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

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

Rafaela C. Baptista[1,2], Ilario Ferrocino[2], Matheus Pavani[3], Tatiane M. Guerreiro[3], Antonio A. Câmara Jr[1], Émilie Lang[1], Juliana L. P. dos Santos[1], Rodrigo R. Catharino[3], Elenilson G. Alves Filho[4], Sueli Rodrigues[4], Edy S. de Brito[4], Magdevis Y. R. Caturla[1], Anderson S. Sant'Ana[1*], Luca Cocolin[2]

[1]Department of Food Science and Nutrition, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil.

[2]Department of Agricultural, Forest and Food Science, University of Turin, Grugliasco, Italy.

[3]Innovare Laboratory, Faculty of Pharmaceutical Science, University of Campinas, Campinas, SP, Brazil.

[4]Department of Food Technology, Federal University of Ceará, Fortaleza, CE, Brazil.

[5]EMBRAPA Tropical Agroindustry, Fortaleza, CE, Brazil.

[#]Corresponding author: Prof. A.S. Sant'Ana: and@unicamp.br Rua Monteiro Lobato, 80. Cidade Universitária Zeferino Vaz. CEP: 13083-862. Campinas, SP, Brazil.

Abstract

This study aimed to assess the bacterial microbiota involved in the spoilage of pacu (*Piaractus mesopotamics*), patinga (female *Piaractus mesopotamics* x male *Piaractus brachypomus*), and tambacu (female *Colossoma macropomum* x male *Piaractus mesopotamics*) during ice and frozen storage. Changes in microbiota during the storage of 22 samples of three fish species were studied through 16S rRNA amplicon-based sequencing and correlated with volatile organic compounds (VOCs) and metabolites assessed by nuclear magnetic resonance (NMR). Fish microbiota comprised mainly of *Pseudomonas sp., Brochothrix sp., Acinetobacter sp., Bacillus sp., Lactiplantibacillus sp., Kocuria sp.*, and *Enterococcus sp.* The relative abundance of *Kocuria*, *P. fragi*, *L. plantarum, Enterococcus*, and *Acinetobacter* was positively correlated with the metabolic pathways of ether lipid metabolism while *B. thermosphacta* and *P. fragi* were correlated with metabolic pathways involved in amino acid metabolism. *P. fragi* was the most prevalent spoilage bacteria in both storage conditions (ice and frozen), followed by *B. thermosphacta*. Moreover, the relative abundance of identified *Bacillus strains* in fish samples was positively correlated with the production of VOCs (1-hexanol, nonanal, octenol, and 2-ethyl-1-hexanol) associated with off-flavors. ^{1}H NMR analysis confirmed that amino acids, acetic acid, and ATP degradation products increase over (ice) storage, and therefore considered chemical spoilage index of fish fillets.

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

1. Introduction

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

Piaractus brachypomus), and tambacu (female *Colossoma macropomum* x male *Piaractus mesopotamics*) have been considered amongst the most expressive in terms of economic importance in Brazilian fish farming (Borges et al., 2013; Dairiki et al., 2010). These species present fast growth, easy adaptation to artificial feed, are well accepted by consumers, and are, therefore, of great interest to farmers (Borges et al., 2013).

However, to date, very few studies focused on fish quality for human consumption, and on the shelf life of pacu and tambacu (Borges et al., 2013, 2014a, 2014b; Bottino et al., 2017; Cossa et al., 2022; Silva et al., 2022). The majority of studies performed with Brazilian round fish aimed at optimizing feeding, treatment, and resistance to diseases, evaluating the factors that can affect the growth and genetic identification of the species' life (Coelho et al., 2022; Girao et al., 2021; Hashimoto et al., 2011; Kumar et al., 2018; Santos et al., 2017; Volkoff et al., 2017).

