UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL - UFRGS
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS - ICTA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS - PPGCTA

TESE DE DOUTORADO

Caracterização e quantificação de bactérias descarboxiladoras de histidina e sua relação com a presença de histamina no pescado

Alessandra Danile de Lira

Porto Alegre
2019

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL - UFRGS
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS - ICTA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS - PPGCTA

TESE DE DOUTORADO

Caracterização e quantificação de bactérias descarboxiladoras de histidina e sua relação com a presença de histamina no pescado

Tese apresentada como requisito parcial para a obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Jeverson Frazzon

Porto Alegre
2019

CIP - Catalogação na Publicação

de Lira, Alessandra Danile
Caracterização e quantificação de bactérias descarboxiladoras de histidina e sua relação com a presença de histamina no pescado / Alessandra Danile de Lira. -- 2019.
106 f.
Orientador: Jeverson Frazzon.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Porto Alegre, BR-RS, 2019.

1. Corvina (Micropogonias furnieri). 2. Sardinha-verdadeira (Sardinella brasiliensis). 3. Histidina descarboxilase. 4. Histamina. 5. Microbioma. I. Frazzon, Jeverson, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

TESE DE DOUTORADO

Caracterização e quantificação de bactérias descarboxiladoras de histidina e sua relação com a presença de histamina no pescado

A Comissão Examinadora, abaixo assinada, aprova a Tese Caracterização e quantificação de bactérias descarboxiladoras de histidina e sua relação com a presença de histamina no pescado por Alessandra Danile de Lira, como requisito parcial para obtenção do Grau de Doutor em Ciência e Tecnologia de Alimentos.

Comissão Examinadora:

_____

Profª. Drª. Juliana Elisa Weeke

_____

Prof.ª. Drª. Katia Rezzadori

_____

Prof.ª. Drª. Priscila de Oliveira Moraes

_____

Drª. Ana Paula Murterle Varela

Porto Alegre, 13 de setembro de 2019

RESUMO

A qualidade do pescado é de grande importância, visto que é um alimento de alto valor nutritivo, entretanto muito suscetível à deterioração e formação de substâncias prejudiciais ao homem. Dentre as substâncias produzidas está a histamina, que é uma amina biogênica encontrada em alimentos. Quando ingerida em grande quantidade pode causar intoxicação alimentar. No pescado, a histamina pode ser produzida no tecido muscular através da descarboxilação do aminoácido histidina. Essa reação é catalisada pela enzima histidina descarboxilase (Hdc) produzida por bactérias. Más práticas de produção, higiene e a manutenção inadequada do pescado na cadeia do frio potencializam a presença de histamina no pescado. Testes para determinar e quantificar a histamina e as bactérias responsáveis pela produção da histamina no pescado é importante para a saúde pública e segurança alimentar. Entre as técnicas, destaca-se a cromatografia líquida de alta eficiência (HPLC), um dos principais métodos de referência para identificação e quantificação de histamina em alimentos. A técnica por HPLC com detector de arranjo de diodos (DAD) permitido a determinação rápida e eficiente de histamina em alimentos. A Reação em Cadeia da Polimerase quantitativa (qPCR) em tempo real foi usada para a identificação e quantificação do gene da enzima histidina descarboxilase (gene *hdc*) presente nas bactérias descarboxiladoras de histidina. A qPCR apresenta inúmeras vantagens, principalmente por detectar células viáveis e não viáveis, é uma técnica mais rápida, específica, sensível e de boa reprodutibilidade. O sequenciamento de alto rendimento (HTS) através da amplificação do gene *16S rRNA* foi um aliado para identificação e caracterização da microbiota presente no tecido muscular dos peixes e relaciona-las com as bactérias produtoras de histamina em peixes. Portanto, o objetivo deste estudo foi detectar e quantificar bactérias capazes de descarboxilar a histidina livre e relacionar a quantidade de histamina e sua microbiota em peixe fresco e congelado. De acordo com os resultados obtidos por HPLC-DAD, a concentração de histamina ficou acima de 200 mg kg^{-1} em todas as amostras de corvinas frescas e das sardinhas frescas e congeladas. O gene da histidina descarboxilase (hdc) de bactérias Gram negativas, *Morganella morganii* e *Enterobacter aerogenes* foi quantificado em todas as amostras de peixes. Os microrganismos mais abundantes presentes nas corvinas frescas foram bactérias pertencentes a família *Moraxellaceae*, enquanto Pseudomonadaceae esteve mais presente nas amostras de inverno. Nas sardinhas frescas os gêneros *Macrococcus*, *Acinetobacter* e *Pseudomonas* foram mais prevalentes, enquanto, nas sardinhas congeladas aos gêneros *Phyllobacterium*, *Pseudomonas* e *Acinetobacter* foram mais presentes. Os métodos utilizados demonstram ser úteis na avaliação da qualidade microbiológica e química dos peixes comercializados no mercado, frescos e congelados e, no futuro, poderão fazer parte do sistema estratégico de rastreabilidade da histamina na cadeia de produção de peixes.

Palavras-chave: corvina (*Micropogonias furnieri*), sardinha-verdadeira (*Sardinella brasiliensis*), histidina descarboxilase, histamina, microbioma.

ABSTRACT

The quality of fish is of great importance, as it is a food of high nutritional value, yet very susceptible to spoilage and formation of substances harmful to man. Among the substances produced is histamine, which is a biogenic amine found in foods. When ingested in large quantities it may cause food poisoning. In fish, histamine can be produced in muscle tissue by decarboxylation of the amino acid histidine. This reaction is catalyzed by the histidine decarboxylase enzyme (Hdc) produced by bacteria. Poor production practices, hygiene and improper maintenance of cold chain fish potentiate the presence of histamine in the fish. Testing to determine and quantify histamine and the bacteria responsible for histamine production in fish is important for public health and food safety. Among the techniques, we highlight the high performance liquid chromatography (HPLC), one of the main reference methods for identification and quantification of histamine in foods. Currently, the diode array detector (DAD) HPLC technique has been studied for fast and efficient determination of histamine in foods. Another analysis is the real-time quantitative polymerase chain reaction (qPCR) being studied for the identification and quantification of the histidine decarboxylase enzyme gene (*hdc* gene) for the identification of histidine decarboxylating bacteria. qPCR has many advantages, mainly because it detects viable and non-viable cells. It is a faster, specific, sensitive and reproducible technique. High Throughput Sequencing (HTS) by amplifying the *16S rRNA* gene can be an ally for identifying the microbiota present in fish muscle tissue and relating them to histamine-producing bacteria in fish. Therefore, the aim of this study was to detect and quantify bacteria capable of decarboxylating free histidine and to relate the amount of histamine and its microbiota in fresh and frozen fish. According to the results obtained by HPLC-DAD, the histamine concentration was above 200 mg kg-1 in all fresh and frozen sardines samples. The histidine decarboxylase (hdc) gene from Gram negative bacteria *Morganella morganii* and *Enterobacter aerogenes* was quantified in all fish samples. The most abundant microorganisms present in fresh Whitemouth croaker were bacteria belonging to the *Moraxellaceae* family, while *Pseudomonadaceae* was more present in winter samples. In fresh sardines the genera Macrococcus, Acinetobacter and *Pseudomonas* were more prevalent, while in frozen sardines the genera *Phyllobacterium*, *Pseudomonas* and *Acinetobacter* were more present. The methods used prove to be useful in assessing the microbiological and chemical quality of fresh and frozen commercially marketed fish and may in future be part of the strategic histamine traceability system in the fish production chain.

Keywords: Whitemouth croaker (*Micropogonias furnieri*), sardine (*Sardinella brasiliensis*), histidine decarboxylase, histamine, microbiome.

LISTA DE SÍMBOLOS

AB	Aminas biogênicas
aa	Aminoácidos
pH	potencial hidrogeniônico
%	Porcentagem
BPH	Bactérias Produtoras de histamina
hdc	Gene da Histidina Descarboxilase
qPCR	Reação em Cadeia da Polimerase quantitativa
HPLC	Cromatografia Líquida de Alta Eficiência
t	toneladas
%	Porcentagem
DTAs	Doenças Transmitidas por Alimentos
°C	Graus Celsius
UFC	Unidade Formadora de Colônias
cm^2	Centímetros quadrado
g	grama
BAL	Bactérias Ácido Lácticas
OMS	Organização Mundial de Saúde
OPAS	Organização Pan-Americana de Saúde
EFSA	*European Food Safety Authority*
STEC	Toxina Shiga
CO_2	Dióxido de Carbono
MAO	Monoaminas-Oxidase
DAO	Diamina-Oxidase
HNMT	N-metiltransferase
NMH	N-metil-histamina
min	minutos
h	hora
mg	miligrama
kg	quilograma
c	Número de aceitação
m	Limite mínimo de histamina
M	Limite máximo superior

FAO	Organização das Nações Unidas para a Alimentação e Agricultura
OMS	Organização Mundial da Saúde
nº	número
MERCOSUL	Mercado Comum do Sul
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
HPLC	Cromatografia líquida de alta eficiência
HPLC-MS	Cromatografia líquida acoplada a espectrometria de massa em tandem
HPLC- DAD	Cromatografia líquida acoplada ao detector de arranjo de diodos
ELISA	Ensaio de Imunoabsorção Enzimática
PLP	Piridoxal-5'-fosfato
Hdc	Enzima Histidina Descarboxilase
GN	Bactérias Gram negativas
GP	Bactérias Gram positivas

LISTA DE TABELAS

REVISÃO DE LITERATURA

Tabela 1. Surtos de origem alimentar comprovados causados pela ingestão de histamina..... 24
Tabela 2. Fonte de Histamina no corpo Humano..... 25
Tabela 3. Peixes associados a uma quantidade elevada concentração de histidina livre no tecido muscular..... 26
Tabela 4. Microrganismos capazes de metabolizar histamina no pescado..... 31
Tabela 5. Oligonucleotídeos utilizados em ensaios de PCR para a detecção do gene *hdc* em bactérias gram negativa em alimentos..... 32

ARTIGO I

Table S1. Oligonucleotides sequence designed for of the gene histidine decarboxylase *(hdc*) of Gram-negative bacteria (GN), *E. aerogenes* (EA) and *M. morganii* (MM)…… 40
Table 1. Values of weight, superficial temperature, pH and histamine of the fresh Whitemouth croaker muscle collected in different seasons…… 44
Table S2. Efficacy and reliability of the qPCR assay for the different *hdc* gene…… 45
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 genes* present in the Whitemouth croaker muscle collected in the different seasons…… 45

ARTIGO II

Table 1. Median, Interquartile interval of weight, pH and histamine of sardines by type of conservation…….. 70
Table 2. Efficacy and reliability of the qPCR assay for the different oligonucleotides of the *hdc* gene…… 70
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……. 71

LISTA DE FIGURAS

REVISÃO DE LITERAURA

Figura 1. Principais Aminas biogênicas e seus aminoácidos precursores..... 21

Figura 2. Estrutura química das aminas biogênicas..... 22

Figura 3. Reação de Dansilação de aminas biogênicas com Cloreto de Dansila (cloreto de 5-dimetilaminoonaftaleno-l-sulfonila), adaptado de SILVA, 2005..... 27

Figura 4. Biossíntese de Histamina..... 29

ARTIGO I

Figure S1. HPLC-DAD chromatogram of Histamine (A) and sardine sample, S15 (B). Analytical curves of the histamine standard (C)..... 45

Figure S2. Calibration curve of the histamine standard…… 45

Figure 1. Comparison of the number of copies of the *hdc* gene (log $_{10}$ g^{-1}) 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 ( ) and winter ( )…… 46

Figure 2. Comparison between histamine concentration and Whitemouth croaker samples collected in different seasons……... 47

Figure 3. Chart showing the interaction between histamine concentration and pH of muscle samples collected in different seasons. Sample: summer and winter …… 49

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%…….. 51

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)…….. 52

LISTA DE FIGURAS

ARTIGO II

Figure S1. HPLC-DAD chromatogram of Histamine (A) and sardine sample, S15 (B). Standard curves of the histamine pattern (C)…….. 70
Figure 1. Alpha diversity measurements of microbial communities in the sardines muscle tissue. Represented by Simpson index, Chao1 diversity index and Shannon diversity index. Boxes span the first to third quartiles; the horizontal line inside the boxes represents the median. Whiskers extending vertically from the boxes indicate variability outside the upper and lower quartiles, and the single circles indicate outliers. No significant difference was found among the three groups ($p > 0.05$) according to the non-parametric Wilcoxon test…….. 72
Figure 2. Principal Coordinate Analysis (PCoA) of bacterial communities. Based on Bray Curtis dissimilarity index with Jaccard index, Weighted-Unifrac and Unweighted-Unifrac regarding conservation type. Color: Blue represents frozen sardines (S1, S2 S4, S7 and S12) and Red represents fresh sardines (S13, S14, S17, S18 and S19).……. 73
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).…… 74
Figure 4. Relative abundance of the dominant bacterial phyla presents in the muscle tissue of sardines. Samples: frozen sardines (S13, S14, S17, S18 and S19) and fresh sardines (S1, S2, S4, S7 and S12).…… 75
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); frozen sardines (S13, S14, S17, S18 and S19).…… 76
Figure 6. Heat map showing non-metric multidimensional scaling (NMDS) (hierarchical grouping of samples and taxon's based on Bray-Curtis distance) of composition of the microbiota present 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, with blue indicating high abundance and dark blue indicating low abundance.… 77
Figure 7. Volcano Plot of ANCOM differential abundance. For analysis, the family-level log-scale OTUs table was used. The x-axis value represents the mean difference of clr transformed in the abundance of a given family between the fresh and frozen sardines groups. A positive x-axis means that the family is differentially abundant in frozen sardines and a negative x-axis means that the family is differentially abundant in fresh sardines. Statistical analysis w represents the number of paired comparisons that were considered significantly different..…… 78

SUMÁRIO

1. INTRODUÇÃO E OBJETIVOS 13
2. CAPÍTULO I - REVISÃO DE LITERATURA 16

2.1 Produção e consumo de pescado 16

2.2 Deterioração Microbiana do Pescado 16

2.3 Doenças Transmitidas por Alimentos (DTAs) 18

2.4 Aminas Biogênicas (AB) 21

2.5 Histamina nos Alimentos 23

2.6 Determinações de histamina em alimentos 26

2.7 Legislação 27

2.8 Bactérias produtoras da enzima histidina descarboxilase (Hdc) 29

CAPÍTULO II 33
ARTIGO I CHARACTERIZATION OF MICROORGANISMS AND QUANTIFICATION OF HISTAMINE PRESENT IN WHITEMOUTH CROAKER (*MICROPOGONIAS FURNIERI*) BY HTS, QPCR AND HPLC-DAD 33
CAPÍTULO III 58
ARTIGO II DETERMINATION OF HISTAMINE AND CHARACTERIZATION OF BACTERIA COMMUNITIES PRESENTS IN SARDINES (*SARDINELLA BRASILIENSIS*) MUSCLE….. 58
CAPÍTULO IV DISCUSSÃO GERAL 87
CAPÍTULO V: CONCLUSÃO 93
REFERÊNCIAS 95

1. INTRODUÇÃO E OBJETIVOS

As aminas biogênicas (AB) são bases orgânicas de baixo peso molecular que são produzidas naturalmente por microrganismos, plantas e animais, por consequência do seu metabolismo. Podem ser formadas de forma exógena por bactérias, fungos e leveduras principalmente pela descarboxilação de aminoácidos (aa) em alimentos de origem vegetal, animal e bebidas. Os precursores das AB são os aa livres fornecidos por alterações proteolíticas de proteínas e/ou peptídeos presente nos alimentos. A formação das AB pode ser influenciada pela composição da matéria-prima, pH, presença de microrganismos, tipo de processamento e armazenamento dos alimentos (Kovacova-Hanuskova et al., 2015; Gardini et al., 2016). Estes compostos têm sido relacionados a baixa qualidade higiênica, por estarem associados a um crescimento considerável de bactérias deterioradoras em alimentos (Tabanelli, Montanari e Gardini, 2019).

A histamina é uma das AB mais importante em alimentos, principalmente o pescado. A intoxicação por histamina, frequentemente denominada "intoxicação por escombrotoxina" é causada por ingestão de espécies de peixes marinhos que contêm altos níveis de histamina. Provocam diversos sintomas, como hipertensão ou hipotensão, dores de cabeça, náuseas, edema, erupção cutânea generalizada, urticária, problemas respiratórios rinite, edema pulmonar (Prester, 2011). A intoxicação por histamina é comum na Europa e outros continentes. Na Europa em 2017, foi terceiro agente mais comum identificado em surtos alimentares. Nos EUA, por exemplo, representa 5% das intoxicações alimentares. Portanto, a histamina é uma toxina bastante relevante para a segurança alimentar (Tortorella et al., 2014; EFSA e ECDC, 2018).

No pescado, a histamina é produzida no tecido muscular dos peixes através da descarboxilação do aa livre histidina, reação catalisada pela descarboxilase exógena microbiana. Esta habilidade está presente em diversas espécies de bactérias gram negativas e gram positivas (Ladero et al., 2010). Os microrganismos descarboxiladores podem ser provenientes da água, ou está presente na pele, brânquias e conteúdo intestinal dos peixes ou serem introduzidos durante processamento e conservação do pescado. As bactérias produtoras de histamina (BPH) são capazes de multiplicarem-se rapidamente em condições de refrigeração inadequadas promovendo a formação da histamina na musculatura dos peixes (Visciano et al., 2014). A enzima histidina decarboxilase (Hdc) uma vez sintetizada, ela pode continuar catalisando a reação de descarboxilação mesmo sobre temperaturas de refrigeração e, ainda, se mantém estável durante o congelamento do peixe, podendo ser reativada logo após o seu

descongelamento. No entanto, a toxina histamina formada no alimento não é eliminada por calor ou congelamento (FDA, 2011).

O objetivo da pesquisa foi identificar, caracterizar e quantificar bactérias descarboxiladoras de histidina livre e relacionar com a quantidade de histamina e a microbiota presente no tecido muscular de corvina (*Micropogonias furnieri*) fresca coletadas em diferentes sazonalidades (verão e inverno) e de sardinhas frescas e congeladas comercializadas em um mercado popular de peixe no município de Porto Alegre, Rio Grande doi Sul.

CAPÍTULO I

REVISÃO DE LITERATURA

2. CAPÍTULO I - REVISÃO DE LITERATURA

2.1 Produção e consumo de pescado

A produção mundial de pescado, em 2016, atingiu 171 milhões de toneladas (t). Desse total, 90,9 milhões t foram provenientes da pesca extrativista, dos quais 87,2 milhões t foram de águas marinhas (79,3%) e 12,8 milhões t de águas interiores (11,6%). A China é o maior produtor na pesca marinha, seguida por Indonésia e Estados Unidos. Dos peixes destinados para consumo humano direto, 31% refere-se a peixe congelado, seguido de peixe vivo, fresco ou refrigerado (45%). O restante da produção, diz respeito a produto preparado (12%) e curado (12%). A percentagem da produção pesqueira mundial utilizada para consumo humano foi de 1484 milhões de t, com um consumo aparente per capita de 20,3 kg (FAO, 2018a).

O consumo mundial de peixe foi de 148,8 t de peso vivo, em 2016. A América Latina e Caribe foram consumidos apenas 19,7 t de peso vivo, com um consumo aparente per capita de 9,80 kg (FAO, 2018a). Em média, o brasileiro consome menos de 10 kg de peixes por ano. Um consumo abaixo do que é recomendado pela Organização das Nações Unidas para a Alimentação e a Agricultura, 12 kg/hab/ano. Entretanto, a média de consumo mundial é superior a 20 kg/ hab/ano (Dellova et al., 2019).

A produção de pescado no Brasil, em 2016 foi de 1.286 mil t, deste total a pesca extrativista obteve um total de 705.000 t, incluindo peixe, mariscos e crustáceos. A pesca extrativista marinha somou 580 500 t (FAO, 2018b). O Rio Grande do Sul apresenta uma extensão de litoral de 622 km, que representa 8,39% do total do litoral brasileiro. No Estado, há apenas um porto de pesca industrial expressivo, o do Rio Grande, situado na Laguna dos Patos, onde são desembarcados os produtos da pescaria provenientes de ambiente estuarino, lacunar, costeiro e alto-mar. As principais espécies desembarcadas, em 2011, no porto de Rio Grande, provenientes da pesca artesanal marinha estuarina (88,5%) foram a corvina, tainha e o camarão-rosa. Já na pesca marinha industrial os principais pescados foram a corvina, castanha e pescada-olhuda (80%) (SUDEP, 2003; IBAMA e CEPERG, 2012).

2.2 Deterioração Microbiana do Pescado

O pescado é um alimento mais perecível entre os produtos de origem animal devido à sua composição biológica. Os altos níveis de umidade, aminoácidos livres, outros compostos

nitrogenados e proteínas de fácil digestão contribuem para a deterioração do pescado. Os tecidos de peixe têm níveis elevados de compostos nitrogenados não proteicos (aa livre, óxido de trimetilamina, creatinina, putrescina, cadaverina), peptídeos e proteínas. Essa perecibilidade do pescado também está relacionada a fatores intrínsecos (atividade da água, pH, teor de nutrientes) e fatores extrínsecos (temperatura de conservação, umidade relativa de armazenamento e atividade microbiana) (Erkmen e Bozoglu, 2016; Majumdar et al., 2018).

Três tipos de deterioração podem ocorrer no pescado, a autolítica, a microbiana e a oxidativa. A perda de qualidade inicial é explicada principalmente pela autólise, especialmente a degradação de nucleotídeos por enzimas autolíticas. As perdas do nucleotídeo intermediário, monofosfato de inosina (IMP), são responsáveis pela perda de aroma do peixe fresco. Estas alterações autolíticas tornam disponível catabólitos para o crescimento bacteriano. As proteases também participam da deterioração e estão relacionadas ao amolecimento da carne e o rompimento da cavidade celomática dos peixes (HUSS, 1999).

As bactérias proteolíticas produzem proteinases extracelulares que hidrolisam proteínas de peixe e fornecem peptídeos e aminoácidos para outras bactérias deterioradoras. Os compostos voláteis da putrefação de proteínas resultarão na formação de diferentes tipos de odor livre, como o peixe (devido à trimetilamina) e odor pútrido. O crescimento bacteriano provoca descoloração de brânquias e olhos em peixe e perda de textura muscular (suavizada devido a proteólise) e produção de muco (Erkmen e Bozoglu, 2016).

A microbiota associada a peixes reflete a microbiota do meio ambiente no momento da captura, mas é modificada pela capacidade de diferentes microrganismos se multiplicarem na superfície da pele, brânquias e conteúdo intestinal. As bactérias isoladas da pele e as brânquias podem ser transitórias (alóctone), uma vez que estão diretamente em contato com a água. Entretanto, a microbiota intestinal pode variar com a complexidade do sistema digestivo do peixe. É constituída principalmente por bactérias anaeróbias obrigatórias, facultativas e podem ser classificadas como autóctone ou alóctone (Ringøa et al., 2003). A manipulação durante a captura, transporte, processamento também pode influenciar na microbiota desses peixes (Remenant et al., 2014).

A quantidade e diversidade bacteriana podem variar de acordo com sazonalidade, devido a variação da temperatura da água e suspensão de sólidos. O pescado de águas mais frias (< 10-15 °C) geralmente produzem contagens de 10^2-10^4 UFC cm^2 na pele e superfície das brânquias, enquanto peixes de águas quentes têm níveis de 10^3-10^6 UFC cm^2. O conteúdo

intestinal varia de 10^2 UFC g^{-1} em peixes não alimentados para 10^8 UFC g^{-1} em espécies alimentadas (Cahill, 1990; Kasuga, 2005; SkrodenytėArbaČIauskiene, 2007).

A poluição ambiental também contribui com essa diversidade microbiana nos peixes, como espécies da família *Enterobacteriaceae*. O intestino dos peixes, por exemplo, pode conter espécies de *Alcaligenes*, *Pseudomonas*, *Flavobacterium* e *Vibrio*. Ainda pode conter bactérias patogênicas contaminantes da água e do solo, como *Bacillus*, *Clostridium*, *Escherichia* e *Serratia* (Erkmen e Bozoglu, 2016).

O comitê do Codex Alimentarius (2018) listou as principais bactérias autóctones dos peixes que podem representar um perigo para a saúde humana, são *Aeromonas hydrophila*, *Clostridium botulinum*, *Vibrio parahaemolyticus*, *V. cholerae*, V. vulnificus e *Listeria monocytogenes*. Além de outras espécies que podem causar Doenças Transmitidas por Alimentos (DTA) e que foram isoladas de peixes, *Edwardsiella tarda*, *Plesiomonas shigelloides* e *Yersinia enterocolitica*.

As bactérias deteriorantes presentes no tecido muscular dos peixes refrigerados geralmente são gram negativas. Principalmente os gêneros *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Chromobacterium*, *Cytophaga*, *Enterobacter*, *Flavobacterium*, *Halobacterium*, *Moraxella*, *Morganella*, *Photobacterium*, *Pseudomonas*, *Shewanella* e *Vibrio*. No entanto, algumas bactérias gram positivas podem estar presentes, *Streptococcus*, *Staphylococcus*, *Microbacterium*, *Corynebacterium*, *Brochothrix*, *Bacillus*, *Arthrobacter*, *Lactobacillus* e *Listeria* (Marshall, 2012).

Muitas bactérias *Shewanella putrefaciens* e *Pseudomonas spp.* são bactérias deterioradoras específicas de peixe fresco, independentemente de sua origem. Peixes marinhos de águas temperadas embalados em atmosfera modificada são contaminados principalmente por *Photobacterium phosphoreum*, enquanto que, as bactérias gram positivas são deterioradoras prováveis presentes em peixes de água doce ou tropicais (Gram e Huss, 1996).

2.3 Doenças Transmitidas por Alimentos (DTAs)

Segundo a Organização Mundial de Saúde (OMS), uma das prioridades global é a inocuidade de alimentos, além de ser uma questão de saúde pública, visto que as DTAs são importante causa de óbitos em todo mundo. As DTAs são causadas pela ingestão de alimentos e água e abrangem uma série de doenças causadas por bactérias, vírus, parasitas, contaminantes químicos, toxinas e biotoxinas. Para diminuir o número de casos, é necessário aplicar normas e práticas de inocuidade a toda cadeia produtiva dos alimentos (WHO, 2015).

Reconhecendo a importância do risco alimentar a saúde em todo o mundo, a OMS aprovou a Estratégia Regional de Segurança Alimentar do Pacífico Ocidental (2011-2015) na resolução WPR/RC62.R513 (2011) estabelecendo como prioridade a inocuidade dos alimentos e a prevenção e o controle de DTAs (OMS, 2011). A Organização Pan-Americana de Saúde (OPAS) também adotou como prioridade a inocuidade dos alimentos como um dos objetivos do seu Plano Estratégico 2014-2019 (WHO, 2015). A OPAS tem um sistema de informação regional sobre surtos de DTAs da América Latina e Caribe. Em um relatório elaborado, pela OPAS em 22 países (1993 a 2010), constatou que, dos 9.180 surtos em que o agente causador havia sido identificado, 69% foram de origem bacteriana, 9,7% de origem viral, 9,5% foram causados por toxinas marítimas, 2,5% por contaminantes químicos, 1,8% por parasitas e 0,5% por toxinas vegetais (OPAS, 2013).

Durante 2009-2015, o Sistema de Vigilância de Surtos de DTAs dos Estados Unidos recebeu relatos de 5.760 surtos que resultaram em 100.939 doenças, 5.699 hospitalizações e 145 mortes. Entre os 2.953 surtos com uma única etiologia confirmada, o Norovírus foi a causa mais comum de surtos (38%), seguido por Salmonela (30%). Entre 1.281 surtos nos quais os alimentos relatados puderam ser classificados em uma única categoria de alimentos, os peixes foram a categoria mais comumente implicada (17%), seguidos por laticínios (11%) e frango (10%). Nesse período foram relatados 215 surtos por biotoxinas marinhas, num total de 739 doentes, 87 hospitalizações e 5 mortes. A intoxicação por histamina foi a mais relata, com 95 casos confirmados (Dewey-Mattia et al., 2018).

O relatório sobre surto alimentar realizado pela *European Food Safety Authority* (EFSA) na União Europeia em 2015 mostrou que a maioria dos surtos alimentares foi causada por agentes bacterianos (33,7% de todos os surtos), em particular Salmonela e *Campylobacter* sp. Em seguida, as toxinas bacterianas, parasitoses e outros agentes. Em particular, a intoxicação por histamina foi relatada em menos de 3% dos surtos (EFSA, 2016). Em 2017, a intoxicação por histamina foi o terceiro agente mais comum em surtos na União Europeia. Observando um aumento de 22% comparando com o ano anterior (21 casos a mais). Esse aumento está relacionado ao aumento significativo do consumo de peixe cru, principalmente na França e Espanha (EFSA, 2018).

