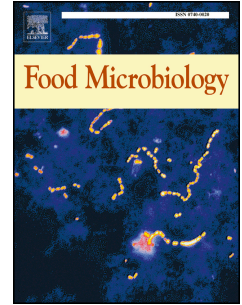


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Discovering microbiota and volatile compounds of *surströmming*, the traditional Swedish sour herring

Luca Belleggia, Lucia Aquilanti, Ilario Ferrocino, Vesna Milanović, Cristiana Garofalo, Francesca Clementi, Luca Cocolin, Massimo Mozzon, Roberta Foligni, M. Naceur Haouet, Stefania Scuota, Marisa Framboas, Andrea Osimani

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1 **Discovering microbiota and volatile compounds of *surströmming*, the traditional Swedish sour herring**

2

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32 **Abstract**

33

34 In this study, the microbiota of ready-to-eat *surströmming* from three Swedish producers were studied using a combined  
35 approach. The pH values of the samples ranged between  $6.67\pm 0.01$  and  $6.98\pm 0.01$ , whereas their  $a_w$  values were  
36 between  $0.911\pm 0.001$  and  $0.940\pm 0.001$ . The acetic acid concentration was between  $0.289\pm 0.009$  g/100 g and  
37  $0.556\pm 0.036$  g/100 g. Very low concentrations of lactic acid were measured. Viable counting revealed the presence of  
38 mesophilic aerobes, mesophilic lactobacilli and lactococci as well as halophilic lactobacilli and lactococci, coagulase-  
39 negative staphylococci, halophilic aerobes and anaerobes. Negligible counts for Enterobacteriaceae, Pseudomonadaceae  
40 and total eumycetes were observed, whereas no sulfite-reducing anaerobes were detected. *Listeria monocytogenes* and  
41 *Salmonella* spp. were absent in all samples. Multiplex real-time PCR revealed the absence of the *bont/A*, *bont/B*, *bont/E*,  
42 *bont/F*, and *4gyrB* (*CP*) genes, which encode botulinic toxins, in all the samples analyzed. Metagenomic sequencing  
43 revealed the presence of a core microbiota dominated by *Halanaerobium praevalens*, *Alkalibacterium gilvum*,  
44 *Carnobacterium*, *Tetragenococcus halophilus*, *Clostridiisalibacter*, and *Porphyromonadaceae*. *Psychrobacter celer*,  
45 *Ruminococcaceae*, *Marinilactibacillus psychrotolerans*, *Streptococcus infantis* and *Salinivibrio costicola* were detected  
46 as minority OTUs. GC-MS analysis of volatile components revealed the massive presence of trimethylamine and sulfur  
47 compounds. Moreover, 1,2,4-trithiolane, phenols, ketones, aldehydes, alcohols, esters and long chain aliphatic  
48 hydrocarbons were also detected. The data obtained allowed pro-technological bacteria, which are well-adapted to  
49 saline environments, to be discovered for the first time. Further analyses are needed to better clarify the extent of the  
50 contribution of either the microbiota or autolytic enzymes of the fish flesh in the aroma definition.

51

52 **Keywords:** fermented fish; 16S rRNA gene amplicon target sequencing; *Tetragenococcus halophilus*; *Carnobacterium*;  
53 *Porphyromonadaceae*; trimethylamine.

54 **1. Introduction**

55

56 The fermentation of fish flesh represents one of the most ancient methods to preserve or even enhance the quality of  
57 seafood (Speranza et al., 2015). Indeed, in some regions of the world, fishing can be strictly affected by seasons; hence,  
58 for some populations, the preservation of caught fish is pivotal. Fermented fish products are actually common in east  
59 and south-east Asia, as well as in Nordic European countries, where the fermentation of fish muscle is often associated  
60 with salting (Skåra et al., 2015; Speranza et al., 2015). In more detail, in the latter countries, principally represented by  
61 those included in the Scandinavian Peninsula and Iceland, salting of fish has been particularly difficult due to the  
62 scarcity of salt; hence, instead of full-salt preservation, low-salt fish preparations have been empirically developed since  
63 the Viking Era (Skåra et al., 2015). The production processes usually vary in accordance with the fish species, salt  
64 availability, environmental temperatures, storage techniques, raw material manipulation, and tradition. Admirable  
65 examples of traditional fermented and ripened fish products in the northern European countries are represented by  
66 *hákarl*, obtained from the Greenland shark (Osimani et al. 2019); *rakfisk* and *gravlax*, both of which are obtained from  
67 salmonid freshwater fish; *surströmming*; and other barrel-salted ripened herring and sprats (Skåra et al., 2015).

68 In particular, the consumption of herring by Scandinavian and other populations on the Baltic and North Sea dates back  
69 to 5,000 years ago (Kurlansky, 2002). During the sixteenth century, on the Swedish coast of the Gulf of Bothnia, the art  
70 of herring preservation by local people gave birth to the so-called *surströmming* (Kurlansky, 2002). The name of this  
71 refined food preparation comes from the fusion of two nouns, “*sur*” (English meaning of sour or acid) and “*strömming*”,  
72 which is the local name for the herring (*Clupea harengus* var. *membras*) caught in the northern regions of the Baltic Sea  
73 (Skåra et al., 2015). It is assumed that the preparation of *surströmming* was invented to overcome the lack of salt, which  
74 is commonly used for the preservation of herrings.

75 In accordance with a royal ordinance, *surströmming* must be obtained from herring caught between April and May,  
76 whereas some authors have reported that herring can be caught until July before spawning (Alm, 1965; Kurlansky,  
77 2002). The preparation of *surströmming* usually starts with a 1-2-day presalting step of the herring in saturated salt  
78 solution. The heads and entrails of the herrings are then removed, whereas the gonads (roe) and pyloric ceca are  
79 retained. Subsequently, the as-prepared herrings are left to ferment in sealed barrels, which can hold 200 pounds of fish,  
80 containing a weak brine (17% salt) from 3-4 to 10-12 weeks at 15-18 °C, depending on the traditional process applied  
81 (Kurlansky, 2002; Skåra et al., 2015). The barrels are sporadically rotated for a few days and then stored. The biological  
82 reactions that occur during such a process lead to the production of gas that escapes from the staves of the barrel. The  
83 herring and brine are then placed in cans, where fermentation can continue, until the formation of a bulge on the top and  
84 bottom of the can (Kurlansky, 2002; Skåra et al., 2015). *Surströmming* is characterized by a wine-colored fermented

85 flesh that, as described by Kurlansky (2002) in a very suggestive way, “fizzes out, bubbling like fermented cider and  
86 smelling like a blend of Parmesan cheese and the bilge water from an ancient fishing vessel”. It is therefore undeniable  
87 that the consumption of *surströmming* represents a real challenge even for the most daring palates.

88 It is already acknowledged that during the fermentation of herring, chemicals and microbiological activities strongly  
89 influence the safety and sensory traits of *surströmming*. In greater detail, as reported by Skåra et al. (2015), autolytic  
90 enzymes (e.g., calpains, cathepsins, proteasomes with caspase, etc.) that naturally occur in fish flesh and in the pyloric  
91 ceca of the gut, combined with the organic acids (e.g., lactate, propionate, butyrate, and acetate) and hydrogen sulfide  
92 that are produced by the metabolic activity of the microbiota, contribute to the definition of the main traits of the final  
93 product. Moreover, the salt contained in the brine should prevent the growth of spoilage bacteria that can lead to the  
94 rotting (protein decomposition) of herrings (Skåra et al., 2015). It is noteworthy that fish gut can contain pathogenic  
95 bacteria, some of them able to grow at low temperature (e.g. *Clostridium botulinum* and *Listeria monocytogenes*) hence,  
96 where fermentation conditions of fish are not correct, pathogenic bacteria may potentially be present in the final  
97 product, thus representing a health threat for the consumers (Köse, 2010; Waisundara et al., 2016).

98 Despite the long history of *surströmming* consumption, a lack of knowledge is available concerning the microbial  
99 consortia involved in herring fermentation. Indeed, to the authors’ best knowledge, only one study that dates back to the  
100 year 2000 is available in the scientific literature (Kobayashi et al., 2000). Although the study of Kobayashi et al. (2000)  
101 shed a first precious light on some of the viable microbial species occurring in *surströmming*, the complex microbial  
102 consortia that are likely involved in the fermentation of such a delicacy remain unknown.

103 At present, a number of reliable microbiological techniques are available to aid in obtaining in-depth knowledge of the  
104 microbial species occurring in fermented food matrices. In more detail, in addition to conventional techniques based on  
105 the use of selective growth media, the study of microbial DNA or RNA prepared directly from the food matrix allows  
106 the detection of both major and minor species. Among the most applied and sensitive molecular methods, next-  
107 generation sequencing and real-time polymerase chain reaction (PCR) provide sound data for microbiological profiling  
108 of foods.

109 Based on these concepts, a combined approach based on the use of selective growth media, 16S rRNA gene amplicon  
110 target sequencing and real-time PCR was adopted to study the microbial species occurring in ready-to-eat *surströmming*  
111 samples collected from three different Swedish producers. Moreover, a first characterization of the *surströmming*  
112 volatilome was carried out via gas chromatography-mass spectrometry (GC-MS) analysis.

113

## 114 **2. Materials and methods**

115

## 116 2.1. Sampling

117

118 Fifteen samples of ready-to-eat (canned) *surströmming* (Figure 1) were purchased during April 2019 from three  
119 different producers located in Sweden. Samples were codified as follows: S1-S5 (expiration date 02.2020) obtained  
120 from Producer 1, S6-S10 (expiration date 12.2019) obtained from Producer 2, and S11-S15 (expiration date 12.2019)  
121 obtained from Producer 3. Each sample consisted of 400 g/can of whole unpasteurized fermented herring in brine stored  
122 at 5°C. No further information on the samples was provided by the producers or from the can label. All the samples  
123 were analyzed before their expiration date.

124

## 125 2.2. Chemical-physical measurements

126

127 pH values of *surströmming* samples were determined with a pH meter equipped with an HI2031 solid electrode (Hanna  
128 Instruments, Padova, Italy).

129 Total titratable acidity (TTA) was determined using 10 g of the *surströmming* samples, which were homogenized in 90  
130 mL of distilled water for 5 min at 260 rpm using a Stomacher 400 Circulator apparatus (VWR International PBI, Milan,  
131 Italy). The results are expressed as the total volume (mL) of 0.1 N NaOH used to achieve a pH of 8.3.

132 The concentration of salt (sodium chloride) was assessed by gravimetric analysis in accordance with the method  
133 suggested by the Italian *Istituto Superiore di Sanità* (ISTISAN, 96/34).

134 The water activity ( $a_w$ ) was measured in accordance with the ISO 21807:2004 standard method using the Aqualab 4TE  
135 apparatus (Meter Group, Pullman, USA).

136 The acetic acid and lactic acid concentrations were measured using the Acetic Acid (Acetate Kinase Manual Format)  
137 test kit and D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit, respectively, from Megazyme (Bray, Ireland).

138 For each sample, the measurements were performed in duplicate, and the results are expressed as the mean  $\pm$  standard  
139 deviation.

140

## 141 2.3. Microbiological analyses

142

143 For the assessment of microbial viable counts, twenty-five grams of each *surströmming* sample were homogenized in  
144 225 mL of sterile peptone water (peptone, 1 g/L) using a Stomacher 400 Circulator apparatus (VWR International PBI,  
145 Milan, Italy) for 5 min at 260 rpm. The obtained homogenates (dilution  $10^{-1}$ ) were further diluted ten-fold and subjected  
146 to viable counts of the following groups of microorganisms: i) total mesophilic aerobes on plate count agar (PCA) (cod.

