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

### This is the author's manuscript

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/2031342> since 2024-11-20T15:41:37Z

*Published version:*

DOI:10.1016/j.fm.2024.104617

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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 *mesopotamicus*), patinga (female *Piaractus mesopotamicus* x male *Piaractus brachypomus*), and  
29 tambacu (female *Colossoma macropomum* x male *Piaractus mesopotamicus*) 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. <sup>1</sup>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.

44

45 **Keywords:** Microbial ecology; fish spoilage; 16S rRNA sequencing; volatile organic  
46 compounds (VOCs).

47

## 48 **1. Introduction**

49 Among the Brazilian native fish species, the “round” (due the rhomboidal body shape)  
50 fishes pacu (*Piaractus mesopotamicus*), patinga (female *Piaractus mesopotamicus* x male

51 *Piaractus brachypomus*), and tambacu (female *Colossoma macropomum* x male *Piaractus*  
52 *mesopotamicus*) 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 °C). *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

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

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

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

93

## 94 **2. Materials and methods**

### 95 **2.1. Fish sample preparation and storage conditions**

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

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  $1$  °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  $-18$  °C. Samples were  
111 stored at  $-18$  °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  $-18$  °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  $-18$  °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  $30$  °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, Kasvi, Roseto degli Abruzzi, Italy) supplemented with 0.5% NaCl  
130 (Dinâmica, São Paulo, Brazil) for Aerobic mesophilic, incubated at  $35 \pm 1^\circ\text{C}$  for  $48 \pm 2$  h; (iii)  
131 PCA with 0.5% NaCl for Psychrotrophic bacteria, incubated at  $7 \pm 1^\circ\text{C}$  for  $48 \pm 2$  h; (iv) Casein  
132 Agar for Proteolytic bacteria, incubated at  $30^\circ\text{C}$  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  $25 \pm 1^\circ\text{C}$  for  $48 \pm 2$  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  $25 \pm 1^\circ\text{C}$  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  $\mu\text{L}$  lysozyme (50 mg/mL) and incubated at  $37^\circ\text{C}$  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/ $\mu\text{L}$ .

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.

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

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

202

## 203 **2.4. Gas chromatography-mass spectrometry analysis - CG-MS**

### 204 2.4.1. VOCs determination by headspace HS-SPME/CG-MS

205 The Headspace Solid Phase Micro-extraction (HS-SPME) technique was performed to  
206 extract the volatile compounds by using a Divinylbenzene Carboxen/Polydimethylsiloxane  
207 (DVB/CAR/PDMS) 100  $\mu\text{m}$  SPME fiber and manual holder (Supelco, USA). After storage at  
208  $-80\text{ }^{\circ}\text{C}$ s, samples were kept at room temperature ( $\sim 22\text{ }^{\circ}\text{C}$ ) 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  $50\text{ }^{\circ}\text{C}$  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  $50\text{ }^{\circ}\text{C}$  and immediately desorbed  
213 in a gas chromatographer injector at  $250\text{ }^{\circ}\text{C}$ .

#### 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  $30\text{ m} \times 0.25\text{ mm ID} \times 1.0\text{ }\mu\text{m } d_f$   
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<sup>TM</sup> 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. <sup>1</sup>H NMR analysis

### 241 2.5.1. Molecular identification and monitoring of metabolites by <sup>1</sup>H NMR spectroscopy

242 Before the <sup>1</sup>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/<sup>15</sup>N-<sup>31</sup>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 <sup>1</sup>H NMR (totaling 24 <sup>1</sup>H NMR spectra) were converted to American Standard Code for  
255 Information Interchange (ASCII) format to matrix construction, which was exported for

256 chemometric analysis using The Unscrambler X™ program 10.4 (CAMO software,  
257 Woodbridge, NJ, USA). Singular Value Decomposition (SVD) algorithm was used to  
258 decompose the matrix (24 samples × 6,947 variables = 166,728 NMR data) in order to reduce  
259 data dimension and to observe trends of the samples variability with a confidence level of 95  
260 %.

### 261 **3. Results**

#### 262 **3.1. Microbiological analysis**

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

273

#### 274 **3.2. Microbiota diversity assessment by 16S rRNA amplicon target sequencing**

275 The bacterial sequencing accounted for 7.333.944 reads. After quality filtering,  
276 3.752.991 reads passed the filters, averaging 57.738 reads/sample and 460 bp of sequence  
277 length. The analyses were satisfactory for all samples, with an average recovery value of 86%.  
278 Adonis and analysis of similarity (ANOSIM) statistical tests showed no significant differences  
279 among fish species (patinga, pacu, and tambacu samples) and neither between the lots (February  
280 and June).

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

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

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

299           The comparison of the relative abundance of the main OTUs between ice and frozen  
300 samples suggested that *A. johnsonii*, *Acinetobacter*, *Enterococcus*, *Kocuria*, *L. plantarum*, and  
301 *P. fragi* were characteristic of frozen samples ( $FDR < 0.05$ ). On the other hand, only *Bacillus*  
302 were associated with ice samples ( $FDR < 0.05$ ). Regarding the inferred metagenome, the  
303 nearest sequenced taxon index (NSTI) score was  $0.048 \pm 0.011$ , indicating an accuracy of  
304 95.2%. The pathway enrichment analysis performed by GAGE showed an enrichment of the  
305 following inferred metabolism: methane (ko00680), propanoate (ko00640), butanoate  
306 (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  $\delta$  1.34 and quadruplet at  $\delta$  4.13), creatine (singlet at  $\delta$  3.05 and  $\delta$   
340 3.95), and taurine (doublet of doublets at  $\delta$  3.30 and  $\delta$  3.44). In the aromatic region ( $\delta$  6.5 –  
341 9.0), the main compounds detected were ATP/ADP (singlet at  $\delta$  8.57) and the decomposition  
342 product inosine (singlet at  $\delta$  8.48). Due to the complexity of the  $^1\text{H}$  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

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 *Flavobacteriaceae* 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 sulphhydryl 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.

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

#### 446 **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:  $0.14 \pm 0.29$  for octenol in tambacu stored in ice for 21 days (Table 2) and  $0.02 \pm 0.13$   
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-ethyl-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 9<sup>o</sup> to 21<sup>o</sup> 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 <sup>1</sup>H NMR), suggesting their potential as chemical spoilage index  
535 candidates of pacu fillets.

536

## 537 ***Acknowledgments***

538 This study was financially supported by *Coordenação de Pessoal de Nível Superior* (CAPES)  
539 (Finance code 001). The authors would like to thank the *Fundação de Amparo à Pesquisa do*  
540 *Estado de São Paulo* (FAPESP) (Grants #2014/25028-8, #2018/19311-0 #2019/16209-2,  
541 #2019/20287-9) and to and to *Conselho Nacional de Desenvolvimento Científico e Tecnológico*  
542 (CNPq, Grants #306644/2021-5, #305804/2017-0, #403865/2013-1, #234405/2014-7).

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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.

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