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ORIGINAL ARTICLE

Assessing the quality of fresh Whitemouth croaker (*Micropogonias furnieri*) meat based on micro-organism and histamine analysis using NGS, qPCR and HPLC-DAD

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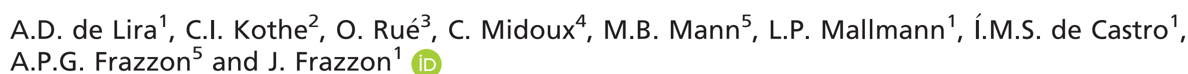

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Keywords
assessment of quality, histamine, HPLC-DAD, NGS, qPCR, whitemouth croaker.

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Abstract

Aims: Quality evaluation of fresh whitemouth croaker (*Micropogonias furnieri*) by histamine determination using the HPLC-DAD method and quantification of histamine-forming bacteria using NGS and qPCR.

Methods and Results: The histamine content of fresh whitemouth croaker was detected by high performance liquid chromatography with diode array detector with a concentration ranging from 258·52 to 604·62 mg kg^{-1} being observed. The number of histidine decarboxylase (*hdc* gene) copies from Gram-negative bacteria and the bacteria *Morganella morganii* and *Enterobacter aerogenes* were quantified by quantitative polymerase chain reaction. All samples were positive, with copy numbers of the *hdc* gene ranging from 4·67 to 12·01 log_{10} per g. The microbial community was determined by sequencing the V4 region of the *16S rRNA* gene using the Ion Torrent platform. The bioinformatics data generated by FROG software showed that the phylum Proteobacteria was the most abundant, with the family Moraxellaceae being more prevalent in samples collected in the summer, whereas the Pseudomonadaceae was more present in the winter.

Conclusions: All fish muscle samples analysed in this study presented histamine values higher than those allowed by CODEX Alimentarius. Additionally, a wide variety of spoilage micro-organisms capable of expressing the enzyme histidine decarboxylase were detected. Thus, improvements in handling and processing are required to minimize the prevalence of histamine-producing bacteria in fish.

Significance and Impact of the Study: Global fish production in 2016 was 171 million tons, with the largest consumer being China, followed by Indonesia and the USA. In Brazil, 1·3 million tons of fish are consumed per year, with whitemouth croaker being the main fish landed. Notably, cases associated with histamine poisoning are quite common. According to the European Food Safety Authority and European Centre for Disease Prevention and Control, a total of 599 HFP outbreaks were identified in the European Union during the period 2010–2017. In the USA, there were 333 outbreaks with 1383 people involved between 1998 and 2008.

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Introduction

Whitemouth croaker (*Micropogonias furnieri*) is one of the most important species in Brazilian fishing, especially in the south and southeast areas of the country, representing the second most captured species at approximately 44 000 tons per year. Nutritionally, whitemouth croaker contains several essential amino acids such as lysine and methionine, and also is an important source of long-chain polyunsaturated fatty acids (omega-ω3), vitamins (A, B and D), as well as minerals (calcium, phosphorus, zinc, iron, iodine and selenium). Although, it has a high nutritional value, this food can easily deteriorate without preventive measures. Such deterioration can occur within the production chain, from gathering to consumption (Béné *et al.* 2015; Svanevik *et al.* 2015; Khalili Tilami and Sampels 2018) due to the presence of deteriorating micro-organisms, resulting in the formation of toxic compounds such as biogenic amines (Olatunde and Benjakul 2018).

Biogenic amines possess low molecular weight and nonvolatile organic nitrogenous bases (Suzzi and Torriani 2015). These compounds are present in small amounts in foods due to amino acids decarboxylation through microbial action during transport, processing and storage. Among the biogenic amines found in foods, histamine has been implicated as the causative agent of several outbreaks of food poisoning (Angelo *et al.* 2017; Ruiz-Capillas and Herrero 2019).

Histamine is the product of the decarboxylation of the histidine amino acid, catalysed by the histidine decarboxylase enzyme, which is a pyridoxal 5'-phosphate and cofactor dependent in Gram-negative bacteria. The presence of these micro-organisms may be associated with wild fish microbiota or bacterial contamination from the processes of capture, processing, distribution and consumption. The most commonly involved bacteria include *Morganella morganii*, *Enterobacter aerogenes*, *Photobacterium phosphoreum* and *Raoultella planticola* (Landete *et al.* 2007; Ladero *et al.* 2010). Deteriorating micro-organisms such as *Clostridium* sp., *Vibrio* sp., *Acinetobacter* sp., *Plesiomonas* sp., *Pseudomonas* sp., *Aeromonas* sp., *Citrobacter* sp. and *Salmonella* sp. may also contribute to the formation of histamine (Huang *et al.* 2010).

In this context, the objective of the present study was to assess the quality of fresh and whole whitemouth croaker sold in a fish market in the South Region of Brazil. To achieve this, the quantification of histamine-forming bacteria, determination of histamine content in fish muscle and the molecular characterization of bacterial diversity were determined using quantitative polymerase chain reaction (qPCR), HPLC-DAD and NGS respectively.

Materials and methods

Sample collection

The fish were collected in the Central Public Market of Porto Alegre, Rio Grande do Sul (Brazil). Twenty-four fresh and whole whitemouth croaker were collected from a single supplier. Twelve fish samples (C1–C12) were collected in summer (February 2017 to March 2017) and 12 samples (C13–C24) in winter (July 2017). The surface temperature of the fish was measured at the time of collection, using a digital infrared thermometer (MT-320 Minipa, São Paulo, Brazil). Afterwards, the fish were identified, packed and then transported in isothermal boxes with recycled ice. The fish were weighed, gutted and parts of the muscle were removed as described by (Frank *et al.* 1981). After the muscle collection, 10 g of the sample was mixed in sterile distilled water (90 ml), homogenized and the pH was determined (Bench pH meter Q400AS; Quimis, São Paulo, Brazil).

