Microbiota diversity of three Brazilian native fishes during ice and frozen storage

(Article begins on next page)

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1   **Microbiota diversity of three Brazilian native fishes during ice and frozen**

2   **storage**

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26   ***Abstract***

27   This study aimed to assess the bacterial microbiota involved in the spoilage of pacu (*Piaractus*

28   *mesopotamics*), patinga (female *Piaractus mesopotamics* x male *Piaractus brachypomus*), and

29   tambacu (female *Colossoma macropomum* x male *Piaractus mesopotamics*) during ice and

30   frozen storage. Changes in microbiota during the storage of 22 samples of three fish species

31   were studied through 16S rRNA amplicon-based sequencing and correlated with volatile

32   organic compounds (VOCs) and metabolites assessed by nuclear magnetic resonance (NMR).

33   Fish microbiota comprised mainly of *Pseudomonas sp., Brochothrix sp., Acinetobacter sp.,*

34   *Bacillus sp., Lactiplantibacillus sp., Kocuria sp.*, and *Enterococcus sp.* The relative abundance

35   of *Kocuria*, *P. fragi, L. plantarum, Enterococcus*, and *Acinetobacter* was positively correlated

36   with the metabolic pathways of ether lipid metabolism while *B. thermosphacta* and *P. fragi*

37   were correlated with metabolic pathways involved in amino acid metabolism. *P. fragi* was the

38   most prevalent spoilage bacteria in both storage conditions (ice and frozen), followed by *B.*

39   *thermosphacta*. Moreover, the relative abundance of identified *Bacillus strains* in fish samples

40   was positively correlated with the production of VOCs (1-hexanol, nonanal, octenol, and 2-

41   ethyl-1-hexanol) associated with off-flavors. ^{1}H NMR analysis confirmed that amino acids,

42   acetic acid, and ATP degradation products increase over (ice) storage, and therefore considered

43   chemical spoilage index of fish fillets.

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45   **Keywords:** Microbial ecology; fish spoilage; 16S rRNA sequencing; volatile organic

46   compounds (VOCs).

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48   **1. Introduction**

49   Among the Brazilian native fish species, the "round" (due the rhomboidal body shape)

50   fishes pacu (*Piaractus mesopotamics*), patinga (female *Piaractus mesopotamics* x male

51   *Piaractus brachypomus*)**,** and tambacu (female *Colossoma macropomum* x male *Piaractus*

52   *mesopotamics*) have been considered amongst the most expressive in terms of economic

53   importance in Brazilian fish farming (Borges et al., 2013; Dairiki et al., 2010). These species

54   present fast growth, easy adaptation to artificial feed, are well accepted by consumers, and are,

55   therefore, of great interest to farmers (Borges et al., 2013).

56   However, to date, very few studies focused on fish quality for human consumption, and

57   on the shelf life of pacu and tambacu (Borges et al., 2013, 2014a, 2014b; Bottino et al., 2017;

58   Cossa et al., 2022; Silva et al., 2022). The majority of studies performed with Brazilian round

59   fish aimed at optimizing feeding, treatment, and resistance to diseases, evaluating the factors

60   that can affect the growth and genetic identification of the species' life (Coelho et al., 2022;

61   Girao et al., 2021; Hashimoto et al., 2011; Kumar et al., 2018; Santos et al., 2017; Volkoff et

62   al., 2017).

63   Fish is a highly perishable food; spoilage involves changes in physicochemical and

64   microbiological parameters (El-Hanafi et al., 2011). Microbial activity is mainly involved in

65   the fish loss of quality and associated with psychrotrophic bacteria which become dominant

66   during fish chilled storage (0 to 7 ℃). *Pseudomonas, Shewanella, Photobacterium,*

67   *Carnobacterium, Lactococcus*, and *Brochothrix* are commonly reported as Specific Spoilage

68   Organisms (SSOs) in fish products (Dalgaard et al., 1993; Gram and Huss, 1996). They are

69   known to produce typical off-odor and off-flavor metabolites associated with food

70   deterioration, such as amines, sulfides, alcohols, aldehydes, ketones, and organic acids (Doyle

71   et al., 2003; Gram et al., 1987; Gram and Huss, 1996).

72   Microbial spoilage is directly influenced by storage conditions, seafood species, and

73   fish farming climatic conditions. Thus, knowing the composition of the initial fish microbiota

74   can strongly contribute to mitigating the losses associated with the contamination of this food

75   matrix. The characterization of SSOs is often performed via classical microbial plating

76   techniques. However, this method is known to not support the cultivation of most

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microorganisms. Therefore, molecular methods, such as high throughput amplicon-based sequencing, are primarily employed for such characterization (Cocolin et al., 2013).

To the authors' best knowledge, there are no studies on the microbial ecology of Brazilian native fish species, and only a few evaluate the impact of storage conditions on their quality (Borges et al., 2013, 2014a, 2014b; Bottino et al., 2017; Castro et al., 2007). For instance, a shelf life of 11 days for tambacu and pacu has been suggested during ice storage (Borges et al., 2013, 2014a, 2014b), while an increase of 50% in the shelf life of tambacu has been described when UV radiation was employed (Bottino et al., 2017).

This study aimed (i) to characterize the microbiota responsible for the spoilage of three Brazilian native fish stored on ice or frozen for up to one year by 16S amplicon-based sequencing (ii) to analyze volatile and nonvolatile metabolites through GC-MS and H-NMR and (iii) to investigate potential correlation of volatile organic compounds (VOCs) and spoilage microbiota in fish. This study contributes to a better understanding of microbial population dynamics during the shelf life and determines the microbial species' role in spoilage. This valuable information also contributes to improving preservation measures and maintaining fish quality and safety.

2. Materials and methods

2.1. Fish sample preparation and storage conditions

Individuals (N=22, weighing ~ 1.5 kg/each) of the three Brazilian native fish species (pacu, tambacu, and patinga) were collected from a fish farm in São Paulo, Brazil. Sample variability was encompassed by collecting two lots: "batches A," referring to the samples collected in February, and "batches B," for the samples collected in June. The slaughter, washing, gutting, filleting, and transportation to the lab were done as previously described (Baptista et al., 2024).

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102   The fresh fish fillets were stored in two conditions: ice and freezing. For the samples

103   stored on ice, three fish fillets of the same species were aseptically cut into small pieces of about

104   23 g for a total of 70 g for each sample. Then, fish fillets were packed in PVC films and stored

105   inside a hermetic cooler with ice. The ratio of fish fillets to ice was 1:1 (one layer of ice and

106   one layer of fish samples). The hermetic cooler was placed inside a refrigerator and kept at $2 \pm$

107    $1\ ^{\circ}C$ until analyses. Fish samples were kept on ice for 18 days and re-iced when necessary. The

108   microbiological analyses were performed in duplicate at 0, 3, 6, 9, 12, 15, and 18 days of

109   storage. For the samples stored in a freezer, 70 g of fillet were packed in PVC films and placed

110   in absorbent trays. The trays were placed in cardboard boxes and stored at $-18\ ^{\circ}C$. Samples were

111   stored at $-18\ ^{\circ}C$ for one year, and the analyses were carried out every 15 days in duplicate. At

112   each time interval planned for analyses, a portion of samples was separated in triplicate and

113   stored at $-18^{\circ}C$ for the following analyses: 10 g for DNA, 30 g for NMR, and 10 g for CG

114   analyses. These analyses were performed within 2 months.

115   The RMN and DNA samples were placed in Petri dishes and covered with PVC film,

116   which had been previously drilled to allow air circulation. The samples were placed at $-18\ ^{\circ}C$

117   overnight before transference to the freeze-dryer (Terroni, LS3000, São Paulo, Brazil) until

118   complete dehydration.

119

120   **2.2. Microbiological analysis**

121   At every sampling point, 10 g of fresh sample were aseptically cut into small pieces and

122   transferred to a stomacher bag containing 90 mL of sterile buffered peptone water (BPW)

123   (Himedia, Mumbai, India) for obtaining a 1:10 ratio, which was homogenized in a Stomacher

124   400 circulator (Model BA7021, Seward, London, England) for 1 min. Serial ten-fold dilutions

125   were prepared and 0.1 mL were spread in duplicate in the following culture plates: (i) Agar

126   tributyrin for Lipolytic bacteria, incubated for 48 h at $30\ ^{\circ}C$, which consisted of 1.3% nutrient

127   broth (Kasvi, Roseto degli Abruzzi, Italy), 1% tributyrin (Sigma-Aldrich, St Louis,

128 Missouri,USA), and 2% agar (Inlab, São Paulo, Brazil) (Ben-Gigireya et al., 2000); (ii) Plate

129 Count Agar (PCA, Kasci, Roseto degli Abruzzi, Italy) supplemented with 0.5% NaCl

130 (Dinâmica, São Paulo, Brazil) for Aerobic mesophilic, incubated at $35 \pm 1°C$ for 48 ± 2 h; (iii)

131 PCA with 0.5% NaCl for Psychrotrophic bacteria, incubated at 7 ± 1 °C for 48 ± 2 h; (iv) Casein

132 Agar for Proteolytic bacteria, incubated at 30 °C for 48 h, prepared with 1.5 g of Beef extract

133 (Acumedia, Neogen, Lansing, Michigan, US), 2.5 g of Bacto Tryptone (Kasvi, Roseto degli

134 Abruzzi, Italy), 0.5 g of Glucose (Sigma Aldrich, Rio de Janeiro, Brazil), 8.5 g of Agar, and

135 12 g of skim milk powder (Nestlé Brazil LTDA, São Paulo, Brazil) (Ben-Gigireya et al., 2000);

136 and, (v) *Pseudomonas* Agar Cetrimide Fucidina Cephaloridine (Oxoid LTD, Basingstone,

137 Hampshire, England) for *Pseudomonas* spp.*,* incubated at 25 ± 1 ºC for 48 ± 2 h. To confirm

138 *Pseudomonas* spp., 5 colonies per plate were randomly chosen and purified in Nutrient Agar

139 (Kasvi, Roseto degli Abruzzi, Italy), incubated at 25 ± 1 °C for 24 h. After incubation, pure

140 colonies were selected for confirmation by the Oxidase test (Laborclin, Rio Grande do Sul,

141 Brazil) and the Kligler Iron Agar test (Oxoid LTDA, Basingstoke, Hampshire, England). The

142 results were expressed as $\log_{10}$ CFU/g.

