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Running title:

Taxonomic RNA-based assessment of beefsteaks spoilage microbiota

Key words: 16S rRNA, Meat, Volatilome, Aqueous ozone, Electrolyzed water, *Pseudomonas fragi*

Abstract

The microbial contamination that occurs during the slaughtering process and during handling of the meat results in a shortening of the shelf-life of meat. In this study, which has had the aim of extending the shelf life of beefsteaks, pilot-scale treatments were carried out with aqueous ozone (AO) and electrolyzed water (EW) before vacuum packaging (VP). The development of the potentially active microbiota and the associated volatilome were followed over 15 days of storage under refrigerated conditions (4 °C), in order to define the potential long-term effects of the treatments and storage condition on microbiota.

The targeted RNA-based amplicon sequencing identified *Pseudomonas fragi* as the most frequent species before

26 and after the treatments with AO and EW, as well as in the untreated control. The tested treatments did not
27 reduce the overall presence of this species, but they affected the intra-species distribution of its oligotypes, albeit
28 slightly. With the progression of the refrigerated storage and the reduction of the oxygen availability,
29 *Lactobacillus sakei*, *Leuconostoc gasicomitatum* and *Lactococcus piscium* became the dominant, potentially
30 active, beef microbiota, as confirmed by microbiological data. When the OTU abundances and volatilome were
31 coupled, a significant association was observed between the organic acids, esters and aldehydes and these lactic
32 acid bacteria species.
33 In spite of the limited effectiveness of the treatments over the short and long term, this study has provided a
34 detailed view of beef spoilage using RNA as the sequencing target, strengthening and confirming the current
35 knowledge based on DNA-amplicon sequencing.
36

37 **Introduction**

38 Apart from abiotic factors (e.g. oxygen and UV radiation) and endogenous autolytic enzymatic reactions, the
39 spoilage of meat is mainly caused by complex microbial dynamics that encompass heterogeneous bacterial taxa,
40 of which the most common are *Pseudomonas sp.*, *Enterobacteriaceae*, *Brochothrix thermosphacta* and
41 psychrotrophic lactic acid bacteria (LAB), all of which are capable of surviving and proliferating in a cold
42 environment (Agapi I Doulgeraki et al., 2012; Doulgeraki et al., 2010; Ercolini et al., 2009; Pothakos et al.,
43 2015). It is well known that several different bacterial groups contaminate meat during the slaughtering
44 processes, although the complexity of the microbiota of meat is reduced when it is sold, due to the selective
45 pressure determined by the storage temperature, the packaging atmospheres and the initial antimicrobial
46 treatments. (de Filippis et al., 2013; La Storia et al., 2012; Stellato et al., 2016). The main problem faced by the
47 meat processing industry is the necessity of efficiently contrasting the development of species capable of
48 producing the volatile organic compounds (VOCs) that are associated with unpleasant odors (Argyri et al.,
49 2015; Casaburi et al., 2015, 2011).
50 Accordingly, the treatment of meat with adequate preservation technologies before the being packaged may
51 represent a feasible solution to extend its shelf-life, and thus to avoid product losses. Several non-thermal

52 treatments have been considered and developed for the sanitization of ready-to-eat portions of meat, and
53 promising results have been achieved through the utilization of supercritical CO_2, gamma radiation, and
54 ultraviolet light (Buckow et al., 2017; Jermann et al., 2015; Sommers et al., 2017). In this frame, low levels of
55 aqueous ozone (AO) and electrolyzed water (EW) may represent economically convenient, environmentally
56 friendly and safe approaches for the sanitization of meat at the end of the slaughtering process, as well as of the
57 slaughter environments, since they are broad-spectrum disinfectants and leave the treated food free of residues.
58 AO and EW have long been known to be detrimental to the bacterial cells that result from the destructive
59 oxidation of membrane-bound respiratory enzymes and lipids, the perturbation of cellular electrical charge
60 maintenance, proteins and peptidoglycan in spore coats and virus capsids (Huang et al., 2008; Miller et al., 2013;
61 Veasey and Muriana, 2016). To date, the decontaminant efficacy of these oxidative agents has only been tested
62 at low concentrations and at a pilot-scale level by spraying or dipping beef and poultry, without observing
63 deterioration of the organoleptic characteristics due to lipid oxidation or irreversible color modification, while
64 the viable counts of several microbial group have been found to be reduced (Duan et al., 2016; Kalchayanand et
65 al., 2008; Pohlman et al., 2002; Veasey and Muriana, 2016). However, the studies carried out so far on beef
66 sprayed with AO and/or EW have been limited to observing the microbial reduction after treatment or the
67 decontaminating effect toward deliberately introduced pathogens, without considering the complex dynamics of
68 spoilage microbiota and the associated volatilome that may develop after the treatments and during storage. So
69 far, the treatment of other food has also followed similar approaches, with attention being focused only on the
70 short term effect of AO and EW (Pinto et al., 2015; Segat et al., 2014). Only recently has the post-treatment
71 effect of aqueous ozone been investigated on wine grapes by means of culture-independent techniques applied
72 during winemaking, and a significant perturbation of the yeast population of the final wine volatilome has
73 been revealed (Cravero et al., 2016).
74 Nowadays, such ecological studies, aimed at unraveling the composition and dynamics of food microbiota,
75 cannot be dealt without the use of high-throughput amplicon target sequencing (HTS) approaches, which may be
76 oriented either toward DNA or RNA to explain the total microbial community (Ercolini, 2013; Ferrocino and
77 Cocolin, 2017; Li et al., 2016). Despite RNA-based amplicon sequencing is susceptible to biases depending on

78 PCR process and presence of rRNA beyond the life cycle of the cells (Rosselli et al., 2016). The decay of rRNA
79 after bacterial death is not generally predictable (Ceuppens et al., 2014), however rRNA remains the most
80 suitable target to detect microbial phylotypes with potentially metabolic activities in the food matrix (Yang et al.,
81 2017). This approach may result in a more reliable correlation between taxa and volatile compounds related to
82 meat spoilage. (De Angelis et al., 2015; De Pasquale et al., 2016).
83 Therefore, the aim of this work was to investigate the effect of AO and EW treatments on the complexity and
84 dynamics of the potential active microbiota of beefsteaks, and their associated volatilome, during storage at 4 °C
85 and in vacuum packaging conditions.
86
87
88 **Materials and methods**
89
90 **Treatments with aqueous (AO) ozone and electrolyzed water (EW)**
91 The studied steaks, weighing about 200 g each, were obtained from three different batches of tender boneless
92 beef, 24 h after slaughtering. Each batch of beefsteaks was divided equally into four parts (7 beefsteaks per part)
93 and treated with (EW) electrolyzed water, (AO) aqueous ozone and (W) water, while a fourth untreated part was
94 used as a control (C) (Fig. 1). The AO was produced using a C32-AG O_3 generator (De Nora S.P.A, Milano,
95 Italia) equipped with an oxygen concentrator, with/which has a nominal production capacity of 32 g O_3/h, and
96 using/considering pure oxygen as an/the input gas. The AO treatment was performed with water containing 6.00
97 ± 0.25 mg/L. EW was produced from salt (KCl) diluted in tap water using an Eva System 100 (De Nora S.P.A.).
98 The system produced EW of approximately 4 g/L free chlorine, pH 9 and 1 % residual KCl. The treatments were
99 performed with diluted EW at 100 mg L^{-1} of free chloride. The water treated samples (W) were treated in the
100 same way using the same time frame and the same type of water used to produce EW and AO, in order to
101 highlight any effect due to the water itself without oxidizing agents. All the treatments were carried out by
102 homogeneously spraying each side of the beefsteaks, placed on a still grid in a dedicated sanitized room of a
103 local slaughterhouse, for 90 sec (Cuneo, Italy). The spraying treatments were performed with a distance of 20

104 cm between the meat and nozzles, and pumping tap water at 4 °C at a constant flux. The treated beefsteaks
105 (AO, EW, W) were left to dry for 20 min on the grids and, together with the untreated control beefsteaks (C),
106 were packed singly in linear low-density polyethylene (LLDPE; oxygen transmission, 0.83 $cm^3 \cdot m^{-2} \cdot h^{-1}$ at
107 23°C, 30 cm X 30 cm) and vacuum packed.
108 The samplings were performed before the treatments, for each treatment and each batch, on the first day and
109 after 5, 9 and 15 days of storage at 4 °C.
110
111 **Microbiological analysis**
112 The packages were aseptically opened on each sampling day. Five surface portions of 1 cm^2 were cut from each
113 side of the beefsteaks, using a sterile scalpel and a cork borer (about 10 g of meat each sample), and were
114 homogenized in 90 ml of Ringer's solution (Oxoid, Basingstoke, Hampshire, UK) for 2 min using a Stomacher®
115 400 Circulator (LAB blender 400; PBI, Milan, Italy). Decimal dilutions were prepared, and aliquots of the
116 appropriate dilutions were spread in triplicate on the following media: (i) plate count agar (PCA, Lab M,
117 Heywood, Lancashire, UK) to establish the total aerobic bacteria incubated for 48 to 72 h at 30 °C; (ii) De Man
118 Rogosa and Sharpe agar (MRS, LabM) to establish the total LAB population, incubated at 30 °C for 48 h; (iii)
119 violet red bile agar (VRBGA, LabM) to establish the *Enterobacteriaceae*, incubated at 30 °C for 24–48 h; (iv)
120 malt extract agar (MEA, LabM) plus tetracycline (0.05 g L^{-1}; Sigma-Aldrich, St. Louis, USA) to establish the
121 yeasts and moulds/molds incubated at 25 °C for five days. The results were calculated as the means of log
122 colony forming units per cm^2 (log CFU/cm^2) of the beefsteak surface for the three batches (± standard error
123 mean).
124 In parallel, two 25 cm^2 surface pieces (about 25 g, one for each beefsteak surface) were cut and minced, and the
125 pH was measured with a pH-meter (Crison, Modena, Italy).
126 ANOVA (One way-Analysis of Variance), coupled with Tukey's post-hoc test and the Kruskal–Wallis non-
127 parametric test, were used to assess the overall variation and differences between the multiple groups. Statistical
128 analyses were performed with Statistica, ver. 7.0, (StatSoft Inc., Tulsa, OK, USA).
129

130 **GC-MS analysis of the volatile compounds (VOCs)**

131 Chemical analyses were performed before the treatment and after 1 and 15 days of storage. A static headspace
132 solid-phase microextraction analysis was carried out as described by Argyri et al. (2015), with some minor
133 modifications. Briefly, in parallel with the microbiological samplings, 3 g of surface pieces (one for each side of
134 the beefsteak) were cut from the beefsteak and then cut into small pieces using a sterile knife. Then, 1 g of
135 minced meat sample was placed in a 20 mL glass vial and mixed with 2 mL of 25 % NaCl solution and 10 µL of
136 internal standard (3-octanol, final concentration of 97 µg/kg).