Determination of histamine by high performance liquid chromatography (HPLC) with diode array detector (DAD)

Preparation of the solutions

The standard histamine dihydrochloride (HPLC grade, >99%, Sigma Aldrich, Darmstadt, Germany) was prepared in stock solutions in 0·1 mol l^{-1} hydrochloric acid (PA, Dinâmica, Brazil) at 1000 mg ml^{-1} and refrigerated at −20°C, in 5·0 ml aliquots. The preparation of the working solutions was carried out by appropriate dilutions of the stock solution in hydrochloric acid, TCA (P.A., Synth) 0·1 mol l^{-1} (Gouveia 2009). The dansyl chloride solution (HPLC grade, >99%, Sigma Aldrich), 10 mg ml^{-1} was prepared by dissolving 100 mg in 10 ml of acetone (HPLC grade, J.T. Baker) and stored under refrigeration at 4°C (Shukla *et al.* 2010).

Histamine extraction from the matrix

For histamine extraction, 100 g of the samples were triturated in a blender and 5 g was directly weighed into 50 ml polypropylene centrifuge tubes. Ten millilitre of 5% TCA was homogenized for 5·0 min in vortex and centrifuged at 3000 ***g*** for 10 min at 4°C; the supernatant was transferred to another centrifuge tube (50 ml). The residue was extracted again with an equal volume of TCA 5% and centrifuged again. Both supernatants were combined, and the final volume was adjusted to 25 ml TCA 5%. Subsequently, the supernatant was filtered through qualitative filter paper, no. 1 (Whatman, UK) and reserved for derivatization.

Derivatization

The derivatization of amines with dansyl chloride was performed according to the method of (Ben-Gigirey *et al.* 1998), with some modifications. One millilitre of each extracted sample or standard solution of histamine were mixed with 200 μl of sodium hydroxide at 2 mol l^{-1}, and 300 μl of saturated P.A. sodium bicarbonate (Synth) solution. Then, 2·0 ml of a solution of dansyl chloride (10 mg ml^{-1} in acetone) was added to the mixture and incubated in a 40°C water bath for 45 min. Then, the solution was mixed to 100 μl of ammonium hydroxide at 28–30%, P.A. (Neon) was added in the reaction to remove the residual dansyl chloride and was incubated for 30 min at room temperature in the dark. After incubation, the final volume was adjusted with 5·0 ml of HPLC grade acetonitrile, >99·9% (J.T. Baker). Finally, the mixture was centrifuged at 2500 ***g*** for 5 min and the supernatant was filtered on a 0·22 μm diameter (Filtrilo, Syringe Filters, 13 mm, 0·22 μm) syringe filter. The filtered supernatant was maintained at −20°C.

Analysis of histamine in muscle samples

The analysis of histamine was based on the methodology of Hu, Li and Yang (2012), with some modifications. Identification and quantification of histamine were performed using a Shimadzu (Kyoto, Japan) HPLC apparatus 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 histamine was identified and quantified using a C18 Merck chromatography column (250 × 4·6 mm) with 5 μm particle size and a 1·0 ml min^{-1} flow at 30°C. The mobile phase consisted of ultrapure water obtained in a MilliQ system, Millipore (Darmstadt, Germany) (solvent A) and HPLC grade acetonitrile (solvent B). The volume injected was 20 μl. The linear gradient ranged from 60% to 76% of B in 6 min and in 1 min reached 100% of B, sustained for 2 min. In the next minute, it returned to 60% of B and was sustained for 3 min, totalizing 13 min of runtime. Spectra were obtained between 200 and 800 nm and the chromatograms were processed at 254 nm.

Quantification of histamine

Histamine quantification in fish muscle samples was performed using the analytical curve of the histamine standard. The standard working solution (200 mg l^{-1}) was prepared with the stock solution of histamine hydrochloride (1000 mg l^{-1} diluted in 0·1 mol l^{-1} hydrochloric acid. The analytical curve was prepared with six concentrations (1·0, 2·0, 3·0, 5·0, 7·0 and 9·0 mg l^{-1}) of histamine. The method was validated by analysing the following performance parameters: linearity ($R^2 > 0{\cdot}99$), limit of detection (LD) and limit of quantification (LQ), according to (Guideline 2006), with Eqns 1 and 2.

$$\mathrm{LD} = 3 \cdot 3 \times \frac{s}{b} \quad (1)$$

$$\mathrm{LQ} = 10 \times \frac{s}{b} \quad (2)$$

where: s = standard error of the analytical curve; b = slope of the analytical curve.

Reference histamine-producing bacterial strains

Reference strains of histamine-producing bacteria, *M. morganii* (ATCC 8019), and *E. aerogenes* (ATCC 13048) were obtained from the Laboratory of Reference Materials of Institute Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil. The bacteria were activated in tryptic soy broth and incubated at 37°C per 24 h. After this period, they were cultivated in a tryptone soy agar plate and incubated at 37°C per 24 h.