Fish is a highly perishable food; spoilage involves changes in physicochemical and microbiological parameters (El-Hanafi et al., 2011). Microbial activity is mainly involved in the fish loss of quality and associated with psychrotrophic bacteria which become dominant during fish chilled storage (0 to 7 ºC). *Pseudomonas, Shewanella, Photobacterium, Carnobacterium, Lactococcus*, and *Brochothrix* are commonly reported as Specific Spoilage Organisms (SSOs) in fish products (Dalgaard et al., 1993; Gram and Huss, 1996). They are known to produce typical off-odor and off-flavor metabolites associated with food deterioration, such as amines, sulfides, alcohols, aldehydes, ketones, and organic acids (Doyle et al., 2003; Gram et al., 1987; Gram and Huss, 1996).

Microbial spoilage is directly influenced by storage conditions, seafood species, and fish farming climatic conditions. Thus, knowing the composition of the initial fish microbiota can strongly contribute to mitigating the losses associated with the contamination of this food matrix. The characterization of SSOs is often performed via classical microbial plating 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).

The fresh fish fillets were stored in two conditions: ice and freezing. For the samples stored on ice, three fish fillets of the same species were aseptically cut into small pieces of about 23 g for a total of 70 g for each sample. Then, fish fillets were packed in PVC films and stored inside a hermetic cooler with ice. The ratio of fish fillets to ice was 1:1 (one layer of ice and one layer of fish samples). The hermetic cooler was placed inside a refrigerator and kept at 2 ± 1 ºC until analyses. Fish samples were kept on ice for 18 days and re-iced when necessary. The microbiological analyses were performed in duplicate at 0, 3, 6, 9, 12, 15, and 18 days of storage. For the samples stored in a freezer, 70 g of fillet were packed in PVC films and placed in absorbent trays. The trays were placed in cardboard boxes and stored at -18 ºC. Samples were stored at -18 ºC for one year, and the analyses were carried out every 15 days in duplicate. At each time interval planned for analyses, a portion of samples was separated in triplicate and stored at -18°C for the following analyses: 10 g for DNA, 30 g for NMR, and 10 g for CG analyses. These analyses were performed within 2 months.

The RMN and DNA samples were placed in Petri dishes and covered with PVC film, which had been previously drilled to allow air circulation. The samples were placed at -18 ºC overnight before transference to the freeze-dryer (Terroni, LS3000, São Paulo, Brazil) until complete dehydration.

2.2. Microbiological analysis

At every sampling point, 10 g of fresh sample were aseptically cut into small pieces and transferred to a stomacher bag containing 90 mL of sterile buffered peptone water (BPW) (Himedia, Mumbai, India) for obtaining a 1:10 ratio, which was homogenized in a Stomacher 400 circulator (Model BA7021, Seward, London, England) for 1 min. Serial ten-fold dilutions were prepared and 0.1 mL were spread in duplicate in the following culture plates: (i) Agar tributyrin for Lipolytic bacteria, incubated for 48 h at 30 °C, which consisted of 1.3% nutrient broth (Kasvi, Roseto degli Abruzzi, Italy), 1% tributyrin (Sigma-Aldrich, St Louis,

Missouri,USA), and 2% agar (Inlab, São Paulo, Brazil) (Ben-Gigireya et al., 2000); (ii) Plate Count Agar (PCA, Kasci, Roseto degli Abruzzi, Italy) supplemented with 0.5% NaCl (Dinâmica, São Paulo, Brazil) for Aerobic mesophilic, incubated at 35 ± 1°C for 48 ± 2 h; (iii) PCA with 0.5% NaCl for Psychrotrophic bacteria, incubated at 7 ± 1 °C for 48 ± 2 h; (iv) Casein Agar for Proteolytic bacteria, incubated at 30 °C for 48 h, prepared with 1.5 g of Beef extract (Acumedia, Neogen, Lansing, Michigan, US), 2.5 g of Bacto Tryptone (Kasvi, Roseto degli Abruzzi, Italy), 0.5 g of Glucose (Sigma Aldrich, Rio de Janeiro, Brazil), 8.5 g of Agar, and 12 g of skim milk powder (Nestlé Brazil LTDA, São Paulo, Brazil) (Ben-Gigireya et al., 2000); and, (v) *Pseudomonas* Agar Cetrimide Fucidina Cephaloridine (Oxoid LTD, Basingstone, Hampshire, England) for *Pseudomonas* spp., incubated at 25 ± 1 ºC for 48 ± 2 h. To confirm *Pseudomonas* spp., 5 colonies per plate were randomly chosen and purified in Nutrient Agar (Kasvi, Roseto degli Abruzzi, Italy), incubated at 25 ± 1 °C for 24 h. After incubation, pure colonies were selected for confirmation by the Oxidase test (Laborclin, Rio Grande do Sul, Brazil) and the Kligler Iron Agar test (Oxoid LTDA, Basingstoke, Hampshire, England). The results were expressed as $\log_{10}$ CFU/g.