137 After an equilibration time of 5 min at 40 °C, the extraction was performed, with stirring (250 rpm), adopting
138 the same temperature for 30 min with a 50/30 µm DVB/CAR/PDMS fiber (Supelco, Milan, Italy) using an
139 SPME autosampler (PAL System, CombiPAL, Switzerland). The fiber was desorbed at 260 °C for 1 min in
140 splitless mode. A GC/MS analysis was performed with a Shimadzu GC-2010 gas chromatograph, equipped with
141 a Shimadzu QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a DB-
142 WAXETR capillary column (30m × 0.25 mm, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA). The
143 carrier gas (He) flow rate was 1 mL/min. The temperature program was started at 40 °C and held for 5 min, and
144 the temperature was then increased at a rate of 10 °C/min to 80 °C and 5 °C/min to 240 °C for 5 min. The
145 injection port temperature was 260 °C, while the ion source temperature and the interface temperature were 240
146 °C. The detection was carried out by electron impact mass spectrometry, in total ion current mode, using an
147 ionization energy of 70 eV. The acquisition range was *m/z* 33–330 amu. The identification of volatile
148 compounds was confirmed by injecting pure standards, and a comparison was made of their retention indices (a
149 mixture of a homologous series of C5–C28) with MS data reported in the literature and in databases (NIST05
150 and http:// webbook.nist.gov/chemistry/). Any compounds for which no pure standards were available were
151 identified on the basis of the mass spectra and retention indices available in the literature. Semi-quantitative data
152 (µg/kg) were obtained by measuring the relative *m/z* peak area of each identified compound in relation to that of
153 the added internal standard.

154 Statistical analyses were performed as described above.

155

156 **RNA extraction and synthesis of cDNA**

157 An aliquot (1 ml) of the first 10-fold serial dilution was collected at each sampling point and centrifuged
158 directly at the maximum speed for 30 s. After removing the supernatants, 2 mL of RNA-later (Ambion, Thermo
159 Scientific, Milan, Italy) was immediately added to the pellet, which was then stored at – 80 °C. Total RNA was
160 extracted using the Master Pure complete DNA and RNA purification kit (Epicentre, Madison, WI, USA)
161 according to the manufacturer's instructions. Three microliters of Turbo DNase (Ambion) was added to digest
162 the DNA in the RNA samples, with an incubation of 3 h at 37°C. The quality of the extracted RNA was
163 evaluated and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Milan, Italy). The cDNA
164 was synthesized from 2 µg of RNA with the Moloney murine leukemia virus (M-MLV) Reverse Transcriptase
165 System (Promega, Milan, Italy). The reaction, in a final volume of 25 µL, contained: 1 µg of random hexamer
166 primers, 0.5 mM of each dNTP, 200 U of M-MLV Reverse Transcriptase enzyme, 25 U of RNase ribonuclease
167 inhibitor and 1 × M-MLV reaction buffer. The RT reactions were performed in an Engine Peltier Thermal Cycler
168 (BioRad, Hercules, CA, USA) according to the following steps: 72 °C for 5 min and 42 °C for 60 min.

169

170 **16S rRNA amplicon target sequencing**

171 The cDNA (2.5uL) was used to assess the potentially active microbiota that had amplified the V3-V4 region of
172 the 16S rRNA gene using the primers and the condition described by Klindworth et al. (2013). Owing to the
173 poor quality of the cDNA, three samples (one replicate of the samplings at 5, 9 and 15 days) were excluded.
174 The PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and
175 the resulting products were tagged with sequencing adapters using the Nextera XT library preparation kit
176 (Illumina Inc, San Diego, CA), according to the manufacturer's instructions. Sequencing was performed using a
177 MiSeq Illumina instrument (Illumina) with V3 chemistry, which generated 2X250 bp paired-end reads,
178 according to the manufacturer's instructions. MiSeq Control Software, V2.3.0.3, RTA, v1.18.42.0, and
179 CASAVA, v1.8.2, were used for the base-calling and Illumina barcode demultiplexing processes.

180

181 **Bioinformatics and statistical analysis**

182 Paired-end reads were first merged using FLASH software (Magoč and Salzberg, 2011) with default parameters.
183 Joint reads were quality filtered (at Phred < Q20) using QIIME 1.9.0 software (Caporaso et al., 2010) and
184 analyzed through the pipeline recently described (Ferrocino et al., 2017).
185 Alpha diversity indices were calculated using the diversity function of the vegan package (Dixon, 2003). The
186 Shannon-Wiener diversity index *H*' was further analyzed using the *t-test* to assess any differences between the
187 three producers and treatment. In order to avoid biases, due to different sequencing depths, all the samples were
188 rarefied at 1165 reads after raw read quality filtering. Weighted UniFrac distance matrices were used to perform
189 Adonis and Anosim statistical tests in the R environment (www.r-project.org). A filtered OTU table was
190 generated at 0.2% abundance, in at least 5 samples, through QIIME. In order to explore the relationship between
191 the microbiota of the meat and the VOC datasets, a principal components analysis (PCA) was carried out on the
192 individual datasets and the results were then integrated using coinertia analysis (CIA), which allows the shared
193 biological trends within the two datasets to be identified. A CIA analysis was performed with the *made4* package
194 in the R environment.
195 Reads assigned to the most abundant OTUs were extracted, and then entropy analysis and oligotyping were
196 carried out, as described by the developers (Eren et al., 2013). The OTUs that show the higher entropy were
197 chosen to compute the oligotypes (-C option): 8, 9, 12, 122, 131, 133, 136, 137, 217, 233, 253, 258, 268, 270,
198 279, 287, 297, 298, 299, 340, 457, 458. In order to minimize the noise, each oligotype was required to appear in
199 at least 5 samples (-s option), to occur in more than 1.0 % of the reads for at least one sample and to represent a
200 minimum of 200 reads (-M option) in all the combined samples (Stellato et al., 2017). Four samples were
201 removed from the analysis because their reads were eliminated during the QC. Pairwise Wilcoxon tests were
202 used to determine any significant differences in specific oligotype abundance according to the treatment.
203 Spearman correlation coefficients were calculated between the oligotype abundance data matrix and the VOCs
204 through the *psych* function of R, and were then plotted through the *made 4* function of R.
205 All the sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology
206 Information (SRP095454).
207

208

209 **Results**

210

211 **Microbial dynamics and volatile compounds (VOCs)**

212 The results of the viable counts of the spoilage-related microorganisms made during the storage of the treated
213 (EW, AO and W) and untreated (C) vacuum-packed beefsteaks are shown in Table 1 together with the pH
214 variations. All the considered microbial populations and the pH were affected more by the storage period than
215 by the undergone treatment, with a progressive increase in counts that became significantly higher than the
216 initial level from the 5th day of storage onwards ($P < 0.05$). Only at the 15th day of storage did all the samples
217 show a significant decrease in the pH values. Overall, LAB were the dominant population after the first day of
218 storage, and they reached the highest values at the end of the shelf-life (on average 6 log/cm^2),.
219 As far as the VOCs in the headspace are concerned, 32 different compounds were detected, of which ethanol,
220 hexanal, acetoin, ethyl acetate and acetic acid showed higher concentrations than 100 µg/kg (Supplementary
221 Table 1).
222 Overall, alcohols were the most numerous compounds present in the headspace, and they were followed by
223 aldehydes, ketones, volatile fatty acids and esters. Amounts of VOCs released from the beefsteaks varied during
224 storage time and, regardless of the treatments and batch of origin. Higher amounts of volatile organic acids,
225 esters and 3-methyl-1-butanol were observed at the end of the storage period, the concentrations of ketones
226 decreased at the same time, while most of the alcohols and aldehydes remained stable over the storage period
227 (Tab. 2). Notably, only ethyl acetate and acetic acid, increased significantly (P <0.05) along the shelf life,
228 whereas 1-pentanol, 1-octen-3-ol and acetoin significantly decreased (P <0.05).

229

230 **Assessment of bacterial population based on RNA**

231 A total of 966.656 raw reads (2x250bp) were obtained after sequencing. After joining the pair end reads, a total
232 of 640.315 reads passed the filters applied by QIIME, with an average value of 13.339 (min 1164 max 56.239)
233 reads/sample and a sequence length of 465 bp. The rarefaction analysis and the estimated sample coverage

234 (Supplementary Table 2) indicated that there was a satisfactory coverage of all the samples (ESC between 85-
235 95%). Moreover, the alpha-diversity showed that there were no differences, in terms of complexity ($P > 0.05$),
236 between the treatment and storage time. Adonis and Anosim statistical tests, based on the Weighted UniFrac
237 distance matrix, showed significant differences over time ($P < 0.001$). Overall, the most frequently detected
238 relative abundant OTUs were *Psychrobacter sp.*, *P. fragi*, *Lactococcus* (*Lc.*) *piscium*, *Lactobacillus* (*Lb.*) *sakei*
239 and *Leuconostoc* (*L.*) *gasicomitatum*, which represented more than 84 % of the total relative abundance at all the
240 sampling points (Fig. 2). The microbiota showed an initial condition dominated by *Psychrobacter sp.*, *P. fragi*,
241 which represented 10 % and 72 % of the relative OTU abundance, respectively. From the first day after the
242 treatments and vacuum packaging, *Psychrobacter sp.* and *P. fragi* remained the most abundant OTUs in all the
243 samples, while *L. gasicomitatum*, *Lc. piscium* and *Lb. sakei* were the main OTUs detected on the 5th, 9th and 15th
244 days.
245 When the relative abundance of the main OTUs was compared across samples, it was possible to observe that
246 *Lc. piscium* and *Lactobacillus sp.* were found to be characteristic in samples treated with AO (g-test $P < 0.01$),
247 while *P. fragi* and *Psychrobacter* were found to be characteristic in the untreated control samples ($P < 0.01$). The
248 co-occurrence/exclusion patterns of the OTUs were also investigated (Supplementary Figure 1), and only the
249 significant correlations are here reported (False Discovery Rate - FDR < 0.05). The most abundant OTUs, *Lb.*
250 *sakei*, *Photobacterium* and *P. fragi,* displayed the highest number of negative correlations. *P. fragi* in particular
251 displayed a strong co-exclusion with *Carnobacterium divergens* and *Lc. piscium*. *C. divergens* instead displayed
252 the highest number of positive correlations with *Lb. sakei*, *Lc. piscium* and *L. gasicomitatum*.
253
254 **Correlations between the potentially active community and volatile compounds (VOCs)**
255 Plotting the correlation between the OTUs and VOCs, it was observed that *P. fragi* and *Acinetobacter lwoffii*
256 showed a positive correlation with octanal, nonanal and the alcohols, while *C. divergens* and *Lc. piscium* were
257 correlated with the short chain ethyl lactate, ethyl acetate and the short and medium chain fatty acids (Fig. 3).
258 A co-inertia analysis was carried out, combining the PCA of the microbiota (OTUs) and the VOCs, in order to
259 establish the relative importance of the OTU vectors that affect the volatilome structures in each sample (Fig. 4).