143

144 **2.3. Microbiota diversity assessment by 16S rRNA amplicon target sequencing**

145 2.3.1. DNA extraction from fish

146 Lyophilized fish samples (1 g) were homogenized with 5 mL of STE Buffer (100 mM

147 NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA) for 1 min in a stomacher (LAB blender 400; PBI,

148 Italy; stomacher bags, Sto-circul-bag; PBI, Italy). One milliliter of the supernatant was mixed

149 with 50 μL lysozyme (50 mg/mL) and incubated at 37 ºC for 30 min. After incubation, DNA

150 was extracted according to the MasterPure DNA purification kit (Epicentre, Madison,

151 Wisconsin), following the manufacturing instructions. DNA concentration was measured by

152 NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy) and standardized at five

153 ng/μL.

154

155 2.3.2. 16S rRNA amplicon target sequencing

156   DNA directly extracted from fish samples was used to assess the microbiota by

157 amplifying the V3-V4 region of the 16S rRNA gene using the primers and protocols described

158 by Klindworth et al. (2013). PCR amplicons were then subject to cleanup steps using an

159 Agencourt AMPure kit (Beckman Coulter, Milan, Italy). The Nextera XT Index Kit (Illumina

160 Inc. San Diego. CA) was used to tag the clean PCR products following the manufacturer's

161 instructions. After the second purification step, amplicon products were quantified by using a

162 QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy). An equimolar amount of amplicons

163 was pooled (4 nM). The pooled library was run on an Experion workstation (Biorad, Milan,

164 Italy) for quality analysis before sequencing. The library was denatured with 0.2 N NaOH,

165 diluted to 12 pM, and combined with 20% (v/v) denatured 12 pM PhiX, prepared according to

166 Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument

167 (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the

168 manufacturer's instructions.

169

170 2.3.3. Bioinformatic and statistical analysis

171   FLASH software (Magoc and Salzberg, 2011) with default parameters merged the

172 paired-end reads. Sequences were quality filtered (at Phred < Q20) using QIIME 1.9.0 software

173 (Caporaso et al., 2010), and short reads (< 250 bp) were discarded through Prinseq (Schmieder

174 and Edwards, 2011). Chimeras were filtered out by USEARCH software version 8.1 (Edgar et

175 al., 2011). Operational Taxonomic Units (OTUs) were picked at 99% similarity by UCLUST

176 algorithms (Edgar, 2010), and representative sequences for each cluster were mapped to the

177 manually updated Greengenes 16S rRNA gene database (v. 2013). Greengenes database was

178 edited to update taxonomic classifications of lactic acid bacteria based on the latest research.

OTU tables were rarefied to the lowest number of sequences per sample. The OTU table displays the higher taxonomy resolution reached; when the taxonomy assignment could not reach the genus, the family name was displayed. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v1.1.1) was used to predict the abundances of KEGG orthologs (KO) (Langille et al., 2013). α-diversity was assessed by Chao1 and Shannon index, using the diversity function of the vegan package v. 2.6-4 (Dixon, 2003) in R environment (http://www.r-project.org) (R Core Team, 2023). OTU table was used to build a principal-component analysis (PCA) as a function of the sample type (frozen or ice) by the made4 v. 3.18 package (Culhane et al., 2005) of R. ADONIS, and the ANOSIM statistical test was used to detect significant differences in the overall microbial community by using the Weighted UniFrac distance matrices and the OTU table. Wilcoxon matched pairs test or Mann-Whitney test was used as appropriate to find a specific association between α-diversity index or microbiota and sample type. The KO abundance table at level 3 of the KEGG annotations was imported in *the gage* Bioconductor package (Luo et al., 2009) to carry out pathway enrichment analysis to identify biological pathways overrepresented or underrepresented between sample types. Pairwise, Spearman's non-parametric correlations were used to study the relationships between the relative abundance of microbiota and inferred metabolic pathways or volatile and nonvolatile metabolites. Log2 transform data was used to normalize datasets. The correlation plots were visualized in R using the corrplot v. 0.92 package (Friendly, 2002) of R. P-values were adjusted for multiple testing using the Benjamini–Hochberg procedure, which assesses the false discovery rate (FDR).

All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (Bioproject accession number: PRJNA1035758).

2.4. Gas chromatography-mass spectrometry analysis - CG-MS

2.4.1. VOCs determination by headspace HS-SPME/CG-MS

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205   The Headspace Solid Phase Micro-extraction (HS-SPME) technique was performed to

206   extract the volatile compounds by using a Divinybenzene Carboxen/Polydimethylsiloxane

207   (DVB/CAR/PDMS) 100 μm SPME fiber and manual holder (Supelco, USA). After storage at

208   -80 °Cs, samples were kept at room temperature (~22 °C) for 30 min, and 3 g of each sample

209   were transferred into headspace vials (40 mL). Six milliliters of sodium chloride (0.36 mg/L)

210   were added, and vials were sealed with polyethylene and silicone septum cap. The vials were

211   placed at 50 ºC for 15 min before SPME analysis to let the sample and headspace equilibrate.

212   Vials headspace were exposed to the SPME fiber for 15 min at 50 ºC and immediately desorbed

213   in a gas chromatographer injector at 250 ºC.

214   2.4.2. GC-MS procedures

215   The chromatographic analysis was performed using an Agilent 7890A linked to an Agilent 7000

216   Triple Quad triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) and

217   helium at a 1 mL/min flow rate. The Agilent MassHunter Workstation Software Qualitative and

218   Quantitative Analysis for QQQ (Agilent Technologies Inc.) and National Institute of Standards

219   and Technology (NIST, 2011) MS database were used for data acquisition and ion

220   fragmentation consultation, respectively. The splitless mode was used for all analyses with a

221   liner suitable for SPME analysis. HP-5ms capillary column 30 m × 0.25 mm ID × 1.0 μm d_f

222   (Agilent Technologies, Palo Alto, CA) were employed. The injector and oven temperature

223   parameters employed were optimized by Xu et al. (2015). Electron ionization (70 eV) ion

224   fragmentation was used to acquire mass spectra, employing nitrogen and helium as collision

225   gas. Data acquisition was performed at Multiple Reaction Monitoring (MRM). The ANOVA

226   single factor ($P < 0.05$ significance level) was performed on semi-quantitative results (peaks

227   area) using the software Origin™ 9.4 to certify statistical differences among the conditions. In

228   the case of significant differences shown by ANOVA means, a comparison was performed

229   using Tukey's and Levene's tests to assess the homogeneity of variance. The combined

230 uncertainties were based on the standard deviation of the sampling and the analytical errors of

231 the replicate of the chromatograms acquisition (Alves Filho et al., 2017).

232 2.4.3. Biomarkers determination

233   The investigation of product ions and all relevant mass transitions (Table 1) was done

234 by acquiring a full scan before MRM analysis using the following conditions: 10 000 amu/s

235 scan speed and 30 – 400 *m/z* mass range. The identified biomarkers were performed during the

236 fish storage time for those samples stored on ice and frozen at three distinct points (initial,

237 intermediate, and final). Biomarker semi-quantification was performed using respective

238 chromatographic peak area counts.

239

240 **2.5. ^{1}H NMR analysis**

241 2.5.1. Molecular identification and monitoring of metabolites by ^{1}H NMR spectroscopy

242   Before the ^{1}H NMR analysis, an adapted experimental method was applied to prepare

243 the fish samples, according to Baptista et al. (2024).

244   The NMR experiments, the equipment employed (Agilent 600 MHz spectrometer

245 equipped with a 5 mm (H-F/^{15}N-^{31}P) inverse detection One Probe™ with actively shielded Z-

246 gradient), acquisition of spectra, processing of spectra, phase correction of the entire spectral

247 range, and the identification of the constituents were detailed in a previous work (Baptista et

248 al., 2024).

249

250 2.5.2. Chemometric analysis

251 Chemometric analysis investigated the variability of the composition of the fish samples stored

252 on ice and frozen during two different seasons (Batches A – February and December and B –

253 June). These analyses were performed as previously described (Baptista et al., 2024). In brief,

254 The 1H NMR (totaling 24 1H NMR spectra) were converted to American Standard Code for

255 Information Interchange (ASCII) format to matrix construction, which was exported for

chemometric analysis using The Unscrambler X™ program 10.4 (CAMO software, Woodbridge, NJ, USA). Singular Value Decomposition (SVD) algorithm was used to decompose the matrix (24 samples × 6,947 variables = 166,728 NMR data) in order to reduce data dimension and to observe trends of the samples variability with a confidence level of 95 %.