De acordo com o último informe da Vigilância Epidemiológica do Ministério da Saúde, em 2018, foram registrados 503 surtos de DTA e 6.803 pessoas doentes e um total de 9 óbitos contra 598 surtos e 9.426 pessoas doentes e 12 óbitos no ano de 2017 (Brasil, 2019). Conforme notificações da Vigilância Epidemiológica entre os anos de 2009 a 2018 a distribuição dos surtos por local de ocorrência, as residências (37,7% - 6.809 casos) são os principais locais de

ocorrência mais associado aos surtos. A distribuição dos alimentos incriminados (2.345 surtos) confirmados nesse período foram os alimentos mistos (25,5%), a água (21,1%) e o pescado contribuiu apenas com 2,1% desses surtos. Dentre os agentes etiológicos identificados como únicos responsáveis pelos surtos confirmados, a *Escherichia coli* (23,4%) foi a mais comum, seguido de *Salmonella* spp (11,3%) e *Staphylococcus aureus* (9,4%) (Brasil, 2019).

No Estado do Rio Grande do Sul entre os anos de 2000 a 2014 foram notificados 2.620 surtos, sendo 854 (51,1%) confirmados. Entre os agentes identificados como responsáveis pelos surtos no estado, observaram-se *Salmonela* spp. (803 casos), *Staphylococcus aureus* (219 casos) e *Bacillus cereus* (129 casos) (Rio Grande do Sul, 2015).

2.4 Aminas Biogênicas (AB)

As AB são compostos orgânicos, básicos, nitrogenados de baixo peso molecular e não voláteis. Podem ser produzidas por bactérias, pela descarboxilação enzimática dos aa livres nos alimentos e bebidas fermentados ou não. Atuam seletivamente em aa específicos na remoção do grupo alfa-carboxila com a formação da correspondente da amina e CO_2 (Gardini et al., 2016). A Figura 1 apresenta as principais AB encontradas como contaminantes de alimentos, seus respectivos aminoácidos precursores, em destaque esta a histamina uma das AB mais importantes.

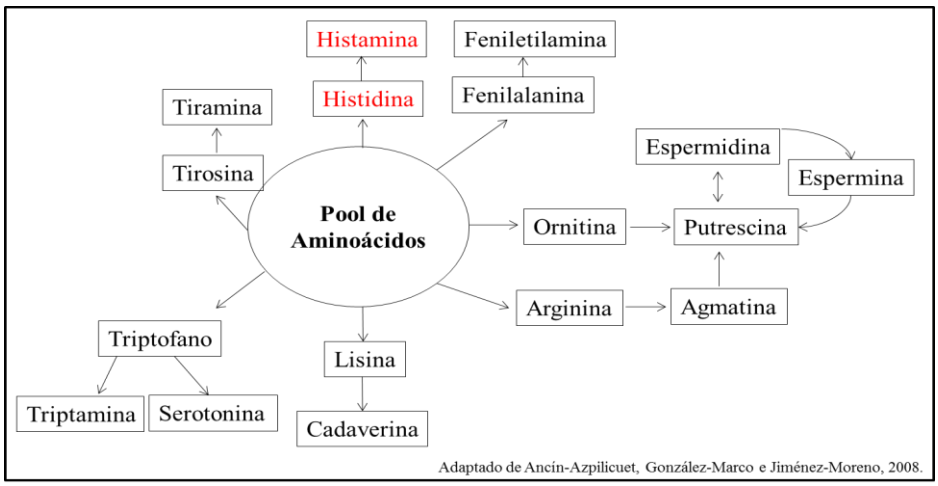
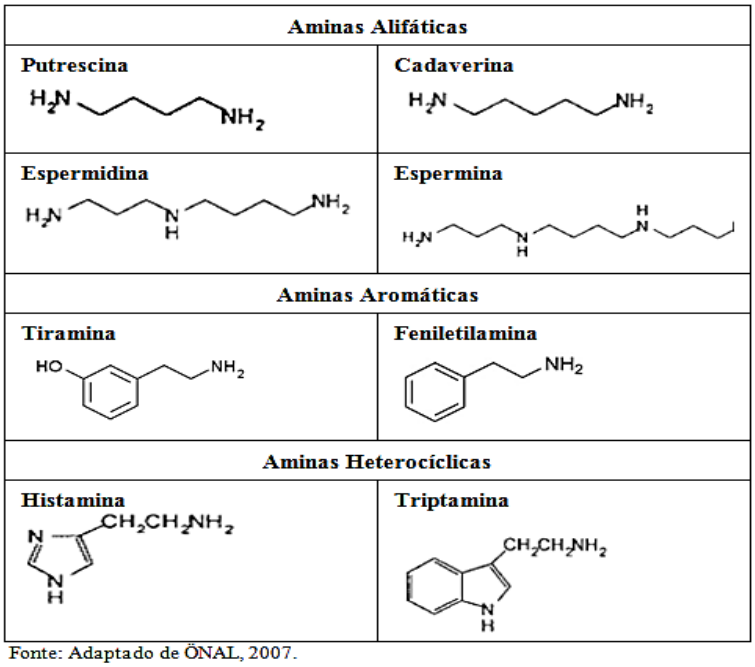


Figura 1. Principais Aminas biogênicas e seus aminoácidos precursores.

As AB ainda podem ser formadas e degradadas em processos fisiológicos normais em humanos, animais e plantas. Funcionam no corpo humano como, neurotransmissores, participando da transmissão sináptica, controle da pressão arterial, regulação da temperatura corporal, volume e pH do estômago, apetite, crescimento celular e resposta alérgica (Danquah, Benjakul E Simpson, 2012). Podem ser classificadas de acordo com sua estrutura química: heterocíclicas (histamina e triptamina), alifáticas (putrescina, cadaverina, espermina e espermidina) e aromáticas (tiramina e feniletilamina) bem como pelo número de amina: monoaminas (tiramina e Feniletilamina), diaminas (Histamina, putrescina e cadaverina) e poliaminas (Espermidina, espermina e agmatina) (Figura 2) (De La Torre e Conte-Junior, 2018).



Fonte: Adaptado de ÖNAL, 2007.

Figura 2. Estrutura química das Aminas Biogênicas.

A ingestão elevada de alimentos contaminados com AB podem causar problemas à saúde humana, levando a sintomas como, hipotensão, hipertensão, distúrbios gastrointestinais e enxaqueca. Assim, é crucial reduzir a formação e os níveis desses compostos nos alimentos (Danquah, Benjakul e Simpson, 2012). Pequenas quantidades de AB são metabolizadas normalmente com a ingestão de alimentos no intestino humano formando produtos fisiologicamente menos ativos. As enzimas Monoamina-Oxidase (MAO) e Diamina-Oxidase (DAO) desempenham um papel fundamental na degradação da AB no corpo humano. Na intoxicação alimentar por AB deve ser considerado, além do nível tóxico da amina no alimento, também a quantidade do alimento ingerido, a presença e concentração de outras aminas (cadaverina, putrescina e triptamina), ingestão de álcool e outros componentes alimentares também são importantes (Önal, 2007).

Para segurança alimentar a histamina e a tiramina são consideradas as mais tóxicas das AB. A putrescina e cadaverina são conhecidas por potencializar o efeito tóxico da histamina. Além disso, essas aminas são termoestáveis, e por isso, não são inativadas por tratamentos térmicos utilizados no processamento e preparação dos alimentos. Atualmente, apenas as estratégias de prevenção e monitoramento permitem o controle da formação de AB nos alimentos durante a cadeia produtiva (Alvarez e Moreno-Arribas, 2014).

Existem duas razões para a determinação de AB em alimentos. A primeira está relacionada ao seu potencial de toxicidade e a segunda é a possibilidade ser usada como

marcador de qualidade em alimentos. As análises de AB podem ser aplicadas no controle de matéria prima, produtos intermediários e finais, monitoramento de processos de fermentação, controle de processos, pesquisa e desenvolvimento de qualidade em alimentos (Önal, 2007).

Medidas de controle secundário podem ser adotadas na cadeia produtiva de alimento para prevenir a formação das AB. Algumas técnicas podem ser adotadas visando principalmente a redução ou eliminação do crescimento de microrganismos descarboxiladores de AB ou degradação das AB, tais como, controle da temperatura (Fan et al., 2016), pressões hidrostáticas, irradiação e embalagem em atmosfera controlada (Krîžek et al., 2017) ou uso de aditivos alimentares (Mohan et al., 2019; Hajimohammadi et al., 2019) e microrganismos degradadores de AB (Alvareza e Moreno-Arribas, 2014; Bäumlisberger et al., 2015).

2.5 Histamina nos Alimentos

A histamina (2-[4-imidazolil] etilamina) é uma substância endógena que ocorre naturalmente no corpo humano, tem funções fisiológicas importantes relacionadas com respostas imunitárias locais, secreção de ácido gástrico, reações alérgicas e neuromodulação. Participam da regulação do sono, administração de energia, alimentação, consumo, liberação de ácido gástrico no estômago, aprendizado, memória e desempenha um papel na proliferação celular. A histamina pode também estar presente em certos alimentos que contêm histidina livre, e gerada por determinadas bactérias deterioradoras e fermentadoras de alimentos. Pode ser proveniente da atividade dos microrganismos residentes no intestino grosso que descarboxilam histidina presente em alimentos proteicos (Leurs et al., 2012; Kovacova-Hanuskova et al., 2015).

A histamina está presente em todos os alimentos que contenham proteínas ou histidina livre e que estão sujeitos a condições que permitem a atividade microbiana (Landete et al., 2008). Os alimentos que podem conter níveis elevados de histamina são peixes, queijos, chucrute, produtos cárneos fermentados. Ainda pode ser encontrada em bebidas, como, suco, chá, cerveja, cidra e vinho (Papageorgiou et al., 2018). A formação de histamina nos alimentos vai depender da presença de aa livres, tipo de microbiota presente no alimento, condições de favoráveis para crescimento de microrganismos produtores da enzima Hdc (pH, temperatura, concentração de oxigênio e teor de sal) e as condições de processamento e armazenamento desse alimento (Gagic et al., 2019). Portanto, é de fundamental importância monitorar a qualidade dos alimentos durante toda cadeia produtiva (Ruiz-Capillas e Herrero, 2019).

O envenenamento por histamina é a principal causa de doença relacionada ao pescado em todo mundo (Yu et al., 2018). O tipo de tecido muscular do peixe influencia a formação e

acumulo de histamina nos peixes. Neste contexto, o peixe de músculo escuro tem níveis mais elevados de histamina em seus tecidos do que os peixes de músculo branco. Isto é devido ao maior conteúdo do aa histidina no tecido de peixe de carne escura (Danquah, Benjakul e Simpson, 2012).

A intoxicação por peixes com histamina é uma intoxicação que pode ser causada pelo consumo de diferentes de peixes marinhos. Raramente os peixes de água doce, crustáceos e moluscos podem causar essa doença (Ryder, Iddya e Ababouch, 2014). A intoxicação por histamina pode ocorrer em indivíduos após a ingestão do alimento com alto teor de histamina (> 100 mg kg^{-1}) levando há um aumento da concentração de histamina sanguínea (Kovacova-Hanuskova et al., 2015). A Tabela 1 mostra os surtos alimentares por histamina ocorrida pela ingestão de pescado em diversos países.

Tabela 1. Surtos de origem alimentar comprovados causados pela ingestão de histamina.

País	Ano	Tipo de Alimento	Histamina (mg kg^{-1})	Referência
Senegal	2010	Atum	4900	Demoncheaux et al., 2012
Brasil	2013	Atum	1076,5 - 1534,7	Takemoto et al., 2014
Espanha	2014	Atum embalado a vácuo	3716	European Commission (2015)
Itália	2014	Posta de atum refrigerado	1.946	European Commission (2015)
Espanha	2015	Filé de atum	2692,18 e 4703,60	European Commission (2016)
Estados Unidos	2016	Tilápia cozida	290	Nordt e Pomeranz, 2016
China	2016	Siri cozido	47,08 a 1.893	Yu et al., 2018
Korea	2016	Isca de Seriola	293	Kang et al., 2018

A histamina ingerida pode ser inativada por duas enzimas no organismo humano. Uma das principais enzimas é a diamina oxidase (DAO ou histaminase) que converte a histamina em Imidazol Acetaldeído e ainda pode ser conjugada com ribose antes da excreção urinária (Schwelberger, 2004). A outra enzima é a N-metiltransferase (HNMT). O produto da metilação de histamina catalisada por HNMT é a N-metil-histamina (NMH), que é subsequentemente transformada pela monoamina oxidase (MAO) em acetaldeído de N-metilimidazol (Maintz e Novak, 2007). Os dois sistemas produzem produtos finais diferentes (Tabela 2), isso proporciona um meio pelo qual o destino da histamina da dieta pode ser caracterizada de forma independente da histamina originada dos processos metabólicos produzida pelo corpo (Joneja e Fellow, 2004).

Tabela 2. Fonte de histamina no corpo humano.

Fonte de histamina	Origem	Catabolismo da histamina	Local do catabolismo
Produto do metabolismo de microrganismos presente nos alimentos *in natura* Pescado, carnes, outros alimentos proteicos inadequadamente refrigerados e vegetais. Alimentos processados e fermentados Leite e derivados, suco, vinho, cerveja e outras bebidas alcoólicas.	Exógena	Catabolizado por Diamina Oxidase	Jejuno, íleo, rim, timo, placenta.
Histamina intrínseca em vários tecidos dependentes da função corporal e necessidade. Degranulação de mastócitos e outros granulócitos por lecitinas, em resposta a anticorpos e fatores neurogênicos.	Endógena	Catabolizado por N-metiltransferase	Estômago, timo, pulmão, rim, cérebro.

Fonte: Adaptado de Joneja e Fellow, 2004.

O efeito da histamina no organismo humano só ocorre quando é liberada em grande quantidade pelo organismo ou quando ocorre uma intoxicação alimentar (Karovicova e Kohajdova, 2005). Os sintomas de intoxicação por histamina podem ocorrer dentro de 30 min à 2h do consumo do alimento suspeito, mas, em raros casos, podem persistir por 12h há alguns dias (Ruethers et al., 2018). A gravidade dos sintomas pode variar, dependendo da quantidade de histamina e da sensibilidade de cada indivíduo (WHO e FAO, 2013). Os sintomas incluem rubor facial, dor abdominal, diarreia, dor de cabeça, palpitações, náuseas, vômitos, urticária, boca seca, tontura, e, raramente, respiração ruidosa ou perda de consciência devido a hipotensão. Alguns pacientes com envenenamento Escombroide também nota um sabor metálico, amargo, ou picante (Feng, Teuber e Gershwin, 2015). Historicamente a intoxicação por histamina é conhecida por "envenenamento por escombrotoxina" pela associação ao consumo de peixes contaminados das famílias *Scombridae* e *Scomberesocidae* (Niraj e Pandey, 2012).

O diagnóstico da intoxicação por histamina é geralmente baseado no histórico alimentar do paciente e os sintomas apresentados. O diagnóstico pode ser confirmado através da detecção de níveis elevados de histamina no alimento implicado, sobra do alimento ou do produto semelhante obtido a partir da mesma fonte. Os sintomas também podem ser confundidos com outras doenças, como alergias alimentares ou outras doenças não relacionadas a alimentos, dificultando seu diagnóstico (Feng, Teuber e Gershwin, 2015). O anti-histamínico é o tratamento de escolha para intoxicação por histamina. Os antagonistas H1 (difenidramina ou

clorfeniramina) são geralmente selecionados para o tratamento de envenenamento por histamina, embora os antagonistas H2 (cimetidina) podem também ser eficazes (Karovicova e Kohajdova, 2005).

O risco de intoxicação por histamina pode ser controlado pela aplicação de boas práticas de fabricação e higiene básica associada a um sistema apropriado de ponto crítico de controle de risco. Os peixes devem ser mantidos a uma temperatura próxima ao ponto de fusão do gelo, logo após a coleta, para atender aos critérios de frescor e evitar o crescimento de bactérias deterioradoras e produtoras de histamina. Todas as operações de processamento e beneficiamento do peixe devem ser realizadas de forma higiênica a bordo dos navios e nos entrepostos de pescado para garantir a qualidade do alimento (Visciano et al., 2014).

2.6 Determinações de histamina em alimentos

A determinação das AB ainda continua sendo um desafio na análise de alimentos, e isto são devido a vários fatores. Como a elevada polaridade e o peso molecular baixo das AB favorecem sua solubilidade em água. Além disso, a ausência de propriedades intrínsecas que podem facilitar sua detecção com métodos espectroscópicos ou eletroquímicos, tornando-o mais difícil sua quantificação. Várias técnicas de detecção foram usadas para a quantificação de AB em amostras de alimentos (Ruiz-Capillas e Herrero, 2019; Önal, Tekkeli e Önal, 2013). A quantificação é realizada principalmente por técnicas cromatográficas, como cromatografia líquida de alta eficiência (HPLC), cromatografia gasosa e cromatografia de camada delgada, cromatografia líquida acoplada à espectrometria de massa (HPLC-MS), cromatografia líquida acoplada detector de arranjo de diodos (HPLC-DAD), cromatografia liquida de ultra eficiência, cromatografia líquida de par iônico, Eletroforese Capilar e Ensaio de Imunoabsorção Enzimática (ELISA). Os métodos oficiais de análise da AOAC propõem o uso de métodos fluorimétricos (AOAC 977.13) ou outros métodos validados cientificamente equivalentes (AOAC, 1990; AOAC, 2012; Ordóñez et al., 2016).

O método mais utilizado para detecção de AB é a cromatografia líquida de alta eficiência (HPLC). Estes métodos são baseados em reações de separação, seguidos da derivatização e detecção ultravioleta ou detecção fluorescente. As amostras sólidas são extraídas com solventes ácidos, tais como ácido tricloroacético, ácido perclórico ou ácido clorídrico (Erim, 2013). HPLC é relatada como método mais eficiente, sensível e reprodutível em comparação com outros métodos analíticos, mas ainda é uma técnica que requer equipamentos e reagentes caros e exige pessoal capacitado (Marcobal, De Las Rivas e Muñoz, 2006).

Devido à baixa volatilidade das AB e a falta de agentes cromóforos, adaptação de procedimentos analíticos baseados na formação de derivados fluorescentes com diferentes agentes de derivatização é necessária para aumentar à sensibilidade da detecção ultravioleta ou detecção fluorescente. A técnica de derivatização pré-coluna é usada com maior frequência que a derivatização pós-coluna devido a detecção ser mais sensível. Vários reagentes foram testados para derivatizar a histamina, como por exemplo, o Cloreto de Dansila, O-ftaldialdeído, Éster de N-hidroxisuccinimida, 4-cloro-3,5-dinitrobenzotrifluoreto, 1,2-naftoquinona-4-sulfonato, 6-aminoquinolil-N-hidroxisiccinimida. O cloreto de dansila é o reagente mais utilizado em análise em amostras de peixes (Tahmouzi et al., 2011; Önal, Tekkeli e Önal, 2013).

O Cloreto de dansila é um composto por uma fração aromática altamente fluorescente (5-dimetilaminonaftaleno) e um grupo reativo (cloreto de sulfonila). O grupo reativo se liga aos analitos por meio de uma reação de substituição nucleofílica para produzir sulfonamidas (dansilamidas) fluorescentes azuis ou verdes azuladas (Figura 3). Os derivados do cloreto de dansila formados em condições alcalinas são estáveis que podem ser facilmente detectados por um detector ultravioleta, fluorescência, DAD, ou MS (Silva, 2005; Zazzu et al., 2019).

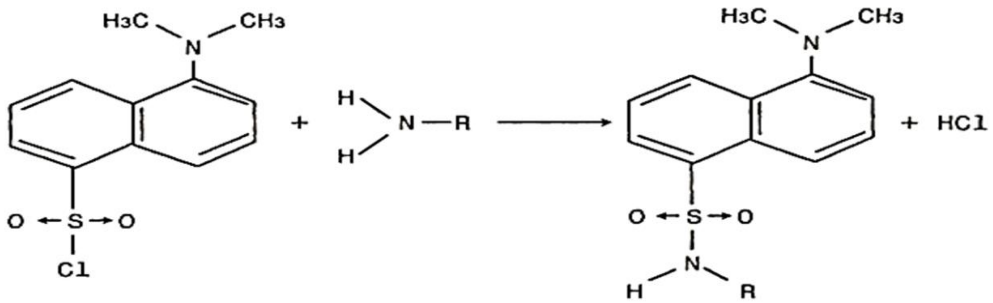


Figura 3. Reação de Dansilação de aminas biogênicas com Cloreto de Dansila (cloreto de 5-dimetilaminoonaftaleno-l-sulfonila), adaptado de Silva, 2005.

2.7 Legislação

A histamina é a principal AB investigada em peixes, sendo a única amina com limites legais estabelecidos para o consumo humano. Em 2018, o comitê do Codex Alimentarius revisou o Código de Prática para Peixes e Produtos da Pesca (CXC 52-2003) restabelecendo o plano de amostragem para coleta de material para análise de histamina e o nível máximo de histamina permitido para as espécies de peixes das famílias *Scombridae*, *Clupeidae*,

Engraulidae, Coryphaenidae, Pomatomidae e Scomberesocidae e seus produtos (Codex Alimentarius, 2018).

A Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) e Organização Mundial da Saúde (OMS) apresentaram uma relação de espécies de peixes que foram associadas a níveis elevados de histidina ou com ocorrência de intoxicação alimentar (WHO e FAO, 2013). A Tabela 3 mostra as principais famílias e espécies associados a alta produção de histamina.

Tabela 3. Peixes associados a uma quantidade elevada concentração de histidina livre no tecido muscular.

Família	**Espécie**	**Nome vulgar**
Scombridae	*Thunnus maccoyii*; *T. obesus*, *T. albacares*, *T. alalunga*, *T. thynnus*, *Katsuvwonus pelamis*; *Acanthocybium solandri* e *Pristis Pectinata*	Atum Albacora Albacora bandolim, albacora laje, albacora branca e albacora azul, bonito de barriga listrada, Cavala e serra
Scombresocidae	*Strongylura marina*	Agulha
Clupeidae	*Sardinella brasiliensis, S. pilchardus*	Sardinha
Coryphaenidae	*Coryphaena hippurus*	Dourado
Pomatomidae	*Pomatomus saltator*	Anchova

A norma técnica nº 40/94 do MERCOSUL e o regulamento técnico nº 185 de Identidade e Qualidade para Peixe Fresco (inteiro e eviscerado) do Ministério da Agricultura, Pecuária e Abastecimento (MAPA) fixa a identidade e a qualidade do pescado fresco destinado ao consumo humano. Nos parâmetros físico-químicos listados deve-se avaliar a histamina no músculo, nas espécies pertencentes às famílias *Scombridae*, *Scombresocidae*, *Clupeidae*, *Coryphaenidae*, *Pomatomidae*, sendo que o teor de histamina deve ser inferior a 100 mg kg^{-1} (MERCOSUL, 1994; MAPA, 1997).

O regulamento técnico de identidade e qualidade de conservas de peixes (nº 45/2011) do MAPA descreve que o nível de histamina no produto final não deve ser superior a 100 mg kg^{-1} de histamina. Nenhuma amostra pode apresentar um resultado superior a 200 mg kg^{-1} para espécies de peixes histaminogênicas (MAPA, 2011).

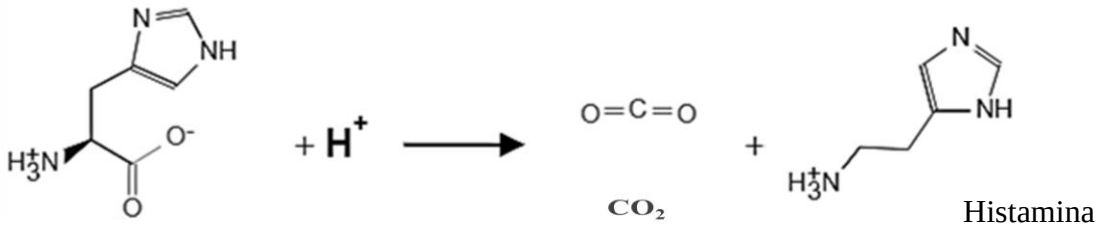
O regulamento técnico de identidade e qualidade para peixe congelado, aprovado pela Instrução Normativa, nº 21/2017, estabelece que os níveis de histamina máximo não devam ser superiores a 100 mg kg^{-1} tomando como base uma amostra composta por 9 unidades amostrais

e nenhuma unidade amostral pode apresentar resultado superior a 200 mg kg^{-1} para os grupos de famílias de peixes já citados (MAPA, 2017).

Norma Técnica do MERCOSUL (nº 40/94) se baseia no método da AOAC Official Methods of Analysis, 1990 para análise de histamina. A legislação brasileira pela Instrução Normativa (n° 25/2011) de métodos analíticos oficiais físico-químicos para controle de pescado e seus derivados se baseia na extração ácida das aminas, derivatização cloreto de dansila em pH alcalino, seguido de separação e quantificação por Cromatografia Líquida de Alta Eficiência com Gradiente de Eluição com detecção ultravioleta (MERCOSUL, 1994; MAPA, 2011).

2.8 Bactérias produtoras da enzima histidina descarboxilase (Hdc)

A maioria da histamina presente nos alimentos é gerada pela descarboxilação do aa histidina através da enzima Hdc presentes nos alimentos (Figura 4). A enzima Hdc pode ser produzida por bactérias endógenas presentes nos alimentos, ou podem ser introduzidas por contaminação bacteriana cruzada durante a manipulação, processamento, limpeza e preparo do alimento (Benkerroum, 2016).



Histidina Histamina

Figura 4. Biossíntese de Histamina. Adaptado de Caspi et al., 2017.

A concentração de histamina nos alimentos depende de vários fatores, como características da matéria prima (composição da carne, pH do músculo e atividade da água), condições de armazenamento, manuseio e uso de aditivos (sal e nitritos), tipo de embalagem. Estas condições afetam direta ou indiretamente as concentrações do aa histidina, microbiota do alimento (espécie, crescimento bacteriano, quantidade e metabolismo) e a atividade da enzima Hdc (Triki et al., 2018). Sabe-se ainda que a síntese da enzima *hdc* por microrganismos e sua ação catalisadora têm uma temperatura ótima a 32,2 °C, mas pode ocorrer a partir de 21,1 °C e atividade ótima do pH entre 2 a 6,5 (FDA, 2011). A presença de quantidades perigosas de

histamina está associada a um crescimento relevante (>7,0 log UFC g^{-1}) de microrganismos descarboxiladores (Gardini et al., 2016).

Nos peixes, as bactérias produtoras de histamina podem estar presentes na pele, brânquias, ou no trato gastrointestinal. A transferência destas bactérias para a carne do peixe, onde os aa livres podem estar presentes, leva ao desenvolvimento de histamina. A transferência pode ocorrer a partir do trato gastrointestinal após a coleta ou despesca dos peixes, através de migração, ou através de ruptura do conteúdo intestinal durante a evisceração. Os microrganismos também podem ser transferidos a partir da pele ou brânquias durante o abate e processamento do pescado (FAO e WHO, 2013). Suabes superficial das brânquias, pele e cavidade anal foram avaliados quanto a presença de bactérias formadoras de histamina em peixes, incluindo as espécies, bonito (*Sarda sarda*), atum (*Thunnus obesus*), anchova (*Pomatomus saltatrix*), peixe-espada indiano (*Alectis indicus*), sarda (*Scomberomorus cavalla*), dourado (*Coryphaena hippurus*), carapau (*Scomberomorus maculatus*), peixe-espada (*Xiphias gladius*), cavala (*Acanthocybium solandri*) e albacora (*Thunnus albacares*). Foram detectadas espécies de *P. damselae*, *M. morganii* e *E. aerogenes* nas brânquias (45, 10 e 7%), na pele (51, 25 e 11 %) e cavidade anal (51, 8 e 2%) dos peixes respectivamente (Bjornsdottir-Butler et al., 2015).

As bactérias capazes de descarboxilar o aa histidina em peixes fresco e resfriado pertencem ao grupo de bactérias gram negativas (GN). A família *Enterobacteriaceae* é o grupo mais importante dessas bactérias. Onde se destacam as espécies *Morganella morganii*, *Enterobacter aerogenes*, *Proteus* spp, *Klebsiella pneumoniae* e *Hafnia alvei.* Estas bactérias estão comumente presentes no ambiente da água salgada e existem naturalmente nas guelras e no intestino dos peixes vivos (James et al., 2013). A Tabela 4 mostra algumas bactérias gram negativas produtores de histamina no pescado (Brink et al., 1990; Brime, 2011; FAO, 2016b).

Tabela 4. Microrganismos capazes de metabolizar histamina no pescado.

