Research article

Evaluating *Sardinella brasiliensis* quality indicators through the quantification of histamine and bacterial communities

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ABSTRACT

Primarily formed by the microbial decarboxylation of the amino acid histidine, histamine is the leading global cause of food poisoning from fish consumption worldwide. In the present work, the quality of 12 fresh and 12 frozen marketed sardines (*Sardinella brasiliensis*) were evaluated for histamine concentration using High-performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD), while the detection and quantification of histamine-producing bacteria were performed via quantitative Polymerase Chain Reaction (qPCR), and the microbiota composition of sardines was assessed through amplification of the *16S rRNA* gene using high-throughput sequencing (HTS). According to the results obtained by HPLC-DAD, histamine concentration ranged from 226.14 to 583.87 mg kg^{-1}. The histidine decarboxylase (*hdc*) genes from gram-negative bacteria (*Morganella morganii*, and *Enterobacter aerogenes*) were identified. The most abundant microorganisms present in fresh sardines belong to the genera *Macrococcus* spp., *Acinetobacter* spp., and *Pseudomonas* spp., while the genera *Phyllobacterium* spp., *Pseudomonas* spp., and *Acinetobacter* spp. were most abundant in frozen sardines.

1. Introduction

Foodborne diseases pose a serious threat to human health in both developed and developing countries. Among these diseases, histamine food poisoning is one of the most severe illnesses transmitted by fish (Yu et al., 2018). In 2017, 56 outbreaks generated by fish consumption were reported by European Union countries (Food Safety Authority et al., 2017), while 85 outbreaks and 250 illnesses were reported in the US due to the consumption of fish contaminated with histamine between 2009 and 2015 (CDC, 2016). Histamine poisoning is related to several fish species of the *Scombridae* family (tuna and mackerel), since these fish have high concentrations of the amino acid histidine. However, other fish families can also produce high levels of histamine including Scombresocidae, Engraulidae, Coryphaenidae, Pomatomidae, and Clupeidae (FAO/WHO, 2018). Belonging to the Clupeidae family, the sardines (*Sardinella brasiliensis*) inhabit the south-eastern coast of Brazil and are

targeted by the fishing industry due to their great economic value (Hariri et al., 2018).

Histamine is produced by the decarboxylation of the amino acid histidine via the exogenous histidine decarboxylase enzyme released by bacteria present in fish. These microorganisms can originate from surrounding water or represent endogenous fish microbiota (James et al., 2013). Bacteria of the Enterobacteriaceae family, such as *Morganella morganii* and *Enterobacter aerogenes*, are primarily responsible for the formation of histamine in fish. Other bacteria capable of producing histamine include *Photobacterium damselae*, *Klebsiella* spp., *Pseudomonas* spp., *Proteus* spp., *Vibrio* spp., *Bacillus* spp., *Staphylococcus* spp., *Citrobacter* spp. and *Clostridium* spp. (Arulkumar et al., 2019; Klanian et al., 2018).

A variety of molecular methods are available to identify and quantify microbiota from different biological sources. Traditionally, culture-based techniques were used to determine the composition of microbiota;

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however, only a small proportion of these microorganisms are cultivable. When associated with bioinformatics tools, partial amplification of the *16S rRNA* gene is proven to be a suitable marker gene for the quantification of microbiota at taxonomic and phylogenetic levels. Quantitative PCR (qPCR) is a fast and sensitive method used to identify and quantify specific microorganisms present in microbiota. A technology known as high-throughput sequencing (HTS) has been widely used to examine the complexity of the gut microbiota due to its speed, scale, and the need for the information it can provides, while qPCR has been successfully used to quantify specific bacterial groups and species. Moreover, high-performance liquid chromatography with diode-array detection (HPLC-DAD) showed accuracy to determine the histamine concentration in the samples.

Therefore, the identification and quantification of histamine-producing bacteria are fundamentally important for consumer food safety. Consequently, the objectives of the present study were: (i) to characterize the microbial communities present in fresh and frozen commercialized sardines, (ii) to quantify histamine-producing bacteria and, (iii) to quantify the histamine present in sardines.

2. Material and methods

2.1. Sample collection

In this study, 24 whole sardine samples (*Sardinella brasiliensis*) were purchased at the Central Public Market of Porto Alegre, Rio Grande do Sul (Brazil) from February to April 2018. Twelve of them were collected fresh (with viscera and gills - S1 to S12) and others 12 sardines were acquired frozen at -18 °C (with viscera and gills - S13 to S24). Although they were purchased at the same establishment, fresh sardines come from the state of Santa Catarina and frozen sardines from the state of Rio de Janeiro. The fish were identified and transported in isothermal boxes with reusable ice packs. The fish were weighed, gutted and parts of the muscle was aseptically removed, (1:10 w/v), mixed in sterile distilled water (90 mL) and, homogenized for pH determination (Q400AS bench pH meter, Quimis, Brazil).

2.2. Histamine determination by HPLC-DAD method

2.2.1. Preparations of solutions

The stock solution of the Histamine dihydrochloride standard (≥99 %, HPLC, Sigma Aldrich) was dissolved in 0.1 mol l^{-1} Hydrochloric acid at 1000 mg mL^{-1} and stored at -20 °C. Work solutions were prepared in different concentrations by diluting the stock solution in 0.1 mol l^{-1} HCl (Gouveia, 2009). The 10 mg mL^{-1} solution of dansyl chloride (≥99 %, HPLC, Sigma Aldrich) was prepared by dissolving 100 mg in 10 mL acetone (≥99 %, HPLC, JT Baker) and stored under refrigeration at 4 °C (Shukla et al., 2010).

2.2.2. Extraction, derivatization, HPLC-DAD separation and histamine quantification

Histamine extraction and derivatization were according to the previously described method (Hu et al., 2012). Histamine identification was performed using HPLC Shimadzu (Kyoto, Japan) equipped with two pumps (LC-20A), a degasser (DGU-20A), an automatic injector (SIL20AHT), a column oven (CTO-20A) and a DAD detector (SPD-M20A). The C18 chromatography column (Merck, Germany) was used for separation. The flow rate was 1.0 mL min^{-1} at 30 °C. The mobile phase consisted of ultrapure water (MilliQ System, Millipore) (solvent A) and acetonitrile (solvent B), and injection volume was of 20 μL. The linear gradient ranged from 60 % to 76 % of solvent B within 6 min and within 1 min reached 100 % of B sustained for 2 min. In the next minute, it returned to 60 % of B and was maintained for 3 min, totaling 13 min. The spectra were obtained from 200 and 800 nm and the chromatograms set at 254 nm. Histamine quantification in sardine muscle was performed using the standard histamine curve at the following concentrations 1.0,

2.0, 3.0, 5.0, 7.0 and 9.0 mg L^{-1} of the histamine solution. To verify the performance of the method, the parameters, linearity, detection limit and limit of quantification were evaluated according to (EMEA, 2006).