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

2.3.1. DNA extraction from fish

Lyophilized fish samples (1 g) were homogenized with 5 mL of STE Buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA) for 1 min in a stomacher (LAB blender 400; PBI, Italy; stomacher bags, Sto-circul-bag; PBI, Italy). One milliliter of the supernatant was mixed with 50 µL lysozyme (50 mg/mL) and incubated at 37 ºC for 30 min. After incubation, DNA was extracted according to the MasterPure DNA purification kit (Epicentre, Madison, Wisconsin), following the manufacturing instructions. DNA concentration was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy) and standardized at five 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

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 ºC) 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

uncertainties were based on the standard deviation of the sampling and the analytical errors of the replicate of the chromatograms acquisition (Alves Filho et al., 2017).

2.4.3. Biomarkers determination

The investigation of product ions and all relevant mass transitions (Table 1) was done by acquiring a full scan before MRM analysis using the following conditions: 10 000 amu/s scan speed and 30 – 400 *m/z* mass range. The identified biomarkers were performed during the fish storage time for those samples stored on ice and frozen at three distinct points (initial, intermediate, and final). Biomarker semi-quantification was performed using respective chromatographic peak area counts.

2.5. ^{1}H NMR analysis

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

Before the ^{1}H NMR analysis, an adapted experimental method was applied to prepare the fish samples, according to Baptista et al. (2024).

The NMR experiments, the equipment employed (Agilent 600 MHz spectrometer equipped with a 5 mm (H-F/^{15}N-^{31}P) inverse detection One Probe™ with actively shielded Z-gradient), acquisition of spectra, processing of spectra, phase correction of the entire spectral range, and the identification of the constituents were detailed in a previous work (Baptista et al., 2024).

2.5.2. Chemometric analysis

Chemometric analysis investigated the variability of the composition of the fish samples stored on ice and frozen during two different seasons (Batches A – February and December and B – June). These analyses were performed as previously described (Baptista et al., 2024). In brief, The 1H NMR (totaling 24 1H NMR spectra) were converted to American Standard Code for 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).

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 ± 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

ice samples (data not shown). When plotting the correlation between OTUs and KO, it was observed that *Kocuria*, *P. fragi*, *L. plantarum*, *Enterococcus*, and *Acinetobacter* were positively correlated with the metabolic pathways (Figure 5) (FDR < 0.05) of ether lipid metabolism. *B. thermosphacta* and *Pseudomonas* sp. were related to inferred metabolic pathways involved in amino acid metabolism (arginine, alanine, and proline metabolism) (FDR < 0.05).

3.3. Volatile organic compounds (VOCs) analysis

In the present study, a screening was performed to determine the most frequent markers (VOCs) and those familiar to different fish species, making the comparison between the species and the storage time possible. The following VOCs identified exhibited an appropriate analytical signal present in our library: 1-hexanol, nonanal, octenol, and 2-ethyl-1-hexanol (Tables 2 and 3). Therefore, a semi-quantitative evaluation of these compounds was developed considering the integration value of the respective peaks area (no calibration curves using analytical standards).

Fluctuation in 1-hexanol concentration was observed on frozen and ice storage without observing a standard behavior. By evaluating the concentration of nonanal, an increase during the frozen and ice storage was observed, followed by the reduction in levels of the volatile in all species studied. However, this reduction was insignificant for tambacu samples stored on ice and freezing. Likewise, 2-ethyl-1-hexanol concentration also tended to increase, followed by ice and frozen storage reduction. The reduction was not significant for the pacu sample under freezing. Additionally, only increasing values of 2-ethyl-1-hexanol ($P < 0.05$) were observed for the pacu sample stored on ice.