3. Results

3.1. Microbiological analysis

The results of microbial counts on selective culture media of patinga, pacu, and tambacu during ice and frozen storage are presented in Figures 1 and 2, respectively. During the storage on ice, all the species showed an initial count of about 4.11 ± 0.32 log CFU/g, increasing during storage. At the end of the storage period (24 days), counts of approximately 9.06 ± 0.45 log CFU/g were obtained for psychotrophic, mesophilic, and *Pseudomonas* and about 8.12 ± 0.28 log CFU/g for proteolytic and lipolytic bacteria (Figure 1). Samples stored under frozen conditions showed an initial count of about 4 log CFU/g, and the bacterial load was kept throughout the year (Figure 2). No significant differences in counts were observed among the three fishes (patinga, pacu, and tambacu) when each group of microorganisms was compared amongst samples stored for the same period of time at same storage conditions (ice or frozen).

3.2. Microbiota diversity assessment by 16S rRNA amplicon target sequencing

The bacterial sequencing accounted for 7.333.944 reads. After quality filtering, 3.752.991 reads passed the filters, averaging 57.738 reads/sample and 460 bp of sequence length. The analyses were satisfactory for all samples, with an average recovery value of 86%. Adonis and analysis of similarity (ANOSIM) statistical tests showed no significant differences among fish species (patinga, pacu, and tambacu samples) and neither between the lots (February and June).

However, significant differences between the storage types (Frozen *vs* Ice, P < 0.001) were observed. Principal Component Analysis (PCA) was performed on the OTUs table, and the results (Figure 3) further confirmed the separation of the samples according to the storage type, ice or frozen. Considering the single sampling point, observing few differences among OTUs was possible.

As shown in Figure 4, *Pseudomonas fragi, Brochothrix thermosphacta, Acinetobacter, Acinetobacter johnsonii, Bacillus, Lactiplantibacillus plantarum, Kocuria*, and *Enterococcus* were the main OTUs shared among the entire datasets. No differences were observed according to the fish species. Variance in the microbiota was observed to be affected by the sampling time in ice samples. In particular, samples on day 3 showed a higher presence (P < 0.05) of several minor OTUs (*Acinetobacter, Bacillus, Bacteroides, Enterococcus, Kurthia,* and *Macrococcus*). Meanwhile, *P. fragi* was characteristic at the end of the trial. On the other hand, no significant differences across the samples were observed.

Regarding samples stored in frozen condition, no difference in the function of the fish species was observed. Considering the sampling time in frozen samples, it was possible to observe a reduction (P < 0.05) in the relative abundance from time 0 till the end of several OTUs such as *Kurthia, Enterococcus, Ralstonia, Staphylococcus*, and *Streptococcus* while *P. fragi* increased as affected by the storage time (P < 0.05).

The comparison of the relative abundance of the main OTUs between ice and frozen samples suggested that *A. johnsonii, Acinetobacter, Enterococcus, Kocuria, L. plantarum,* and *P. fragi* were characteristic of frozen samples (FDR < 0.05). On the other hand, only *Bacillus* were associated with ice samples (FDR < 0.05). Regarding the inferred metagenome, the nearest sequenced taxon index (NSTI) score was 0.048 ± 0.011, indicating an accuracy of 95.2%. The pathway enrichment analysis performed by GAGE showed an enrichment of the following inferred metabolism: methane (ko00680), propanoate (ko00640), butanoate (ko00650), sulfur (ko00920), arginine and proline (ko00330) in frozen samples compared to

307   ice samples (data not shown). When plotting the correlation between OTUs and KO, it was

308   observed that *Kocuria*, *P. fragi*, *L. plantarum*, *Enterococcus*, and *Acinetobacter* were positively

309   correlated with the metabolic pathways (Figure 5) (FDR < 0.05) of ether lipid metabolism. *B.*

310   *thermosphacta* and *Pseudomonas* sp. were related to inferred metabolic pathways involved in

311   amino acid metabolism (arginine, alanine, and proline metabolism) (FDR < 0.05).

312

313   **3.3. Volatile organic compounds (VOCs) analysis**

314   In the present study, a screening was performed to determine the most frequent markers

315   (VOCs) and those familiar to different fish species, making the comparison between the species

316   and the storage time possible. The following VOCs identified exhibited an appropriate

317   analytical signal present in our library: 1-hexanol, nonanal, octenol, and 2-ethyl-1-hexanol

318   (Tables 2 and 3). Therefore, a semi-quantitative evaluation of these compounds was developed

319   considering the integration value of the respective peaks area (no calibration curves using

320   analytical standards).

321   Fluctuation in 1-hexanol concentration was observed on frozen and ice storage without

322   observing a standard behavior. By evaluating the concentration of nonanal, an increase during

323   the frozen and ice storage was observed, followed by the reduction in levels of the volatile in

324   all species studied. However, this reduction was insignificant for tambacu samples stored on

325   ice and freezing. Likewise, 2-ethyl-1-hexanol concentration also tended to increase, followed

326   by ice and frozen storage reduction. The reduction was not significant for the pacu sample under

327   freezing. Additionally, only increasing values of 2-ethyl-1-hexanol (P < 0.05) were observed

328   for the pacu sample stored on ice.

329   The octenol showed the lowest concentrations compared with the other VOCs analyzed

330   among the studied species and the storage forms. On ice storage, pacu samples showed a

331   tendency to increase the octanol concentration, followed by a reduction in concentration levels

332   (P < 0.05); in tambacu samples, a reduction in the concentration was the trend observed (P <

333 0.05); in patinga samples was, observed an increase in concentration over the storage (P < 0.05).

334 No change in octenol concentration was observed for the frozen samples, except for pacu

335 samples, which tended to reduce the concentration of this volatile (P < 0.05).

336

337 **3.4. NMR analysis**

338   The major compounds identified in frozen and ice fillets of pacu, tambacu, and patinga

339 were lactic acid (doublet at δ 1.34 and quadruplet at δ 4.13), creatine (singlet at δ 3.05 and δ

340 3.95), and taurine (doublet of doublets at δ 3.30 and δ 3.44). In the aromatic region (δ 6.5 –

341 9.0), the main compounds detected were ATP/ADP (singlet at δ 8.57) and the decomposition

342 product inosine (singlet at δ 8.48). Due to the complexity of the ^{1}H NMR dataset and the

343 similarity among the fish composition, the PCA method was applied to investigate the

344 variability of the organic compounds in three species of fish (pacu, tambacu, and patinga).

345   Figure 6 presents the scores graph (a) and loadings plotted in lines form (b) from fish

346 species pacu (6-a), tambacu (6-b), and patinga (6-c), respectively, during three storage periods:

347 in the beginning (0 days) after 12 days; and after 21 days of storing in ice. Figures with main

348 variations according to the first two principal components (PC1 and PC2) with 79.9%, 95.3%,

349 and 85.7% of the total variance, respectively.

350   All the loadings graphs in ice storage showed a positive correlation among

351 phosphocreatine creatine and taurine and their opposite behavior with fatty acids, acetic acid,

352 leucine, isoleucine, and valine. For pacu species (Figure 6-a), the loadings graph (b) illustrates

353 mainly the decreased tendency in the amount of creatine and phosphocreatine in fish fillets and

354 presented increased tendencies in amounts of acetic and fatty acids, leucine, isoleucine, and

355 valine, and PC2 loadings revealed decrease in the amount of lactic acid after both storages (12

356 and 21 days). After 12 days of storage, tambacu species samples presented higher decrease

357 tendencies in amounts of creatine phosphocreatine and lactic acid (Figure 6-b). Furthermore,

358 after 12 days of storage, amounts of acetic and fatty acids, leucine, isoleucine, and valine

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359 increased in fish fillets. Samples before storage presented higher amounts of creatine and

360 phosphocreatine, taurine, and lactic acid based on PC1 and PC2 loadings. In patinga samples,

361 negative loadings of PC1 presented decreased tendencies in amounts of creatine,

362 phosphocreatine, and lactic acid, mainly for fish meats after storage in February (12 or 21 days),

363 and an increase in the amount of acetic and fatty acids (Figure 6-c).

364

365 **3.5. Correlation between microbiota and volatile and nonvolatile compounds**

366 When plotting the Spearman correlation between microbiota and metabolites, it was

367 possible to observe that phosphocreatine/creatine showed the highest number of positive

368 correlations (FDR < 0.05) with the microbiota (Figure 7). Conversely, all the volatile

369 metabolites were negatively correlated with the microbiota. Only *Bacillus* showed a positive

370 correlation with the volatile metabolites. In addition, a positive correlation was observed

371 between *P. fragi* and taurine, while *Flavobacteriacea* was correlated with the production of

372 lactic acid (Figure 7).

373

374 **4. Discussion**

375 **4.1. Microbiological and 16S rRNA analysis**

376 The present study aimed to monitor the microbiological microbiota community of three

377 native Brazilian fishes (pacu, tambacu, and patinga) during ice and frozen storage. Fish stored

378 on ice had a shelf life of approximately 15 days, when microbial counts reached 7 logs CFU/g

379 as established as a microbiological standard count in refrigerated fish (ICMSF, 1986).

