308

### 309 3.2.2. Yeast and filamentous fungal communities

310 A DNA fragment of approximately 250 bp containing the partial D1/D2 domain of the 26S rRNA gene was  
311 successfully amplified from all of the samples, except H1. DGGE analysis of the PCR products showed distinctive  
312 patterns characterized by intense and clearly defined fragments (Supplementary Figure 2). With the goal of identifying  
313 the microbial species present in *hákarl*, the main DGGE bands were excised, sequenced and matched to species by  
314 using BLAST and phylogenetic trees analyses (Table 3 and Figure 2). The sequences matched the yeast species  
315 *Candida tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. zeylanoides*, *Saccharomyces cerevisiae* and the yeast genera  
316 *Debaryomyces*, *Torulaspota*, *Yamadazyma*, *Sporobolomyces*. Figure 3 shows the relative percentages of the most  
317 abundant fungal genera detected in the different samples. For each sample, the percentage was calculated by dividing  
318 the number of fragments referring to a genus by their total number. Each *hákarl* sample showed a different yeast  
319 composition, with *Debaryomyces* occurring in all the samples, although at different levels. In three *hákarl* samples (H2,  
320 H6 and H7), sequences related to filamentous fungal species were also found. In particular, *Cladosporium tenuissimum*  
321 occurred in the H2 sample, while *Moristroma quercinum* was found in the H7 sample. The H6 sample was  
322 characterized by the presence of *Alternaria*, and genera belonging to the family Didymellaceae, such as *Phoma* and  
323 *Epicoccum*.

324

### 325 3.3. 16S rRNA gene amplicon target sequencing

326

327 The total number of paired sequences obtained from the 16S rRNA gene sequencing reached 3,208,571 raw reads. After  
328 merging, a total of 997,224 reads passed the filters applied through QIIME, with an average value of  $49.861,2 \pm$   
329  $17.399,72$  reads/sample, and a mean sequence length of 456 bp. The rarefaction analysis and Good's coverage,  
330 expressed as a median percentage (95%), also indicated satisfactory coverage for all samples (Supplementary Table 1).  
331 Alpha-diversity indicated a higher level of complexity and the highest number of OTUs when only taking into the  
332 account the sample H3 ( $P < 0.05$ ). Adonis and analysis of similarity (ANOSIM) statistical tests based on weighted and  
333 unweighted UniFrac distance matrices showed significant differences among the samples ( $P < 0.001$ ;  $R = 0.980$ ).  
334 Differences between the samples were further demonstrated by principal-component analysis (PCA) based on the

335 relative abundance of the main OTUs (Fig. 4). The PCA clearly showed a separation between samples (ANOSIM  
336 statistical test  $P < 0.01$ ). As shown in Fig. 7, the main OTUs shared among the data set were *Tissierella* (78.6% of the  
337 relative abundance) (Table 4), *Pseudomonas* (8.4%), *Abyssivirga* (4.0%), *Oceanobacillus* (6.7%) and *Lactococcus*  
338 (0.2%), and based on the size of the edges, it was possible to see that *Tissierella* can be considered a core OTU of the  
339 *hákarl* (Fig. 5 and Table 4). Several minor OTUs (<1%) were also detected, including *Listeria*, *Staphylococcus*,  
340 *Photobacterium* and *Acinetobacter* (Table 4).

341

### 342 3.4. Real-time PCR analyses

343

344 Fluorescence signals resulting from real-time PCR assays were analyzed by manually positioning the cycle threshold at  
345 the take-off point of the positive control's amplification curve relative to the gene under investigation.

346 All DNA samples analyzed yielded negative results for both pathogens of interest. Every reaction mixture, regardless of  
347 the type of master mix or internal amplification control used, yielded positive signals in the yellow channel, thus  
348 ensuring the absence of inhibition and excluding false negative results.

349 As for STEC strain, the first detection step (aimed at revealing the two Shiga toxin-encoding genes *stx1* and *stx2*) were  
350 performed in singleplex, since the specific probes were labeled with the same fluorophore. All of the DNA samples  
351 tested negative for both sequences; therefore, no further analysis of *eae* or serogroups was necessary according to the  
352 ISO/TS 13136:2012. The reference strain used in this experiment was EURL-VTEC D07 *E. coli* O26 (*stx1+*, *stx2+*,  
353 *eae+*).

354

## 355 4. Discussion

356

357 Among the fermented fish products of northern European countries, *hákarl* represents a masterful example of a delicacy  
358 and niche product that, in former times, nourished the Icelandic populations (Skåra et al., 2015). It is noteworthy that  
359 only a few producers are currently carrying out the production of *hákarl* in accordance with ancient traditions that  
360 maintain the use of Greenland shark flesh.

361 Greenland shark is a slow growing, coldwater shark that can reach 21 feet in length. As reported by MacNeil et al.  
362 (2012), the physiology of the Greenland shark is not generally well studied. It is noteworthy that high levels of  
363 trimethylamine N-oxide (TMAO) have been detected in its flesh by different authors (Anthoni et al., 1991; Bedford et  
364 al., 1998; Goldstein et al., 1967; Seibel and Walsh, 2002). The role of such a compound is not completely understood;  
365 nevertheless, it is thought that the high concentrations found in polar fish suggest that this osmolyte may contribute to

366 the enhancement of osmotic concentrations, thus lowering the freezing point of the bodily fluids (MacNeil et al., 2012).  
367 Moreover, both TMAO and its reduced form TMA represent low-density molecules that can increase the buoyancy of  
368 the shark. It is also thought that, due to the high urea concentrations present in elasmobranchs like the Greenland shark,  
369 TMAO might act as a counteracting solute that protects proteins from destabilization (MacNeil et al., 2012; Seibel and  
370 Walsh, 2002).

371 The attention of the food industry and consumers towards locally produced traditional food is constantly increasing.  
372 Although both the ancient and modern processing steps used to obtain *hákarl* from Greenland shark are mostly  
373 recognized and standardized, less is known about the chemical and microbiological traits of such a food product. Based  
374 on the physiology of the Greenland shark, it is thought that the microbiota occurring in *hákarl* might be strongly  
375 influenced by the peculiarities of the flesh used as raw material. A 30-year-old study attempted to identify the bacterial  
376 species in *hákarl* (Magnússon and Guðbjörnsdóttir, 1984) with no mention of the possible occurrence of eumycetes.  
377 Hence, to our knowledge, the microbiology of such a fermented food product has not yet been unveiled. The present  
378 study aimed to reveal the microbial species occurring in ready-to-eat *hákarl* samples using a combination of traditional  
379 microbiological culture-dependent (viable counts) and -independent methods (namely, PCR-DGGE, amplicon-based  
380 sequencing and qualitative real-time PCR).

381 The samples under study were first subjected to pH measurements. The detected values were in agreement with those  
382 reported by Skåra et al. (2015) for dried ready-to-eat *hákarl*. High pH values can be explained by microbial metabolic  
383 activities that led to the conversion of urea, which is naturally present in Greenland shark flesh, into ammonia (Skåra et  
384 al., 2015). Interestingly, Jang et al. (2017) reported similar pH values (8.4-8.9) in alkaline-fermented skate, which  
385 represents a typical fermented seafood in South Korea. It is known that skates retain urea and trimethylamine N-oxide  
386 in their muscles; hence, similarly to *hákarl*, the fermentation of skate, which is carried out at low temperature, leads to  
387 the production of a distinctive odor due to the ammonia and trimethylamine produced during fermentation (Reynisson  
388 et al., 2012).

389 In the present study, the viable counts of total mesophilic aerobes, presumptive mesophilic lactobacilli and lactococci,  
390 Pseudomonadaceae, Enterbacteriaceae and eumycetes were assessed.

391 Regarding the total mesophilic aerobes, the counts were generally lower than the value of  $8 \log \text{cfu g}^{-1}$  reported by  
392 Skåra et al. (2015) in *hákarl* at the end of ripening.

393 As far as the counts of lactic acid bacteria are concerned, little is known about the magnitude of their presence in  
394 *hákarl*, though this microbial group is known to play a primary role in the production of many other traditional  
395 fermented fish, such as *jeotgal* from Korea, *shidal* from India, *rakfisk* from Norway, and numerous fermented fish  
396 products from China (François, 2010; Majumdar et al., 2016; Skåra et al., 2015; Thapa et al., 2016; Xu et al., 2018;

397 Zang et al., 2018a). In addition to this, the occurrence of lactic acid bacteria in the marine environment has been  
398 reported (Gómez-Sala et al., 2016). Of note, the differences in the counts of presumptive lactococci in comparison with  
399 those of presumptive mesophilic lactobacilli in *hákarl*. This difference agrees well with the results from metagenomic  
400 sequencing that highlighted the sole presence of lactococci among lactic acid bacteria. Such a difference might  
401 tentatively be ascribed to both the higher adaptation of lactococci to shark flesh fermentation conditions and their  
402 acknowledged higher competitiveness in protein-rich substrates explained by their higher proteolytic and peptidase  
403 activities in respect with lactobacilli (Requena et al., 1993; Quigley et al., 2013; Terzic-Vidojevic et al., 2014),  
404 especially under alkaline conditions (Addi and Guessas, 2016).

405 The presence of Pseudomonadaceae has already been described in alkaline-fermented skate, where *Pseudomonas* was  
406 reported to be among the dominant genera (Jang et al., 2017; Reynisson et al., 2012).

