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Tracking spoilage bacteria in the tuna microbiome

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Abstract :

Like other seafood products, tuna is highly perishable and sensitive to microbial spoilage. Its consumption, whether fresh or canned, can lead to severe food poisoning due to the activity of specific microorganisms, including histamine-producing bacteria. Yet many grey areas persist regarding their ecology, conditions of emergence and proliferation in fish. In this study, we used 16S rDNA barcoding to investigate post-mortem changes in the bacteriome of fresh and brine-frozen yellowfin tuna (Thunnus albacares), until late stages of decomposition (i.e. 120 h). The results revealed that despite standard refrigeration storage conditions (i.e. 4°C), a diverse and complex spoilage bacteriome developed in the gut and liver. The relative abundance of spoilage bacterial taxa increased rapidly in both organs, representing 82% of the bacterial communities in fresh yellowfin tuna, and less than 30% in brine-frozen tuna. Photobacterium was identified as one of the dominant bacterial genera, and its temporal dynamics were positively correlated with histamine concentration in both gut and liver samples, which ultimately exceeded the recommended sanitary threshold of 50 ppm in edible parts of tuna. The results from this study show that the sanitary risks associated with the consumption of this widely eaten fish are strongly influenced by post-capture storage conditions.

Keywords : fish, histamine, Photobacterium, spoilage microorganisms, Yellowfin

Introduction

Like other living organisms, fish live in close association with a diverse assemblage of microorganisms, including bacteria, viruses, archaea and microeukaryotes, which constitute their microbiome. Increasing attention has been paid to the fish microbiome in recent years, and we now know that it ensures a number of essential functions for the health and fitness of the host (Egerton et al. 2018; Sehnal et al. 2021). It has also been shown to be highly heterogeneous throughout the body, with specific microbial signatures in different fish organs, including the gut, gills, skin, liver, etc. (Apprill 2017; Egerton et al. 2018; Ross et al. 2019; Gadoin et al. 2021). Numerous studies have reported that the composition of the fish microbiome depends on various factors, such as species (Larsen et al. 2013; Chiarello et al. 2015, 2018; Givens et al. 2015), stage of individual development (Hansen & Olafsen 1999), sex (Dhanasiri et al. 2011), diet (Cordero et al. 2015; Parata et al. 2019), geographical location (Chiarello et al. 2019; Xavier et al. 2020) or captive state (Dhanasiri et al. 2011;

Parata et al. 2019). However, little is known about the evolution of this microbiome in different organs after the death of the fish, which may influence its quality before consumption. After a fish dies, numerous physical and chemical alterations take place (i.e. decrease in pH, cellular lysis), inducing taxonomic and functional shifts in the bacterial community initially present in the organism (Boziaris & Parlapani 2017; Duarte et al. 2020; Gram & Huss 1996).

In the last three decades, numerous studies have analysed the diversity and activity of spoilage microorganisms in many seafood products, mainly using a culture-based approach (reviewed in Boziaris & Parlapani 2017; Gram & Huss 1996; Gram & Dalgaard 2002). The results of these studies have identified microorganisms such as *Pseudomonas*, *Psychrobacter*, *Lactobacillus* and other lactic acid bacteria (LAB), *Shewanella*, *Photobacterium* and *Aeromonas* as specific spoilage organisms (SSOs) due to their ability to produce metabolites and off-odours/flavours in seafood flesh (Boziaris & Parlapani 2017; Gram & Dalgaard 2002; Parlapani 2021). Such microorganisms produce specific metabolites (trimethylamine oxide, ammonia, biogenic amines, organic acids, acetate and sulphur) that lead to organoleptic rejection of the food product (Boziaris & Parlapani 2017; Gram & Dalgaard 2002). The levels of these SSOs in the host organism are mainly dependent on fish evisceration procedures and on storage conditions (Huss 1995; Indergård et al. 2014; Odeyemi et al. 2018; Antunes-Rohling et al. 2019; Zhuang et al. 2021), but can also vary depending on the fish species (Parlapani et al. 2013, 2018; Reynisson et al. 2010), geographical location (Parlapani et al. 2018), and the composition of the initial microbiome (Boziaris & Parlapani 2017; Gram & Dalgaard 2002; Parlapani 2021). Preservation methods such as chilling, freezing and reduced oxygen are all known to delay bacterial growth and thus extend shelf-life (Dawson, Al-Jeddawi & Remington 2018; Ghaly 2010; Sivertsvik, Jeksrud & Rosnes 2002).

To date, the majority of studies on the spoilage microbiome in fish have been conducted on flesh (Kuuliala et al. 2018; Parlapani et al. 2018; Syropoulou et al. 2021; Wang et al. 2017; Antunes-Rohling et al. 2019; Eliasson et al. 2019; Zotta et al. 2019), while viscera such as the gut and liver have received less attention. Yet the latter are recognized as important microbial reservoirs: the digestive tract of fish is known to host specific bacterial taxa that play key roles in the digestion, immunity and fitness of the host (Egerton et al. 2018;

Ghanbari, Kneifel & Domig 2015). More recently, diverse microbial communities have also been discovered in the liver of several fish species, including tuna, mullet, sardinella and Randall's threadfin bream (Meron et al. 2020; Gadoin et al. 2022), showing the importance of including this organ in microbiome studies on marine organisms. As is the case with fish flesh, enteric and hepatic bacterial communities are likely to evolve rapidly on the death of the fish, but these mechanisms remain poorly understood to date. Although the gut and liver are generally not consumed, it remains important to study their microbiological development, as these organs could contaminate others (in particular the flesh) during the multiple stages of handling from fish capture to consumption.