147 85941, VWR Chemicals, Milan, Italy) incubated at 30°C for 48 h; ii) total halophilic aerobes enumerated on PCA with  
148 8% NaCl after incubation at 30°C for 7 days; iii) total halophilic anaerobes counted on PCA with 8% NaCl after  
149 incubation in anaerobic jars using AnaeroGen 2.5 L Atmosphere Generation Systems (cod. AN0025, Thermo Scientific,  
150 Massachusetts, USA) at 30°C for 7 days; iv) mesophilic lactic acid bacteria (LAB) on De Man, Rogosa and Sharpe  
151 (MRS) agar (cod. 84607, VWR Chemicals) incubated at 30 °C for 48 h and M17 agar (cod. CM0785, Oxoid Limited,  
152 Basingstoke, UK) incubated at 22 °C for 72 h for enumeration of presumptive lactobacilli and lactococci, respectively;  
153 v) halophilic LAB on MRS and M17 agar with 8% NaCl incubated at 30 °C for 7 days and at 22 °C for 10 days for  
154 enumeration of presumptive halophilic lactobacilli and lactococci, respectively; vi) Enterobacteriaceae counted on  
155 Violet Red Bile Glucose Agar (VRBGA) (cod. 85845, VWR Chemicals) after incubation at 37°C for 24 h (Cardinali et  
156 al., 2017); vii) Pseudomonadaceae enumerated on Pseudomonas Agar Base (PAB) (cod. CM0559, Oxoid Limited) with  
157 ceftrimide-fucidin-cephalosporin (CFC) selective supplement (cod. CM0559, Oxoid Limited) and incubated at 30 °C for  
158 24–48 h; viii) staphylococci enumerated on Mannitol Salt Agar (MSA) (cod. LIOF610029, Liofilchem, Roseto degli  
159 Abruzzi, Italy) (Chapman, 1945) and incubated at 37°C for 24-48 h; ix) total eumycetes enumerated on Yeast Extract  
160 Peptone Dextrose Agar (YPD) (cod. J903, VWR Chemicals) (Ausubel et al., 1994) incubated at 25°C for 72 h; and x)  
161 halophilic eumycetes counted on YPD agar added with 8% NaCl after incubation at 25°C for 72 h. MRS and M17 agar  
162 were supplemented with cycloheximide (cod. 441892A, VWR Chemicals) (250 mg/L) to prevent eumycete growth,  
163 while YPD agar was supplemented with chloramphenicol (cod. 0230-EU-100G, VWR Chemicals) (100 mg/L) to  
164 prevent bacterial growth. For counting sulfite-reducing clostridia, homogenates were treated in a water bath at 80 °C for  
165 10 min and cooled in iced water. Aliquots (0.1 mL) of each dilution were spread on Tryptone Sulfite Neomycin (TSN)  
166 agar (cod. 610074, Liofilchem) and incubated at 37 °C for 24 h under anaerobic conditions using the AnaeroGen 2.5  
167 System.

168 Finally, a miniVIDAS apparatus (bioMérieux, Marcy l'Etoile, France) was used to assess the presence/absence of *L.*  
169 *monocytogenes* and *Salmonella* spp. through the enzyme-linked fluorescent assay (ELFA) method, in accordance with  
170 the AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively (Haouet et al., 2017). *L.*  
171 *monocytogenes* detection was carried out through pre-enrichment in half-Fraser broth (cod. 84721, VWR Chemicals)  
172 with incubation for 24 to 26 hours at 30°C ± 1°C, followed by enrichment in Fraser broth (cod. VWRC84730.0001,  
173 VWR Chemicals) with incubation for 24 hours at 37°C ± 1°C. *Salmonella* spp. detection was carried out through pre-  
174 enrichment in Buffered Peptone Water (cod. 881314TA, VWR Chemicals) with incubation for 16 to 22 hours at 37°C ±  
175 1°C, followed by enrichment in SX 2 broth (cod. 42121, bioMérieux) with incubation for 24 hours at 42°C ± 1°C.

176

177 2.4. DNA extraction from surströmming samples

178

179 The 1.5-mL aliquots of each sample homogenate (containing approximately 150 mg of the sample) (dilution  $10^{-1}$ )  
180 prepared as described in paragraph 2.3 were centrifuged for 5 min at 16000 g, and the obtained cell pellets were stored  
181 at  $-20^{\circ}\text{C}$  until use. The total microbial DNA was extracted from the cell pellets using an E.Z.N.A. soil DNA kit (Omega  
182 Bio-tek, Norcross, GA, USA) following the manufacturer's instructions. The quantity and purity of the extracted DNAs  
183 were checked using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). DNA extracts were then  
184 subjected to 16S rRNA gene amplicon target sequencing (Osimani et al., 2019).

185

#### 186 *2.5. DNA library preparation and sequencing*

187

188 Microbiota were studied by amplifying the V3 and V4 regions of 16S rRNA using primers and conditions previously  
189 described by Klindworth et al. (2013). Library preparation was performed according to the Illumina metagenomic  
190 procedure. Sequencing was performed using a MiSeq instrument (Illumina) with V3 chemistry and the generated 250-  
191 bp paired-end reads, following the producer's instructions.

192

#### 193 *2.6. Bioinformatics*

194

195 After the sequencing reads were assembled, they were quality-filtered and processed using QIIME 1.9.0 software  
196 (Caporaso et al., 2010) and the pipeline described by Ferrocino et al. (2017). Centroid sequences of each cluster were  
197 manually check using the Blast tool to confirm the taxonomic assignment. QIIME was used to rarefy the OTU table at  
198 the lowest number of sequences per sample and to build it (filtered at 0.2 % in at least 2 samples). The OTU table  
199 displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level,  
200 genus or the family name was displayed. The statistical package Kruskal–Wallis was used to find differences ( $\text{FDR} <$   
201  $0.05$ ) in microbial taxa abundance profiles and in the alpha diversity index according to producers. As a measure of the  
202 association between microbial OTUs and volatilome variables, Spearman's rank correlation coefficient was obtained  
203 through the function psych and plotted using the corrplot package in R ( $\text{FDR} < 0.05$ ). To explore the relationship  
204 between microbiota volatilome profiles, a principal component analysis (PCA) was carried out on the individual  
205 datasets, and the results were then integrated using coinertia analysis (CIA). CIA analysis was performed and is  
206 presented as a plot derived using the made4 package in the R environment.

207 The 16S sequences are available at the Sequence Read Archive of NCBI (accession number SRP217047).

208



## 209 2.6. Real-time PCR analyses for the detection of botulinic toxin genes

210

211 *C. botulinum* was analyzed in accordance with the multiplex real-time PCR method of the Italian National Reference  
212 Centre for botulism (<http://old.iss.it/binary/cnrb/cont/CNRB31.010.pdf>) for detection of the target genes *bont/A*, *bont/B*,  
213 *bont/E*, *bont/F*, and *4gyrB* (CP) that codify for botulinic toxins.

214 Briefly, 25 g of *surströmming* sample was blended in 225 mL Triptone Peptone Glucose Yeast extract (Microbiol  
215 Diagnostici, Italia), incubated under anaerobiosis at 30°C for 96 h and then extracted with 6% Chelex-100 (Bio-Rad,  
216 Milan, Italy). The amplification was carried out using the QuantiTect multiplex No Rox Kit (Qiagen) in a Stratagene  
217 Mx3005P (Agilent Technologies) thermal cycler, and the primer and probe nucleotide sequences are listed in  
218 Supplementary Table 1. Two different master mixes were used concurrently, with the following thermal profile: 1 cycle  
219 at 95°C for 15 min, followed by 40 cycles at 94°C for 30 s and 56°C for 90 s.

220 Real-time PCR analyses were performed on a RotorGene Q thermal cycler (Qiagen, Hiden, Germany) exploiting  
221 TaqMan chemistry. All target probes employed were dual-labeled with 5'-FAM and a 3'-nonfluorescent quencher (as  
222 specified below). The oligonucleotides were purchased from Thermo Fisher Scientific (Milan, Italy) and from LCG  
223 Biosearch Technologies (Petaluma, CA, USA). The reaction mixtures were all prepared in a final 25- $\mu$ l reaction  
224 volume. Molecular-grade H<sub>2</sub>O was included in each analytical session as a negative control, as well as DNA from  
225 reference strains as positive controls. Fluorescence was measured in the green channel for the target genes and in the  
226 yellow channel for the internal amplification control.

227

## 228 2.7. GC-MS analysis of volatile components

229

230 Solid phase micro extraction (SPME) was used to collect volatiles, according to Savini et al. (2017). A Varian 3900 gas  
231 chromatograph coupled to a Saturn 2100T ion trap mass detector (Varian Analytical Instruments, Walnut Creek, CA)  
232 was used to separate and identify aroma components. The GC conditions were as follows: fused silica capillary column  
233 ZB-5 (30 m L, 0.25 mm ID, 0.25  $\mu$ m FT; Phenomenex, Torrance, CA); oven temperature increasing from 40 to 220 °C,  
234 at a constant rate of 6 °C/min; carrier gas (He) flow 1.0 mL/min (constant flow mode); transfer line and ion trap  
235 temperatures 220 °C.

236 Experiments of both electronic impact fragmentation (EI 70 eV) and chemical ionization (CI) (reagent gas: methanol)  
237 were performed. Full-scan MS data were acquired in the mass range from 31-250 amu. Identification of  
238 chromatographic peaks was accomplished by comparison to Kovats retention indices (RIs) and mass fragmentation  
239 patterns of pure analytical standards; comparison to MS data published in the NIST/EPA/NIH Mass Spectral Library

240 Version 2.0a, built July 1, 2002 (National Institute of Standards and Technology); and analysis of the CI mass spectra  
241 using RI data reported in the published literature and listed in several authentic online databases  
242 (<http://webbook.nist.gov>, <https://pubchem.ncbi.nlm.nih.gov>, <http://www.flavornet.org>). RIs were determined for SPME-  
243 GC/MS by using a series of n-hydrocarbons (C6-C20) (Sigma-Aldrich, St. Louis, MO).  
244 Experimental data (volatile component abundance) were subjected to analysis of variance (ANOVA), and the  
245 significance of means was evaluated by Tukey-Kramer's Honest Significant Difference (HSD) test ( $P < 0.05$ ).  
246 Statistical analysis was performed using the software JMP® Version 10 (SAS Institute Inc., Cary, NC).

247

## 248 2.8. Statistical analysis

249

250 The Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used to evaluate  
251 differences within samples by one-way analysis of variance (ANOVA). The software JMP Version 11.0.0 (SAS  
252 Institute Inc., Cary, NC) was used to carry out all tests.

253

## 254 3. Results

255

### 256 3.1. Physicochemical measurements

257

258 The results of the physicochemical analyses of ready-to-eat *surströmming* samples are reported in Table 1. pH values  
259 ranged between  $6.67 \pm 0.01$  (sample S6) and  $6.98 \pm 0.01$  (sample S8), whereas  $a_w$  values were between  $0.911 \pm 0.001$   
260 (samples S9 and S10) and  $0.940 \pm 0.001$  (sample S2). The concentration of NaCl was between  $8.88 \pm 0.21$  g/100 g  
261 (sample S12) and  $6.49 \pm 0.16$  g/100 g (sample S5). TTA values measured in the analyzed *surströmming* samples ranged  
262 between  $7.4 \pm 0.42$  mL of 0.1 N NaOH (sample S9) and  $4.0 \pm 0.00$  mL of 0.1 N NaOH (sample S14). The acetic acid  
263 concentration was between  $0.289 \pm 0.009$  g/100 g (sample S1) and  $0.556 \pm 0.036$  g/100 g (sample S15). Finally, very low  
264 concentrations of lactic acid were measured among the samples, with most of the values being  $< 0.00002$  g/100 g and  
265 the maximum value being  $0.041 \pm 0.006$  g/100 g (sample S15).