Extraction and quantification of bacterial DNA isolated from whitemouth croaker muscle

From the muscle samples, 10 g were transferred to an Erlenmeyer (250 ml) containing autoclaved distilled water (90 ml) and then incubated at 37°C per 2 h in an orbital shaker (Shaker SL-222; Solab, São Paulo, Brazil). Afterwards, 1·0 ml of the sample and centrifuged at 3000 ***g*** for 3 min (320R Refrigerated bench centrifuge; Hettich, Tuttlingen, Germany). The supernatant was discarded, and the process was repeated twice. For DNA extractions the kit Invitrogen™ PureLink™ Genomic DNA (Thermofisher, Waltham, MA) was used. All DNA samples were quantified through the Quibit® 2.0 fluorometer (Invitrogen, CA) according to the manufacturer's protocols.

Oligonucleotides

In order to perform the real-time qPCR assay, an oligonucleotide set previously described in the literature was used for histidine decarboxylase-producing (*hdc* gene) in Gram-negative (GN) bacteria (Bjornsdottir-Butler *et al.* 2011). Two more oligonucleotides were designed to detect *hdc* gene in *M. morganii* (*hdc* MM) and *E. aerogenes* (*hdc* EA) using the Genscript (Table S1).

The oligonucleotides were analysed *in silico* through the nucleotide database, Basic Local Alignment Search Tool (BLAST, NCBI). The oligonucleotide for *hdc* gene of MM and EA obtained 100% of identity for the *M. morganii* accesses in the GenBank (KP728801.1, KC771251.1, CP004345.1, AB259290.1, J02577.1 and KP728802.1) and for the *E. aerogenes* accesses in GenBank (KP728798.1,

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KP728797.1, FJ469567.1, M62745.1 and CP014748.1) respectively.

Quantitative polymerase chain reaction

For the qPCR reactions (StepOne™ Real-Time PCR System, 96-well; Thermo Fisher Scientific, Waltham, MA) SYBR Green (Applied Biosystems™, Waltham, MA) was used. Amplification of the DNA by qPCR was performed in 15 μl reaction volume containing 2 ng μl^{-1} of the DNAg, 1x PCR buffer (200 mmol l^{-1} Tris-HCl, pH 8·4, 500 mmol l^{-1} KCl); 0·75 μl $MgCl_2$ (50 mmol l^{-1}), *hdc* F and *hdc* R oligonucleotides (10 μmol l^{-1}); 0·1 μl dNTP (10 mmol l^{-1}); SYBR Green 1x, Taq DNA Polymerase Platinum® Invitrogen 5 U μl^{-1}, concentration of ultrapure water to complete the volume. The conditions for the qPCR were initial denaturation of 94°C for 5 min, 35 cycles at 94°C for 30 s, 58°C for 30 s (*hdc* GN and *hdc* EA), 55°C for 30 s (*hdc* MM) and 72°C for 30 s and a final extent of 72°C for 5 min. The melting curve was determined after the last amplification cycle. Negative control was used in all runs. The quantification of the *hdc* gene by qPCR was based on (Biosystems 2013). The standard curve was generated for each oligonucleotide from serial dilutions (10^1 to 10^6 μl^{-1} copies) of the bacterial DNA with a dilution factor of (1 : 10). Each point of the standard curve was performed in triplicate. Quantification values, Ct (cycle threshold) points generated in the qPCR of the samples were related to the Ct of the standard curve. The absolute quantification was obtained by comparing the Ct values of the samples with the standard curve. Using this equation, Ct was derived,

$$(\log_{10}):\ N = 10\left(\frac{Ct - b}{a}\right)$$

where, N = number of copies; Ct = threshold cycle, b = corresponds to the point of intersection of the axes of the curve; a = represents the slope of the curve.

The result of the analysis was the amount of nucleic acid (number of copies, $\log_{10}$) per given amount of sample (per g of sample, per μg of total DNA).

Statistical analysis

The study variables were described through median and interquartile intervals. Wilcoxon test was used to evaluate the differences between the seasonal periods. The linear regression model was used to evaluate the relationship between histamine and other variables tested (season, weight, pH, temperature, Ct value and *hdc* gene number). The significance level considered was 5% (P value $<0{\cdot}05$). Statistical analyses were conducted in software R ver. 3.4.2 (R Core Team 2017).

Amplification of the *16S rRNA* gene from muscle samples of whitemouth croaker collected at different seasons

Six samples of DNA extracted from the muscle of whitemouth croaker were selected. The V4 region of the *16S rRNA* gene was amplified and sequenced, according to (Dobbler *et al.* 2018) methodology. We performed the analysis by the PGM™ Ion Torrent (Thermo Fisher Scientific) platform using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') according to the protocol described by (Caporaso *et al.* 2012).

Data analysis

Quality control of the raw data was assessed using FastQC (Andrews 2010) and the individual reports were grouped with MultiQC (Ewels *et al.* 2016). The adapters involved in the sequences were removed with cutadapt ver. 2.3 (Martin 2011) and sequences without the adapters were removed. Afterwards, the data were imported into the FROGS (Find Rapidly Operational Taxonomic Units, OTUs, with Galaxy Solution) pipeline (Escudié *et al.* 2017) to obtain OTUs. The sequences were filtered by length (250–300 bp) and then pooled into OTUs with SWARM (Mahé *et al.* 2015) with a distance parameter of 3. Chimeras were removed with VSEARCH (Rognes *et al.* 2016) and OTUs with low abundance were eliminated (filter applied to the OTUs with <5 sequences). Taxonomic affiliation was performed using the Silva 132 pintail 100 databases (Quast *et al.* 2013). Alpha diversity and microbial composition were analysed using the R Phyloseq package (McMurdie and Holmes 2013). Sequencing data were deposited in the Sequence Read Archive of the European Nucleotide Archive (ENA, Cambridgeshire, UK), access number PRJEB33351.