Of the main fish species consumed worldwide, tuna show one of the highest risks of food poisoning (Hungerford 2010; Tortorella et al. 2014). From a microbiological perspective, tuna, like other members of the Scombridae family, are an interesting study model, as the consumption of these species can lead to histamine poisoning (Hungerford 2010, 2021). Histamine is produced when specific spoilage-associated bacteria decarboxylate free histidine, an amino acid present in high concentrations in Scombridae (Gram & Dalgaard 2002; Jørgensen et al. 2000; Bjornsdottir-Butler et al. 2015). It has been clearly established that storage temperature is a major factor influencing the production of histamine by these histamine-producing bacteria (HPB) (Economou et al. 2007; Guizani et al. 2005; Hungerford 2021; Mahusain et al. 2017; Silva et al. 1998). Yet while these HPB have been identified and the production mechanisms of this biogenic amine are relatively well known, their ecology and development within the post-mortem microbiome of tuna remain poorly documented, especially in digestive organs such as the liver or gut (Bjornsdottir-Butler et al. 2016; Gadoin et al. 2022).

In this study, we chose to conduct our investigations on a particular species – the yellowfin tuna (*Thunnus albacares*) – which is found in tropical waters worldwide and is the second most consumed tuna species in the world (FAO 2020). Our objective was to understand how the bacteriome of this key species evolves after fish capture/death by examining two major bacterial reservoirs: the gut and the liver. We used a metabarcoding approach to depict the dynamics of the whole bacterial community as well as the emergence of more specific spoilage bacteria and HPB. The results are discussed in the light of fish conditioning

processes by comparing the development of the bacteriome in fresh and brine-frozen tuna captured by artisanal and industrial techniques respectively.

Material and methods

Sampling

The yellowfin tuna (*Thunnus albacares*) in our study were captured using two different fishing techniques and post-capture storage conditions: (1) artisanal fishing with immediate storage on ice of fresh individuals, and (2) industrial fishing followed by immediate brine-freezing treatment. In the first case, 12 individual yellowfin were captured around fish-aggregating devices located in the Gulf of Guinea (Ivory Coast, N04°55'00", W03°42'19.97'') on 20–21 November 2019. The capture and euthanasia of the fish were performed by professional fishermen. Three tuna were dissected on board immediately after their capture (see 'Experimental design'), while the remaining nine individuals were individually placed in plastic bags and kept on ice until they reached the laboratory, less than 5 h after death. The mean fork length of the individuals was 49.5 cm (min 45.7 cm – max 52.3 cm), and the average weight was 2.1 kg (min 1.7 kg – max 2.6 kg).

In the second case, 12 individual yellowfin were collected at the Abidjan tuna port (Ivory Coast) by the Exploited Tropical Pelagic Ecosystem Observatory (IRD, Ob7, certified ISO 9001:2015) within the framework of multiannual European fishery data collection (DCF, financed by the European Maritime and Fisheries Fund, Article 77). All individuals were caught by purse seine vessels between May and December 2019 in the Eastern Atlantic Ocean (Gulf of Guinea and off the coast of Senegal) and immediately chilled in brine to lower their temperature to around -15°C. The fish remained frozen in the tanks until landing in the Port of Abidjan and were then thawed at 4°C in the laboratory, 24 hours before the beginning of the experiment (Fig. 1). The mean fork length of these brine-frozen individuals was 63.4 cm (min 58.0 cm – max 70.0 cm), and the average weight was 4.4 kg (min 3.1 kg – max 5.9 kg).

Experimental design

From each sample (fresh and brine-frozen), a batch of three yellowfin individuals was dissected at the beginning of the experiment (T_0) to analyse their liver and gut microbiota, as well as the histamine concentration (see sampling procedure below) (Fig. 1). For brine-frozen tuna, T_0 corresponded to 24 h after thawing at 4°C, which is considered the standard temperature for home storage. For fresh tuna, T_0 corresponded to the time of death of the fish since they were dissected directly onboard after their capture. The nine remaining fish in each batch were kept at 4°C in temperature-controlled refrigerators. Every 48 h until the end of the 120-h experiment (i.e. T_{120}), three individuals from each batch were randomly selected to sample their hepatic and intestinal microbiota (Fig. 1).

Sampling the gut and liver microbiota

Gut

The dissection of the tuna involved extracting the gastrointestinal tract after cutting from below the stomach to the rectum using sterile tools. Each gut was opened, squeezed, and its inner surface entirely rubbed to expel the contents (minimum volume of 5 mL) on a sterile surface. The contents were homogenized before sampling (Gadoin et al. 2021).

Liver

A 2 x 0.2 x 2 cm (L x W x H) piece was trimmed from the right lobe of each tuna liver using sterile tools. Liver samples were rinsed with distilled water filtered through a 0.2 µm filter to avoid any contamination from other internal organs or fluids.

