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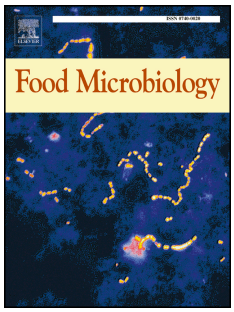

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Discovering microbiota and volatile compounds of *surströmming*, the traditional Swedish sour herring

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Sampling

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

2.2. Chemical-physical measurements

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

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

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

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

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

For each sample, the measurements were performed in duplicate, and the results are expressed as the mean ± standard deviation.

2.3. Microbiological analyses

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

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

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

2.4. DNA extraction from surströmming samples

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

2.5. DNA library preparation and sequencing

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

2.6. Bioinformatics

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

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

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

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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-µl reaction
224 volume. Molecular-grade H_2O 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*

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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 µm 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

Version 2.0a, built July 1, 2002 (National Institute of Standards and Technology); and analysis of the CI mass spectra using RI data reported in the published literature and listed in several authentic online databases (http://webbook.nist.gov, https://pubchem.ncbi.nlm.nih.gov, http://www.flavornet.org). RIs were determined for SPME-GC/MS by using a series of n-hydrocarbons (C6-C20) (Sigma-Aldrich, St. Louis, MO).

Experimental data (volatile component abundance) were subjected to analysis of variance (ANOVA), and the significance of means was evaluated by Tukey-Kramer's Honest Significant Difference (HSD) test ($P < 0.05$). Statistical analysis was performed using the software JMP® Version 10 (SAS Institute Inc., Cary, NC).

2.8. Statistical analysis

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

3. Results

3.1. Physicochemical measurements

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

3.2. Microbiological analyses

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Furthermore, no samples revealed the presence of *L. monocytogenes* or *Salmonella* spp. in 25 g of product. Finally, multiplex real-time PCR revealed the absence of the target genes *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *4gyrB (CP*), encoding botulinic toxins, in all the samples analyzed.

3.3. 16S rRNA gene amplicon target sequencing

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

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

3.4. GC-MS analysis of volatile components

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

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

3.5. Correlations between microbiota and volatilome profiles

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

Data regarding coagulase-negative staphylococci collected in the present study are in accordance with the results of 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 study of Gassem (2019), among the detected species, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus xylosus*, and *Staphylococcus saprophyticus* were those dominating the fermentation process. It is noteworthy that, in the analyzed *surströmming* samples, the presence of *Staphylococcus epidermidis* was also confirmed by metagenomic sequencing, although they presented a minority OTU. As reported by Zeng et al. (2017), protease and lipase activities of coagulase-negative staphylococci produce various flavor compounds, which can be responsible for the texture and flavor development in salted fermented fish.

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

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

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

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

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

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

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

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

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

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

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

Regarding *Clostridiisalibacter*, species belonging to this genus have already been detected in deep-sea hydrothermal environments (Jiang et al., 2015); moreover, *Clostridiisalibacter paucivorans* has been isolated from olive mill wastewater (Liebgott et al., 2008). This moderately halophilic spore-forming bacterium was observed to grow at NaCl 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, with an optimum of 6.8. *Clostridiisalibacter* was also detected on the surface of semi-hard Danish Danbo (Ryssel et al., 2015) and Raclette type cheeses (Roth et al., 2010), together with *Marinilactibacillus psychrotolerans*, where the latter species, found as minority in *surströmming*, exerted potential antilisterial activity in the analyzed cheeses (Roth et al., 2010).