Results

Identification and quantification of histamine by HPLC-DAD

Histamine was identified in the samples on the basis of the retention time in comparison to standard solutions. The histamine was separated in 1·3 min of run time and exhibited good peak resolution, sharpness and symmetry (Fig. S1). The proposed analytical method for determining histamine in whitemouth croaker muscle samples was validated under optimized conditions. The analytical curve was linear, with correlation coefficients of 0·9957 (Fig. S2). The LD was 0·77 mg kg^{-1} and the LQ was 2·0 mg kg^{-1}. This result indicates that the analytical

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method of HPLC-DAD with derivatization was very accurate for the determination of histamine.

Determination of the temperature, pH and histamine of whitemouth croaker

The interquartile medians and ranges of weight, superficial temperature, pH and histamine concentration in the whitemouth croaker muscle collected in different seasons are present in Table 1. Statistically significant differences between the weight medians ($P = 0{\cdot}002$), fish superficial temperature ($P = 0{\cdot}004$) and muscle pH ($P = 0{\cdot}05$) between whitemouth croaker were observed. Histamine was detected in all whitemouth croaker samples, but there was no statistical difference in histamine concentrations ($P = 0{\cdot}488$) in the whitemouth croaker (Table 1). All samples contained high histamine levels, ranging from 417·63 to 604·62 mg kg^{-1} for whitemouth croaker collected during summer and 258·52 to 533·24 mg kg^{-1} for whitemouth croaker collected during winter.

Determination of qPCR efficiency and the absolute quantification of histamine-producing bacteria in whitemouth croaker

To evaluate the sensitivity and efficiency of the qPCR assay, standard curves were determined through serial dilutions of the DNA (10^1–10^6 copies) of *M. morganii* (*hdc* GN and *hdc* MM) and *E. aerogenes* (*hdc* EA). A linear relationship between the amount of DNA from histamine-producing bacteria in the qPCR reaction was obtained for each oligonucleotide (Table S2). The high values of efficiency and curve regression coefficient obtained facilitated the direct quantification of the histidine decarboxylase-producing bacteria present in the samples. In all histamine-producing bacteria with positive samples quantified by qPCR, the presence of histamine was confirmed by HPLC-DAD. The Wilcoxon statistical test showed a significant difference ($P < 0{\cdot}001$) between the medians of whitemouth croaker samples collected in different seasons based on cycle threshold (Ct) and the number of copies of *hdc* gene ($\log_{10}$ per g) (Table 2).

All fresh whitemouth croaker samples collected in different seasons ($n = 24$) were positive for *hdc* genes, although their wide range of Ct values, indicates differences in oligonucleotide concentrations among the samples. Notably, the qPCR assays had high *hdc* gene quantification capacity. The number of copies transformed into $\log_{10}$ for the GN *hdc* gene (7·9–12·1 $\log_{10}$ per g) was higher when compared to the number of copies of the specific gene from *M. morganii* (4·67–10·06 $\log_{10}$ per g) and *E. aerogenes* (6·32–8·23 $\log_{10}$ per g). A greater level of variability was observed in the concentrations of histamine-producing Gram-negative bacteria in summer samples, but no significant difference was observed between seasonality ($P = 0{\cdot}002$). Significant differences were also observed between the summer samples when compared to the medians ($P = 0{\cdot}001$) of the *hdc* MM copy numbers (Table 2 and Fig. 1). However, there was no statistical difference ($P = 0{\cdot}002$) for the *hdc* EA copy numbers.

These results highlight the importance of Gram-negative bacteria, including *M. morganii* and *E. aerogenes* in the formation of histamine in fish. Despite the high concentrations of free histamine in whitemouth croaker muscle (258·52–604·62 mg kg^{-1}), no case of food poisoning by histamine has ever been reported for this species. In this context, the early detection of histamine can be used as a predictive method to identify a potential fish risk factor during processing, storage and commercialization.

The relationship of histamine and seasons with the other variables studied

A regression analysis was performed to determine the relationship between the histamine concentration and season, weight, superficial temperature and pH of the evaluated whitemouth croaker. There was a positive correlation between the histamine concentration in muscle and the different seasons ($\beta = 40{\cdot}15$, $P <$ 2e-16). This correlation suggests that histamine may increase by 40 mg kg^{-1} when fish are contaminated in the summer. Notably, the amount of histamine among fish collected in the summer showed a lower variability in histamine concentration (Fig. 2).

Table 1 Values of weight, superficial temperature, pH and histamine of the fresh whitemouth croaker muscle collected in different seasons

Parameters	Number of fishes (n = 24)*,†	Summer fishes (n = 12)*,‡	Winter fishes (n = 12)*,†	P value‡
Weight (kg)	1·86 (1·52; 2·88)	1·57 (1·45; 1·66)	2·88 (2·46; 3·19)	0·002
Superficial temperature (°C)	0·90 (−0·07; 1·42)	1·45 (0·90; 1·85)	0·10 (−0·90; 0·40)	0·004
Muscle pH	6·58 (6·45; 7·05)	6·54 (6·41; 6·89)	6·86 (6·53; 7·16)	0·050
Histamine (mg kg^{-1})	492 (469; 515)	493 (478; 515)	491 (450; 508)	0·488

*n = number of samples.
†Results expressed in median (1ª Quartile and 3ª Quartile).
‡P value <0·05.