266

### 267 3.2. Microbiological analyses

268

269 The results of viable counts for total mesophilic aerobes, presumptive mesophilic lactobacilli and lactococci,  
270 Enterobacteriaceae, Pseudomonadaceae, coagulase-negative staphylococci, sulfite-reducing anaerobes and total  
271 eumycetes are reported in Table 2.

272 Total mesophilic aerobes showed viable counts between  $5.67 \pm 0.04$  log cfu/g (sample S2) and  $4.08 \pm 0.089$  log cfu/g  
273 (sample S14); means among producers were significantly higher for producers A and B, whereas producer C showed  
274 the lowest value.

275 Presumptive mesophilic lactobacilli counts were characterized by values that ranged between  $4.57 \pm 0.18$  log cfu/g  
276 (sample S3) and  $<1$  log cfu/g (samples from S6 to S14). A significantly higher mean value was recorded for producer  
277 A, whereas producer B showed the lowest value.

278 Presumptive mesophilic lactococci showed counts between  $4.80 \pm 0.20$  log cfu/g (sample S2) and  $<1$  log cfu/g (samples  
279 from S6 to S14). Producer A showed the highest mean value, whereas producer B showed the lowest value.

280 Very low counts were observed for Enterobacteriaceae and Pseudomonadaceae, both presenting  $<1$  log cfu/g for all  
281 samples.

282 As for coagulase-negative staphylococci, counts between  $5.77 \pm 0.07$  log cfu/g (sample S5) and  $2.60 \pm 0.43$  cfu/g (sample  
283 S8) were detected. A higher mean value was reported for producer A, whereas producers B and C showed the lowest  
284 values.

285 For all samples, sulfite-reducing anaerobe counts were  $<2$  log cfu/g.

286 Finally, excluding sample S4, which showed viable counts of  $1.92 \pm 1.30$  log cfu/g, total eumycetes were  $<1$  log cfu/g in  
287 the remaining samples. The highest mean count was detected in samples from producer A.

288 Counts of total halophilic aerobes and anaerobes, presumptive halophilic lactobacilli and lactococci, and halophilic  
289 eumycetes are reported in Table 3.

290 In more detail, total halophilic aerobes counts ranged between  $6.74 \pm 0.18$  log cfu/g (sample S8) and  $4.99 \pm 0.06$  log cfu/g  
291 (sample S1), with samples from producer B showing the highest mean value.

292 Total halophilic anaerobes had values of  $6.98 \pm 0.05$  log cfu/g (sample S10) and  $5.61 \pm 0.13$  log cfu/g (S14), with samples  
293 from producer B showing the highest mean value.

294 The counts of presumptive halophilic lactobacilli were between  $7.06 \pm 0.10$  log cfu/g (sample S15) and  $5.85 \pm 0.19$  log  
295 cfu/g (S13). Producer B showed the highest mean value, whereas both producers A and C showed the lowest values.

296 Regarding presumptive halophilic lactococci, all samples from producer A and B showed values  $<1$  log cfu/g. Viable  
297 counts from producer C showed values between  $5.59 \pm 0.06$  log cfu/g (sample S15) and  $4.00 \pm 0.06$  log cfu/g (sample  
298 S13).

299 No halophilic eumycetes were detected, with all the counts being  $<1$  log cfu/g.

300 Furthermore, no samples revealed the presence of *L. monocytogenes* or *Salmonella* spp. in 25 g of product.

301 Finally, multiplex real-time PCR revealed the absence of the target genes *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *4gyrB*  
302 (*CP*), encoding botulinic toxins, in all the samples analyzed.

303

### 304 3.3. 16S rRNA gene amplicon target sequencing

305

306 The total number of paired sequences obtained from *surströmming* samples reached 1,475,590 raw reads. After quality  
307 filtering, a total of 623,326 reads were used, with an average value of  $20,777 \pm 8812$  reads/sample, and a mean sequence  
308 length of 465 bp. The alpha diversity index showed satisfactory coverage for all samples (> 95%). Alpha diversity  
309 showed a different level of complexity based on the producers. The highest level of complexity was observed in  
310 samples from producer B, followed by samples from producers A and C. The latter showed a low level of complexity  
311 and smaller number of observed species (FDR < 0.05), as shown in Figure 2.

312 Taking into the account the composition of the microbiota at the highest taxonomic level (Figure 3), we could observe a  
313 core microbiota composed of *Alkalibacterium gilvum* (approximately 8, 4 and 24 % of the relative abundance in  
314 samples from producers B, A, and C, respectively), *Carnobacterium* (16, 24 and 18 %), *Tetragenococcus halophilus* (9,  
315 63 and 16 %), *Halanaerobium praevalens* (8, 1 and 9 %), *Clostridiisalibacter* (2, 0.15 and 15 %) and  
316 *Porphyromonadaceae* (48, 0.09 and 19 %). It should also be noted that several minor OTUs were observed in the three  
317 producers that varied significantly across the producers (Figure 4, FDR<0.05). Producer A was characterized by  
318 *Porphyromonadaceae*, *Psychrobacter celer* and *Ruminococcaceae*, samples from producer B showed the presence of  
319 *Alkalibacterium gilvum*, *Clostridiisalibacter*, *Marinilactibacillus psychrotolerans* and *Streptococcus infantis*, whereas  
320 producer C was defined by *Salinivibrio costicola* and *Tetragenococcus halophilus*.

321

### 322 3.4. GC-MS analysis of volatile components

323

324 Thirty-five substances were consistently or tentatively identified in sample aromas (Table 4). Trimethylamine (TMA)  
325 and sulfur compounds (mainly dimethyl, methyl ethyl, and bis[1-(methylthio)ethyl] disulfides, and dimethyl trisulfide)  
326 dominated the volatilome profile of samples from producers A and B and were abundantly represented in the samples  
327 from producer C. The 1,2,4-trithiolane was detected only in samples from producer C. Phenols and ketones were the  
328 most represented compound in samples from producer C, whereas aldehydes were more represented in samples from  
329 producer A. Alcohols (2-methyl-2-butanol, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol, 2-penten-1-ol, 1-octen-3-ol)

330 were more represented in samples from producer C than from producers A and B. Esters (ethyl acetate, ethyl butyrate,  
331 ethyl 2-methylbutyrate, propyl butyrate, butyl butyrate) were more abundant in samples from producer B, whereas  
332 neither volatile fatty acids (from C1 to C5) nor volatile alcohols (ethanol, propanol, 2-propanol) were detected. Finally,  
333 some long-chain aliphatic hydrocarbons (dodecane, tridecane, heptadecane) were detected in all analyzed samples.

334

### 335 3.5. Correlations between microbiota and volatilome profiles

336

337 Plotting the correlation between the OTUs and VOCs (Figure 5, FDR<0.05), it was observed that *Alkalibacterium*  
338 *gilvum*, *Marinilactibacillus psychrotolerans* and *Psychrobacter celer* showed a positive correlation with  
339 1,3-ditert-butylbenzene, 2-methyl-2-butanol and dodecane, while *Porphyromonadaceae* correlated with  
340 2-heptanone, 3-methyl-1-butanol, ethyl 2-methylbutyrate and heptanal. Coinertia analysis was carried out, combining  
341 the PCA of the microbiota (OTUs) and VOCs, to evaluate differences among the three producers (Fig. 6). The analysis  
342 revealed a significant relationship between the microbiota and VOCs (RV coefficient = 0.55; Monte Carlo P = 0.02).  
343 The first horizontal component accounted for 55.33% of the variance, and a second vertical component accounted for  
344 another 31.71%. We observed a clear separation of the samples according to the producers (Fig. 6).

345

## 346 4. Discussion

347

348 As reported by Skåra et al. (2015), the annual production of *surströmming* is not negligible, being approximately 600  
349 tons. Notwithstanding, the physical-chemical and microbiological characteristics of this fermented fish have mostly  
350 been unexplored. To the authors' knowledge, only one study on the microbiota of *surströmming* is actually available in  
351 the scientific literature (Kobayashi et al., 2000), together with one dealing with the detection of chemical contaminants  
352 (organoarsenic compounds) in the same product (Richter et al., 2012).

353 In more detail, the study by Kobayashi et al. (2000) focused on the identification of strictly anaerobic halophiles  
354 isolated from only two cans of *surströmming*. Although limited, the study of Kobayashi et al. (2000) provided a first  
355 interesting glimpse of the bacterial species involved in the microbiological activities that lead to the production of such  
356 a peculiar Swedish food.

357 The *surströmming* samples analyzed in the present study were all characterized by pH values ranging between 6.67 and  
358 6.98, hence close to neutrality; the recorded values were in accordance with those reported by Kobayashi et al. (2000) in  
359 the same food matrix. NaCl values measured in the present study were slightly lower than those previously reported by  
360 Kobayashi et al. (2000), who reported values from 9-9.5 %. It is noteworthy that salt interacts with myofibrillar

361 proteins, thus affecting the water-holding capacity (Laub-Ekgreen et al., 2019). In salted foods, salt is transferred by the  
362 concentration gradient, whereas water is transferred by osmotic pressure between the muscle and the salting medium;  
363 furthermore, in salted fish, fat and skin can influence the results of the salting process (Laub-Ekgreen et al., 2019). The  
364  $a_w$  detected in the analyzed *surströmming* samples was between 0.911 and 0.940. Regarding pH and  $a_w$  values,  
365 *surströmming* was found to be a ready-to-eat food that is potentially able to support the growth of *L. monocytogenes*.  
366 Indeed, as reported by Regulation (EC) 2073/2005, and amended by Regulation (EC) 1441/2007, on microbiological  
367 criteria for foodstuffs, products that are unable to support the growth of *L. monocytogenes* are those with a  $\text{pH} \leq 4.4$  or  
368  $a_w \leq 0.92$ , or with a  $\text{pH} \leq 5.0$  and  $a_w \leq 0.94$ . It is noteworthy that all the analyzed samples showed pH values above the  
369 limits established by the above-mentioned regulatory agencies, and 3 out of 15 samples showed  $a_w$  values  $> 0.92$ .  
370 Notwithstanding, all the samples revealed the absence of *L. monocytogenes* in 25 g of product. Further studies are  
371 needed to better clarify whether the final pH values detected in *surströmming* were constantly maintained throughout  
372 fermentation or had been influenced by a microbial or enzymatic deacidifying activity during production.

373 The values of acetic and lactic acids were almost in accordance with those reported by Kobayashi et al. (2000) for  
374 *surströmming*. Interestingly, the samples analyzed in the present study and those analyzed by Kobayashi et al. (2000)  
375 revealed higher amounts of acetic acid with respect to lactic acid.

376 Despite a scarcity of knowledge about the microbiota involved in the production of *surströmming*, it is acknowledged  
377 that the preparation of this Swedish sour herring is carried out without the use of starter cultures. Hence, the  
378 autochthonous microorganisms present in *surströmming* are likely of environmental or animal origin (e.g., fish gut or  
379 skin).

380 The high counts of total mesophilic aerobes highlight intense microbial activity during the shelf life of the product.  
381 Such microbial groups encompass a large proportion of either pro-technological, spoilage or pathogenic bacteria. The  
382 presence of an active microbial fraction was also verified by the swollen cans, which were deformed due to internal gas  
383 formation. Moreover, the total halophilic aerobe counts were in accordance with the values reported by Gassem et al.  
384 (2019) for salted fermented mullet fish (Hout-Kasef), for which halophilic bacteria counts were between 3.26 and 5.14  
385 log cfu/g, with an average value of 4.32 log cfu/g. Lower counts for halophilic bacteria were detected by Wawire et al.  
386 (2019) in salted pressed spotted sardine (*Amblygaster sirm*), with values between 2.0 and 2.8 log cfu/g.

