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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 presence of histamine-producing bacteria and other specific spoilage organisms (SSOs) found in the tuna microbiome. Such bacteria generally develop in dead fish after their capture if conservation conditions are deficient. However, many grey areas persist regarding their ecology, their conditions of emergence and proliferation, and their distribution within different organs. In this study, we used 16S rDNA barcoding to investigate post-mortem changes in the tuna necrobiome until the advanced stages of decomposition (i.e. 120 h). The analyses were performed on fresh and brine-frozen yellowfin tuna (*Thunnus albacares*) captured in the tropical Atlantic Ocean. The results revealed that despite standard refrigeration storage conditions (i.e. 4°C), a diverse and complex spoilage bacteriome continued to develop in the gut and liver. In general, the relative abundance of SSOs increased rapidly in both organs, representing 82% of the bacterial communities in fresh yellowfin tuna, and less than 30% in brine-frozen ones. Interestingly, *Photobacterium* was identified as a major bacterial genus, and its temporal dynamics were positively correlated with histamine concentrations, which ultimately, in fresh tunas, exceeded the recommended sanitary level of 50 ppm established by the United States Food and Drug Administration. Finally, the study of the tuna necrobiome shows that the sanitary risks associated with the consumption of this widely eaten fish is strongly influenced by the post-capture storage conditions.

Keywords: necrobiome, tuna, histamine, *Photobacterium*, microbiome, spoilage microorganisms

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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 in 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 (Chiarello et al. 2015, 2018; Givens et al. 2015; Larsen et al. 2013), stage of 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 the different organs after the death of the fish, which nevertheless partly conditions its sanitary quality for consumption. After a fish dies, numerous physical and chemical alterations take place in the body (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). The microbial assemblage that grows in a dead fish and leads to its decomposition is known as the necrobiome, from the Greek word *nekrós* for 'death'.

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). These microorganisms, referred to as specific spoilage organisms (SSOs), typically belong to the bacterial genera *Aeromonas, Vibrio, Photobacterium, Shewanella* or *Enterobacteriaceae*, to cite a few, and they are commonly found in the flesh of fish and seafood products (Boziaris & Parlapani 2017; Gram & Dalgaard 2002). In general, most SSOs are known to produce specific metabolites (trimethylamine oxide, ammonia, biogenic amines, organic acids, acetate and sulphur) leading to the organoleptic rejection of the seafood product during quality control checks (Boziaris & Parlapani 2017; Gram & Dalgaard 2002). Among SSOs, several species such as *Shewanella* spp*., Vibrio* spp., *Salmonella* and *Listeria monocytogenes*

are also human pathogens (Parlapani 2021). The levels of these SSOs in the host organism are mainly dependent on storage conditions and the previous fish evisceration (Huss 1995). Chilling, freezing and vacuum storage can reduce the production of degrading metabolites by SSOs and thus increase the shelf-life of seafood products (Dawson, Al-Jeddawi & Remington 2018; Ghaly 2010; Sivertsvik, Jeksrud & Rosnes 2002). The diversity of SSOs also varies according to the fish species (Parlapani et al. 2013; Parlapani et al. 2018; Reynisson et al. 2010), the geographical location (Parlapani et al., 2018), and the composition of the initial microbiome (Boziaris & Parlapani 2017; Gram & Dalgaard 2002).

The majority of studies on the spoilage microbiome in fish have been conducted on flesh (Antunes-Rohling et al. 2019; Chytiri et al. 2004; Eliasson et al. 2019; Taliadourou et al. 2003; Wang et al. 2017; Zotta et al. 2019), while the 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 (Gadoin et al. in rev.; Meron et al. 2020), showing the importance of including this organ in microbiome studies on marine organisms.

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, is an interesting study model, as the consumption of this species can lead to histamine poisoning (Hungerford 2010, 2021). Histamine is produced by specific SSOs (Gram & Dalgaard 2002; Jørgensen et al. 2000) called histamine-producing bacteria (HPB), from a precursor amino acid (histidine) present in high concentrations in *Scombridae,* that HPB catalyse with the enzyme histidine decarboxylase (HDC) (Prester 2011). It has been clearly established that storage temperature is a major factor influencing the production of histamine by HPB (Economou et al. 2007; Guizani et al. 2005; Hungerford 2021; Mahusain et al. 2017; Silva et al. 1998). Yet, while these histamine-producing bacteria have been identified and the production mechanisms of this biogenic amine are relatively well known, the ecology and development of HPB within the post-mortem microbiome of tuna remain poorly understood.