2.3. Determination and quantification of histamine producing bacteria by qPCR

Bacterial reference strains, DNA extraction of sardine muscle, and *hdc* gene quantification by qPCR followed methodology described in (de Lira et al., 2019). The oligonucleotides used for the qPCR assay in this study targeted the gram-negative bacteria *hdc* gene (*hdc* GN) was described by (Bjornsdottir-Butler et al., 2011). The *hdc* MM (MM; *M. morganii*) and *hdc* EA (EA; *E. aerugiosa*) oligonucleotides were designed in (de Lira et al., 2019).

2.4. PCR amplification of 16S rRNA gene and HTS

In order to characterize the bacterial composition of the samples, five samples of fresh sardines (S1, S2, S4, S7 and S12) and five samples of frozen sardines (S13, S14, S17, S18 and S19) were selected. The V4 hypervariable regions of the *16S rRNA* gene was amplified using primers 515F and 806R (Caporaso et al., 2012). The PCR reaction were carried out in a total volume of 25 containing 12.50 ng of the extracted DNA, 1x PCR buffer, 1.5 mmol MgCl$_2$, 0.2 μmol of each oligonucleotide, 10 mmol dNTP, 2U Taq Platinum™ DNA Polymerase (Invitrogen™, USA) and 1x reaction buffer. Amplification was performed in a BioRad MyCycler thermocycler (BioRad, USA) following program: 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 72 °C for 1.0 min and a final cycle for 72 °C for 5.0 min. The products were purified using Agencount AMPure XP beads (Beckman Counter life Sciences, Indianapolis, IN, USA) following the manufacturer's instructions. Library constructions was performed according to Illumina protocols. The sequencing was conducted on a MiSeq ™ system (Illumina, USA) with a MiSeq™ v2, 500 cycle sequencing reagent kit (Illumina, USA).

2.5. Data analysis

2.5.1. Statistical analysis of histamine, pH and qPCR

For the statistical analysis of the investigated variables in sardine samples (histamine quantification, pH value and *hdc* gene quantification) was used the Wilcoxon non-parametric hypothesis test. The variables were described through the median and interquartile ranges. The test also was used to evaluate the differences between the conservation status (frozen and fresh) of sardines. The significance level considered was 5 % (p value < 0.05). Analyzes were performed using R software version 3.4.2.

2.5.2. Processing of 16S rRNA sequencing analyses

Bioinformatics analysis of *16S rRNA* amplicons were performed using QIIME 2 2019.4 (Bolyen et al., 2019). Raw sequence data were quality filtered and denoised, dereplicated and chimera filtered using the q2-dada2 plugin with DADA2 pipeline (Callahan et al., 2016). A total 1, 000.000 reads were used for training the DADA2 error model of each sequencing run. The 5′ end 10 nucleotide bases were trimmed from forward and reverse read sequences due to low quality. Reads with a number of expected errors higher than 2 were discarded. Read length filtering was applied and the reads were trimmed at the first instance of a quality score less than or equal to 11. The resulting reads with nucleotide overlap between the forward and reverse reads below 20 and shorter than 240 bp length were discarded. Chimera removal was performed using the consensus method.

The amplicon sequence variants (ASVs) obtained by DADA2 pipeline were merged into a single feature table using the q2-feature-table plugin. The ASV's were aligned with MAFFT (via q2-alignment) (Katoh, 2002) and used to construct a phylogeny with fasttree2 (Price et al., 2010). Taxonomy was assigned to ASV's (Bokulich et al., 2018) classify-sklearn

naïve Bayes taxonomy classifier. The classifier was trained using extracted Greengenes 13_8 reference sequences with 99% similarity truncated at 250 bp length from *16S rRNA* variable region 4 (V4). The resulting feature table, rooted tree from reconstructed phylogeny, and taxonomy classification were imported from QIIME2 to R v3.6.1 environment for further data analysis using Microbiome v1.6.0 and Phyloseq v1.28.0 R packages (McMurdie and Holmes, 2013). Sequencing data were deposited in the Sequence Read Archive of the National Center of Biotechnology Information (NCBI, USA), access number PRJNA558404.

For Taxonomic analysis, feature table was transformed to compositional data for taxa bar plot composition visualization of the 10 most abundant genera using plot composition function from Microbiome R package. A heatmap was plotted using features transformed to $\log_{10}$ frequency by plot heatmap function from Phyloseq R package (McMurdie and Holmes, 2013).

For Community Analysis, Alpha-diversity metrics (Shannon, Simpson Chao1), beta diversity metrics Weighted UniFrac, unweighted UniFrac (Lozupone et al., 2007), Jaccard distance, and Bray-Curtis dissimilarity, were estimated using Microbiome and Phyloseq packages in R. MDS ordination was applied to beta diversity chosen metrics for Principle Coordinate Analysis (PCoA) using plot ordination function from Phyloseq. Alpha diversity significance was estimated with a pairwise comparison using a non-parametric test Wilcoxon (Kotz and Johnson, 1998), using function from Microbiome R package. Beta diversity significance were estimated with a permutational multivariate analysis of variance using distance matrices obtained by MDS ordination previously described with Permutational Multivariate Analysis of variance test (PERMANOVA), Adonis function of Vegan R package. Compositional biplot PCoA were estimated using Aitchison distance with DEICODE QIIME2 plug in (Sparse et al., 2019).

In relation to differential abundance analysis, feature table was filtered to remove singletons using the q2-feature-table plugin. The ASV's, that was observed less than two samples and less than 10 abundance frequency, were removed from the feature table. The resulting filtered features were grouped at family level. Differential abundance analysis was performed with ANCOM, with mean difference as fold difference in feature abundances across groups and centered log-ratio (clr) as transform-function for volcano plot. ANCOM is calculated pairwise log ratios between all features and performing a significance test to determine if there is a significant difference in feature ratios with respect to the variable of interest. "W" is the W-statistic, or number of features that a single feature is tested to be significantly different against (Mandal et al., 2015). R script for alpha diversity, beta diversity and taxonomic analysis were added as Supplementary material.

3. Results

3.1. Identification of histamine by HPLC-DAD

The linearity of the standard curve was determined by injecting six concentrations of the standard histamine solution (1.0–9.0 mg L^{-1}) into HPLC-DAD. The analytical curve was linear (retention time 1.30 min), with correlation coefficients of 0.9954. The limit of detection was 0.77 mg kg^{-1} and the limit of quantification was 2.0 mg kg^{-1}. This result indicated that the analytical method of HPLC-DAD with dansyl chloride

derivatization was accurate for the identification of histamine in sardine samples (Supplementary Figure S1).