The octenol showed the lowest concentrations compared with the other VOCs analyzed among the studied species and the storage forms. On ice storage, pacu samples showed a tendency to increase the octanol concentration, followed by a reduction in concentration levels ($P < 0.05$); 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

increased in fish fillets. Samples before storage presented higher amounts of creatine and phosphocreatine, taurine, and lactic acid based on PC1 and PC2 loadings. In patinga samples, negative loadings of PC1 presented decreased tendencies in amounts of creatine, phosphocreatine, and lactic acid, mainly for fish meats after storage in February (12 or 21 days), and an increase in the amount of acetic and fatty acids (Figure 6-c).

3.5. Correlation between microbiota and volatile and nonvolatile compounds

When plotting the Spearman correlation between microbiota and metabolites, it was possible to observe that phosphocreatine/creatine showed the highest number of positive correlations (FDR < 0.05) with the microbiota (Figure 7). Conversely, all the volatile metabolites were negatively correlated with the microbiota. Only *Bacillus* showed a positive correlation with the volatile metabolites. In addition, a positive correlation was observed between *P. fragi* and taurine, while *Flavobacteriacea* was correlated with the production of lactic acid (Figure 7).

4. Discussion

4.1. Microbiological and 16S rRNA analysis

The present study aimed to monitor the microbiological microbiota community of three native Brazilian fishes (pacu, tambacu, and patinga) during ice and frozen storage. Fish stored on ice had a shelf life of approximately 15 days, when microbial counts reached 7 logs CFU/g as established as a microbiological standard count in refrigerated fish (ICMSF, 1986).

Amplicon sequencing of the samples demonstrated the dominant OTUs, *P. fragi, B. thermosphacta, Acinetobacter, Acinetobacter johnsonii, Bacillus, Lactiplantibacillus plantarum, Kocuria*, and *Enterococcus.*

Pseudomonas spp. are commonly reported in the literature as a dominant microorganism in chilled fish under aerobic growth (Ben Mhenni et al., 2023; Mikš-Krajnik et

al., 2016; Parlapani et al., 2014). In this study, *P. fragi* was the most prevalent spoiler in all fish samples analyzed in ice and frozen storage. This species is commonly found in aquatic environments, in different climates, e.g., tropical and subtropical areas, and in different salinity of marine and freshwater (Gram, 1993; Liston, 1992). *P. fragi* is often associated as a spoilage microorganism in fish at refrigerated conditions, due to its proteolytic and lipolytic activity with considerable heterogeneity (Ercolini et al., 2010). Moreover *P. fragi* produces odors reminiscent of spoiling tropical fruit (a mix of ethyl esters of acetate, butyrate, and hexanoate) and sulphydryl compounds (Cann, 1974; Gillespie, 1981).

Several authors have positively correlated the presence of *P. fragi*, in meat samples, with the production of the VOCs studied in the present study: nonanal, 1-octen-3-ol, 2-ethyl-1-hexanol and 1-hexanol (Casaburi et al., 2011; Edwards et al., 1987; Ercolini et al., 2011; Ferrocino et al., 2013). These VOCs have been associated with both triglyceride and amino acid catabolism. However, in the present study, the activity of *Pseudomonas spp.* was only associated to the inferred metabolic pathway of amino acids, and no significant correlation was observed between *P. fragi* and VOCs. On the other hand, a clear correlation was observed between *P. fragi* and taurine. According to Shimamoto and Berk (1979), *Pseudomonas* spp. can use taurine as a source of carbon and nitrogen for energy metabolism. Several strains of *Pseudomonas* present the enzyme taurine pyruvate aminotransferase, capable of catabolizing taurine and pyruvate in forming sulfoacetaldehyde and alanine.

B. thermosphacta has been reported in fish stored in aerobic conditions (Mikš-Krajnik et al., 2016; Nowak et al., 2012), characterized by producing a mild and sour odor.