Alimento	**Fontes de histamina no intestino**	**Grupo 1 Bactérias Produtoras de Histamina (Histidina descarboxilase)**
Pescado	Atividade da histidina descarboxilase microbiana na deterioração de alimentos	*Aeromonas hydrophila, Bacillus cereus, B. subtilis, Brevibacterium linens, Citrobacter diversus, C. freundii, Citrobacter sp., Clostridium perfringens, Enterobacter aerogenes, E. cloacae, E. liquefaciens, Enterobacter sp, Escherichia coli, E. intermedia, Hafnia alvei, Klebsiella pneumoniae, Klebsiella* sp., *Lactobacillus brevis, L. buchneri, L. fermentum, Lactobacillus* sp., *Pediococcus cerevisiae, Proteus mirabilis, P. morgani, P. rettgeri, P. vulgarisi, Proteus* sp., *Providencia alcalifaciens, Pseudomonas aeruginosa, P. fluorescens, P. reptilivora, Pseudomonas* sp., *Salmonella arizonae, S. cholerasuis, S. enteritidis, S. paratyphi, S. schottmuelleri, S. typhi, S. typhimurium, Salmonella* sp., *Serratia marcescens, Serratia sp., Shigella boydii, Staphylococcus aureus, S. bovis, S. faecalis, S. faecalis subsp. Liquefaciens, S. faecium, Streptococcus* sp.
Alimento	**Fontes de histamina no intestino**	**Grupo 2 Produtores de histamina isolados do trato digestivo (Saliva, conteúdo estomacal, trato intestinal, fezes)**
Alimentos ricos em histidina Pescado	Descarboxilação microbiana de histidina em resíduos alimentares no intestino grosso	*E. coli, Lactobacillus* sp., *M. morganii, Proteus* sp., *Pseudomonas reptilivora, Streptococcus* sp.

Adaptado de Joneja e Fellow, 2004; Taylor et al., 1978.

A atividade da enzima histidina descarboxilase foi avaliada em bactérias isoladas de três espécies marinhas de peixes frescos da Austrália (*Mugil cephalus*, *Pagrus auratus* e *Pseudocaranx dentex*). A histidina não foi descarboxilada por nenhum dos 288 isolados de bactérias gram positivas (GP) (*Staphylococcus*, *Micrococcus*, *Bacillus*, *Streptococcus*, *Corynebacterium*, *Carnobacterium*, *Exiguobacterium* e *Vagococcus*) confirmando que peixes marinhos frescos não têm o potencial para formar histamina (Al Bulushi et al., 2018). Entretanto as bactérias gram positivas podem estar presentes em peixes fermentados, como, *Enterococcus* sp, *Vagococcus* sp, *Tetragenococcus* sp e *Bacillus subtilis* isolados de anchovas salgadas enlatadas com óleo de cozinha (Kobayashi et al., 2016).

Diversos autores identificaram o gene *hdc* como o codificador da enzima catalisadora da reação de descarboxilação da histidina em bactérias (Fernandez et al., 2006; Bjornsdóttir-Butler et al., 2010; Bjornsdottir-Butler et al., 2011; Postollec et al., 2011). Sabe-se ainda, que duas diferentes famílias de enzimas codificadas por este gene podem ser distinguidas: a enzima Hdc piridoxal-5'-fosfato (PLP) dependente encontrada em bactérias GN e ao passo que a enzima Hdc de bactérias GP utiliza enzimas heterométrica que contêm um grupo piruvoil essencial, mas não PLP (Kung; 2012).

Métodos moleculares vêm sendo desenvolvidos e têm mostrado ser mais rápidos, sensíveis e mais confiáveis para a identificação de bactérias produtoras de histamina, através do gene histidina descarboxilase em alimentos. Vários oligonucleotídeos foram descritos para detectar genes *hdc* por PCR (Tabela 5). Uma vez que os oligonucleotídeos desenhados são baseados em regiões de aminoácidos preservados do gene *hdc* (Marcobal, de Las Rivas, Muñoz, 2006).

Tabela 5. Oligonucleotídeos utilizados em ensaios de PCR para a detecção do gene hdc em bactérias gram negativa em alimentos.

Gene	Oligonucleotídeos	pb	Referência
hdc	CL1 + CL2	150	Le Jeune et al.,1995
	JV16HC + JV17HC	367	Le Jeune et al.,1995 Alves et al., 2002 Marcobal et al., 2005a De las Rivas et al., 2005 Costantini et al., 2006
	CL1 + JV17HC	458	Alves et al., 2002 Le Jeune et al., 1995
	hdc-f + hdc-r	709	Takahashi et al., 2003
	106 + 107	534	De las Rivas et al., 2005
	CL1mod + JV17HC	450	Landete, Ferrer, Pardo (2005)
	HDC3 + HDC4	435	Coton e Coton, 2005
	PHDC1 + PHDC2	497	Costantini et al., 2006

* *hdc*: gene histidina descarboxilase, Pb: número de pares de bases. Adaptado: Marcobal, De Las Rivas, Muñoz, 2006.

CAPÍTULO II

ARTIGO I Characterization of microorganisms and quantification of histamine present in Whitemouth croaker (*Micropogonias furnieri*) by HTS, qPCR and HPLC-DAD

Article type: Original Article

Characterization of microorganisms and quantification of histamine present in Whitemouth croaker (*Micropogonias furnieri*) by NGS, qPCR and HPLC-DAD

Alessandra Danile de Lira[1], Caroline Isabel Kothe[2], Olivier Rué[3], Cédric Midoux[4], Michele Bertoni Mann[5], Luana Peixoto Mallmann[1], Ícaro Maia Santos de Castro[1], Ana Paula Guedes Frazzon[5] and Jeverson Frazzon[1*]

[1]Institute of Food Science and Technology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

[2] MICALIS, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France

[3] MAIAGE, INRA, Université Paris-Saclay, Jouy-en-Josas, France

[4] Irstea, UR HBAN, F-92761 Antony, France

[5] Department of Microbiology, UFRGS, Porto Alegre, Brazil

Running head: Food poisoning by histamine in fish

*****Correspondent author

Jeverson Frazzon, Ph.D., Av. Bento Gonçalves 9500, building 43212 – room 208, Porto Alegre, Brazil - ZIPCode 91501-970

e-mail: jeverson.frazzon@ufrgs.br - phone: +555133087115

Abstract

Aims: Evaluate the quality of fresh Whitemouth croaker collected in different seasons through quantification of histamine-forming bacteria, determination histamine content and characterization of the microorganism communities.

Methods and Results: The histamine content was detected by HPLC-DAD with a concentration ranging from 258.52 to 604.62 mg kg^{-1}. The number of copies of histidine decarboxylase (*hdc* gene) of gram-negative bacteria and the bacteria *Morganella morganii* and *Enterobacter aerogenes* were quantified by qPCR. All samples were positive, with copy numbers of the *hdc* gene ranging from 4.67 to 12.01 $\log_{10}$ g^{-1}. 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 the FROG software show that the phylum Proteobacteria was the most abundant, being the family *Moraxellaceae* more prevalent in samples collected in the summer, whereas the *Pseudomonadaceae* was more present in the winter.

Conclusions: All fish muscle samples analyzed in this study presented histamine values higher than those allowed by CODEX Alimentarius. In addition, a wide variety of spoilage microorganisms capable of expressing the enzyme histidine decarboxylase have been 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: World fish production in 2016 was 171 million tons, with China followed by Indonesia and the USA as the largest consumers. In Brazil, 1.3 million tons are consumed per year, with Whitemouth croaker being the main fish landed. Cases associated with histamine poisoning are quite common. According to the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 599 HFP outbreaks were notified in the European Union (EU) during the period 2010–17, in the USA, between 1998 and 2008 there were 333 outbreaks and 1383 people involved.

Keywords: Food poisoning, Whitemouth croaker, Histamine, NGS, HPLC-DAD, qPCR

Introduction

Whitemouth croaker (*Micropogonias furnieri)* is one of the most important species in Brazilian fishing, especially in the South and Southeast of the country, being the second most captured species, with approximately 44 thousand tons/year. Nutritionally, Whitemouth croaker contains several essential amino acids in the composition as lysine and methionine, 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 the gathering to consumption (Béné et al. 2015; Svanevik et al. 2015; Khalili Tilami and Sampels 2018) due presence of deteriorating microorganisms, resulting in the formation of toxic compounds such as biogenic amines (Olatunde and Benjakul 2018).

Biogenic amines possess low molecular weight and non-volatile 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, catalyzed by the histidine decarboxylase enzyme, which in gram-negative bacteria is a pyridoxal 5'-phosphate (PLP) cofactor dependent. The presence of this microorganisms may be associated with the wild fish microbiota or bacterial contamination from the processes of capture, processing,

distribution and consumption. The most involved bacteria including *Morganella morganii*, *Enterobacter aerogenes*, *Photobacterium phosphoreum* and *Raoultella planticola* (Landete *et al.* 2007; Ladero *et al.* 2010). Deteriorating microorganisms such as *Clostridium sp.*, *Vibrio* sp., *Acinetobacter* sp., *Plesiomonas sp.*, *Pseudomonas sp.*, *Aeromonas spp.*, *Citrobacter sp.*, *Salmonella sp*. may also contribute to the formation of histamine (Huang *et al.* 2010).

In this context, the objective of this study was to evaluate the quality of fresh and whole Whitemouth croaker sold in a fish market in the South of Brazil. In this way, for quantification of the histamine-forming bacteria, determination of the histamine content in their muscles and molecular characterization the bacterial diversity, qPCR, HPLC-DAD and NGS was applied, respectively.

Material 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 the summer period (February to March/2017) and twelve 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, then packed, and 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, ten grams 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) 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. 10 ml 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, n° 1 (Whatman, Maidstone, England) and reserved for derivatization.

Derivatization

The derivatization of amines with dansyl chloride was performed according the method of (Ben-Gigirey et al. 1998), with some modifications. One milliliter 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 x 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 (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 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 analyzing the following performance parameters: linearity ($R^2 > 0.99$), limit of detection (LD) and limit of quantification (LQ), according to (Guideline 2006), with equations 1 and 2.

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

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

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, *Morganella morganii* (ATCC 8019), and *Enterobacter 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 (TSB) and incubated at 37°C/24 h. After this period, they were cultivated in a tryptone soy agar (TSA) plate and incubated at 37°C/24 h.

Extraction and quantification of bacterial DNA isolated from Whitemouth croaker muscle

From the muscle samples, ten grams were transferred to an Erlenmeyer (250 ml) containing autoclaved distilled water (90 ml) and then incubated at 37°C/ 2h in an orbital shaker (Shaker SL-222, SOLAB, São Paulo, Brazil). It was collected 1.0 ml of the sample and centrifuged at 3000 x *g* for 3 min (**320R** Refrigerated bench centrifuge, Hettich). The supernatant was discarded, and the process was repeated twice. For DNA extractions was used the kit Invitrogen™ PureLink™ Genomic DNA (Thermofisher, USA). All DNA samples were quantified through the Quibit® 2.0 fluorometer (Invitrogen, California, USA) according to the manufacturer's protocol.

Oligonucleotides

In order to perform the real-time quantitative Polymerase Chain Reaction (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).

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

Bacteria	Oligonucleotídes (*hdc*)†	Sequence (5'- 3')	Amplicon (pb)‡	Reference
Gram-negative	*hdc* GN (Forward)	TCHATYARYAACTGYGGTGACTG*	139	Bjornsdottir-Butler et al. (2011)
	hdc GN (Reverse)	CCRTTRGTNACRTAVCCCCA*		
E. aerogenes	*hdc* EA (Forward)	GGATCCACAACCGTTTACCT	106	This work
	hdc EA (Reverse)	TTTCTTGGCTACCACATTGC		
M. morganii	*hdc* MM (Forward)	GGGAAGCTATCCGTTCACAT	86	This work
	hdc MM (Reverse)	ACGGTCAACAGCGTACTGAG		

†*hdc*= histidine decarboxylase,
* Oligonucleotídes degenerate: H can be Adenine (A) ou Citosine (C) ou Tiamine (T); R can be A or Guanine (G); Y can be C or T,
‡bp = base pairs.

The oligonucleotides were analyzed *in silico* through the nucleotide database, Basic Local Alignment Search Tool (BLAST, NCBI, USA). 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, KP728797.1, FJ469567.1, M62745.1and CP014748.1), respectively.

Quantitative Polymerase Chain Reaction (qPCR)

For the qPCR reactions (StepOne™ Real-Time PCR System, 96-well, Thermo Fisher Scientific) SYBR Green (Applied Biosystems™, USA) 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 ºC for 5 min, 35 cycles at 94 ºC for 30 s, 58 ºC for 30 s (*hdc* GN and *hdc* EA), 55 ºC for 30 s (*hdc* MM) and 72 ºC for 30 s and a final extent of 72 ºC 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. By using this equation, Ct was derived,

$$(_{\log 10}): N = 10(\frac{Ct-b}{a})$$

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 <0.05). Statistical analyzes were conducted in software R version 3.4.2 (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, Waltham, MA, USA) 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 v2.3 (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 less than 5 sequences). Taxonomic affiliation was performed using the Silva 132 pintail 100 databases (Quast *et al.* 2013). Alpha diversity and microbial composition were analyzed 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 by comparison with standard solutions. The histamine was separated in 1.3 min run time and exhibited good peak resolution, sharpness and symmetry (Figure S1). The proposed analytical method for determining histamine in the Whitemouth croaker muscle samples was validated under optimized conditions.

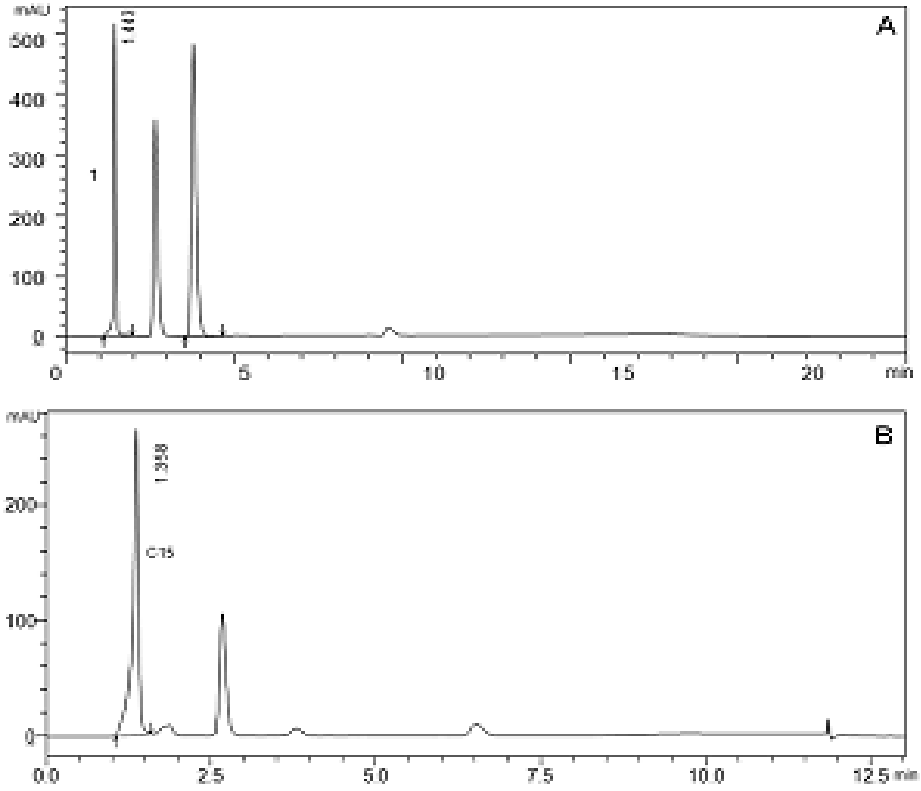


Figure S1. (A) Chromatography of the histamine standard, 9.0 μl^{-1} (1), and (B) histamine identified in fresh Whitemouth croaker muscle (C15).

The analytical curve was linear, with correlation coefficients of 0.9957 (Figure S2). The LD was 0.77 mg kg-1 and LQ was 2.0 mg kg^{-1}. This result indicated that the analytical method of HPLC-DAD with derivatization was very accurate for the determination of histamine.

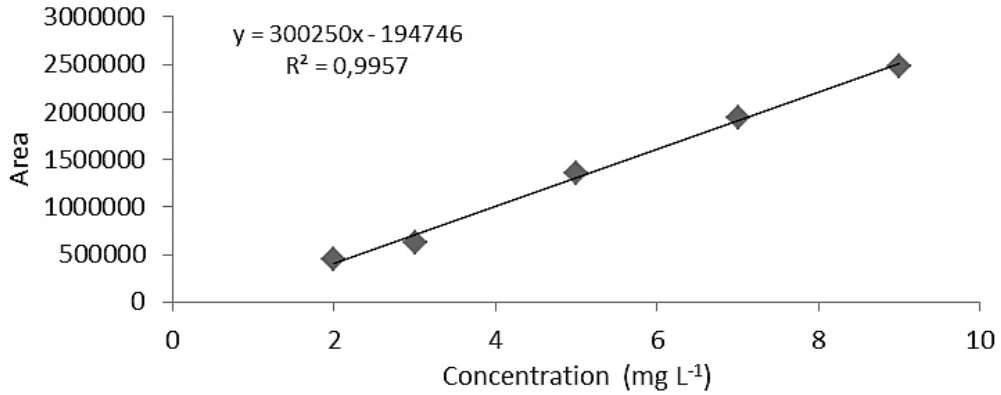


Figure S2. Calibration curve of the histamine standard.

Determination of temperature, pH and histamine of Whitemouth croaker

The values of the interquartile median and range of weight, superficial temperature, pH and histamine concentration in the Whitemouth croaker muscles collected in different seasons are

shown in Table 1. There was a statistical difference between the weight medians (p = 0.002), fish superficial temperature (p = 0.004), and muscle pH (p = 0.05) between Whitemouth croaker. Histamine was detected in all Whitemouth croaker samples, but there was no statistical difference in histamine concentrations (p = 0,488) in the Whitemouth croaker (Table 1). All samples obtained high histamine levels, ranging from 417.63 to 604.62 mg kg^{-1} for the Whitemouth croaker collected during summer and 258.52 to 533.24 mg kg^{-1} for the Whitemouth croaker collected during winter.

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)*ǂ	Summer fishes (n= 12)*ǂ	Winter fishes (n = 12)*ǂ	p valueŀ
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.
ǂ Results expressed in median [1ª Quartile e 3ª Quartile].
ŀ p value < 0.05.

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

To evaluate the sensitivity and efficiency of the qPCR assay, standard curves were determined through the DNA serial dilutions (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 of histamine-producing bacteria in the qPCR reaction was obtained for each oligonucleotide (Table S2).

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

hdc gene*	Y-Inter	Slop Ɨ (-3.58 a -3.10)ǂ	R^2 Ɨ (> 0.99)ǂ	Efficiency Ɨ (90 a 110%)ǂ	Equation
hdc GN	44.2	-3.10	0.99	110%	Ct = -3.10x + 44.20
hdc MM	32.3	-3.50	0.99	103%	Ct = -3.08x + 32.90
hdc EA	32.9	-3.10	0.99	100%	Ct = -3.54x + 32.35

* *hdc* = histidine decarboxylase, GN = Gram-negative bacteria, MM = *M. morganii*, EA= *E. aerogenes*,
Ɨ R^2 = linear regression coefficient, Ct= cycle threshold,
ǂ Reference value.

The high values of efficiency and of the curve regression coefficient obtained allowed the direct quantification of the histidine decarboxylase-producing bacteria present in the samples. In all histamine-producing bacteria positive samples quantified by qPCR, the presence of histamine was confirmed by HPLC-DAD. The Wilcoxon statistical test shows a significant difference ($p<0.001$) between the medians of the Whitemouth croaker samples collected in different seasons with the different parameters analyzed cycle threshold (Ct) and number of copies of *hdc* gene ($\log_{10}$ g^{-1}) (Table 2).

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 genes* present in the Whitemouth croaker muscle collected in the different seasons.

Parameters*	Number of fishes (n = 24)Ɨ	Summer fishes (n= 12)Ɨ	Winter fishes (n = 12)Ɨ	p valueǂ
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 ($\log_{10}$ g^{-1})	11,2 [10,2; 11,6]	10,1 [8,29; 11,2]	11,6 [11,2; 11,8]	0,002
hdc MM ($\log_{10}$ g^{-1})	7,35 [6,65; 7,69]	6,78 [6,30; 7,02]	7,72 [7,52; 7,84]	0,001
hdc EA ($\log_{10}$ g^{-1})	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*,
Ɨ n = number of samples, Results expressed in median [1ª Quartile e 3ª Quartile],
ǂ $p < 0,001$.

All fresh Whitemouth croaker samples collected in the different seasons (n=24) were positive for *hdc* genes, although with a wide range of Ct values, indicating differences in oligonucleotide concentrations in the samples. The qPCR assays had high *hdc* gene quantification capacity. The amount of the number of copies transformed into $\log_{10}$ for GN *hdc* gene (7.9 to 12.1 $\log_{10}$ g^{-1}) were higher when compared to the quantity of copies of the specific gene, *M. morganii* (4.67 to 10.06 $\log_{10}$ g^{-1}) and *E. aerogenes* (6.32 to 8.23 $\log_{10}$ g^{-1}). There was

a greater variability in concentrations of histamine-producing Gram- negative bacteria in summer samples, but there was no statistical difference between seasonality (p = 0.002). There were also differences between the summer samples when compared to the medians (p = 0.001) of the *hdc* MM copy numbers (Table 2 and Figure 1). However, for the *hdc* EA copy numbers there was no statistical difference (p = 0.002).

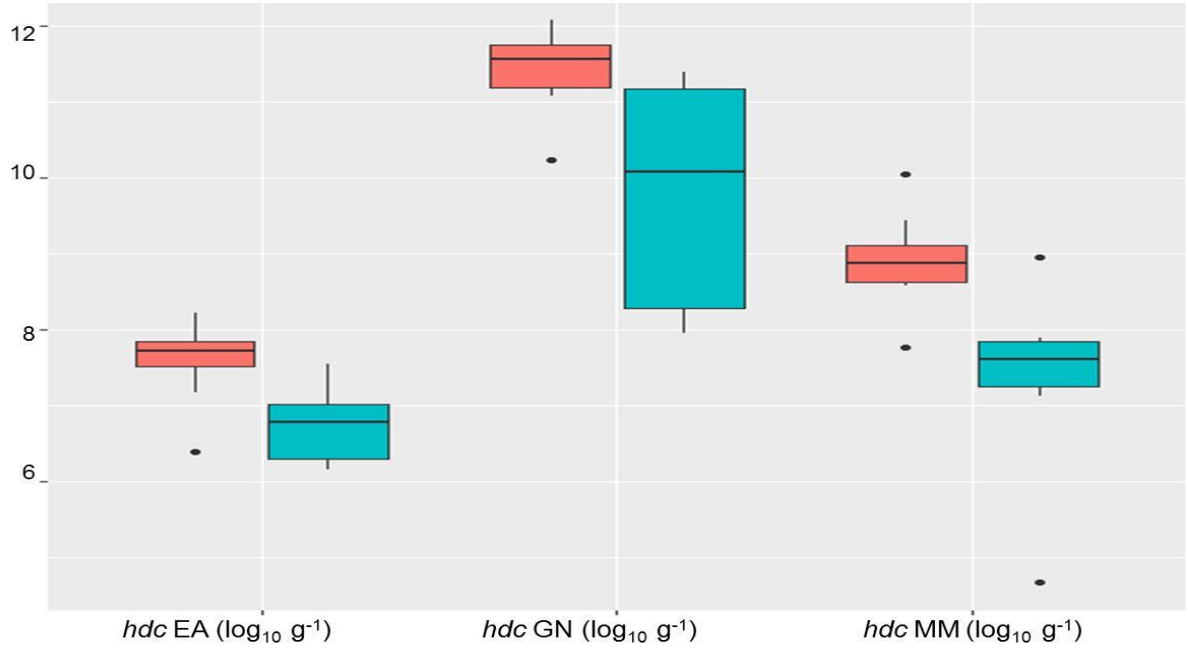


Figure 1. Comparison of the number of copies of the *hdc* gene (log $_{10}$ g^{-1}) of histamine-producing Gram-negative bacteria (*hdc* GN), *M. morganii* (*hdc* MM) and *E. aerogenes* (*hdc* EA) in fresh Whitemouth croaker at different seasons. Sample: summer ( ) and winter ( ).

importance of gram-negative bacteria, including *M. morganii* and *E. aerogenes* in the formation of histamine in fish. Despite high concentrations of free histamine in Whitemouth croaker muscle (258.52 to 604.62 mg kg^{-1}), no case of food poisoning by histamine has ever been reported with this species. In this context, early detection of histamine can be used as a predictive method to identify a potential fish risk factor during processing, storage and commercialization.

Relationship of Histamine and seasons with the other variables studied

A regression analysis was performed to determine the relationship between histamine concentration and season, weight, superficial temperature and pH of the evaluated Whitemouth croaker. There was a positive correlation between the histamine concentration in the muscles and the different seasons studied (ß = 40.15, p <2e-16). This correlation explains that histamine

may have an increase of 40 mg kg^{-1} when fish is contaminated in the summer. As can be observed, the amount of histamine in the fish collected in the summer showed a lower variability in histamine concentrations (Figure 2).

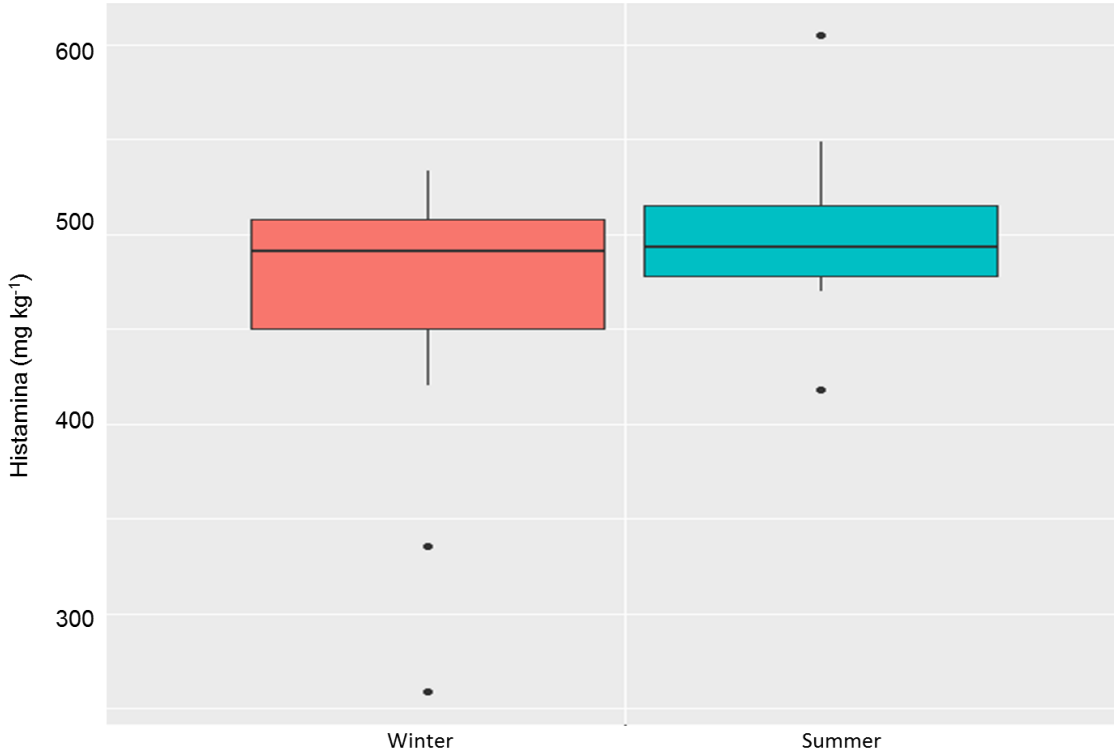


Figure 2. Comparison between histamine concentration and Whitemouth croaker samples collected in different seasons.

Another evaluated linear correlation was the histamine concentration and the fish muscle pH. By relating these variables, the pH would increase on average 61.27 mg kg^{-1} of histamine in the muscle ($p = 0.097$). When the season factor was added to these variables, this relation was increased to 96.02 mg kg^{-1} ($p = 0.0102$) in samples of fish collected in the summer. In this work, it was observed that pH influenced the concentration of histamine in the Whitemouth croaker gathered at different seasons, but the pH factor contributes with a higher initial concentration of histamine in the summer samples (Figure 3).