3.2. Histamine levels and pH in sardine samples

Table 1 presents the mean fish weight, pH value, and histamine concentration for fresh and frozen sardines, respectively. The weight of whole sardines ranged from 65.45 to 176.46 g. Fresh sardines had a significantly (p < 0.001) higher weight. The same trend was observed with the pH value of fish (p < 0.001), which ranged from 6.22 to 7.18 among samples. Fish pH was higher in fresh sardine samples (approaching pH 7.0, which can be explained by the freshness of these fish). All evaluated fresh and frozen sardine samples were contaminated with histamine. A significant difference (p < 0.05) was observed between histamine concentration in frozen and fresh fish samples, with higher histamine concentration levels being observed in frozen sardines (342.74–583.87 mg kg^{-1}) than fresh sardines (226.14–579.83 mg kg^{-1}).

3.3. Quantification of the hdc gene by qPCR

A linear correlation was observed between the Ct values of the *hdc* genes. Notably, the curves showed a correlation coefficient (R^2) greater than 0.99 and an efficiency ~100% (Table 2).

The primers *hdc* MM and *hdc* EA were shown to be specific to the identification of *M. morganii* and *E. aerogenes*. A significant difference (p = 0.001) was obtained between *hdc* GN and *hdc* EA in the frozen samples. However, no significant (p = 0.947) difference was observed between frozen and fresh samples for the Ct value of the *hdc* MM gene. Significant differences were observed in the number of copies for *hdc* GN (p = 0.001), *hdc* MM (p = 0.048) and *hdc* EA (p = 0.049) in the frozen samples. Based on the qPCR results, all 24 samples were positive for the *hdc* gene, which produces histamine in sardines (Table 3).

3.4. Microbiota analysis of fresh and frozen sardine samples

3.4.1. Alpha and beta diversity

After pre-processing the sequences using the DADA2 pipeline, a total of 691738 high-quality sequences were analyzed. These sequences were assigned to 307 amplicon sequence variants (ASVs) based on 99 % similarity using the Greengenes database. Alpha diversity indices were used to compare and characterize microbial diversity in each sample (Figure 1). No significant differences were observed among the Simpson (p = 0.31), Chao1 (p = 0.69), and Shannon diversity (p = 0.84) indices. According to a non-parametric Wilcoxon statistical test, the Shannon diversity index exhibited no difference in species richness between fresh and frozen sardine samples; however, the diversity present in frozen samples was more homogeneous. A greater equability in the distribution of species abundance among frozen samples was observed in the Simpson index values, which showed larger variability in microbiota when fish was stored fresh. A violin plot was created based on the Simpson and Chao1 diversity index data for frozen sardine samples. The shape of this plot presents a distribution (extremely thin at each end and broad in the middle) indicating that the microbiota is highly concentrated around the median.

Beta diversity analysis was performed using the Bray-Curtis similarity index, Jaccard index, weighted-UniFrac and unweighted-UniFrac metrics

Table 1. Median, interquartile interval of weight, pH and histamine of sardines by type of preservation.

Parameters	Number of sardines (n = 24)*	Frozen sardines (n = 12)*	Fresh sardines (n = 12)*	p value**
Weight (g)	133 [79.9; 161]	79.2 [73.6; 91.3]	161 [148; 172]	<0.001
Muscle pH	6.69 [6.38; 6.79]	6.38 [6.34; 6.42]	6.79 [6.74; 6.85]	<0.001
Histamine (mg kg^{-1})	413 [353; 485]	443 [392; 497]	371 [338; 413]	0.033

**p value <0.05.
* n = number of samples, results expressed in median [1^a Quartile e 3^a Quartile].

Table 2. Efficacy and reliability of the qPCR assay for the different oligonucleotides of the *hdc* gene.

Primer**	Bacteria	Linear range (copies number)	Slop (-3,58/-3,10)*	R² (>0.99)*	Efficiency (90/110 %)*	Standard curve*
hdc GN	*M. morganii*	10^1–10^6	-3.08	0.990	100.6 %	Ct = -3.08x + 44.22
hdc MM	*M. morganii*	10^1–10^6	-3.23	0.998	103.8 %	Ct = -3.23x + 35.33
hdc EA	*E. aerogenes*	10^1–10^6	-3.30	0.990	100.8 %	Ct = -3.30x + 37.63

** hdc = histidine decarboxylase gene, GN = Gram-negative bacteria, MM = *M. morganii*, EA = *E. aerogenes*.
* Reference value: Life technologies (2016), R² = linear regression coefficient, Ct = cycle threshold.

Table 3. Median, Interquartile interval of Ct corresponding to the standard curve derived from the qPCR test and the number of copies of the *hdc* genes.

Parameters*	Number of sardine (n = 24)*	Frozen sardine (n = 12)*	Fresh sardine (n = 12)*	p value**	Sardine evaluated
Ct value (*hdc* GN)	29.8 [25.5; 32.2]	32.2 [31.7; 32.4]	25.3 [24.7; 27.0]	0.001	24
Ct value (*hdc* MM)	17.4 [15.0; 19.1]	17.1 [15.6; 18.7]	17.7 [14.7; 19.2]	0.947	22
Ct value (*hdc* EA)	15.9 [15.3; 19.2]	19.5 [17.2; 22.1]	15.3 [15.1; 15.6]	0.001	23
hdc GN ($\log_{10} g^{-1}$)	8.33 [7.51; 9.09]	7.82 [7.35; 8.16]	9.09 [8.81; 9.70]	0.001	24
hdc MM ($\log_{10} g^{-1}$)	7.18 [6.73; 8.28]	7.84 [7.40; 8.34]	6.80 [6.41; 7.29]	0.048	22
hdc EA ($\log_{10} g^{-1}$)	7.79 [7.17; 8.32]	6.94 [6.38; 7.83]	7.98 [7.65; 8.38]	0.049	23