Table 2 Median, interquartile interval of Ct corresponding to the standard curve derived from the qPCR test and the number of copies of the *hdc* gene present in the whitemouth croaker muscle collected in the different seasons

Parameters*	Number of fishes (n = 24)†	Summer fishes (n = 12)†	Winter fishes (n = 12)†	P value‡
Ct (*hdc* GN)	15·8 (15·1; 21·0)	22·6 (18·5; 30·2)	15·1 (14·2; 15·3)	<0·001
Ct (*hdc* MM)	16·3 (14·0; 18·4)	18·3 (18·0; 19·1)	14·0 (13·7; 14·3)	0·001
Ct (*hdc* EA)	11·5 (11·3; 14·8)	14·9 (14·5; 15·4)	11·3 (11·1; 11·4)	<0·001
hdc GN ($\log_{10}$ per g)	11·2 (10·2; 11·6)	10·1 (8·29; 11·2)	11·6 (11·2; 11·8)	0·002
hdc MM ($\log_{10}$ per g)	7·35 (6·65; 7·69)	6·78 (6·30; 7·02)	7·72 (7·52; 7·84)	0·001
hdc EA ($\log_{10}$ per g)	7·88 (7·60; 8·90)	7·61 (7·26; 7·85)	8·88 (8·63; 9·11)	0·002

**Ct* value = cycle threshold, $\log_{10}$ *hdc* gene copies number in 1·0 g de muscle, hdc = histidine decarboxylase, GN = gram-negative bacteria, MM = *M. morganii*, *EA* = *E. aerogenes.*

†n = number of samples, results expressed in median (1ª Quartile and 3ª Quartile).

‡P < 0·001.

Another evaluated linear correlation was histamine concentration and the fish muscle pH. By relating these variables, the pH would increase by an average of 61·27 mg kg^{-1} of histamine in muscle (P = 0·097). When season was added to these variables as a factor, this relation increased to 96·02 mg kg^{-1} (P = 0·0102) in fish samples collected during the summer. In this study, it was observed that pH influenced the concentration of histamine in whitemouth croaker gathered during different seasons; however, the pH factor contributed to a higher initial concentration of histamine in the summer samples (Fig. 3).

Furthermore, the linear regression analysis showed positive correlations when compared to the histamine concentration of the samples with fish weight (β = 80·64, P = 0·000109), seasonality (β = 157·15, P = 0·0000063) and muscle pH (β = 80·64, P = 0·000758). Additionally, the surface temperature of the fish had a significant influence when compared with the seasonality (β = 152·62, P = 0·000133), weight (β = 79·59, P = 0·000246) and muscle pH (β = 93·90, P = 0·0010).

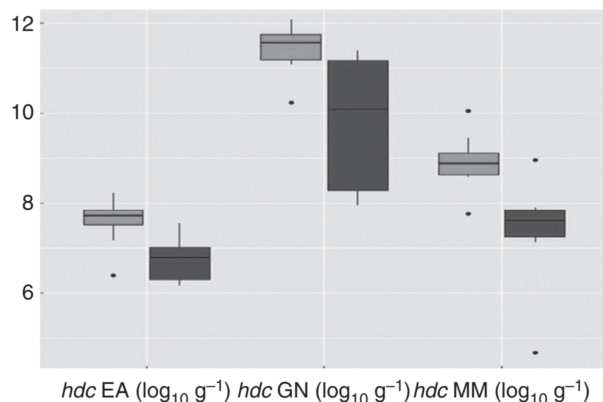

Figure 1 Comparison of the number of copies of the *hdc* gene ($\log_{10}$ per g) of histamine-producing Gram-negative bacteria (*hdc* GN), *M. morganii* (*hdc* MM) and *E. aerogenes* (*hdc* EA) in fresh whitemouth croaker at different seasons. Sample: Summer (grey) and winter (black).

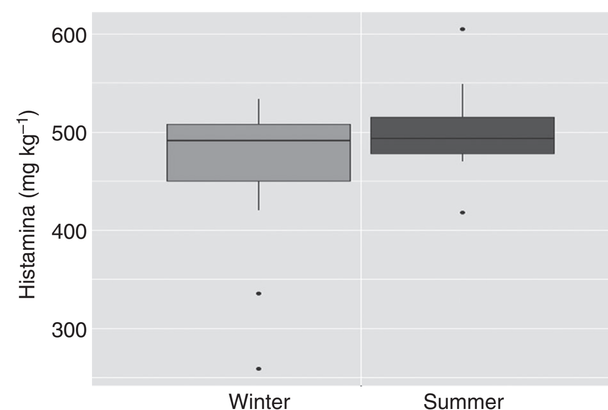

Figure 2 Comparison between histamine concentration and whitemouth croaker samples collected in different seasons. Winter (grey) and summer (black).

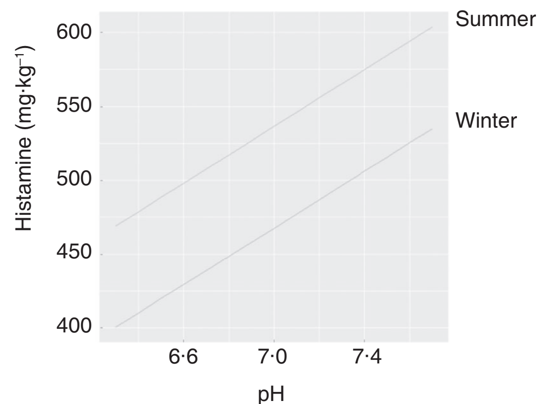

Figure 3 Chart showing the interaction between histamine concentration and pH of muscle samples collected in different seasons. Sample: Summer (upper line) and Winter (lower line).