380 Amplicon sequencing of the samples demonstrated the dominant OTUs, *P. fragi, B.*

381 *thermosphacta, Acinetobacter, Acinetobacter johnsonii, Bacillus, Lactiplantibacillus*

382 *plantarum, Kocuria,* and *Enterococcus.*

383 *Pseudomonas* spp. are commonly reported in the literature as a dominant

384 microorganism in chilled fish under aerobic growth (Ben Mhenni et al., 2023; Mikš-Krajnik et

385 al., 2016; Parlapani et al., 2014). In this study, *P. fragi* was the most prevalent spoiler in all fish

386 samples analyzed in ice and frozen storage. This species is commonly found in aquatic

387 environments, in different climates, e.g., tropical and subtropical areas, and in different salinity

388 of marine and freshwater (Gram, 1993; Liston, 1992). *P. fragi* is often associated as a spoilage

389 microorganism in fish at refrigerated conditions, due to its proteolytic and lipolytic activity with

390 considerable heterogeneity (Ercolini et al., 2010). Moreover *P. fragi* produces odors

391 reminiscent of spoiling tropical fruit (a mix of ethyl esters of acetate, butyrate, and hexanoate)

392 and sulphydryl compounds (Cann, 1974; Gillespie, 1981).

393   Several authors have positively correlated the presence of *P. fragi*, in meat samples,

394 with the production of the VOCs studied in the present study: nonanal, 1-octen-3-ol, 2-ethyl-1-

395 hexanol and 1-hexanol (Casaburi et al., 2011; Edwards et al., 1987; Ercolini et al., 2011;

396 Ferrocino et al., 2013). These VOCs have been associated with both triglyceride and amino acid

397 catabolism. However, in the present study, the activity of *Pseudomonas spp.* was only

398 associated to the inferred metabolic pathway of amino acids, and no significant correlation was

399 observed between *P. fragi* and VOCs. On the other hand, a clear correlation was observed

400 between *P. fragi* and taurine. According to Shimamoto and Berk (1979), *Pseudomonas* spp. can

401 use taurine as a source of carbon and nitrogen for energy metabolism. Several strains of

402 *Pseudomonas* present the enzyme taurine pyruvate aminotransferase, capable of catabolizing

403 taurine and pyruvate in forming sulfoacetaldehyde and alanine.

404   *B. thermosphacta* has been reported in fish stored in aerobic conditions (Mikš-Krajnik

405 et al., 2016; Nowak et al., 2012), characterized by producing a mild and sour odor.

406   In this study, *B. thermosphacta* was positively correlated with metabolic pathways

407 involved in amino acid metabolism (FDR < 0.05). Likewise, Dainty and Mackey (1992)

408 showed that *B. thermosphacta* was responsible for degrading some amino acids in meat, such

409 as valine, leucine, and isoleucine, resulting in the production of isobutyric, isovaleric, and 2-

410 methylbutyric acids, respectively. And also capable of producing acetamin and acetic acid.

411   *Acinetobacter* spp. is a spoilage bacteria on freshwater fish (González et al., 2001; Gram

412   and Huss, 1996). It is a nutritionally versatile bacterium, capable of use some amino acids and

413   fatty acids as carbon sources. Within the genus *Acinetobacter*, *A. johnsonii* was the most

414   abundant OTU found on fish samples*,* as reported by other authors (Dabadé et al., 2015;

415   Kozińska et al., 2014). However, the low abundance of *Acinetobacter* and *A. johnsonii* in our

416   study suggests that they contribute on a smaller scale to the spoilage of the analyzed fishes.

417   Enterococci are ubiquitous and can occur in a wide variety of environmental niches,

418   such as soil, aquatic environments or infected humans (Kusuda and Salati, 1999; Michel et al.,

419   2007; Taučer-Kapteijn et al., 2013). *Enterococcus* species may also be present in the

420   gastrointestinal tract, gills, and on the surface of freshwater fish (Austin, 2002; Hatha, 2002;

421   Radu et al., 2003; Vivekanandhan et al., 2005). Their contamination is often associated with

422   contaminated water or unhygienic handling and poor personal hygiene, originate from

423   materials, food operators, or the environment throughout the processing chain (Lampel et al.,

424   1999; Lopez-Sabater et al., 1994). *E. faecalis*, *E. faecium* and *E. casseliflavus* are associated

425   with fish spoilage and with the catabolism of carbohydrates to lactic acid (Dalgaard et al., 2003;

426   Tomé et al., 2008). These species were recently isolated on a fish farm in Brazil (Araújo et al.,

427   2021). When present in protein matrices, *Enterococcus* spp. can degrade amino acids to

428   biogenic amines, with highlights for tyramine production (Chong et al., 2011). As for

429   *Acinetobacter* spp.*,* a low rate of *Enterococcus* spp. was observed in the fish matrix, indicating

430   only an additional contribution of this genus to fish spoilage.

431   *L. plantarum, Kocuria* spp., and *Bacillus* spp. are reported as relevant fish flora, being

432   naturally present in the fish intestine (Austin, 2006; Grayfer et al., 2014). *L. plantarum* is a

433   species with high proteolytic activity which can contribute to the degradation of short-chain

434   fatty acids (Ztaliou et al., 1996). *Kocuria* species can be isolated from various environments,

435   including marine ones (Jorgensen et al., 2001; Parlapani et al., 2017). Fish from tropical water

436   usually have a slightly higher concentration of Gram-positive bacteria, such as *Bacillus* spp.

17

437　and lactic acid bacteria, when compared to fish from temperate water (Liston, 1980). *Bacillus*

438　spp. has been identified in fish species and the aquatic environment (Popović et al., 2017;

439　Rasheeda et al., 2017). They present high proteolytic and lipolytic activity, contributing to fish

440　spoilage. *Bacillus* spp. are known for their proteolytic and amylolytic activity. In protein

441　matrices, they can release proteases, degrading proteins into amino acids (Liu et al., 2018;

442　Zheng et al., 2011). In the present study, the genus *Bacillus* was positively correlated with the

443　production of all VOCs identified (octanol, nonanal, 1-hexanol, and 2-ethyl-1-hexanol) in fish

444　samples.

445

4.2. Volatile organic compounds analysis

447　In general, the VOCs studied were negatively correlated with the microbiota (relative

448　abundance of selected taxa), except the *Bacillus* genus. All the alcohols evaluated are reported

449　as products of oxidation of unsaturated fatty acids. They are formed by a lipoxygenase-initiated

450　peroxidation of the n−3 and n−6 polyunsaturated fatty acids (Selli and Cayhan, 2009); while

451　saturated aldehydes, as nonanal, come from the oxidation of n-6 and n-9 polyunsaturated fatty

452　acid (Duflos et al., 2006; Soncin et al., 2009), contributing to the production of off-flavors in

453　the fish matrix.

454　Among the VOCs studied, the smallest quantifications (peak areas) were observed as

455　follows: 0.14 ± 0.29 for octenol in tambacu stored in ice for 21 days (Table 2) and 0.02 ± 0.13

456　for nonanal in patinga stored frozen for 12 months (Table 3). Unsaturated alcohols and

457　aldehydes are known to present a low active odor threshold, such as 0.0075 and 1 ppm, for

458　octenol and nonanal, respectively, As a result, when present in trace amounts, they can affect

459　the flavor. For 1-hexanol, the reported limit of active odors is 8,000 ppm (Guth, 1997; Marco

460　et al., 2007). Moreover, nonanal contributes to the following fish odors: green, fruity, gas,

461　chlorine, floral, waxy, sweet, melon, soapy, fatty, and citrus fruit (Ganeko et al., 2008).

462   The fluctuation in 1-hexanol concentration observed in this study is also reported by

463   different authors, confirming the non-standard behavior during the ice and frozen storage

464   (Odeyemi et al., 2018; Polo et al., 2014). The compounds 1-octen-2-ol and 2-ethil-1-hexanol

465   showed an increase during chilled pacu storage, as reported by Iglesias et al. (2009) and Tuckey

466   et al. (2013). However, in this study, it was observed a decrease in the compounds at the end of

467   shelf life (between the 9º to 21º day of ice storage), which may be associated to the oxidation

468   of secondary alcohols or the esterification of alcohols with carboxylic acids (Padda et al., 2001;

469   Peterson and Chang, 1982). Alasalvar et al. (2005) report that C6-C10 saturated and unsaturated

470   alcohols and carbonyl compounds are naturally found in several fish species, detected in

471   controls and inoculated fish. Similar results were reported for nonanal.

472   The significant differences observed in the VOCs peak area among the fish species (P

473   < 0.05) must result from the lipid profile between the species. It is known that Brazilian

474   ''round'' fish have high levels of n-3 and n-6 polyunsaturated fatty acids (Tanamati et al.,

475   2009). However, only one study has been published on the profile of fatty acids in pacu (Castro

476   et al., 2007).

477

478   **4.3. NMR analysis**

479   Overall, the nonvolatile compounds showed reduced concentrations of creatine and

480   phosphocreatine, taurine, and lactic acid. Additionally, an increase in concentrations of acetic

481   acid, fatty acid, and amino acids leucine, isoleucine, and valine was observed during ice storage

482   for all fish species.

483   The reduction in taurine creatine and phosphocreatine concentration was already

484   expected. As previously mentioned, taurine was positively correlated with the activity of *P.*

485   *fragis*, which can catabolize taurine and pyruvate during the formation of sulfoacetaldehyde

486   and alanine, contributing to the reduction of compound (Shimahara et al., 1989).

487   Phosphocreatine is the primary energy reserve after fish slaughter. It is instantly cleaved by the

488 enzyme phosphocreatine kinase to reconstitute the ATP molecule from ADP, resulting in the

489 formation of creatine. Therefore, the peaks observed in the present study are mainly due to the

490 contribution of creatine due to the rapid degradation of phosphocreatine (Savorani et al., 2010;

491 Wyss and Kaddurah-Daouk, 2000). In particular, creatine and phosphocreatine contribute to

492 the thickness and mouthfulness (Shah et al., 2010).