**p value <0.05.
* Ct = cycle threshold, n = number of samples, results expressed in median [1ª Quartile e 3ª Quartile], $\log_{10}$ number of *hdc* gene copies in 1.0 g of muscle.
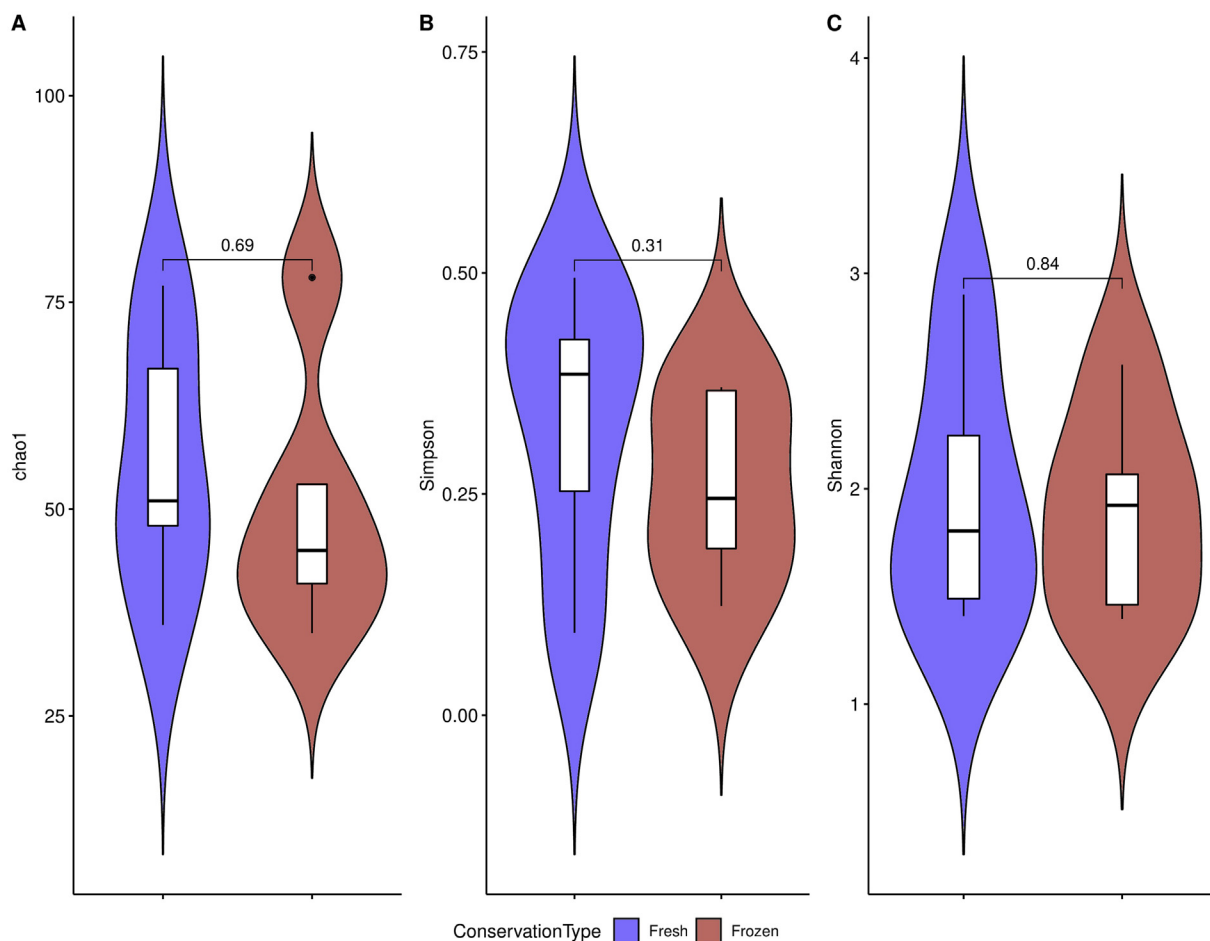

Figure 1. Alpha diversity measurements of microbial communities in the fresh and frozen sardine samples. Represented by (A) Simpson, (B) Chao1 and (C) Shannon diversity indexes. Boxes span the first to third quartiles, while horizontal lines inside the boxes represent median values. Whiskers extending vertically from the boxes indicate variability outside the upper and lower quartiles, while the single circles indicate outliers. No significant differences were observed among the three groups (p > 0.05) according to the non-parametric Wilcoxon statistical test.
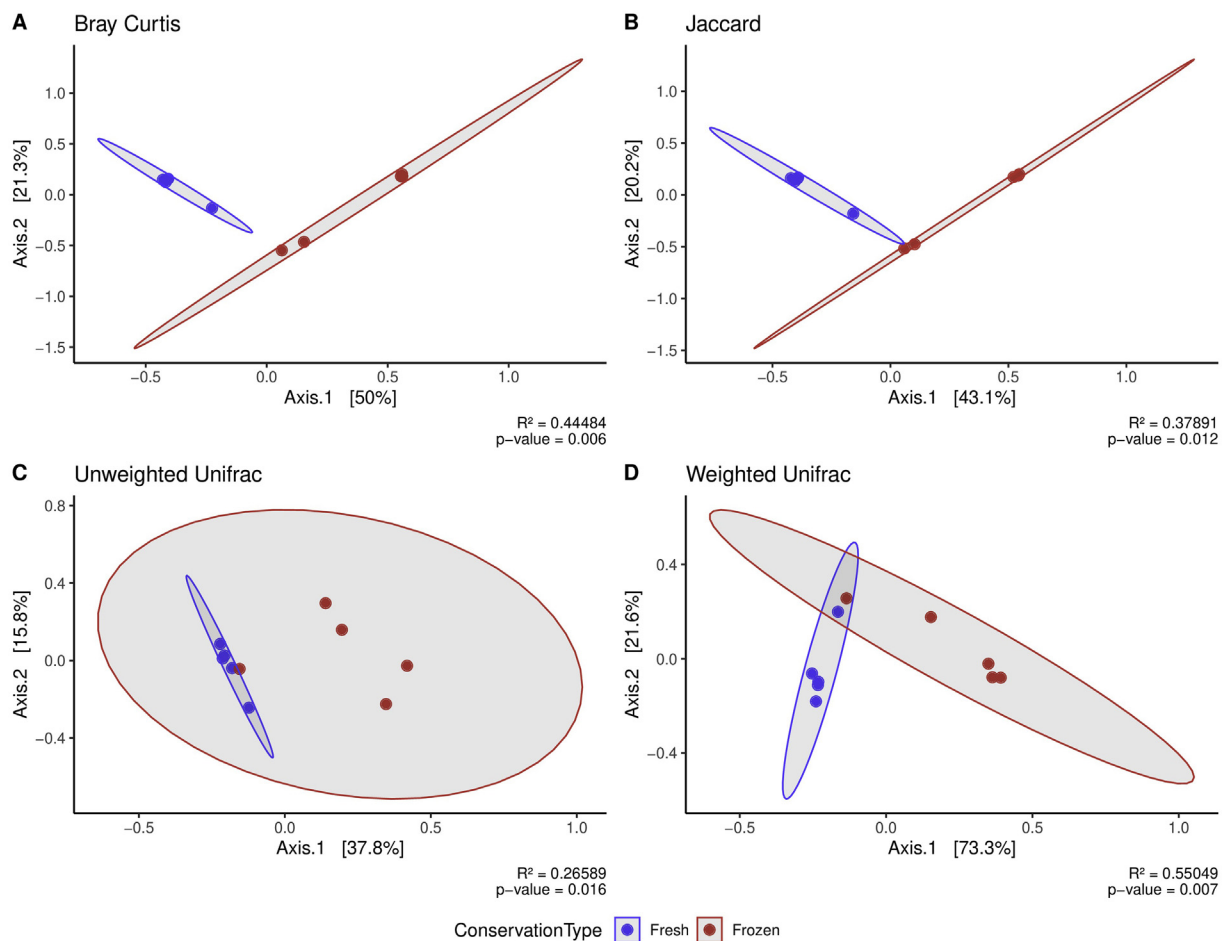
Figure 2. Principal coordinate analysis (PCoA) of bacterial communities based on (A) the Bray Curtis dissimilarity index with (B) Jaccard index, (C) weighted-UniFrac, and (D) unweighted-UniFrac regarding conservation type. Colors: blue represents frozen sardines (S1, S2 S4, S7, and S12) and red represents fresh sardines (S13, S14, S17, S18, and S19).

to determine the distance between samples based on microbiota profiles using principal coordinates analysis (PCoA) for data visualization (Figure 2). Significant differences were observed among the Bray-Curtis similarity index (p = 0.009, R^2 = 0.44484), Jaccard index (p = 0.01, R^2 = 0.37891), weighted-UniFrac (p = 0.009, R^2 = 0.55049), and unweighted-UniFrac (p = 0.023, R^2 = 0.26589) based on the non-parametric permutational multivariate statistical variance test (PERMA-NOVA, Adonis, statistical test with 999 permutations). The results of beta diversity analysis indicated that microbiome composition between the frozen and fresh sardine groups was distinct.