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Likewise, the linear relationship between histamine and Ct (qPCR) and the number of copies of the *hdc* gene in whitemouth croaker samples was evaluated. A significant difference for the Ct of the *hdc* MM gene ($P = 0{\cdot}0617$) was only observed when related to the histamine concentration ($P = 2{\cdot}92\text{e-}08$) and summer samples ($P = 0{\cdot}0356$). This result highlights the contribution of *M. morganii* to the production of histamine among whitemouth croaker collected in the summer, thus indicating that the presence of this bacterium can greatly reduce fish quality and directly contribute to histamine production. Moreover there was a significant difference among the seasons, histamine and the number of copies of the *hdc* GN gene ($\beta = 537{\cdot}52$, $P = 0{\cdot}0040$) and *hdc* MM gene ($\beta = 439{\cdot}55$, $P = 3{\cdot}84\text{e-}11$). When histamine was related to all variables (season, weight, pH, temperature and *hdc* copy number), a significant difference was observed in histamine concentration among summer samples ($P = 0{\cdot}00799$), pH ($P = 0{\cdot}00799$), weight ($P = 0{\cdot}00799$) and the number of copies of *hdc* GN gene ($P = 0{\cdot}0890$).

Bacterial diversity of fresh whitemouth croaker collected in different seasons

In this study, the *16S rRNA* gene was amplified in six fresh whitemouth croaker samples to comparatively explore the bacterial communities collected in the different seasons. A total of 132 423 *16S rRNA* sequences were generated for the six whitemouth croaker samples analysed. These sequences were pooled into 2596 bacterial OTUs, where taxonomic designation was possible up to the family level. These OTUs were numbered and classified according to the taxonomy of each studied sample. Overall, 16 phyla were identified with an abundance of $\geq 1{\cdot}0\%$.

The relative abundance of the bacterial community at the phylum level is present in Fig. 4. Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were the most abundant phyla in fresh whitemouth croaker. Proteobacteria was the most predominant phylum, representing 68·60 and 75·00% of the bacterial composition in the samples collected during summer and winter respectively. Although, Bacteroidetes was the second most represent phylum, there was a greater abundance among the samples C7, C8 and C22. Regarding, the phylum Firmicutes, greater abundance was obtained in samples C10, C15 and C24.

Figure 5 highlights bacterial diversity at the family level. Nearly all families present in the samples collected during the winter were present in samples collected during the summer in greater or lesser quantity, except for the family Pseudoalteromonadaceae, which was only identified in a single sample collected in the summer (C10—2·97%). When analysing at the family taxonomic level, Moraxellaceae, Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Shewanellaceae and Aeromonadaceae were the most abundant families of the Proteobacteria phylum. Moraxellaceae was more prevalent in whitemouth croaker collected in the summer (average of 19·34%), while Pseudomonadaceae was more present in winter samples (average of 35·55%). Burkholderiaceae were proportionally present in the different seasons, while the Enterobacteriaceae family was dominant in the winter period. Histamine-producing bacteria in fish, including Moraxellaceae, Pseudomonadaceae, Enterobacteriaceae, Shewanellaceae and Aeromonadaceae, represent over half of the composition of analysed fish (51% for summer and 77% for winter). A similar pattern was observed when evaluating the bacterial community using hierarchical clustering in Fig. 6. Considering Unweighted Unifrac distances between samples, the C22 sample was distantly related among other samples. In contrast C14, C15 and C10 were closely related, thereby suggesting conservation in microbial communities.

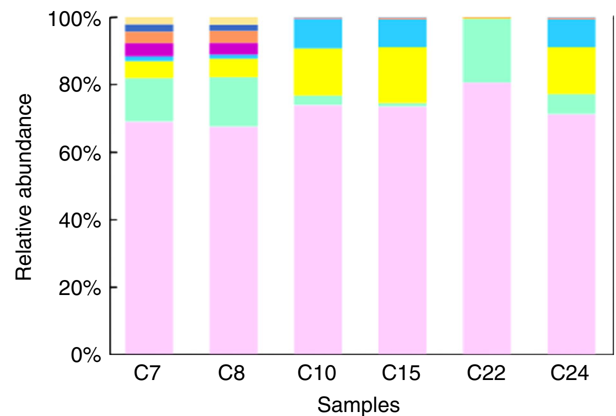

Figure 4 Histogram of the percentage of the phylum level in OTUs per sample of whitemouth croaker muscle collected in different seasons. Whitemouth croaker collected in summer (C7, C8 and C10) and winter (C15, C22 and C24). Only OTUs present in at least two samples, relatively abundant >1% (□ Proteobacteria; □ Bacteroidetes; □ Firmicutes; □ Actinobacteria; □ Verrucomicrobia; □ Acidobacteria; □ Chloroflexi; □ Planctomycetes). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

Utilizing methods for the early and rapid detection of histamine-producing bacteria and the detection of histamine is important for preventing the accumulation of this toxic substance in food. Molecular approaches, such as qPCR, to detect histamine-forming bacteria in fish and