All the gut and liver samples were placed in 5-mL sterile cryovials, frozen in liquid nitrogen and stored at -80°C in the laboratory until the extraction of bacterial nucleic acid.

Bacterial DNA extraction, amplification and sequencing

The bacterial DNA was extracted from 250 ± 0.5 mg of the gut (n= 24) and liver (n= 24) samples. All extractions were performed with the PowerSoil DNA Isolation Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. DNA quality and quantity were assessed by spectrophotometry (NanoDrop®, Wilmington, DE, USA). Blank extractions were performed in duplicate in order to identify potential contaminants within the reagents. The V3-V4 region of the 16S rDNA gene was amplified using universal bacterial primers modified

for Illumina sequencing: 343F (5'- ACGGRAGGCAGCAG) (Klindworth et al. 2013) and 784R (5'-TACCAGGGTATCTAATCCT) (Andersson et al. 2008). The reaction mixture consisted of 12.5 µL of 2X Phusion Mix (New England Biolabs®, Ipswich, MA, USA), 1 µL of each primer at 10 µM (Eurofin®, Luxembourg), 10 ng of DNA template and enough molecular-grade H_2O (Qiagen®) to reach a final volume of 25 µL. All samples were amplified in triplicate to avoid PCR bias in the taxonomic diversity of the community (Perreault et al. 2007). Negative controls to check for the contamination of the PCR reactions were performed and blank extractions were also amplified (n = 2). For all samples and controls, the success of PCR amplification was confirmed on 2% agarose gel in TAE buffer using a 100 bp DNA ladder. Successfully amplified samples (n = 50) were sequenced on the Illumina platform using 2x250 bp MiSeq chemistry (accession number GenBank: SUB11887845, BioProject THE MOME, PRJNA674773).

Bacterial sequence processing and analysis

A total of 16,277,785 reads were obtained. Raw reads were processed with *R*Studio (R version 3.5.3) using the DADA2 package (v1.10.1) (Callahan et al. 2016) following the authors' tutorial (https://benjjneb.github.io/dada2/tutorial.html). The quality of forward and reverse reads was analysed before removing adaptors and primers based on their length. Using the DADA2 tutorial with default parameters, reads were then filtered, trimmed and merged into 8312 amplicon sequence variants (ASVs). Chimaeras were removed, and sequences were aligned to the SILVA 138 database to access their taxonomy (Quast et al. 2013; https://www.arb-silva.de/). Analyses were performed on a random subsample of 2337 sequences per sample, corresponding to the sample with the smallest number of sequences after trimming and quality processing. Using the *phyloseq* package (McMurdie & Holmes 2013), final taxonomic and ASV tables were linked to sample metadata (including biological compartment, sampling time and preservation conditions). The relative abundance of ASVs in each sample was assessed by *phyloseq,* and ASVs assigned to non-prokaryotes, archaea, chloroplasts and mitochondria were removed, as well as the ASVs found in the negative sequencing controls. Using the *phyloseq* and *ggplot2* packages, the composition and diversity of bacterial communities were then represented at the class level, based on the relative abundance of ASVs in each sample. Among the different ASVs detected in the gut and liver of tuna, those corresponding to known spoilage bacterial genera in seafood products were listed (see Table 1). A list of putative histamine-producing bacteria genera

was also established from the literature, and their presence in our samples was assessed by comparing these to our taxonomy table.

Histamine concentration

In all the gut and liver samples, histamine concentration was assessed by enzyme-linked immunosorbent assays (ELISA) using the Veratox® kit for tuna histamine (Neogen®, Lansing, MI, USA) following the manufacturer's instructions. Samples were suspended in distilled water, filtered and diluted 10X prior to the ELISA tests. Assays were performed under sterile conditions and the optical density was measured at 650 nm using a TECAN Infinite M200 Pro (Tecan®, Männedorf, Switzerland). The optical densities of the six standards available in the kit allowed us to trace the standard curve against which the optical density of a sample was plotted to calculate its histamine concentration in parts per million (ppm).

Statistical analysis

All statistical analyses were performed with *R*Studio. For both gut and liver samples, the variability in the bacteria community structure between the three individuals in a batch (see Fig. 1) was tested using PERMANOVAs with 999 permutations on the Bray–Curtis dissimilarity matrix, using the "adonis" function of the vegan package (Dixon 2003). Since no significant inter-fish variability could be detected in any of the batches (PERMANOVA, p value > 0.05), the mean relative abundance of each ASV was calculated from each triplicate in order to analyse the effect of storage conditions, for each organ, in both fresh and frozen samples and for all sampling occasions. The effect of time and post-catch storage conditions on the composition of hepatic and intestinal bacterial communities was determined by single-factor and multiple-factor PERMANOVAs. Correlations between histamine concentration and the relative abundance of potential HPB were evaluated using a Spearman correlation test performed in *R*Studio.

Results

Short-term dynamics of the tuna bacteriome

The results revealed that the composition of the tuna bacteriome changed significantly over time in both the gut and liver (Tab. 2).