To compare the similarities and differences between fresh and frozen sardine samples, a hierarchical cluster analysis based on distance was also performed (Figure 3). A difference was observed between fresh and frozen sardine microbiota after excluding samples of fresh (S7) and frozen (S18) sardines that had a greater distance from their groups but were similar to each other.

3.4.2. Microbial community

The relative abundance of species in fresh and frozen sardine samples at the phylum level is presented in Figure 4. These results indicate differentiation between the phylum-level microbiota according to sample preservation type. Proteobacteria was the predominant phylum in frozen sardines and represented at least 94.84 % of the bacterial diversity. In fresh sardines, the dominant phylum was Firmicutes (65.86 %), followed by Proteobacteria (33.90 %).

The family relative abundance of the main phyla among fish is presented in Figure 5. In the fresh sardine samples, the predominant families were Staphylococcaceae (50.32 %), Moraxellaceae (17.41 %), Pseudomonadaceae (7.02 %), Enterococcaceae (7.44 %), and Aeromonadaceae (5.87 %), while the predominant families in frozen sardines were Phyllobacteriaceae (47.82 %), Pseudomonadaceae (18.94 %), Moraxellaceae (17.70 %), and Enterobacteriaceae (7.18 %).

A heatmap was constructed to characterize the dynamics of bacterial community patterns (Figure 6). The hierarchical grouping of evaluated samples based on the relative abundance of 20 families showed that the conservation type (i.e. fresh or frozen) influenced the taxonomic groups at the family level. The Sphingomonadaceae family was present in frozen samples (S13, S14, S17 and S19) and a single sample of fresh sardines (S12). Notably, Acetobacteraceae and Lactobacillaceae were found only in frozen samples (S13, S14, S17, and S19), while Enterobacteriaceae and Micrococcaceae were observed in all samples except for one fresh (S4) and one frozen sardine samples (S19), respectively. Notably, Pseudomonadaceae, Staphylococcaceae, Flavobacteriaceae, Listeriaceae, and Moraxellaceae were present in all fresh and frozen sardines.

An analysis of microbiome composition (ANCOM) was used to evaluate differences in the average rate of microbial abundance between fresh and frozen sardines (Figure 7). A significant difference was observed in the logarithmic abundance of the 12 families detected between groups, specifically in the families Lactobacillaceae (w = 6), Sphingomonadaceae (w = 5), Enterobacteriaceae (w = 5), Acetobacteraceae (w = 5), and Comamonadaceae (w = 4) in frozen sardines samples

Cluster Dendrogram

Weighted_unifrac
hclust (*, "average")

Figure 3. Hierarchical cluster dendrogram of bacterial communities of fresh or frozen sardines based on unweighted-UniFrac distance. Samples: frozen sardines (S13, S14, S17, S18, and S19) and fresh sardines (S1, S2, S4, S7, and S12).
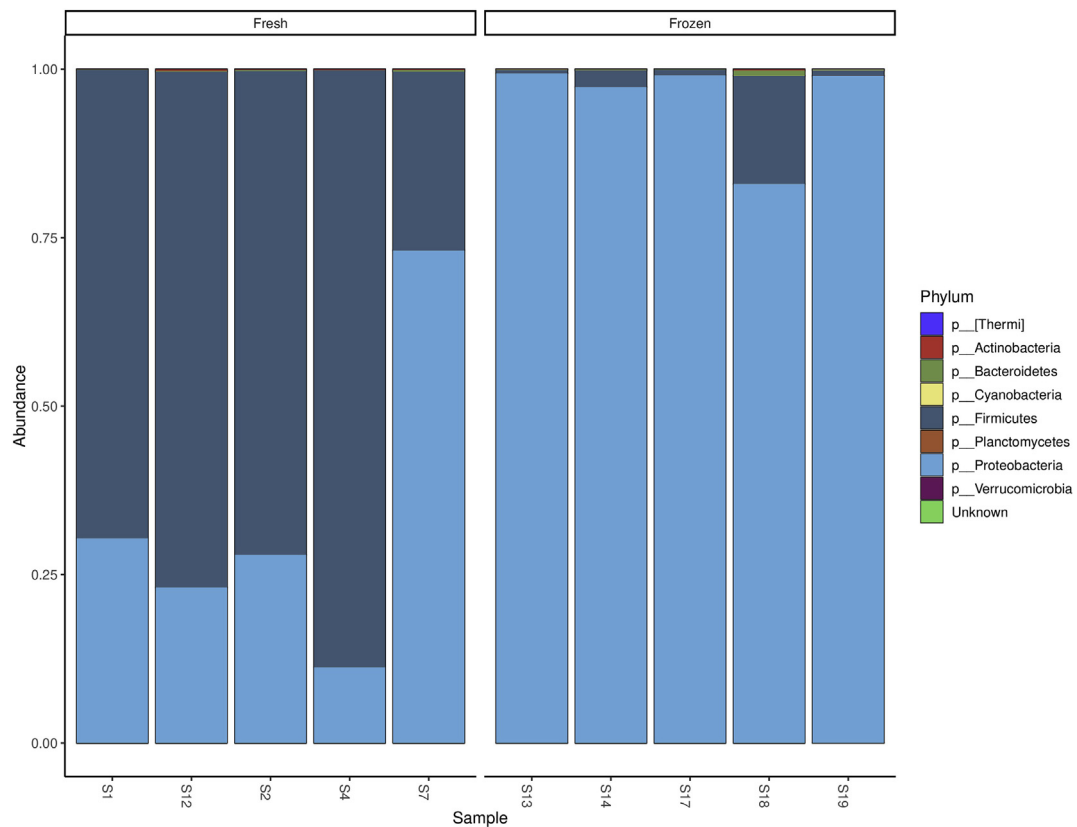