387 Among lactic acid bacteria, halophilic lactobacilli and lactococci were prevalent with respect to those cultivated on  
388 growth media without NaCl supplementation. Lactic acid bacteria constitute a large group of microorganisms that  
389 produce lactic acid as the major metabolite of carbohydrate fermentation (Françoise, 2010). Their metabolic activities  
390 lead to the production of a wide range of fermented products of either vegetable or animal origin, representing one of  
391 the most important groups of pro-technological microorganisms. Marine lactic acid bacteria are known to colonize

392 extreme environmental niches, such as sea water. Lactic acid bacteria are normally included in the gut microbiota of  
393 fish, and their occurrence is influenced by water salinity or environmental stress (Ringo and Storm, 1994). In further  
394 detail, lactobacilli have already been detected in Atlantic salmon, pollock, Arctic char, cod and rainbow trout;  
395 moreover, the presence of *Leuconostoc*, *Lactococcus*, *Vagococcus*, *Streptococcus*, and *Weissella* has also been reported  
396 (Françoise, 2010). Among halophilic lactic acid bacteria, the genera *Staphylococcus* and *Tetragenococcus* usually  
397 dominate during the production of fermented seafood (Taira et al., 2007), thus contributing to lowering the pH and  
398 reducing the risk of putrefaction; however, a component of the flesh degradation is also performed by endogenous fish  
399 enzymes (Françoise, 2010).

400 Enterobacteriaceae encompass either spoilage or pathogenic bacteria; the low viable counts generally recorded in  
401 *surströmming* for this bacterial family, as well as the absence of *Salmonella* spp., confirm the establishment of  
402 unsuitable environmental conditions for their survival in salted fermented fish, as previously reported by other authors  
403 (Alfonzo et al. 2018; Gassem, 2019; Wawire et al., 2019).

404 Regarding Pseudomonadaceae, it is known that members of the genus *Pseudomonas* can be naturally present in the fish  
405 environment, being the causative agent of fish infection or spoilage of processed fish (Kačániová et al., 2017). In the  
406 analyzed samples, low Pseudomonadaceae counts were consistently recorded. Notwithstanding, it is known that some  
407 *Pseudomonas* species have strong lipolytic activity that could have interacted with the fat of canned herrings, thus  
408 contributing to the flavor definition of *surströmming*. Moreover, Osimani et al. (2019) have recently suggested that  
409 *Pseudomonas* species can have a role in the reduction of content of TMA in fish flesh. Further research is needed to  
410 better understand the dynamics of such bacterial families during the fermentation of *surströmming*.

411 Data regarding coagulase-negative staphylococci collected in the present study are in accordance with the results of  
412 Gassem (2019), which reported counts between 2.71 and 3.85 log cfu/g, with an average value of 3.23 log cfu/g. In the  
413 study of Gassem (2019), among the detected species, *Staphylococcus epidermidis*, *Staphylococcus hominis*,  
414 *Staphylococcus xylosum*, and *Staphylococcus saprophyticus* were those dominating the fermentation process. It is  
415 noteworthy that, in the analyzed *surströmming* samples, the presence of *Staphylococcus epidermidis* was also confirmed  
416 by metagenomic sequencing, although they presented a minority OTU. As reported by Zeng et al. (2017), protease and  
417 lipase activities of coagulase-negative staphylococci produce various flavor compounds, which can be responsible for  
418 the texture and flavor development in salted fermented fish.

419 Counts of halophilic anaerobes were in accordance with the data published by Kobayashi et al. (2000), who reported  
420 counts between 6.30 and 6.70 log cfu/g, thus confirming the strong association of such a microbial group with the salty  
421 and anaerobic environmental conditions established inside the *surströmming* cans.

422 Counts of total eumycetes were lower than the values reported by Gassem (2019) for the same microbial group, which,  
423 in salted-fermented mullet fish, showed an average value of 1.33 log cfu/g. Eumycete counts in *surströmming* were also  
424 lower than those reported by Wawire et al. (2019) in salted pressed spotted sardine, where total fungal counts showed a  
425 maximum value of 3.60 log cfu/g. Yeasts of marine origin have been previously isolated from seawater, seaweeds,  
426 marine fish and mammals, as well as seabirds (Zaky et al., 2014); hence, further research is needed to better elucidate  
427 their eventual contribution during herring fermentation.

428 Interestingly, no sulfite-reducing anaerobes were detected. Anaerobic sulfite-reducing bacteria are generally considered  
429 to be indicators of clostridial contamination. Notwithstanding, as reported by Prevost et al. (2013), such a bacterial  
430 group is not supported by any taxonomical consideration; hence, their significance should probably be reconsidered.  
431 Indeed, many other bacterial genera can present a sulfite-reducing phenotype, including *Aeromonas*, *Citrobacter*,  
432 *Enterobacter*, *Enterococcus*, *Escherichia*, *Hafnia*, *Klebsiella*, *Tissierella*, and *Veillonella*, (Prevost et al., 2013).

433 Regarding clostridia, the absence of the target genes *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *4gyrB (CP)*, encoding botulinic  
434 toxins, in all the analyzed samples revealed no risks associated with the presence of *C. botulinum* strains in the analyzed  
435 *surströmming* samples. *C. botulinum* is the causative agent of foodborne intoxication caused by the consumption of  
436 preformed toxins, which are responsible for a severe neuroparalytic disease called botulism. The pathogen is a spore-  
437 forming, obligately anaerobic, mesophilic bacterium that is able to grow at temperatures between 12 °C and 37 °C.  
438 Moreover, psychrotrophic strains are also able grow at 3.0 °C (Carter and Peck, 2015). As reviewed by Carter and Peck  
439 (2015), genes that encode neurotoxins are situated together in one of two conserved neurotoxin clusters. Under  
440 anaerobic conditions, spore germination may occur, followed by bacterial multiplication, thus releasing toxins  
441 (Thwaites, 2017). *C. botulinum* spores are extremely heat resistant; hence, to assure the safety of low-acid canned food,  
442 which are particularly at risk, the thermal process is usually aimed at reducing the spore concentration (Ramaswamy et  
443 al., 2013). As reported by Gauthier (2015), *C. botulinum* can occur naturally in the gut of both marine and freshwater  
444 fish. Such a human pathogen can produce up to seven recognized toxin types (A–G), among which type E is mostly  
445 involved in human foodborne diseases related to fish consumption (Gauthier, 2015). As reported by Françoise (2010),  
446 the growth of *C. botulinum* type E can be inhibited by the combination of NaCl (3.5%), low temperature (< 5 °C) and  
447 low pH (< 4.5). Recent cases of foodborne botulisms from seafood were caused by the consumption of raw “*muktuk*”  
448 (skin and blubber from beluga whale, stored in sealed plastic bags) and *rakfisk*, a commercial vacuum-packed, hot-  
449 smoked whitefish (Carter and Peck, 2015). It is noteworthy that smoked fish produced in Arctic and northern temperate  
450 regions has also been associated with cases of human botulism (Gauthier, 2015). Hence, monitoring of seafood  
451 regarding the presence of *C. botulinum* must be constantly conducted to protect the health of consumers, especially  
452 when canned foods not subjected to strong heating treatment are considered.



453 The application of 16S rRNA gene amplicon target sequencing to DNA directly extracted from the *surströmming*  
454 samples allowed major and minor taxa to be detected, thus permitting the depiction of the first in-depth overview of the  
455 complex, previously undiscovered, microbiota occurring in such an intriguing traditional food. It is noteworthy that the  
456 amplification of the V3–V4 region, applied in the present study, has already been suggested by Klindworth et al. (2013)  
457 for the study of marine bacteria. Moreover, the V3–V4 region shows the best overall coverage for different applications  
458 and, being widely used by the scientific community, its study allows comparisons among available datasets to be  
459 performed (Klindworth et al., 2013).

460 Regarding the *surströmming* under study, *Alkalibacterium gilvum* was found in all samples. This bacterial species is a  
461 slightly halophilic, highly halotolerant and alkaliphilic lactic acid bacterial species that was first isolated from soft and  
462 semi-hard cheeses (Ishikawa et al., 2013). Such microorganisms can grow at NaCl concentrations between 0–1% and  
463 15–17.5 % (w/v), with an optimum between 2.0% and 5.0%; moreover, the optimal pH for growth is between 8.5 and  
464 9.5 (Ishikawa et al., 2013). The abovementioned physiological features likely explain the presence of *Alkalibacterium*  
465 *gilvum* in the analyzed samples that showed pH and NaCl values in accordance with those optimal for such bacterial  
466 species. Indeed, *Alkalibacterium* includes species that are highly adapted to brine, with members of this genus detected  
467 in green olive brine containing 10–11% (w/v) NaCl (Lucena-Padrós and Ruiz-Barba, 2019). The major fermentation  
468 product of *Alkalibacterium gilvum*, from D-glucose, is lactic acid; moreover, formic acid, acetic acid and ethanol with a  
469 molar ratio of approximately 2:1:1 can also be produced, without gas formation (Ishikawa et al., 2013). Interestingly the  
470 presence of *Alkalibacterium gilvum* has also been reported by Schornsteiner et al. (2014) in cheese rind, with a  
471 hypothesized antilisterial activity (Roth et al., 2010). Although this latter feature should be further investigated, such  
472 activity could likely contribute to assure the safety of *surströmming*, which, due to its  $a_w$  and pH values, can support the  
473 growth of *L. monocytogenes*. It is noteworthy that the genus *Alkalibacterium* has also been detected in salted and  
474 fermented seafoods as jeotgal (Guan et al., 2011), in saeu-jeot, a traditional Korean salted seafood (Jung et al., 2013), as  
475 well as in *hákarl* samples (Osimani et al., 2019) and marine environments (Jang et al., 2017), thus confirming the high  
476 adaptation of such a bacterial genus to alkaline and saline conditions. To the authors' knowledge, no other report of  
477 *Alkalibacterium gilvum* is available in the scientific literature for further data comparison.

478 *Carnobacterium* was widely distributed in all the samples. This lactic acid bacteria genus comprises rods that were  
479 isolated for the first time from poultry meat stored at a low temperature (Stiles and Holzapfel, 1997). Indeed,  
480 carnobacteria have been massively detected in vacuum-packaged meats stored at a refrigerated temperature, with the  
481 ability to decarboxylate one or more amino acids (Casaburi et al., 2011; Kołożyn-Krajewska and Dolatowski, 2012).  
482 Species of *Carnobacterium* have also been isolated from lightly preserved seafood products, such as salted lumfish,  
483 cold-smoked salmon, gravad rainbow trout, shrimp brine (Françoise, 2010), and alkaline-fermented skate (Jang et al.,

484 2017). Interestingly, Zhang et al. (2019) recently demonstrated that both bacteriocins and organic acids (mainly formate  
485 and acetate) produced by *Carnobacterium* species are key antimicrobial activity factors exerted in foodstuffs, thus  
486 representing a potential biopreservative. Regarding this latter feature of carnobacteria, Tulini et al. (2014) demonstrated  
487 that a *Carnobacterium maltaromaticum* strain, isolated from minimally processed smoked vacuum-packed fish, was  
488 able to produce carnobacteriocins B1, BM1, and an antimicrobial peptide encoded by the gene *cbnX*, with potent  
489 antilisterial activity (Alves et al., 2005; Reis et al., 2011). Similarly, Sahnouni et al. (2016) isolated carnobacteria strains  
490 from the gut of Atlantic horse mackerel, European pilchard and Atlantic bonito with antibacterial activity towards  
491 *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria innocua*, *Salmonella* spp. and *Vibrio* sp.  
492 (Marti-Quijal et al., 2019). Hence, the presence of *Carnobacterium* species in *surströmming* (deriving from either the  
493 gut or skin of herrings) could be pivotal for inhibition of the survival or prevention of the growth of potential foodborne  
494 pathogens in such foods.