407 Regarding Enterobacteriaceae, it is noteworthy that their minimum pH for growth is 3.8, with an upper limit of  
408 approximately 9.0; hence, even if they were present, the high pH of the samples did not allow Enterobacteriaceae to  
409 grow in the fermented flesh. Among Enterobacteriaceae, STEC strains represent a major health treat for humans due to  
410 their ability to produce food-borne infections (EFSA and ECDC, 2016). Although *E. coli* is not naturally harbored by  
411 fish, STEC strains have recently been isolated by Cardozo et al. (2018) from the fish *Nile tilapia*. Of note is the absence  
412 of STEC strains in all of the analyzed *hákarl* samples, as shown by real-time PCR assays.

413 In the present study, viable counts  $< 1 \log \text{cfu g}^{-1}$  were reported for eumycetes.

414 The use of PCR-DGGE and amplicon sequencing allowed major and minor microbial species to be detected in the  
415 analyzed *hákarl* samples.

416 In more detail, a massive presence of *Tissierella* was detected by both PCR-DGGE and amplicon sequencing in all the  
417 analyzed samples. *Tissierella* belongs to the *Tissierellia* classis nov., which includes the genera *Anaerococcus*,  
418 *Anaerosphaera*, *Finegoldia*, *Gallicola*, *Helcococcus*, *Murdochiella*, *Parvimonas*, *Peptoniphilus*, *Soehngenia*,  
419 *Sporanaerobacter*, and *Tepidimicrobium*. Moreover, the misclassified species *Bacteroides coagulans* and *Clostridium*  
420 *ultunense* are also included.

421 Members of the class *Tissierellia* are Gram-positive or Gram-variable anaerobic obligate cocci and rods. As reported by  
422 Chen et al. (2018), the genus *Tissierella* comprises protein-degrading anaerobes that can produce volatile fatty acids  
423 such as acetic, butyric, and propionic acids. Moreover, it is acknowledged that *Tissierella creatinophila* grows on  
424 creatinine as sole source of carbon and energy (Harms et al., 1998a). *Tissierella* species can degrade creatinine via  
425 creatine, sarcosine, and glycine into monomethylamine, ammonia, carbon dioxide, and acetyl phosphate (Harms et al.,  
426 1998b). Interestingly, active *Tissierella* cells have already been reported among the major genera involved in the  
427 production of alkaline-fermented skate (Jang et al., 2017), thus confirming the possible adaptation of this microbial



428 genus to saline and alkaline conditions, as suggested by Jang et al. (2017). It is noteworthy that species belonging to the  
429 *Tissierella* genus have also been described as psychrotolerant microorganisms (Prevost et al., 2013), thus likely  
430 explaining the presence of members of such genus in the analyzed *hákarl* samples.

431 Regarding the presence of *Oceanobacillus*, which was detected only through amplicon sequencing, it is worth noting  
432 that this bacterial genus comprises Gram-positive, spore forming rods that are obligate aerobes, extremely halotolerant  
433 and facultatively alkaliphilic (Lu et al., 2001). Moreover, *Oceanobacillus* species can grow at temperatures between 15  
434 and 42 °C and in pH range from 6.5 to 10, with an optimum pH between 7.0 and 9.5 (Lu et al., 2001). Kumar et al.  
435 (2012) demonstrated that halotolerant bacteria, including *Oceanobacillus*, can produce enzymes that are salt stable and  
436 active under extreme conditions. Interestingly, Yukimura et al. (2009) reported the isolation of *Oceanobacillus* strains  
437 from a glacial moraine in Qaanaaq, Greenland, and this finding likely explains the presence of these spore forming  
438 bacteria in the analyzed *hákarl* samples.

439 Amplicon sequencing supported the detection of *Abyssivirga*. This bacterial genus belongs to the Lachnospiraceae  
440 family and comprises strictly anaerobic, mesophilic and syntrophic organisms. To the our best knowledge, the sole  
441 species that belongs to this genus is represented by *Abyssivirga alkaniphila*, which is an alkane-degrading, anaerobic  
442 bacterium recently isolated from a deep-sea hydrothermal vent system (Catania et al., 2018; Schouw et al., 2016). This  
443 observation explains the presence of this bacterium in the marine environment and, hence, in the *hákarl* samples. This  
444 species can grow between 14–42 °C and within a pH range between 7.0 and 8.2. As reported by Schouw et al. (2016),  
445 who first described this species, *A. alkaniphila* can ferment carbohydrates, peptides and aliphatic hydrocarbons.

446 As for the presence of *Pseudomonas*, which was detected through amplicon sequencing, it is noteworthy that this  
447 bacterial genus has already been reported among the most abundant isolates in fermented skate (Jang et al., 2017). The  
448 genus *Pseudomonas* encompasses many alkaliphiles; among these, Yumoto al. (2001) reported the isolation of a novel  
449 facultatively psychrotrophic alkaliphilic species of *Pseudomonas*, being *Pseudomonas alcaliphila* sp. nov. This species  
450 of marine origin can grow at 4 °C and at pH 10 in the presence of NaCl; these features likely explain the presence of the  
451 genus *Pseudomonas* in the analyzed *hákarl* samples. Moreover, it is widely acknowledged that the *Pseudomonas*  
452 species responsible for fresh fish spoilage can also be present in fish-based foods (Stanborough et al., 2018). In this  
453 regard, Liffourrena et al. (2010) reported that some species of *Pseudomonas* possess TMA degradation pathways. More  
454 specifically, in *Pseudomonas aminovorans* TMAO is oxidized by a trimethylamine monooxygenase (TMA  
455 monooxygenase), whereas TMA can be directly dehydrogenated to formaldehyde and dimethylamine (DMA) by a  
456 trimethylamine dehydrogenase (TMA dehydrogenase). Similarly, in *Pseudomonas putida* the same enzymes, namely,  
457 TMA monooxygenase and TMA dehydrogenase, oxidize TMA under aerobic conditions (Liffourrena et al., 2010). On  
458 the one hand, these metabolic pathways represent a selective advantage for *Pseudomonas*, and on the other hand, they

459 lead to the removal of TMA (Liffourrena et al., 2010). Although the amounts of TMAO and TMA were not assessed in  
460 the present study, it can be hypothesized that the metabolic activity of *Pseudomonas* species could presumably lead to  
461 the detoxification of TMAO or TMA in the shark meat or during fermentation.

462 Of note, the pathogenic species *P. aeruginosa* was absent in all of the samples, as shown by qualitative real-time PCR  
463 assays.

464 In the analyzed *hákarl* samples, the presence of *Lactococcus* was also detected through amplicon sequencing. The  
465 presence of lactococci in the marine environment has already been demonstrated (Gómez-Sala et al., 2016); moreover,  
466 Alonso et al. (2018) recently reported the isolation of *Lactococcus* strains from the gut of marine fishes. As reported by  
467 Guan et al. (2011), lactic acid bacteria were among the dominant genera isolated from *saeu-jeot*, a Korean salted  
468 fermented food produced made with shrimp (*Acetes japonicas*). Although their role has not yet been fully clarified,  
469 lactococci were also detected in fermented skate and in *narezushi*, which is produced through the fermentation of salted  
470 fish with rice (Jang et al., 2017). As reported by Françoise (2010), lactic acid bacteria are generally less competitive in  
471 fish flesh than psychrotrophic Gram-negative bacteria; nevertheless, Ji et al. (2017) highlighted the essential role of  
472 lactic acid bacteria (including *Lactococcus*) in flavor definition during fish fermentation. It is also noteworthy that lactic  
473 acid bacteria can exert a potential biopreservative activity in seafood products (Ghanbari et al., 2013).

474 Regarding *Alkalibacterium*, the presence of this marine lactic acid bacteria has already been reported in marine  
475 environments and organisms (Jang et al., 2017) as well as in marine-based foods such as *jeotgal* (Guan et al., 2011).  
476 Interestingly, species belonging to the *Alkalibacterium* genus were found in fermented Spanish-style green table-olives  
477 and blue-veined raw milk cheese, thus confirming the high adaptation of this genus to halophilic and alkaline conditions  
478 (Lucena-Padrós et al., 2015; Yunita and Dodd, 2018).

479 *Photobacterium*, detected through amplicon sequencing, encompasses species that are naturally present in the marine  
480 environment with both symbiotic or pathogenic relationships with marine organisms (Labella et al., 2018). This  
481 bacterial genus was also detected by Reynisson et al. (2012) in fermented skate, where it was thought to play a key role  
482 in the fish flesh fermentation.

483 Although found in a limited number of samples, *Proteiniclasticum* and *Anaerobacillus* were also detected. The former  
484 genus includes anaerobic bacteria (e.g., *Proteiniclasticum ruminis*) with proteolytic activity (Zhang et al., 2010),  
485 whereas the latter genus includes species that can grow in alkaline environments (e.g., *Anaerobacillus alkalilacustre*)  
486 (Zavarzina et al., 2009).