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

Although a minority OTU, *Arcobacter* was found in all the samples, highlighting potential safety issues for the consumers. This emerging foodborne and waterborne pathogen can serve as the causative agent of different gastrointestinal diseases (e.g., abdominal cramps and watery diarrhea) (Hsu and Lee, 2015). *Arcobacter* has been previously detected in various foodstuffs, including seafood (e.g., shellfish), with an average prevalence of 32.3 %, and the water environment, including seawater (Rivas et al., 2004; Collado et al., 2009; Ghane, 2014; Levican et al., 2014; Hsu and Lee, 2015; Leoni et al., 2017). As reported by Zhang et al. (2019), information concerning the prevalence of *Arcobacter* in retail seafood products is still scarce; hence, data obtained in the present study for *surströmming* can contribute to improving knowledge related to the occurrence of such pathogens in ready-to-eat foods. Interestingly, Zhang et al. (2019) found that among a total of 318 samples that included bivalves, shrimps and cephalopods collected from local retail seafood markets in Germany, 17.6% of the analyzed samples were positive for the presence *Arcobacter*. Among the detected species, *Arcobacter butzleri* was predominant, followed by *Arcobacter venerupis*, *Arcobacter cryaerophilus*, *Arcobacter aquimarinus*. *Arcobacter skirrowii* and *Arcobacter thereius* (Zhang et al., 2019).

Regarding volatile compounds detected in *surströmming*, it is well known that aroma components of fermented fish mainly originate from microbial proteolytic and lipolytic activities; further oxidation and metabolism of fatty acids and amino acid catabolism lead to the final products. Although the pathways of aroma component generation have been studied in some fermented foods, such as wine, beer and dairy products (Smit et al., 2005), research on the flavor of fermented fish is still limited to identification of the volatiles produced by different microorganisms (Zang et al., 2019). In such a context, the use of mass spectrometry is essential for analyzing complex mixtures of natural substances, in order to provide detailed and reproducible information about the molecular structure of unknown components that can be compared with mass spectrum libraries (Mozzon et al., 2015). In fact, due to the complexity of food aromas, the chromatographic behavior (retention indexes) is far from enough for unambiguous identification of chromatographic peaks. For this reason, chemical ionization experiments were also carried out since the lower energy impact allows the analytes to be less fragmented and therefore the molecular weight of an unknown analyte can be more easily determined.

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

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

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

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

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

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

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

5. Conclusions

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

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

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

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

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Conflict of interests

The authors declare no conflict of interests

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FIGURE CAPTIONS

Figure 1. Ready-to-eat *surströmming*

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

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

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

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

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

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

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

Only significant associations between OTUs and VOCs are shown (FDR< 0.05).
The intensity of the colors represents the degree of correlation, where blue represents a positive degree of correlation and red represents a negative correlation.

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

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).

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

Sample	pH	a_w	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
Mean values						
Producer A	6.86±0.07^{a}	0.933±0.001^{b}	7.54±0.88^{a}	4.6±0.33^{ab}	0.316±0.023^{a}	< 0.00002^{a}
Producer B	6.79±0.12^{a}	0.915±0.001^{a}	7.42±0.42^{a}	5.8±1.19^{b}	0.459±0.284^{b}	< 0.00002^{a}
Producer C	6.83±0.05^{a}	0.918±0.001^{a}	8.45±0.51^{a}	4.2±0.26^{a}	0.368±0.100^{ab}	0.009±0.018^{b}

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
Mean values								
Producer A	5.02±0.40^{a}	4.24±0.32^{c}	4.60±0.25^{c}	0.21±0.46^{a}	0.20±0.45^{a}	4.77±0.64^{b}	0.00±0.00^{a}	0.38±0.86^{a}
Producer B	5.43±0.08^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.20±0.45^{a}	3.02±0.33^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Producer C	4.60±0.50^{a}	0.33±0.74^{b}	2.52±0.15^{b}	0.26±0.58^{a}	0.84±0.86^{a}	3.17±0.53^{a}	0.00±0.00^{a}	0.00±0.00^{a}

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
Mean values					
Producer A	5.59±0.42^{a}	5.90±0.16^{a}	5.91±0.20^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Producer B	6.61±0.13^{b}	6.79±0.20^{b}	6.72±0.15^{b}	0.00±0.00^{a}	0.00±0.00^{a}
Producer C	5.89±0.46^{a}	6.06±0.50^{a}	6.12±0.53^{a}	4.61±0.69^{b}	0.00±0.00^{a}