260 The analysis revealed a significant relationship between microbiota composition and VOCs (RV
261 coefficient=0.63; Monte Carlo P=0.001). The first horizontal component accounted for 84.5% of the variance,
262 and a second vertical component accounted for another 9.8 %. Clustering the samples according to (A) the time,
263 (B) the batch and (C) the treatments, a clear separation of the samples was observed on the basis of the storage
264 time and also, although to a lesser extent, of the origin of the batch (Fig. 4).
265
266 **Intra-species oligotype analysis**
267 The oligotype analysis was performed on the main OTUs observed but only with *P. fragi* we found a significant
268 Shannon entropy level able to detect sub-OTU (or oligotype). Overall a total of 32 oligotypes were identified.
269 The Pairwise Wilcoxon test was used to identify specific oligotypes associated with a specific treatment.
270 Oligotypes P1, P13, P18, P2, P30, P4, P7, P8 and P9 were associated with EW treated meat, P11 and P26 were
271 associated with the AO treatment, and P22, P24 and P3 were associated with the control samples ($P < 0.05$). The
272 relative abundance of the oligotypes, calculated in relation to the total abundance of *P. fragi,* resulted to be very
273 fragmented, with the highest percentage of abundance being shown by the P1 oligotype (5.7 % on average for
274 all the samples), and the lowest being observed for the P20 oligotype, with 1.8 % of abundance (Supplementary
275 Table 3).
276 When the correlation between *P. fragi* oligotype abundance and VOC profiles was plotted (Fig. 4), it was
277 possible to observe a cluster of oligotypes (P23, P28, P16, P32, P2, P12, P20 and P22) that were closely
278 correlated with the aldehydes, the primary and secondary alcohols and the acetoin. The acetoin was positively
279 correlated with the P22 oligotype, which in turn was significantly associated with the meat samples treated with
280 water (W). Another group of seven oligotypes (P29, P5, P31, P14, P17, P15 and P30) was positively correlated
281 with the organic acids, esters and 3-methyl-1-butanol. The significance of the correlation observed is reported in
282 the Supplementary Table 4. Oligotypes P29, P5 and P14 in particular showed a strong correlation with butanoic,
283 acetic and hexanoic acid, respectively, while P31 was closely related to the 3-methyl-1-butanol alcohol.
284 However, none of them was associated with a specific treatment.
285

286 **Discussion**

287 This study has investigated the microbiota of VP beefsteaks treated with electrolyzed water and aqueous ozone,
288 and it offers a detailed view of the evolution of the potentially active community after treatments thanks to the
289 use of the RNA-based HTS of the 16S rRNA and headspace analysis of the VOCs.

290 A progressive growth of all the microbial groups considered during the storage time was observed, with the LAB
291 population taking over after the 5th day, probably due to the limited oxygen condition established after the
292 treatments, and resulting in an increased final acidity, as can be expected in VP meat (Doulgeraki et al., 2012).
293 The dominant group the present survey was properly detected by the selective media, unlike the results of
294 previous investigations on VP beef (Ercolini et al., 2010b; Ferrocino et al., 2013). Several olfactory indicators of
295 spoilage were observed through the headspace analysis, but none of them reached the respective odor thresholds,
296 not even the alcohols, which are generally the most abundant VOC family in VP meat (Casaburi et al., 2015).
297 Together, the microbiological and VOC profiles depicted an acceptable meat quality of the beefsteaks over the
298 entire storage time (Ercolini et al., 2011), regardless of the AO and EW treatments, which did not therefore
299 seem to have had any impact on the initial microbiological situation of the beefsteaks, or on the subsequent fate
300 of the microbial counts throughout VP storage.

301 Previous DNA-based HTS investigations highlighted a limited microbial complexity of the final meat portions in
302 comparison to the initial condition of the carcasses, cuts and slaughter environments due to the different storage
303 conditions, which may favor certain groups of bacteria at the expense of others (de Filippis et al., 2013; Ercolini
304 et al., 2011; Stellato et al., 2016). This low complexity was here confirmed by analyzing the total live microbiota
305 composition.

306 Overall, the relative abundance of OTUs during the storage period and the co-occurrence/co-exclusion analysis
307 highlighted a clear shift from an initial population, dominated by psychrophilic gram-negative bacteria, to a
308 final condition in which the LAB species were the dominant OTUs favored by the anaerobic conditions
309 (Doulgeraki et al., 2012).

310 As expected, *Pseudomonas sp*., *Brochothrix sp*., *Psychrobacter sp.*, *Lactobacillus sp.*, and *Acinetobacter sp*.
311 were identified as the core microbiota of the raw meat before the treatments, since they are commonly reported

312 as contaminants in beef from processing environments (de Filippis et al., 2013; Stellato et al., 2016).
313 *Pseudomonas fragi* was the dominant OTU before the treatments, and it remained likely active over the whole
314 storage period in VP, with a progressive decrease in abundance. Unfortunately counts of *Pseudomonas* spp.
315 were not performed in this study, thereby this hypothesis remains to be demonstrated. Within the *Pseudomonas*
316 genus, *P. fragi* is recognized as the dominant spoiler species in beef and the main species responsible for
317 spoilage in aerobic conditions, but it may grow in the absence of oxygen by limiting its catabolism to the
318 consumption of glucose and lactic acid (Casaburi et al., 2015; Doulgeraki et al., 2012; Ercolini et al., 2010a;
319 Pennacchia et al., 2011). However, the presence of other aerobe spoilage bacteria, such as *Psychrobacter sp*. and
320 *A. lwoffii*, on the first day of VP storage, suggests that low levels of oxygen were still available in the products
321 (Hernández-Macedo et al., 2011) and were sufficient to keep these aerobic bacteria metabolically active, at least
322 for the first 24 hours. Overall, these OTUs and *B. thermosphacta* were positively correlated with several
323 alcohols, aldehydes (nonanal and octanal) and ketones. Notably the concentration of the most important ketone,
324 acetoin, decreased at the end of the storage period as previously observed in MAP minced beef (Argyri et al.,
325 2015). This leads us to speculate its possible reduction to 2,3-butendiol by the butanediol fermentation activity of
326 *Serratia* sp., *Enterobacter* sp. and *L. gasicomitatum* (Jääskeläinen et al., 2015; Radoš et al., 2016), although it
327 remains unclear why this end product has not been detected among the VOCs the fiftieth day. Overall, acetoin
328 confers an unpleasant buttery/creamy flavor to meat, and its production had previously been associated with *A.*
329 *lwoffii*, *B. thermosphacta* and *P. fragi* (Ercolini et al., 2011; Ferrocino et al., 2013). However, as also observed
330 by Ercolini et al. (2009) on inoculated meat, *P. fragi* in the present study was more correlated with nonanal,
331 octanal and 1-octen-3-ol, which can be derived from the hydrolysis of triglycerides or from amino acid
332 degradation. *P. fragi* is recognized as major food spoilers (Ercolini et al., 2011) and an oversimplified
333 classification of *P. fragi* in a homogeneous OTU cannot disclose the possible strain-specific response to
334 treatments, or the strain-specific relationship to VOCs, an attempt has here been made to overcome the limits of
335 the OTU clustering method through the use of an oligotyping pipeline (De Filippis et al., 2016; Eren et al.,
336 2013). *Pseudomonas sp*. from dairy and meat processing environments have recently been investigated at a sub-
337 species level, and a relatively low number of dominant oligotypes has been revealed in both environments and

338 in the related food (Stellato et al., 2017). However, a fragmented distribution and a high number of oligotypes of
339 *P. fragi* have been observed in the present analyses, and only a few of these oligotypes have been significantly
340 associated with the EW and AO treatments. A minimum selective pressure of the treatments was therefore
341 observed at the *P. fragi* sub-species level, without however highlighting any effective dominance of these
342 treatment-associated oligotypes on the others. Nevertheless, different oligotypes showed distinctive correlation
343 patterns with their volatilome, in accordance with the strain-related volatilome of the *P. fragi* species (Casaburi
344 et al., 2015; Ercolini et al., 2010a).
345 As far as LAB are concerned, *Lc. piscium* and *Lb. sakei* have recently been found to be the most abundant
346 OTUs in beef burgers packaged with nisin-activated films (Ferrocino et al., 2016) and have been identified, by
347 means of culture-dependent methods, in a variety of meat products under MAP conditions (Rahkila et al., 2012).
348 On the other hand, *L. gasicomitatum* is a psychrotrophic LAB that is associated with the spoilage of several cold-
349 storage foods, and in particular with meat packaged in high-oxygen MAP, as a result of its respiratory capability
350 when heme is available (Jääskeläinen et al., 2013; Susiluoto et al., 2003). It is therefore possible to state that the
351 presence of oxygen inside packaging favors the predominance of *L. gasicomitatum* compared to the
352 *Lactobacillus* and *Lactococcus* species at the end of meat storage (Rahkila et al., 2012), unlike what was
353 observed in the here examined VP beefsteaks. In accordance with the final volatilome of the beefsteaks, the *L.*
354 *gasicomitatum*, *Lc. piscium*, *Lb. sakei* and *C. divergens* metabolisms were closely associated with the production
355 of short chain esters and acids, while a significant correlation was here observed between 3-methyl-1-butanol
356 and the gram negative *Photobactrium angustum* and *Photobactrium phosphoreum* (Casaburi et al., 2015). This
357 alcohol confers a pungent ethereal odor, and it has been associated with the metabolic activities of *P.*
358 *phosphoreum* in MAP packaged pork meat (Nieminen et al., 2016), although it has so far mainly been
359 associated with the metabolic activity of *Enterobacteriaceae* and LAB species in VP spoiled meat (Ercolini et
360 al., 2009; Hernández-Macedo et al., 2012). Despite *Enterobacteriaceae* having been reported as being
361 particularly important during the spoilage process of VP meat (Hernández-Macedo et al., 2012), in the present
362 experiment, their relative abundance has been found to be very low and limited to a few taxa, as previously
363 reported for the potentially active microbiota of VP beefburgers (Ferrocino et al., 2016).