487 The low occurrence of *Staphylococcus* suggests a possible contamination during the processing of *hákarl*, even though  
488 Ji et al. (2017) suggested a possible role of this genus in the bacterial amino acid metabolism occurring during the  
489 fermentation of the fish *Siniperca chuatsi*, together with *Acinetobacter*. Of note is that although found as a minority

490 species in the *hákarl* samples analyzed in the present study, Magnússon and Guðbjörnsdóttir (1984) reported  
491 *Acinetobacter* as one of the fermentation-driving microorganisms during the production, along with *Lactobacillus*.  
492 Regarding *Ochrobactrum*, it is noteworthy that members belonging to this halophilic genus have recently been isolated  
493 by Jamal and Pugazhendi (2018) in Red Sea saline water and sediments.  
494 *Erysipelothrix* was sporadically detected in the analyzed *hákarl* samples through metagenomic sequencing.  
495 Interestingly, species of this genus have already been detected in soils collected from the Ross Sea region of Antarctica,  
496 which is characterized by extreme low temperatures and high water salinity (Aislabie et al., 2008).  
497 *Listeria* was found as minority OTU by amplicon sequencing; notwithstanding, no viable cells belonging to the  
498 pathogenic species *L. monocytogenes* were found in any of the analyzed *hákarl* samples in accordance with the results  
499 reported by Jang et al. (2017) in fermented skate. It is noteworthy that in seafood from cold environments *L.*  
500 *monocytogenes* represents a foodborne pathogen of increasing public health and food safety concern (Elbashir et al.,  
501 2018; Jami et al., 2014).  
502 Although the bacterial biota in *hákarl* has already tentatively been studied by Magnússon and Guðbjörnsdóttir back in  
503 1984, to our knowledge, the present study represents the first attempt to gain insight into the fungal biota present in this  
504 fermented fish product.  
505 The assessment of the diversity of yeast communities present in the *hákarl* samples revealed the occurrence of 4 yeast  
506 genera: *Debaryomyces*, *Candida*, *Saccharomyces*, *Torulospora*, which are commonly found in several traditional  
507 fermented beverages and food products, including fermented fish (Tamang et al., 2016), along with two other genera,  
508 *Yamadazyma* and *Sporobolomyces*. The occurrence of sequences affiliated to *Debaryomyces* in all of the *hákarl*  
509 samples suggests that this genus may be the main organism responsible for the late stages of *hákarl* fermentation. This  
510 yeast, retrieved from the skin and inside the intestines of fresh fish by Andlid et al. (1995) and Gatesoupe (2007), was  
511 also reported to be able to grow at extremely high salt concentrations and low water activity ( $a_w$ ) (Asefa et al., 2009;  
512 Viljoen and Greyling, 1995), characteristics of the ripened *hákarl*.  
513 Our findings are consistent with previous reports showing the occurrence of this genus in salted and traditionally  
514 fermented fish from Thailand and Ghana (Paludan-Muller et al., 2002; Sanni et al., 2002). The yeasts of the genus  
515 *Debaryomyces* might positively contribute to the development of the sensory qualities of fermented fish, as they are  
516 known to occur in cheeses and dry-cured meat products. More specifically, in cheeses with high salt content, *D.*  
517 *hansenii* was found to predominate, being responsible for the acceleration of lipolysis and proteolysis (Andrade et al.,  
518 2009).  
519 Several sequences affiliated with different species from the genus *Candida* were retrieved in almost all the *hákarl*  
520 samples. In particular, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. zeylanoides* were identified. Our results agree

521 with a previous report showing the dominance of the genus *Candida* in ripened *Suan yu*, a Chinese traditional fermented  
522 fish (Zang et al., 2018b) and a study demonstrating the presence of *C. zeylanoides* and *C. tropicalis* in a salted and  
523 fermented traditional fish called “*adjuevan*”, which is produced in Ivory Coast (Clementine et al., 2012).

524 *Candida* was reported to produce more flavoring substances than other yeasts by metabolizing branched-chain amino  
525 acids (BCAAs) through the Ehrlich pathway (O’Toole, 1997). In particular, *C. tropicalis* was also isolated from  
526 *Burukutu*, a Nigerian traditional fermented beer, and it was shown to produce protease, phytase, lipase and esterase  
527 enzymes (Ogunremi et al., 2015). These last two enzymes improve the aromatic profile of fermented foods by  
528 increasing their free fatty acid content, which are precursors to the formation of different aromatic compounds (Arroyo-  
529 Lopez et al., 2012).

530 Three sequences affiliated with *Saccharomyces* were retrieved from the nine *hákarl* samples, confirming the presence  
531 of such yeasts in fermented fish (Clementine et al., 2012; Zang et al. 2018a).

532 Close relatives of *Yamadazyma* and *Sporobolomyces* were also detected in the H9 and H10 *hákarl* samples,  
533 respectively. These two yeast genera were not previously recovered from fermented fish products, although  
534 *Sporobolomyces* is a marine yeast commonly found in deep-sea waters (Kutty and Philip, 2008).

535 As for the occurrence of filamentous fungi, sequences affiliated with the genera *Alternaria*, *Cladosporium*,  
536 *Phoma/Epicoccum*, and *Moristroma* were retrieved from the analyzed *hákarl* samples. Among such fungal genera,  
537 *Cladosporium* was previously found during the fermentation of the Chinese traditional fermented fish *Suan yu* (Zang et  
538 al. 2018). The presence of filamentous fungi, such as *Aspergillus*, *Penicillium* and *Mucor*, was also reported from some  
539 Japanese fermented fish products, *i.e.*, *Katsuobushi* and *Narezushi*, where they produced enzymes such as amylase,  
540 protease, and lipase, which are important for the improvement of the nutritional and functional traits of fermented goods  
541 (Fukuda et al., 2014).

542

## 543 **Conclusions**

544

545 Overall, the combination of culture-dependent and -independent methods allowed major and minor microbial species  
546 harbored by the ready-to-eat *hákarl* samples to be detected. The culture-dependent approach provided insight into the  
547 viable microbial species, whereas the culture-independent methods were pivotal in preventing the possible  
548 underestimation of bacterial diversity caused by culturing biases or the presence of microbial cells in the “viable but  
549 non culturable” (VBNC) state. It is noteworthy that although amplicon sequencing was essential for detecting major and  
550 minor components of the bacterial biota, the use of PCR-DGGE led to the identification of both bacterial and fungal

551 populations at the species level, thus contributing to the development of a first overview of the microbiota occurring in  
552 this poorly studied food product.

553 Based on our results, *hákarl* revealed a complex and heterogeneous biodiversity. The bacterial community was  
554 characterized by species well adapted to alkaline and saline environments; the dominant genus was *Tissierella*, followed  
555 by *Oceanobacillus*, *Pseudomonas* and *Abyssivirga*. Moreover, based on the presence of *Pseudomonas* in the analyzed  
556 samples, a role of this bacterial genus in the detoxification of TMAO or TMA in shark meat during fermentation may be  
557 hypothesized. The fungal community was mainly represented by *Debaryomyces*, *Candida* and, to a lesser extent,  
558 *Saccharomyces* species, which through interactions with the bacterial community might play key roles in the late stages  
559 of *hákarl* fermentation, especially contributing to the development of the sensory qualities of the end product. Further  
560 studies are needed to establish the roles and the viabilities of the detected microbial species occurring during shark  
561 fermentation, as well as their interactions and relationships with the physical-chemical and rheological parameters of  
562 *hákarl*. Moreover, the occurrence of spore-forming bacteria should also be evaluated since the presence of these  
563 microorganisms has already been reported by different authors in fresh or fermented fish products (Metcalf et al., 2011,  
564 Reynisson et al., 2012). It is noteworthy that, among spore forming bacteria, *Clostridium botulinum* type E spores and  
565 toxins (produced even at low temperatures) can commonly be found in seafood (Elbashir et al., 2018; Iwamoto et al.,  
566 2010), thus representing a serious health threat for the consumers.

567

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569

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572

#### 573 **References**

574

575 Aislabie, J.M., Jordan, S., Barker, G.M. 2008. Relation between soil classification and bacterial diversity in soils of the  
576 Ross Sea region, Antarctica. *Geoderma* 144, 9–20.

577 Addi, N., Guessas, B., 2016. Characterization of protease activity of *Lactococcus lactis* species isolated from raw  
578 camel's milk. *J. Biol. Sci.* 16 (6), 215-220.

579 Alessandria, V., Dolci, P., Rantsiou, K., Pattono, D., Dalmaso, A., Civera, T., Coccolin, L., 2010. Microbiota of the  
580 Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde  
581 Islands. *World J. Microbiol. Biotechnol.* 26, 2211–2221.