In this study, *B. thermosphacta* was positively correlated with metabolic pathways involved in amino acid metabolism (FDR < 0.05). Likewise, Dainty and Mackey (1992) showed that *B. thermosphacta* was responsible for degrading some amino acids in meat, such as valine, leucine, and isoleucine, resulting in the production of isobutyric, isovaleric, and 2-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 ± 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 ($P < 0.05$) 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

enzyme phosphocreatine kinase to reconstitute the ATP molecule from ADP, resulting in the formation of creatine. Therefore, the peaks observed in the present study are mainly due to the contribution of creatine due to the rapid degradation of phosphocreatine (Savorani et al., 2010; Wyss and Kaddurah-Daouk, 2000). In particular, creatine and phosphocreatine contribute to the thickness and mouthfulness (Shah et al., 2010).

Although an increase in the concentration of lactic acid is expected throughout storage due to the anaerobic catabolism of glucose, a reduction in the concentration of the compound was observed. Likewise, Shumilina et al. (2016) report a fluctuation in the lactic acid concentration in fish by-products (Backbones, Viscera, and heads), while Tan et al. (2018) observed a reduction in concentration in fish muscle. These findings may be an indicative of the microbiological activity. It is known that some microorganisms can consume or produce lactate.

The increase in the concentration of the amino acids valine, isoleucine, and leucine is consistent among several authors (Savorani et al., 2010; Tan et al., 2018). Amino acid formation results from the hydrolysis of the protein. At the same time, the proteolytic activity may be associated with endogenous enzymes (such as cathepsin and calpain) and bacterial proteinases, such as lactic acid bacteria, *Pseudomonas* spp., *Bacillus* spp., and *Aeromonas* spp. (Rao et al., 1998).

The accumulation of free fatty acids in the fish matrix also contributes to the loss of quality, resulting in a rancid flavor. The lipolytic action results in the cleavage of the triglycerides and is associated with the activity of endogenous enzymes or lipolytic bacteria (i.e., *P. fragi*, *L. plantarum*) (Huss, 1995).

Acetic acid has been reported as an indicator of quality, being absent or present in low quantity in fresh food, increasing proportionally the deterioration of the food quality (Mikš-Krajnik et al., 2016), in agreement with the observation in the present study. Acetic acid is 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

537 ***Acknowledgments***

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

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 *q*NMR 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., Máralém, 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., Máralém, 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

multivariate analysis of gene expression data. Bioinformatics, 21, 2789-90. doi:10.1093/bioinformatics/bti394.

Dabadé, D.S., den Besten, H.M.W., Azokpota, P., Nout, M.J.R., Hounhouigan, D.J., Zwietering, M.H., 2015. Spoilage evaluation, shelf-life prediction, and potential spoilage organisms of tropical brackish water shrimp (*Penaeus notialis*) at different storage temperatures. Food Microbiol. 48, 8–16. https://doi.org/10.1016/j.fm.2014.11.005

Dainty, R.H., Mackey, B.M., 1992. The relationship between the phenotypic properties of bacteria from chilled-stored meat and spoilage processes. Soc Appl Bacteriol Symp Ser. 21, 103–114. https://doi.org/10.1111/j.1365-2672.1992.tb03630.x

Dairiki, J.K., Baldessin Junior, I., Pena, S. V., Cyrino, J.E.P., 2010. Manual técnico de extensão Pacu e tambacu, (2nd ed). Piracicaba: ESALQ.

Dalgaard, P., Gram, L., Huss, H.H., 1993. Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres. Int. J. Food Microbiol. 19, 283–294. https://doi.org/10.1016/0168-1605(93)90020-H

Dalgaard, P., Vancanneyt, M., Vilalta, N.E., Swings, J., Fruekilde, P., Leisner, J.J., 2003., Identification of lactic acid bacteria from spoilage associations of cooked and brined shrimps stored under modified atmosphere between 0°C and 25°C. J. Appl. Microbiol. 94, 80–89. https://doi.org/10.1046/j.1365-2672.2003.01806.x

Dixon, P.V., 2003., VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Doyle, M.P., Beuchat, L.R., Montville, T.J., Press, A., 2003. Food Microbiology : Frontiers, in Trends in Food Science & Technology. pp. 390–391.