The gut bacteriome

In fresh tuna, the initial enteric bacteriome was comprised of numerous taxa that included the genera *Cutibacterium, Enhydrobacter, BD1-7 clade* and *Neorickettsia*, as well as several known spoilage-associated bacteria genera such as *Photobacterium, Shewanella, Pseudomonas* and *Vibrio* (Tab. 1). Over the 120-h period, the abundance of *Photobacterium* then rapidly increased to reach almost 90% of the total abundance of bacteria, while most of the other genera decreased (Fig. 2).

In brine-frozen yellowfin, significant changes in the composition of the gut bacteriome were also observed during the experiment (Fig. 2). In addition, in these fish, the presence of spoilage bacteria that were not found in fresh tuna were identified (e.g. *Lactococcus*, *Lactobacillus*, *Psychrobacter*, *Psychrilyobacter* and *Proteus).* The occurrence of certain taxa such as *Psychrobacter, Lactococcus* and *Shewanella* increased throughout the experiment. At T_{96}, *Photobacterium, Lactobacillus, BD1-7 clade* and *Mycoplasma* were the most abundant bacterial genera, but taxa with a relative abundance of less than 2% represented more than 25% of the community. Their proportion increased at T_{120}, when the spoilage genera *Shewanella, Psychrobacter, Proteus, Pseudomonas, Photobacterium, Lactobacillus* and *Psychrilyobacter* were detected and together represented 22.6% of the bacterial community.

The liver bacteriome

The composition of the bacterial community in the liver was highly diverse and was significantly different from that of the gut (Fig. 2, Permanova, p = 0.003). At T_0, the bacteriome in fresh tuna was mostly comprised of *Enhydrobacter, Micrococcus, Neorickettsia* and *Massilia*. In contrast with gut samples, the liver of fresh yellowfin initially hosted few spoilage bacteria, but these proliferated rapidly over time. The only spoilage bacteria genus observed in liver samples at the beginning of the experiment was *Pseudomonas*, but at T_{48} the relative abundance of other genera such as *Photobacterium, Shewanella, Psychrobacter* and *Vibrio* began to increase. By T_{96}, spoilage bacteria were dominant within the liver bacteriome, representing 76% of the bacterial community. They remained the major component of the liver bacteria until the end of the experiment, when

other genera such as *Salegentibacter, Sporosarcina, Enhydrobacter* and *Cutibacterium* were also detected.

The treatment prior to storage greatly impacted the composition of the bacteriome in this organ. The liver-associated bacteria in brine-frozen tuna evolved in a different way than in fresh tuna (Tab. 1, Fig. 2). For example, the genus *Photobacterium*, which was highly dominant in the liver bacteriome of fresh fish, was much less abundant in brine-frozen fish. Generally, although the relative abundance of spoilage bacteria increased over time, their occurrence remained lower in brine-frozen than in fresh samples. At the beginning of the experiment, hepatic bacterial communities were composed of *Enhydrobacter*, *Cutibacterium*, *Brachybacterium, Macrococcus, Halomonas, Acinetobacter* and *Methylobacterium*, as well as two main genera (*Photobacterium* and *Pseudomonas*), and potential pathogens such as *Staphylococcus* and *Corynebacterium*. At the end of the experiment (T_{120}), the liver bacteriome hosted several other spoilage taxa including *Proteus, Psychrobacter, Photobacterium, Shewanella* and *Psychrilyobacter*, which together represented 29% of the bacterial community.

Diversity of histamine-producing bacteria and histamine concentrations

In general, the relative abundance of HPB was much higher in fresh than in brine-frozen tuna. *Photobacterium* ASVs were generally dominant in gut samples, while other HPB genera (*Pseudomonas* and *Acinetobacter*) were also present in the liver in equivalent proportions (Fig. 3). Interestingly, the genus *Proteus* was only detected at the late stage of fish decomposition (T_{120}) and exclusively in brine-frozen samples.

In fresh yellowfin, the temporal dynamics of *Photobacterium* ASVs were significantly correlated with histamine concentration in both gut and liver samples (Pearson, p <0.05). Other potential HPB genera such as *Pseudomonas, Vibrio, Acinetobacter* and *Enterobacter* were also detected, but at low levels (Fig. 3).

Fresh and brine-frozen tuna exhibited contrasting patterns of histamine concentration. In fresh fish, histamine concentration increased abruptly after T_{48} to reach a maximum at T_{96} in the gut (mean = 676 ppm) and at T_{120} in the liver (mean = 59 ppm), thus exceeding the 50 ppm sanitary threshold established by the United States Food and Drug Administration (FDA 2021) but which concerns only the edible parts (i.e. the flesh) of the fish (Fig. 3). Conversely,

in brine-frozen fish, histamine concentrations remained below that threshold throughout the experiment.

Discussion

Modifications in microbiome composition following an animal's death are normal phenomena resulting from physical and chemical changes, as well as the loss of immune response (Benbow, Receveur & Lamberti 2020). In fish, however, the evolution of the post-mortem bacteriome over time has been poorly studied. In this study, we sought to begin to address this gap by exploring the tuna spoilage bacteriome and the dynamics of bacterial communities in two major bacterial reservoirs: the gut and the liver. We compared the influence of post-capture storage conditions (fresh or brine-frozen) on the development of these spoilage bacteria.