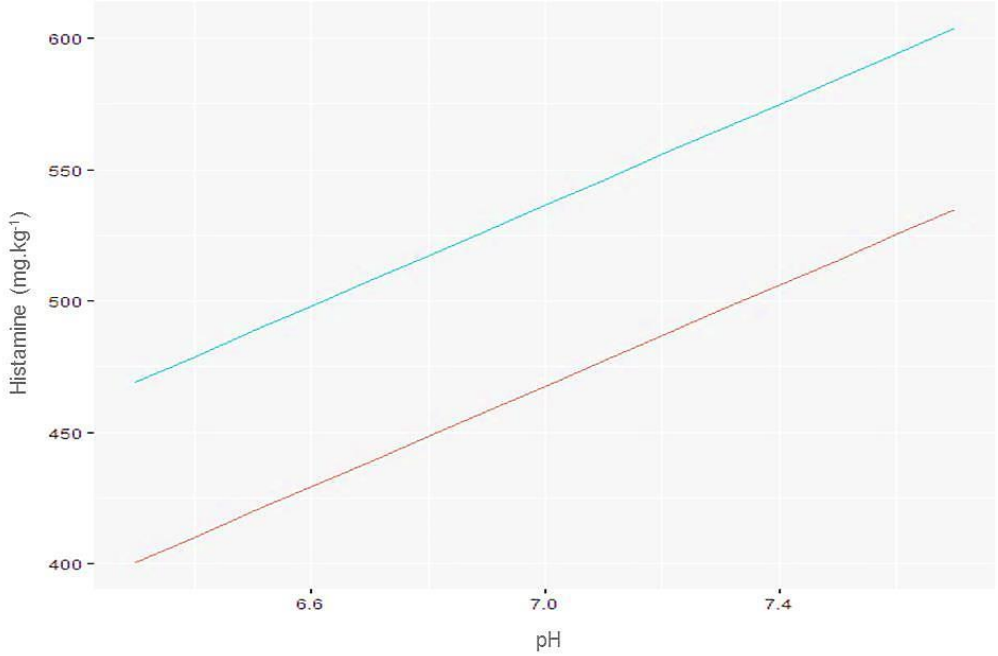


Figure 3. Chart showing the interaction between histamine concentration and pH of muscle samples collected in different seasons. Sample: summer ( ) and winter ( ).

The linear regression analysis showed positive correlations when compared to the histamine concentration of the samples with fish weight (ß = 80.64, p = 0.000109), seasonality (ß = 157.15, p = 0.0000063), muscle pH (ß = 80.64, p = 0.000758). In addition, the surface temperature of the fish had a significant influence when compared with the seasonality (ß = 152.62, p = 0.000133), weight (ß = 79.59, p = 0.000246) and pH of the muscle (ß = 93.90, p = 0.0010).

Likewise, the linear relationship between histamine and Ct (qPCR) and the amount of copies of *hdc* gene in Whitemouth croaker samples was evaluated. There was only statistical difference for the Ct of the *hdc* MM gene (p = 0.0617) when related to the histamine concentration (p = 2.92e-08) and summer samples (p = 0.0356). This result shows the contribution of the *M. morganii* to the production of histamine in the Whitemouth croaker collected in the summer, indicating that the presence of this bacterium can greatly reduce fish quality and contribute directly to histamine production. Also, there was a significant difference between the seasons, histamine and amount of copies of *hdc* GN gene (ß = 537.52, p = 0.0040) and *hdc* MM gene (ß = 439.55, p = $3.84e^{-11}$). When histamine was related to all variables (season, weight, pH, temperature and *hdc* copy amount), there was a statistical difference in histamine concentration in summer samples (p = 0.00799), pH (p = 0.00799), weight (p = 0.00799) and amount of copies of *hdc* GN gene (p = 0.0890).

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 analyzed. 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, sixteen phyla were identified with abundance $\geq 1.0\%$.

The relative abundance of the bacterial community at the phylum level was shown in Figure 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. Bacteroidetes was the second most represent phylum, however, there was a greater abundance between the samples (C7, C8 and C22). As for the phylum Firmicutes, greater abundance was obtained in samples (C10, C15 and C24).

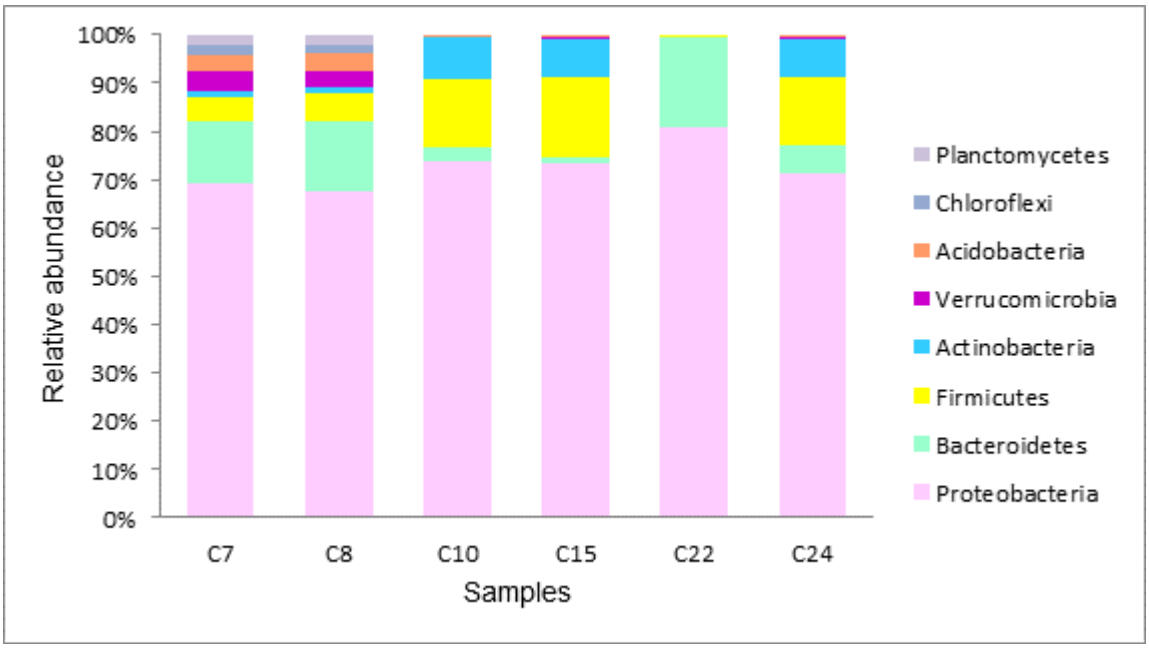


Figure 4. Histogram of the percentage of the phylum level in OTUs per sample of Whitemouth croaker muscle collected in different seasons. Whitemouth croaker collected in summer (C7, C8 and C10) and winter (C15, C22, and C24). Only OTUs present in at least two samples, relatively abundant > 1%.

Figure 5 highlights the bacterial diversity at the family level. Almost all the families present in the samples collected during the winter were present in the samples collected in the summer in greater or lesser quantity. Except the family *Pseudoalteromonadaceae* was identified only in a single sample collected in the summer (C10 - 2.97%). When analyzing at the family taxonomic level, *Moraxellaceae*, *Pseudomonadaceae*, *Burkholderiaceae*, *Enterobacteriaceae*, *Shewanellaceae* and *Aeromonadaceae* were the most abundant families in 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 more than half the composition of the fish analyzed (51% for summer and 77% for winter).

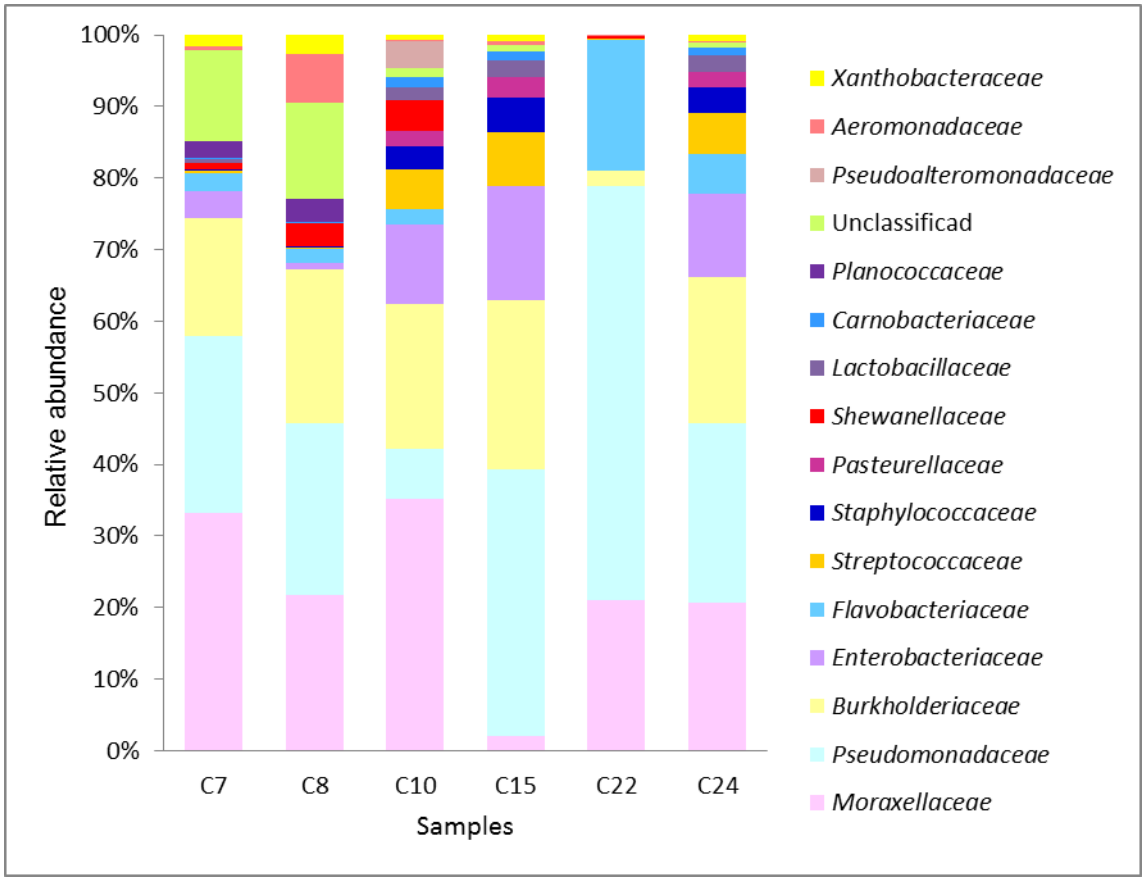


Figure 5. Plots of relative abundance (≥1%) composition of OTUs generated by the amplification of 16S rRNA at the family level within the phylum Proteobacteria. Samples collected in the summer (C7, C8 and C10) and samples collected in the winter (C15, C22 and C24).

Discussion

The use of methods for the early and rapid detection of histamine-producing bacteria and the detection of histamine is important to prevent accumulation of this toxic substance in food. The use of the molecular approach, as qPCR, for the detection of histamine-forming bacteria in fish and determination of presence of histamine by HPLC, has been applied concomitantly in several studies. In particular, real-time quantitative PCR (qPCR) offers the possibility of rapid detection and quantification of bacteria and detection of toxins with greater specificity, sensitivity and reliability than traditional methods of culture (Postollec *et al.* 2011; Rodríguez-Lázaro *et al.* 2014). 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 most frequently associated microorganisms 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 a high potential to enzyme histidine decarboxylase expression. The identification of these bacteria is of fundamental importance for food safety in order to avoid possible deterioration and formation of histamine in the fish (Zou and Hou 2017). Studies have shown that histamine is only detectable in fully decomposed fish or associated with a relevant growth, 10^7 CFU g^{-1}, of bacteria in the fish muscle (Gardini *et al.* 2016).

Histamine is one of the most important biogenic amines current in fish and is the only one with established legal limits for human consumption. In this work, all Whitemouth croaker samples tested showed concentrations above 200 mg kg^{-1} of histamine, values established as legal maximum limit by (NATIONS 2018). This legislation has been applied to fish of the *Scombridae, Clupeidae, Engraulidae, Coryphaenidae, Pomatomidae*, and *Scomberesocidae* families. Several foods were implicated in food poisoning by histamine, for example, it was identified 239 mg g^{-1} of histamine 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 recommended temperature of 0.0 °C to 2.0 °C and, may remain at an acceptable temperature of up to 4.0 °C (NATIONS 2018). In addition, the enzyme histidine decarboxylase present in fish can continue to produce histamine, even with bacterial inactivation caused by cooling (Food and Administration 2011). Muscle pH has a direct influence on the deteriorating microbiota present and consequently histamine formation in fish. The formation of lactic acid caused by the consumption of glycogen in the post-mortem muscle causes acidification, reducing the pH of the muscle. Like consequence of acid stress, the bacteria produce the decarboxylation of amino acids with excretion of biogenic amines to try 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). The enzyme histidine decarboxylase from *E. aerogenes* and *M. morganii* bacteria has better activity, pH 6.5 (Wendakoon and Sakaguchi 1995).

One of the main histamine-producing families are the *Enterobacteriaceae*, this group of bacteria can grow at low temperatures, its abundance decreases during storage in the cold chain, possibly because their growth rate is lower than in other gram-negative psychrotrophic decaying bacteria (Bahmani *et al.* 2011). Mesophilic bacteria considered as proliferative

histamine producers such as *M. morganii, Morganella psychrotolerans, E. aerogenes* can produce dangerous levels of histamine in a short period of time when stored under inadequate temperature (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 microorganisms in these foods (Parlapani *et al.* 2018).

Next generation sequencing (NGS) is the current method that allow to identify the profile of microbial communities, monitoring population fluctuations and characterization of 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 amplification of the *16S rRNA* gene it was possible to identify bacteria in fresh Whitemouth croaker muscles. The main phyla, Proteobacteria, Bacteriodetes, Firmicutes and Actinobacterium, identified in Whitemouth croaker samples are commonly found in the autochthonous microbiota of the skin, gills, gut and intestinal fish contents (Llewellyn *et al.* 2014).

Among these groups of spoilage microorganisms, 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 the fresh Whitemouth croaker muscle samples in this study were represented mostly by gram negative bacteria, members of the family *Moraxellaceae, Pseudomonadaceae, Flavobacteriaceae, Burkholderiaceae* and, *Enterobacteriaceae*. In smaller percentage the families *Shewanellaceae, Aeromonadaceae, Pasteurellaceae* and *Xanthomonadaceae.* Enterobacteria may occur in fish products as a result of fecal 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 presented (Hazards 2011). In addition, species belonging to the genus *Clostridium, Vibrio, Acinetobacter, Plesiomonas, Pseudomonas, Aeromonas* and *Photobacterium* have also been reported as producers of histamine (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 microorganisms 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 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 and intraspecific interactions and interspecies (Andreevskaya *et al.* 2018). This occurs with species of the family *Lactobacillaceae* that are in low concentration in fish commercialized fresh and refrigerated, but they are predominant when they are stored in 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. On the other hand, the extrinsic parameters are related to the location of capture, processing, storage and sanitary conditions in which these fish were submitted. In addition, the deterioration will depend on the initial microbiota amount and interaction between the microorganisms present in the fish (Llewellyn *et al.* 2014; Marshall 2014; Webster *et al.* 2018). The methodologies used in this study (qPCR, HPLC-DAD and NGS) allowed the molecular characterization of food spoilage bacteria, quantification of histamine-producing bacteria and presence of histamine in fresh corvine muscle. These techniques together may be used in the future as a predictive method to identify a potential risk in the formation of histamine in the entire fish production chain.

Acknowledgements

The authors acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, DF, Brazil, for the Fellowship. JF is grateful for CNPq for Financial support (#401714/2016-0 and # 303603/2015-1).

Conflict of interest

The authors have no conflict of interest to declare.

Reference

1. Alimentarius, Códex (2018) Revision of the code of practice for fish and fishery products (CXC 52-3002) and revisions of the section on sampling, examination and analyses related to histamine food safety. *Revision of the code of practice for fish and fishery products (CXC 52-3002) and revisions of the section on sampling, examination and analyses related to histamine food safety* **Fiftieth Session**, 26.
2. Andreevskaya, M., Jääskeläinen, E., Johansson, P., Ylinen, A., Paulin, L., Björkroth, J. and Auvinen, P. (2018) Food spoilage-associated Leuconostoc, Lactococcus, and

Lactobacillus species display different survival strategies in response to competition. *Appl Environ Microbiol* **84**, e00554-00518.
3. Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data: Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.
4. Angelo, K., Nisler, A., Hall, A., Brown, L. and Gould, L. (2017) Epidemiology of restaurant-associated foodborne disease outbreaks, United States, 1998–2013. *Epidemiology & Infection* **145**, 523-534.
5. Bahmani, Z.-A., Rezai, M., Hosseini, S., Regenstein, J., Böhme, K., Alishahi, A. and Yadollahi, F. (2011) Chilled storage of golden gray mullet (Liza aurata). *LWT-Food Science and Technology* **44**, 1894-1900.
6. Barbosa, R.G., Gonzaga, L.V., Lodetti, E., Olivo, G., Costa, A.C.O., Aubourg, S.P. and Fett, R. (2018) Biogenic amines assessment during different stages of the canning process of skipjack tuna (Katsuwonus pelamis). *International journal of food science & technology* **53**, 1236-1245.
7. Ben-Gigirey, B., Vieites Baptista de Sousa, J.M., VILLA, T.G. and BARROS-VELAZQUEZ, J. (1998) Changes in biogenic amines and microbiological analysis in albacore (Thunnus alalunga) muscle during frozen storage. *Journal of Food Protection* **61**, 608-615.
8. Béné, C., Barange, M., Subasinghe, R., Pinstrup-Andersen, P., Merino, G., Hemre, G.-I. and Williams, M. (2015) Feeding 9 billion by 2050–Putting fish back on the menu. *Food Security* **7**, 261-274.
9. Biji, K., Ravishankar, C., Venkateswarlu, R., Mohan, C. and Gopal, T.S. (2016) Biogenic amines in seafood: a review. *Journal of food science and technology* **53**, 2210-2218.
10. Biosystems, A. (2013) Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR. *F Hoffmann-La Roche Ltd*.
11. Bjornsdottir-Butler, K., Jones, J.L., Benner, R. and Burkhardt III, W. (2011) Development of a real-time PCR assay with an internal amplification control for detection of Gram-negative histamine-producing bacteria in fish. *Food microbiology* **28**, 356-363.
12. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L. and Bauer, M. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* **6**, 1621.
13. Dobbler, P.C.T., Laureano, Á.M., Sarzi, D.S., Cañón, E.R.P., Metz, G.F., de Freitas, A.S., Takagaki, B.M., Barbosa, C., Pylro, V.S. and Copetti, A.C. (2018) Differences in bacterial composition between men's and women's restrooms and other common areas within a public building. *Antonie van Leeuwenhoek* **111**, 551-561.
14. Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., Maman, S., Hernandez-Raquet, G., Combes, S. and Pascal, G. (2017) FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics* **34**, 1287-1294.
15. Ewels, P., Magnusson, M., Lundin, S. and Käller, M. (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **32**, 3047-3048.
16. Fan, H., Liu, X., Hong, H., Shen, S., Xu, Q., Feng, L. and Luo, Y. (2016) Quality changes and biogenic amines accumulation of black carp (Mylopharyngodon piceus) fillets stored at different temperatures. *Journal of food protection* **79**, 635-645.
17. Food and Administration, D. (2011) *Fish and fishery products hazards and controls guidance*: US Department of Health and Human Services Food and Drug Administration ….

18. Frank, H.A., Yoshinaga, D.H. and Nip, W.-K. (1981) Histamine formation and honeycombing during decomposition of skipjack tuna, Katsuwonus pelamis, at elevated temperatures. *Mar Fish Rev* **43**, 9-14.
19. Gale, E.F. (1946) The bacterial amino acid decarboxylases. *Advances in enzymology and related areas of molecular biology* **6**, 1-32.
20. Gardini, F., Özogul, Y., Suzzi, G., Tabanelli, G. and Özogul, F. (2016) Technological factors affecting biogenic amine content in foods: a review. *Frontiers in microbiology* **7**, 1218.
21. Gouveia, N.N.F.d. (2009) Desenvolvimento de uma metodologia analítica para determinação de aminas biogénicas em tunídeos: Universidade da Madeira.
22. Guideline, I.H.T. (2006) Impurities in new drug substances Q3A (R2). In *Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland*.
23. Hazards, E.P.o.B. (2011) Scientific opinion on risk based control of biogenic amine formation in fermented foods. *Efsa Journal* **9**, 2393.
24. Huang, Y.-R., Liu, K.-J., Hsieh, H.-S., Hsieh, C.-H., Hwang, D.-F. and Tsai, Y.-H. (2010) Histamine level and histamine-forming bacteria in dried fish products sold in Penghu Island of Taiwan. *Food Control* **21**, 1234-1239.
25. Huss, H.H. (1995) *Quality and quality changes in fresh fish*: FAO Rome.
26. Jacobsen, C., Nielsen, H.H., Jørgensen, B. and Nielsen, J. (2010) Chemical processes responsible for quality deterioration in fish. In *Chemical deterioration and physical instability of food and beverages*. pp.439-465: Elsevier.
27. Kang, C.R., Kim, Y.Y., Lee, J.I., Joo, H.D., Jung, S.W. and Cho, S.-I. (2018) An Outbreak of Scombroid Fish Poisoning Associated with Consumption of Yellowtail Fish in Seoul, Korea. *Journal of Korean medical science* **33**.
28. Khalili Tilami, S. and Sampels, S. (2018) Nutritional value of fish: lipids, proteins, vitamins, and minerals. *Reviews in Fisheries Science & Aquaculture* **26**, 243-253.
29. Kung, H.-F., Lee, Y.-C., Lin, C.-W., Huang, Y.-R., Cheng, C.-A., Lin, C.-M. and Tsai, Y.-H. (2017) The effect of vacuum packaging on histamine changes of milkfish sticks at various storage temperatures. *journal of food and drug analysis* **25**, 812-818.
30. Ladero, V., Calles-Enríquez, M., Fernández, M. and A Alvarez, M. (2010) Toxicological effects of dietary biogenic amines. *Current Nutrition & Food Science* **6**, 145-156.
31. Landete, J.M., de las Rivas, B., Marcobal, A. and Muñoz, R. (2007) Molecular methods for the detection of biogenic amine-producing bacteria on foods. *International journal of food microbiology* **117**, 258-269.
32. Laudadio, I., Fulci, V., Palone, F., Stronati, L., Cucchiara, S. and Carissimi, C. (2018) Quantitative assessment of shotgun metagenomics and 16S rDNA amplicon sequencing in the study of human gut microbiome. *Omics: a journal of integrative biology* **22**, 248-254.
33. Levin, R.E. (2016) Assessment of seafood spoilage and the microorganisms involved. *Safety Analysis of Foods of Animal Origin*, 463.
34. Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in microbiology* **5**, 207.
35. Macé, S., Mamlouk, K., Chipchakova, S., Prévost, H., Joffraud, J.-J., Dalgaard, P., Pilet, M.-F. and Dousset, X. (2013) Development of a rapid real-time PCR method as a tool to quantify viable Photobacterium phosphoreum bacteria in salmon (Salmo salar) steaks. *Appl Environ Microbiol* **79**, 2612-2619.
36. Mahé, F., Rognes, T., Quince, C., de Vargas, C. and Dunthorn, M. (2015) Swarm v2: highly-scalable and high-resolution amplicon clustering. *PeerJ* **3**, e1420.

37. Marshall, D. (2014) SPOILAGE OF ANIMAL PRODUCTS| Seafood.
38. Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet journal* **17**, 10-12.
39. McMurdie, P.J. and Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* **8**, e61217.
40. Olatunde, O.O. and Benjakul, S. (2018) Natural preservatives for extending the shelf-life of seafood: a revisit. *Comprehensive Reviews in Food Science and Food Safety* **17**, 1595-1612.
41. Ordóñez, J.L., Troncoso, A.M., García-Parrilla, M.D.C. and Callejón, R.M. (2016) Recent trends in the determination of biogenic amines in fermented beverages–A review. *Analytica chimica acta* **939**, 10-25.
42. Özogul, F. and Özogul, Y. (2005) Formation of biogenic amines by Gram-negative rods isolated from fresh, spoiled, VP-packed and MAP-packed herring (Clupea harengus). *European Food Research and Technology* **221**, 575-581.
43. Parlapani, F., Michailidou, S., Anagnostopoulos, D., Sakellariou, A., Pasentsis, K., Psomopoulos, F., Argiriou, A., Haroutounian, S. and Boziaris, I. (2018) Microbial spoilage investigation of thawed common cuttlefish (Sepia officinalis) stored at 2° C using next generation sequencing and volatilome analysis. *Food microbiology* **76**, 518-525.
44. Podeur, G., Dalgaard, P., Leroi, F., Prevost, H., Emborg, J., Martinussen, J., Hansen, L.H. and Pilet, M.-F. (2015) Development of a real-time PCR method coupled with a selective pre-enrichment step for quantification of Morganella morganii and Morganella psychrotolerans in fish products. *International journal of food microbiology* **203**, 55-62.
45. Postollec, F., Falentin, H., Pavan, S., Combrisson, J. and Sohier, D. (2011) Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food microbiology* **28**, 848-861.
46. Pothakos, V., Devlieghere, F., Villani, F., Björkroth, J. and Ercolini, D. (2015) Lactic acid bacteria and their controversial role in fresh meat spoilage. *Meat science* **109**, 66-74.
47. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glockner, F.O. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**, D590-596.
48. Ringo, E., Hossein, S., Ghosh, K., Doan, H.V., Beck, B.R. and Song, S. (2018) Lactic acid bacteria in finfish–an update. *Frontiers in microbiology* **9**, 1818.
49. Rodríguez-Lázaro, D., Hernández, M., Demain, A.L. and Vaishnav, P. (2014) IDENTIFICATION METHODS| Real-Time PCR.
50. Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahe, F. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584.
51. Ruiz-Capillas, C. and Herrero, A.M. (2019) Impact of biogenic amines on food quality and safety. *Foods* **8**, 62.
52. Shukla, S., Park, H.-K., Kim, J.-K. and Kim, M. (2010) Determination of biogenic amines in Korean traditional fermented soybean paste (Doenjang). *Food and Chemical Toxicology* **48**, 1191-1195.
53. Suzzi, G. and Torriani, S. (2015) Editorial: Biogenic amines in foods. *Frontiers in microbiology* **6**, 472.
54. Svanevik, C.S., Roiha, I.S., Levsen, A. and Lunestad, B.T. (2015) Microbiological assessment along the fish production chain of the Norwegian pelagic fisheries sector–results from a spot sampling programme. *Food microbiology* **51**, 144-153.
55. Team, R.C. (2017) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http s. *www R-proje ct org*.

56. Triki, M., Herrero, A., Jiménez-Colmenero, F. and Ruiz-Capillas, C. (2018) Quality Assessment of Fresh Meat from Several Species Based on Free Amino Acid and Biogenic Amine Contents during Chilled Storage. *Foods* **7**, 132.
57. Trip, H., Mulder, N.L. and Lolkema, J.S. (2012) Improved acid stress survival of Lactococcus lactis expressing the histidine decarboxylation pathway of Streptococcus thermophilus CHCC1524. *Journal of Biological Chemistry* **287**, 11195-11204.
58. Visciano, P., Schirone, M., Tofalo, R. and Suzzi, G. (2012) Biogenic amines in raw and processed seafood. *Frontiers in microbiology* **3**, 188.
59. Webster, T.M.U., Consuegra, S., Hitchings, M. and de Leaniz, C.G. (2018) Interpopulation variation in the Atlantic salmon microbiome reflects environmental and genetic diversity. *Appl Environ Microbiol* **84**, e00691-00618.
60. Wendakoon, C.N. and Sakaguchi, M. (1995) Inhibition of amino acid decarboxylase activity of Enterobacter aerogenes by active components in spices. *Journal of food protection* **58**, 280-283.
61. Wongsariya, K., Bunyapraphatsara, N., Yasawong, M. and Chomnawang, M.T. (2016) Development of molecular approach based on PCR assay for detection of histamine producing bacteria. *Journal of food science and technology* **53**, 640-648.
62. Yu, Y., Wang, P., Bian, L. and Hong, S. (2018) Rare death via histamine poisoning following crab consumption: a case report. *Journal of forensic sciences* **63**, 980-982.
63. Zou, Y. and Hou, X. (2017) Histamine production by Enterobacter aerogenes in chub mackerel (Scomber japonicus) at various storage temperatures. *Food Science and Technology* **37**, 76-79.