Duflos, G., Coin, V.M., Cornu, M., Antinelli, J.F., Malle, P., 2006., Determination of volatile compounds to characterize fish spoilage using headspace/mass spectrometry and solid-phase microextraction/gas chromatography/mass spectrometry. J. Sci. Food Agric. 86, 600–611. https://doi.org/10.1002/jsfa.2386

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.,

2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1. https://doi.org/10.1093/nar/gks808

Kozińska, A., Paździor, E., Pękala, A., Niemczuk, W., 2014. *Acinetobacter johnsonii* and *Acinetobacter lwoffii* - The emerging fish pathogens. Bull. Vet. Inst. Pulawy 58, 193–199. https://doi.org/10.2478/bvip-2014-0029

Kumar, A., Pradhan, P.K., Das, P.C., Srivastava, S.M., Lal, K.K., Jena, J.K., 2018. Growth performance and compatibility of pacu, *Piaractus brachypomus* with Indian major carps in polyculture system. Aquaculture 490, 236–239. https://doi.org/10.1016/j.aquaculture.2018.02.052

Kusuda, R., Salati, F., 1999. *Enterococcus seriolicida* and *Streptococcus iniae*, in: Woo, P.T.K. (Ed.), Fish diseases and disorders. viral, bacterial and fungal infections. CAB International, Wallingford, pp. 375–396.

Lampel, K.A., Sandlin, R.C., Formal, S., 1999. Shigella, in: Richard, K.R. (Ed.), Encyclopedia of Food Microbiology. Introduction and Detection by Classical Cultural Techniques. Elsevier, Oxford, pp. 2015–2020.

Langille, M.G.I., Zaneveld, J., Caporaso, J.G., Mcdonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821. https://doi.org/10.1038/nbt.2676

Liston, J., 1992. Bacterial spoilage of fish, in: Huss, H., Jacobsen, M., Liston, J. (Eds.), Quality Assurance in the Fish Industry. Elsevier Science Publishers, Amsterdam, pp. 93–105.

Liston, J., 1980. Microbiology in fishery science, in: Connell, J.J. (Ed.), Advances in Fishery Science and Technology. Fishing News Books, Farnham, England, pp. 138–157.

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,

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

Polo, D., Feal, X., Varela, M.F., Monteagudo, A., Romalde, J.L., 2014. Depuration kinetics of murine norovirus in shellfish. Food Res. Int. 64, 182–187. https://doi.org/10.1016/j.foodres.2014.06.027

Popović, N.T., Kazazić, S.P., Strunjak-Perović, I., Čož-Rakovac, R., 2017. Differentiation of environmental aquatic bacterial isolates by MALDI-TOF MS. Environ. Res. 152, 7–16. https://doi.org/10.1016/j.envres.2016.09.020

Radu, S., Ahmad, N., Ling, F.H., Reezal, A., 2003. Prevalence and resistance to antibiotics for *Aeromonas* species from retail fish in Malaysia. Int. J. Food Microbiol. 81, 261–266. https://doi.org/10.1016/S0168-1605(02)00228-3

Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V. V., 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 3, 597–635. https://doi.org/10.1128/mmbr.62.3.597-635.1998

Rasheeda, M.K., Rangamaran, V.R., Srinivasan, S., Ramaiah, S.K., Gunasekaran, R., Jaypal, S., Gopal, D., Ramalingam, K., 2017. Comparative profiling of microbial community of three economically important fishes reared in sea cages under tropical offshore environment. Mar. Genomics 34, 57–65. https://doi.org/10.1016/j.margen.2017.04.003

R Core Team (2023). _R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

Santos, V.B., Oliveira, M.W.M., Salomão, R.A.S., Santos, R. de S., Paula, T.G., Silva, M.D.P., Mareco, E.A., 2017. Influence of temperature and exercise on growth performance, muscle, and adipose tissue in pacus (*Piaractus mesopotamicus*). J. Therm. Biol. 69, 221–227. https://doi.org/10.1016/j.jtherbio.2017.08.004

Savorani, F., Picone, G., Badiani, A., Fagioli, P., Capozzi, F., Engelsen, S.B., 2010. Metabolic profiling and aquaculture differentiation of gilthead sea bream by ^{1}H NMR 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,