- 582 Alonso, S., Castro, C., Berdasco, M., García de la Banda, I., Moreno-Ventas, X., Hernández de Rojas, A., 2018.  
583 Isolation and Partial Characterization of Lactic Acid Bacteria from the Gut Microbiota of Marine Fishes for  
584 Potential Application as Probiotics in Aquaculture. *Probiotics Antimicro.*, [https://doi.org/10.1007/s12602-018-](https://doi.org/10.1007/s12602-018-9439-2)  
585 [9439-2](https://doi.org/10.1007/s12602-018-9439-2)
- 586 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.*  
587 215, 403–410.
- 588 Amagliani, G., Schiavano, G.F., Stocchi, V., Bucci, G., Brandi, G., 2013. Application of real-time PCR to *Pseudomonas*  
589 *aeruginosa* monitoring in a public swimming pool. *Microchem. J.* 110, 656–659.
- 590 Ampe, F., Ben Omar, N., Moizan, C., Wachter, C., Guyot, J.P., 1999. Polyphasic study of the spatial distribution of  
591 microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent  
592 methods to investigate traditional fermentations. *Appl. Environ. Microbiol.* 65, 5464–5473.
- 593 Andlid, T., Juárez, R.V., Gustafsson, L., 1995. Yeast colonizing the intestine of rainbow trout (*Salmo gairdneri*) and  
594 turbot (*Scophthalmus maximus*). *Microb. Ecol.* 30, 321-334.
- 595 Andrade, M.J., Cordoba, J.J., Sanchez, B., Casado, E.M., Rodriguez, M., 2009. Evaluation and selection of yeasts  
596 isolated from dry-cured Iberian ham by their volatile compound production. *Food Chem.* 113, 457-463.
- 597 Anthoni, U., Christophersen, C., Gram, L., Nielsen, N.H., Nielsen, P., 1991. Poisonings from flesh of the Greenland  
598 shark *Somniosus microcephalus* may be due to trimethylamine. *Toxicon* 29, 1205-1212.
- 599 Aquilanti, L., Santarelli, S., Babini, V., Osimani, A., Clementi, F., 2013. Quality evaluation and discrimination of semi-  
600 hard and hard cheeses from the Marche region (Central Italy) using chemometric tools. *Int. Dairy J.* 29 (1), 42-  
601 52.
- 602 Aquilanti, L., Santarelli, S., Silvestri, G., Osimani, A., Petruzzelli, A., Clementi, F., 2007. The microbial ecology of a  
603 typical Italian salami during its natural fermentation. *Int. J. Food Microbiol.* 120, 136–145.
- 604 Arroyo-Lopez, F.N., Romero-Gil, V., Bautista-Gallego, J., Rodriguez-Gomez, F., Jimenez-Diaz, R., Garcia-Garcia, P.,  
605 Querol, A., Garrido-Fernandez, A., 2012. Yeasts in table olive processing: desirable or spoilage microorganisms?  
606 *Int. J. Food Microbiol.* 160, 42-49.
- 607 Asefa, D.T., Møretrø, T., Gjerde, R.O., Langsrud, S., Kure, C.F., Sidhu, M.S., Nesbakken, T., Skaar, I., 2009. Yeast  
608 diversity and dynamics in the production processes of Norwegian dry-cured meat products. *Int. J. Food*  
609 *Microbiol.* 133, 135-140.
- 610 Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: an open source software for exploring and manipulating networks.  
611 In Proceedings of the Third International Conference on Weblogs and Social Media.

- 612 Bedford, J.J., Harper, J.L., Leader, J.P., Smith, R.A.J., 1998. Identification and measurement of methylamines in  
613 elasmobranch tissues using proton nuclear magnetic resonance (H-1-NMR) spectroscopy. *J. Comp. Physiol. B*  
614 168, 123–131.
- 615 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G.,  
616 Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A.,  
617 McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A.,  
618 Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of highthroughput  
619 community sequencing data. *Nat. Methods* 7, 335–336.
- 620 Cardozo, M.V., Borges, C.A., Beraldo, L.G., Maluta, R.P., Pollo, A.S., Borzi, M.M., Dos Santos, L.F., Kariyawasam,  
621 S., Ávila, F.A., 2018. Shigatoxigenic and atypical enteropathogenic *Escherichia coli* in fish for human  
622 consumption. *Braz. J. Microbiol.* pii: S1517-8382(17)30946-2.
- 623 Catania, A., Cappello, S., Di Giorgi, V., Santisi, S., Di Maria, R., Mazzola, A., Vizzini, S., Quatrini, P., 2018. Microbial  
624 communities of polluted sub-surface marine sediments. *Mar. Pollut. Bull.* 131, 396–406.
- 625 Chen, S., He, J., Wang, H., Dong, B., Li, N., Dai, X., 2018. Microbial responses and metabolic pathways reveal the  
626 recovery mechanism of an anaerobic digestion system subjected to progressive inhibition by ammonia. *Chem.*  
627 *Engin. J.* 350, 312–323.
- 628 Clementine, K.A., Mohamed, C., Epiphane, K., Kouakou, D.B., Dje, M.K., Montet, D., 2012. Identification of yeasts  
629 associated with the fermented fish, adjuevan, of Ivory Coast by using the molecular technique of PCR-denaturing  
630 gradient gel electrophoresis (DGGE). *Afr. J. Microbiol. Res.* 6, 4138-4145.
- 631 Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. *FEMS*  
632 *Microbiol. Lett.* 189, 81-87.
- 633 Dixon, P., 2003. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927-930.
- 634 Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461.
- 635 Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of  
636 chimera detection. *Bioinformatics* 27, 2194–2200.
- 637 Elbashir, S., Parveen, S., Schwarz, J., Rippen, T., Jahneke, M., DePaola, A., 2018. Seafood pathogens and information  
638 on antimicrobial resistance: A review. *Food Microbiol.* 70, 85-93.
- 639 EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2017. The  
640 European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in  
641 2016. *EFSA Journal* 2017;15(12):5077, 228 pp. <https://doi.org/10.2903/j.efsa.2017.5077>
- 642 Ferrocino, I., Bellio, A., Romano, A., Macori, G., Rantsiou, K., Decastelli, L., Cocolin, L., 2017. RNA-based amplicon

- 643 sequencing reveals the microbiota development during ripening of artisanal vs. industrial Lard d'Arnad. Appl.  
644 Environ. Microbiol. 83, (16) e00983-17.
- 645 Franco, W., Johanningsmeier, S., Lu, J., Demo, J., Wilson, E., Moeller, L., 2016. Cucumber Fermentation. Lactic Acid  
646 Fermentation of Fruits and Vegetables, pp. 107-155, Paramithiotis, S., Ed., CRC Press, Taylor & Francis Group,  
647 Milton Park, Abingdon, United Kingdom.
- 648 Françoise, L., 2010. Occurrence and role of lactic acid bacteria in seafood products. Food Microbiol. 27, 698-709.
- 649 Fukuda, T., Furushita, M., Shiba, T., Harada, K., 2014. Fish Fermented Technology by Filamentous Fungi. J. National  
650 Fish. Univ. 62, 163-168.
- 651 Garofalo, C., Bancalari, E., Milanović, V., Cardinali, F., Osimani, A., Savo Sardaro, M.L., Bottari, B., Bernini, V.,  
652 Aquilanti, L., Clementi, F., Neviani, E., Gatti, M., 2017a. Study of the bacterial diversity of foods: PCR-DGGE  
653 versus LH-PCR. Int. J. Food Microbiol. 242, 24–36.
- 654 Gatesoupe, F.J., 2007. Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health  
655 and development. Aquaculture 267, 20-30.
- 656 Ghanbari, M., Jami, M., Domig, K.J., Kneifel, W., 2013. Seafood biopreservation by lactic acid bacteria: A review.  
657 LWT - Food Sci. Technol. 54, 315-324.
- 658 Goldstein, L., Hartman, S.C., Forster, R.P., 1967. On origin of trimethylamine oxide in spiny dogfish *Squalus acanthias*.  
659 Comp. Biochem. Physiol. 21, 719–722.
- 660 Gómez-Sala, B., Herranz, C., Díaz-Freitas, B., Hernández, P.E., Sala, A., Cintas, L.M., 2016. Strategies to increase  
661 the hygienic and economic value of fresh fish: Biopreservation using lactic acid bacteria of marine origin. Int. J.  
662 Food Microbiol. 223, 41–49.
- 663 Guan, L., Cho, K.H., Lee, J.H., 2011. Analysis of the cultivable bacterial community in jeotgal, a Korean salted and  
664 fermented seafood, and identification of its dominant bacteria. Food Microbiol. 28, 101-113.
- 665 Halsted, B.W., 1962. Biotoxications, allergies and other disorders. In: Borgstrom G, editor. Fish as food. New York  
666 (USA): Academic Press; p. 521-540.
- 667 Harms, C., Schleicher, A., Collins, M.D., Andreesen, J.R., 1998a. *Tissierella creatinophila* sp. nov., a gram-positive,  
668 anaerobic, non-spore-forming, creatinine-fermenting organism. Int. J. Syst. Bacteriol. 3, 983-993.
- 669 Harms, C., Ludwig, U., Andreesen, J.R., 1998b. Sarcosine reductase of *Tissierella creatinophila*: purification and  
670 characterization of its components. Arch. Microbiol. 170, 442–450.
- 671 Huang, Y., 2016. Fermented Food and Ancient Civilization. 2016 3rd International Conference on Social Science (ICSS  
672 2016), 1307-1309.