493   Although an increase in the concentration of lactic acid is expected throughout storage

494 due to the anaerobic catabolism of glucose, a reduction in the concentration of the compound

495 was observed. Likewise, Shumilina et al. (2016) report a fluctuation in the lactic acid

496 concentration in fish by-products (Backbones, Viscera, and heads), while Tan et al. (2018)

497 observed a reduction in concentration in fish muscle. These findings may be an indicative of

498 the microbiological activity. It is known that some microorganisms can consume or produce

499 lactate.

500   The increase in the concentration of the amino acids valine, isoleucine, and leucine is

501 consistent among several authors (Savorani et al., 2010; Tan et al., 2018). Amino acid formation

502 results from the hydrolysis of the protein. At the same time, the proteolytic activity may be

503 associated with endogenous enzymes (such as cathepsin and calpain) and bacterial proteinases,

504 such as lactic acid bacteria, *Pseudomonas* spp*., Bacillus* spp*.,* and *Aeromonas* spp. (Rao et al.,

505 1998).

506   The accumulation of free fatty acids in the fish matrix also contributes to the loss of

507 quality, resulting in a rancid flavor. The lipolytic action results in the cleavage of the

508 triglycerides and is associated with the activity of endogenous enzymes or lipolytic bacteria

509 (i.e., *P. fragi*, *L. plantarum*) (Huss, 1995).

510   Acetic acid has been reported as an indicator of quality, being absent or present in low

511 quantity in fresh food, increasing proportionally the deterioration of the food quality (Mikš-

512 Krajnik et al., 2016), in agreement with the observation in the present study. Acetic acid is

513 exclusively related to the growth (Parlapani et al., 2017) of *B. thermosphacta* and lactic acid

514   bacteria, but also *S. putrefaciens* and *P. phosphoreum* resulting in a sour odor (Macé et al.,

515   2013; Nychas et al., 2008).

516   This study showed that the microbiota diversity of the three analyzed fishes was

517   directly influenced by storage conditions (ice or frozen) rather than by fish species or different

518   lot/sampling seasons. These results confirmed that microbiota analysis along with the storage

519   condition can help in the early detection of the main actors involved in spoilage. Ultimately,

520   these findings, together with the correlation of microbiota and metabolites, are crucial for

521   mitigating the loss of quality in this food matrix.

522

523   5. Conclusions

524   In this study, 16S rRNA analysis significantly contributed to identifying the main OTU's

525   potential impact on the microbiological microbiota community associated with the loss of

526   quality of t Brazilian native fish. *P. fragi* was the most prevalent spoilage bacteria in the three

527   fishes stored in ice and frozen conditions, followed by *B. thermosphacta*. Therefore, both

528   species can contribute to determining the final characteristics of fish products. Moreover, all

529   the volatile compounds studied were positively correlated with *Bacillus* spp. However, a wide

530   further exploitation must be realized on VOCs diversity released during spoilage of native fish

531   to make available their correlation with other microorganisms. The data may be useful for a

532   more effective analysis of fish spoilage. An increase in some nonvolatile compounds (i.e.,

533   amino acids, acetic acid, ATP degradation products, etc.) has been observed during fish storage

534   (metabolite analysis using 1H NMR), suggesting their potential as chemical spoilage index

535   candidates of pacu fillets.

536

References

Alasalvar, C., Taylor, K.D.A., Shahidi, F., 2005. Comparison of volatiles of cultured and wild sea bream (*Sparus aurata*) during storage in ice by dynamic headspace analysis/gas chromatography-mass spectrometry. J. Agric. Food Chem. 53, 2616–2622. https://doi.org/10.1021/jf0483826

Alves Filho, E.G., Silva, L.M.A., Teo, E.M., Larsen, F.H., Brito, E.S. De, 2017. Genotype evaluation of cowpea seeds (*Vigna unguiculata*) using ^{1}H qNMR combined with exploratory tools and solid-state NMR. Food Res. Int. 91, 140–147. https://doi.org/10.1016/j.foodres.2016.12.007

Araújo, A., Grassotti, T.T., Frazzon, A., 2021. Characterization of *Enterococcus* spp. isolated from a fish farming environment in southern Brazil. Brazil. J. Biol. 81, 954–961. https://doi.org/10.1590/1519-6984.232503

Austin, B., 2002. The bacterial microflora of fish. Sci. World J. 2, 558–572. https://doi.org/10.1100/tsw.2002.137

Austin, B., 2006. The bacterial microflora of fish, Revised. Sci. World J. 6, 931–945. https://doi.org/10.1100/tsw.2006.181

Baptista, R. C., Oliveira, R. B. A., Câmara, A. A., Lang, É., dos Santos, J. L. P., Pavani, M., Guerreiro, T. M., Catharino, R. R., Filho, E. G. A., Rodrigues, S., de Brito, E. S., Alvarenga, V. O., Bicca, G. B., & Sant'Ana, A. S., 2024. Chilled pacu (*Piaractus Mesopotamicus*) fillets: Modeling *Pseudomonas* spp. and psychrotrophic bacteria growth and monitoring spoilage indicators by 1H NMR and GC–MS during storage. Int. J. Food

564   Microbiol. 415, 110645. doi:10.1016/j.ijfoodmicro.2024.110645.

565   Ben-Gigireya, B., Sousa, J.M.V.B., Villa, T.G., Barros-Velazquez, J., Vieites, J.M., Sousa, B.

566   De, Villa, T.G., 2000. Characterization of biogenic amine-producing *Stenotrophomonas*

567   *maltophilia* strains isolated from white muscle of fresh and frozen albacore tuna. Int. J.

568   Food Microbiol. 57, 19–31. https://doi.org/10.1016/S0168-1605(00)00240-3

569   Ben Mhenni, N., Alberghini, G., Giaccone, V., Truant, A., Catellani, P. 2023. Prevalence and

570   antibiotic resistance phenotypes of *Pseudomonas* spp. in fresh fish fillets. Foods. 12,

571   950. https://doi.org/10.3390/foods12050950

572   Borges, A., Conte-Junior, C.A., Franco, R.M., Freitas, M.Q., 2013. Quality Index Method

573   (QIM) developed for pacu *Piaractus mesopotamicus* and determination of its shelf life.

574   Food Res. Int. 54, 311–317. https://doi.org/10.1016/j.foodres.2013.07.012

575   Borges, A., Conte-Junior, C.A., Franco, R.M., Mársico, E.T., Freitas, M.Q., 2014a. Quality

576   Index Method (QIM) for the hybrid tambacu (*Colossoma macropomum×Piaractus*

577   *mesopotamicus*) and the correlation among its quality parameters. LWT - Food Sci.

578   Technol. 56, 432–439. https://doi.org/10.1016/j.lwt.2013.12.008

579   Borges, A., Conte-Junior, C.A., Franco, R.M., Mársico, E.T., Freitas, M.Q., 2014b. Quality

580   parameters of pacu (*Piaractus mesopotamicus*) and tambaqui (*Colossoma macropomum*)

581   gutted and stored on ice for different periods. Int. Food Res. J. 21, 589–596.

582   Bottino, F.D.O., Rodrigues, B.L., de Nunes Ribeiro, J.D., Lázaro, C.A. de la T., Conte-Junior,

583   C.A., 2017. Influence of UV-C radiation on shelf life of vacuum package tambacu

584   (*Colossoma macropomum×Piaractus mesopotamicus*) fillets. J. Food Process. Preserv.

585   41, 1–11. https://doi.org/10.1111/jfpp.13003

586   Cann, D.C., 1974. Bacteriological aspects of tropical shrimp, in: Kreuzer, R. (Ed.), Fishety

587   Products. Fishing News Books, Surrey, pp. 338–344.

588   Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.,

589   Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights,

590   D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M.,

591   Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T.,

592   Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community

593   sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

594 Casaburi, A., Nasi, A., Ferrocino, I., Mauriello, G., Villani, F., Ercolini, D., 2011. Spoilage-

595   related activity of *Carnobacterium maltaromaticum* strains in air-stored and vacuum-

596   packed meat. Appl. Environ. Microbiol. 77, 7382–7393.

597   https://doi.org/10.1128/AEM.05304-11

598 Castro, F.A.F., Pinheiro Sant'Ana, H.M., Campos, F.M., Costa, N.M.B., Silva, M.T.C.,

599   Salaro, A.L., Franceschini, S. do C.C., 2007. Fatty acid composition of three freshwater

600   fishes under different storage and cooking processes. Food Chem. 103, 1080–1090.

601   https://doi.org/10.1016/j.foodchem.2006.10.002

602 Chong, C.Y., Bakar, F.A., Russly, A.R., Jamilah, B., Mahyudin, N.A., 2011. The effects of

603   food processing on biogenic amines formation. Int. Food Res. J. 18, 867–876.

604 Cocolin, L., Alessandria, V., Botta, C., Gorra, R., Filippis, F. De, Ercolini, D., Rantsiou, K.,

605   Agraria, D., Ii, F., 2013. NaOH-Debittering induces changes in bacterial ecology during

606   table olives fermentation. PLoS One 8, e69074.

607   https://doi.org/10.1371/journal.pone.0069074

608 Coelho, MEG, Pedrazzani, AS, Quintiliano, MH, Bolfe, F, and Molento, CFM. 2022, Fish

609   slaughter practices in Brazilian aquaculture and their consequences for animal welfare.