Figure 4. Relative abundance of the dominant bacterial phyla presents in the muscle tissue of sardines. Samples: fresh sardines (S1, S2, S4, S7, and S12) and frozen sardines (S13, S14, S17, S18, and S19).
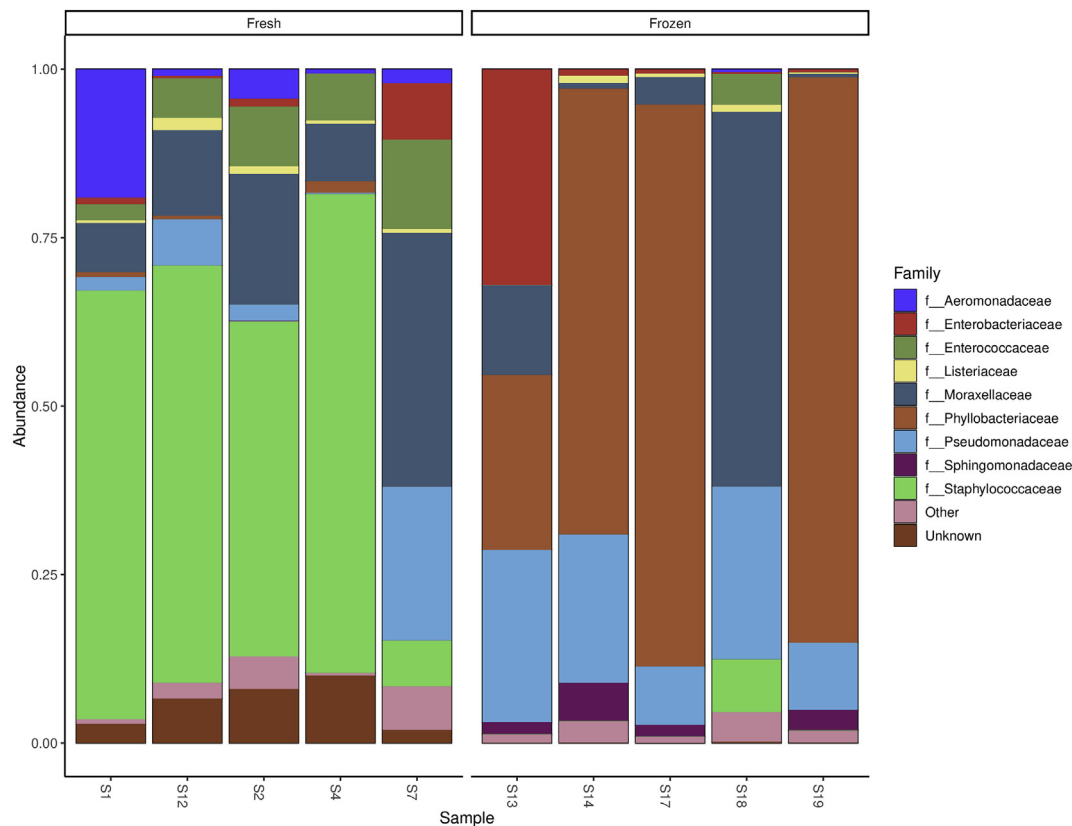
Figure 5. Relative abundance of dominant bacterial families presents in fresh or frozen sardine muscle tissue. Samples: fresh sardines (S1, S2, S4, S7, and S12) and frozen sardines (S13, S14, S17, S18, and S19).

and Enterococcaceae (w = 19), Aeromonadaceae (w = 16), Micrococcaceae (w = 9), Pseudomonadaceae (w = 9), Planococcaceae (w = 9), Campylobacteraceae (w = 5), and Listeriaceae (w = 4) in flesh samples. This group of bacteria represented 13.84 % and 28.59 % of the relative abundance in fresh and frozen sardine samples, respectively. These results may infer that the type of fish conservation method used can select the group of bacteria present in fish.

The main genera of bacteria found in fresh and frozen sardines are described in Figure 8. The genera most commonly present in fresh samples were *Macrococcus* spp. (49.88 %), *Acinetobacter* spp. (11.11 %), *Pseudomonas* spp. (6.98 %), *Psychrobacter* spp. (6.14 %), *Aeromonas* spp. (5.82 %), and *Vagococcus* spp. (5.56 %). In frozen sardines, the predominant genera were *Phyllobacterium* spp. (47.73 %), *Pseudomonas* spp. (16.76 %), *Acinetobacter* spp. (9.75 %), and *Psychrobacter* spp. (7.91 %). *Phyllobacterium* spp. represented >83.43 % of two frozen sardines samples (S17 and S19), while the genus *Macrococcus* spp. (>61.83 %) was predominant in three fresh sardine samples (S1, S4, and S12).

Major histamine-producing bacteria in fish were identified and had a relative abundance of less than 1 %. *M. morganii* bacteria were present in fresh (0.02 %) and frozen sardines (0.08 %), while *Photobacterium damselae* was only identified in fresh samples (0.08 %). Bacteria considered to have a low histamine production capacity in fish (i.e. *Shewanella* spp., *Aeromonas* spp., *Janthinobacterium* spp., *Proteus* spp., *Vibrio* spp., *Flavobacterium* spp., and *Serratia* spp.) were also identified.

The decaying microbiota in fish consisted of several psychrotrophic gram-negative bacteria, mainly *Pseudomonas* spp., *Macrococcus* spp., *Acinetobacter* spp., *Psychrobacter* spp., and *Aeromonas* spp., representing 79.93 and 36.52 % of the fresh and frozen sardine microbiomes, respectively. The genera *Shewanella* spp., *Janthinobacterium* spp., *Proteus* spp., *Serratia* spp., *Flavobacterium* spp., and *Photobacterium* spp. were also identified with relative abundances greater than 1 %.

Food-spoiling gram-positive bacteria were also found in lower relative abundance (>1 %) in fresh and frozen sardines and were represented by the genera *Arthrobacter, Bacillus, Brochothrix, Carnobacterium, Clostridium, Enterococcus, Kurthia, Lactobacillus, Lactococcus, Leuconostoc, Staphylococcus, Streptococcus*, and *Micrococcus*.

Some food spoilage bacteria were also identified at the species level, including *Pseudomonas caeni, P. veronii, P. viridiflava, Psychrobacter pulmonis, P. sanguinis, P. arenosus, P. marincola, P. pacificensis, Macrococcus caseolyticus, Acinetobacter johnsonii, Flavobacterium succinicans, Kurthia gibsonii, Janthinobacterium lividum, Carnobacterium viridans*, and *Lactobacillus delbrueckii*.

4. Discussion

Histamine detection and quantification methods are significant for food safety and the avoidance of food poisoning among consumers. The Codex Alimentarius has established a maximum histamine level of 200 mg kg^{-1} (FAO/WHO, 2018), while an EU Regulation (2019/627) included histamine control in fish landings, the processing industry, and the wholesale and retail fish markets to assess the compliance as well as the hygiene and sanitary conditions of establishments. In this sense, the presence of histamine in fish activates a warning signal among consumers.