CAPÍTULO III

ARTIGO II Determination of histamine and characterization of bacteria communities presents in sardines (*Sardinella brasiliensis*) muscle

Determination of histamine and characterization of bacteria communities presents in sardines (*Sardinella brasiliensis*) muscle

Alessandra Danile de Lira[1], Ícaro Maia Santos de Castro[3], Michele Bertoni Mann[2], Luana Peixoto Mallmann[1], Ana Paula Muterle Varela[2], Ana Paula Guedes Frazzon[2] and Jeverson Frazzon[1*]

[1]Institute of Food Science and Technology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil
[2] Department of Microbiology, UFRGS, Porto Alegre, Brazil
3 Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, Brazil

*****Correspondent author
Jeverson Frazzon, Ph.D., Av. Bento Gonçalves 9500, building 43212 – room 208, Porto Alegre, Brazil – ZIP Code 91501-970
e-mail: jeverson.frazzon@ufrgs.br, phone: +555133087115

Abstract
Primarily formed by the microbial decarboxylation of the amino acid histidine, histamine is the leading cause of food poisoning from fish consumption worldwide. In this work, the quality of fresh and frozen marketed sardines was evaluated for histamine concentration by HPLC-DAD; the identification and quantification of histamine producing bacteria by qPCR and composition of the microbiota present in muscle tissue by amplification of the *16S rRNA* gene by high throughput sequencing (HTS). According to the results obtained by HPLC-DAD, the histamine concentration ranged from 226.14 to 583.87 mg kg^{-1} was detected in all samples. The histidine decarboxylase (*hdc*) gene from Gram negative bacteria, *Morganella morganii* and *Enterobacter aerogenes* were quantified in all samples. The most abundant microorganisms present in fresh sardines belong to the genera *Macrococcus*, *Acinetobacter* and *Pseudomonas* and in frozen sardines to genera *Phyllobacterium*, *Pseudomonas* and *Acinetobacter*. The methods used show to be useful in evaluating the microbiological and chemical quality of fresh and frozen marketed sardine and, in future, could be part of strategic on histamine traceability system in the fish production chain.
Keywords: Histamine, Histidine decarboxilase, High Throughput Sequencing (HTS), microbiome, Food poising.

1. Introduction

Foodborne diseases pose a serious threat to human health in developed and underdeveloped countries. In the United States, an estimated 9.4 million food-borne illnesses occur annually (Dewey-Mattia et al., 2018). Among these diseases, histamine food poisoning is one of the most severe illnesses transmitted by fish (Yu et al., 2018). In 2017, 56 food outbreaks generated by the fish consumption were reported by European Union countries (EFSA and ECDC, 2018), followed by 12 cases of food poisoning by histamine reported in 2016 in the United States (CDC, 2018).

Histamine poisoning is related to several fish species of the *Scombridae* family (tuna and mackerel), as they have high concentrations of histidine amino acid in muscle tissue. However, other fish families also can produce high levels of histamine, such as *Scombresocidae*, *Engraulidae*, *Coryphaenidae*, *Pomatomidae* and *Clupeidae* (WHO and FAO, 2012). Belong to

Clupeidae family, the *Sardinella brasiliensis* (inhabit the south-eastern coast of Brazil) is targeted by the fishing industry, due to its great economic value (Dias-Neto and Dias, 2015); however, this species is known to contain large quantities of histamine (FAO and WHO, 2018; El Hariri et al., 2018).

Histamine is produced by the decarboxylation of the amino acid histidine by the exogenous histidine decarboxylase enzyme released by bacteria present in the fish. These microorganisms may come from water or the endogenous fish microbiota (James et al., 2013). The bacteria of the *Enterobacteriaceae* family are mainly responsible for the formation of histamine in fish, among them, the species *Morganella morganii* and *Enterobacter aerogenes*. Other bacterial species capable of producing histamine include *M. psychrotropicus*, *Photobacterium damselae*, *Klebsiella* sp., *Pseudomonas* sp., *Proteus* sp., *Vibrio* sp., *Bacillus* spp., *Staphylococcus* sp., *Citrobacter* spp. and *Clostridium* sp. (Johnson and Schantz, 2017, Klanian et al., 2018; Arulkumar et al., 2019). Bacterial enzymatic decarboxylation depends on several factors, such as the availability of the amino acid histidine and the presence of histamine producing microorganisms. In addition factors such as muscle pH, storage temperature, oxygen availability and the microbiota composition present in the muscle could interfere in the histamine production.(Landete et al., 2008; Danquah, Benjakul and Simpson, 2012).

Therefore, the identification and quantification of histamine producing bacteria is of fundamental importance for consumers' food safety. Consequently, the objectives of the present study were: (i) to characterize the microbial community present in fresh and frozen commercialized sardines, (ii) to quantify histamine-producing bacteria and (iii) to identify and quantify histamine present in fish muscle tissue.

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 sardines (with viscera and gills) were collected fresh (S1-S12) and others sardines frozen at -18 °C (S13-S24). The fish were identified and transported in isothermal boxes with recycled ice. The fish were weighed, gutted and parts of the muscle was aseptically removed, (1:10 w/v). of the muscle sample was 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 M 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 M 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 and derivatization of histamine

Histamine extraction in sardine muscle tissue was based on the methodology of Hu et al. (2012). One hundred grams of muscle tissue was ground in a blender and5.0 g was mixed with 10 mL of Trichloroacetic acid (TCA) 5.0% (P.A., Synth). The mix was vortexed for 5.0 min and centrifuged at 3000 g for 10 min at 4.0 °C. This step was repeated once again with the residue. The supernatants were adjusted to 25 mL of 5.0% TCA volume and filtered on n° 1 filter paper (Whatman, England). The filtrate and the standard histamine solution were derivatized with dansyl chloride according to the methodology of Ben-Gigirey et al. (1998) with some modifications. Briefly, 1.0 mL of each extracted sample or histamine standard solution was mixed with 200 µL of 2 M sodium hydroxide solution and 300 µL of saturated sodium bicarbonate solution (P.A., Synth, Brazil). 2.0 mL of the solution dansyl chloride was added to the mixture and incubated in a water bath at 40 °C for 45 min. Then 100 µL of ammonium hydroxide solution (28-30%, P.A., Neon, Brazil) was mixed into the solution to remove residual dansyl chloride and incubated for 30 min at room temperature in the dark. After incubation, the final volume was adjusted with 5.0 mL of acetonitrile (HPLC, ≥99%, J.T. Baker, USA). Finally, the mixture was centrifuged at 2500 *g* for 5.0 min and the supernatant was filtered on a 0.22 µm syringe filter (Filtrilo, Brazil). The filtered supernatant was refrigerated at -20 ° C until HPLC-DAD analysis.

2.2.3 HPLC-DAD histamine separation and quantification

Histamine determination was based on the methodology of Hu et al. (2012), with some modifications. 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). Histamine was quantified using the C18 chromatography column (Merck, Germany). 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), 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 between 200 and 800 nm and the chromatograms processed 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 ICH (2006).

2.3 Determination and Quantification of Histamine Producing Bacteria by qPCR

2.3.1 Bacterial Reference Strains

The reference bacteria *Morganella morganii* (ATCC 8019) and *Enterobacter aerogenes* (ATCC 13048) were provided by the Oswaldo Cruz Institute Reference Materials Laboratory (FIOCRUZ, Brazil). They were grown in Soy Tryptone Broth (TSB) and Soy Tryptone Agar (TSA) and then incubated at 37 °C for 24 h.

2.3.2 DNA extraction and quantification

Ten grams of sardine muscle tissue was mixed with 90 mL of sterile distilled water and incubated at 37 °C for 2 h at 1000 rpm (Shaker SL-222, SOLAB, Brazil). Subsequently, 3.0 ml of the supernatant was collected and centrifuged at 3000 *g* for 3 min (320R, Hettich, Germany). For the extraction of DNA from the reference bacteria, 2.0 mL of the bacterial culture broth in TSB after incubation and performed the same processing of muscle tissue. The Invitrogen™ PureLink™ Genomic DNA Kit (Thermofisher, USA) was used to extract DNA from fish and

bacteria. All DNA samples were quantified using the Quibit® 2.0 fluorometer (Invitrogen, California, USA) according to the manufacturer's protocol.

2.3.3 *hdc* gene quantification by qPCR

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). In addition, two more *hdc* gene oligonucleotides were designed for *M. morganii* bacteria, GenBank accession (J02577.1) and *E. aerogenes*, GenBank access (M62745.1) using GenScript Biotech Corp. (New Jersey, USA). The sequences were: *hdc* MM forward (5'-GGGAAGCTATCCGTTCACAT-3') and *hdc* MM reverse (5'-ACGGTCAACAGCGTACTGAG-3'), with an amplicon of 86 bp and *hdc* EA forward (5'-GGATCCACAACCGTTTACCT-3') and *hdc* EA reverse (5'-TTTCTTGGCTACCACATTGC-3'), with an amplicon of 106 bp.

qPCR reactions (StepOnePlus™ System, USA) SYBR Green I (Applied Biosystems™, USA) was used. DNA amplification by qPCR was carried out in 15 μL reaction volume containing 10 ng DNA, 1x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl); 1.5 mM $MgCl_2$, 0.2 μM *hdc* primers ; 0.2 μM dNTP ; SYBR Green I 1x, 2U Taq DNA polymerase Platinum® Invitrogen, ultrapure water to complete volume. The following conditions for qPCR were used: initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s (*hdc* MM), 58 °C for 30 s (*hdc* GN and *hdc* EA) and 72 °C for 30 s and a final extension of 72 °C for 5 min. Melting curve was performed after the last amplification cycle. Negative control was used in all reactions. Amplification was performed in Step One Plus™ System (USA) based on the methodology of Applied Biosystems (2003). A standard curve was generated for each oligonucleotide from serial dilutions (10^1 to 10^6 μL^{-1} copies) of bacterial DNA and used to absolute quantification by Ct value comparation (cycle threshold). The Ct value generated in qPCR, the correlation coefficient (R^2) and the amplification efficiency (E) were obtained by StepOne v2.3 software (Applied Biosystems, USA). The result of the analysis was the amount of nucleic acid (copy number, $\log_{10}$) per given amount of sample (per g sample, per μg total DNA).

2.4.1 PCR amplification of *16S rRNA* gene and HTS sequencing

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 domain of the bacterial 16S rRNA gene was amplified using oligonucleotides 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'GACTACHVGGGTWTCTAAT-3'), both containing an Illumina adapter region as described by Caporaso et al. (2012). Amplification was performed in a 25 µL mixture consisting of a DNA concentration of 12,50 ng DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM of each oligonucleotide (515F and 806R), 10 mM dNTP, 2U Taq High Fidelity Platinum TM DNA Polymerase (Invitrogen™, USA) and 1x reaction buffer. Amplification was performed in a BioRad MyCycler thermocycler (BioRad, USA) according to the following program: initial denaturation at 94 ° C for 3 min, followed by 30 cycles of 94 °C at 45 s, 56 °C at 45s, 72 °C at 1.0 min and a final cycle at 72 °C for 5.0 min. Amplicons were purified using Agencourt AMPure XP beads (Beckman Couter, USA) following the manufacturer's instructions. Indexes were added to the DNA libraries following the manufacturer's instructions (Illumina, USA). 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 muscle 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 (R Core Team, 2019).

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., 2018). Raw sequence data were quality filtered and denoised, dereplicated and

chimera filtered using the q2-dada2 plugin with DADA2 pipeline Callahan (Callahan et al., 2016). 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, in which chimeras are detected in samples individually, and sequences found chimeric in a sufficient fraction of samples were removed assuming at least 1.0-fold change of potential parents of a sequence being tested as chimeric.

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 et al., 2002) and used to construct a phylogeny with fasttree2 (via q2-phylogeny) (Price, Dehal and Arkin, 2010). Taxonomy was assigned to ASV's using the q2-feature-classifier (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 (Lahti et al., 2017) 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 (Lozupone, 2005), 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 (Wilcoxon, 1945), using function from Microbiome R package. Beta diversity significance were estimated with a permutacional multivariate analysis of variance (Marti,

2001) using distance matrices obtained by MDS ordination previously described with Permutacional Multivariate Analysis of variance test (PERMANOVA), Adonis function of Vegan R package (Oksanen et al., 2007). Compositional biplot PCoA were estimated using Aitchison distance with DEICODE Qiime2 plugin (Martino 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 using q2-taxa plugin . Differential abundance analysis were performed with ANCOM using q2-composition plugin, 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).

3. Results

3.1 Determination of Histamine by HPLC-DAD

Figure S1.A and S1.B shows a typical chromatogram of the histamine standard (9.0 mg L-1) and a frozen sardine sample (S15), respectively, obtained by optimum adequate chromatographic separation. Peaks of the samples were separated and could be identified by the retention time of the histamine standard (1.30 min). The chromatogram exhibited good peak resolution, sharpness and symmetry. 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, with correlation coefficients of 0.9954 (Figure S1.C). 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 determination of histamine in sardine muscle samples.

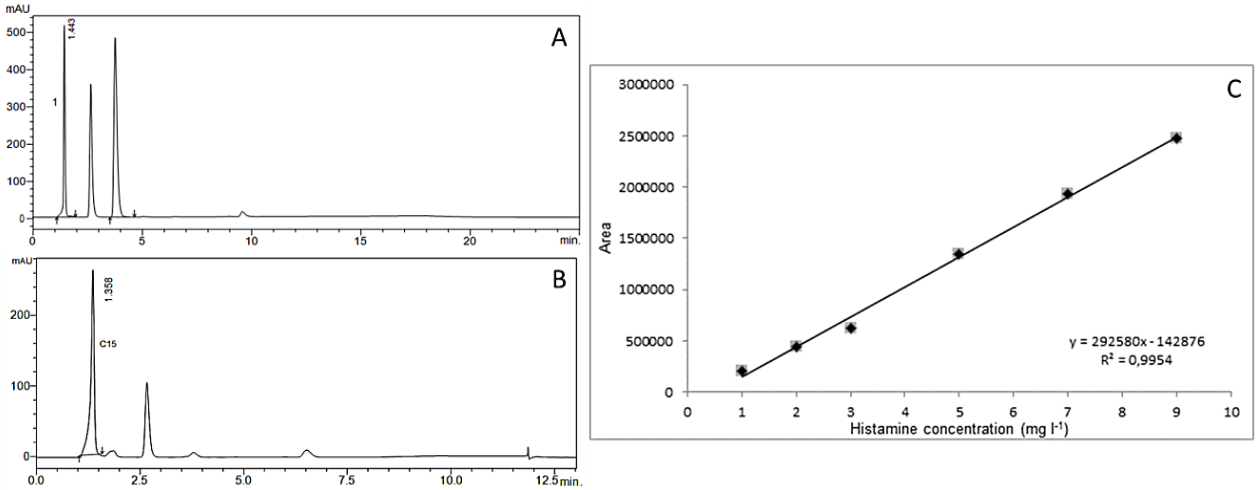


Figure S1. HPLC-DAD chromatogram of Histamine (A) and sardine sample, S15 (B). Analytical curves of the histamine standard (C).

3.2 Histamine levels and pH in sardine muscle samples

Table 1 shows the mean and interquartile interval of fish weight, pH value, and histamine concentration in sardine muscle in different types of conservation. The weight of whole sardines ranged from 65.45 to 176.46 g. Fresh sardines had higher weight, with statistically significant difference ($p < 0.001$). The same occurred with the pH value of fish muscle ($p < 0.001$), ranging from 6.22 to 7.18 between samples. Fish muscle pH was higher in fresh sardines samples, this can be explained by the freshness of these fish, since the pH was close to 7.0.

All fresh and frozen sardines samples evaluated were contaminated with histamine. Significant difference ($p < 0.05$) was observed between histamine concentration in frozen and fresh fish samples. With higher histamine concentration level in frozen sardines (342.74 - 583.87 mg kg-1) compared to fresh sardines (226.14 - 579.83 mg kg-1). The high concentration of histamine in fish could be associated with these fish being sold whole (with guts and gills), contributing to high level of deteriorating bacteria in muscle and consequently histidine decarboxylase enzyme formation.

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

Parameters	Number of sardine (n=24) *	Frozen sardine (n=12) *	Fresh sardine (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

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

3.3 Quantification of *hdc* gene by qPCR

There was a linear correlation between the Ct values of the *hdc* genes and the genomic DNA concentrations of the *M. morganii* and *E. aerogenes* bacterial samples. The curves showed a good correlation coefficient (R^2> 0.99) and efficiency (100%). (Table 2).

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) *	R^2 (> 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), R^2 = linear regression coefficient, Ct= cycle threshold.

Primers *hdc* EA and *hdc* MM, were specific for identification of *M. morganii* and *E. aerogenes* species, respectively, in sardine samples. Significant difference (p = 0.001) was obtained between *hdc* GN and *hdc* EA in the frozen samples. However, there was no statistical difference between frozen and fresh samples for the Ct value of the *hdc* MM gene (p = 0.947). There was a statistical difference in the number of copies of the hdc for *hdc* GN bacteria (p = 0.001), *hdc* MM (p=0.048) and *hdc* EA (p=0.049) in the frozen samples. All samples were positive for *hdc* gene by qPCR produced histamine in fish muscle (Table 3).

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

*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.
**p value < 0.05.

3.4 Microbiota analysis of fresh and frozen sardines muscle samples

3.4.1 Alpha and beta diversity

After pre-processing the sequences with the DADA2 pipeline, a total of 691738 good quality sequences were analysed. 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 difference was found among the Simpson (p=0.31), Chao1 (p=0.69) and Shannon diversity index (p= 0.84) according to non-parametric Wilcoxon statistical test Shannon index showed no difference between species richness of fresh and frozen sardines samples, however, the diversity awing frozen samples is more homogeneous. It was observed in the Simpson Index, a greater equability in the distribution of species abundance among frozen samples, showing a greater variability in microbiota when the fish is stored fresh. Violin plot was observed in frozen sardine samples through the Simpson and Chao1 diversity index. Chao1 diversity index, even though there was no statistical difference due to plot distribution, it was observed that there was greater richness variability in fresh samples. Violin plot was observed in frozen sardine samples through the Simpson and Chao1 diversity index. This shape shows the distribution (extremely thin at each end and broad in the middle) indicates that the microbiota is highly concentrated around the median.

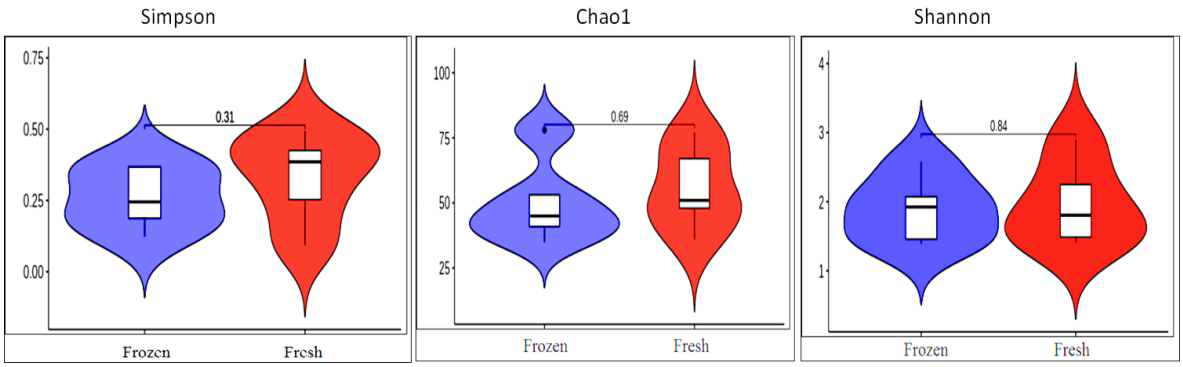


Figura 1. Alpha diversity measurements of microbial communities in the sardines muscle tissue. Represented by Simpson, Chao1 and Shannon diversity index. Boxes span the first to third quartiles; the horizontal line inside the boxes represents the median. Whiskers extending vertically from the boxes indicate variability outside the upper and lower quartiles, and the single circles indicate outliers. No significant difference was found among the three groups ($p > 0.05$) according to the non-parametric Wilcoxon statistical test.

Beta diversity analysis was performed using the Bray-Curtis similarity index, Jaccard index, Weighted-Unifrac and Unweighted-Unifrac metrics to determine the distance between samples based on microbiota profile from muscle using Principal Coordinate Analysis (PCoA) for data visualization (Figure 2). Significant difference was found 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 ($p=0.023$, $R^2=0.26589$) by the non-parametric permutational multivariate statistical variance test (PERMANOVA, Adonis, statistical test with 999 permutations). The results of the beta diversity analysis indicated that the microbiome composition was distinct between the frozen and fresh sardines groups.

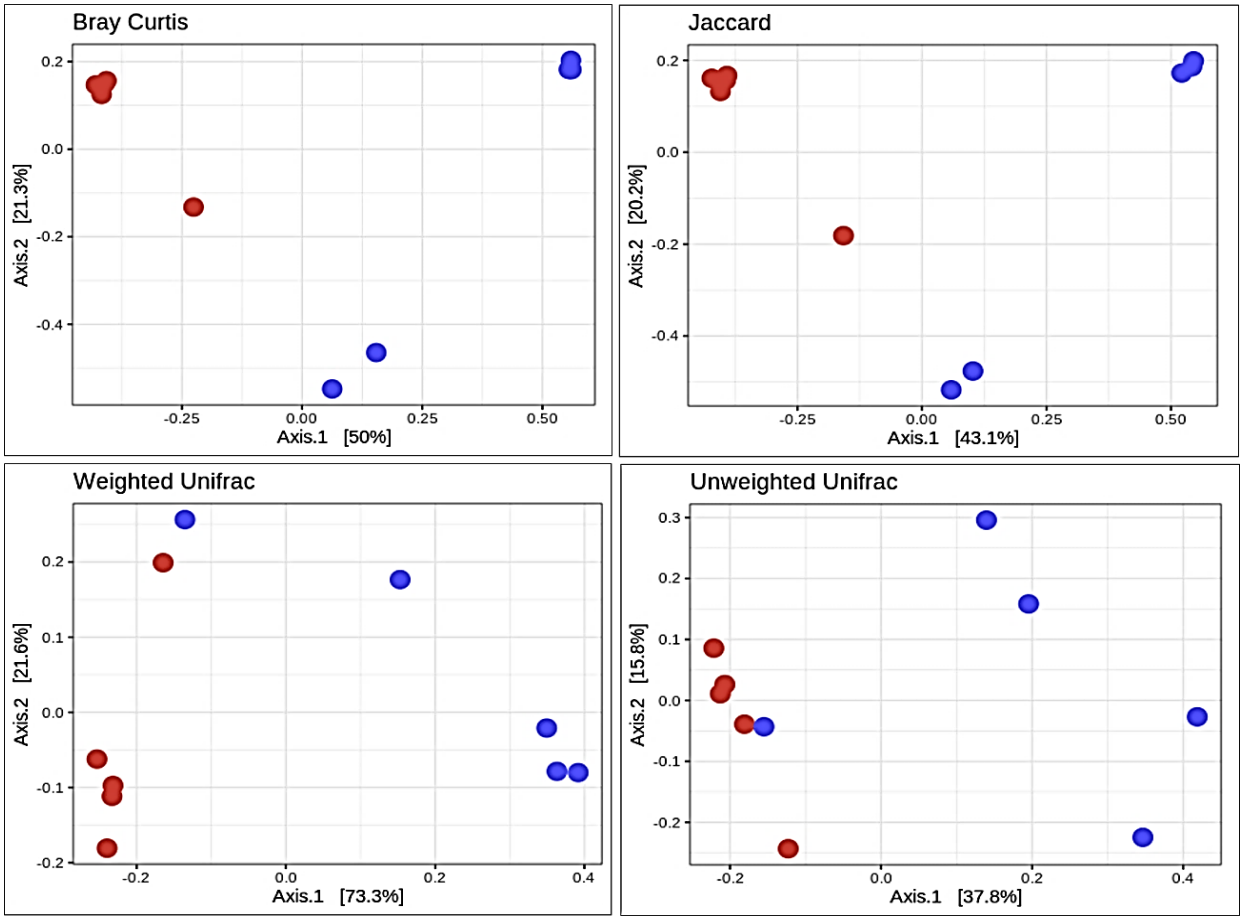


Figure 2. Principal Coordinate Analysis (PCoA) of bacterial communities. Based on Bray Curtis dissimilarity index with Jaccard index, Weighted-Unifrac and Unweighted-Unifrac regarding conservation type. Color: Blue represents frozen sardines (S1, S2 S4, S7 and S12) and Red represents fresh sardines (S13, S14, S17, S18 and S19).

To compare the similarity and difference between fresh and frozen sardine samples, a hierarchical cluster analysis based on Unweighted-Unifrac distance was also performed (Figure 3). It was observed that there is a difference in sardine microbiota according to the type of conservation of these fish. Excluding samples of fresh (S7) and frozen (S18) sardines that have a greater distance from their groups but are similar to each other.

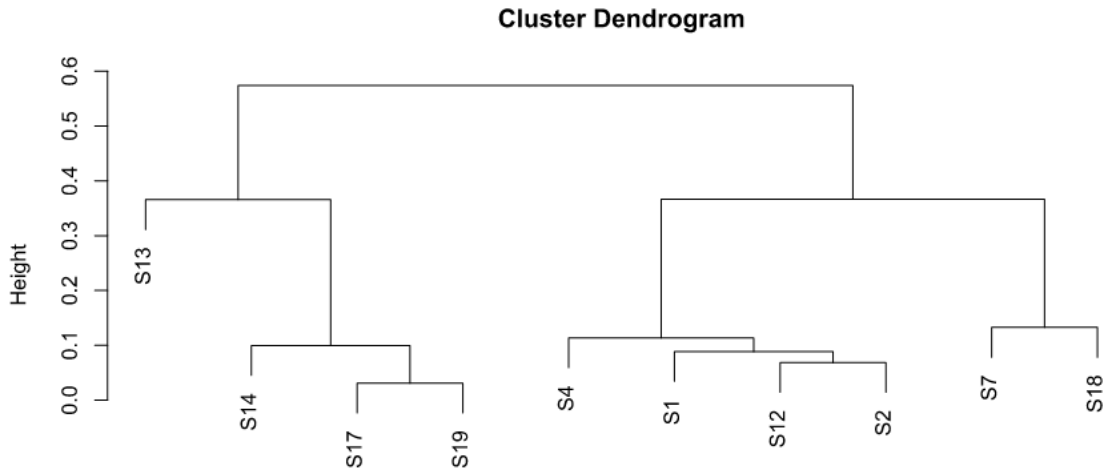


Figure 3. Hierarchical cluster dendrogram of bacterial communities of fresh or frozen sardines based on Unweighted-Unifrac distance. Samples: frozen sardines (S13, S14, S17, S18 and S19) and fresh sardines (S1, S2, S4, S7 and S12).

3.4.2 Microbial Community

The relative abundance of species in fresh and frozen sardine muscle samples at the phylum level is shown in Figure 4. These results show that there was a differentiation between the phylum level microbiota according to the preservation type of the samples. Proteobacteria was the predominant phylum in the frozen sardines and represented at least 94.84% of the bacterial diversity. While in fresh sardines the dominant phylum was Firmicutes with 65.86%, followed from phylum Proteobacteria (33.90%).

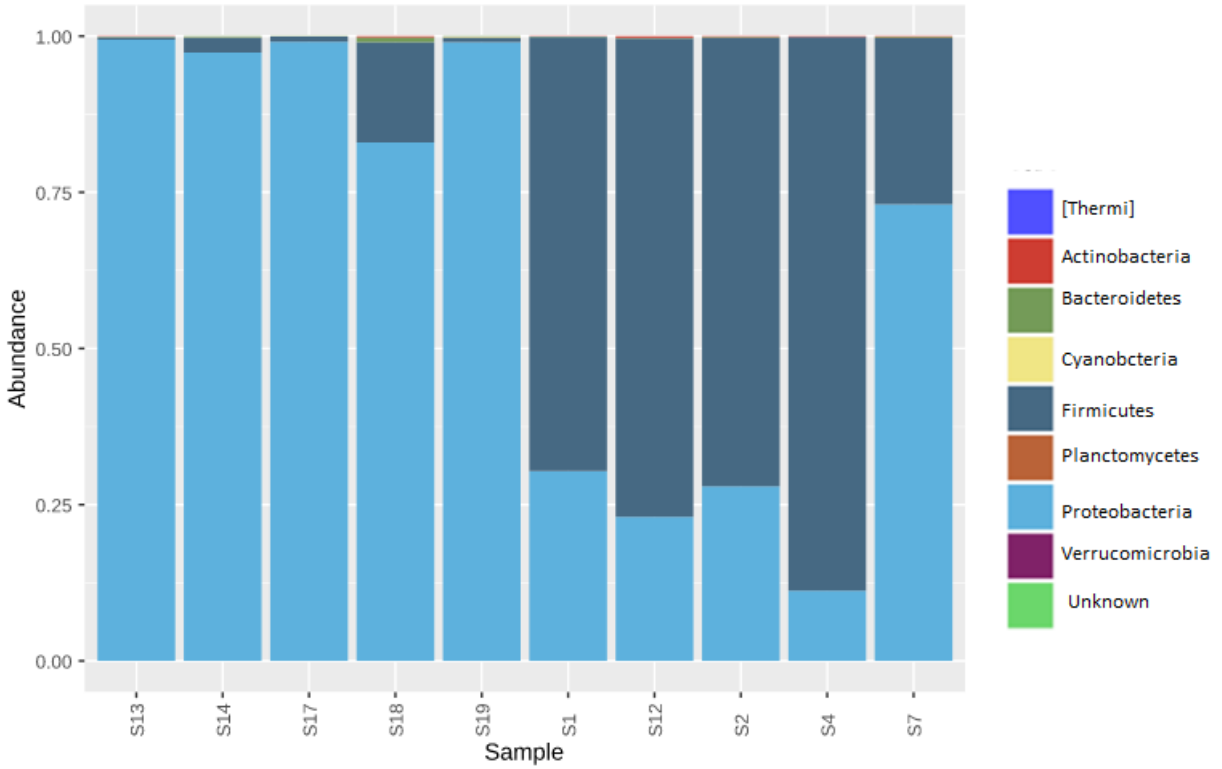


Figure 4. Relative abundance of the dominant bacterial phyla presents in the muscle tissue of sardines. Samples: frozen sardines (S13, S14, S17, S18 and S19) and fresh sardines (S1, S2, S4, S7 and S12).