495 *Tetragenococcus* represents one of the major lactic acid bacteria genera involved in the fermentation of fishery products  
496 (Ly et al., 2018) and one the main species (*Tetragenococcus halophilus*) detected in *surströmming* by Kobayashi et al.  
497 (2000). The presence of this moderately halophilic and homofermentative microorganism, which is able to grow in 18%  
498 NaCl (e.g., *T. halophilus* and *Tetragenococcus muriaticus*), has been reported in traditional Japanese squid liver sauce  
499 (Satomi et al., 1997; Kimura et al., 2001), *prahok* and *kapi*, produced in Cambodia with mudfish and shrimps,  
500 respectively (Chuon et al., 2014), in *pa-daek* and *pla-ra* produced in Laos and Thailand, respectively, with freshwater  
501 fish (Marui et al., 2015), in anchovy sauce (Kim et al., 2019), and in *moromi*, a fish-sauce mush produced with  
502 mackerel (Fukami et al., 2004). Different authors have reported that *Tetragenococcus* aminopeptidases play a pivotal  
503 role in amino acid production and flavor definition in salted fermented fish (Fukami et al., 2004; Udomsil et al., 2010).  
504 Interestingly, Kobayashi et al. (2000) isolated *T. halophilus* from puffer fish ovaries, thus suggesting the natural  
505 occurrence of this bacterial species in the fish body and, hence, likely explaining its presence in *surströmming*. It is  
506 noteworthy that species of *Tetragenococcus* can exert some beneficial effects on health, such as immunomodulatory  
507 activity and amelioration of atopic diseases (Chun et al., 2019). Notwithstanding, the metabolic activities of *T.*  
508 *halophilus* during the fermentation of salted foods can lead to the production of biogenic amines (e.g., histamine,  
509 cadaverine, putrescine, and tyramine), thus representing a potential health threat for consumers (Chun et al., 2019).  
510 Very recently, Kim et al. (2019) isolated a *T. halophilus* strain (MJ4) that is able to repress the formation of cadaverine  
511 during the fermentation of *saeu-jeot* (salted shrimp), thus representing a potential starter culture for the reduction of  
512 biogenic amines in salted fermented fish.

513 As previously reported by Kobayashi et al. (2000), *Halanaerobium prevalens* was one of the main species detected in  
514 *surströmming*. Such species of strictly anaerobic halophiles produce acetic, propionic and lactic acids, thus likely

515 contributing to the specific aroma of *surströmming* (Kobayashi et al., 2000). Moreover, *Halanaerobium prevalentis* is  
516 able to produce hydrogen and carbon dioxide, leading to the swelling of *surströmming* cans. *Halanaerobium prevalentis*  
517 is a non-spore-forming rod that is able to grow at pH values between 6 and 9 with an optimum at 7, thus explaining its  
518 presence in the analyzed *surströmming* samples with pH values between 6.67 and 6.98 (Zeikus et al., 1983). Moreover,  
519 *Halanaerobium prevalentis* has demonstrated the ability to grow at NaCl concentrations up to 20%, with an optimum at  
520 13%, but with no significant growth at NaCl values <2% or ≥30% (Zeikus et al., 1983). Species of the genus  
521 *Halanaerobium* emerged as those dominating saeu-jeot (Jung et al., 2013) and myeolchi-aekjeot, a traditional fermented  
522 fish sauce prepared by fermenting anchovies in high concentrations of salt (Singh et al., 2017). In this latter preparation,  
523 *Halanaerobium*, together with *Tetragenococcus* and *Staphylococcus*, causes the salt-fermentative hydrolysis of protein  
524 components in anchovies, with the consequent release of amino acid through exogenous and endogenous proteases  
525 (Singh et al., 2017).

526 Regarding *Clostridiisalibacter*, species belonging to this genus have already been detected in deep-sea hydrothermal  
527 environments (Jiang et al., 2015); moreover, *Clostridiisalibacter paucivorans* has been isolated from olive mill  
528 wastewater (Liebgott et al., 2008). This moderately halophilic spore-forming bacterium was observed to grow at NaCl  
529 concentrations between 10 and 100 g/L, with an optimum of 50 g/L. The pH range for growth was between 5.5 and 8.5,  
530 with an optimum of 6.8. *Clostridiisalibacter* was also detected on the surface of semi-hard Danish Danbo (Ryssel et al.,  
531 2015) and Raclette type cheeses (Roth et al., 2010), together with *Marinilactibacillus psychrotolerans*, where the latter  
532 species, found as minority in *surströmming*, exerted potential antilisterial activity in the analyzed cheeses (Roth et al.,  
533 2010).

534 The Porphyromonadaceae family includes the following bacterial genera, namely, *Falsiporphyromonas*,  
535 *Fermentimonas*, *Gabonia*, *Gabonibacter*, *Lascolabacillus*, *Macellibacteroides*, *Massilibacteroides*, *Microbacter*,  
536 *Petrimonas*, *Porphyromonas*, and *Sanguibacteroides*. Members of this bacterial family, found as part of the core  
537 microbiota of all *surströmming* samples, have already been detected in the gut of Nile tilapia (Yu et al., 2019; Zheng et  
538 al., 2019). As reported by different authors, some Porphyromonadaceae genera may represent a threat to both humans  
539 (e.g., periodontal diseases caused by *Porphyromonas gingivalis*) and aquatic organisms (Kontani et al., 1999;  
540 Summanen et al., 2009; Lawson et al., 2010; Russel et al., 2015). It is noteworthy that many of the genera belonging to  
541 Porphyromonadaceae have been previously detected during the anaerobic digestion processes of sludge from  
542 wastewater treatment plants (Kong et al., 2019; Jin et al., 2019; Sánchez-Andrea et al., 2014). Other  
543 Porphyromonadaceae genera have also been detected in fecal samples or tissues of animal or human origin (Wagener et  
544 al., 2014; Mourembou et al., 2015, 2016). Further investigation is needed to better clarify the roles of this bacterial  
545 family in the analyzed *surströmming* samples.

546 Although a minority OTU, *Arcobacter* was found in all the samples, highlighting potential safety issues for the  
547 consumers. This emerging foodborne and waterborne pathogen can serve as the causative agent of different  
548 gastrointestinal diseases (e.g., abdominal cramps and watery diarrhea) (Hsu and Lee, 2015). *Arcobacter* has been  
549 previously detected in various foodstuffs, including seafood (e.g., shellfish), with an average prevalence of 32.3 %, and  
550 the water environment, including seawater (Rivas et al., 2004; Collado et al., 2009; Ghane, 2014; Levican et al., 2014;  
551 Hsu and Lee, 2015; Leoni et al., 2017). As reported by Zhang et al. (2019), information concerning the prevalence of  
552 *Arcobacter* in retail seafood products is still scarce; hence, data obtained in the present study for *surströmming* can  
553 contribute to improving knowledge related to the occurrence of such pathogens in ready-to-eat foods. Interestingly,  
554 Zhang et al. (2019) found that among a total of 318 samples that included bivalves, shrimps and cephalopods collected  
555 from local retail seafood markets in Germany, 17.6% of the analyzed samples were positive for the presence  
556 *Arcobacter*. Among the detected species, *Arcobacter butzleri* was predominant, followed by *Arcobacter venerupis*,  
557 *Arcobacter cryaerophilus*, *Arcobacter aquimarinus*, *Arcobacter skirrowii* and *Arcobacter thereius* (Zhang et al., 2019).  
558 Regarding volatile compounds detected in *surströmming*, it is well known that aroma components of fermented fish  
559 mainly originate from microbial proteolytic and lipolytic activities; further oxidation and metabolism of fatty acids and  
560 amino acid catabolism lead to the final products. Although the pathways of aroma component generation have been  
561 studied in some fermented foods, such as wine, beer and dairy products (Smit et al., 2005), research on the flavor of  
562 fermented fish is still limited to identification of the volatiles produced by different microorganisms (Zang et al., 2019).  
563 In such a context, the use of mass spectrometry is essential for analyzing complex mixtures of natural substances, in  
564 order to provide detailed and reproducible information about the molecular structure of unknown components that can  
565 be compared with mass spectrum libraries (Mozzon et al., 2015). In fact, due to the complexity of food aromas, the  
566 chromatographic behavior (retention indexes) is far from enough for unambiguous identification of chromatographic  
567 peaks. For this reason, chemical ionization experiments were also carried out since the lower energy impact allows the  
568 analytes to be less fragmented and therefore the molecular weight of an unknown analyte can be more easily  
569 determined.

570 Regarding the analyzed *surströmming* samples, high levels of TMA were detected. TMA (rotten fish and ammoniacal  
571 smells) is produced by the reduction or/and demethylation of trimethylamine N-oxide (TMAO), which is an osmolyte in  
572 saltwater fish (Jung et al., 2013). TMA was the only nitrogen-containing compound detected, despite previous findings  
573 by other authors who evidenced the presence of a variety of pyrazines (Giri et al., 2010; Udomsil et al., 2010). As  
574 reported by Ndaw et al. (2008), the TMA content represents one of the most widely used parameters for the evaluation  
575 of spoilage in seafood. The flesh of fresh fish naturally contains trimethylamine oxide (TMAO), a tasteless nonprotein  
576 nitrogen compound with varying contents depending on the fish species, size and age (Huss, 1995; Ozogul et al., 2006).

577 Spoilage of fish is influenced by the presence of TMAO, particularly under anaerobic conditions. A number of spoilage  
578 bacteria are able to utilize TMAO as the terminal electron acceptor in anaerobic respiration, resulting in off-odors and  
579 flavors due to the formation of TMA (Dalgaard et al., 1993). As reported by Broekaert et al. (2013), *Psychrobacter*  
580 shows the ability to produce slight amounts of TMA in brown shrimps.

581 Polysulfides have been previously reported in different fermented fish products (Giri et al., 2010a, 2010b; Udomsil et  
582 al., 2010) to be responsible for undesirable, offensive, fecal notes; furthermore, 1,2,4-trithiolane, detected in samples  
583 from producer C, has already been associated with a shiitake mushroom flavor (Politowicz et al., 2018). Ketones,  
584 detected in samples from producer C, have been associated with a cheesy note (acetone, 2-pentanone, 2-heptanone, 2,3-  
585 octanedione). According to Udomsil et al. (2010), 3-methyl butanal has already been described as a compound that is  
586 responsible for a meaty note, whereas aliphatic aldehydes (2-hexenal, heptanal), derived from fatty acid autoxidation  
587 and enzymatic oxidation, are usually responsible for herbaceous and grassy aromas.

588 Branched chain alcohols, which were mainly detected in samples from producer C, could originate from carbohydrates  
589 by the Embden-Meyerhof-Parnas pathway and from amino acids via the Ehrlich pathway, whereas 1-octen-3-ol  
590 (mushroom odor) is considered to be an oxidation product of unsaturated fatty acids (Xu et al., 2018).

591 Esters (ethyl acetate, ethyl butyrate, ethyl 2-methylbutyrate, propyl butyrate, butyl butyrate) contribute to fruity and  
592 buttery notes (Udomsil et al., 2010), and they were abundantly detected in samples from producer B, although neither  
593 volatile fatty acids (from C1 to C5) nor volatile alcohols (ethanol, propanol, 2-propanol) were detected.