610   Anim Welf. 31:187–92. https://doi.org/10.7120/09627286.31.2.003

611 Cossa, M. de A. V.; Bilck, A. P.; Yamashita, F.; Mitterer-Daltoé, M. L., 2022 Biodegradable

612   packaging as a suitable protectant for the conservation of frozen pacu (*Piaractus*

613   *mesopotamicus*) for 360 days of storage at -18°C. J. Food Process. and Preser. 46, 1–11.

614   https://doi.org/10.1111/jfpp.16133

615 Culhane, AC, Thioulouse J, Perriere G, Higgins DG, 2005. MADE4: an R package for

616 multivariate analysis of gene expression data. Bioinformatics, 21, 2789-90.

617 doi:10.1093/bioinformatics/bti394.

618 Dabadé, D.S., den Besten, H.M.W., Azokpota, P., Nout, M.J.R., Hounhouigan, D.J.,

619 Zwietering, M.H., 2015. Spoilage evaluation, shelf-life prediction, and potential spoilage

620 organisms of tropical brackish water shrimp (*Penaeus notialis*) at different storage

621 temperatures. Food Microbiol. 48, 8–16. https://doi.org/10.1016/j.fm.2014.11.005

622 Dainty, R.H., Mackey, B.M., 1992. The relationship between the phenotypic properties of

623 bacteria from chilled-stored meat and spoilage processes. Soc Appl Bacteriol Symp Ser.

624 21, 103–114. https://doi.org/10.1111/j.1365-2672.1992.tb03630.x

625 Dairiki, J.K., Baldessin Junior, I., Pena, S. V., Cyrino, J.E.P., 2010. Manual técnico de

626 extensão Pacu e tambacu, (2nd ed). Piracicaba: ESALQ.

627 Dalgaard, P., Gram, L., Huss, H.H., 1993. Spoilage and shelf-life of cod fillets packed in

628 vacuum or modified atmospheres. Int. J. Food Microbiol. 19, 283–294.

629 https://doi.org/10.1016/0168-1605(93)90020-H

630 Dalgaard, P., Vancanneyt, M., Vilalta, N.E., Swings, J., Fruekilde, P., Leisner, J.J., 2003.,

631 Identification of lactic acid bacteria from spoilage associations of cooked and brined

632 shrimps stored under modified atmosphere between 0°C and 25°C. J. Appl. Microbiol.

633 94, 80–89. https://doi.org/10.1046/j.1365-2672.2003.01806.x

634 Dixon, P.V., 2003., VEGAN, a package of R functions for community ecology. J. Veg. Sci.

635 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

636 Doyle, M.P., Beuchat, L.R., Montville, T.J., Press, A., 2003. Food Microbiology : Frontiers,

637 in Trends in Food Science & Technology. pp. 390–391.

638 Duflos, G., Coin, V.M., Cornu, M., Antinelli, J.F., Malle, P., 2006., Determination of volatile

639 compounds to characterize fish spoilage using headspace/mass spectrometry and solid-

640 phase microextraction/gas chromatography/mass spectrometry. J. Sci. Food Agric. 86,

641 600–611. https://doi.org/10.1002/jsfa.2386

25

642   Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST.

643   Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

644   Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves

645   sensitivity and speed of chimera detection. Bioinform. 27, 2194–2200.

646   https://doi.org/10.1093/bioinformatics/btr381

647   Edwards, R.A., Dainty, R.H., Hibbard, C.M., 1987. Volatile compounds produced by meat

648   *Pseudomonads* and related reference strains during growth on beef stored in air at chill

649   temperatures. J. Appl. Bacteriol. 62, 403–412. https://doi.org/10.1111/j.1365-

650   2672.1987.tb02669.x

651   El-hanafy, A.E.A., Shawky, H.A., Ramadan, M.F., 2011. Preservation of *Oreochromis*

652   *niloticus* fish using frozen green tea extract: impact on biochemical, microbiological and

653   sensory characteristics. J. Food Process. Preserv. 35, 639–646.

654   https://doi.org/10.1111/j.1745-4549.2011.00513.x

655   Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Ferranti, P., Mauriello, G., Villani, F., 2010.

656   Different molecular types of *Pseudomonas fragi* have the same overall behaviour as

657   meat spoilers. Int. J. Food Microbiol. 142, 120–131.

658   https://doi.org/10.1016/j.ijfoodmicro.2010.06.012

659   Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., Storia, A. La, Laghi, L.,

660   Mauriello, G., Guerzoni, M.E., Villani, F., 2011. Monitoring of microbial metabolites

661   and bacterial diversity in beef stored under different packaging conditions. Appl Environ

662   Microbiol. 77, 7372–7381. https://doi.org/10.1128/AEM.05521-11

663   Ferrocino, I., La Storia, A., Torrieri, E., Musso, S.S., Mauriello, G., Villani, F., Ercolini, D.,

664   2013. Antimicrobial packaging to retard the growth of spoilage bacteria and reduce the

665   release of volatile metabolites in meat stored under a vacuum at 1°C. J. Food Prot. 76,

666   52–58. https://doi.org/10.4315/0362-028X.JFP-12-257

667   Friendly, M., 2002. Corrgrams: Exploratory displays for correlation matrices. The American

668   Statistician, 56, 316–324.

669   Ganeko, N., Shoda, M., Hirohara, I., Bhadra, A., Ishida, T., Matsuda, H., Takamura, H.,

670   Matoba, T., 2008. Analysis of volatile flavor compounds of sardine (*Sardinops*

671   *melanostica*) by solid phase microextraction. J. Food Sci. 73, 83–88.

672   https://doi.org/10.1111/j.1750-3841.2007.00608.x

673   Gillespie, N.C., 1981. A numerical taxonomic study of *Pseudomonas*-like bacteria isolated

674   from fish in southeastern Queensland and their association with spoilage. J. Appl.

675   Bacteriol. 50, 29–44. https://doi.org/10.1111/j.1365-2672.1981.tb00868.x

676   Girao, P. J. M., Oliveira Filho, P. R. C., Melo, M. P., Andrade, H. A., & Viegas, E. M. M.,

677   2021. Slaughtering procedures on the flesh quality of frozen stored pacu. Rev. Cienc.

678   Agron. 52, e20164845-8.

679   González, César, J., Santos, J.A., García-López, M.-L., González, N., Otero, A., 2001.

680   Mesophilic *Aeromonads* in wild and aquacultured freshwater fish. J. Food Prot. 64, 687–

681   691. https://doi.org/10.4315/0362-028X-64.5.687

682   Gram, L., Trolle, G., Huss, H.H., 1987. Detection of specific spoilage bacteria from fish

683   stored at low (0° C) and high (20° C) temperatures. Int. J. Food Microbiol. 4, 65–72.

684   https://doi.org/10.1016/0168-1605(87)90060-2

685   Gram, L., 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas*

686   strains isolated from spoiled and fresh fish. Appl Environ Microbiol. 59, 2197–2203.

687   https://doi.org/10.1128/aem.59.7.2197-2203.1993

688   Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. Int. J. Food

689   Microbiol. 33, 121–137. https://doi.org/10.1016/0168-1605(96)01134-8

690   Grayfer, L., Hodgkinson, J.W., Belosevic, M., 2014. Antimicrobial responses of teleost

691   phagocytes and innate immune evasion strategies of intracellular bacteria. Dev. Comp.

692   Immunol. 43, 223–242. https://doi.org/10.1016/j.dci.2013.08.003

693   Guth, H., 1997. Quantitation and sensory studies of character impact odorants of different

694   white wine varieties. J. Agric. Food Chem. 45, 3027-3032.

695   https://doi.org/10.1021/jf970280a

696 Hashimoto, D.T., Mendonça, F.F., Senhorini, J.A., de Oliveira, C., Foresti, F., Porto-Foresti,

697   F., 2011. Molecular diagnostic methods for identifying Serrasalmid fish (Pacu,

698   Pirapitinga, and Tambaqui) and their hybrids in the Brazilian aquaculture industry.

699   Aquaculture 321, 49–53. https://doi.org/10.1016/j.aquaculture.2011.08.018

700 Hatha, A., 2002. Transitory and resident microflora of the gastrointestinal tract of farm-raised

701   freshwater fishes in relation to habitat microflora. Asian J. Microbiol. Biotechnol.

702   Environ. 4, 277–282.

703

704 Huss, H.H., 1995. Quality and quality changes in fresh fish. FAO Fisheries Technical Paper,

705   No: 348. Rome.

706 ICMSF., 1986. Sampling plans for fish and shellfish. In: Microorganisms in Foods, In

707   International Union of Biological Societies (ed.), Sampling for microbiological analysis:

708   principles and specific applications. Blackwell Scientific Publications, Oxford, UK, pp.

709   181–196.

710 Iglesias, J., Medina, I., Bianchi, F., Careri, M., Mangia, A., Musci, M., 2009. Study of the

711   volatile compounds useful for the characterization of fresh and frozen-thawed cultured

712   gilthead sea bream fish by solid-phase microextraction gas chromatography-mass

713   spectrometry. Food Chem. 115, 1473–1478.

714   https://doi.org/10.1016/j.foodchem.2009.01.076

715 Jorgensen, L. V., Huse, H.H., Dalgaard, P., 2001. Significance of volatile compounds

716   produced by spoilage bacteria\rin vacuum-packed cold-smoked salmon (*Salmo salar*)

717   analyzed by\rgc-ms and multivariate regression. J. Agric. Food Chem. 49, 2376–2381.