- 673 ISO/TS 13136:2012 Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method  
674 for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing  
675 *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups.
- 676 Iwamoto, M., Ayers, T., Mahon, B.E., Swerdlow, D.I., 2010. Epidemiology of seafood associated infections in the  
677 United States. *Clin. Microbiol. Rev.* 23, 399-411.
- 678 Jamal, M.T., Pugazhendi, A. 2018. Degradation of petroleum hydrocarbons and treatment of refinery wastewater under  
679 saline condition by a halophilic bacterial consortium enriched from marine environment (Red Sea), Jeddah, Saudi  
680 Arabia. *3 Biotech.* 8(6), 276.
- 681 Jami, M., Ghanbari, M., Zunabovic, M., Domig, K.J., Kneifel, W., 2014. *Listeria monocytogenes* in aquatic food  
682 products - a review. *Compr. Rev. Food Sci. Food Saf.* 13, 798–813.
- 683 Jang, G.I., Kim, G., Hwang, C.Y., Cho, B.C., 2017. Prokaryotic community composition in alkaline-fermented skate  
684 (*Raja pulchra*). *Food Microbiol.* 61, 72-82.
- 685 Ji, C., Zhang, J., Lin, X., Han, J., Dong, X., Yang, S., Yan, X., Zhu, B., 2017. Metaproteomic analysis of microbiota in  
686 the fermented fish, *Siniperca chuatsi*. *LWT - Food Sci. Technol.* 80, 479-484.
- 687 Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies  
688 of nucleotide sequences. *J. Mol. Evol.* 16, 111-120.
- 689 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general  
690 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies.  
691 *Nucleic Acids Res.* 41, e1. <https://doi.org/10.1093/nar/gks808>.
- 692 Kumar, S., Karan, R., Kapoor, S., Singh, S.P., Khare, S.K., 2012. Screening and isolation of halophilic bacteria  
693 producing industrially important enzymes. *Braz. J. Microbiol.* 43, 1595–1603.
- 694 Kutty, S.N., Philip, R., 2008. Marine yeasts-a review. *Yeast* 25, 465-483.
- 695 Labella, A.M., Castro, M.D., Manchado, M., Lucena, T., Arahál, D.R., Borrego, J.J., 2018. *Photobacterium*  
696 *malacitanum* sp. nov., and *Photobacterium andalusiense* sp. nov., two new bacteria isolated from diseased farmed  
697 fish in Southern Spain. *Syst. Appl. Microbiol.* <https://doi.org/10.1016/j.syapm.2018.04.005>
- 698 Liffourrena, A.S., Salvano, M.A., Lucchesi, G.I., 2010. *Pseudomonas putida* A ATCC 12633 oxidizes trimethylamine  
699 aerobically via two different pathways. *Arch. Microbiol.* 192, 471–476.
- 700 Lu, J., Nogi, Y., Takami, H., 2001. *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and  
701 alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS Microbiol. Lett.* 205, 291-297.

- 702 Lucena-Padrós, H., Jiménez, E., Maldonado-Barragán, A., Rodríguez, J.M., Ruiz-Barba J.L., 2015. PCR-DGGE  
703 assessment of the bacterial diversity in Spanish-style green table-olive fermentations. *International Journal of*  
704 *Food Microbiol.* 205, 47–53.
- 705 Luz, C., Ferrer, J., Mañes, J., Meca, G., 2018. Toxicity reduction of ochratoxin A by lactic acid bacteria. *Food Chem.*  
706 *Toxicol.* 112, 60–66.
- 707 MacNeil, M.A., McMeans, B.C., Hussey, N.E., Vecsei, P., Svavarsson, J., Kovacs, K.M., Lydersen, C., Treble, M.A.,  
708 Skomal, G.B., Ramsey, M., Fisk, A.T., 2012. Biology of the Greenland shark *Somniosus microcephalus*. *J. Fish*  
709 *Biol.* 80, 991–1018.
- 710 Magnússon, H., Gudbjörnsdóttir, B., 1984. Örveru-og efnabreytingar við verkun hkarls. Reykjavik (Iceland): Icelandic  
711 Fisheries Laboratories.
- 712 Magoc, T., Salzberg, S.L., 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies.  
713 *Bioinformatics* 27, 2957–2963.
- 714 Majumdar, R.K., Roy, D., Bejjanki, S., Bhaskar, N., 2016. An overview of some ethnic fermented fish products of the  
715 Eastern Himalayan region of India. *J. Ethn. Foods* 3, 276-283.
- 716 Metcalf, D., Avery, B.P., Janecko, N., Matic, N., Reid-Smith, R., Weese, J.S., 2011. *Clostridium difficile* in seafood and  
717 fish. *Anaerobe* 17, 85-86.
- 718 Milanović, V., Osimani, A., Garofalo, C., De Filippis, F., Ercolini, D., Cardinali, F., Taccari, M., Aquilanti, L.,  
719 Clementi, F., 2018. Profiling white wine seed vinegar bacterial diversity through viable counting, metagenomic  
720 sequencing and PCR-DGGE. *Int. J. Food Microbiol.* 286, 66-74.
- 721 Ogunremi, O., Sanni, A., Agrawal, R., 2015. Probiotic potentials of yeasts isolated from some cereal-based Nigerian  
722 traditional fermented food products. *J. Appl. Microbiol.* 119, 797-808.
- 723 Osimani, A., Babini, V., Aquilanti, L., Tavoletti, S., Clementi, F., 2011. An eight-year report on the implementation of  
724 HACCP in a university canteen: impact on the microbiological quality of meals. *Int. J. Environ. Health Res.* 21  
725 (2), 120–132.
- 726 Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., Clementi, F., 2015. Unpasteurised commercial boza as a source  
727 of microbial diversity. *Int. J. Food Microbiol.* 94, 62–70.
- 728 Osimani, A., Milanović, V., Cardinali, F., Garofalo, C., Clementi, F., Pasquini, M., Riolo, P., Ruschioni, S., Isidoro, N.,  
729 Loreto, N., Franciosi, E., Tuohy, K., Petruzzelli, A., Foglini, M., Gabucci, C., Tonucci, F., Aquilanti, L., 2018.  
730 The bacterial biota of laboratory-reared edible mealworms (*Tenebrio molitor* L.): from feed to frass. *Int. J. Food*  
731 *Microbiol.* 272, 49–60.
- 732 O'Toole, D.K., 1997. The role of microorganisms in soy sauce production. *Adv. Appl. Microbiol.* 45, 87-152.

- 733 Palla, M., Cristani, C., Giovannetti, M., Agnolucci, M., 2017. Identification and characterization of lactic acid bacteria  
734 and yeasts of PDO Tuscan bread sourdough by culture dependent and independent methods. *Int. J. Food*  
735 *Microbiol.*, 250, 19-26.
- 736 Paludan-Muller, C., Madsen, M., Sophanodora, P., Gram, L., Peter, L.M., 2002. Fermentation and microflora of plaasom, a Thai fermented fish product prepared with different salt concentrations. *Int. J. Food Microbiol.* 73, 61-70.
- 737
- 738 Petruzzelli, A., Amagliani, G., Micci, E., Foglini, M., Di Renzo, E., Brandi, G., Tonucci, F., 2013. Prevalence  
739 assessment of *Coxiella burnetii* and verocytotoxin-producing *Escherichia coli* in bovine raw milk through  
740 molecular identification. *Food Control* 32, 532–536.
- 741 Prevost, S., Cayol, J.-L., Zuber, F., Tholozan, J.-L., Remize, F., 2013. Characterization of clostridial species and sulfite-  
742 reducing anaerobes isolated from foie gras with respect to microbial quality and safety. *Food Control* 32, 222-  
743 227.
- 744 Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex  
745 microbiota of raw milk. *FEMS Microbiol. Rev.* 37, 664-698.
- 746 Rajauria, G., Sharma, S., Emerald, M., Jaiswal, A.K., 2016. Novel Fermented Marine-Based Products. *Novel Food*  
747 *Fermentation Technologies*, K. Shikha Ojha, Brijesh K. Tiwaripp, eds., pp 235-262. Springer International  
748 Publishing AG, Cham, Switzerland.
- 749 Requena, T., Pelaez, C., Fox, P.F., 1993. Peptidase and proteinase activity of *Lactococcus lactis*, *Lactobacillus casei*  
750 and *Lactobacillus plantarum*. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 196, 351-355.
- 751 Reynisson, E., Þór Marteinsson, V., Jónsdóttir, R., Magnússon, S.H., Hreggvidsson, G.O., 2012. Bacterial succession  
752 during curing process of a skate (*Dipturus batis*) and isolation of novel strains. *J. Appl. Microbiol.* 113, 329-338.
- 753 Sanni, A.I., Asieduw, M., Ayernorw, G.S., 2002. Microflora and Chemical Composition of Momoni, a Ghanaian  
754 Fermented Fish Condiment. *J. Food Compos. Anal.* 15, 577-583.
- 755 Schouw, A., Leiknes Eide, T., Stokke, R., Pedersen, R., Steen, I., Bødtker, G., 2016. *Abyssivirga alkaniphila* gen. nov.,  
756 sp. nov., an alkane-degrading, anaerobic bacterium from a deep-sea hydrothermal vent system, and emended  
757 descriptions of *Natranaerovirga pectinivora* and *Natranaerovirga hydrolytica*. *Int. J. Syst. Evol. Microbiol.* 66 (4),  
758 1724–1734.
- 759 Seibel, B.A., Walsh, P.J., 2002. Trimethylamine oxide accumulation in marine animals, relationship to acylglycerol  
760 storage. *J. Exp. Biol.* 205, 297–306.
- 761 Shiferaw Terefe, N., 2016. Food Fermentation. Reference Module in Food Science, Elsevier, Amsterdam, The  
762 Netherlands.