364 The CIA, which correlated the VOC and OTU abundances, apart from confirming the microbiological dynamics,
365 has also highlighted a segregation of the samples, regardless of the AO and EW treatments. We observed a clear
366 separation of the samples based to the storage time and origin of the batches. Meat spoilage microbiota is known
367 to show a high lot-to-lot variation that decrease progressively along the storage time (Säde et al., 2017). As
368 observed by Ferrocino et al. (2016) the antimicrobial treatments may by more or less effective as a function of
369 the initial microbiota composition.. Therefore, the different initial microbiota of the three batches here might
370 have react differently to the AO and EW treatments, according to the susceptibility to the treatments. Whether
371 future and further experimentation will deal the use of these sanization treatments, this aspect have to be
372 considered carefully. However, the limiting factors for applying AO and EW to raw beef remain undoubtedly
373 their concentrations and exposure times, which were here chosen on the basis of the acceptability of the color
374 of the meat after treatments and considering the effectiveness of treatments performed by spraying AO (Chawla
375 et al., 2007; Chen et al., 2014; Crowe et al., 2012; Kalchayanand et al., 2008) and EW (Duan et al., 2016; Purnell
376 et al., 2014; Veasey and Muriana, 2016) on different meats and seafood products.
377 In short, the here performed treatments with EW and AO were not able to reduce the initial microbial counts of
378 the products. Moreover, they were incapable of modifying the microbiota composition, dynamics and the related
379 volatilome to any great extent during chilled VP storage. In spite of this, the RNA-based analysis, integrated
380 with the volatilome, has helped to unravel the complexity of the potentially active microbiota, in this way
381 expanding the current knowledge on the spoilage dynamics of vacuum packaged beefsteaks.
382

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386
387
388
389

390 **Figure legends**

391

392 **Figure 1.**

393 Schematic layout of the experimental plan.

394

395 **Figure 2.**

396 Relative abundance of the OTUs detected by means of 16S amplicon target sequencing. Only OTUs which
397 showed an incidence above 0.2% in at least 5 samples are shown. The abundances of the OTUs in the 3
398 biological replicates was averaged (n=3), except for the 5, 9 and 15 day sampling points, in which one replicate
399 was excluded because of the poor cDNA quality (n=2).

400

401 **Figure 3.**

402 Correlation between the abundance of VOCs (µg/kg) and OTUs that occurred at 0.2% in at least 2 samples. The
403 rows and columns are clustered according to Ward's linkage hierarchical clustering. The intensity of the colors
404 represents the degree of correlation between the samples and VOCs, as measured by Spearman's correlations.

405

406 **Figure 4.**

407 Co-inertia analysis (CIA) of the microbial community (OTUs) and volatilome (VOCs) of the samples before the
408 treatments, on the first day and at the end of the storage period. Samples projected onto the first two axes and
409 grouped according to the (A) time, (B) batch and (C) treatments; (D) loading plot with the OTU vectors and
410 VOCs. Plot A: before the treatments (0); on the first day (1) and at the end of the shelf-life (15). Plot B: batch
411 O1; batch O2 and batch M. Plot C: beefsteaks before the treatments (T0); treated with AO (O); treated with EW
412 (E); treated with water (W) and untreated meat (d).

413

414 **Figure 5.**

415 Correlation between the abundance of VOCs and *Pseudomonas fragi* oligotypes. The rows and columns are
416 clustered according to Ward's linkage hierarchical clustering. The intensity of the colors represents the degree of
417 correlation between the samples and VOCs, as measured by Spearman's correlations. The row bar is colored in
418 according to the significance of the association (Pairwise Wilcoxon; $P < 0.05$) between the oligotypes and
419 treatments: AO (green); EW (red); W (blue). No significant association was observed for the untreated control.
420

421

422 **Table 1.**

423 Viable counts of the different meat spoilage microbial groups and pH on the beefsteak surfaces treated with aqueous ozone (AO), electrolyzed water
424 (EW), water (W) and the untreated control (C). Samplings were performed before the treatments (day 0) and during storage of vacuum-packed
425 beefsteaks at 4 °C for 15 days. The data are the means (± SD) of the three independent batches (n=3). The lower case letters in each row (a, b, c, d)
426 indicate significant differences ($P < 0.05$; ANOVA with Tukey's post-hoc test or Kruskal–Wallis test) between the sampling points (0, 1, 5, 9, 15).

	Treatments	Day 0	Day 1	Day 5	Day 9	Day 15
pH	**Control**	**5.60 ± 0.01 [a]**	**5.63 ± 0.00 [a]**	**5.51 ± 0.04 [a]**	**5.43 ± 0.04 [a]**	**5.38 ± 0.02 [b]**
	AO	5.60 ± 0.01 [a]	5.58 ± 0.02 [a]	5.51 ± 0.00 [a]	5.46 ± 0.04 [a]	5.41 ± 0.04 [b]
	EW	5.60 ± 0.01 [a]	5.58 ± 0.03 [a]	5.56 ± 0.02 [a]	5.45 ± 0.02 [a]	5.35 ± 0.03 [b]
	W	5.60 ± 0.01 [a]	5.62 ± 0.01 [a]	5.55 ± 0.02 [a]	5.39 ± 0.07 [a]	5.39 ± 0.06 [b]
Total bacterial counts (PCA)	**Control**	**3.82 ± 0.13 [a]**	**3.85 ± 0.49 [a]**	**3.62 ± 0.87 [b]**	**4.60 ± 0.32 [c]**	**5.13 ± 0.25 [c]**
	AO	3.82 ± 0.13 [a]	4.79 ± 0.22 [a]	3.95 ± 0.91 [b]	4.60 ± 0.20 [c]	5.15 ± 0.17 [c]
	EW	3.82 ± 0.13 [a]	5.29 ± 0.20 [a]	3.83 ± 0.77 [b]	4.76 ± 0.03 [c]	5.21 ± 0.04 [c]
	W	3.82 ± 0.13 [a]	5.18 ± 0.05 [a]	4.33 ± 0.18 [b]	5.13 ± 0.22 [c]	5.23 ± 0.14 [c]
LAB (MRS)	**Control**	**2.64 ± 0.18 [a]**	**2.81 ± 0.37 [a]**	**4.19 ± 0.21 [b]**	**5.45 ± 0.17 [c]**	**6.03 ± 0.21 [d]**
	AO	2.64 ± 0.18 [a]	2.49 ± 0.52 [a]	4.08 ± 0.36 [b]	5.28 ± 0.20 [c]	6.06 ± 0.20 [d]
	EW	2.64 ± 0.18 [a]	3.19 ± 0.84 [a]	3.88 ± 0.10 [b]	5.05 ± 0.23 [c]	6.00 ± 0.12 [d]
	W	2.64 ± 0.18 [a]	2.75 ± 0.50 [a]	4.16 ± 0.08 [b]	5.44 ± 0.18 [c]	6.10 ± 0.10 [d]
Total coliforms (VRBA)	**Control**	**1.94 ± 1.86 [a]**	**1.94 ± 1.86 [a]**	**1.16 ± 0.51 [b]**	**2.41 ± 0.85 [b,c]**	**2.90 ± 0.62 [c]**
	AO	1.94 ± 1.86 [a]	1.41 ± 1.13 [a]	1.60 ± 1.50 [b]	2.52 ± 0.28 [b,c]	3.24 ± 1.10 [c]
	EW	1.94 ± 1.86 [a]	1.00 ± 0.44 [a]	1.43 ± 0.92 [b]	3.05 ± 0.20 [b,c]	3.43 ± 0.65 [c]

	W	1.94 ± 1.86 [a]	2.08 ± 1.78 [a]	1.53 ± 0.64 [b]	2.60 ± 0.21 [b,c]	3.23 ± 0.46 [c]
Yeasts (MEA)	**Control**	**2.21 ± 0.43 [a]**	**2.11 ± 0.53 [a]**	**3.31 ± 0.61 [b]**	**4.72 ± 0.74 [c]**	**5.37 ± 0.62 [d]**
	AO	2.21 ± 0.43 [a]	2.13 ± 0.86 [a]	3.15 ± 0.55 [b]	4.40 ± 0.64 [c]	5.33 ± 0.59 [d]
	EW	2.21 ± 0.43 [a]	2.47 ± 1.03 [a]	3.10 ± 0.42 [b]	4.56 ± 0.61 [c]	5.45 ± 0.61 [d]
	W	2.21 ± 0.43 [a]	2.25 ± 0.95 [a]	3.16 ± 0.46 [b]	4.67 ± 1.07 [c]	5.29 ± 0.91 [d]

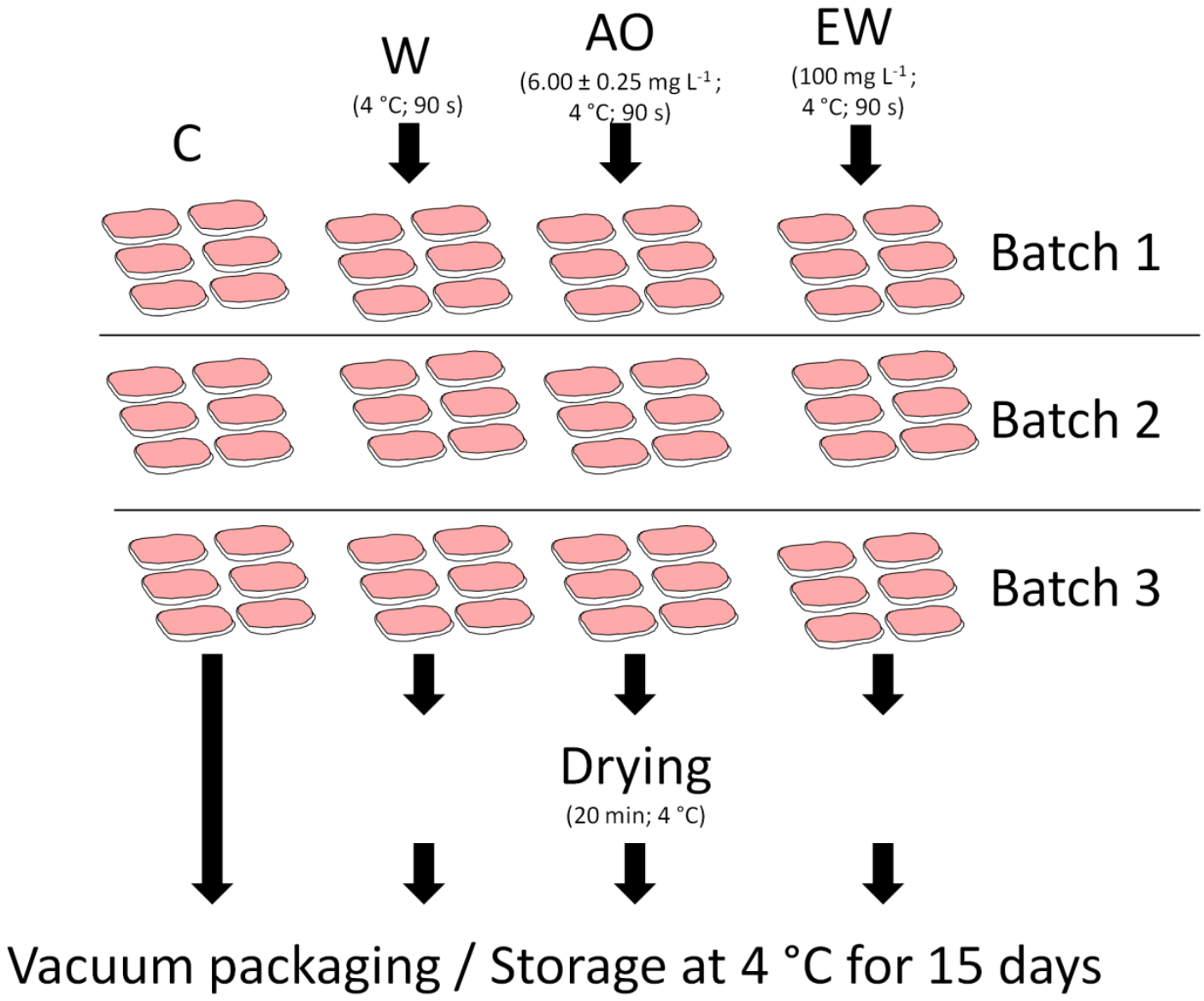

C
W
(4 °C; 90 s)
AO
(6.00 ± 0.25 mg L-1 ;
4 °C; 90 s)
EW
(100 mg L-1 ;
4 °C; 90 s)
Batch 1
Batch 2
Batch 3
Drying
(20 min; 4 °C)
Vacuum packaging / Storage at 4 °C for 15 days