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 necrobiome 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 depict the dynamics of the whole bacterial community as well as the emergence of more specific SSOs and HPBs. The results are discussed in the light of fish conditioning process by comparing the development of the necrobiome in fresh and brine-frozen tuna fished by artisanal and industrial techniques, respectively.

Material and methods

Sampling

The yellowfin tuna (*Thunnus albacares*) 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. For freshly caught yellowfin, 12 individuals were captured around fish-aggregating devices (FADs) 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. The tuna 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).

For the brine-frozen yellowfin tuna, 12 individuals 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 brine to lower their temperature to around -15°C. The fish remained frozen in the tanks until their 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 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

For each fresh and brine frozen lots, three yellowfin individuals were dissected and sampled 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 as 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. The 12 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 tuna were dissected, 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= 30) and liver (n= 30) 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). The V3-V4 region of the

16S rDNA gene was amplified using universal bacterial primers modified for Illumina sequencing: 343F (5'- ACGGRAGGCAGCAG) (Economou et al. 2007; Guizani et al. 2005; Hungerford 2021; Mahusain et al. 2017) 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). Successfully amplified samples (n= 30) were sequenced on the Illumina platform using 2x250 bp MiSeq chemistry.

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 123 database (Quast et al. 2012) to access their taxonomy. 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 conservation conditions). The relative abundance of ASVs in each sample were assessed by *phyloseq,* and ASVs assigned to non-prokaryotes, archaea, chloroplasts and mitochondria were removed. 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. Referring to the literature, a list of putative histamine-producing bacteria (HPB) genera was established, and their presence/absence in our samples was assessed by comparing the list of HPB 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. 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 PERMANOVA with 999 permutations on the Bray–Curtis dissimilarity matrix, using the "adonis" function of the *vegan* package (Dixon 2003). 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 necrobiome

The results revealed that the composition of the tuna necrobiome changed significantly over time in both the gut and liver (Tab. 1).

The gut microbiome

In fresh tuna, the initial enteric microbiome was comprised of numerous taxa that included the genera *Cutibacterium, Enhydrobacter, BD1-7 clade* and *Neorickettsia*, as well as several potential SSO genera such as *Photobacterium, Shewanella, Pseudomonas, Novosphingobium* and *Vibrio*. 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 necrobiome were also observed during the experiment (Fig. 2). In addition, in these fish, the presence of

several potential SSOs that were not found in fresh tuna were identified (*Lactococcus*, *Lactobacillus*, *Psychrobacter*, *Psychrilyobacter* and *Proteus).* The occurrence of certain SSOs 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 potential SSO genera *Shewanella, Psychrobacter, Proteus, Pseudomonas, Photobacterium, Lactobacillus* and *Psychrilyobacter* were detected and together represented 22.6% of the bacterial community.

The liver microbiome

The composition of the bacterial community in the liver was highly diverse and was significantly different from that of the intestine (Fig. 2, Permanova, p = 0.003). At T_0, the microbiome 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 SSOs, but these proliferated rapidly over time. The only SSO genus observed in liver samples at the beginning of the experiment was *Pseudomonas*, but at T_{48} the relative abundance of other SSO genera such as *Photobacterium, Shewanella, Psychrobacter* and *Vibrio* began to increase. By T_{96}, SSO genera were dominant within the liver necrobiome, representing 76% of the bacterial community. They remained the major component of the liver microbiota until the end of the experiment, when other genera such as *Salegentibacter, Sporosarcina, Enhydrobacter* and *Cutibacterium* were also detected.

The storage conditions greatly impacted the composition of the necrobiome 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 microbiome of fresh fish, was much less abundant in brine-frozen fish. Generally, although the relative abundance of SSO genera 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 SSO genera (*Photobacterium* and *Pseudomonas*), and potential pathogens such as

Staphylococcus and *Corynebacterium*. At the end of the experiment (T_{120}), the liver microbiome hosted several other potential SSO genera 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)(Fig. 3). Conversely, in brine-frozen fish, histamine concentrations remained below that threshold throughout the experiment.

Discussion

Modifications in animal's microbiome composition are normal phenomena following their death, 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 explored the tuna necrobiome by examining the dynamics of the main SSOs together with the production of histamine in two major bacterial reservoirs: the gut and the liver. We compared the incidence of post-capture storage conditions (fresh and brine-frozen individuals) on the development of these spoilage bacteria.