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 ± SD (TIC arbitrary units ×10^4) (n = 5)		
					Producer A	Producer B	Producer C
	60 $[M+1]^+$		Trimethylamine	Nitrogen compounds	1067 ± 66^b	1307 ± 142^c	899 ± 49^a
	59 $[M+1]^+$	Yes	Acetone	Ketones	74 ± 13	195 ± 30	217 ± 198
617	89 [M+1]	Yes	Ethyl acetate	Esters	8 ± 4^b	66 ± 33^a	25 ± 18^b
639			2-Methyl-2-Butanol	Alcohols	0 ± 0^b	0 ± 0^b	13 ± 7^a
654	69 $[M+1-H_2O]^+$		Butanal, 3-methyl	Aldehydes	12 ± 7	11 ± 2	19 ± 5
681	69 $[M+1-H_2O]^+$		1-Penten-3-ol	Alcohols	44 ± 11	26 ± 23	67 ± 35
688	87 [M+1]		2-pentanone	Ketones	58 ± 22	70 ± 20	480 ± 942
738	71 $[M+1-H_2O]^+$		3-Methyl-1-butanol	Alcohols	144 ± 34^a	25 ± 6^b	99 ± 34^a
751	95 $[M+1]^+$		Disulphide, dimethyl-	Sulphur compounds	2756 ± 1123^a	1647 ± 576^a	810 ± 453^b
774	71 $[M+1-H_2O]^+$	Yes	1-Pentanol	Alcohols	46 ± 11^{ab}	32 ± 15^b	82 ± 39^a
777	69 $[M+1-H_2O]^+$		2-Penten-1-ol	Alcohols	26 ± 7	16 ± 6	28 ± 25
806	117 $[M+1]^+$	Yes	Ethyl butyrate	Esters	213 ± 59^{ab}	786 ± 154^a	277 ± 144^b
844	109 $[M+1]^+$		Methyl ethyl disulphide	Sulphur compounds	164 ± 56	106 ± 26	85 ± 66
857			Ethyl 2-methylbutyrate	Esters	8 ± 3^a	3 ± 2^b	4 ± 1^b
860	99 $[M+1]^+$	Yes	(E)-2-hexenal	Aldehydes	18 ± 7^a	6 ± 4^b	7 ± 4^b
869	107 $[M+1]^+$		Ethylbenzene	Aromatic compounds	1 ± 1	4 ± 3	2 ± 3
876	107 $[M+1]^+$	Yes	p/o-Xylene	Aromatic compounds	12 ± 6	10 ± 4	13 ± 8
893	119 $[M+1]^+$		S-Methyl thiobutyrate	Sulphur compounds	13 ± 5	15 ± 6	11 ± 13
894			2-Heptanone	Ketones	29 ±7^a	3 ± 2^b	13 ± 8^b
895	105 $[M+1]^+$		Styrene	Aromatic compounds	1 ± 0^b	1 ± 0^b	10 ± 6^a
896	123 $[M+1]^+$		Disulphide, methyl-isopropyl-	Sulphur compounds	0 ± 0^b	8 ± 2^a	0 ± 0^b
901	130 $[M]^+$		Propyl butyrate	Esters	14 ± 5^b	33 ± 7^a	16 ± 6^b
903	97 $[M+1-H_2O]^+$	Yes	Heptanal	Aldehydes	52 ± 6^a	23 ± 4^b	29 ± 11^b
977	127 $[M+1]^+$		Trisulfide, dimethyl-	Sulfur compounds	766 ± 1178	105 ± 75	45 ± 33
983	111 $[M+1-H_2O]^+$	Yes	1-Octen-3-ol	Alcohols	11 ± 6	11 ± 4	12 ± 5
986	143 $[M+1]^+$		2,3-Octanedione	Ketones	7 ± 3	10 ± 5	7 ± 6
991	95 $[M+1]^+$	Yes	Phenol	Aromatic compounds	440 ± 13^b	169 ± 84^b	2233 ± 1335^a
997	144 $[M]^+$		Butyl butyrate	Esters	0 ± 0^b	164 ± 44^a	0 ± 0^b
1034		Yes	Limonene	Monoterpene hydrocarbons	5 ± 2	7 ± 7	5 ± 10
1096			1,2,4-Trithiolane	Sulphur compounds	0 ± 0	0 ± 0	18 ± 25
1176	107 $[M/2]^+$		Disulphide, bis[1-(methylthio)ethyl]	Sulphur compounds	132 ± 37	84 ± 24	175 ± 180
1200		Yes	Dodecane	Alkanes	1 ± 0	0 ± 0	5 ± 6
1259			1,3-Ditert-butylbenzene	Aromatic compounds	5 ± 1^b	3 ± 1^b	45 ± 31^a
1300		Yes	Tridecane	Alkanes	0 ± 0	1 ± 0	1 ± 1
1702	60 $[M+1]^+$	Yes	Heptadecane	Alkanes	2 ± 1^b	4 ± 3^{ab}	9 ± 6^a