Fig. 1

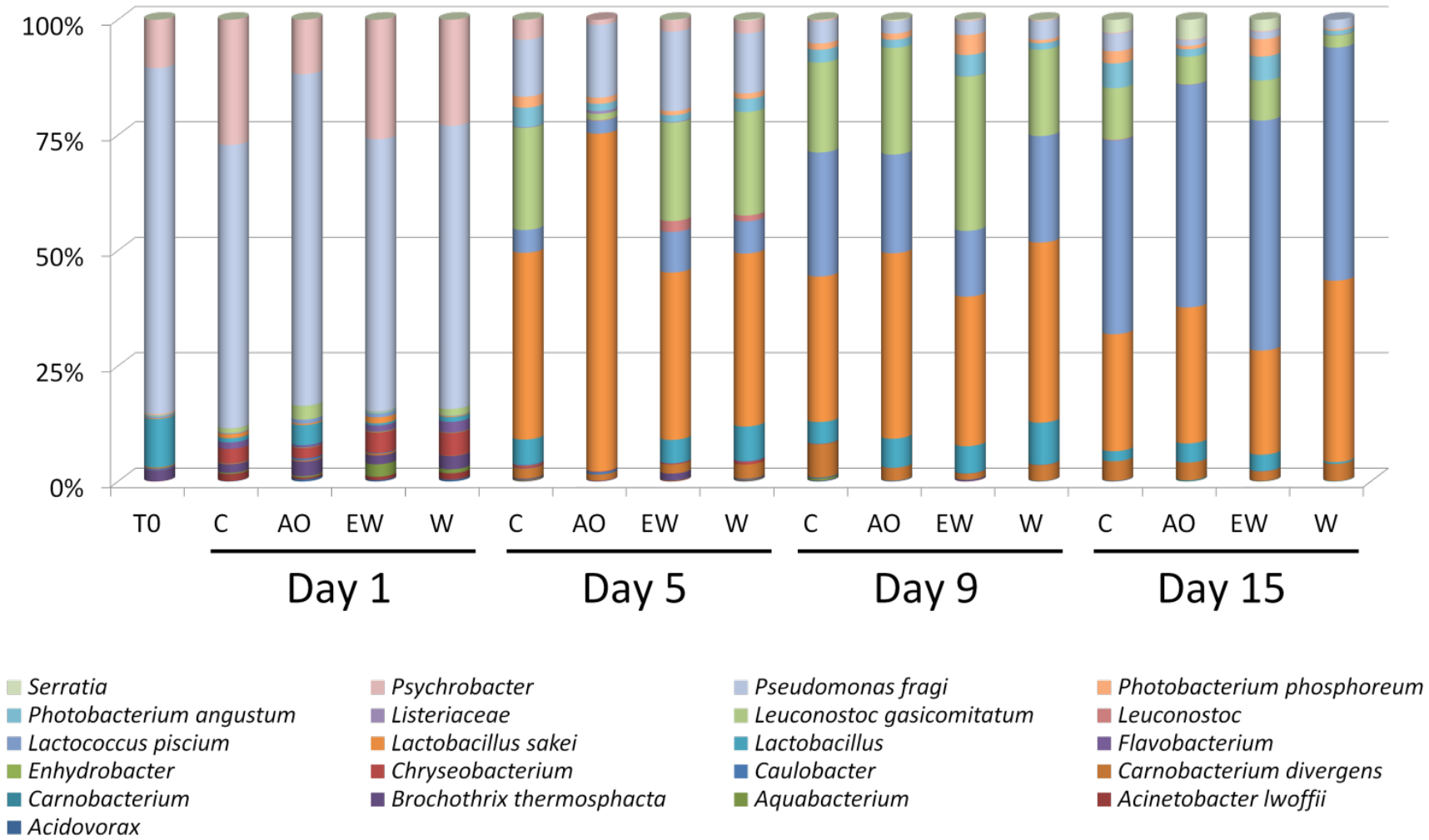
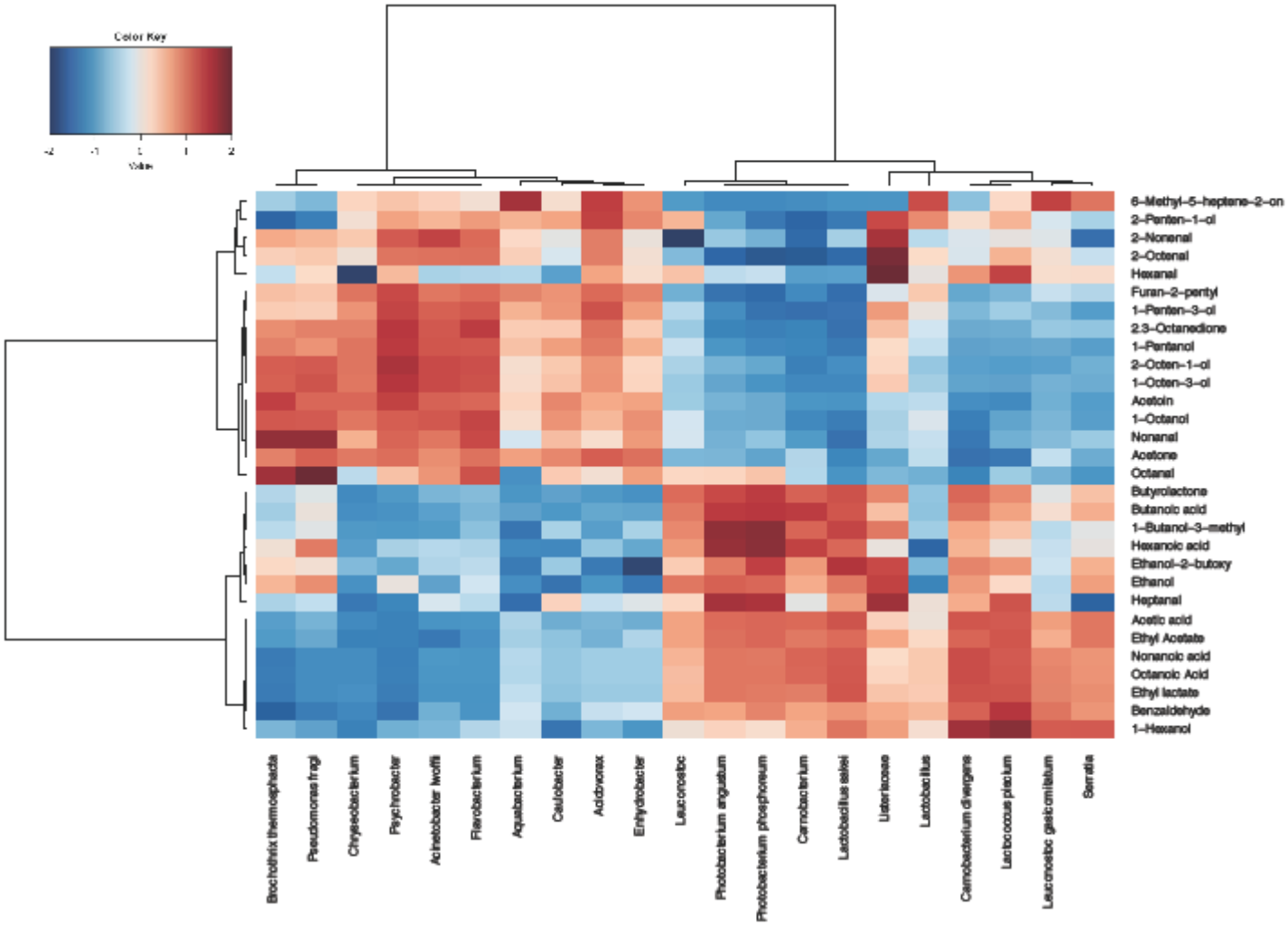

100%
75%
50%
25%
0%
T0
C
AO
EW
W
C
AO
EW
W
C
AO
EW
W
C
AO
EW
W
Day 1
Day 5
Day 9
Day 15
Serratia
Psychrobacter
Pseudomonas fragi
Photobacterium phosphoreum
Photobacterium angustum
Listeriaceae
Leuconostoc gasicomitatum
Leuconostoc
Lactococcus piscium
Lactobacillus sakei
Lactobacillus
Flavobacterium
Enhydrobacter
Chryseobacterium
Caulobacter
Carnobacterium divergens
Carnobacterium
Brochothrix thermosphacta
Aquabacterium
Acinetobacter lwoffii
Acidovorax

Fig. 2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1
2
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2-Nonenal
2-Octenal
Hexanal
Furan-2-pentyl
1-Penten-3-ol
2,3-Octanedione
1-Pentanol
2-Octen-1-ol
1-Octen-3-ol
Acetoin
1-Octanol
Nonanal
Acetone
Octanal
Butyrolactone
Butanoic acid
1-Butanol-3-methyl
Hexanoic acid
Ethanol-2-butoxy
Ethanol
Heptanal
Acetic acid
Ethyl Acetate
Nonanoic acid
Octanoic Acid
Ethyl lactate
Benzaldehyde
1-Hexanol
Brochothrix thermosphacta
Pseudomonas fragi
Chryseobacterium
Psychrobacter
Acinetobacter lwoffii
Flavobacterium
Aquabacterium
Ceulobacter
Acidovorax
Enhydrobacter
Leuconostoc
Photobacterium angustum
Photobacterium phosphoreum
Carnobacterium
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Listeriosae
Lactobacillus
Carnobacterium divergens
Lactococcus piscium
Leuconostoc gasicomitatum
Serratia