594 Regarding long chain aliphatic hydrocarbons, heptadecane has been previously detected in fermented fish products by  
595 Giri et al (2010); the same authors suggested that n-alkanes could result from the decarboxylation of higher nonvolatile  
596 fatty acids. However, due to their low volatility and high perception threshold, their contribution to sample aromas  
597 could be negligible. As recently reported by Lu et al. (2019), TMA, ketones, aldehydes, and alcohols are also the main  
598 odor components in Asian and American carp meat, confirming the presence of such compounds in fish flesh.

599 The present study provides a first glimpse into the volatilome of *surströmming*, which requires further in-depth analyses  
600 to better clarify the extent of the contribution of either the microbiota or autolytic enzymes of the fish flesh in the aroma  
601 definition.

602

## 603 **5. Conclusions**

604

605 Data obtained from a combination of culture-dependent and -independent techniques allowed major and minor  
606 microbial species harbored in ready-to-eat *surströmming* samples to be discovered for the first time. In further detail,  
607 pro-technological bacteria, which are well-adapted to saline environments, characterized the microbial population of all

608 the samples. On the one hand, the presence of *Halanaerobium praevalens* as one of the dominant species of halophilica  
609 bacteria was confirmed; on the other hand, the occurrence of *Alkalibacterium gilvum*, *Carnobacterium*,  
610 *Tetragenococcus halophilus*, *Clostridiisalibacter*, and *Porphyromonadaceae* revealed a core microbiota characterized  
611 by complex microbial associations that require further investigation. In addition to potential safety issues arising from  
612 the presence of *Acrobacter*, the absence of *L. monocytogenes*, *Salmonella* and *C. botulinum* is noteworthy in all  
613 samples.

614 Moreover, volatile compounds were detected in fermented fish, thus representing the first attempt to characterize the  
615 strong and peculiar aroma of *surströmming*. In more detail, high levels of TMA and sulfur compounds were detected in  
616 all samples, followed by phenol, ketones, aldehydes, alcohols, esters and long-chain aliphatic hydrocarbons.

617 Further in-depth research is needed to establish the roles of the microbial species that are present during herring  
618 fermentation, as well as their influence on the chemical-physical and volatile features of *surströmming*.

619

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621

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623

#### 624 **Conflict of interests**

625

626 The authors declare no conflict of interests

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- 876

877 **FIGURE CAPTIONS**

878

879 **Figure 1.** Ready-to-eat *surströmming*

880

881 **Figure 2.** Boxplots showing the  $\alpha$ -diversity measures of the *surströmming* microbiota of Producer A, Producer B, and  
882 Producer C samples. Individual points and brackets represent the richness estimate and the theoretical standard error  
883 range, respectively.

884

885 Producer A, samples S1-S5; Producer B, samples S6-S10; Producer C, samples S11-S15

886

887 **Figure 3.** Relative abundance of the microbiota in *surströmming*. Only OTUs that showed an incidence greater than  
888 0.2% in at least 2 samples are shown.

889

890 Producer A, samples S1-S5; Producer B, samples S6-S10; Producer C, samples S11-S15

891

892 **Figure 4.** Boxplots showing the relative abundance of differentially abundant OTUs based on the Kruskal–Wallis test  
893 (FDR < 0.05) in the *surströmming* microbiota of Producer A, Producer B, and Producer C samples.

894

895 Producer A, samples S1-S5; Producer B, samples S6-S10; Producer C, samples S11-S15

896

897 **Figure 5.** Correlation plot showing Spearman's correlation between microbial OTUs and the volatilome profile of  
898 *surströmming*.

899

900 Only significant associations between OTUs and VOCs are shown (FDR &lt; 0.05).

901 The intensity of the colors represents the degree of correlation, where blue represents a positive degree of correlation  
902 and red represents a negative correlation.

903

904 **Figure 6.** Coinertia analysis (CIA) of the microbial community (OTUs) and volatilome (VOCs) in *surströmming*  
905 samples.

906

907 Samples projected onto the first two axes and grouped according to producers A (samples S1-S5), B (samples S6-S10),  
908 and C (samples S11-S15).

Journal Pre-proof



**Table 1.** Physico-chemical parameters of *Surströmming*.

Sample	pH	a <sub>w</sub>	NaCl (g/100g)	TTA (mL of 0.1N NaOH)	Acetic acid (g/100g)	Lactic acid (g/100g)
S1	6.93±0.01	0.929±0.003	6.92±0.57	5.2±0.07	0.289±0.009	< 0.00002
S2	6.80±0.01	0.940±0.001	8.28±0.00	4.7±0.21	0.346±0.004	< 0.00002
S3	6.83±0.01	0.937±0.001	8.59±0.37	4.7±0.21	0.322±0.002	< 0.00002
S4	6.95±0.01	0.935±0.001	7.43±0.16	4.3±0.21	0.324±0.030	< 0.00002
S5	6.79±0.01	0.928±0.001	6.49±0.16	4.5±0.49	0.297±0.005	< 0.00002
S6	6.67±0.01	0.917±0.000	6.79±0.16	5.0±0.21	0.491±0.048	< 0.00002
S7	6.83±0.01	0.914±0.002	7.38±0.08	5.7±0.78	0.474±0.030	< 0.00002
S8	6.98±0.01	0.922±0.001	7.36±0.24	4.5±0.14	0.474±0.025	< 0.00002
S9	6.72±0.01	0.911±0.001	7.91±0.14	7.4±0.42	0.438±0.008	< 0.00002
S10	6.76±0.00	0.911±0.001	7.69±0.01	6.7±0.28	0.422±0.021	< 0.00002
S11	6.81±0.01	0.916±0.003	7.57±0.13	4.3±0.28	0.296±0.026	< 0.00002
S12	6.77±0.01	0.921±0.001	8.88±0.21	4.6±0.49	0.314±0.003	< 0.00002
S13	6.84±0.01	0.922±0.002	8.46±0.17	4.0±0.14	0.349±0.008	0.001±0.001
S14	6.84±0.01	0.917±0.001	8.72±0.07	4.0±0.00	0.328±0.006	0.004±0.002
S15	6.91±0.01	0.916±0.001	8.62±0.06	4.4±0.14	0.556±0.036	0.041±0.006
<b>Mean values</b>						
Producer A	6.86±0.07 <sup>a</sup>	0.933±0.001 <sup>b</sup>	7.54±0.88 <sup>a</sup>	4.6±0.33 <sup>ab</sup>	0.316±0.023 <sup>a</sup>	< 0.00002 <sup>a</sup>
Producer B	6.79±0.12 <sup>a</sup>	0.915±0.001 <sup>a</sup>	7.42±0.42 <sup>a</sup>	5.8±1.19 <sup>b</sup>	0.459±0.284 <sup>b</sup>	< 0.00002 <sup>a</sup>
Producer C	6.83±0.05 <sup>a</sup>	0.918±0.001 <sup>a</sup>	8.45±0.51 <sup>a</sup>	4.2±0.26 <sup>a</sup>	0.368±0.100 <sup>ab</sup>	0.009±0.018 <sup>b</sup>

Values are expressed as means ± standard deviation.

For each producer (A, B, C) means followed by different letters are significantly different ( $P < 0.05$ ).

Producer A, samples S1-S5

Producer B, samples S6-S10

Producer C, samples S11-S15

**Table 2.** Results of viable counting of bacteria and eumycetes in *Surströmming*.

Sample*	Total mesophilic aerobes	Mesophilic presumptive lactobacilli	Mesophilic presumptive lactococci	Enterobacteriaceae	Pseudomonadaceae	Coagulase-negative staphylococci	Sulfite-reducing anaerobes	Total eumycetes
S1	4.66±0.05	4.19±0.02	4.80±0.11	1.04±1.47	< 1.00	4.57±0.13	< 2.00	< 1.00
S2	5.67±0.04	3.77±0.16	4.20±0.08	< 1.00	< 1.00	4.18±0.26	< 2.00	< 1.00
S3	4.96±0.02	4.57±0.18	4.80±0.20	< 1.00	< 1.00	4.31±0.44	< 2.00	< 1.00
S4	4.74±0.12	4.18±0.00	4.52±0.04	< 1.00	< 1.00	5.02±0.56	< 2.00	1.92±1.30
S5	5.09±0.07	4.50±0.55	4.67±0.41	< 1.00	1.00±0.00	5.77±0.07	< 2.00	< 1.00
S6	5.40±0.02	< 1.00	< 1.00	< 1.00	< 1.00	3.04±0.19	< 2.00	< 1.00
S7	5.56±0.01	< 1.00	< 1.00	< 1.00	< 1.00	2.96±0.26	< 2.00	< 1.00
S8	5.41±0.11	< 1.00	< 1.00	< 1.00	< 1.00	2.60±0.43	< 2.00	< 1.00
S9	5.35±0.06	< 1.00	< 1.00	< 1.00	< 1.00	3.52±0.25	< 2.00	< 1.00
S10	5.41±0.10	< 1.00	< 1.00	< 1.00	1.00±0.00	3.00±0.00	< 2.00	< 1.00
S11	4.71±0.06	< 1.00	2.68±0.14	< 1.00	1.00±0.00	3.33±0.18	< 2.00	< 1.00
S12	4.70±0.00	< 1.00	2.50±0.20	< 1.00	1.13±1.59	3.22±0.06	< 2.00	< 1.00
S13	4.18±0.04	< 1.00	2.39±0.04	< 1.00	< 1.00	2.65±0.07	< 2.00	< 1.00
S14	4.08±0.89	< 1.00	2.37±0.23	< 1.00	< 1.00	2.69±0.09	< 2.00	< 1.00
S15	5.35±0.63	1.65±0.07	2.67±0.23	1.30±0.00	2.05±0.21	3.94±0.16	< 2.00	< 1.00
<b>Mean values</b>								
Producer A	5.02±0.40 <sup>a</sup>	4.24±0.32 <sup>c</sup>	4.60±0.25 <sup>c</sup>	0.21±0.46 <sup>a</sup>	0.20±0.45 <sup>a</sup>	4.77±0.64 <sup>b</sup>	0.00±0.00 <sup>a</sup>	0.38±0.86 <sup>a</sup>
Producer B	5.43±0.08 <sup>b</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.20±0.45 <sup>a</sup>	3.02±0.33 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>
Producer C	4.60±0.50 <sup>a</sup>	0.33±0.74 <sup>b</sup>	2.52±0.15 <sup>b</sup>	0.26±0.58 <sup>a</sup>	0.84±0.86 <sup>a</sup>	3.17±0.53 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>

Values are expressed as log cfu/g ± standard deviation.

For each producer (A, B, C) means followed by different letters are significantly different ( $P < 0.05$ ).