* 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

Fig. 2

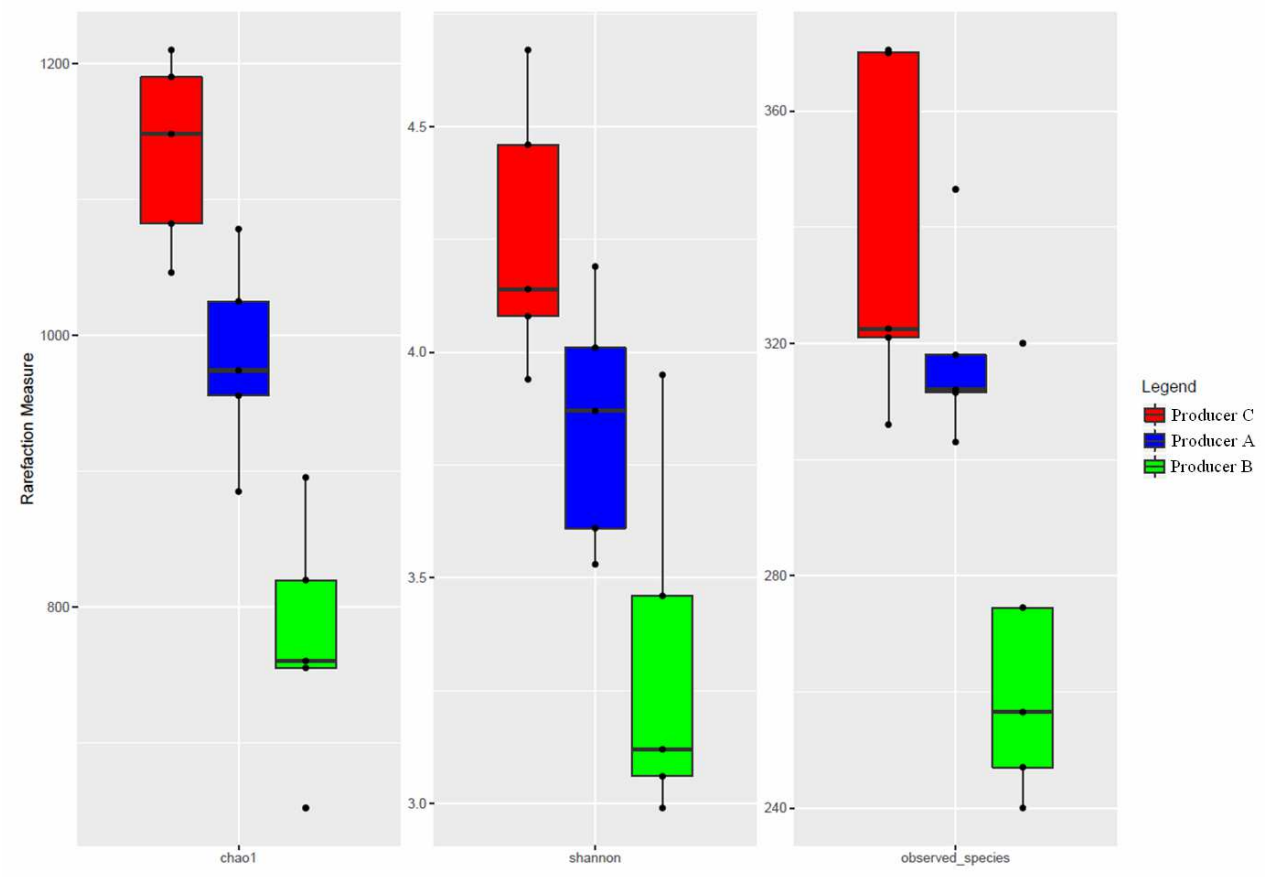


Fig. 3

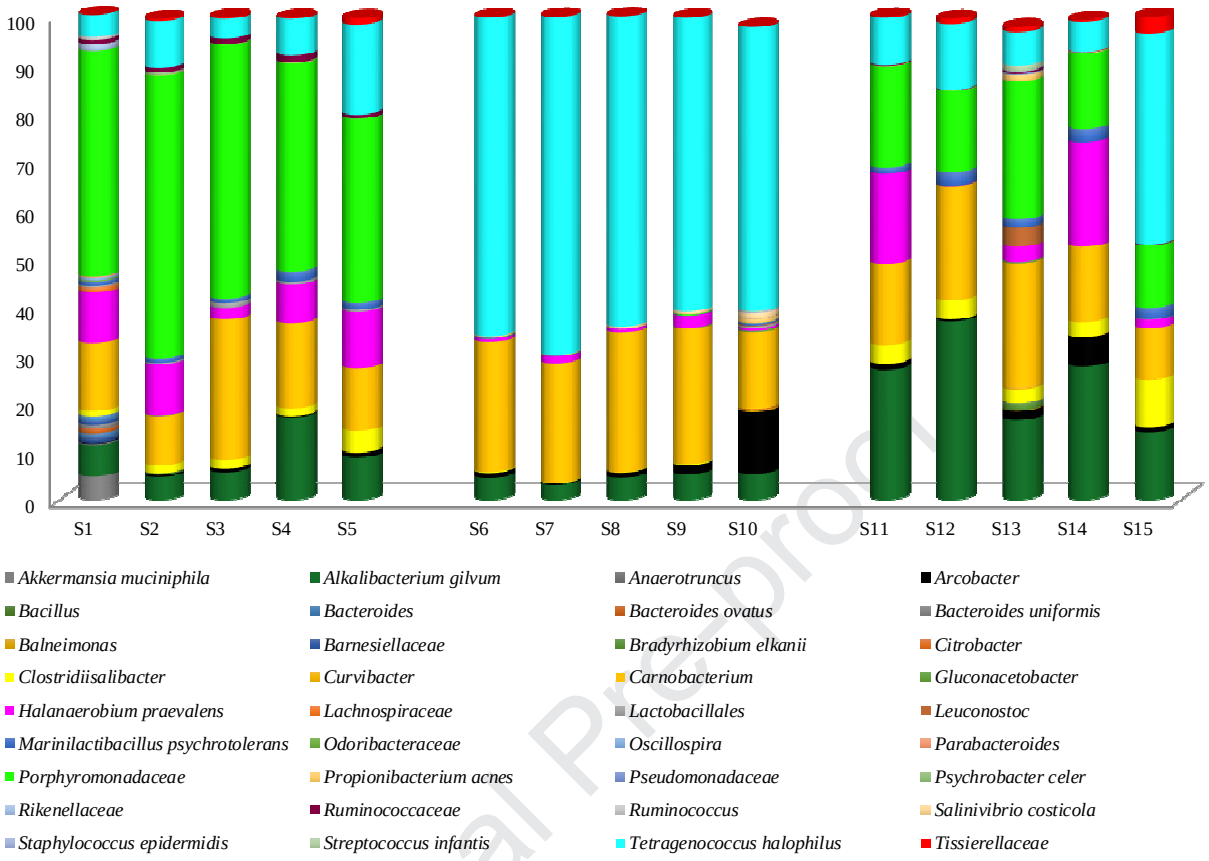


Fig. 4