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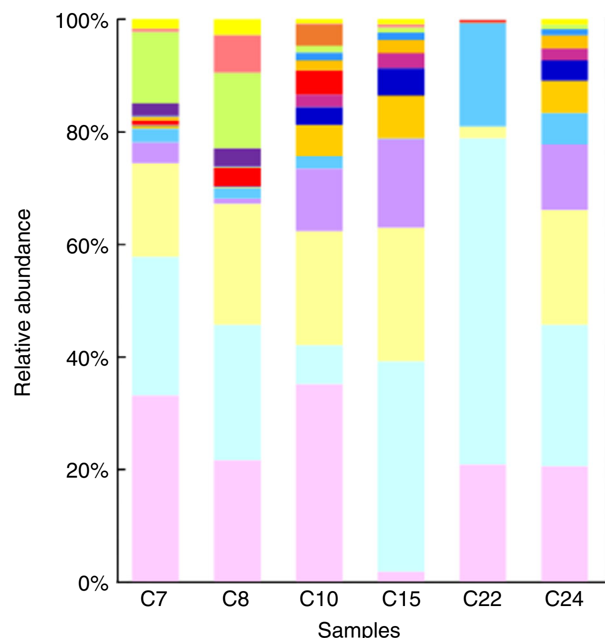

Figure 5 Plots of relative abundance (≥1%) composition of OTUs generated by the amplification of *16S rRNA* at the family level within the phylum Proteobacteria. Samples collected in the summer (C7, C8 and C10) and samples collected in the winter (C15, C22 and C24) (☐ Moraxellaceae; ☐ Pseudomonadaceae; ☐ Burkholderiaceae; ☐ Enterobacteriaceae; ☐ Flavobacteriaceae; ☐ Streptococcaceae; ☐ Staphylococcaceae; ☐ Pasteurellaceae; ☐ Shewanellaceae; ☐ Lactobacillaceae; ☐ Carnobacteriaceae; ☐ Planococcaceae; ☐ Unclassified; ☐ Pseudoalteromonadaceae; ☐ Aeromonadaceae; ☐ Xanthobacteraceae). [Colour figure can be viewed at wileyonlinelibrary.com]

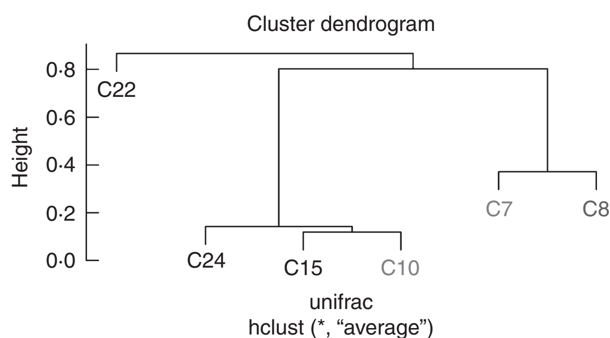

Figure 6 Hierarchical clustering with Unifrac distance between samples using average agglomeration method. Grey samples collected in the summer (C7, C8 and C10) and black samples collected in the winter (C15, C22 and C24).

using HPLC to determine the presence of histamine have been applied concomitantly in several studies. In particular, qPCR offers the possibility for the rapid detection and quantification of bacteria added of HPLC detection of toxins with greater specificity, sensitivity and reliability than traditional methods of culture (Postollec *et al.* 2011; Rodríguez-Lázaro *et al.* 2013). These techniques may represent good predictive methods for identifying a potential risk factor in fish products during processing, storage and marketing and may be used to investigate risk control strategies (Barbosa *et al.* 2018).

The micro-organisms most frequently associated with histamine poisoning in fish are Gram-negative bacteria, mainly Enterobacteriaceae (Biji *et al.* 2016) followed by species as *M. morganii* and *E. aerogenes*, which show high potential for enzyme histidine decarboxylase expression. The identification of these bacteria is of fundamental importance for food safety in order to avoid the possible deterioration and formation of histamine in fish (Zou and Hou 2017). Previous studies have shown that histamine is only detectable in fully decomposed fish or associated with a relevant growth (10^7 CFU per gram) of bacteria in fish muscle (Gardini *et al.* 2016).

Histamine is one of the most important biogenic amines in fish and is the only one with established legal limits for human consumption. Notably, all whitemouth croaker samples tested in the present study exhibited concentrations above 200 mg kg^{-1} of histamine the value established as the legal maximum limit by (NATIONS W.H.O.A.A.O.O.T.U. 2018). This legislation has been applied to fish of the Scombridae, Clupeidae, Engraulidae, Coryphaenidae, Pomatomidae and Scomberesocidae families. Furthermore, several foods have been implicated in food poisoning by histamine; for example, 239 mg g^{-1} of histamine was identified in Yellowtail fillet (*Seriola quinqueradiata*) supplied at a school restaurant in Seoul, Korea (Kang *et al.* 2018). Another study reported one death after ingestion of cooked crab with 470·8 mg g^{-1} of histamine (Yu *et al.* 2018).

The combination of time and temperature is the main determinant for the formation of histamine in food. Fish that are sold fresh are kept on ice at the recommended temperature range of 0·0–2·0°C and, may remain at an acceptable temperature of up to 4·0°C (NATIONS W.H.O.A.A.O.O.T.U. 2018). Moreover the enzyme histidine decarboxylase present in fish can continue to produce histamine, even with bacterial inactivation caused by cooling (Food and Administration 2011). Notably, muscle pH has a direct influence on the deteriorating microbiota present in fish and consequently affects histamine formation. The formation of lactic acid caused by the consumption of glycogen in post-mortem muscle causes acidification, reducing the pH of the muscle. As a consequence of acid stress, bacteria produce the decarboxylation of amino acids with the excretion of biogenic amines in an attempt to increase pH (Jacobsen *et al.* 2010; Trip *et al.* 2012; Fan *et al.* 2016). Optimal pH values for decarboxylating bacteria range from 2·5 to 6·0 (Gale 1946), though the enzyme histidine decarboxylase from *E. aerogenes* and *M.*

morganii bacteria exhibits better activity at pH 6·5 (Wendakoon and Sakaguchi 1995).