Bacterial diversity within the spoilage bacteriome

As expected, the relative abundance of spoilage-associated microorganisms (see Table 1) increased significantly in the gut and liver over the duration of the experiment. This trend was particularly marked in fresh tuna. In both organs, we observed an increase in abundance of *Psychrobacter, Pseudomonas, Proteus, Aeromonas*, *Lactobacillus*, *Shewanella* and *Photobacterium,* which have all been previously detected in the flesh of various fish species such as haddock, Atlantic salmon, gilthead sea bream, European sea bass and yellowfin tuna (Dalgaard et al. 2006; Fogarty et al. 2019; Jääskeläinen et al. 2019; Parlapani et al. 2018; Reynisson et al. 2010; Syropoulou et al. 2020).

The development of spoilage bacteria in fish and seafood products is well documented, and their proliferation typically depends on the applied storage conditions (Boziaris & Parlapani 2017). Several studies have demonstrated that the diversity of such bacteria in the flesh of different fish species varies depending on conditions such as chilling, vacuum packaging or thawing temperature (Reynisson et al. 2010; Odeyemi et al. 2018; Antunes-Rohling et al. 2019; Syropoulou et al. 2021). Bacteria from the *Shewanella*, *Photobacterium* and *Pseudomonas* genera are known for their ability to produce respectively high quantities of H_2S, trimethylamine and volatile nitrogenous compounds (Boziaris & Parlapani 2017; Carrascosa et al. 2014; Chinivasagam et al. 1998).

It should be noted that the spoilage activity of bacteria is a relatively complex mechanism that has multiple determinants. The production of spoiling metabolites is clearly species dependent and can also vary according to storage conditions such as temperature (Antunes-Rohling et al. 2019; Parlapani & Boziaris 2016) and atmosphere (Emborg, Laursen & Dalgaard 2005; Silbande et al. 2016; Sivertsvik et al. 2002), as well as microbial interactions between communities (Joffraud et al. 2006; Zotta et al. 2019). Various analyses such as the detection of spoilage genes and the quantification of spoilage metabolites are usually required to assess the spoilage potential of specific spoilage organisms (Fu et al. 2018; Tang et al. 2019; Syropoulou et al. 2020); these analyses would be valuable to perform in future studies.

The effect of storage conditions on the tuna bacteriome

The relative abundance and dynamics of spoilage-associated bacteria greatly varied according to the initial storage conditions (fresh vs brine-frozen). In fresh tuna, spoilage ASVs represented on average 82% (for both organs studied) of the total number of bacterial ASVs at the end of the experiment, and less than 30% in brine-frozen samples. The influence of storage conditions on the composition of the fish microbiome has long been a subject of investigation (Ghaly 2010; Zhuang et al. 2021). For example, delayed development of spoilage microorganisms was reported in frozen fillets of Atlantic cod, mackerel and salmon compared to fresh samples (Fagan, Ronan Gormley & Mhuircheartaigh 2003; Sørensen et al. 2020). Low-temperature chilling is known to decrease the growth of microorganisms, while freezing between -18 and -30°C kills between 10% and 60% of viable bacteria (Berkel, Boogaard & Heijnen 2004; Rahman 1999). In addition, the presence of sodium chloride (for example, in brine) is also known to inactivate autolytic enzymes in fish, as well as to negatively impact the growth of several spoilage bacteria (Ghaly 2010; Henney et al. 2010; Mejlholm, Devitt & Dalgaard 2012; Turan & Erkoyuncu 2012). This may partially explain why brine-frozen yellowfin exhibited a limited abundance of spoilage bacteria in the gut and liver microbiota compared to fresh tuna.

Spoilage bacteria and histamine production in tuna

Of the spoilage-associated bacteria detected in the tuna microbiome, histamine-producing bacteria are of particular interest, as they have been implicated in cases of food poisoning worldwide (Torido et al. 2012; Bjornsdottir-Butler et al. 2016; Hungerford 2010, 2021). We

identified several HPB genera in the gut and liver samples, including *Acinetobacter, Enterobacter, Morganella, Proteus, Pseudomonas* and *Vibrio*, but *Photobacterium* was the most abundant, especially in fresh fish, where it rapidly dominated the bacterial community in both organs. The genus *Photobacterium* is ubiquitous in marine environments and is composed of several species (Thyssen & Ollevier 2005). It has been described as commensal in various fish species (Torido et al. 2012; Bjornsdottir-Butler et al. 2015; Estruch et al. 2015; Givens et al. 2015; Egerton et al. 2018), but some *Photobacterium* species such as *P. damselae* and *P. piscicida* are known as fish and human pathogens (Rivas, Lemos & Osorio 2013; Romalde 2002). *Photobacterium* has also been identified as an HPB in Atlantic cod (Kuuliala et al. 2018), haddock (Reynisson et al. 2010) and Atlantic salmon (Jääskeläinen et al. 2019). Indeed, several *Photobacterium* species are able to synthetize histamine, including *P. angustum*, *P. aquimaris, P. kishitanii, P. damselae* and *P. phosphoreum*, which are designated as high histamine producers (> 200ppm) (Bjornsdottir-Butler et al. 2018). While histamine-production capacity has been demonstrated to vary across different *Photobacterium* species, this capacity is also influenced by temperature (Bjornsdottir-Butler et al. 2018; Morii & Kasama 2004; Takahashi et al. 2015). Insufficiently cold temperatures are known to favour the production of histamine, so maintaining the cold chain is essential to prevent its formation (Hungerford 2021; Torido et al. 2012). Some psychrotrophic HPB, such as *P. phosphoreum* and *Morganella psychrotolerans,* are able to synthesize histamine at temperatures between 0° and 5°C (Bjornsdottir-Butler et al. 2018; Emborg et al. 2005; Kanki et al. 2004; Wang et al. 2020). Interestingly, our results revealed the existence of positive and significant correlations between histamine concentration and the relative abundance of bacteria belonging to the genus *Photobacterium*, both in the gut and liver of fresh tuna (see Fig. 3). Although our data did not allow us to identify the potential HPB down to species level, such correlations suggest that these *Photobacterium* taxa are probably HPB, a hypothesis that remains to be confirmed.