In the present study, we demonstrated that all sardine samples used for analysis were above the legal limit for histamine levels in fish. One of the predisposing factors for histamine formation is inadequate fish preservation temperature. Temperature variation during fish storage enables the growth of deteriorating microorganisms, thereby increasing proteolytic activity and the formation and activity of the histidine decarboxylase enzyme (Ruiz-Capillas and Herrero, 2019). Even with bacterial growth retardation by cooling, histamine can still be formed at
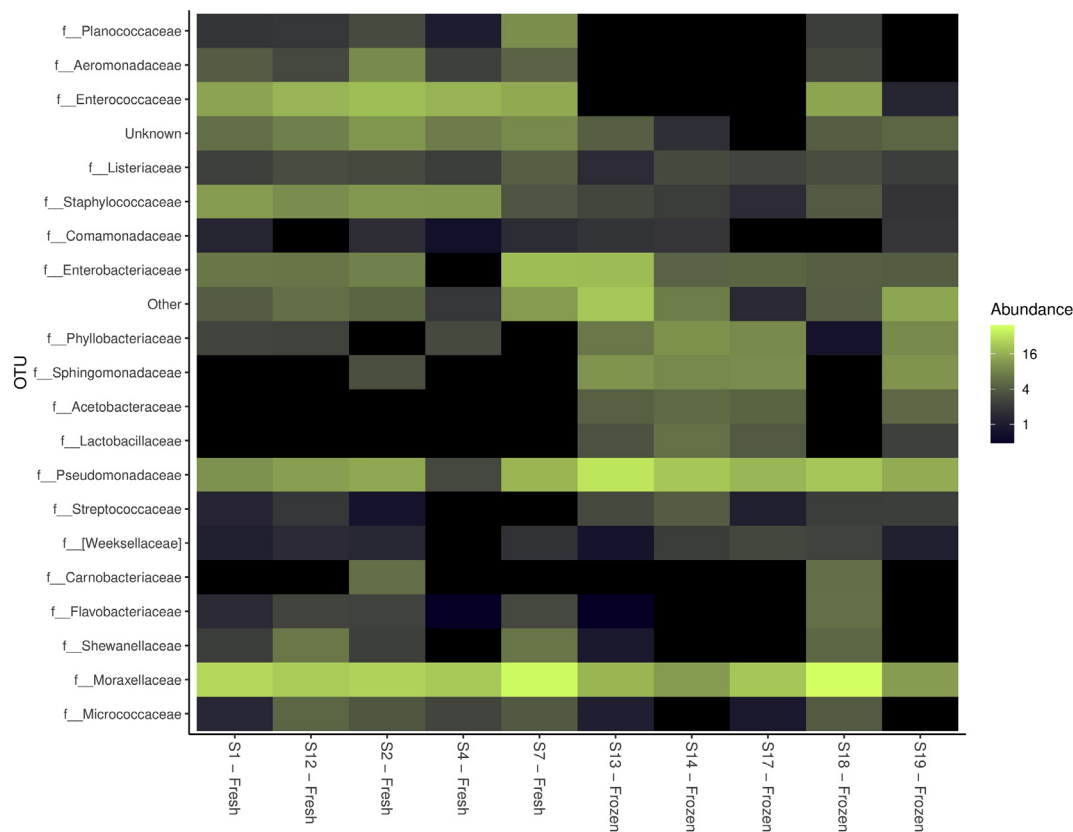
Figure 6. Heat map showing non-metric multidimensional scaling (NMDS) (hierarchical grouping of samples and taxa based on Bray-Curtis distance) of the microbiota composition in frozen (S13, S14, S17, S18, and S19) and fresh (S1, S2, S4, S7, and S12) sardines. Normalization of the frequency of the family level in log10. The color scale represents the stepped abundance of each variable, indicated by the score. Colors: blue indicates high abundance and dark blue indicates low abundance.

lower concentrations through the action of this enzyme. Under freezing conditions, the enzyme remains stable and becomes active after thawing (FAO/WHO, 2012).

The fresh sardines used in this study were acquired with viscera and gills, which may have influenced the high histamine concentration observed in this study. However, while fish meat is considered sterile, self-contamination or cross-contamination of bacteria may occur during cleaning and evisceration when fish are sold whole (de Bruijn et al., 2018).

Another predisposing factor for histamine formation is the pH of fish. Fresh sardines had higher pH values (6.70–7.18) when compared to frozen sardines (6.22–6.69). The control of pH is important for maintaining the sensory and microbiological quality of fish meat. This variation in pH is influenced by fish species, diet, seasonality, and post-capture stress (Ocaño-Higuera et al., 2009). Most sardines evaluated in this study had adequate pH for histidine decarboxylase activity since optimal activity occurs around pH 6.5 (Wendakoon and Morihiko, 1995).

The genus *Macrococcus* dominated the microbiota of fresh sardines (49.88 %), with *Macrococcus* sp. appearing to be part of the initial chilled fish microbiota (Parlapani et al., 2015). Previous studies have shown that this genus also dominated vacuum-packaged common carp (*Cyprinus carpio*) fillet microbiota. In frozen sardines, *Phyllobacterium* was the predominant genus (47.97 %), which encompasses gram-negative, nitrogen-fixing microorganisms that are easily found in plants and soil (León-Barrios et al., 2018).

Another predominant genus in sardines was *Acinetobacter* spp., encompassing 12.65 and 10.28 % of the microbiota in fresh and frozen sardines, respectively. Moreover, the genus *Pseudomonas* spp. was less common in fresh sardines (7.80 %) and was found at higher concentrations in frozen sardines (18.25 %). These bacteria belong to the resident
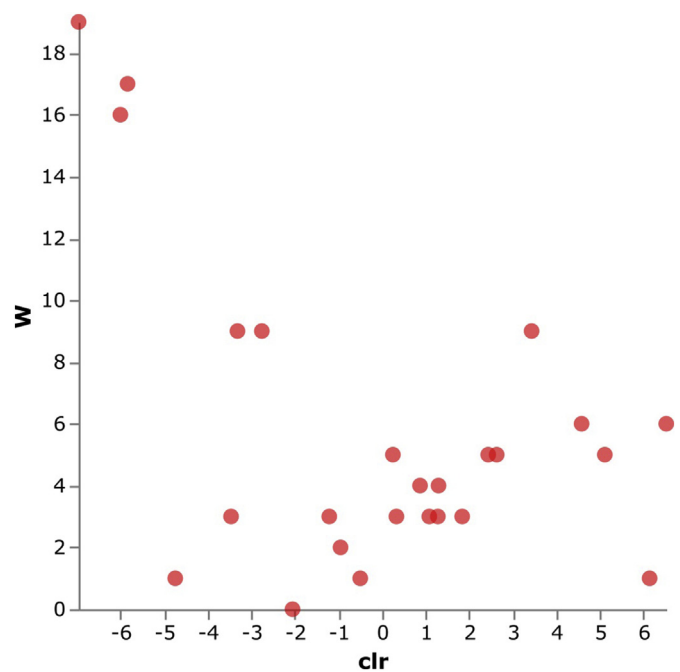