- 763 Simidu, W., 1961. Nonprotein nitrogenous compounds. In: Borgstrom G, editor. Fish as food: production, biochemistry,  
764 and microbiology. New York (USA): Academic Press; p. 353-384.
- 765 Skåra, T., Axelsson, L., Stefánsson, G., Ekstrand, B., Hagen, H., 2015. Fermented and ripened fish products in the  
766 northern European countries. *J. Ethn. Foods* 2, 18-24.
- 767 Stanborough, T., Fegan, N., Powell, S.M., Singh, T., Tamplin, M., Chandry, S., 2018. Genomic and metabolic  
768 characterization of spoilage-associated *Pseudomonas* species. *Int. J. Food Microbiol.* 268, 61–72.
- 769 Taccari, M., Aquilanti, L., Polverigiani, S., Osimani, A., Garofalo, C., Milanović, V., Clementi, F., 2016. Microbial  
770 diversity of type I sourdoughs prepared and backslopped with wholemeal and refined soft (*Triticum aestivum*)  
771 wheat flours. *J. Food Sci.* 81, 1996–2005.
- 772 Tamang, J.P., Watanabe, K., Holzapfel, W.H., 2016. Diversity of microorganisms in global fermented foods and  
773 beverages. *Front. Microbiol.* 7, 377.
- 774 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar S., 2013. MEGA6: Molecular Evolutionary Genetics  
775 Analysis Version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.
- 776 Terzic-Vidojevic, A., Mihajlovic, S., Uzelac, G., Veljovic, K., Tolinacki, M., Nikolic, M., Topisirovic, L., Kojic, M.,  
777 2014. Characterization of lactic acid bacteria isolated from artisanal Travnik young cheeses, sweet creams and  
778 sweet kajmaks over four seasons. *Food Microbiol.* 39, 27-38.
- 779 Thapa, N., 2016. Ethnic fermented and preserved fish products of India and Nepal. *J. Ethn. Foods* 3, 69-77.
- 780 Viljoen, B.C., Greyling, T., 1995. Yeasts associated with Cheddar and Gouda making. *Int. J. Food Microbiol.* 28, 79-88.
- 781 Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic  
782 study. *J. Bacteriol.* 173, 697-703.
- 783 Xu, Y., Li, L., Regenstein, J.M., Gao, P., Zang, J., Xia, W., Jiang, Q., 2018. The contribution of autochthonous  
784 microflora on free fatty acids release and flavor development in low-salt fermented fish. *Food Chem.* 256, 259–  
785 267.
- 786 Yukimura, K., Nakai, R., Kohshima, S., Uetake, J., Kanda, H., Naganuma, T., 2009. Spore-forming halophilic bacteria  
787 isolated from Arctic terrains: Implications for long-range transportation of microorganisms. *Polar Sci.* 3, 163-  
788 169.
- 789 Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Suemori, A., Nakajima, K., Inoue, N., Kawasaki, K., 2001.  
790 *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. *Int. J.*  
791 *Syst. Evol. Microbiol.* 51, 349-355.
- 792 Yunita, D., Dodd, C.E.R., 2018. Microbial community dynamics of a blue-veined raw milk cheese from the United  
793 Kingdom. *J. Dairy Sci.* 101 (6), 4923-4935.

- 794 Zang, J., Xu, Y., Xia, W., Jiang, Q., Yang, F., Wang, B., 2018a. Phospholipid molecular species composition of  
795 Chinese traditional low-salt fermented fish inoculated with different starter cultures. *Food Res. Int.* 111, 87-96.
- 796 Zavarzina, D.G., Tourova, T.P., Kolganova, T.V., Boulygina, E.S., Zhilina, T.N., 2009. Description of *Anaerobacillus*  
797 *alkalilacustre* gen. nov., sp. nov.—Strictly anaerobic diazotrophic bacillus isolated from soda lake and transfer of  
798 *Bacillus arseniciselenatis*, *Bacillus macyae*, and *Bacillus alkalidiazotrophicus* to *Anaerobacillus* as the new  
799 combinations *A. arseniciselenatis* comb. nov., *A. macyae* comb. nov., and *A. alkalidiazotrophicus* comb. nov.  
800 *Microbiology.* 78 (6), 723–731.
- 801 Zhang, K., Song, L., Dong, X., 2010. *Proteiniclasticum ruminis* gen. nov., sp. nov., a strictly anaerobic proteolytic  
802 bacterium isolated from yak rumen. *Int. J. Syst. Evol. Microbiol.* 60, 2221-2225.
- 803 Zang, J., Xu, Y., Xia, W., Yu, D., Gao, P., Jiang, Q., Yang, F., 2018b. Dynamics and diversity of microbial community  
804 succession during fermentation of Suan yu, a Chinese traditional fermented fish, determined by high throughput  
805 sequencing. *Food Res. Int.* 111, 565-573.

806 **FIGURE LEGENDS**

807

808 **Fig. 1** Ready-to-eat *hákarl*

809

810 **Fig. 2.** Affiliation of the sequences retrieved from DGGE gel fragments (marked in Supplementary Figure 2) with the  
811 existing sequences of the partial D1/D2 region of the large sub-unit rRNA gene.

812

813 Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the kimura 2-parameter model.  
814 Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA6. The  
815 sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are  
816 indicated by their corresponding band number and their accession number.

817

818 **Fig. 3.** Relative abundance (%) of yeast and filamentous fungal genera detected in each *hákarl* sample.

819

820 For each sample the percentage was calculated as follows: the number of fragments referring to a genus divided by their  
821 total number.

822

823 **Fig. 4** PCA based on the OTU abundance of the *hákarl* grouped as a function of the samples.

824

825 The first component (horizontal) accounts for the 40.66% of the variance and the second component (vertical) accounts  
826 for the 22.53 %

827

828 **Fig. 5** OTU network summarizing the relationships between taxa and samples.

829

830 Only OTUs occurring at 0.2% in at least 2 samples are shown. The abundances of OTUs in the two biological replicate  
831 were averaged. The sizes of the OTUs were made proportional to weighted degree (i.e., for OTUs, this measures the  
832 total occurrence of an OTU in the whole data set) using a power spline. OTUs and samples are connected with a line  
833 (i.e., edge) to a sample node, and its thickness is made proportional to the abundance of an OTU in the connected  
834 sample.

**Table 1** Results of pH and viable counting (log cfu per gram) of bacteria and eumycetes in ready-to-eat *hákarl* samples

Sample	pH	Total mesophilic aerobes	Presumptive mesophilic lactobacilli	Presumptive mesophilic lactococci	Pseudomonadaceae	Enterobacteriaceae	Eumycetes
H1	8.07±0.06	3.61±0.02	<1	4.51±0.04	<1	<1	<1
H2	8.23±0.01	5.71±0.09	1.60±0.43	4.39±0.01	1.54±0.34	<1	<1
H3	8.20±0.01	1.24±0.34	<1	4.27±0.05	<1	<1	<1
H4	8.09±0.01	1.30±0.00	<1	3.84±0.08	1.59±0.16	<1	<1
H5	8.37±0.01	2.40±0.02	<1	3.20±0.17	1.30±0.00	<1	<1
H6	8.53±0.04	2.01±0.15	<1	3.22±0.31	1.15±0.21	<1	<1
H7	8.41±0.00	1.15±0.21	<1	1.95±0.07	1.00±0.00	<1	<1
H8	8.41±0.01	1.00±0.00	<1	2.39±0.06	<1	<1	<1
H9	8.46±0.01	2.24±0.09	<1	3.30±0.08	1.24±0.34	<1	<1
H10	8.76±0.00	2.01±0.15	<1	4.04±0.15	<1	<1	<1

Values are expressed as means ± standard deviation

**Table 2.** Sequencing results of the bands excised from the DGGE gel obtained from the amplified fragments of bacterial DNA extracted directly from the pooled *hákarl* samples

Sample	Band <sup>a</sup>	Identification	% Identity <sup>b</sup>	Most closely related GeneBank sequence
H1	1	<i>Tissierella creatinophila</i>	98%	NR_037028
	2	<i>Anaerosalibacter massiliensis</i>	97%	NR_144694
	3	<i>Anaerosalibacter</i> sp.	93%	LT598565
	4	<i>Tissierella creatinophila</i>	97%	NR_117377
	5	<i>Tissierella creatinophila</i>	95%	NR_117377
H2	6	<i>Anaerosalibacter massiliensis</i>	97%	NR_144694
	7	<i>Tissierella creatinophila</i>	97%	NR_117377
H3	8	<i>Tissierella creatinophila</i>	85%	NR_117377
H4	9	<i>Tissierella creatinophila</i>	98%	NR_117377
H5	10	<i>Murdochiella massiliensis</i>	97%	NR_148568
	11	<i>Tissierella creatinophila</i>	98%	NR_117377
H6	12	<i>Tissierella creatinophila</i>	96%	NR_117377
	13	<i>Sporanaerobacter acetigenes</i>	97%	NR_117381
	14	<i>Tissierella creatinophila</i>	95%	NR_117377
H7	15	<i>Tissierella creatinophila</i>	97%	NR_117377
	16	<i>Tissierella creatinophila</i>	94%	NR_117377
H8	17	<i>Tissierella creatinophila</i>	97%	NR_117377
H9	18	<i>Tissierella creatinophila</i>	97%	NR_117377
	19	<i>Tissierella creatinophila</i>	90%	NR_117377
H10	20	<i>Tissierella creatinophila</i>	97%	NR_117377
	21	<i>Pontibacillus marinus</i>	97%	LT992038
	22	<i>Anaerosalibacter massiliensis</i>	97%	NR_144694
	23	<i>Anaerosalibacter massiliensis</i>	93%	NR_144694

<sup>a</sup> Bands are numbered as indicated in Supplementary Figure 1

<sup>b</sup> Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database.



**Table 3** Identification of yeasts and filamentous fungi occurring in the *hákarl* by sequencing the fragments obtained from DGGE profiles.