\*Producer A, samples S1-S5; Producer B, samples S6-S10; Producer C, samples S11-S15

**Table 3.** Results of viable counting of halophilic bacteria and eumycetes in *Surströmming*.

Sample*	Total halophilic aerobes	Total halophilic anaerobes	Presumptive halophilic lactobacilli	Presumptive halophilic lactococci	Halophilic eumycetes
S1	4.99±0.06	5.69±0.11	5.99±0.09	< 1.00	< 1.00
S2	6.08±0.04	6.09±0.07	5.88±0.05	< 1.00	< 1.00
S3	5.58±0.10	5.91±0.05	5.59±0.09	< 1.00	< 1.00
S4	5.43±0.20	5.83±0.03	5.93±0.05	< 1.00	< 1.00
S5	5.90±0.36	6.01±0.25	6.15±0.21	< 1.00	< 1.00
S6	6.45±0.00	6.52±0.06	6.52±0.03	< 1.00	< 1.00
S7	6.74±0.05	6.91±0.03	6.72±0.02	< 1.00	< 1.00
S8	6.74±0.18	6.64±0.14	6.68±0.04	< 1.00	< 1.00
S9	6.53±0.08	6.88±0.09	6.75±0.03	< 1.00	< 1.00
S10	6.60±0.03	6.98±0.05	6.95±0.03	< 1.00	< 1.00
S11	6.07±0.13	6.00±0.07	5.94±0.00	5.09±0.20	< 1.00
S12	5.92±0.13	5.87±0.09	5.88±0.16	4.16±0.06	< 1.00
S13	5.32±0.29	5.89±0.27	5.85±0.19	4.00±0.06	< 1.00
S14	5.61±0.53	5.61±0.13	5.86±0.28	4.21±0.13	< 1.00
S15	6.52±0.01	6.92±0.04	7.06±0.10	5.59±0.06	< 1.00
<b>Mean values</b>					
Producer A	5.59±0.42 <sup>a</sup>	5.90±0.16 <sup>a</sup>	5.91±0.20 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>
Producer B	6.61±0.13 <sup>b</sup>	6.79±0.20 <sup>b</sup>	6.72±0.15 <sup>b</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>
Producer C	5.89±0.46 <sup>a</sup>	6.06±0.50 <sup>a</sup>	6.12±0.53 <sup>a</sup>	4.61±0.69 <sup>b</sup>	0.00±0.00 <sup>a</sup>

Values are expressed as log cfu/g ± standard deviation.

For each producer (A, B, C) means followed by different letters are significantly different ( $P < 0.05$ ).

\*Producer A, samples S1-S5; Producer B, samples S6-S10; Producer C, samples S11-S15

**Table 4.** Volatile compounds detected in ready-to-eat *surströmming* samples

RI*	CI base peak**	Standard***	Name	Category	Mean $\pm$ SD (TIC arbitrary units $\times 10^4$ ) (n = 5)		
					Producer A	Producer B	Producer C
	60 [M+1] <sup>+</sup>		Trimethylamine	Nitrogen compounds	1067 $\pm$ 66 <sup>b</sup>	1307 $\pm$ 142 <sup>c</sup>	899 $\pm$ 49 <sup>a</sup>
	59 [M+1] <sup>+</sup>	Yes	Acetone	Ketones	74 $\pm$ 13	195 $\pm$ 30	217 $\pm$ 198
617	89 [M+1]	Yes	Ethyl acetate	Esters	8 $\pm$ 4 <sup>b</sup>	66 $\pm$ 33 <sup>a</sup>	25 $\pm$ 18 <sup>b</sup>
639			2-Methyl-2-Butanol	Alcohols	0 $\pm$ 0 <sup>b</sup>	0 $\pm$ 0 <sup>b</sup>	13 $\pm$ 7 <sup>a</sup>
654	69 [M+1-H <sub>2</sub> O] <sup>+</sup>		Butanal, 3-methyl	Aldehydes	12 $\pm$ 7	11 $\pm$ 2	19 $\pm$ 5
681	69 [M+1-H <sub>2</sub> O] <sup>+</sup>		1-Penten-3-ol	Alcohols	44 $\pm$ 11	26 $\pm$ 23	67 $\pm$ 35
688	87 [M+1]		2-pentanone	Ketones	58 $\pm$ 22	70 $\pm$ 20	480 $\pm$ 942
738	71 [M+1-H <sub>2</sub> O] <sup>+</sup>		3-Methyl-1-butanol	Alcohols	144 $\pm$ 34 <sup>a</sup>	25 $\pm$ 6 <sup>b</sup>	99 $\pm$ 34 <sup>a</sup>
751	95 [M+1] <sup>+</sup>		Disulphide, dimethyl-	Sulphur compounds	2756 $\pm$ 1123 <sup>a</sup>	1647 $\pm$ 576 <sup>a</sup>	810 $\pm$ 453 <sup>b</sup>
774	71 [M+1-H <sub>2</sub> O] <sup>+</sup>	Yes	1-Pentanol	Alcohols	46 $\pm$ 11 <sup>ab</sup>	32 $\pm$ 15 <sup>b</sup>	82 $\pm$ 39 <sup>a</sup>
777	69 [M+1-H <sub>2</sub> O] <sup>+</sup>		2-Penten-1-ol	Alcohols	26 $\pm$ 7	16 $\pm$ 6	28 $\pm$ 25
806	117 [M+1] <sup>+</sup>	Yes	Ethyl butyrate	Esters	213 $\pm$ 59 <sup>ab</sup>	786 $\pm$ 154 <sup>a</sup>	277 $\pm$ 144 <sup>b</sup>
844	109 [M+1] <sup>+</sup>		Methyl ethyl disulphide	Sulphur compounds	164 $\pm$ 56	106 $\pm$ 26	85 $\pm$ 66
857			Ethyl 2-methylbutyrate	Esters	8 $\pm$ 3 <sup>a</sup>	3 $\pm$ 2 <sup>b</sup>	4 $\pm$ 1 <sup>b</sup>
860	99 [M+1] <sup>+</sup>	Yes	(E)-2-hexenal	Aldehydes	18 $\pm$ 7 <sup>a</sup>	6 $\pm$ 4 <sup>b</sup>	7 $\pm$ 4 <sup>b</sup>
869	107 [M+1] <sup>+</sup>		Ethylbenzene	Aromatic compounds	1 $\pm$ 1	4 $\pm$ 3	2 $\pm$ 3
876	107 [M+1] <sup>+</sup>	Yes	p/o-Xylene	Aromatic compounds	12 $\pm$ 6	10 $\pm$ 4	13 $\pm$ 8
893	119 [M+1] <sup>+</sup>		S-Methyl thiobutyrate	Sulphur compounds	13 $\pm$ 5	15 $\pm$ 6	11 $\pm$ 13
894			2-Heptanone	Ketones	29 $\pm$ 7 <sup>a</sup>	3 $\pm$ 2 <sup>b</sup>	13 $\pm$ 8 <sup>b</sup>
895	105 [M+1] <sup>+</sup>		Styrene	Aromatic compounds	1 $\pm$ 0 <sup>b</sup>	1 $\pm$ 0 <sup>b</sup>	10 $\pm$ 6 <sup>a</sup>
896	123 [M+1] <sup>+</sup>		Disulphide, methyl-isopropyl-	Sulphur compounds	0 $\pm$ 0 <sup>b</sup>	8 $\pm$ 2 <sup>a</sup>	0 $\pm$ 0 <sup>b</sup>
901	130 [M] <sup>+</sup>		Propyl butyrate	Esters	14 $\pm$ 5 <sup>b</sup>	33 $\pm$ 7 <sup>a</sup>	16 $\pm$ 6 <sup>b</sup>
903	97 [M+1-H <sub>2</sub> O] <sup>+</sup>	Yes	Heptanal	Aldehydes	52 $\pm$ 6 <sup>a</sup>	23 $\pm$ 4 <sup>b</sup>	29 $\pm$ 11 <sup>b</sup>
977	127 [M+1] <sup>+</sup>		Trisulfide, dimethyl-	Sulfur compounds	766 $\pm$ 1178	105 $\pm$ 75	45 $\pm$ 33
983	111 [M+1-H <sub>2</sub> O] <sup>+</sup>	Yes	1-Octen-3-ol	Alcohols	11 $\pm$ 6	11 $\pm$ 4	12 $\pm$ 5
986	143 [M+1] <sup>+</sup>		2,3-Octanedione	Ketones	7 $\pm$ 3	10 $\pm$ 5	7 $\pm$ 6
991	95 [M+1] <sup>+</sup>	Yes	Phenol	Aromatic compounds	440 $\pm$ 13 <sup>b</sup>	169 $\pm$ 84 <sup>b</sup>	2233 $\pm$ 1335 <sup>a</sup>
997	144 [M] <sup>+</sup>		Butyl butyrate	Esters	0 $\pm$ 0 <sup>b</sup>	164 $\pm$ 44 <sup>a</sup>	0 $\pm$ 0 <sup>b</sup>
1034		Yes	Limonene	Monoterpene hydrocarbons	5 $\pm$ 2	7 $\pm$ 7	5 $\pm$ 10
1096			1,2,4-Trithiolane	Sulphur compounds	0 $\pm$ 0	0 $\pm$ 0	18 $\pm$ 25
1176	107 [M/2] <sup>+</sup>		Disulphide, bis[1-(methylthio)ethyl]	Sulphur compounds	132 $\pm$ 37	84 $\pm$ 24	175 $\pm$ 180
1200		Yes	Dodecane	Alkanes	1 $\pm$ 0	0 $\pm$ 0	5 $\pm$ 6
1259			1,3-Ditert-butylbenzene	Aromatic compounds	5 $\pm$ 1 <sup>b</sup>	3 $\pm$ 1 <sup>b</sup>	45 $\pm$ 31 <sup>a</sup>
1300		Yes	Tridecane	Alkanes	0 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 1
1702	60 [M+1] <sup>+</sup>	Yes	Heptadecane	Alkanes	2 $\pm$ 1 <sup>b</sup>	4 $\pm$ 3 <sup>ab</sup>	9 $\pm$ 6 <sup>a</sup>

\* Kovats Retention indices (RI) calculated for DB-5 type column using *n*-alkanes (C6-C20); \*\*Chemical Ionization (CI) experiments using methanol as reagent gas; \*\*\*Pure standard matching RI and mass spectrum. TIC, Total ion current.