718   https://doi.org/10.1021/jf0009908

719 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O.,

720   2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and

721   next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1.

722   https://doi.org/10.1093/nar/gks808

723 Kozińska, A., Paździor, E., Pękala, A., Niemczuk, W., 2014. *Acinetobacter johnsonii* and

724   *Acinetobacter lwoffii* - The emerging fish pathogens. Bull. Vet. Inst. Pulawy 58, 193–

725   199. https://doi.org/10.2478/bvip-2014-0029

726 Kumar, A., Pradhan, P.K., Das, P.C., Srivastava, S.M., Lal, K.K., Jena, J.K., 2018. Growth

727   performance and compatibility of pacu, *Piaractus brachypomus* with Indian major carps

728   in polyculture system. Aquaculture 490, 236–239.

729   https://doi.org/10.1016/j.aquaculture.2018.02.052

730 Kusuda, R., Salati, F., 1999. *Enterococcus seriolicida* and *Streptococcus iniae*, in: Woo,

731   P.T.K. (Ed.), Fish diseases and disorders. viral, bacterial and fungal infections. CAB

732   International, Wallingford, pp. 375–396.

733 Lampel, K.A., Sandlin, R.C., Formal, S., 1999. Shigella, in: Richard, K.R. (Ed.),

734   Encyclopedia of Food Microbiology. Introduction and Detection by Classical Cultural

735   Techniques. Elsevier, Oxford, pp. 2015–2020.

736 Langille, M.G.I., Zaneveld, J., Caporaso, J.G., Mcdonald, D., Knights, D., Reyes, J.A.,

737   Clemente, J.C., Burkepile, D.E., Thurber, R.L.V., Knight, R., Beiko, R.G., Huttenhower,

738   C., 2013. Predictive functional profiling of microbial communities using 16S rRNA

739   marker gene sequences. Nat. Biotechnol. 31, 814–821. https://doi.org/10.1038/nbt.2676

740 Liston, J., 1992. Bacterial spoilage of fish, in: Huss, H., Jacobsen, M., Liston, J. (Eds.),

741   Quality Assurance in the Fish Industry. Elsevier Science Publishers, Amsterdam, pp. 93–

742   105.

743 Liston, J., 1980. Microbiology in fishery science, in: Connell, J.J. (Ed.), Advances in Fishery

744   Science and Technology. Fishing News Books, Farnham, England, pp. 138–157.

745 Liu, Z., Wang, Z., Lv, X., Zhu, X., Chen, L., Ni, L., 2018. Comparison study of the volatile

746   profiles and microbial communities of Wuyi Qu and Gutian Qu, two major types of

747   traditional fermentation starters of Hong Qu glutinous rice wine. Food Microbiol. 69,

748   105–115. https://doi.org/10.1016/j.fm.2017.07.019

749 Lopez-Sabater, E.I., Rodriguez-Jerez, J.J., Roig-Sagues, A.X., Mora-Ventura, M.A.T., 1994.

750   Bacteriological quality of tuna fish (*Thunnus thynnus*) destined for canning: Effect of

751   tuna handling on presence of histidine decarboxylase bacteria and histamine level. J.

752   Food Prot. 57, 318–323. https://doi.org/10.4315/0362-028X-57.4.318

753 Luo, W., Friedman, M.S., Shedden, K., Hankenson, K.D., Woolf, P.J., 2009. GAGE:

754   generally applicable gene set enrichment for pathway analysis. BMC Bioinformatics 10,

755   1–17. https://doi.org/10.1186/1471-2105-10-161

756 Macé, S., Joffraud, J., Cardinal, M., Malcheva, M., Cornet, J., Lalanne, V., Chevalier, F.,

757   Sérot, T., Pilet, M., Dousset, X., 2013. Evaluation of the spoilage potential of bacteria

758   isolated from spoiled raw salmon (*Salmo salar*) fillets stored under modified atmosphere

759   packaging. Int. J. Food Microbiol. 160, 227–238.

760   https://doi.org/10.1016/j.ijfoodmicro.2012.10.013

761 Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve

762   genome assemblies Tanja Mago c. Bioinform. 27, 2957–2963.

763   https://doi.org/10.1093/bioinformatics/btr507

764 Marco, A., Navarro, J.L., Flores, M., 2007. Quantitation of selected odor-active constituents

765   in dry fermented sausages prepared with different curing salts. J. Agric. Food Chem. 55,

766   3058–3065. https://doi.org/10.1021/jf0631880

767 Michel, C., Pelletier, C., Boussaha, M., Douet, D.G., Lautraite, A., Tailliez, P., 2007.

768   Diversity of lactic acid bacteria associated with fish and the fish farm environment,

769   established by amplified rRNA gene restriction analysis. Appl. Environ. Microbiol. 73,

770   2947–2955. https://doi.org/10.1128/AEM.01852-06

771 Mikš-Krajnik, M., Yoon, Y.J., Ukuku, D.O., Yuk, H.G., 2016. Volatile chemical spoilage

772   indexes of raw Atlantic salmon (*Salmo salar*) stored under aerobic condition in relation

773   to microbiological and sensory shelf lives. Food Microbiol. 53, 182–191.

774   https://doi.org/10.1016/j.fm.2015.10.001

775 Nowak, A., Rygala, A., Oltuszak-Walczak, E., Walczak, P., 2012. The prevalence and some

776   metabolic traits of *Brochothrix thermosphacta* in meat and meat products packaged in

777   different ways. J. Sci. Food Agric. 92, 1304–1310. https://doi.org/10.1002/jsfa.4701

778 Nychas, G.E., Skandamis, P.N., Tassou, C.C., Konstantinos, K.P., 2008. Meat spoilage during

779   distribution. Meat Sci. 78, 77–89. https://doi.org/10.1016/j.meatsci.2007.06.020

780 Odeyemi, O.A., Burke, C.M., Bolch, C.J.S., Stanley, R., 2018. Evaluation of spoilage

781   potential and volatile metabolites production by *Shewanella baltica* isolated from

782   modified atmosphere packaged live mussels. Food Res. Int. 103, 415–425.

783   https://doi.org/10.1016/j.foodres.2017.10.068

784 Padda, R.S., Pandey, K.K., Kaul, S., Nair, V.D., Jain, R.K., Basu, S.K., Chakrabarti, T., 2001.

785   A novel gene encoding a 54 kDa polypeptide is essential for butane utilization by

786   *Pseudomonas* sp. IMT37. Microbiol. 147, 2479–2491.

787   https://doi.org/10.1099/00221287-147-9-2479

788 Parlapani, F.F., Mallouchos, A., Haroutounian, S.A., Boziaris, I.S., 2017. Volatile organic

789   compounds of microbial and non-microbial origin produced on model fish substrate un-

790   inoculated and inoculated with gilt-head sea bream spoilage bacteria. LWT - Food Sci.

791   Technol. 78, 54–62. https://doi.org/10.1016/j.lwt.2016.12.020

792 Parlapani, F.F., Mallouchos, A., Haroutounian, S.A., Boziaris, I.S., 2014. Microbiological

793   spoilage and investigation of volatile profile during storage of sea bream fillets under

794   various conditions. Int. J. Food Microbiol. 189, 153–163.

795   https://doi.org/10.1016/j.ijfoodmicro.2014.08.006

796 Peterson, R.J., Chang, S.S., 1982. Identification of volatile flavor compounds of fresh, frozen

797   beef stew and comparison of these with those of canned beef stew. J. Food Sci. 47,

798   1444–1448. https://doi.org/10.1111/j.1365-2621.1982.tb04957.x

799 Polo, D., Feal, X., Varela, M.F., Monteagudo, A., Romalde, J.L., 2014. Depuration kinetics of

800   murine norovirus in shellfish. Food Res. Int. 64, 182–187.

801   https://doi.org/10.1016/j.foodres.2014.06.027

802 Popović, N.T., Kazazić, S.P., Strunjak-Perović, I., Čož-Rakovac, R., 2017. Differentiation of

803   environmental aquatic bacterial isolates by MALDI-TOF MS. Environ. Res. 152, 7–16.

804   https://doi.org/10.1016/j.envres.2016.09.020

805 Radu, S., Ahmad, N., Ling, F.H., Reezal, A., 2003. Prevalence and resistance to antibiotics

806   for *Aeromonas* species from retail fish in Malaysia. Int. J. Food Microbiol. 81, 261–266.

807   https://doi.org/10.1016/S0168-1605(02)00228-3

808 Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V. V., 1998. Molecular and

809   biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 3, 597–635.

810   https://doi.org/10.1128/mmbr.62.3.597-635.1998

811 Rasheeda, M.K., Rangamaran, V.R., Srinivasan, S., Ramaiah, S.K., Gunasekaran, R., Jaypal,

812   S., Gopal, D., Ramalingam, K., 2017. Comparative profiling of microbial community of

813   three economically important fishes reared in sea cages under tropical offshore

814   environment. Mar. Genomics 34, 57–65. https://doi.org/10.1016/j.margen.2017.04.003

815 R Core Team (2023). _R: A Language and Environment for Statistical Computing. R

816   Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

817 Santos, V.B., Oliveira, M.W.M., Salomão, R.A.S., Santos, R. de S., Paula, T.G., Silva,

818   M.D.P., Mareco, E.A., 2017. Influence of temperature and exercise on growth

819   performance, muscle, and adipose tissue in pacus (*Piaractus mesopotamicus*). J. Therm.