DGGE fragments <sup>a</sup>	Identification	Identity (%) <sup>b</sup>	Most closely related GeneBank sequence
H2-1	<i>Candida tropicalis</i> CBS:2320	99%	KY106851.1
H2-2	<i>Torulaspora delbrueckii</i> CBS 1146	100%	NG_058413.1
	<i>Torulaspora pretoriensis</i> CBS 11124		KY109883.1
	<i>Torulaspora quercuum</i> CBS 11403		NG_058413.1
H2-3	<i>Cladosporium tenuissimum</i> QCC:M024/17	100%	KY781762.1
H3-4	<i>Candida parapsilosis</i> CBS:2915	100%	Y102317.1
H3-5	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H3-6	<i>Saccharomyces cerevisiae</i> CBS:7961	100%	KY109313.1
H4-7	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H6-8	<i>Candida zeylanoides</i> CBS:947	100%	KY106918.1
H6-9	<i>Alternaria</i> sp strain QCC/M011/17	99%	KY744118.1
H6-10	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H6-11	<i>Alternaria</i> sp. isolate 1A1	100%	MF379649.1
H6-12	<i>Epicoccum</i> sp CN018	100%	KX954392.1
	<i>Phoma herbarum</i> JN0408		MG004796.1
H7-13	<i>Moristroma quercinum</i> BN1678	98%	AY254051.1
H7-14	<i>Candida zeylanoides</i> CBS:947	100%	KY106918.1
H7-16	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H7-17	<i>Candida glabrata</i> CBS:859	99%	KY106478.1
H8-18	<i>Candida parapsilosis</i> CBS:2193	99%	KY102320.1
H9-19	<i>Yamadazyma mexicana</i> CBS 7066	97%	NG_058439.1
H9-20	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H9-21	<i>Saccharomyces cerevisiae</i> CBS:7961	100%	KY109313.1
H10-22	<i>Debaryomyces hansenii</i> CBS:11096	100%	KY107560.1
	<i>Debaryomyces prosopidis</i> JCM 9913		NG_055701.1
	<i>Debaryomyces subglobosus</i> JCM 1989		NG_055699.1
	<i>Debaryomyces fabryi</i> CBS:4373		KY107483.1
H10-23	<i>Sporobolomyces salmonesus</i> CBS:488	100%	KY109767.1
	<i>Sporobolomyces roseus</i> OL10		KF273854.1
H10-24	<i>Saccharomyces cerevisiae</i> CBS:7961	100%	KY109313.1

<sup>a</sup> Bands are numbered as indicated in Supplementary Figure 2

<sup>b</sup> Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database.

**Table 4** Incidence of the major taxonomic groups detected by 16S amplicon target sequencing. Only OTUs with an incidence above 0.2% in at least 2 biological replicate were averaged.

<b>OTU</b>	<b>H1</b>	<b>H2</b>	<b>H3</b>	<b>H4</b>	<b>H5</b>	<b>H6</b>	<b>H7</b>	<b>H8</b>	<b>H9</b>	<b>H10</b>
<i>Abyssivirga</i>	0.19	2.88	2.92	5.73	3.56	5.52	2.93	6.88	4.81	4.41
<i>Acinetobacter</i>	0.00	0.04	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Alkalibacterium</i>	0.06	0.15	0.10	0.24	0.13	0.19	0.18	0.18	0.21	0.23
<i>Anaerobacillus</i>	0.00	0.91	0.01	0.00	0.40	0.06	0.00	0.02	0.00	0.00
<i>Erysipelothrix</i>	0.05	0.15	0.23	0.20	0.13	0.13	0.05	0.11	0.13	0.02
<i>Lactococcus</i>	0.46	0.14	0.05	0.26	0.07	0.11	0.09	0.06	0.29	0.67
<i>Listeria</i>	0.00	0.29	0.00	0.00	0.16	0.05	0.00	0.02	0.01	0.00
<i>Oceanobacillus</i>	43.69	1.84	2.17	3.76	0.71	3.32	5.08	1.52	4.05	1.15
<i>Ochrobactrum</i>	0.00	0.57	0.00	0.00	0.27	0.06	0.00	0.01	0.01	0.00
<i>Photobacterium</i>	0.01	0.67	0.07	0.10	0.14	1.00	0.35	0.10	0.20	0.20
<i>Proteiniclasticum</i>	0.00	0.14	0.43	0.35	0.24	0.03	0.00	0.08	0.13	0.00
<i>Pseudomonas</i>	42.99	0.29	0.31	0.25	0.65	8.24	13.04	4.65	2.57	10.96
<i>Staphylococcus</i>	0.00	0.17	0.00	0.00	2.71	0.00	0.00	0.00	0.00	0.00
<i>Tissierella</i>	12.37	87.77	92.56	88.66	90.42	80.75	77.82	86.07	87.09	82.11