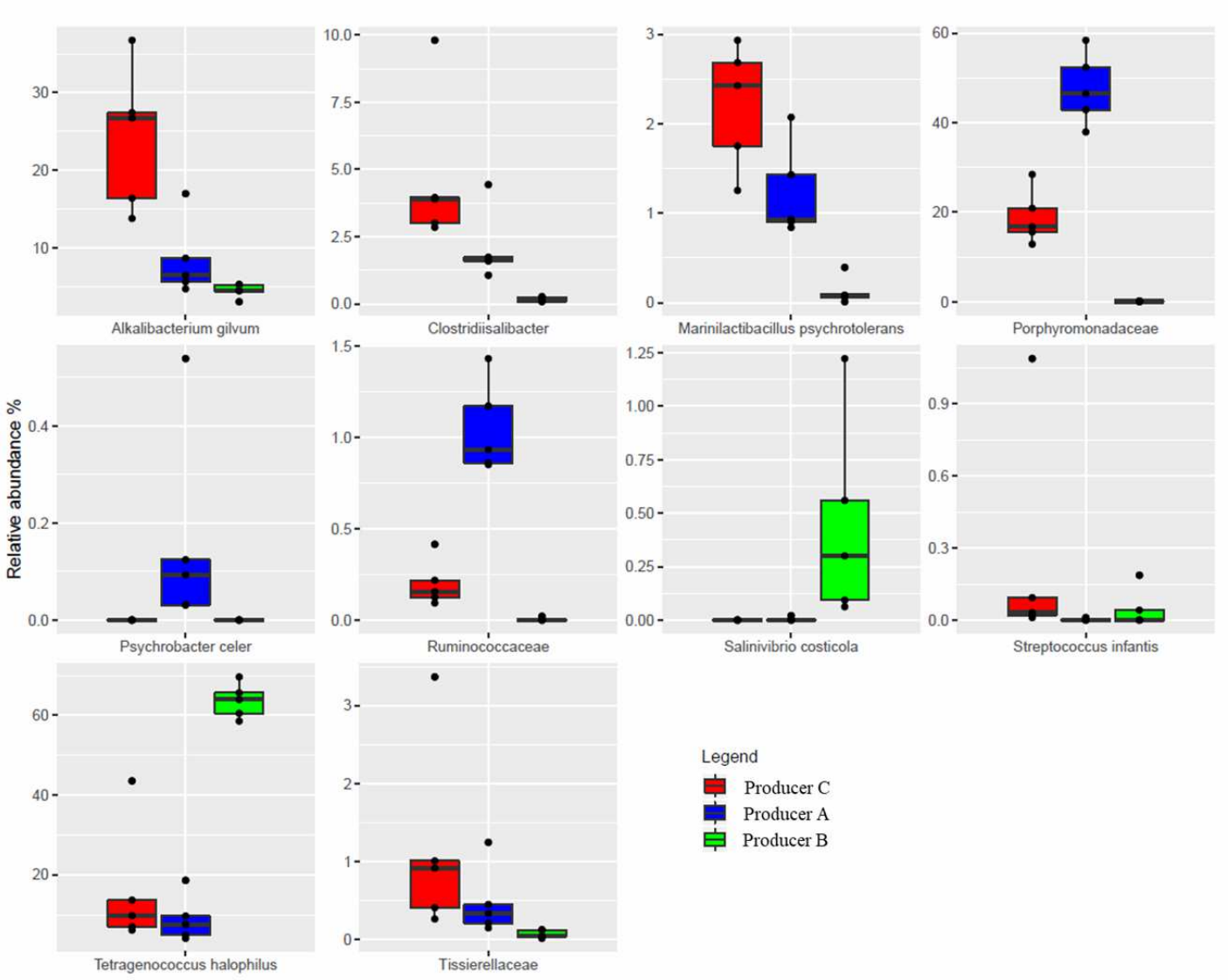

Relative abundance %
Alkalibacterium gilvum
Clostridiisalibacter
Marinilactibacillus psychrotolerans
Porphyromonadaceae
Psychrobacter celer
Ruminococcaceae
Salinivibrio costicola
Streptococcus infantis
Tetragenococcus halophilus
Tissierellaceae
Legend
Producer C
Producer A
Producer B