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

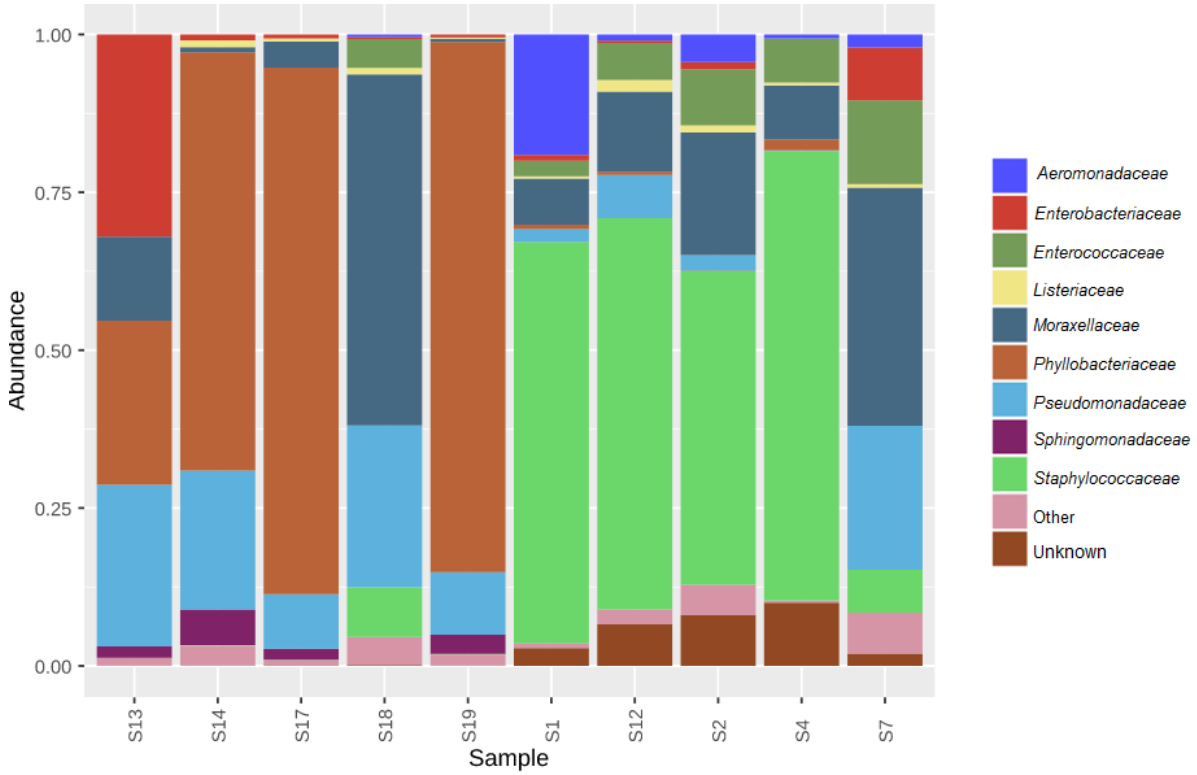


Figure 5. Relative abundance of dominant bacterial families presents in fresh or frozen sardine muscle tissue. Samples: fresh sardines (S1, S2, S4, S7 and S12); frozen sardines (S13, S14, S17, S18 and S19).

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

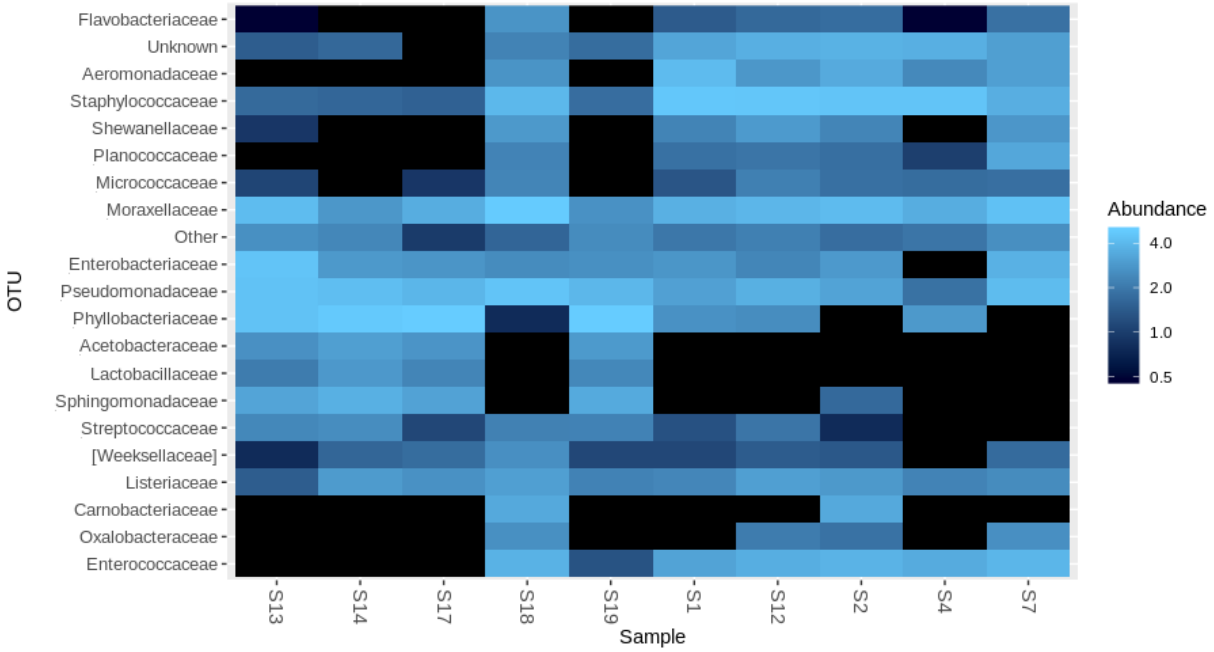


Figure 6. Heat map showing non-metric multidimensional scaling (NMDS) (hierarchical grouping of samples and taxon's based on Bray-Curtis distance) of composition of the microbiota present in frozen (S13, S14, S17, S18 and S19) and fresh (S1, S2, S4, S7 and S12) sardines. Normalization of the frequency of the family level in log10. The color scale represents the stepped abundance of each variable, indicated by the score, with blue indicating high abundance and dark blue indicating low abundance.

An analysis of microbiome composition (ANCOM) was used to evaluate differences in the average rate of microbial abundance between fresh and frozen sardines (Figure7). We observed a significant difference in logarithmic abundance of twelve families detected between groups. In particular, the families *Lactobacillaceae* (w = 6), *Sphingomonadaceae* (w = 5), *Enterobacteriaceae* (w = 5), *Acetobacteraceae* (w = 5) and *Comamonadaceae* (w = 4) in frozen sardines samples. While in fresh samples were *Enterococcaceae* (w = 19), *Aeromonadaceae* (w = 16), *Micrococcaceae* (w = 9), *Pseudomonadaceae* (w = 9), *Planococcaceae* (w = 9), *Campylobacteraceae* (w = 5), *Listeriaceae* (w = 4). This group of bacteria represented Representing 13.84% and 28.59% of the relative abundance of fresh and frozen sardine samples respectively. These results may infer that the type of fish conservation can select the group of bacteria present in fish.

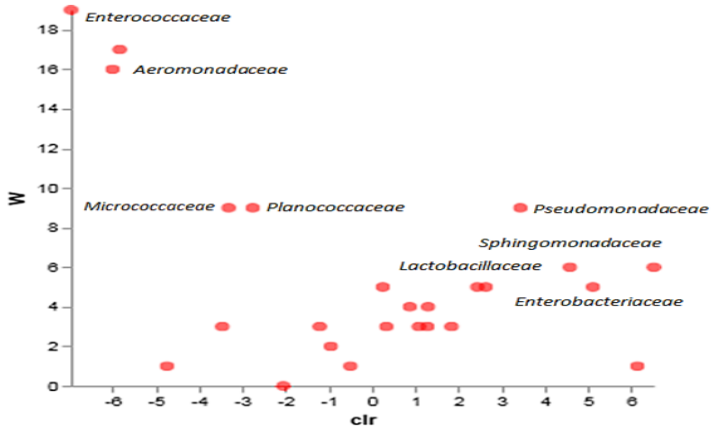


Figure 7. Volcano Plot of ANCOM differential abundance. For analysis, the family-level log-scale OTUs table was used. The x-axis value represents the mean difference of clr transformed in the abundance of a given family between the fresh and frozen sardines groups. A positive x-axis means that the family is differentially abundant in frozen sardines and a negative x-axis means that the family is differentially abundant in fresh sardines. Statistical analysis w represents the number of paired comparisons that were considered significantly different.

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

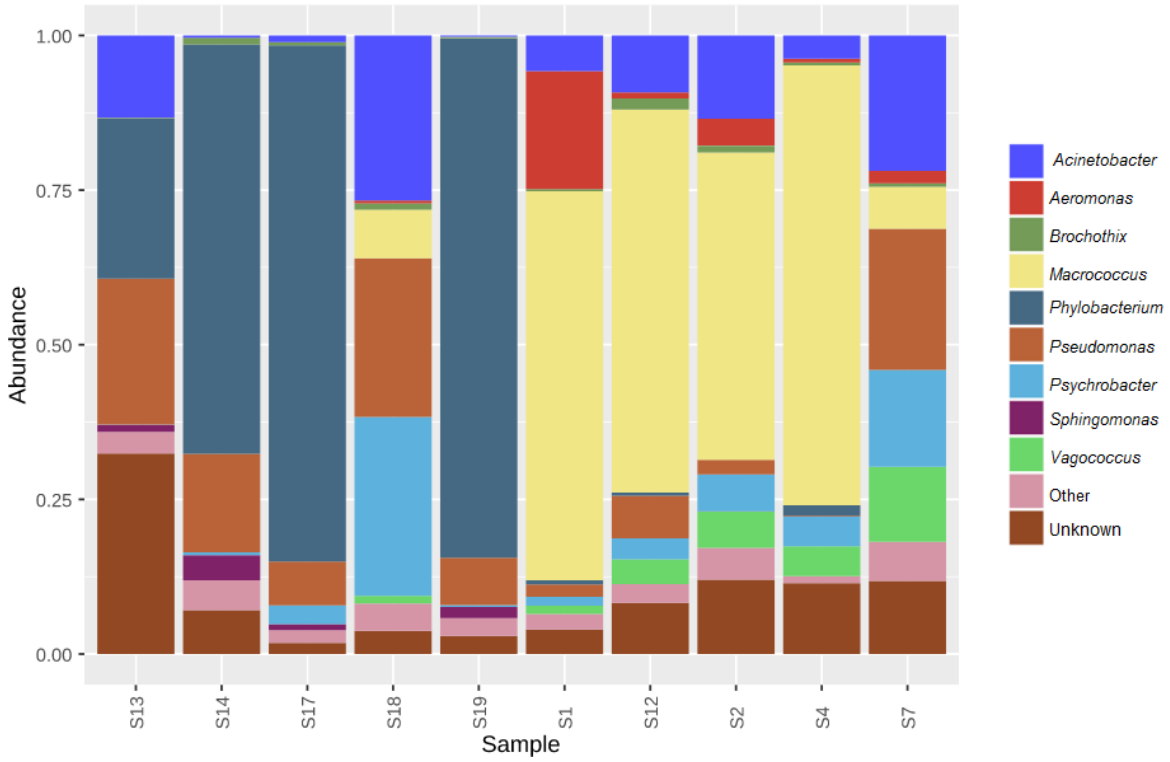


Figure 8. Taxonomic classification of sardine muscle tissue microbiome at genera level. Samples: fresh sardines (S1, S2, S4, S7 and S12) and frozen sardines (S13, S14, S17, S18 and S19).

Gram negative histamine producing bacteria were found to represent 31.12% of fresh sardines and 34.97% of frozen sardines. The genera *Acinetobacter* (11.11% and 9.75%), *Pseudomonas* (6.98% and 16.76%), *Psychrobacter* (6.14% and 7.91%) were identified with the highest relative abundance, respectively. *Aeromonas* was observed in higher percentage in fresh sardines (5.82%).

Bacteria considered to be major histamine-producing bacteria in fish tissue with relative abundance of less than 1% were identified. In particular the *M. morganii* bacteria present in fresh sardines (0.02%) and frozen sardines (0.08%) and *Photobacterium damselae* identified only in fresh samples (0.08%). Bacteria considered to have low histamine production capacity in fish, *Shewanella, Aeromonas, Janthinobacterium, Proteus, Vibrio, Flavobacterium, Serratia* were also identified.

The decaying microbiota in fish consisted of several psychrotrophic Gram negative bacteria, mainly *Pseudomonas*, *Macrococcus*, *Acinetobacter*, *Psychrobacter* and *Aeromonas*, representing 79.93% and 36.52% of the fresh and frozen sardines microbiome respectively. The genera *Shewanella, Janthinobacterium, Proteus, Serratia, Flavobacterium* and *Photobacterium* with relative abundance> 1% were also identified.

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

Some food spoilage bacteria have been identified at the species level, for example; *Pseudomonas caeni, P. veronii, P. viridiflava, Psychrobacter pulmonis, P. sanguinis, P. arenosus, P. marincola, P. pacificensis, Macrococcus caseolyticus, A. johnsonii, Flavobacterium succinicans, Kurthia gibsonii, J. lividum, Carnobacterium viridans* and *Lactobacillus delbrueckii.*

4. Discussion

Histamine detection and quantification methods are of great importance for food safety and, consequently, to avoid food poisoning among consumers. The Codex Alimentarius (2018) has established a maximum histamine level of 200 mg kg^{-1}, while the EU Regulation (2019/627) included histamine control in fish landings, the processing industry and the wholesale and retail fish market to assess compliance and hygiene and sanitary conditions of establishments (EC, 2019). In this sense, the presence of histamine in fish turns on the warning signal among consumers.

In this 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 the inadequate temperature in fish conservation. The temperature variation during fish storage enables the growth of deteriorating microorganisms, increasing the proteolytic activity in the muscle and the formation and activity of the histidine decarboxylase enzyme (Ruiz-Capillas and Herrero, 2019). Moreover, even with the bacterial growth retardation by cooling, histamine can still be formed at a lower concentration by the action of the enzyme. Under freezing conditions, the enzyme remains stable and after thawing becomes active (FDA, 2011).

The sardines used in this study were acquired with viscera and gills, which may have influenced the high histamine concentration observed in this study. The bacterial concentration in the digestive tract can range from 10^4-10^9 CFU g^{-1} (Skrodenytė-ArbaČIauskiene, 2007), while in the gills can achieve 10^6 CFU g^{-1}(Austin, 2006). However, the muscle tissue of the fish is considered sterile, but when these fish are sold whole, self-contamination or cross-contamination of bacteria may occur at the time of cleaning and evisceration (Gatti Junior et al., 2014; Bruijn et al., 2018).

Another predisposing factor for histamine formation is the pH of fish muscle tissue. In this study, muscle pH in fresh sardines had higher values (6.70 to 7.18) when compared to frozen

sardines (6.22 to 6.69). Control of muscle pH is important for maintaining the sensory and microbiological quality of fish meat. This variation in muscle pH is influenced by fish species, muscle tissue type, 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 your best activity is around pH 6.5 (Wendakoon and Sakaguchi, 1995).

The genus *Macrococcus* dominated the microbiota of fresh sardines (49.88%). *Macrococcus* sp. appears to be part of the initial chilled fish microbiota (Parlapani et al., 2014). Others studies showed that this genus also dominated the vacuum-packaged common carp fillets microbiota (*Cyprinus carpio*) and Sea bream fillets (*Sparus aurata*) fillets (Parlapani et al., 2014; Zhang et al., 2017). The *M. caseolyticus* was identified in all sardine samples, a higher proportion in fresh sardines (46.6%) and in a smaller proportion in frozen sardines (1.33%). These results corroborate with other studies that identified *M caseolyticus* in other foods such as fermented fish (Zang et al., 2018), semi-dried shrimp (Karani et al., 2015) and frozen catfish products (*Pangasius hypophthalmus*) (Thi et al., 2016). In addition, *Phyllobacterium* was the predominant genus in frozen sardines (47.97%). They are gram-negative, nitrogen-fixing organisms easily found in plants and soil (León-Barrios et al., 2018). This genus was isolated in previously studies from the intestinal microbiota of Atlantic salmon (*Salmo salar*) and Catfish (*Ictalurus punctatus*) (McInnis et al., 2015; Raats et al., 2011).

Other predominant genera in sardine muscle tissue were *Acinetobacter*, with 12.65% in fresh sardines and 10.28% in frozen sardines. In addition to the genus *Pseudomonas*, less in fresh sardines (7.80%) and higher concentration in frozen sardines (18.25%). These bacteria are widely present in water and soil and can be found in refrigerated fresh foods (Deák, 2010). In addition to belonging to the resident gut microbiota and therefore can potentially contaminate the fish during processing (Egerton et al., 2018). These genera are often reported in fish processing plants (Møretrø and Langsrud, 2017). The main deteriorating psychrotrophic microorganisms in cold fish are the genera *Pseudomonas*, and *Acinetobacter* (Odeyemi et al., 2018). Other spoilage bacteria were found to a lesser extent in fresh and frozen sardines in this study. Like the bacteria *Aeromonas*, *Brochothrix*, *Photobacterium*, *Psychrobacter*, *Flavobacterium*, Lactic Acid Bacteria (BAL), *Staphylococcus*, *Micrococcus*, *Listeria*, *Vagococcus* and *Kurthia* (Remenant et al., 2015; Bjerke et al., 2019).

Fish decomposing bacteria play a key role in histamine production in fish (Takahashi et al., 2003). Many of these bacteria are part of the endogenous fish microbiota (FAO and WHO, 2013). However, these bacteria can contaminate post-capture fish and throughout fish processing, marketing and consumption (Lehane and Olley, 2000). Among the

Enterobacteriaceae family, the species *M. morganii*, *E. aerogenes* and *P. angustum* are important producers of the enzyme histidine decarboxylase in fish (Bjornsdottir-Butler et al., 2016). In addition to the microorganisms described, other bacteria have positive decarboxylase activity in fish, such as *Aeromonas* sp, *Shewanella* sp, *Proteus mirabilis*, *P. vulgaris*, *Vibrio vulnificus*, *Shigella* sp, *Psychrobacter* sp (Houicher et al., 2013; Tembhurne et al., 2013). In the present study, these bacteria were found in fresh and frozen sardines, corresponding to 26.52% and 37.57% of sardine microbiota respectively.

The quantification of qPCR-based histamine-producing bacteria has been showing a potential method for detecting these microorganisms in food (Hattori and Seifert, 2017). However, the presence of histidine decarboxylase enzyme does not determine the presence or formation of histamine in fish muscle tissue. Therefore, this technique could be used as a risk indicator for histamine production in fish (Bover-Cid et al., 2014). qPCR, through the Ct value, allowed to identify the presence of the *hdc* gene in sardine muscle tissue. The *hdc* GN gene (Ct 24 to 36.3) presented 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 number in sardine muscle tissue samples. This can be observed by the Ct value that is inversely related to the amount of qPCR reaction amplicons (Schmittgen and Livak, 2008).

5. Conclusion

With the methodologies employed it was possible to evaluate the microbiological and chemical quality of fresh and frozen sardines sold in a public market in Porto Alegre. These methods could be used as part of a histamine traceability system in the fish production chain. HPLC-DAD has been shown to be a good technique for identifying and quantifying histamine in fish. qPCR was a fast and reliable method to evaluate the quantification of histamine-producing gram-negative bacteria in fish. Moreover, with the amplification of the 16S rRNA gene and sequencing by HTS, it was possible to characterize important spoilage bacteria present in the sardines analysed.

6. Acknowledgements

The authors acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, DF, Brazil, for the Fellowship. JF is grateful for CNPq for Financial support (#401714/2016-0 and # 305497/2018-6).

7. Conflict of interest

No conflict of interest declared.

8. References

Altieri, I., Semeraro, A., Scalise, F., Calderari, I. and Stacchini, P. (2016). European official control of food: Determination of histamine in fish products by a HPLC–UV-DAD method. Food Chemistry, 211, 694-699.

Applied Biosystem (2003). Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR. 117GU18-01, p. 1-8.

Arulkumar, A.; Paramasivam, S., Rameshthangam, P., Paramithiotis, S. (2019) Evaluation of psychrophilic, mesophilic, histamine forming bacteria and biogenic amine content in the muscle of mud spiny lobster, *Panulirus polyphagus* (HERBST, 1793) during ice storage. J Food Saf.; 39:e12582.

Austin, B. (2006). The Bacterial Microflora of Fish, Revised. The Scientific World Journal 6, 931-945.

Ben-Gigirey, B., Tomas, J.M.V.B., VILLA, G., and Barros-Velazquez, J. (1998) Changes in Biogenic Amines and Microbiological Analysis in Albacore (*Thunnus alalunga*) Muscle during Frozen Storage. Journal of Food Protection, 61, 5, 608-615.

Bio-Rad Laboratories (2006). Real-Time PCR Applications Guide. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf

Bjerke, G.A., Rudi, K., Avershina, E., Moen, B., Blom, H., Axelsson, L. (2019). Exploring the Brine Microbiota of a Traditional Norwegian Fermented Fish Product (Rakfisk) from Six Different Producers during Two Consecutive Seasonal Productions. Foods, 8, 72.

Bjornsdóttir-Butler, K.; Jones, J. L.; Benner Jr., R.A.; Burkhardt III, W. (2011). Development of a real-time PCR assay with an internal amplification control for detection of Gram-negative histamine producing bacteria in fish. Food Microbiology, 28, 356-363.

Bjornsdottir-Butler, K., McCarthy, S.A., Dunlap, P.V., Benner Jr., R.A. (2016). *Photobacterium angustum* and *Photobacterium kishitanii*, Psychrotrophic High-Level Histamine-Producing Bacteria Indigenous to Tuna. Applied and Environmental Microbiology, 82, 7.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Harriet, A. (2018). QIIME 2: Reproducible, Interactive, Scalable, and Extensible Microbiome Data Science. PeerJ Preprints, e27295v1.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Caporaso, J. G. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome, 6, 1, 90.

Bover-Cid, S., Latorre-Moratalla, M.L., Veciana-Nogués, M.T., Vidal-Carou, M.C. (2014). Processing contaminants Biogenic Amines. Encyclopedia of Food Safety, v. 2.

Bruijn, I., Liu, Y., Wiegertjes, G.F., Raaijmakers, J.M. (2018). Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiology Ecology, 94, fix161.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods, 13, 7, 581–583. doi:10.1038/nmeth.3869

Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J.A.; Smith, G.; Knight, B. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Short communication. International Society for Microbial Ecology, 6, 1621-1624.

CDC, Centers for Disease Control and Prevention (2018). Surveillance for Foodborne Disease Outbreaks, United States, 2016, Annual Report. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC, 2018.

Codex Alimentarius (2018). Revision of the code of practice for fish and fishery products (CXC 52-3002) and revisions of the section on sampling, examination and analyses related to histamine food safety. Joint FAO and WHO Food Standards Programme Codex Committee on Food Hygiene, Fiftieth Session, Panama.

Danquah, A.O., Benjakul, S., Simpson, B.K. (2012). Biogenic Amines in Foods. In: Simpson, B.K. Food Biochemistry and Food Processing, 43, 820-832.

Deák, T. (2010). Current taxonomy of common foodborne bacteria. Acta Alimentaria, 39, 4, 471–487. doi:10.1556/aalim.39.2010.4.8

Dewey-Mattia, D., Karunya, M.I., Manikonda, M.P.H., Aron, J.H., DVM2 Matthew E. Wise, J. Crowe, S.J. Surveillance for Foodborne Disease Outbreaks - United States, 2009-2015. Centers for Disease Control and Prevention (CDC), U.S., Department of Health and Human Services, Atlanta, GA 30329-4027. MMWR Surveill Summ, 2018, 67.

Dias-Neto, J., Dias, J.F.O. (2015). O uso da biodiversidade aquática no Brasil: uma avaliação com foco na pesca. Brasília, Ibama, pp. 288.

EC, Commission Regulation. Microbiological criteria for foodstuffs. Commission Regulation, nº 2073, 15 november 2005.

EC, Commission Regulation (2019). Laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation nº 2074/2005 as regards official controls. Commission Implementing Regulation, 2019/627.

EFSA and ECDC, European Food Safety Authority and European Centre for Disease Prevention and Control (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA Journal, 16, 12, 5500, 1-262.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R. P. (2018). The Gut Microbiota of Marine Fish. Frontiers in Microbiology, 9, 873, 1-17. doi:10.3389/fmicb.2018.00873

El Hariri, O., Bouchriti, N., Baamal, L., Bengueddour, R. (2018). Fish Consumption, Purchasing Behaviour and Perception of Fish Quality with Especial Reference to Sardine. Int J Pharma Bio Sci, 9, 4, 89-101.

FDA, Food and Drug Administration (2011). Fish and Fishery Products Hazards and Controls Guidance, Department of Health and Human Services Public Health Service Food and Drug Administration Center for Food Safety and Applied Nutrition Office of Food Safety, Fourth Edition, 476, april.

FAO and WHO, Food and Agriculture Organization of the United Nations and World Health Organization (2018). Histamine in Salmonids. Literature Review.

FAO and WHO, Food and Agriculture Organization of the United Nations (FAO) or of the World Health Organization (2013). Public Health Risks of Histamine and other Biogenic Amines from Fish and Fishery Products. Meeting report, FAO headquarters, Rome Italy, 138 p.

FDA, Food and Drug Administration (2011). Fish and Fishery Products Hazards and Controls Guidance, fourth edition. U.S. Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition, Silver Spring, MD, 4th ed, cap. 7, p 113-152. https://www.fda.gov/media/80637/download

Gatti Junior, P., Assunção, A. W. A., Baldin, J. C., & Amaral, L. A. (2014). Microbiological quality of whole and filleted shelf-tilapia. Aquaculture, 433, 196–200.

Gouveia, N.N.F. Desenvolvimento de uma metodologia analítica para determinação de aminas biogênicas em tunídeos. 2009. Dissertação (Mestrado em Bioquímica Aplicada), Universidade da Madeira, Funchal, 2009.

Hattori, Y., Seifert, R. (Eds.). (2017). Histamine and Histamine Receptors in Health and Disease. Handbook of Experimental Pharmacology, 241.

Houicher, A.; Kuley, E.; Bendeddouche, B. (2013). Histamine and Tyramine Production by Bacteria Isolated from Spoiled Sardine (*Sardina Pilchardus*). African Journal of Biotechnology, 12, 3288.

Hu, Y.; Huang, Z.; Li, J.; Yang, H. (2012). Concentrations of biogenic amines in fish, squid and octopus and their changes during storage. Food Chemistry, 135, 2604-2611.

ICH, International Conference on Harmonisation. ICH Harmonised Tripartite Guideline Impurities in New Drug Substances Q3A (R2). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Current Step 4 version, 2006.

James, C., Derrick, S., Purnell, G., James, S.J. (2013). Review of the risk management practices employed throughout the fish processing chain in relation to controlling histamine formation in at-risk fish Species. Food Refrigeration and Process Engineering Research Centre, Grimsby Institute, Nuns Corner, Grimsby, North East Lincolnshire, UK, DN34 5BQ, FS241055.

Johnson, E.A., Schantz, E.J. (2017). Seafood Toxins. In: Dodd, C.E.R., Aldsworth, T., Stein, R.A. Foodborne Diseases, third edition, 17, 345-366.