The vast majority of studies investigating Scombroid (histamine) poisoning have been conducted on tuna flesh or in processed products such as filets or canned tuna (Emborg et al. 2005; Guizani et al. 2005; Kim et al. 2002; Kung et al. 2009; Silva et al. 2011). Our study extends this by revealing the presence of histamine and HPB in both gut and liver samples. These organs have been previously identified as important reservoirs of HPB in tuna (Taylor

& Speckhard 1983; Bjornsdottir-Butler, Bowers & Benner 2015; Gadoin et al. 2022), but few studies have considered the liver and gut in their investigations on histamine formation in scombroid fish. Glória et al. (1999) observed that the intestinal wall of yellowfin tuna contained a substantial concentration of histamine. Similarly, Fernández-Salguero & Mackie (1979) reported significant histamine concentration in the liver of mackerel, in an even greater proportion than in muscle. In light of our results and the sanitary risks associated with histamine synthesis in scombroid fish, it would be very useful to characterize more precisely the activity of the potential HPB identified in tuna digestive organs.

Another key finding was that histamine was not detected in the gut or liver samples of brine-frozen tuna, despite the presence of potential HPB genera. This suggests that the brine-freezing treatment may alter the capacity of HPB to produce histamine in these two organs. Freezing has been previously observed to limit the production of this biogenic amine in tuna fillets (Tahmouzi et al. 2013). In addition, brine immersion is known to inhibit the activity of the histidine decarboxylase enzyme in HPB, and therefore to limit the synthesis of histamine from its precursor histidine (Hwang et al. 2020; Morii & Kasama 2004; Tabanelli et al. 2012). Overall, in line with previous studies, our results confirm the usefulness of applying a brine-freezing treatment to tuna to prevent the formation of histamine, and thus reduce the health risk associated with their consumption.

Conclusion

Using a metagenomic approach, our study provides new information on the presence of spoilage microorganisms in the gut and liver of tuna, and their modifications after the death of the fish. The results show not only that the evolution of this spoilage bacteriome was specific to each of these digestive organs, but also highly variable according to the storage conditions of the fish, with the potential risk of rapid local development of histamine-producing bacteria. From a sanitary perspective, these metagenomic results demonstrate the importance of removing tuna viscera as soon as possible before consumption. These findings highlight the need to take into account the gut and liver in investigations on the ecology of HPB and other SSOs in scombroid fish.

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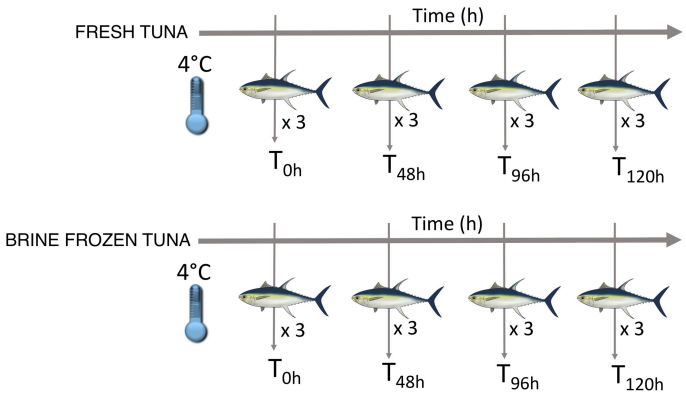

Figure 1

Figure 1. Experimental design to study the post-mortem microbiome of yellowfin tuna stored at 4°C. Gut and liver samples were collected in triplicate at the beginning of the experiment (T_0) and after 48, 96 and 120 hours, on fresh (A) and brine-frozen (B) individuals.