OTU Operational Taxonomic Units

Fig. 1



ACCEPTED MANUSCRIPT

Fig. 2

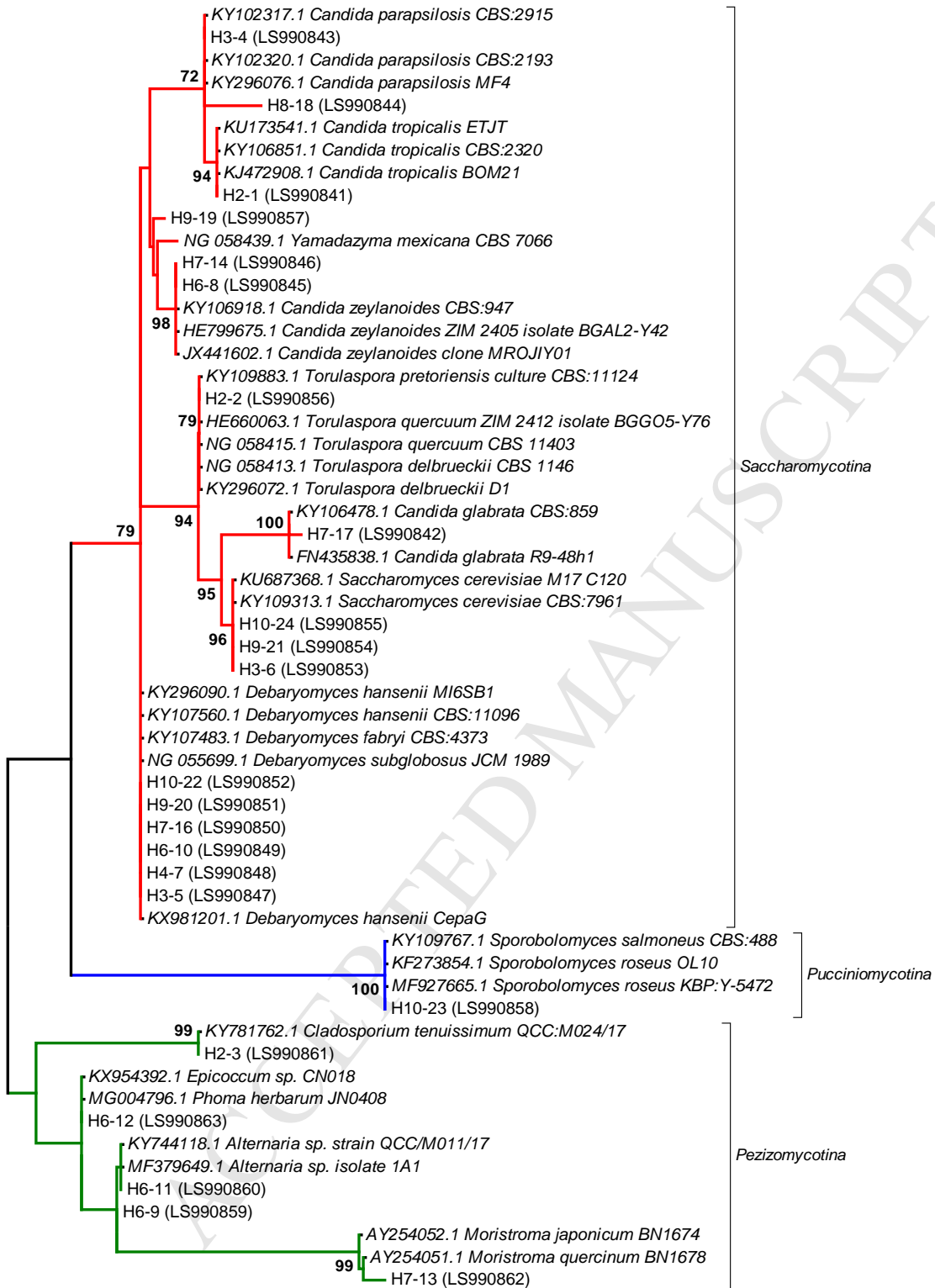


Fig. 3

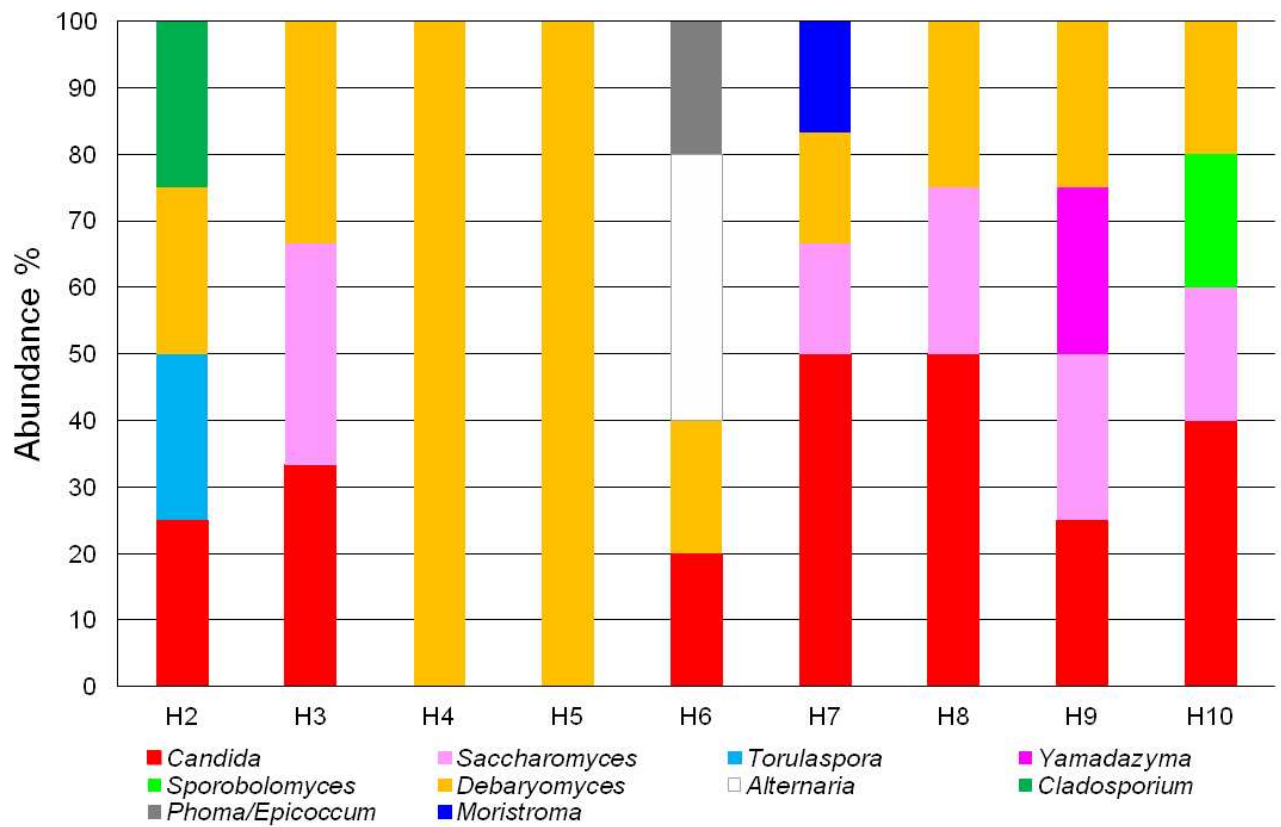


Fig. 4

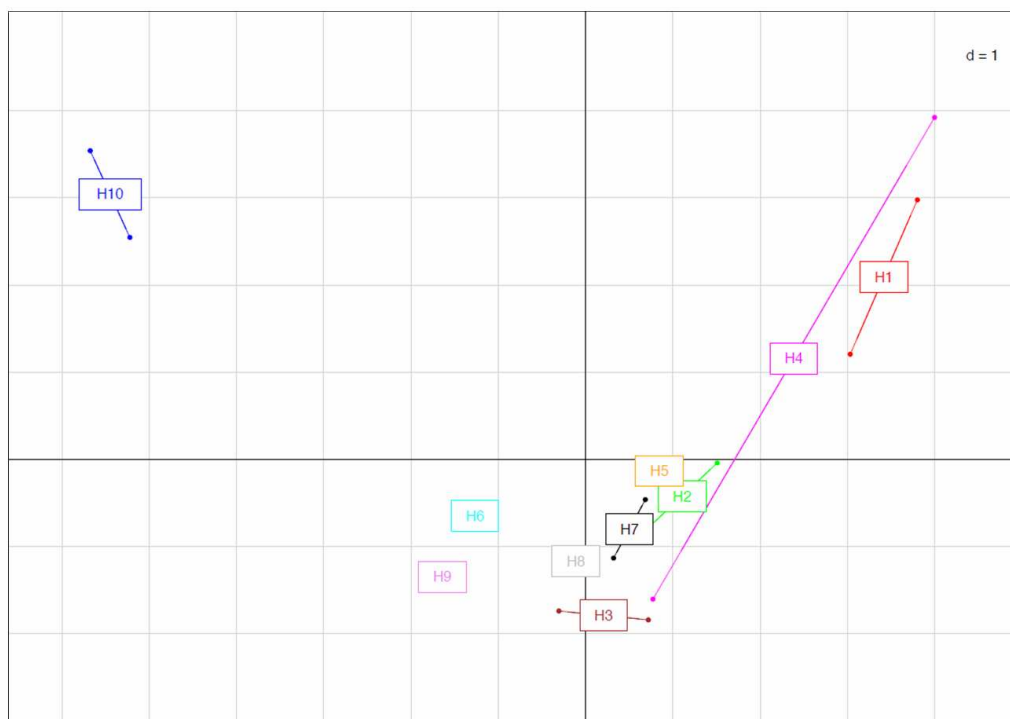
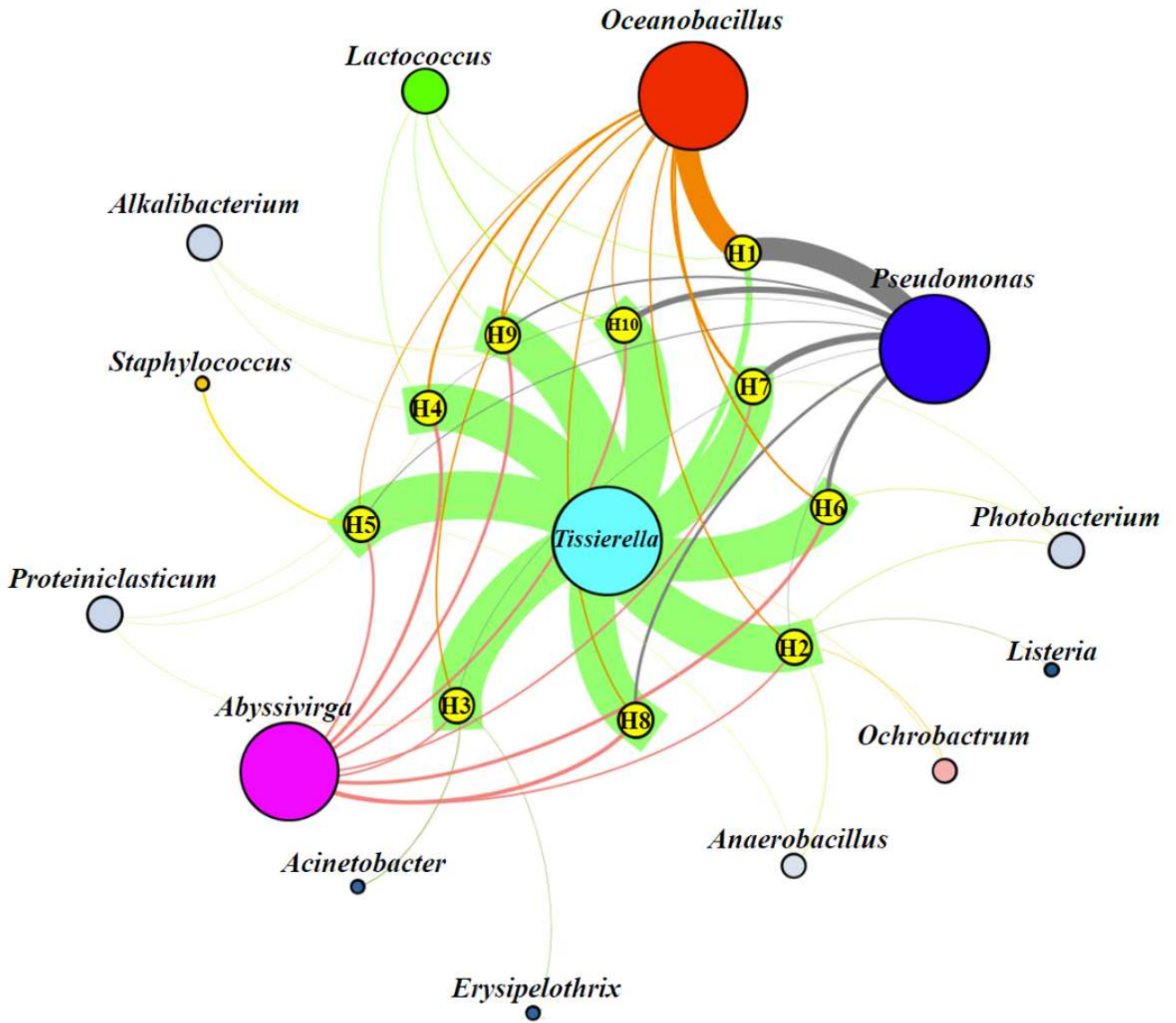


Fig. 5



**Highlights**

The microbiota of *hákarl* was studied using a polyphasic approach

Both metagenomic sequencing and PCR-DGGE highlighted the dominance of *Tissierella* sp.

The contribution of *Pseudomonas* in the detoxification of shark meat was hypothesized

The fungal community was mainly represented by *Debaryomyces* and *Candida* species

Real-time PCR showed the absence STEC and *Pseudomonas aeruginosa* in all the samples