Katoh K, Misawa K, Kuma Ki, Miyata T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic acids research, 30, 14, 3059-66.

Klanian, M.G., Díaz, M.D. and Solís, M.J.S. (2018). Molecular Characterization of Histamine-Producing Psychrotrophic Bacteria Isolated from Red Octopus (*Octopus maya*) in Refrigerated Storage. High-Throughput, 7, 25.

Kruskal, W. H., & Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. Journal of the American statistical Association, 47, 260, 583-621.

Lahti L., Shetty, S., Blake, T., Salojarvi, J. (2017). Microbiome R package. Tools Microbiome Anal R.

Landete, J.M., De Las Rivas, B., Marcobal, A., Muñoz, R. (2008). Updated Molecular Knowledge about Histamine Biosynthesis by Bacteria. Critical Reviews in Food Science and Nutrition, 48, 697-714.

Lehane, L., and Olley, J. (2000). Histamine fish poisoning revisited. International Journal of Food Microbiology, 58(1-2), 1–37.

León-Barrios, M., Ramírez-Bahena, M.H., Igual, J.M., Peix A., Velázquez, E. (2018). *Phyllobacterium salinisoli* sp. nov., isolated from a *Lotus lancerottensis* root nodule in saline soil from Lanzarote. Int J Syst Evol Microbiology, 68,108.

Life technologies (2016). Real-time PCR handbook. Life Technologies Corporation, CO019861 0216.

Lozupone C, Knight R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol., 71, 8228-8235.

Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol., 73, 5, 1576-85.

Marti, A.J. (2001). A new method for non-parametric multivariate analysis of variance. Austral ecology, 26, 1, 32-46.

Martino, C., Morton, J.T., Marotz, C.A., Thompson, L.R., Tripathi, A., Knight, R., Zengle, K. (2019). A Novel Sparse Compositional Technique Reveals Microbial Perturbations. mSystems. 4:e00016-19.

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., and Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. Microbial ecology in health and disease, 26, 1, 27663.

McInnis, E.A., Kalanetra, K.B., Mills, D.A., Maga, E.A. (2015). Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. Food Microbiology 46, 121-131.

McMurdie, P.J., Holmes, S. (2013). "Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data." PLoS ONE, 8, 4, e61217.

Møretrø, T., Langsrud, S. (2017). Residential Bacteria on Surfaces in the Food Industry and Their Implications for Food Safety and Quality. Comprehensive Reviews in Food Science and Food Safety, 16, 5, 1022–1041.

Ocaño-Higuera, V. M., Marquez-Ríos, E., Canizales-Dávila, M., Castillo-Yáñez, F. J., Pacheco-Aguilar, R., Lugo-Sánchez, M. E., García-Orozco, K.D., Graciano-Verdugo, A. Z. (2009). *Post mortem* changes in cazon fish muscle stored on ice. Food Chemistry, 116, 4, 933-938.

Odeyemi, O.A., Burke, C.M., Bolch, C.C.J., Stanley, R. (2018). Seafood spoilage microbiota and associated volatile organic compounds at different storage temperatures and packaging conditions. Int. Jour. of Food Microb., 280, 87-99.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., Suggests, M.A.S.S. (2007). The vegan package. Community ecology package, 10, 631-637.

Parlapani, F. F., Kormas, K. A., Boziaris, I. S. (2014). Microbiological changes, shelf life and identification of initial and spoilage microbiota of sea bream fillets stored under various conditions using 16S rRNA gene analysis. Journal of the Science of Food and Agriculture, 95, 12, 2386-2394.

Price, M.N., Dehal, P.S5, Arkin, A.P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS ONE., 5:e9490.

Raats, D., Offek, M., Minz, D., Halpern, M. (2011). Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiology, 28, 3, 465–471.

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M.-F., & Zagorec, M. (2015). Bacterial spoilers of food: Behavior, fitness and functional properties. Food Microbiology, 45, 45-53.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Acessado: 24 de junho de 2019. Available in: https://www.R-project.org/.

Ruiz-Capillas, C.; Herrero, A.M. (2019). Impact of Biogenic Amines on Food Quality and Safety. Foods, 8, 62, 1-16.

Shukla, S., Park, H.-K., Kim, J.-K., Kim, M. (2010). Determination of biogenic amines in Korean traditional fermented soybean paste (Doenjang). Food and Chemical Toxicology, 48, 5, 1191-1195.

Skrodenytė-ArbaČIauskiene, V. (2007). Enzymatic activity of intestinal bacteria in roach *Rutilus rutilus* L. Fisheries Science, 73, 964–966.

Takahashi, H., Kimura, B., Yoshikawa, M. and Fujii, T. (2003). Cloning and Sequencing of the Histidine Decarboxylase Genes of Gram-Negative, Histamine-Producing Bacteria and Their Application in Detection and Identification of These Organisms in Fish. Applied and Environmental Microbiology, 2568-2579, 69, 5.

Tembhurne, M., Ghag, A., Sanathkumar, H., Nayak, B.B. Dominance of Enterobacteria among Histamine-Producing Bacteria Isolated from Indian Mackerel. *Advances in Microbiology*, 2013, 3, 537-542.

Thi, A.N.T.; Samapundo, S., Devlieghere, F., Heyndrickx, M. (2016). Microbiota of frozen Vietnamese catfish (*Pangasius hypophthalmus*) marketed in Belgium. International Journal of Food Contamination, 3, 17.

Wendakoon, C.N.; Sakaguchi, M. (1995). Inhibition of Amino Acid Decarboxylase Activity of Enterobacter aerogenes by Active Components in Spices. Journal of Food Protection, 58, 3, 280-283.

Wilcoxon, F. (1945). Individual comparisons by ranking methods. Biometrics Bulletin, 1, 6, 80-83.

WHO and FAO. (2012). World Health Organization, Food and Agriculture Organization of the United Nations. Code of practice for fish and fishery products. Secretariat of the Codex Alimentarius Commission, CAC/RCP 52-2003, Rome.

Yesudhason, P., Al-Zidjali, M., Al-Zidjali, A., Al-Busaidi, M., Al-Waili, A., Al-Mazrooei, N., Al-Habsi, S. (2013). Histamine levels in commercially important fresh and processed fish of Oman with reference to international standards. Food Chemistry, 140, 777–783.

Yu, Y., M.D.; Wang, M.M.P.; Bian, M.D.L.; Hong, M.D.S. (2018). Rare death via Histamine Poisoning following crab consumption: A Case Report. J Forensic Sci, 63, 3.

Zhang, Y., Li, D., Ly, J., Li, Q., Kong, C., Luo, Y. (2017). Effect of cinnamon essential oil on bacterial diversity and shelf-life in vacuum-packaged common carp (*Cyprinus carpio*) during refrigerated storage. International Journal of Food Microbiology, 249, 1–8.

Zang, J., Xu, Y., Xia, W., Yu, D., Gao, P., Jiang, Q., Yang, F. (2018). Dynamics and diversity of microbial community succession during fermentation of Suan yu, a Chinese traditional fermented fish, determined by high throughput sequencing. Food Research International, 111, 565–573.

CAPÍTULO IV DISCUSSÃO GERAL

CAPÍTULO IV

DISCUSSÃO GERAL

A histamina é uma das aminas biogênicas mais importantes presentes nos peixes e é a única com limites legais estabelecidos para o consumo humano. Neste trabalho, todas as amostras de corvinas frescas (24 amostras) e sardinhas frescas (12 amostras) e congeladas (12 amostras) testadas apresentaram concentrações acima de 200 mg kg^{-1} de histamina, valores estabelecidos como limite máximo legal pelo Códex Alimentarius, 2018. Esta legislação só faz referências aos peixes das famílias *Scombridae*, *Clupeidae*, *Engraulidae*, *Coryphaenidae*, *Pomatomidae* e *Scomberesocidae*. Todas as amostras de corvina obtiveram altos níveis de histamina, variando de 417,63 a 604,62 mg kg^{-1} para o corvina coletada durante o verão foi de 258,52 a 533,24 mg kg^{-1} para a corvina coletada durante o inverno. Já nas amostras de sardinhas frescas variou de 226,14 a 484,00 mg kg^{-1} e as sardinhas congeladas variou de 342,74 a 533,28 mg kg^{-1}.

As legislações vigente no Brasil, como a norma técnica nº 40/1994 do MERCOSUL e o regulamento técnico de Identidade e Qualidade para Peixe Fresco do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), nº 185, fixam o nível máximo de 100 mg kg^{-1} de histamina no tecido muscular de peixes Escombroide (MERCOSUL, 1994; MAPA, 1997). A legislação mais recente para peixes congelados, a Instrução Normativa, nº 21/2017. Estabelece que os níveis de histamina máximo não devam ser superiores a 100 mg kg^{-1} tomando como base uma amostra composta por 9 unidades amostrais e nenhuma unidade amostral pode apresentar resultado superior a 200 mg kg^{-1} para os grupos de famílias de peixes já citados (MAPA, 2017).

Em todas as amostras positivas de bactérias produtoras de histamina quantificadas por qPCR, a presença de histamina foi confirmada por HPLC-DAD. Todas as amostras frescas de corvina e das sardinhas frescas e congeladas foram positivas para os genes *hdc*, embora com uma ampla gama de valores de Ct, indicando diferenças nas concentrações de oligonucleotídeos nas amostras. Os ensaios de qPCR tinham alta capacidade de quantificação do gene *hdc*. A quantidade do número de cópias transformadas em log_{10} para o gene *hdc* nas amostras de corvina para bactérias GN variou de 7,9 a 12,1 log_{10} g^{-1} foi maior quando comparada à quantidade de cópias do gene específico, *M. morganii* (4,67 a 10,06 log_{10} g^{-1}) e *E. aerogenes* (6,32 a 8,23 log^{10} g^{-1}). O gene *hdc* GN nas sardinhas frescas e congeladas variou de 5,31 a 9,8 log_{10} g^{-1}, cópias do gene específico, para *M. morganii* foi de 5,96 a 9,58 log_{10} g^{-1} e para *E. aerogenes* variou de 4,90 a 8,60 log_{10} g^{-1} valores bem menores do que os observados

nas corvinas congeladas. Os microrganismos mais frequentemente associados ao envenenamento por histamina em peixes são bactérias gram-negativas, principalmente *Enterobacteriaceae* (Biji et al. 2016), principalmente as espécies *M. morganii* e *E. aerogenes*, que apresentam alto potencial para a expressão da enzima histidina descarboxilase e podem produzir níveis perigosos de histamina em um curto período de tempo, quando armazenados em temperatura inadequada (Podeur et al. 2015). A identificação dessas bactérias é de fundamental importância para a segurança alimentar, a fim de evitar possíveis deteriorações e formação de histamina nos peixes (Zou e Hou 2017).

As altas concentrações de histamina nesses peixes podem estar relacionadas à microbiota presente nesses peixes. Além disso, as corvinas e sardinhas utilizadas neste estudo foram adquiridas com vísceras e brânquias. Existem diferentes níveis de microrganismos nesses órgãos. A concentração bacteriana no trato digestivo é maior e pode variar de 10^4 a 10^9 UFC g^{-1} (SkrodenytėArbaČIauskiene, 2007). Enquanto nas brânquias (10^6 UFC g^{-1}), há uma população menor de bactérias (Austin, 2006). No entanto, o tecido muscular do peixe é considerado estéril, mas quando esses peixes são vendidos inteiros, pode ocorrer auto contaminação ou contaminação cruzada de bactérias no momento da limpeza e evisceração (Gatti Junior et al., 2014; Bruijn et al., 2018). Estudos demonstraram que a histamina é detectável apenas em peixes em decomposição ou associados a um crescimento relevante, 10^7 UFC g^{-1}, de bactérias no músculo dos peixes (Gardini et al. 2016).

Um dos fatores predisponentes para a formação de histamina é a temperatura inadequada na conservação dos peixes. Essa variação de temperatura permite o crescimento de microrganismos em deterioração, o aumento da atividade proteolítica no músculo e a formação e atividade da enzima histidina descarboxilase (Ruiz-Capillas e Herrero, 2019). Além disso, mesmo com a inativação bacteriana por resfriamento, a histamina ainda pode ser formada em menor concentração pela ação da enzima. Sob condições de congelamento, a enzima permanece estável e após o descongelamento se torna ativa (FDA, 2011).

Outro fator predisponente para a formação de histamina é o pH do tecido muscular dos peixes. Neste estudo, o pH do músculo nas sardinhas frescas apresentou valores mais altos (6,70 a 7,18) quando comparado às sardinhas congeladas (6,22 a 6,69), proporcionando uma melhor microbiota. (Fan et al., 2016). O controle do pH muscular é importante para manter a qualidade sensorial e microbiológica da carne de peixe. Essa variação no pH muscular é influenciada pelas espécies de peixes, tipo de tecido muscular, dieta, sazonalidade e estresse pós-captura (Ocaño-Higuera et al., 2009). O valor do pH do músculo influencia diretamente a

atividade da enzima histidina descarboxilase. A enzima histidina descarboxilase das bactérias *E. aerogenes* e M. morganii apresenta melhor atividade a pH 6,5 (Wendakoon e Sakaguchi, 1995). A maioria das sardinhas e corvinas avaliadas neste estudo apresentou pH adequado para a atividade da histidina descarboxilase.

Devido à complexidade da microbiota de peixes, os métodos de detecção dependentes da cultura podem ser insuficientes, portanto, métodos alternativos são necessários para entender a diversidade dos microrganismos nesses alimentos (Parlapani et al. 2018). O High Throughput Sequencing (HTS) é o método atual que permite identificar o perfil das comunidades microbianas, monitorando as flutuações populacionais e caracterizando as bactérias nas matrizes alimentares. O perfil taxonômico de uma comunidade microbiana de alimentos pode ser obtido através da amplificação do gene 16S rRNA (Laudadio et al. 2018). Através da amplificação do gene 16S rRNA foi possível identificar bactérias nos músculos corvina frescas e das sardinhas congeladas e frescas. Os principais filos encontrados nas corvinas foram Proteobacteria (68,60% coletadas no verão e 75,00% no inverno), Bacteriodetes, Firmicutes e Actinobacterium. O filo Proteobacteria foi predominante nas sardinhas congeladas e representaram pelo menos 94,84% da diversidade bacteriana. Enquanto em sardinha fresca, o filo dominante foi Firmicutes com 65,86%, seguido pelo filo Proteobacteria (33,90%). Esses resultados mostram que houve uma diferenciação entre a microbiota do nível do filo de acordo com o tipo de preservação das sardinhas. Estás bactérias são comumente encontrados na microbiota autóctone dos conteúdos de pele, brânquias, intestinos e intestinos de peixes (Llewellyn et al. 2014).

As bactérias dominantes nas amostras de corvina foram as famílias *Moraxellaceae, Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Shewanellaceae e Aeromonadaceae* foram as famílias mais abundantes no filo de Proteobacteria. *Moraxellaceae* foi mais prevalente na corvina coletada no verão (média de 19,34%), enquanto *Pseudomonadaceae* esteve mais presente nas amostras de inverno (média de 35,55%). *Burkholderiaceae* esteve presente proporcionalmente nas diferentes estações do ano, enquanto a família *Enterobacteriaceae* foi dominante no inverno. As bactérias produtoras de histamina em peixes, incluindo *Moraxellaceae, Pseudomonadaceae, Enterobacteriaceae, Shewanellaceae* e *Aeromonadaceae*, representam mais da metade da composição dos peixes analisados (51% no verão e 77% no inverno).

O gênero *Macrococcus* dominou a microbiota de sardinha fresca (49,87%). *Macrococcus* sp. parece fazer parte da microbiota inicial de peixes refrigerados. Essas bactérias

dominaram os filetes de microbiota de carpa comum embalados a vácuo (*Cyprinus carpio*) e filé de dourada (*Sparus aurata*) (Parlapani et al., 2014; Zhang et al., 2017). A espécie *M. caseolyticus* foi identificada em todas as amostras de sardinha, maior proporção de 65,05% nas sardinhas frescas e menor em sardinha congelada (3,1%). Esses resultados corroboram com outros estudos que identificaram as espécies em outros alimentos, como peixe fermentado (Zang et al., 2018), camarão semi-seco (Karani et al., 2015) e produtos congelados de peixe-gato (*Pangasius hypophthalmus*) (Thi et al. 2016). *Phyllobacterium* foi o gênero predominante nas sardinhas congeladas (47,97%). Estas bactérias são organismos Gram negativos e fixadores de nitrogênio, facilmente encontrados nas plantas e no solo (León-Barrios et al., 2018). No entanto, eles foram isolados na microbiota intestinal do salmão do Atlântico (*Salmo salar*) e do peixe-gato (*Ictalurus punctatus*) (McInnis et al., 2015; Raats et al., 2011).

Outros gêneros predominantes no tecido muscular da sardinha foram *Acinetobacter*, com 12,65% nas sardinhas frescas e 10,28% nas sardinhas congeladas. Além do gênero *Pseudomonas*, nas sardinhas frescas (7,80%) e maior concentração nas sardinhas congeladas (18,25%). Essas bactérias estão amplamente presentes na água e no solo e podem ser encontradas em alimentos frescos refrigerados (Deák, 2010). Além de pertencer à microbiota intestinal residente e, portanto, pode potencialmente contaminar o peixe durante o processamento (Egerton et al., 2018). Esses gêneros são frequentemente relatados em plantas de processamento de peixes (Møretrø e Langsrud, 2017). Os principais microrganismos psicrotróficos em deterioração em peixes frios são os gêneros Pseudomonas e Acinetobacter (Odeyemi et al., 2018). Outras bactérias deteriorantes foram encontradas em menor grau nas sardinhas frescas e congeladas neste estudo. Como as bactérias *Aeromonas*, *Brochothrix*, *Photobacterium*, *Psychrobacter*, *Flavobacterium*, Bactérias ácido-lácticas (BAL), *Staphylococcus*, *Micrococcus*, *Listeria*, *Vagococcus* e *Kurthia* (Remenant et al., 2015; Bjerke et al., 2019).

Entre esses grupos de microrganismos deteriorantes, as bactérias Gram negativas são as principais bactérias deterioradoras e formadoras de histamina em peixes frescos (Macé et al., 2013). As principais famílias de bactérias destruidoras de alimentos encontradas nas amostras frescas do músculo corvina neste estudo foram representadas principalmente por bactérias Gram negativas, membros da família *Moraxellaceae, Pseudomonadaceae, Flavobacteriaceae, Burkholderiaceae* e *Enterobacteriaceae*. Em menor porcentagem, as famílias *Shewanellaceae, Aeromonadaceae, Pasteurellaceae* e *Xanthomonadaceae.* As enterobactérias podem ocorrer em produtos de peixe como resultado de contaminação fecal, poluição da água ou contaminação

durante o processamento (Huss, 1995). Nesse grupo, são apresentadas as espécies *M. morganii*, *Klebsiella pneumoniae*, *Hafnia alvei*, *Proteus vulgaris*, *E. aerogenes* e *E. cloacae* (Hazards 2011). Além disso, espécies pertencentes ao gênero *Clostridium*, *Vibrio*, *Acinetobacter*, *Plesiomonas*, *Pseudomonas*, *Aeromonas* e *Photobacterium* também foram relatadas como produtoras de histamina (Visciano et al. 2012; Wongsariya et al. 2016). Sob condições de armazenamento aeróbico, vários grupos de bactérias Gram negativas, particularmente *Pseudomonas* sp., *Aeromonas* sp. e *Enterobacteriaceae* são microrganismos dominantes na deterioração em peixes de água doce e salgada (Kung et al. 2017). No entanto, espécies de *Moraxellaceae* não possuem a enzima histidina descarboxilase (Özogul e Özogul 2005).

O uso de métodos para a detecção precoce e rápida de bactérias produtoras de histamina e a detecção de histamina é importante para evitar o acúmulo dessa substância tóxica nos alimentos. O uso da abordagem molecular, como qPCR, para a detecção de bactérias formadoras de histamina em peixes e determinação da presença de histamina por HPLC, foi aplicado concomitantemente em vários estudos. Em particular, a PCR quantitativa em tempo real (qPCR) oferece a possibilidade de detecção e quantificação rápidas de bactérias e detecção de toxinas com maior especificidade, sensibilidade e confiabilidade do que os métodos tradicionais de cultura (Postollec et al. 2011; Rodríguez-Lázaro et al. 2014). Essas técnicas podem representar bons métodos preditivos para identificar um potencial fator de risco em produtos de peixe durante o processamento, armazenamento e comercialização e podem ser usadas para investigar estratégias de controle de risco (Barbosa et al. 2018).

CAPÍTULO V: CONCLUSÃO

CAPÍTULO V

CONCLUSÃO

Considerando nossos resultados obtidos ao longo do desenvolvimento deste trabalho, as concentrações de histamina encontradas foram superiores ao nível preconizado pela legislação, ultrapassando o valor máximo de 200 mg kg^{-1}, tanto nas amostras de corvina fresca, bem como, nas sardinhas inteira fresca ou congeladas.

A detecção e quantificação de bactérias produtoras de histamina usando a amplificação do gene *hdc* por meio da técnica da qPCR foi especifica para as bactérias Gram negativas, *M. morganii* e *E. aerogenes*. Tais bactérias foram detectadas em todas as amostras de tecido muscular da corvina e sardinhas frescas ou congeladas. A identificação rápida da histamina e das bactérias produtoras da enzima Hdc é importante para prevenir a deterioração dos alimentos e um potencial perigo para a saúde do consumidor.

O manejo apropriado do peixe desde a captura até sua comercialização e consumo é crucial para controlar a formação e acumulação dessa amina biogênica na carne de peixes. É de fundamental importância acompanhar a temperatura de conservação em toda cadeia produtiva do pescado, para evitar a formação de histamina nos peixes. Um fator importante é que os peixes analisados no estudo foram comercializados inteiros, com vísceras e brânquias, o que pode ter contribuído para a contaminação do tecido muscular com essas bactérias, já que esses órgãos possuem bactérias produtoras da enzima Hdc.

A amplificação do gene *16S rRNA*, mostrou a presença de bactérias deterioradoras e formadoras de histamina nas corvinas e sardinhas frescas ou congeladas. Nas corvinas frescas foram identificadas uma porcentagem significativa da abundância relativa de bactérias Gram negativas pertencentes à família *Moraxellaceae* (19.34% nas amostras de verão) e *Pseudomonadaceae* (35,55% nas amostras de inverno). As bactérias produtoras de histamina em peixes, incluindo *Moraxellaceae, Pseudomonadaceae, Enterobacteriaceae, Shewanellaceae* e *Aeromonadaceae,* representam mais da metade da composição das corvinas analisados (51% no verão e 77% no inverno). Enquanto as sardinhas frescas a maior abundância relativa foi de bactérias Gram positiva pertencentes aos gêneros *Macrococcus* nas sardinhas frescas (49,88%) e as sardinhas congeladas foram compostas pela família *Phyllobacterium* (47,73%). Verificou-se que bactérias produtoras de histamina gram-negativas representavam 31,06% em sardinha fresca e 34,97% em sardinha congelada. Bactérias com alta capacidade de produção de histamina foram identificadas em peixes, como Morganella (representada por M. morganii) e

Photobacterium (representada P. damselae). Além de outras bactérias com baixa capacidade de produção de histamina, como Proteus, *Psychrobacter* (*P. pulmonis*), *Shewanella*, *Aeromonas*, *Stenotrophomonas* (*S. geniculata*), *Acinetobacter*, *Pseudomonas*, *Janthinobacterium*.

Novos estudos devem ser desenvolvidos para acompanhar a formação de histamina durante todo processo produtivo do pescado, para podermos ver os principais pontos críticos para possível formação de histamina. A utilização dessas técnicas pode ser padronizada para no futuro poder ser usadas nos laboratórios e na indústria da pesca para prever o risco de formação da histamina.

REFERÊNCIAS

Al Bulushi, I. M., Poole, S., Deeth, H., C. Dykes, G., A. (2018). Evaluation the spoilage and biogenic amines formation potential of marine Gram-positive bacteria. International Food Research Journal, 25, 5, 2143-2148.

Alvarez, M.A.; Moreno-Arribas, M.V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. Trends in Food Science and Technology, n. 39, p. 146-155.

Alves, R. T., Dos Santos, A. T., Martins, M. F. Detection of histamine-producing bacteria using polymerase chain reaction techniques and DNA probes. Journal European Food Research *and* Technology, *v.* 214, p. 178-180, 2002.

AOAC (1990). Association of Official Analytical Chemists. 977.13 Histamine in Sea food Fluorometric Method First Action 1977 Final Action 1987 Codex-Adopted.

AOAC (2012). Association of Official Analytical Chemists. Official Method. J. AOAC, n. 60, p. 1125-1131, 1977. CAS-51-45-6 (histamine). Revised: June 2003, September, November.

Barbosa, R.G., Gonzaga, L.V., Lodetti, E., Olivo, G., Costa, A.C.O., Aubourg, S.P. and Fett, R. (2018) Biogenic amines assessment during different stages of the canning process of skipjack tuna (*Katsuwonus pelamis*). International journal of food science and technology, n. 53, p. 1236-1245.

Bäumlisberger, M.; Moellecken, U.; König, H.; Claus, H. (2015). The Potential of the Yeast Debaryomyces hansenii H525 to Degrade Biogenic Amines in Food. Microorganisms, v. 3, n. 4, p. 839-850, 2015.

Benkerroum, N. (2016). Biogenic amines in dairy products: origin, incidence, and control means. Comp. Rev. Food Sci. Food Saf. 15, 801-826.

Biji, K.B.; Ravishankar, C.N.; Venkateswarlu, R.; Mohan, C.O.; Gopal, T.K.S. (2016). Biogenic amines in seafood: a review. Journal Food Science Technology, 53, 5, 2210-2218.

Bjerke, G.A., Rudi, K., Avershina, E., Moen, B., Blom, H., Axelsson, L. (2019). Exploring the Brine Microbiota of a Traditional Norwegian Fermented Fish Product (Rakfisk) from Six Different Producers during Two Consecutive Seasonal Productions. Foods, 8, 72.

Bjornsdottir-Butler, K., Bowers, J. C., Benner, R. A. (2015). Prevalence and Characterization of High Histamine–Producing Bacteria in Gulf of Mexico Fish Species. Journal of Food Protection, 78, 7, 1335-1342.

Brasil. Manual Integrado de vigilância, Prevenção e Controle de Doenças Transmitidas por Alimentos. Ministério da Saúde. Secretária de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Editora do Ministério da Saúde, 2010.

Brasil. Surtos de Doenças Transmitidas por alimentos. Informe 2018. Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis. Ministério da Saúde, Coordenação Geral de Doenças Transmissíveis. Unidade de Vigilância das Doenças de Transmissão Hídrica e Alimentar. Brasília, 2019. http://portalarquivos2.saude.gov.br/images/pdf/2019/fevereiro/15/Apresenta----o-Surtos-DTA---Fevereiro-2019.pdf

Brasil. (2017). Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal (RIISPOA). Decreto nº 9.013, de 29 de março de 2017. Instituída pela Lei nº 1.283, de 18 de dezembro de 1950, e pela Lei nº 7.889, de 23 de novembro de 1989, Ministério da Agricultura, Pecuária e Abastecimento (MAPA).

Brime, L. Chemical Food Safety. Ed. Modular Texts Series, First Edition, p. 211-212, 2011.

Brink, B.T.; Damink, C.; Joosten, H.M.L.J.; Veld, J.H.J. (1990). Occurrence and formation of biologically active amines in foods. International Journal of Food Microbiology, v. 11, p. 73-84.

Bruijn, I., Liu, Y., Wiegertjes, G.F., Raaijmakers, J.M. (2018). Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiology Ecology, 94, fix161.

Bunka, F.; Budinský, P.; Zimáková, B.; Merhaut, M.; Flasarová, R.; Pachlová, V.; Kubán, V.; Bunková, L. (2013). Biogenic amines occurrence in fish meat sampled from restaurants in region of Czech Republic. Food Control, v. 31, p. 49-52.

Cahill, M.M. (1990). Bacterial Flora of Fishes: A Review. Microbial Ecology, 19, 1, 21-41.
Carmo, F.B.T.; Mársico, E.T.; São Clemente, S.C.; Carmo, R.P.; Freitas, M.Q. Histamina em conservas de sardinha. Ciência Animal Brasileira, Goiânia, v. 11, n. 1, p. 174-180, jan/mar, 2010.

Caspi, R., Billington, R., Fulcher, C. A., Keseler, I. M., Kothari, A., Krummenacker, M., Latendresse, M., Midford, P.E., Ong, Q., Ong, W.K., Paley, S., Subhraveti, P., Karp, P. D. (2017). The MetaCyc database of metabolic pathways and enzymes. Nucleic Acids Research, 46(D1), D633–D639.