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

Tan, C., Huang, Y., Feng, J., Li, Z., Cai, S., 2018. Chemical freshness assessment of intact fish via 2D 1 H J -resolved NMR spectroscopy combined with pattern recognition methods. Sensors Actuators B. Chem. 255, 348–356. https://doi.org/10.1016/j.snb.2017.08.060

Tanamati, A., Stevanato, F.B., Visentainer, J.E.L., Matsushita, M., de Souza, N.E., Visentainer, J.V., 2009. Fatty acid composition in wild and cultivated pacu and pintado fish. Eur. J. Lipid Sci. Technol. 111, 183–187. https://doi.org/10.1002/ejlt.200800103

Taučer-Kapteijn, M., Medema, G., Hoogenboezem, W., 2013. Comparison between Rapid ID 32 Strep system, matrix-assisted laser desorption ionization-time of flight mass spectrometry, and 16S rRNA gene sequence analysis for the species identification of *Enterococcus* spp. isolated from water. Water Sci. Tech.-Water Supply 13, 1383–1389. https://doi.org/10.2166/ws.2013.152

Tomé, Tomé, E., Gibbs, P.A., Teixeira, P.C., 2008. Growth control of *Listeria* innocua 2030c on vacuum-packaged cold-smoked salmon by lactic acid bacteria. Int. J. Food Microbiol. 121, 285–294. https://doi.org/10.1016/j.ijfoodmicro.2007.11.015

Tuckey, N.P.L., Day, J.R., Miller, M.R., 2013. Determination of volatile compounds in New Zealand Greenshell ™ mussels (*Perna canaliculus*) during chilled storage using solid phase microextraction gas chromatography–mass spectrometry. Food Chem. 136, 218–223. https://doi.org/10.1016/j.foodchem.2012.07.118

Vivekanandhan, G., Hatha, A.A.M., Lakshmanaperumalsamy, P., 2005. Prevalence of *Aeromonas hydrophila* in fish and prawns from the seafood market of Coimbatore, South India. Food Microbiol. 22, 133–137. https://doi.org/10.1016/j.fm.2004.01.015

Volkoff, H., Esatevan Sabioni, R., Coutinho, L.L., Cyrino, J.E.P., 2017. Appetite regulating factors in pacu (*Piaractus mesopotamicus*): Tissue distribution and effects of food quantity and quality on gene expression. Comp. Biochem. Physiol. -Part A Mol. Integr.

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

Wagner, L., Trattner, S., Pickova, J., Gómez-requeni, P., Moazzami, A.A., 2014. H NMR-based metabolomics studies on the effect of sesamin in Atlantic salmon (*Salmo salar*). Food Chem. 147, 98–105. https://doi.org/10.1016/j.foodchem.2013.09.128

Wyss, M., Kaddurah-Daouk, R., 2000., Creatine and creatinine metabolism. Physiol. Rev. 80, 1107–1213. https://doi.org/10.1152/physrev.2000.80.3.1107

Xu, Y., Liu, Y., Zhang, C., Li, X., Yi, S., Li, J., 2015., Physicochemical responses and quality changes of turbot (*Psetta maxima*) during refrigerated storage. Int. J. Food Prop. 19, 196–209. https://doi.org/10.1080/10942912.2015.1022260

Zheng, W. jiang, Hu, Y. hua, Sun, L., 2011., The two Dps of *Edwardsiella tarda* are involved in resistance against oxidative stress and host infection. Fish Shellfish Immunol. 31, 985–992. https://doi.org/10.1016/j.fsi.2011.08.018

Ztaliou, I., Tsakalidou, E., Tzanetakis, N., Kalantzopoulos., G., 1996. *Lactobacillus plantarum* strains isolated from traditional Greek cheese. Taxonomic characterization and screening for enzyme activities. Le Lait. 76, 209-216. https://hal.science/hal-00929481

Figure captions

Figure 1: Counts of different fish spoilage microbial groups for species of tambacu (a), pacu (b), and patinga (c) in ice storage for up to 24 days.

Figure 2: Counts of different fish spoilage microbial groups for species of tambacu (a), pacu (b), and patinga (c) in frozen storage for up to 12 months.

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