Fig. 3

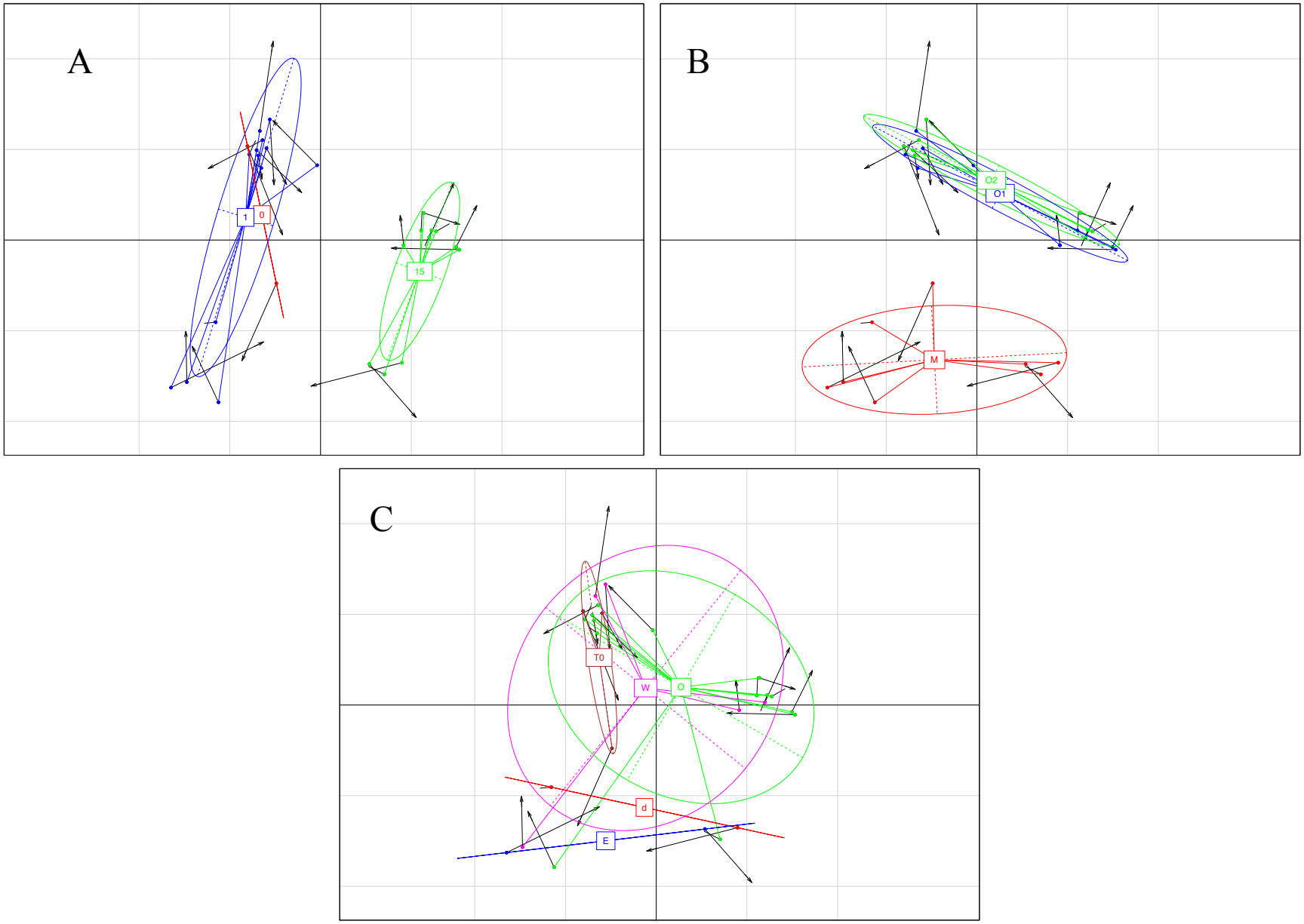
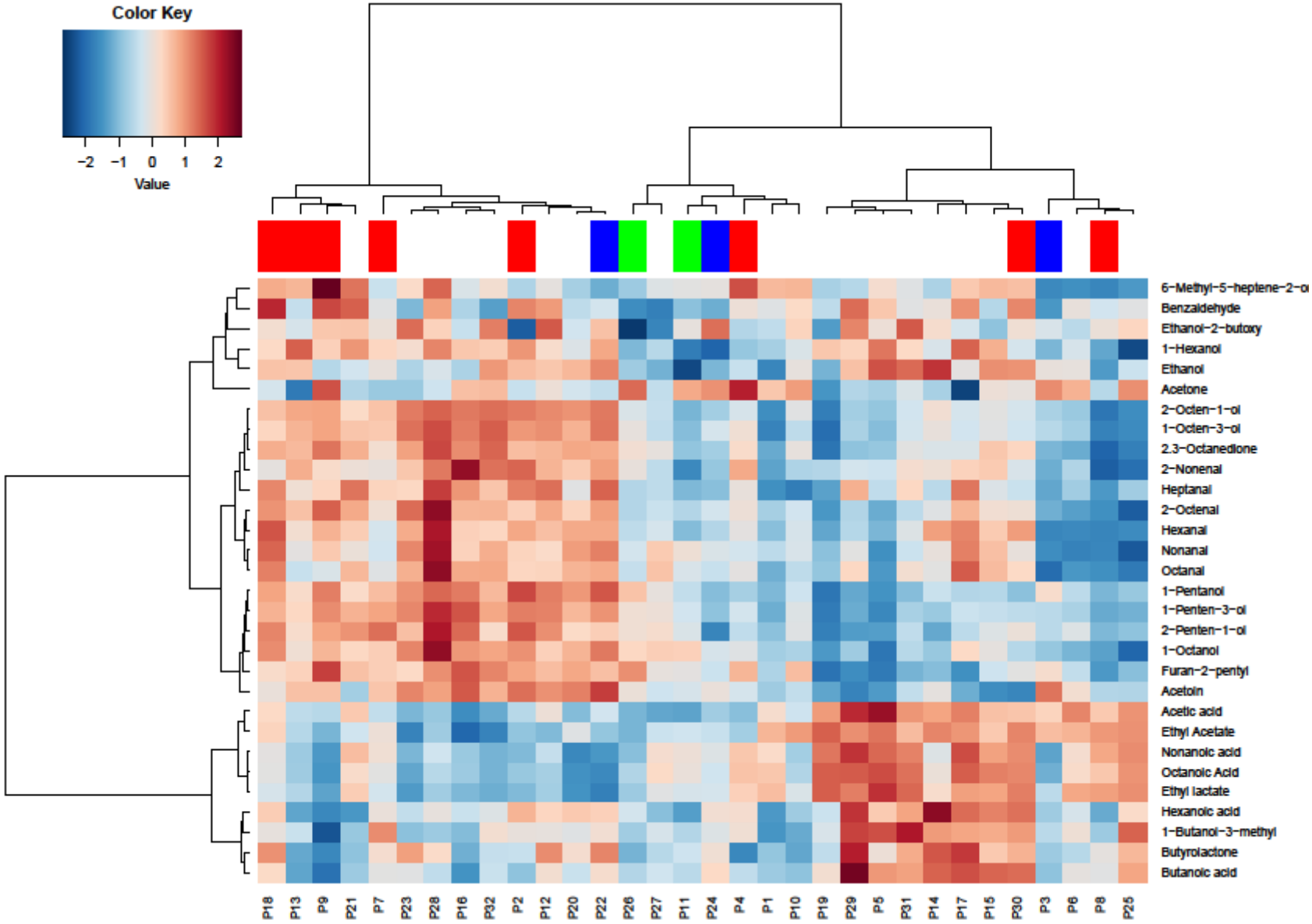


Fig. 4


Color Key
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Value
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Benzaldehyde
Ethanol-2-butoxy
1-Hexanol
Ethanol
Acetone
2-Octen-1-ol
1-Octen-3-ol
2,3-Octanedione
2-Nonenal
Heptanal
2-Octenal
Hexanal
Nonanal
Octanal
1-Pentanol
1-Penten-3-ol
2-Penten-1-ol
1-Octanol
Furan-2-pentyl
Acetoin
Acetic acid
Ethyl Acetate
Nonanoic acid
Octanoic Acid
Ethyl lactate
Hexanoic acid
1-Butanol-3-methyl
Butyrolactone
Butanoic acid
P18 P13 P9 P21 P7 P23 P28 P16 P32 P2 P12 P20 P22 P26 P27 P11 P24 P4 P1 P10 P19 P29 P5 P31 P14 P17 P15 P30 P3 P6 P8 P25

Fig. 5

445 **Supplementary data**

446

447

448

449 **Supplementary Table 1.**

450 Complete dataset of the VOCs (µg/kg) detected in the meat beefsteaks before the treatments and in vacuum-packed beefsteaks after 1 and 15 days of

451 storage at 4 °C. The data are the means (± SD) of the three independent batches (n=3). The lowercase letters in each row highlight significant

452 differences ($P < 0.05$; ANOVA with Tukey's post-hoc test or the Kruskal–Wallis test) between the values in the three sampling points considered (0,

453 1, 15).