Fig. 5

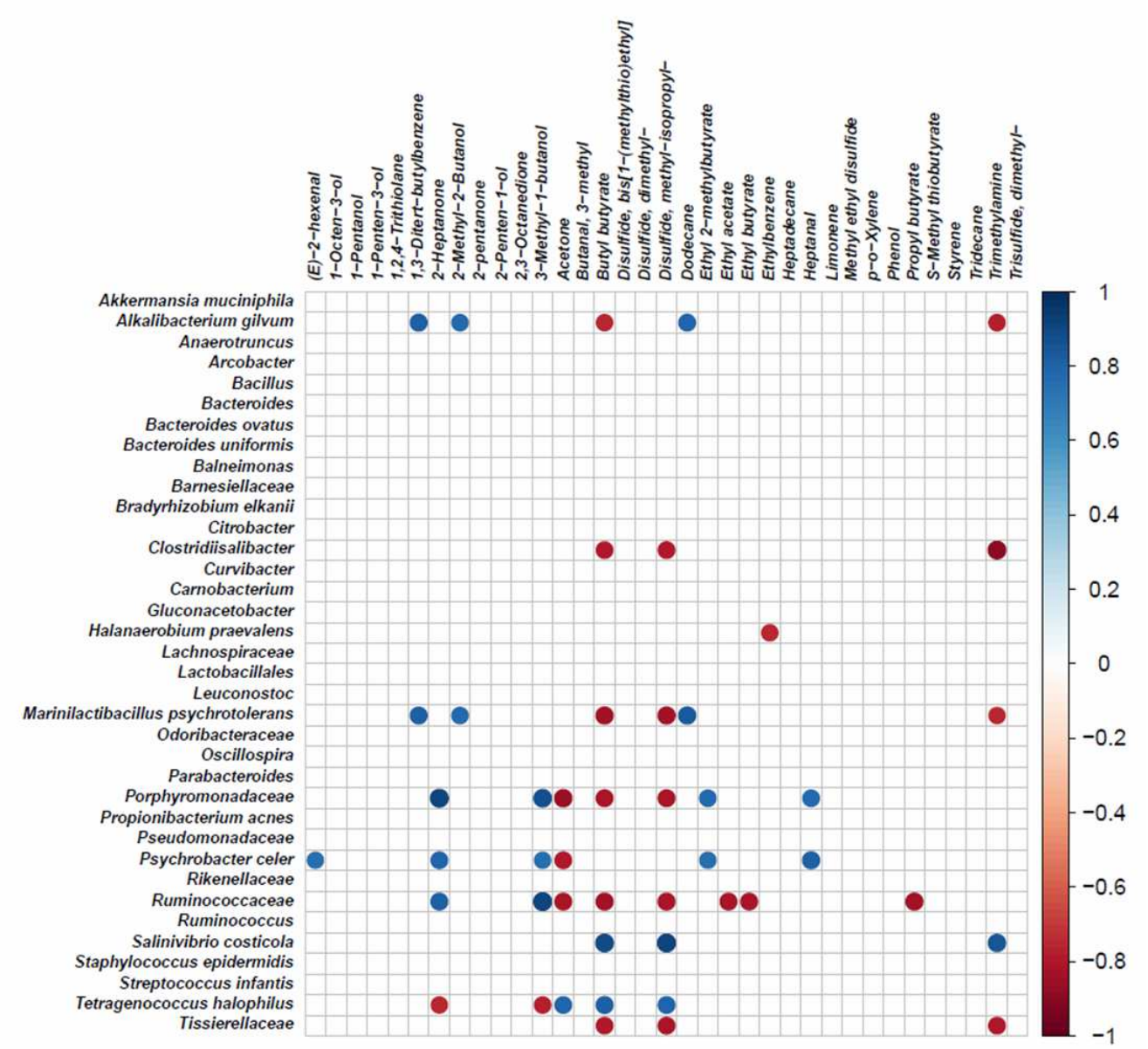
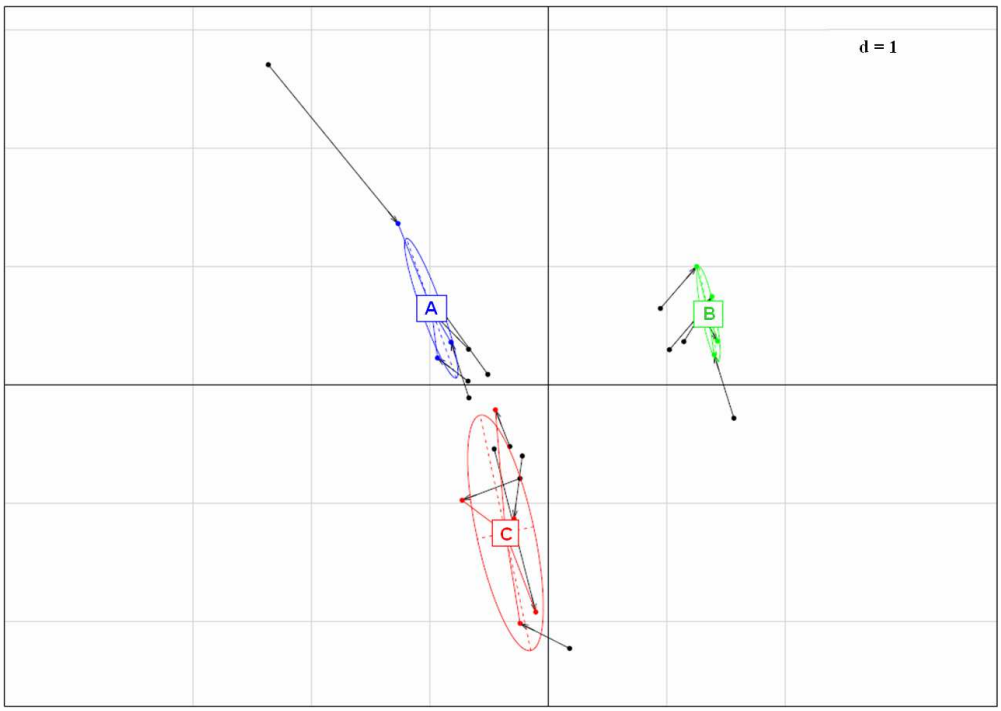