One of the main histamine-producing families is Enterobacteriaceae. This group of bacteria can grow at low temperatures, and their abundance decreases during storage in the cold chain, possibly since their growth rate is lower than that of other Gram-negative psychrotrophic decaying bacteria (Bahmani *et al.* 2011). Mesophilic bacteria considered as proliferative histamine producers, such as *M. morganii*, *Morganella psychrotolerans* and *E. aerogenes* can produce dangerous levels of histamine in a short period of time when stored at inadequate temperatures (Podeur *et al.* 2015).

Due to the complexity of the fish microbiota, culture-dependent detection methods may be insufficient; therefore, alternative methods are necessary to understand the diversity of the micro-organisms in these foods (Parlapani *et al.* 2018).

NGS is a modern sequencing method that allows us to identify the profile of microbial communities, monitor population fluctuations and characterize bacteria in food matrices. The taxonomic profile of a food microbial community can be obtained through the amplification of the *16S rRNA* gene (Laudadio *et al.* 2018). Through the amplification of the *16S rRNA* gene it was possible to identify bacteria in fresh whitemouth croaker muscle. The main phyla identified in whitemouth croaker samples (Proteobacteria, Bacteroidetes, Firmicutes and Actinobacterium) are commonly found in the autochthonous microbiota of the skin, gills, gut and intestinal contents (Llewellyn *et al.* 2014).

Among these groups of spoilage micro-organisms, Gram-negative bacteria are the main spoilage and histamine-forming bacteria in fresh fish (Macé *et al.* 2013). The main families of food spoilage bacteria found in fresh whitemouth croaker muscle samples in this study were largely represented by Gram-negative bacteria, members of the family Moraxellaceae, Pseudomonadaceae, Flavobacteriaceae, Burkholderiaceae and Enterobacteriaceae. A smaller proportion of the families Shewanellaceae, Aeromonadaceae, Pasteurellaceae and Xanthomonadaceae was also observed. Enterobacteria may occur in fish products as a result of faecal contamination, water pollution or contamination during processing (Huss 1995). In this group the species *M. morganii, Klebsiella pneumoniae, Hafnia alvei, Proteus vulgaris, E. aerogenes* and *E. cloacae* are present (Hazards EPoB 2011). In addition, species belonging to the genus *Clostridium, Vibrio, Acinetobacter, Plesiomonas, Pseudomonas, Aeromonas* and *Photobacterium* have also been reported as histamine producers (Visciano *et al.* 2012; Wongsariya *et al.* 2016). Under aerobic storage conditions, various groups of Gram-negative bacteria, particularly *Pseudomonas* sp., *Aeromonas* sp. and Enterobacteriaceae dominate deteriorating micro-organisms in freshwater and saltwater fish (Kung *et al.* 2017). However, species of the Moraxellaceae do not have the enzyme histidine decarboxylase (Özogul and Özogul 2005).

The acid-lactic bacteria found in this study may be present in the microbiota of fish intestinal contents (Ringo *et al.* 2018). The psychrotrophic BAL bacteria, *Leuconostoc, Lactobacillus* and *Carnobacterium* are responsible for the deterioration of fresh meat packed in a modified atmosphere and vacuum (Pothakos *et al.* 2015). The species composition and metabolic activity of such deteriorating communities are determined by the nature of the product, storage conditions as well as intra and inter-specific interactions (Andreevskaya *et al.* 2018). Notably, this occurs with species of the family Lactobacillaceae that are in low concentration in fish; however, they are predominant when stored in a modified atmosphere (Levin 2016). In addition, BAL can produce nitrogenous compounds such as biogenic amines in different refrigerated, frozen, fermented and smoked foods (Biji *et al.* 2016; Ordóñez *et al.* 2016; Triki *et al.* 2018).

Several intrinsic and extrinsic factors may influence the deteriorating microbiota in fish. Among the intrinsic parameters are mainly natural physical barriers (skin and mucus), microbiota present in fish (gut, gills and skin), chemical composition, pH and fish muscle water activity. The extrinsic parameters are related to the location of capture, processing, storage and sanitary conditions in which these fish were submitted. In addition, the level of deterioration will depend on the initial microbiota amount and interactions between the micro-organisms present in the fish (Llewellyn *et al.* 2014; Marshall 2014; Webster *et al.* 2018).

The methodologies used in the present study (qPCR, HPLC-DAD and NGS) facilitated the molecular characterization of food spoilage bacteria, quantification of histamine-producing bacteria to ensure the assessment of quality in fresh fish muscle. These techniques may be used together in the future as a predictive method to identify potential risks in the formation of histamine throughout the entire fish production chain.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Chromatogram of the histamine standard, 9·0 μl^{-1} (1), and (b) histamine identified in fresh Whitemouth croaker muscle (C15).

Figure S2. Calibration curve of the histamine standard.

Table S1. Oligonucleotides sequence designed for the gene histidine decarboxylase (*hdc*) of Gram-negative bacteria (GN), *E. aerogenes* (EA) and *M. morganii* (MM).

Table S2. Efficacy and reliability of the qPCR assay for the different *hdc* gene.

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