Figure 7. Volcano plot of ANCOM differential abundance. The family-level log-scale ASVs table was used for the analysis. The x-axis value represents the mean difference of clr transformed in the abundance of a given family between the fresh and frozen sardine groups. A positive x-axis indicates that the family is differentially abundant in frozen sardines, while a negative x-axis indicates that the family is differentially abundant in fresh sardines. The statistical analysis the represents the number of paired comparisons that were considered significantly different.
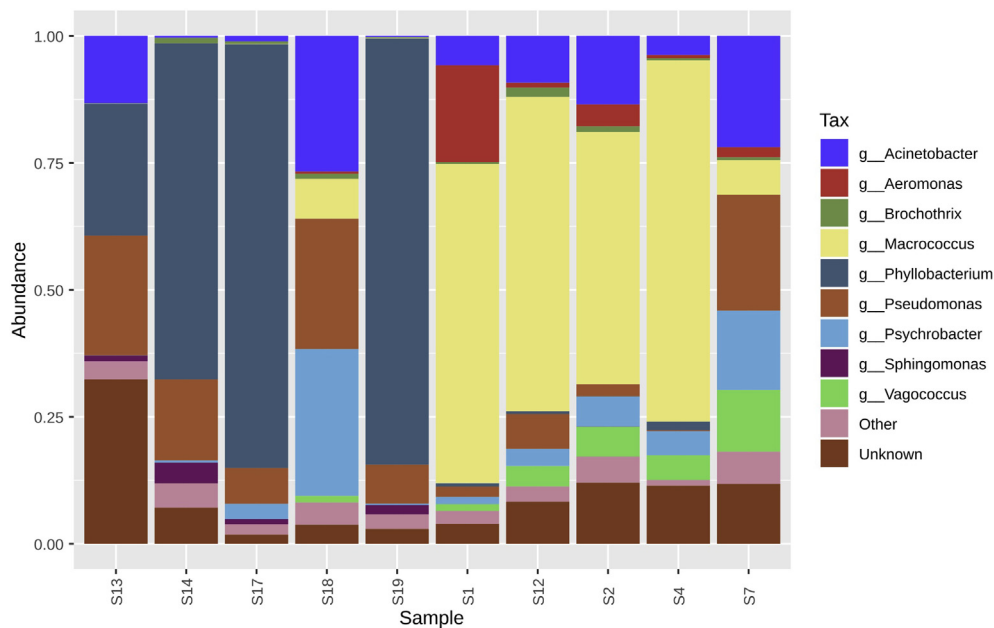
Figure 8. Taxonomic classification of sardine muscle tissue microbiomes at the genus level. Samples: fresh sardines (S1, S2, S4, S7, and S12) and frozen sardines (S13, S14, S17, S18, and S19).

gut microbiota of fish and are widely present in water and soil, can be found in refrigerated fresh foods, and can contaminate fish during processing (Deák, 2010; Egerton et al., 2018). The main deteriorating psychrotrophic microorganisms in refrigerated fish include the genera *Pseudomonas* spp., and *Acinetobacter* spp. (Odeyemi et al., 2018). To a lesser extent, other spoilage bacteria were also found in the fresh and frozen sardines analysed in the present study. These bacteria include *Aeromonas* spp., *Brochothrix* spp., *Photobacterium* spp., *Psychrobacter* spp., *Flavobacterium* spp., bacteria lactic acid (BAL), *Staphylococcus* spp., *Micrococcus* spp., *Listeria* spp., *Vagococcus* spp.*,* and *Kurthia* spp. (Bjerke et al., 2019).

Fish decomposing bacteria serve a key role in histamine production in fish (Takahashi et al., 2003). Many of these bacteria form part of the endogenous fish microbiota. However, these bacteria can contaminate post-capture fish throughout fish processing, marketing, and consumption (Lehane and Olley, 2000). Among the *Enterobacteriaceae* family, the species *M. morganii*, *E. aerogenes*, and *P. angustum*, which belong to the Vibrionaceae family, are important producers of the enzyme histidine decarboxylase in fish (Bjornsdottir-Butler et al., 2011).

The quantification of qPCR-based histamine-producing bacteria has been demonstrated as a potential method for detecting these microorganisms in food. However, the presence of the histidine decarboxylase enzyme does not determine the presence or formation of histamine in fish. Therefore, this technique could be a risk indicator for histamine production in fish (Bover-Cid et al., 2014) Using Ct values, qPCR facilitated the identification of the presence of the *hdc* gene presence in sardines. The *hdc* GN gene (Ct 24 to 36.3) presented a lower copy number, while the *hdc* MM (Ct 11.5 to 20.6) and *hdc* EA (Ct 14.3 to 27.5) genes presented higher copy numbers in sardine samples. This trend can be observed by the Ct values being inversely related to the number of qPCR reaction amplicons (Schmittgen and Livak, 2008).

In the present research, the molecular approaches of qPCR and HPLC with diode array detector proved quite accurate, which was also observed by de Lira et al. (2019) in their assessment of fresh whitemouth croaker quality. While, the qPCR method detects and quantifies the presence of histamine-forming bacteria, HPLC-DAD quantifies the concentration of histamine in fish. Both methods have been shown to exhibit greater specificity, sensitivity, and reliability than traditional methods. Considering the high number of food poisoning outbreaks caused by histamine consumption, the application of these methods provides an

important tool for tracing relevant microorganisms to reduce toxin production in susceptible foods. This would positively impact consumers and the public health system by reducing the number of food poisoning cases.

5. Conclusion

With the methodologies employed in the present study, it was possible to evaluate the microbiological and chemical quality of fresh and frozen sardines sold in a public market in Porto Alegre, Brazil. Notably, HPLC-DAD has been demonstrated as a reliable technique for identifying and quantifying histamine in fish, while qPCR represent a rapid and reliable method to quantify the histamine-producing gram-negative bacteria in fish. With the amplification of the *16S rRNA* gene and sequencing by HTS, it was possible to characterize important spoilage bacteria present in the analyzed sardines.

Although, HTS has shown that most of the microorganisms present in the samples belong to deteriorating microbiota with the potential for histamine formation, the qPCR results facilitated the identification and quantification of two known histamine-forming species via histidine decarboxylation even when present in low concentrations. Accordingly, the methods used in the present work proved to be very appropriate and accurate.

Finally, the authors highlight the importance of this research work and warn that food poisoning outbreaks caused by histamine intake are common. Therefore, improvements in the handling and processing of sardines using the proposed methodology can improve food safety for consumers.

Declarations

Author contribution statement

Alessandra Danile de Lira, Ícaro Maia Santos de Castro: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Michele Bertoni Mann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Luana Peixoto Mallmann: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Caroline Isabel Kothe: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ana Paula Muterle Varela: Performed the experiments; Analyzed and interpreted the data.

Ana Paula Guedes Frazzon: Analyzed and interpreted the data; Wrote the paper.

Jeverson Frazzon: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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