**Fig. 1**



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Fig. 2

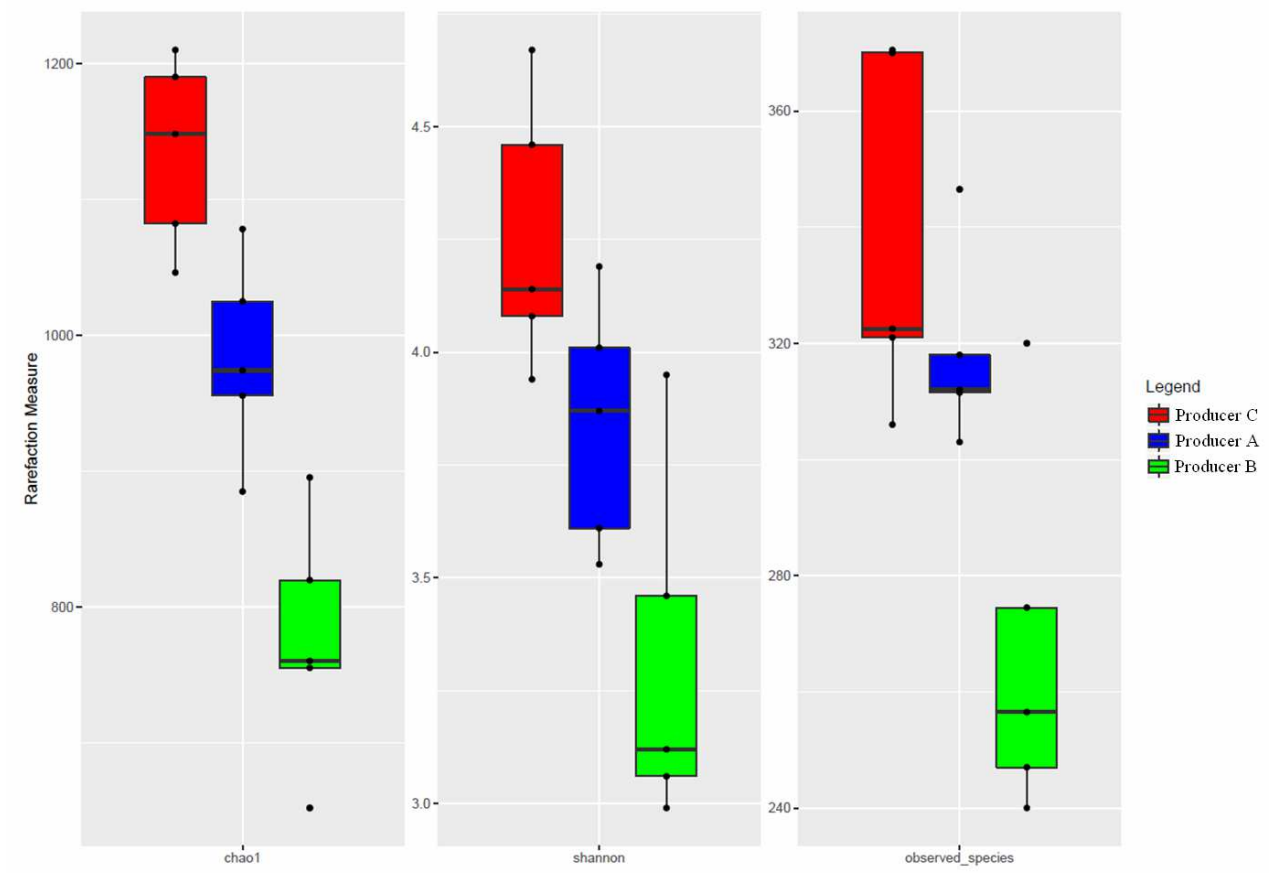


Fig. 3

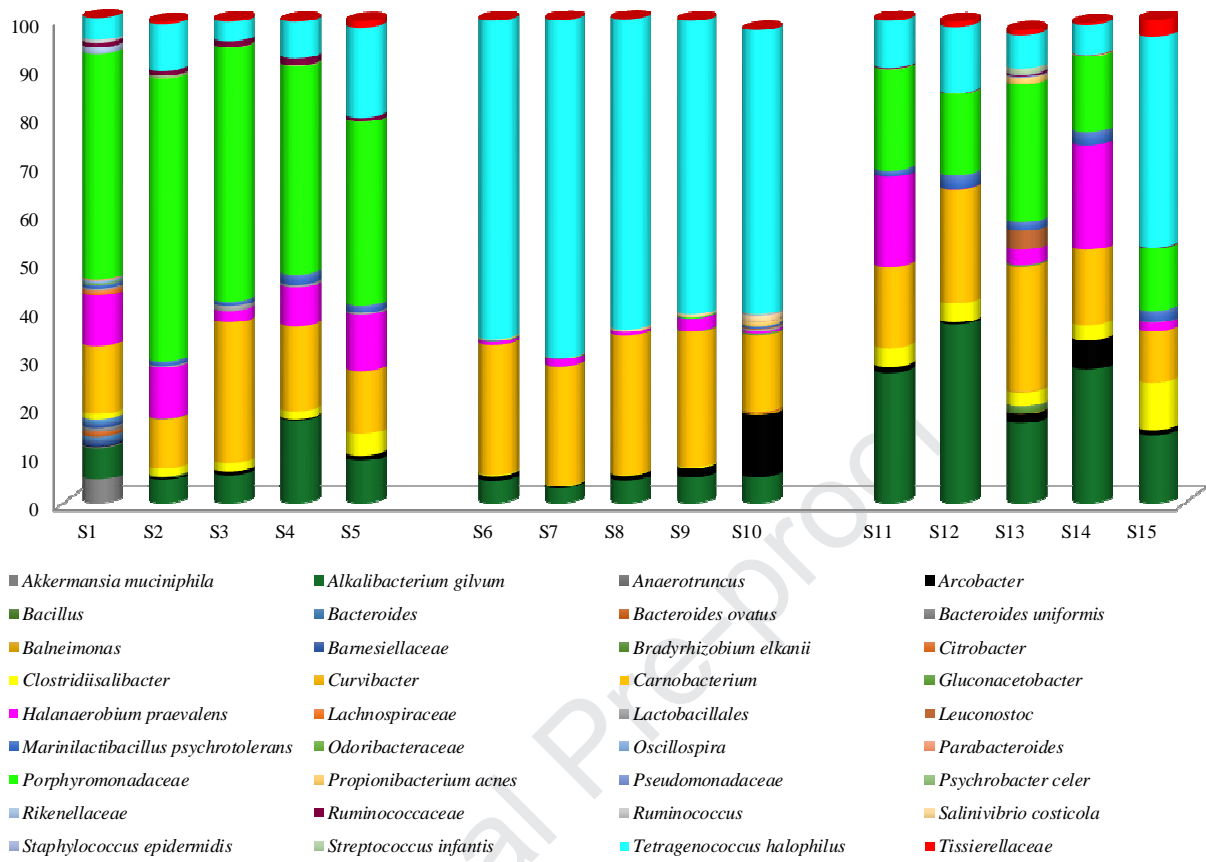


Fig. 4

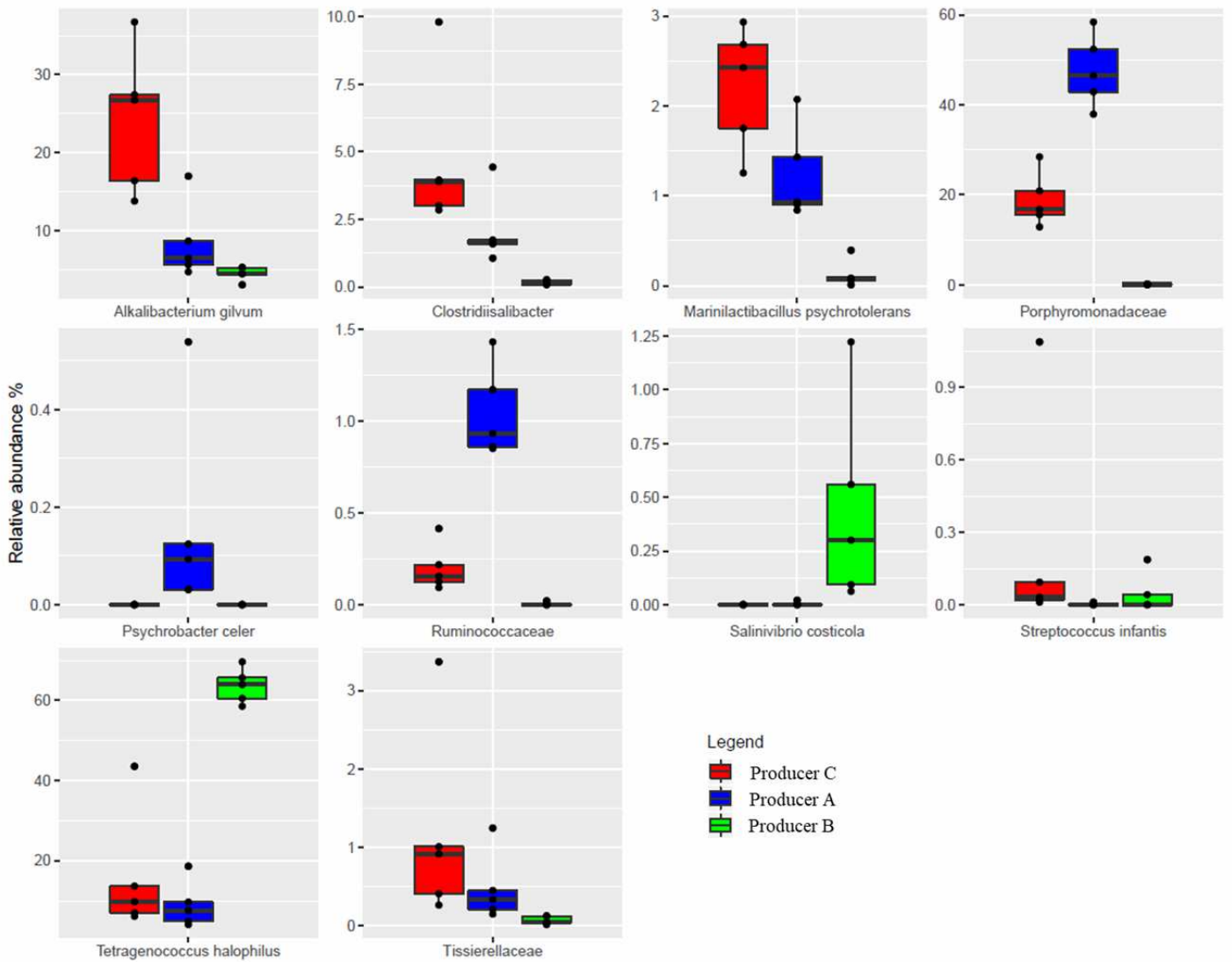
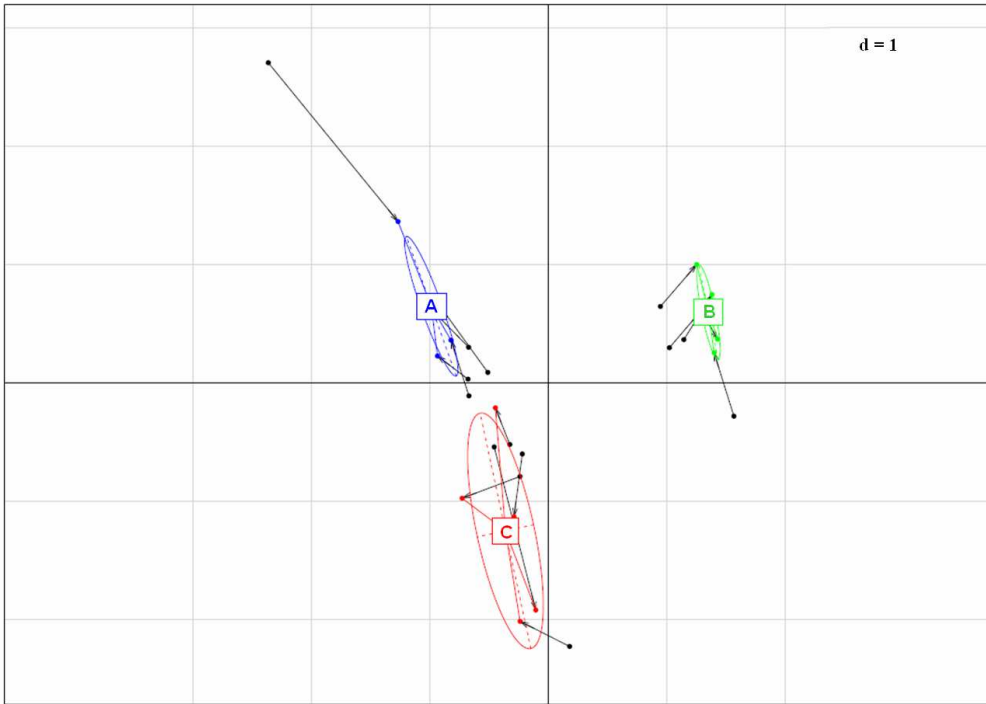






Fig. 6



## Highlights

- The microbiota of *surströmming* samples from three Swedish producers was studied
- *Alkalibacterium*, *Carnobacterium* and *Tetragenococcus* were detected in all the samples
- *Halanaerobium*, *Clostridiisalibacter* and *Porphyromonadaceae* were also found
- *Listeria monocytogenes*, *Salmonella* and botulinic toxins genes were never detected
- The massive presence of trimethylamine and sulphur compounds was detected

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To the Editor of  
Food Microbiology

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Dear Editor,

regarding the manuscript titled “**Discovering microbiota and volatile compounds of *surströmming*, the traditional Swedish sour herring**” submitted for publication in Food Microbiology, the authors declare that NO conflict of interest exists.

Kind regards

On behalf of all the authors  
The corresponding author  
Andrea Osimani