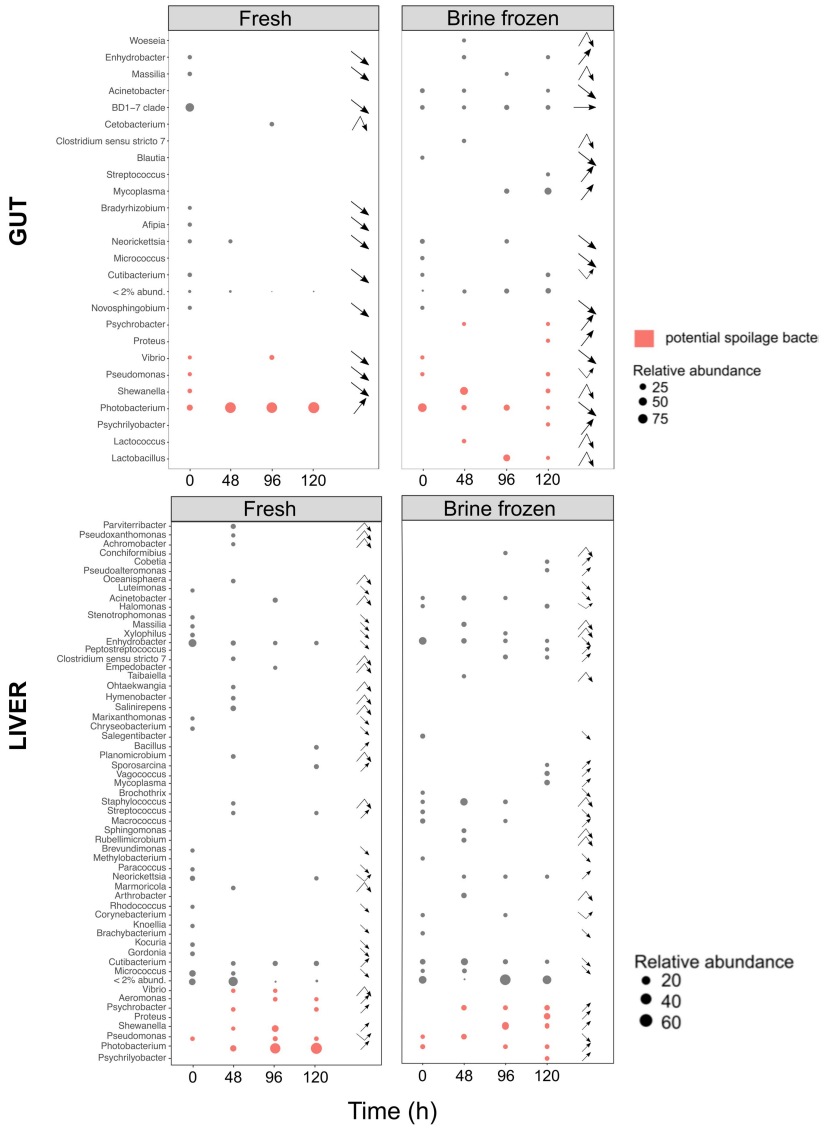

Figure 2

Figure 2. Temporal variation in the relative abundance of the main bacterial genera in the gut and liver samples of fresh and brine-frozen yellowfin tuna. The size of the dot is proportional to the relative abundance of each bacterial genus from T_{0h} to T_{120h}. Genera identified as spoilage organisms (see Table 1) are coloured in red. Arrows represent the overall development of each bacterial genus during the experiment. Bacterial genera with a relative abundance inferior to 2% were grouped and designated as < 2% abund.

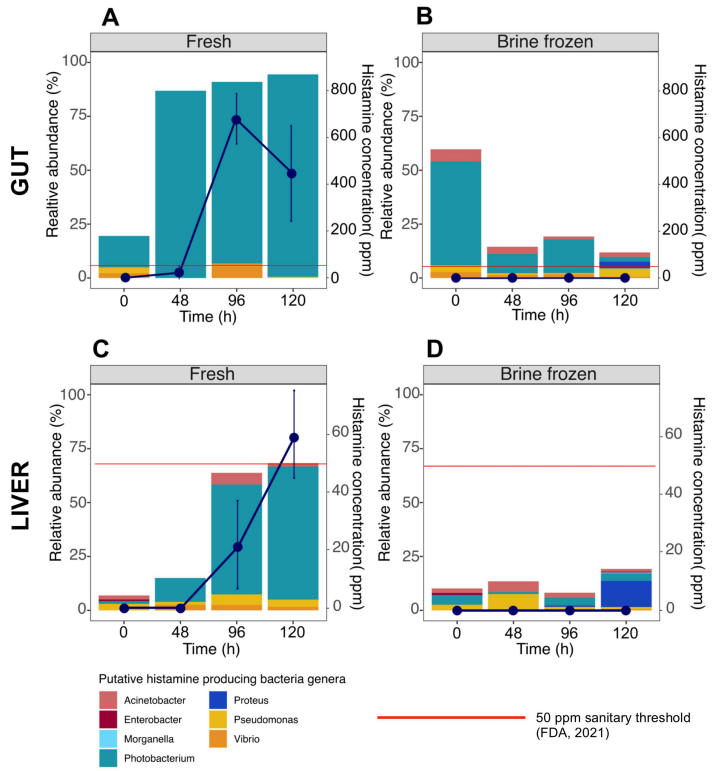

Figure 3

Figure 3. Dynamics of histamine concentration (ppm) (right abscissa) and relative abundance (left abscissa) of the main putative histamine-producing bacteria (HPB) found in the gut (A,B) and liver (C,D) of fresh (B,D) and brine-frozen (A,C) yellowfin tuna. The red horizontal bar represents the sanitary threshold of 50 ppm established by the United States Food and Drug Administration (FDA 2021).

Table 1. List of bacterial genera detected in the tuna gut and liver samples, and reported as spoilage taxa in seafood products.