454

Compound families	VOCs	Sign.	Before treatment	After treatment (Day 1)				End of stoarge time (Day 15)			
				C	W	EW	AO	C	W	EW	AO
Esters	Ethyl Acetate	<0.05	30.97 ± 11.30 a,b	0.22 ± 0.14 a	25.49 ± 24.23 a.b	8.79 ± 7.50 a	0.80 ± 0.65 a	137.30 ± 56.85 b	130.66 ± 74.67 b	126.21 ± 38.05 b	73.66 ± 7.76 a,b
	Ethyl-lactate	N.S.	n.d.	n.d.	n.d.	n.d.	n.d.	64.23 ± 27.73	56.32 ± 21.02	59.88 ± 18.75	30.42 ± 5.12
Alcohols	Ethanol	N.S.	216.88 ± 51.37	206.93 ± 20.60	504.12 ± 297.55	152.92 ± 8.96	180.61 ± 33.94	248.72 ± 63.52	248.15 ± 55.28	229.32 ± 59.35	175.10 ± 4.38
	1-Penten-3-ol	N.S.	7.44 ± 0.70	18.25 ± 4.25	15.30 ± 4.11	8.63 ± 1.84	10.27 ± 2.49	6.16 ± 2.82	9.81 ± 4.85	5.56 ± 1.97	8.72 ± 2.90
	1-Butanol, 3-methyl-	N.S.	13.15 ± 1.71	14.45 ± 12.34	7.30 ± 2.85	3.21 ± 0.61	11.99 ± 6.96	56.20 ± 39.46	71.85 ± 40.61	50.12 ± 37.40	15.57 ± 8.06
	1-Pentanol	<0.05	20.74 ± 0.92 a,b	42.06 ± 13.17 c	36.18 ± 7.45 b,c	23.84 ± 3.94 a,b,c	21.89 ± 2.84 a,b	12.26 ± 5.12 a	14.22 ± 5.42 a	10.20 ± 3.11 a	15.14 ± 4.33 a
	2-Penten-1-ol	N.S.	0.56 ± 0.08	1.15 ± 0.29	0.94 ± 0.48	0.52 ± 0.18	0.66 ± 0.21	0.65 ± 0.25	1.02 ± 0.50	0.45 ± 0.28	0.77 ± 0.24
	1-Hexanol	N.S.	3.77 ± 0.41	15.55 ± 10.46	7.28 ± 1.27	4.65 ± 0.82	6.84 ± 1.43	6.41 ± 1.94	7.40 ± 0.89	8.80 ± 0.80	8.44 ± 0.76
	2-Butoxy-ethanol	N.S.	1.56 ± 0.17	2.57 ± 0.81	1.66 ± 0.34	3.64 ± 1.95	1.78 ± 0.42	5.71 ± 4.01	4.82 ± 1.57	1.87 ± 0.28	2.07 ± 0.70
	1-Octen-3-ol	<0.05	34.57 ± 3.95 a,b	69.83 ± 14.01 c	61.21 ± 13.04 b.c	42.48 ± 15.51 a,b,c	43.59 ± 4.66 a,b,c	23.38 ± 12.75 a	26.04 ± 10.81 a	19.55 ± 9.62 a	22.58 ± 4.36 a
	1-Octanol	N.S.	2.29 ± 1.03	2.98 ± 0.87	2.23 ± 0.25	1.73 ± 0.28	3.53 ± 0.33	1.16 ± 0.17	1.32 ± 0.23	1.32 ± 0.17	1.93 ± 0.31
	2-Octen-1-ol	N.S.	0.80 ± 0.18	1.56 ± 0.37	1.32 ± 0.27	1.03 ± 0.22	1.06 ± 0.05	0.64 ± 0.18	0.66 ± 0.21	0.61 ± 0.26	0.61 ± 0.08
Aldehydes	Hexanal	N.S.	149.79 ± 33.51	229.90 ± 100.92	395.48 ± 48.79	204.83 ± 68.12	188.55 ± 68.86	196.79 ± 105.87	301.51 ± 115.45	235.37 ± 142.98	219.91 ± 42.71
	Heptanal	N.S.	3.78 ± 1.83	10.83 ± 3.46	8.09 ± 0.27	3.95 ± 1.27	5.18 ± 1.75	3.93 ± 1.99	7.84 ± 0.72	6.95 ± 2.53	6.33 ± 2.33
	Octanal	N.S.	2.14 ± 0.84	3.85 ± 1.78	3.63 ± 0.25	1.96 ± 0.93	4.29 ± 0.34	2.09 ± 0.65	2.76 ± 0.85	3.25 ± 1.19	2.96 ± 0.54
	Nonanal	N.S.	8.38 ± 4.01	9.90 ± 3.97	10.43 ± 1.19	6.85 ± 3.23	17.46 ± 2.74	5.18 ± 1.42	5.83 ± 1.86	6.52 ± 1.53	10.11 ± 1.85
	2-Octanal	N.S.	0.45 ± 0.04	1.00 ± 0.27	1.05 ± 0.27	0.61 ± 0.32	0.78 ± 0.19	0.72 ± 0.59	0.93 ± 0.41	0.54 ± 0.20	0.77 ± 0.16
	Benzaldehyde	N.S.	0.47 ± 0.15	0.73 ± 0.22	0.82 ± 0.15	0.82 ± 0.35	0.49 ± 0.16	0.90 ± 0.04	1.07 ± 0.12	0.89 ± 0.11	0.96 ± 0.20
	2-Nonenal	N.S.	0.19 ± 0.03	0.56 ± 0.19	0.47 ±0.11	0.37 ± 0.14	0.35 ± 0.08	0.19 ± 0.16	0.43 ± 0.16	0.36 ± 0.11	0.17 ± 0.09
Ketons	Acetone	N.S.	37.15 ± 10.02	46.23 ± 17.04	49.09 ± 13.08	27.86 ± 14.13	29.41 ± 11.51	37.68 ± 19.80	25.05 ± 11.83	12.28 ± 5.24	6.21 ± 0.54
	Acetoin	<0.05	185.96 ± 34.76 a	283.58 ± 71.06 a	258.54 ± 48.81 a	237.93 ± 47.84 a	233.33 ± 38.07 a	53.73 ± 12.40 b	66.94 ± 15.67 b	30.43 ± 1.76 b	47.13 ± 12.36 b
	2,3-Octanedione	N.S.	20.42 ± 1.88	44.35 ± 7.16	51.48 ± 13.55	22.11 ± 3.64	31.89 ± 5.33	25.85 ± 18.23	17.81 ± 9.04	18.66 ± 10.12	14.74 ± 1.97
	6-Methyl-5-hepten-2-one	N.S.	1.14 ± 0.18	1.25 ± 0.38	1.83 ± 0.58	0.86 ± 0.46	1.46 ± 0.49	1.28 ± 0.23	0.93 ± 0.37	1.44 ± 0.15	1.38 ± 0.24
Volatile fatty acids	Acetic acid	<0.05	4.74 ± 1.25 a	6.90 ± 2.19 a,b	9.79 ± 4.35 a,b	10.00 ± 4.03 a,b	3.46 ± 1.52 a	100.99 ± 42.04 c	63.13 ± 14.22 c	95.61 ± 11.23 c	56.40 ± 9.13 b,c
	Butanoic acid	N.S.	12.68 ± 6.47	7.47 ± 3.85	13.29 ± 7.39	12.27 ± 6.09	10.72 ± 7.10	73.32 ± 36.21	41.55 ± 27.45	41.35 ± 16.90	42.32 ± 25.30
	Hexanoic acid	N.S.	3.77 ± 1.35	5.25 ± 1.83	7.78 ± 2.00	9.49 ± 3.89	2.88 ± 0.54	10.54 ± 4.01	6.54 ± 4.02	7.65 ± 3.01	4.95 ± 1.81
	Octanoic Acid	N.S.	n.d.	n.d.	n.d.	n.d.	n.d.	1.26 ± 0.18	0.88 ± 0.35	2.97 ± 1.87	0.88 ± 0.11
	Nonanoic acid	N.S.	n.d.	n.d.	n.d.	n.d.	n.d.	1.15 ± 0.17	1.05 ± 0.50	3.10 ± 1.93	1.21 ± 0.13
Aromatic compounds	Furan, 2-pentyl-	N.S.	1.98 ± 0.09	4.29 ± 1.40	4.38 ± 2.56	1.77 ± 0.20	2.49 ± 1.00	2.02 ± 1.69	1.50 ± 0.34	0.89 ± 0.40	1.45 ± 0.40
Lactones	Butyrolactone	N.S.	1.49 ± 0.60	1.73 ± 0.48	2.15 ± 1.15	3.04 ± 0.95	1.81 ± 0.58	3.32 ± 1.02	3.77 ± 1.41	2.57 ± 0.56	3.75 ± 0.76

Hydrocarbons	1,3-Hexadiene, 3-ethyl-2-methyl-	N.S.	0.40 ± 0.05	1.18 ± 0.27	0.97 ± 0.31	0.83 ± 0.18	0.70 ± 0.20	0.27 ± 0.16	0.36 ± 0.16	0.22 ± 0.09	0.36 ± 0.12
	2,5,5-Trimethyl-2-hexene	N.S.	1.73 ± 1.07	1.31 ± 0.60	0.91 ± 0.16	0.78 ± 0.22	0.49 ± 0.07	3.52 ±0.30	3.05 ± 1.07	4.10 ± 0.31	3.03 ± 1.10

457

458 **Supplementary Table 2.**

459 Observed diversity and estimated sample coverage for the 16S rRNA amplicons. *ESC estimate sample coverage.

Samples code	ESC	PD_whole_tree	chao1	observed_species	shannon
M_O_15	92.53	4.05	595.63	128	3.97
M_d_15	92.53	3.95	288.88	145	4.33
M_E_15	92.78	4.75	415.50	125	3.82
O1_O_15	93.99	4.18	259.06	117	3.73
O1_E_15	91.41	4.59	459.38	150	4.23
O1_d_15	92.27	5.34	364.50	142	3.99
O2_O_15	93.81	3.86	274.75	115	3.19
O2_E_15	93.13	3.92	333.67	123	3.65
O2_W_15	93.99	3.39	305.25	104	3.16
O2_d_15	92.53	4.82	396.21	129	3.83
M_O_9	92.87	5.61	345.87	119	3.79
M_W_9	91.58	6.75	456.87	140	4.02
M_d_9	90.98	5.81	599.00	144	4.12
O1_O_9	92.96	3.40	302.79	128	3.46
O1_E_9	91.75	4.77	444.00	140	3.80
O1_d_9	93.38	5.57	304.88	122	3.67
O2_O_9	91.92	4.37	533.36	136	3.78
O2_W_9	92.18	4.80	354.50	127	3.28
O2_d_9	92.53	4.71	420.77	133	3.87
O1_W_9	91.58	4.21	460.87	144	3.86
M_E_5	91.07	6.03	389.87	157	4.23
M_W_5	90.98	7.83	446.37	159	4.13
M_d_5	93.21	5.73	308.24	127	3.86
O1_O_5	90.55	9.87	404.58	174	4.36
O1_E_5	90.55	7.48	489.06	156	4.14
O1_W_5	89.43	6.06	743.15	166	4.22

O1_d_5	93.04	4.85	449.00	125	3.75
O2_O_5	94.24	3.60	260.93	103	2.75
O2_E_5	90.98	5.49	537.00	147	4.00
O2_W_5	92.44	4.30	403.43	130	3.77
O2_d_5	91.92	7.17	355.14	147	3.94
O2_W_1	95.02	3.52	320.14	84	3.04
O1_d_1	93.64	5.75	301.93	109	3.47
M_O_1	85.22	13.56	927.45	259	6.29
M_E_1	89.69	8.50	468.62	194	5.54
M_W_1	88.14	10.63	473.38	231	6.01
M_d_1	90.64	8.50	369.36	191	5.53
O1_O_1	93.47	4.89	294.13	116	3.74
O1_E_1	94.67	4.21	238.07	103	3.54
O1_W_1	93.90	4.56	284.50	107	3.47
O2_O_1	94.85	4.68	234.15	98	3.07
O2_E_1	93.81	4.92	385.00	101	3.41
O2_d_1	94.67	4.14	405.17	90	2.94
M_T0	90.46	7.58	541.56	160	4.21
O1_T0	93.47	3.96	293.13	115	3.69
O2_T0	93.90	5.05	299.15	108	3.33
M_E_9	93.04	4.96	364.23	115	3.60
O1_W_15	93.13	4.78	309.88	124	3.55