820   Biol. 69, 221–227. https://doi.org/10.1016/j.jtherbio.2017.08.004

821 Savorani, F., Picone, G., Badiani, A., Fagioli, P., Capozzi, F., Engelsen, S.B., 2010.

822   Metabolic profiling and aquaculture differentiation of gilthead sea bream by ^{1}H NMR

823   metabonomics. Food Chem. 120, 907–914.

824   https://doi.org/10.1016/j.foodchem.2009.10.071

825   Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic

826   datasets. Bioinform. 27, 863–864. https://doi.org/10.1093/bioinformatics/btr026

827   Selli, S., Cayhan, G.G., 2009. Analysis of volatile compounds of wild gilthead sea bream

828   (*Sparus aurata*) by simultaneous distillation-extraction (SDE) and GC-MS. Microchem.

829   J. 93, 232–235. https://doi.org/10.1016/j.microc.2009.07.010

830   Shah, A.K.M.A., Ogasawara, M., Egi, M., Kurihara, H., Takahashi, K., 2010. Identification

831   and sensory evaluation of flavor enhancers in Japanese traditional dried herring (*Clupea*

832   *pallasii*) fillet. Food Chem. 122, 249–253.

833   https://doi.org/10.1016/j.foodchem.2010.02.072

834   Shimahara, K., Takiguchi, V., Kobayashi, T., Uda, K., Sannan, T., 1989. Screening of

835   *Mucoraceae* strains suitable for chitosan production, in: Skjak-Braek, G., Anthosen, T.,

836   Sandford, P. (Eds.), Chitin and Chitosan: Source, Chemistry, Biochemistry, Physical

837   Properties and Application. Elsevier Applied Science, London, London, pp. 171–177.

838   Shimamoto, G., Berk, R.S., 1979. Catabolism of taurine in *Pseudomonas aeruginosa*.

839   Biochim. Biophys. Acta 569, 287–292. https://doi.org/10.1016/0005-2744(79)90064-0

840   Shumilina, E., Slizyte, R., Mozuraityte, R., Dykyy, A., Stein, T.A., Dikiy, A., 2016. Quality

841   changes of salmon by-products during storage: assessment and quantification by NMR.

842   Food Chem. 211, 803–811. https://doi.org/10.1016/j.foodchem.2016.05.088

843   Silva, S. M., Ramirez, J. R. B., Silva, S. M., Dantas Filho, J. V., Marmentini, R. P., Schons, S.

844   V., & Cavali, J., 2022. Quality assessment of amazonian fish from fish farming stored on

845   ice. Acta Vet. Bras. 16, 134-140. https://doi.org/10.21708/avb.2022.16.2.10492.

846   Soncin, S., Chiesa, M.L., Panseri, S., Biondi, P., Cantoni, C., 2009. Determination of volatile

847   compounds of precooked prawn (*Penaeus vannamei*) and cultured gilthead sea bream

848   (*Sparus aurata*) stored in ice as possible spoilage markers using solid-phase

849   microextraction and gas chromatography/mass spectrometry. J. Sci. Food Agric. 89,

850   436–442. https://doi.org/10.1002/jsfa.3466

851 Tan, C., Huang, Y., Feng, J., Li, Z., Cai, S., 2018. Chemical freshness assessment of intact

852   fish via 2D 1 H J -resolved NMR spectroscopy combined with pattern recognition

853   methods. Sensors Actuators B. Chem. 255, 348–356.

854   https://doi.org/10.1016/j.snb.2017.08.060

855 Tanamati, A., Stevanato, F.B., Visentainer, J.E.L., Matsushita, M., de Souza, N.E.,

856   Visentainer, J.V., 2009. Fatty acid composition in wild and cultivated pacu and pintado

857   fish. Eur. J. Lipid Sci. Technol. 111, 183–187. https://doi.org/10.1002/ejlt.200800103

858 Taučer-Kapteijn, M., Medema, G., Hoogenboezem, W., 2013. Comparison between Rapid ID

859   32 Strep system, matrix-assisted laser desorption ionization-time of flight mass

860   spectrometry, and 16S rRNA gene sequence analysis for the species identification of

861   *Enterococcus* spp. isolated from water. Water Sci. Tech.-Water Supply 13, 1383–1389.

862   https://doi.org/10.2166/ws.2013.152

863 Tomé, Tomé, E., Gibbs, P.A., Teixeira, P.C., 2008. Growth control of *Listeria* innocua 2030c

864   on vacuum-packaged cold-smoked salmon by lactic acid bacteria. Int. J. Food Microbiol.

865   121, 285–294. https://doi.org/10.1016/j.ijfoodmicro.2007.11.015

866 Tuckey, N.P.L., Day, J.R., Miller, M.R., 2013. Determination of volatile compounds in New

867   Zealand Greenshell ™ mussels (*Perna canaliculus*) during chilled storage using solid

868   phase microextraction gas chromatography–mass spectrometry. Food Chem. 136, 218–

869   223. https://doi.org/10.1016/j.foodchem.2012.07.118

870 Vivekanandhan, G., Hatha, A.A.M., Lakshmanaperumalsamy, P., 2005. Prevalence of

871   *Aeromonas hydrophila* in fish and prawns from the seafood market of Coimbatore, South

872   India. Food Microbiol. 22, 133–137. https://doi.org/10.1016/j.fm.2004.01.015

873 Volkoff, H., Esatevan Sabioni, R., Coutinho, L.L., Cyrino, J.E.P., 2017. Appetite regulating

874   factors in pacu (*Piaractus mesopotamicus*): Tissue distribution and effects of food

875   quantity and quality on gene expression. Comp. Biochem. Physiol. -Part A Mol. Integr.

876   Physiol. 203, 241–254. https://doi.org/10.1016/j.cbpa.2016.09.022

877   Wagner, L., Trattner, S., Pickova, J., Gómez-requeni, P., Moazzami, A.A., 2014. H NMR-

878   based metabolomics studies on the effect of sesamin in Atlantic salmon (*Salmo salar*).

879   Food Chem. 147, 98–105. https://doi.org/10.1016/j.foodchem.2013.09.128

880   Wyss, M., Kaddurah-Daouk, R., 2000., Creatine and creatinine metabolism. Physiol. Rev. 80,

881   1107–1213. https://doi.org/10.1152/physrev.2000.80.3.1107

882   Xu, Y., Liu, Y., Zhang, C., Li, X., Yi, S., Li, J., 2015., Physicochemical responses and quality

883   changes of turbot (*Psetta maxima*) during refrigerated storage. Int. J. Food Prop. 19,

884   196–209. https://doi.org/10.1080/10942912.2015.1022260

885   Zheng, W. jiang, Hu, Y. hua, Sun, L., 2011., The two Dps of *Edwardsiella tarda* are involved

886   in resistance against oxidative stress and host infection. Fish Shellfish Immunol. 31,

887   985–992. https://doi.org/10.1016/j.fsi.2011.08.018

888   Ztaliou, I., Tsakalidou, E., Tzanetakis, N., Kalantzopoulos., G., 1996. *Lactobacillus*

889   *plantarum* strains isolated from traditional Greek cheese. Taxonomic characterization

890   and screening for enzyme activities. Le Lait. 76, 209-216. https://hal.science/hal-

891   00929481

892

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894   ***Figure captions***

895

896   **Figure 1:** Counts of different fish spoilage microbial groups for species of tambacu (a), pacu

897   (b), and patinga (c) in ice storage for up to 24 days.

898

899   **Figure 2:** Counts of different fish spoilage microbial groups for species of tambacu (a), pacu

900   (b), and patinga (c) in frozen storage for up to 12 months.

901

902   **Figure 3:** Principal Component Analysis (PCA) for OTUs of greater relative abundance for

903   frozen (red) and ice (blue) storages. The first component (horizontal) accounts for 21.09% of

904   the variance, and the second (vertical) accounts for 20.15%.

905

906   **Figure 4:** Incidence of the major taxonomic groups detected by sequencing. Only OTUs with

907   an incidence above 5% in at least two samples are shown. The abundance of OTUs in the two

908   biological replicates for each sampling time was averaged. Samples are labeled according to

909   time on ice [I] (0, 1, 3, 5, 7, 14, and 21 days) and frozen [F] (0,4, 8, and 12 months), batch (A

910   and B), and fish species (pacu [P], patinga [PC] and tambacu [T]).

911

912   **Figure 5:** Heat plot showing Spearman's correlations between OTUs occurring at 5% in at

913   least two samples and predicted metabolic pathways, filtered for KO gene sample presence 1

914   in at least five samples, related to amino acid (red squares), vitamin (green squares), lipid

915   (brown squares), and carbohydrate (blue squares) metabolism. Rows and columns are clustered

916   by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of

917   correlation between the OTUs and KO as measured by Spearman's correlations.

918

919   **Figure 6:** PC1 × PC2 scores coordinate system and respective loadings plotted in lines for (a)

920   pacu, (b) tambacu and (c) patinga fish fillets sampled during June (triangle) and February

921   (circle) before the storage (0 days – black color), after 12 days (red), and after 21 days (green)

922   in ice storage.

923

924

925   **Figure 7:** Correlation between microbiota and volatile or nonvolatile compounds. The color of

926   the scale bar denotes the nature of the correlation, with 1 indicating a positive correlation (blue)

927   and −1 indicating a negative correlation (red). Only significant correlations (FDR 0.05) are

928   shown.

929

930