Bacterial genera	Marine species/Seafood product	References
Aeromonas spp.	Atlantic salmon (*Salmon salar*)	Jakobsen *et al.* 2020
	Common carp (*Cyprinus carpio*)	Beaz-Hidalgo *et al.* 2015
	Sea bass (*Dicentracus labrax*)	Carrascosa et al. 2014; Parlapani *et al.* 2013
	Sea salmon (*Pseudopercis semifasciata*)	Hozbor *et al.* 2006
Lactobacillus spp.	Cold-smoked salmon	Olofsson *et al.* 2007
	Marinated and smoked herring (*Clupea harengus*)	Gancel *et al.* 1997; Lyhs *et al.* 2001
	'Gravad' rainbow trout	Lyhs *et al.* 2002
Lactococcus spp.	Common carp (*Cyprinus carpio*)	Li *et al.* 2018
	Grass carp (*Ctenopharyngodon idellus*)	Zhang *et al.* 2019
Photobacterium spp.	Atlantic cod (*Gadus morhua*)	Hovda *et al.* 2007; Kuuliala *et al.* 2018
	Atlantic salmon (*Salmon salar*)	Emborg *et al.* 2002; Fogarty *et al.* 2019; Jääskeläinen *et al.* 2019; Macé *et al.* 2012
	Garfish (*Belone belone*)	Dalgaard *et al.* 2006
	Haddock (*Melanogrammus aeglefinus*)	Reynisson *et al.* 2010
	Sea bream (*Sparus aurata*)	Parlapani & Boziaris 2016
Proteus spp.	Sardine (*Sardina pilchardus*)	Houicher *et al.* 2013
	Spoiled fish (mackerel, sardine, anchovy and rainbow trout)	Yazgan *et al.* 2019
	Canned tuna	Ahmed 2019
	Yellow croaker (*Pseudosciaena crocea*)	Guo Quanyou *et al*. 2018
Pseudomonas spp.	Atlantic cod (*Gadus morhua*)	Hovda *et al.* 2007
	Atlantic salmon (*Salmon salar*)	Xie *et al.* 2018
	Gilt-head sea bream (*Sparus aurata*)	Parlapani *et al.* 2013; Tryfinopoulou *et al.* 2002
	Norway lobster (*Nephrops norvegicus*)	Bekaert *et al.* 2015
	Sea bass (*Dicentracus labrax)*	Parlapani *et al.* 2015
	Yellow croaker (*Pseudosciaena crocea*)	Ge *et al.* 2017
	Yellowfin tuna (*Thunnus albacares*)	Jääskeläinen *et al.* 2019
	Tilapia fillets (*Oreochromis niloticus*)	Duan *et al.* 2018
Psychrobacter spp.	Brown shrimp (*Crangon crangon*)	Broekaert *et al.* 2013
	Salt-cured cod ((*Gadus morhua*)	Bjørkevoll *et al.* 2003
	Norway lobster (*Nephrops norvegicus*)	Bekaert *et al.* 2015
	Cuttlefish (*Sepia officinalis*)	Parlapani *et al.* 2018
	Hake fillet (*Merluccius merluccius*)	Antunes-Rohling *et al.* 2019
	Deepwater rose shrimp (*Parapenaeus longirostris*)	Parlapani *et al.* 2020
Psychrilyobacter spp.	Mandarin fish (*Siniperca chuatsi*)	Wang *et al.* 2021
	Pacific oyster (*Crassostrea gigas*)	Fernandez-Piquer *et al.* 2012
Shewanella spp.	Common carp (*Cyprinus carpio*)	Beaz-Hidalgo *et al.* 2015
	Sea bream (*Sparus aurata*)	Tryfinopoulou *et al.* 2007
	Atlantic horse mackerel (*Trachurus trachurus*)	Alfaro & Hernandez 2013
	Sea bass (*Dicentracus labrax)*	Carrascosa *et al.* 2014; Parlapani *et al.* 2018
	Tilapia fillets (*Oreochromis niloticus*)	Duan *et al.* 2018
	Sea salmon (*Pseudopercis semifasciata*)	Hozbor *et al.* 2006
	Hake (*Merluccius merluccius*)	López-Caballero *et al.* 2001
	Yellow croaker (*Pseudosciaena crocea*)	Ge *et al.* 2017
	Mussels	Odeyemi *et al.* 2018
Vibrio spp.	Pacific oyster (*Crassostrea gigas*)	Madigan *et al.* 2014
	Blue crab (*Callinectes sapidus*)	Parlapani *et al.* 2019
	Hake fillet (*Merluccius merluccius*)	Antunes-Rohling *et al.* 2019
	Tropical shrimp (*Penaeus vannamei*)	Macé *et al.* 2014

Table 2. Results of permutational ANOVAS (PERMANOVA, 999 permutations) performed on Bray-Curtis dissimilarities matrices to test the variation of bacterial community composition with time and post-capture preservation conditions in gut and liver samples. Bold values indicate a significant effect of the tested factor ($p < 0.05$).

Community dissimilarity						
	Time			Post-mortem preservation		
	p value	r^2	df	*p* value	r^2	df
Gut	**0.001**	0.21	2	0.255	0.05	1
Liver	**0.023**	0.13	2	**0.003**	0.09	1