Codex Alimentarius (2018). Revision of the code of practice for fish and fishery products (CXC 52-3002) and revisions of the section on sampling, examination and analyses related to

histamine food safety. Joint FAO/WHO Food Standards Programme Codex Committee on Food Hygiene, Fiftieth Session, Panama.

Coton, M.; Coton, E. (2005). Multiplex PCR for colony direct detection of Gram positive histamine- and tyramine-producing bacteria. Journal of Microbiological Methods, v.63, p. 296-304.

Costantini, A., Cersosimo, M., Del Prete, V., García-Moruno, E. (2006). Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. Journal Food Protection, n. 69, p. 391-396.

Dadáková, E.; Pelikánová, T.; Kalac, P. Content of biogenic amines and polyamines in some species of European wild-growing edible mushrooms. Journal European Food Research and Technology, n. 230, p. 163-171, 2009.

Danquah, A.O.; Benjakul, S.; Simpson, B.K. Biogenic Amines in Foods (2012). In. Simpson, B.K.; Nollet, L.M.L.; Toldrá, F.; Benjakul, S.; Paliyath, G.; Y.H. Hui. Food Biochemistry and Food Processing, A John Wiley and Sons, Ltd., Publication, Second Edition, cap. 43, p. 825-832.

Deák, T. (2010). Current taxonomy of common foodborne bacteria. Acta Alimentaria, 39, 4, 471–487. doi:10.1556/aalim.39.2010.4.8

De Las Rivas, B., Marcobal, A.; Muñoz, R. (2005). Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS Microbiology Letters, v. 244, p. 367-72.

De La Torre, C.A.L., Conte-Junior, C.A. (2018). Detection of biogenic Amines: Quality and Toxicity Indicators in Food of Animal Origin. In: Holban, A.M.; Grumezescu, A.M. Food Control and Biosecurity, Handbook of Food Bioengineering, 225-257.

Dellova, D., França, D., Donati, G., Cezário, G., Marini, H., Real, J.V., Lino, J., Priebsch, K.,Albuquerque, L., Oliveira, M., Vieira, R. (2019). Anuário Peixe BR da Piscicultura 2019. Associação Brasileira da Piscicultura, Peixe BR.

Demoncheaux, J.P., Michel, R., Mazenot, C., Duflos, G., Iacini, C., Delaval, F., Saware, E.M., Renard, J.-C. (2012) A large outbreak of scombroid fish poisoning associated with eating yellowfin tuna (*Thunnus albacares*) at a military mass catering in Dakar, Senegal. Epidemiol Infect, 140, 1008–1012.

Dewey-Mattia, D.; Manikonda, K.; Hall, A.J.; Wise, M.E.; Crowe, S.J. Surveillance for Foodborne Disease Outbreaks, United States, 2009-2015. Centers for Disease Control and Prevention (CDC), Surveillance Summaries, v. 67, n. 10, p. 1-11, 2018.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R. P. (2018). The Gut Microbiota of Marine Fish. Frontiers in Microbiology, 9, 873, 1-17. doi:10.3389/fmicb.2018.00873

European Commission (2015). RASFF for safer food. The Rapid Alert System for Food and Feed - 2014 annual report, European Union, 2015.

European Commission (2016). RASFF for safer food. The Rapid Alert System for Food and Feed - 2015 annual report, European Union, 2016.

EFSA, European Food Safety Authority (2011). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. EFSA Panel on Biological Hazards (BIOHAZ). European Food Safety Authority (EFSA), European Food Safety Authority Journal, Parma, Italy, 9, 10, 1-93.

EFSA. European Food Safety Authority (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. European Food Safety Authority and European Centre for Disease Prevention and Control, European Food Safety Authority Journal, v. 14, n. 12, p.4634.

EFSA and ECDC, European Food Safety Authority and European Centre for Disease Prevention and Control (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017, EFSA-Q-2017-00751.

Erim, F.B. Review: Recent analytical approaches to the analysis of biogenic amines in food samples. Trends in Analytical Chemistry, v. 52, p. 239-247, 2013.

Erkmen, O.; Bozoglu, T.F. (2016). Spoilage of Fish and Other Seafoods, ch. 18, p. 301-304. In: Food Microbiology: principles into practice, 458 p.

FDA, Food and Drug Administration. (2011). Scombrotoxin (Histamine) Formation. Chap. 7, p. 113-152. In: Fish and fishery products hazards and controls guidance. Washington, Office of Seafood, 4.

Fan, H.; Liu, X.; Hong, H.; Shen, S.; Xu, Q.; Feng, L.; Luo Y. (2016). Quality Changes and Biogenic Amines Accumulation of Black Carp (*Mylopharyngodon piceus*) Fillets Stored at Different Temperatures. Journal of Food Protection, v. 79, n. 4, p. 635-645.

FAO (2011). Food and Agriculture Organization of the United Nations. Scombrotoxin (Histamine) Formation. In: Book Fish and Fishery Products, chap. 7, p. 113-152. Departament of Health and Human Services Public Health Service Food and Drog Administration Center for food Safety and Applied Nutrition Office of Food Fafety. Food and Drog Administration, Fourth Edition, apr.

FAO and WHO (2012). Code of Practice for Fish and Fishery products. Secretariat of the Codex Alimentarius Commission Joint FAO/WHO Food Standards Programme, p. 211-238.

FAO, Food and Agriculture Organization of the United Nations (2016a). El estado mundial de la pesca y la acuicultura 2016. Contribución a la seguridad alimentaria y la nutrición para todos. Roma, 224 p.

FAO, Food and Agriculture Organization of the United Nations (2016b). Safety of fish and fish products. Food and Agriculture Organization of the United Nations. Fisheries and Aquaculture Department. Acessado em: 08 de janeiro de 2016. Disponível em: < http://www.fao.org/fishery/topic/1522/en>

FAO, Food and Agriculture Organization (2018a). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome, CC BY-NC-SA 3.0 IGO.

FAO. Food and Agriculture Organization of the United Nations (2018b). Fishery and Aquaculture Statistics 2016. Estadísticas de Pesca y Acuicultura 2016. FAO yearbook, Rome, 104 p.

FDA, Food and Drug Administration (2011). Fish and Fishery Products Hazards and Controls Guidance, Department of Health and Human Services Public Health Service Food and Drug Administration Center for Food Safety and Applied Nutrition Office of Food Safety, Fourth Edition, 476, april.

Fernandez, M.; Del Río, B.; Linares, D. M.; Martín, M. C.; Alvarez, M. A. Real-time polymerase chain reaction for quantitative detection of histamine-producing bacteria: use in cheese production. Journal of Dairy Science, v. 89, p. 3763-3769, 2006.

Feng, C., Teuber, S., Gershwin, M. E. (2015). Histamine (*Scombroid*) Fish Poisoning: A Comprehensive Review. Clinical Reviews in Allergy and Immunology, v. 50, n. 1, p. 64-69.

Gagic, M., Jamróz, E., Krizkova, S., Milosavljevic, V., Kopel, P., Adam, V. (2019). Current Trends in Detection of Histamine in Food and Beverages. Journal of Agricultural and Food Chemistry. J. Agric. Food Chem., 67, 773−783.

Gardini, F.; Özogul, Y.; Suzzi, G.; Tabanell, G.; Özogul, F. (2016).Technological Factors Affecting Biogenic Amine Content in Foods: A Review. Frontiers in Microbiology, v. 7, n. 1218, p. 1-18.

Gram, L.; Huss, H.H. (1996). Microbiological spoilage of fish and fish products. International Journal of Food Microbiology, v. 33, p. 121-137.

Gatti Junior, P., Assunção, A. W. A., Baldin, J. C., Amaral, L. A. (2014). Microbiological quality of whole and filleted shelf-tilapia. Aquaculture, 433, 196–200.

Hajimohammadi, B.; Raeisi, M.; Eftekhar, E.; Mohebat, R.; Saffari, Azade, H. (2019). Studying the effect of *Allium sativum* and *Bunium persicum* essential oils on histamine production in Mahyaveh, an Iranian seasoned fish sauce. Journal of Food Safety, n.39, p. 1.7.

Halisz, A.; Barith, A.; Simon-Sarkadia, L.; Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. Trends in Food Science and Technology, v. 5.

Hazards, E.P.O.B. (2011) Scientific opinion on risk based control of biogenic amine formation in fermented foods. Efsa Journal 9, 2393.

Huss, H.H. El Pescado Fresco: Su Calidad y Cambios de su Calidad. FAO documento técnico de pesca, Organización de las Naciones Unidas para la Agricultura y la Alimentación. Laboratorio Tecnológico, Ministerio de Pesca, Dinamarca, n. 348, 1999. Acessado em: 10 de outubro de 2019. Disponível em: <http://www.fao.org/docrep/v7180s/v7180s06.htm#5.3 cambios bacteriológicos>.

HUSS, H. H. Quality and quality changes in fresh fish. Technological Laboratory Ministry of Agriculture and Fisheries Denmark. Food and Agriculture Organization of the United Nations, Rome, FAO, 1995. Acessado em: 10 de dezembro de 2018. Disponível em: <http://www.fao.org/docrep/v7180e/V7180E00.HTM#Contents>.

IBAMA e CEPERG, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Centro de Pesquisa e Gestão dos Recursos Pesqueiros Estuarinos e Lagunares (2012). Desembarque de pescado no Rio Grande do Sul. Instituto Brasileiro do Meio Ambiente e dos Recursos naturais Renováveis; Centro de Pesquisa e Gestão dos Recursos Pesqueiros Lagunares e Estuarinos; Projeto Estatístico Pesqueiro. Rio Grande, 40 p.

Joneja, J.M.; Fellow, H.R. (2004). Histamine Intolerance, Diamine Oxidase Activity, and Probiotics. School of Biosciences University of Birmingham, U.K. Acessado em 19 de janeiro de 2018, disponível em: http://www.allergynutrition.com/wp-content/uploads/2014/05/Histamine-DAO-and-Probiotics-Revised.pdf

James, C., Derrick, S., Purnell, G., James, S.J. (2013). Review of the risk management practices employed throughout the fish processing chain in relation to controlling histamine formation in at-risk fish Species. Food Refrigeration and Process Engineering Research Centre, Grimsby Institute, Nuns Corner, Grimsby, North East Lincolnshire, UK, DN34 5BQ, FS241055.

Leurs, R.; Hough, L.B.; Blandina, P.; Haas, H.L. Histamine. In: Brady, S.T.; Siegel, G.J.; Albers, R.W.; Price, *D.L.* Basic Neurochemistry, Principles of Molecular, Cellular, and Medical Neurobiology, Eighth Edition, c. 16, p. 323-341, 2012.

Ladero, V.; Calles, M.; Fernández, M.; Alvarez, M. A. (2010). Toxicological effect of dietary biogenic amines. Current Nutrition & Food Science, 6, 145-156.

Landete, J. M., Ferrer, S., Pardo, I. (2005). Which lactic acid bacteria are responsible for histamine production in wine? Journal of Applied Microbiology, v. 99, p. 580-586.

Landete, J. M.; Rivas, B.; Marcobal, A.; Muñoz, R. (2008). Updated Molecular Knowledge about Histamine Biosynthesis by Bacteria. Critical Reviews in Food Science and Nutrition, v. 48, p. 697-714.

Laudadio, I.; Fulci, V.; Palone, F.; Stronati, L.; Cucchiara, S.; Carissimi, C. (2018). Quantitative Assessment of Shotgun Metagenomics and 16S rDNA Amplicon Sequencing in the Study of Human Gut Microbiome. OMICS, 22, 4, 248-254.

Le Jeune, C., Lonvaud-Funel, A., Ten Brink, B., Hofstra, H., Van Der Vossen, J. M. B. M. (1995). Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. Journal of Applied Microbiology, v.78, p. 316-326.

León-Barrios, M., Ramírez-Bahena, M.H., Igual, J.M., Peix A., Velázquez, E. (2018). *Phyllobacterium salinisoli* sp. nov., isolated from a *Lotus lancerottensis* root nodule in saline soil from Lanzarote. Int J Syst Evol Microbiology, 68,108.

Llewellyn, M.S.; Boutin, S.; Hoseinifar, S.H.; Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology, 5, 207, 1-17.

Kang, C.R., Kim, Y.Y., Lee, J.I., Joo, H.D., Jung, S.W., Cho, S. (2018). Fish Poisoning Associated with Consumption of Yellowtail Fish in Seoul, Korea. J. Korean Med. Sci., 17, 33, 38, e235.

Karani, M., Shashidhar, R., Kakatkar, A., Gautam, R.K., Sukhi, S., Pansare-Godambe, L., Bandekar, J. (2015) . Radiation-resistant *Macrococcus caseolyticus* (A) isolated from radiation-processed semidried prawns. Canadian Journal of Microbiology, v. 61, p. 1-4.

Karovicova, J.; Kohajdova, Z. (2005). Biogenic Amines in Food. Chemical Papers, v. 59, n. 1, p. 70-79.

Kasuga, F. Fish and fish products. In: ICMSF. Microbial Ecology of Food Commodities. International Commission on Microbiological Specifications for Foods. Kluwer Academic, Plenum Publishers, New York, Boston, Dordrecht, London, Moscow, cap. 3, sec. 2, 174-236, 2005.

Kovacova-Hanuskova, E., Buday, T., Gavliakova, S., Plevkova, J. (2015). Histamine, histamine intoxication and intolerance. Allergologia et Immunopathologia, v. 43, n. 5, 498-506.

Kobayashi, T., Wang, X., Shigeta, N., Taguchi, C., Ishii, K., Shozen, K., Harada, Y., Imada, C., Terahara, T., Shinagawa, A. (2016). Distribution of histamine-producing lactic acid bacteria in canned salted anchovies and their histamine production behavior. Annals of Microbiology, v. 66, n. 3, 1277-1284.

Kung, H.F.; Wang, T. Y.; Huang, Y. R.; Lin, C. S.; Wua, W. S.; Lin, C. M.; Tsai; Y.H. (2009). Isolation and identification of histamine-forming bacteria in tuna sandwiches. Food Control, v. 20, p. 1013-1017.

Kung, H.F; Lee, T.M.; Lin, C.S.; Hong, T.Y.; Tsai, Y.H. (2012). Polymerase Chain Reaction for the Detection of Histamine-Producing Bacteria Isolated from Taiwanese Foods. J. of Food and Drug Anaiysis, v. 20, n. 1.

Krîžek, M.; Dadáková, E.; Vácha, F.; Pelikánová, T. (2017). Comparison of the formation of biogenic amines in irradiated and smoked fish. European Food Research and Technology, v. 243, n. 11, p. 1989-1995.

Macé, S., Mamlouk, K., Chipchakova, S., Prévost, H., Joffraud, J.-J., Dalgaard, P., Pilet, M.-F., Dousset, X. (2013). Development of a Rapid Real-Time PCR Method as a Tool to Quantify Viable *Photobacterium phosphoreum* Bacteria in Salmon (*Salmo salar*) Steaks. Applied and Environmental Microbiology, v. 79, n. 8, p. 2612–2619.

Marshall, D.L. (2014). Spoilage of animal products. Seafood. Elsevier Ltd., p. 453-458.

Majumdar, A., Pradhan, N., Sadasivan, J., Acharya, A., Ojha, N., Babu, S., Bose, S. (2018). Food Degradation and Foodborne Diseases: A Microbial Approach. Microbial Contamination and Food Degradation, chap. 5, 109–148.

Maintz, l.; Novak, N. Histamine and histamine intolerance. The American Journal of Clinical Nutrition, v. 85, p. 1185-96, 2007.

Marcobal, A., De Las Rivas, B., Moreno-Arribas, M. V.; Muñoz, R. (2005). Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. Journal Food Protection, v. 68 p. 874-878.

Marcobal, A.; De Las Rivas, B.; Muñoz, R. (2006). Methods for the Detection of Bacteria Producing Biogenic Amines on Foods: A Survey. Journal of Consumer Protection and Food Safety, v.1, p. 187-196.

MAPA (1997). Ministério da Agricultura, Pecuária e Abastecimento. Regulamento Técnico de Identidade e Qualidade para Peixe Fresco (Inteiro e Eviscerado). Ministério da Agricultura e do Abastecimento, MAPA, portaria nº 185, maio, 1997.

MAPA (2011). Ministério da Agricultura, Pecuária e Abastecimento. Regulamento Técnico de Identidade e Qualidade de Conservas de Peixes. Ministério da Agricultura, Pecuária e Abastecimento, Secretaria de Defesa Agropecuária. Instrução Normativa, SDA, n° 45, dezembro, 2011.

MAPA. (2017). Ministério da Agricultura, Pecuária e Abastecimento. Regulamento Técnico de Identidade e Qualidade para Peixe Congelado. Ministério da Agricultura e do Abastecimento. Instrução Normativa, n° 21, MAPA, 31 de maio de 2017.

McInnis, E.A., Kalanetra, K.M., Mills, D.A., Maga, E.A. (2015). Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. Food Microbiology, n. 46, 121-131.

MERCOSUL. Identidad y calidad de pescado fresco. Normas Técnicas, Mercosul/GMC/RES, nº 40/94, 1994.

Møretrø, T., Langsrud, S. (2017). Residential Bacteria on Surfaces in the Food Industry and Their Implications for Food Safety and Quality. Comprehensive Reviews in Food Science and Food Safety, 16, 5, 1022–1041.

Niraj, M. M. G.; Pandey, S. (2012). Histamine Biosensor: A Review. International Journal of Pharmaceutical Sciences and Research, v. 3, n. 11, p. 4158-4168.

Nordt, S. P., Pomeranz, D. (2016). Scombroid poisoning from tilapia. The American Journal of Emergency Medicine, 34, 2, 339e1–339.e2. doi:10.1016/j.ajem.2015.06.017

Ocaño-Higuera, V. M., Marquez-Ríos, E., Canizales-Dávila, M., Castillo-Yáñez, F. J., Pacheco-Aguilar, R., Lugo-Sánchez, M. E., García-Orozco, K.D., Graciano-Verdugo, A. Z.

(2009). *Post mortem* changes in cazon fish muscle stored on ice. Food Chemistry, 116, 4, 933-938.

Odeyemi, O.A., Burke, C.M., Bolch, C.C.J., Stanley, R. (2018). Seafood spoilage microbiota and associated volatile organic compounds at different storage temperatures and packaging conditions. Int. Jour. of Food Microb., 280, 87-99.

OMS. Organização Mundial da Saúde. Dia Mundial da Saúde 2015 do Campo à Mesa, Obtendo Alimentos Seguros. Seus Alimentos são seguros? Organização Mundial da Saúde, p. 1-10, abril, 2015. Acessado em 18 de dezembro de 2015. Disponível: <https://nacoesunidas.org/wp-content/uploads/2015/04/WDH-2015_ConceptNote_Port.pdf>

Önal, A. A review: Current analytical methods for the determination of biogenic amines in foods. Analytical, Nutritional and Clinical Methods, Food Chemistry, n. 103, p. 1475-1486, 2007.

Önal, A.; Tekkeli, S.E.K.; Önal, C. Analytical Methods A review of the liquid chromatographic methods for the determination of biogenic amines in foods. Food Chemistry, v. 138, p. 509-515, 2013.

OPAS. Plano Estratégico da Organização Pan-Americana da Saúde 2014-2019: "Em Prol da Saúde: Desenvolvimento Sustentável e Equidade". Organização Pan-Americana da Saúde. Escritório Regional da Organização Mundial da Saúde para as Américas. Tema 4.1 da Agenda Provisória, Washington, D.C., EUA, p. 113-114, set., 2013.

Ordóñez, J. L.; Troncoso, A.M.; García-Parrill; M.D.C.; Callejón, R.M. (2016). Recent trends in the determination of biogenic amines in fermented beverages - A review. Analytica Chimica Acta, n. 939, p. 10-25.

Owen, P., Fraser, Sumar, S. (1998). Compositional changes and spoilage in fish (part II) - microbiological induced deterioration. Nutrition e Food Science, 98, 6, 325-329.

Papageorgiou, M., Lambropoulou, D., Morrison, C., Kłodzińska, E., Namieśnik, J., Płotka-Wasylka, J. (2018). Literature update of analytical methods for biogenic amines determination in food and beverages. TrAC Trends in Analytical Chemistry, 98, 128-142.

Parlapani, F. F., Kormas, K. A., Boziaris, I. S. (2014). Microbiological changes, shelf life and identification of initial and spoilage microbiota of sea bream fillets stored under various conditions using 16S rRNA gene analysis. Journal of the Science of Food and Agriculture, 95, 12, 2386-2394.

Prester (2011). Biogenic amines in fish, fish products and shellfish: a review. Food Additives and Contaminants, 28, 11, 1547-1560.

Podeur, G.; Dalgaard, P.; Leroi, F.; Prévost, H.; Emborg, J.; Martinussen, J.; Hansen, L.H.; Pilet, M.-F. (2015). Development of a real-time PCR method coupled with a selective pre-enrichment step for quantification of *Morganella morganii* and *Morganella psychrotolerans* in fish products. International Journal of Food Microbiology, 203, 55-62.

Postollec, F.; Falentin, H.; Pavan, S.; Combrisson, J.; Sohier, D. (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiology, v. 28, p. 848-861.

Raats, D., Offek, M., Minz, D., Halpern, M. (2011). Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiology, 28, 3, 465–471.

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M-F, Zagorec, M. (2014). Bacterial spoilers of food: Behavior, fitness and functional properties. Food Microbiology, 1-9.

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M.-F., Zagorec, M. (2015). Bacterial spoilers of food: Behavior, fitness and functional properties. Food Microbiology, 45, 45-53.

Rio Grande do Sul (2015). Vigilância epidemiológica das doenças de transmissão hídrica e alimentar- VE-DTHA. Doença de Transmissão alimentar e Hídrica. Secretária Estadual de Saúde do Rio Grande do Sul.

Rivas, B., Marcobal, A., Munoz, R. (2005). Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. Federation of European Microbiological Societies, FEMS Microbiology Letters, v. 244, p. 367-372.

Ringøa, E.; Olsenb, R.E.; Mayhewc, T.M.; Myklebustd, R. (2003). Electron microscopy of the intestinal microflora of fish. Aquaculture, 227, 395-415.

Rodríguez-Lázaro, D., Hernández, M., Demain, A.L. and Vaishnav, P. (2014). Real-time PCR in Food Science: PCR Diagnostics. Curr. Issues Mol. Biol., n. 15, p. 39-44.

Ryder, J. Iddya, K., Ababouch, L. (2014). Assessment and management of seafood safety and quality - current practices and emerging issues. FAO Fisheries and Aquaculture Technical Paper, 574.

Ruethers, T., Taki, A. C., Johnston, E. B., Nugraha, R., Le, T. T. K., Kalic, T., Kamath, S.D., Lopata, A. L. (2018). Seafood allergy: A comprehensive review of fish and shellfish allergens. Molecular Immunology, n. 100, 28–57.

Ruiz-Capillas, C., Herrero, A. (2019). Impact of Biogenic Amines on Food Quality and Safety. Foods, v. 8, n. 2, 62.

Salihah, N.T.; Hossain, M.M.; Lubis, H.; Ahmed, M.U. (2016). Trends and advances in food analysis by real-time polymerase chain reaction. Journal of Food Science and Technology, v. 53, n. 5, p. 2196-2209.

Schwelberger, H.G. Diamine oxidase (DAO) enzyme and gene (2004). In: Falus A. Histamine: biology and medical aspects. Budapest: SpringMed Publishing, p. 43-52.

Schmittgen, T.D.; Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Publishing Group. Nature Protocols, v. 3, n. 6, p. 1101-1108.

Silva, M. (2005). Quantitation by HPLC of Amines as Dansyl Derivatives, p. 445-470. In: Molnár-Perl, I. Quantitation of Amino Acids and Amines by Chromatography - Methods and Protocols, ed. Elsevier Science, 1 ed., p. 1-668.

Skrodenytė-ArbaČIauskiene, V. (2007). Enzymatic activity of intestinal bacteria in roach *Rutilus rutilus* L. Fisheries Science, 73, 964–966.

SUDEP. Diagnóstico do Setor Pesqueiro do Rio Grande do Sul. Superintendência do Desenvolvimento da Pesca Agência SUDEP, Rio Grande, Ministério da Agricultura, RS, maio, 1988, revisado jan., 2003.

Tabanelli, G.; Montanari, C.; Gardini, F. (2018). Biogenic Amines in Food: A Review of Factors Affecting Their Formation. Reference Module in Food Science, p. 1-6.

Tabanelli, G.; Montanari, C.; Gardini, F. (2019) Biogenic Amines in Food: A Review of Factors Affecting Their Formation. Reference Module in Food Science, p. 337-343.

Thi, A.N.T.; Samapundo, S., Devlieghere, F., Heyndrickx, M. (2016). Microbiota of frozen Vietnamese catfish (*Pangasius hypophthalmus*) marketed in Belgium. International Journal of Food Contamination, 3, 17.

Tahmouzi, S.; Khaksar, R.; Ghasemlou, M. (2011). Development and validation of an HPLC-FLD method for rapid determination of histamine in skipjack tuna fish (*Katsuwonus pelamis*). Food Chemistry, v. 126, n. 2, p. 756-761.

Takemoto, E., EvangelistaII, W.P., Minazzi-RodriguesI, R.S., D.A.P., MarsigliaI, de Oliveira, C.A.F., Glória, M.A.G. (2014). Histamine intoxication outbreak associated to canned tuna intake in the State of São Paulo, Brazil. Boletim Epidemiológico Paulista, v. 11, n. 126, 29-32.

Taylor, S.L.; Guthertz, L.S.; Leatherwood, M.; Tillman, F.; Lieber E.R. (1978). Histamine production by food-borne bacterial species. Journal of Food Safety, n.1, v. 173-187.

Triki, M., Herrero, A., Jiménez-Colmenero, F. and Ruiz-Capillas, C. (2018) Quality Assessment of Fresh Meat from Several Species Based on Free Amino Acid and Biogenic Amine Contents during Chilled Storage. *Foods* **7**, 132

Tortorella, V.; Masciari, P.; Pezzi, M.; Mola, A.; Tiburzi, S. P.; Zinzi, M. C.; Scozzafava, A. M.; Verre, M. (2014). Case Report: Histamine Poisoning from Ingestion of Fish or Scombroid Syndrome. Hindawi Publishing Corporation. Case Reports in Emergency Medicine, 1-5.

Visciano, P.; Schirone, M.; Tofalo, R.; Suzzi, G. (2014). Histamine poisoning and control measures in fish and fishery products. Frontiers in Microbiology, 5, 500.

Wendakoon, C.N.; Sakaguchi, M. (1995). Inhibition of Amino Acid Decarboxylase Activity of Enterobacter aerogenes by Active Components in Spices. Journal of Food Protection, 58, 3, 280-283.

WHO, World Health Organization (2011).Western Pacific Regional Food Safety Strategy (2011–2015). Regional Committee for the Western Pacific. WPR/RC62.R5, October, p. 1-3.

WHO and FAO. World Health Organization and Food and Agriculture Organization of the United Nations. (2013). Joint FAO and WHO expert meeting on the public health risks of histamine and other biogenic amines from fish and fishery products: meeting report. World Health Organization.

Wongsariya, K., Bunyapraphatsara, N., Yasawong, M. and Chomnawang, M.T. (2016) Development of molecular approach based on PCR assay for detection of histamine producing bacteria. Journal of food science and technology, n 53, p 640-648.

Yu, Y., Wang, P.M.D., Bian, L.M.D.; Hong, S.M.D. (2018). Rare death via Histamine poisoning Following Crab Consumption: A Case Report. J Forensic Sci, v. 63, n. 3.

Zhang, Y., Li, D., Ly, J., Li, Q., Kong, C., Luo, Y. (2017). Effect of cinnamon essential oil on bacterial diversity and shelf-life in vacuum-packaged common carp (*Cyprinus carpio*) during refrigerated storage. International Journal of Food Microbiology, 249, 1–8.

Zang, J., Xu, Y., Xia, W., Yu, D., Gao, P., Jiang, Q., Yang, F. (2018). Dynamics and diversity of microbial community succession during fermentation of Suan yu, a Chinese traditional fermented fish, determined by high throughput sequencing. Food Research International, 111, 565–573.

Zhai, H.; Yang, X.; Li, L.; Xia, G.; Cen, J.; Huang, H.; Hao, S. (2012). Biogenic amines in commercial fish and fish products sold in southern China, Food Control, v. 25, p. 303-308.

Zazzu, C.; Addis, M.; Caredda, M.; Scintu, M.F.; Piredda, G.; Sanna, G. (2019). Biogenic Amines in Traditional Fiore Sardo PDO Sheep Cheese: Assessment, Validation and Application of an RP-HPLC-DAD-UV Method. Separations, n. 6, v. 11.

ZOU, Y.; HOU, X. (2017). Histamine production by *Enterobacter aerogenes* in chub mackerel (*Scomber japonicus*) at various storage temperatures. Food Science Technology, 37, 1, 76-79.