(E)−2−hexenal
1−Octen−3−ol
1−Pentanol
1−Penten−3−ol
1,2,4− Trithiolane
1,3−Ditert−butylbenzene
2−Heptanone
2−Methyl−2−Butanol
2−pentanone
2−Penten−1−ol
2,3−Octanedione
3−Methyl−1−butanol
Acetone
Butanal, 3−methyl
Butyl butyrate
Disulfide, bis[1−(methylthio)ethyl]
Disulfide, dimethyl−
Disulfide, methyl−isopropyl−
Dodecane
Ethyl 2−methylbutyrate
Ethyl acetate
Ethyl butyrate
Ethylbenzene
Heptadecane
Heptanal
Limonene
Methyl ethyl disulfide
p−o−Xylene
Phenol
Propyl butyrate
S−Methyl thiobutyrate
Styrene
Tridecane
Trimethylamine
Trisulfide, dimethyl−
Akkermansia muciniphila
Alkalibacterium gilvum
Anaerotruncus
Arcobacter
Bacillus
Bacteroides
Bacteroides ovatus
Bacteroides uniformis
Balneimonas
Barnesiellaceae
Bradyrhizobium elkanii
Citrobacter
Clostridiisalibacter
Curvibacter
Carnobacterium
Gluconacetobacter
Halanaerobium praevalens
Lachnospiraceae
Lactobacillales
Leuconostoc
Marinilactibacillus psychrotolerans
Odoribacteraceae
Oscillospira
Parabacteroides
Porphyromonadaceae
Propionibacterium acnes
Pseudomonadaceae
Psychrobacter celer
Rikenellaceae
Ruminococcaceae
Ruminococcus
Salinivibrio costicola
Staphylococcus epidermidis
Streptococcus infantis
Tetragenococcus halophilus
Tissierellaceae
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

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

Ancona, 24-09-2019

To the Editor of
Food Microbiology

Prof. Andrea Osimani, Ph.D.
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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