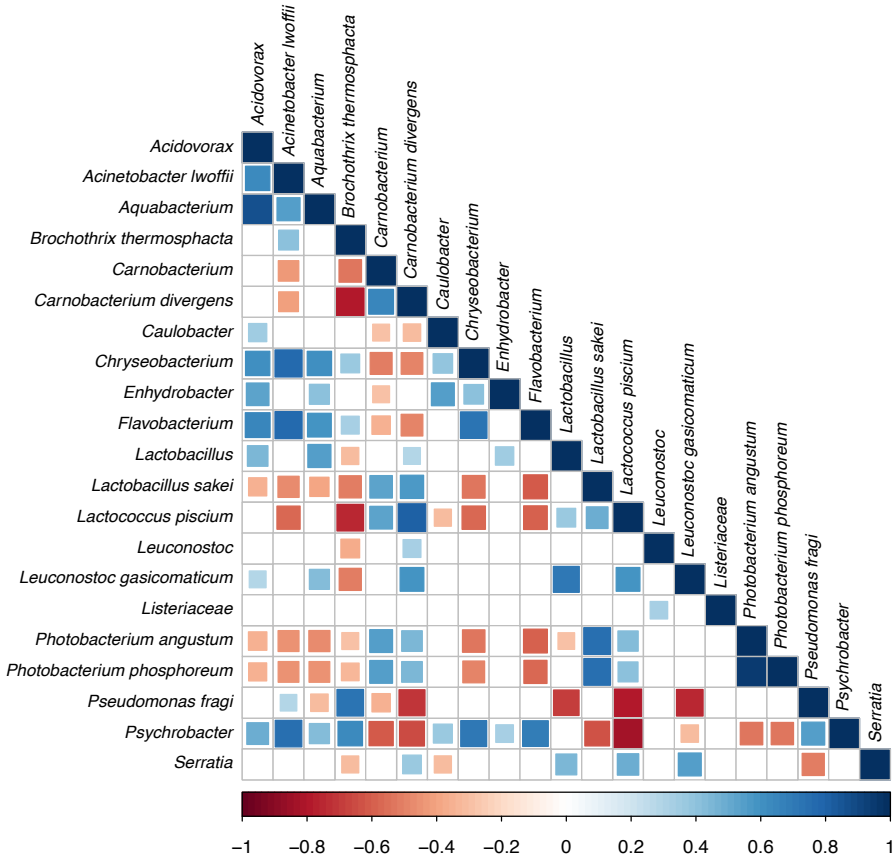
Supplementary Table 3. Average abundances of the oligotypes detected in the *P. fragi* species

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23
Control_0	6.1	3.8	4.2	4.7	4.0	3.5	3.5	4.2	3.7	3.2	3.4	3.0	3.6	3.0	2.9	2.0	2.3	2.8	2.5	1.9	2.9	3.1	
C_1	6.2	3.5	3.1	5.3	4.2	3.9	2.2	2.8	4.5	3.6	3.7	3.9	2.2	1.4	4.0	5.1	0.8	2.7	0.8	1.3	3.6	3.0	
EW_1	5.7	5.2	4.3	2.3	2.4	6.1	4.4	4.7	2.1	5.5	3.9	2.0	2.3	2.3	1.7	4.1	2.3	1.8	4.2	3.9	1.7	2.1	
AO_1	5.9	3.0	3.7	4.1	3.9	4.2	3.6	4.5	2.8	2.6	3.8	2.9	3.2	2.3	3.0	3.6	3.0	1.9	3.5	2.0	2.8	2.2	
W_1	3.5	7.6	3.0	6.9	7.8	2.4	12.1	2.0	7.5	2.2	1.9	1.8	6.7	2.3	2.0	1.8	2.3	2.0	1.9	2.0	1.8	2.4	
C_15	6.4	3.4	4.3	4.5	5.5	4.8	3.0	4.5	2.4	2.8	3.6	2.6	2.2	3.7	4.1	1.5	2.3	3.1	3.1	1.6	1.8	1.3	
EW_15	6.8	2.7	3.8	3.2	6.0	4.9	2.7	7.4	4.6	4.6	1.8	1.3	7.4	1.7	3.2	2.3	3.0	1.8	5.8	1.3	4.6	0.7	
AO_15	5.7	3.9	2.6	3.5	3.3	2.5	4.8	5.2	3.5	1.7	4.7	4.9	3.1	1.8	2.5	2.2	3.8	2.6	2.2	1.0	3.4	2.7	
W_15	5.0	3.0	3.9	6.5	4.3	5.2	3.3	6.3	1.5	1.1	2.8	3.3	2.6	2.6	2.4	3.5	1.3	1.1	2.8	1.1	3.0	2.8	
Average	5.7	4.0	3.7	4.6	4.6	4.2	4.4	4.6	3.6	3.0	3.3	2.9	3.7	2.3	2.9	2.9	2.3	2.2	3.0	1.8	2.9	2.3	

Supplementary Table 4.

Statistic significance of the association between the *P. fragi* oligotype abundance and VOCs by means of the Pairwise Wilcoxon test. The significant associations are highlighted in yellow ($P < 0.05$).

																VOCs		
	Ethyl Acetate	Ethyl lactate	Ethanol	1-Penten-3-ol	1-Butanol-3-n	1-Pentanol	2-Penten-1-ol	1-Hexanol	Ethanol-2-butoxy	1-Octen-3-ol	1-Octanol	2-Octen-1-ol	Hexanal	Heptanal	Octanal	Nonanal	2-Octenal	E
P1	0.44	0.55	0.08	0.13	0.24	0.19	0.29	0.38	0.88	**0.03**	0.50	**0.04**	0.26	0.08	0.31	0.78	0.34	
P2	0.44	0.31	0.39	0.21	0.74	0.07	0.26	0.35	0.09	0.22	0.26	0.12	0.24	0.17	0.48	0.50	0.62	
P3	0.50	0.63	0.86	0.46	0.84	0.92	0.50	0.21	0.94	0.51	0.43	0.39	**0.04**	0.16	**0.04**	0.14	0.12	
P4	0.54	0.70	0.78	0.65	0.66	0.36	0.53	0.31	0.79	0.89	0.96	0.76	0.98	0.98	0.82	0.98	0.94	
P5	0.17	0.05	**0.04**	**0.04**	**0.04**	0.06	0.15	0.22	0.77	0.21	0.06	0.26	0.20	0.62	0.18	0.15	0.11	
P6	0.43	0.40	0.89	0.37	0.67	0.49	0.73	0.73	0.84	0.37	0.30	0.30	**0.04**	0.40	0.17	0.10	0.07	
P7	0.87	0.80	0.86	0.41	0.14	0.58	0.35	0.81	0.78	0.55	0.64	0.50	0.82	0.67	0.88	0.97	0.91	
P8	0.29	0.36	0.15	0.12	0.63	0.18	0.25	0.15	0.77	**0.02**	0.20	**0.01**	**0.03**	0.11	0.14	0.12	**0.04**	
P9	0.33	0.19	0.63	0.27	**0.03**	0.22	0.59	0.77	0.49	0.26	0.45	0.27	0.30	0.71	0.93	0.42	**0.05**	
P10	0.30	0.85	0.90	0.74	0.36	0.62	0.74	0.75	0.58	0.56	0.56	0.89	0.86	**0.04**	0.81	0.96	0.70	
P11	0.84	0.83	**0.01**	0.63	0.79	0.54	0.69	**0.04**	0.81	0.21	0.65	0.16	0.24	0.24	0.94	0.70	0.44	
P12	0.37	0.47	0.47	0.24	0.76	0.22	0.48	0.92	0.11	0.18	0.64	0.16	0.39	0.12	0.47	0.58	0.30	
P13	0.65	0.56	0.46	0.85	0.96	0.89	0.96	0.15	0.96	0.37	0.86	0.30	0.77	0.89	0.89	0.83	0.47	
P14	0.22	0.83	**0.02**	0.28	0.18	0.58	0.19	0.73	0.68	0.99	0.44	0.89	0.19	0.58	0.84	0.78	0.85	
P15	0.57	0.38	0.16	0.53	0.25	0.45	0.72	0.51	0.59	0.91	0.98	0.91	0.49	0.86	0.30	0.35	0.89	
P16	**0.05**	0.30	0.57	0.10	0.49	0.21	0.33	0.68	0.92	0.12	0.29	0.11	0.54	0.25	0.20	0.48	0.37	
P17	0.28	0.36	0.93	0.58	0.22	0.43	0.51	0.16	0.97	0.72	0.73	0.74	0.10	0.14	**0.03**	0.10	0.38	
P18	0.66	0.82	0.44	0.51	0.76	0.39	0.44	0.86	0.70	0.68	0.24	0.49	**0.02**	0.19	0.08	0.05	0.18	
P19	0.12	0.13	0.28	**0.02**	0.90	**0.02**	0.07	0.69	0.34	**0.01**	0.16	**0.02**	0.13	0.14	0.48	0.46	**0.05**	
P20	0.99	0.14	0.36	0.55	0.84	0.34	0.99	0.76	1.00	0.35	0.43	0.20	0.24	0.93	0.25	0.18	0.66	
P21	0.84	0.67	0.86	0.50	0.59	0.88	0.51	0.35	0.48	0.51	0.71	0.74	0.50	0.13	0.28	0.74	0.29	
P22	0.45	0.08	0.14	0.32	0.64	0.10	0.89	0.46	0.45	0.10	0.18	0.11	0.25	0.09	0.22	0.10	0.23	
P23	0.11	0.16	0.59	0.26	0.53	0.30	0.77	0.96	0.15	0.09	0.22	0.12	0.26	0.72	0.23	0.12	0.07	
P24	0.78	0.81	0.30	0.23	0.85	0.21	0.08	**0.01**	0.15	0.75	0.88	0.35	0.52	0.29	0.89	0.94	0.71	
P25	0.27	0.29	0.81	0.14	0.06	0.29	0.30	**0.00**	0.58	**0.04**	**0.04**	**0.04**	**0.04**	0.42	0.06	**0.02**	**0.00**	
P26	0.34	0.44	0.47	0.96	0.66	0.64	0.90	0.22	**0.04**	0.98	0.71	0.79	0.60	0.52	0.87	0.99	0.44	
P27	0.80	0.92	0.26	0.94	0.93	0.88	0.95	0.49	0.22	0.57	0.63	0.60	0.83	0.60	0.35	0.43	0.61	
P28	0.52	0.46	0.94	**0.03**	0.62	0.14	0.11	0.28	0.56	**0.03**	**0.01**	0.06	**0.00**	**0.04**	**0.00**	**0.00**	**0.00**	
P29	0.26	0.23	0.42	0.12	**0.03**	0.12	0.15	0.80	0.20	0.41	0.47	0.34	0.53	0.39	0.50	0.85	0.41	
P30	0.22	0.23	0.18	0.49	0.18	0.24	0.84	0.70	0.73	0.70	0.62	0.73	0.19	0.70	0.53	0.78	0.93	
P31	0.49	0.16	0.08	0.35	**0.01**	0.21	0.53	0.86	0.10	0.73	0.63	0.71	0.93	0.71	0.63	0.91	0.50	
P32	0.12	0.25	0.19	0.43	0.63	0.55	0.98	0.73	0.18	0.05	0.36	0.08	0.60	0.55	0.20	0.25	0.36	



Supplementary Figure 1

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