Occurrence and diversity of SSOs

As expected, the proportion of potential specific spoilage organisms (SSOs) increased significantly throughout the experiment in the gut and the liver (Fig. 2). This trend was particularly marked in fresh tunas. 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) (Fig. 2). The development of SSOs in fish and seafood products is well documented, and their proliferation typically depends on conservation conditions (Boziaris & Parlapani 2017). Several studies have demonstrated that SSO diversity in the flesh of different fish species varies between chilling, vacuum packaging or thawing temperature (Antunes-Rohling et al. 2019; Odeyemi et al. 2018; Reynisson et al. 2010; Syropoulou et al. 2021). Bacteria from the *Shewanella*, *Photobacterium* and *Pseudomonas* genera are known for their ability to produce high quantities of H_2S, trimethylamine and volatile nitrogenous compounds respectively (Boziaris & Parlapani 2017; Carrascosa et al. 2014; Chinivasagam et al. 1998), while species such as *S. putrefaciens, Proteus mirabilis* and *P. damselae* are bacteria potentially pathogenic to humans (Gennari, Tomaselli & Cotrona 1999; Ozogul et al. 2020; Speranza et al. 2013).

It should be noted that the spoilage activity of SSOs is a relatively complex mechanism that has multiple determinants. The production of spoiling metabolites is clearly species-dependent and varies according to the storage conditions, such as temperature (Antunes-Rohling et al. 2019; Parlapani & Boziaris 2016), atmosphere conditions (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, the growth of SSOs on selective media and the quantification of spoilage metabolites are usually required to assess the spoilage potential of SSOs (Fu et al. 2018; Syropoulou et al. 2020; Tang et al. 2019). Although these analyses were not performed in this study, the taxonomic identification of SSO genera in the two digestive organs raises questions about their dispersion from the viscera to the flesh after fish death (Shen & Wang 2020).

The effect of storage conditions on the tuna necrobiome

One of the main findings of this study was that the relative abundance and dynamics of SSOs greatly varied according to the initial storage conditions (Fig. 2). At the end of the experiment, they represented on average (for the two organs) 82% of the bacterial community in fresh tuna, in contrast to less than 30% in brine-frozen samples (Fig. 2). The influence of storage conditions on the composition of the fish microbiome has long been investigated (Ghaly 2010; Zhuang et al. 2021). For example, a delayed development of SSOs was reported in frozen fillets of Atlantic cod, mackerel and salmon compared to fresh samples (Fagan, Ronan Gormley & Mhuircheartaigh 2003; Sørensen et al. 2020). While low-temperature chilling is known to decrease the growth of microorganisms, 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 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 SSOs in the gut and liver microbiota compared to fresh tuna.

SSOs and histamine production in tuna

Among the potential SSO genera detected in the tuna necrobiome, histamine-producing bacteria (HPB) are of particular interest, as they have been implicated in cases of food poisoning worldwide (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 (Fig. 3). 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 (Egerton et al. 2018; Estruch et al. 2015; Givens et al. 2015), 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 SSO 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 and maintaining the cold chain is essential to prevent its formation (Hungerford 2010, 2021). Some psychrotrophic HPB, such as *P. phosphoreum* and *Morganella psychrotolerans,* are able to synthetize 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). Although our data did not allow us to identify the potential HPB down to the species level, we can consider that the *Photobacterium* taxa observed in both the gut and liver of fresh yellowfin tuna were HPB, as their temporal dynamics were positively correlated with the increase in histamine concentration in the different incubations (Fig. 3). As early as 96h after their capture, histamine concentration in the gut and liver of fresh yellowfin exceeded the United States Food and Drug Administration (FDA 2021) recommendations of 50 ppm.

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 (Bjornsdottir-Butler et al. 2015; Taylor & Speckhard 1983; Gadoin et al. 2021), 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 muscles.

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 (Fig. 3). 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 (Hungerford 2021).

Conclusion

Our results highlight the sanitary risks associated with the development of SSOs and of histamine concentrations in tuna's digestive organs, reminding the importance of removing their viscera prior to consumption. Such sanitary risks were much more elevated with fresh than brine-frozen fishes, confirming the incidence of the storage conditions on the evolution of the tuna necrobiome. Finally, this study confirms the need to take into account the gut and liver in further investigations on the ecology of HPB in scombroid fish.

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FIGURE LEGENDS

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. 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 potential specific spoilage organisms (SSOs) 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. 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).

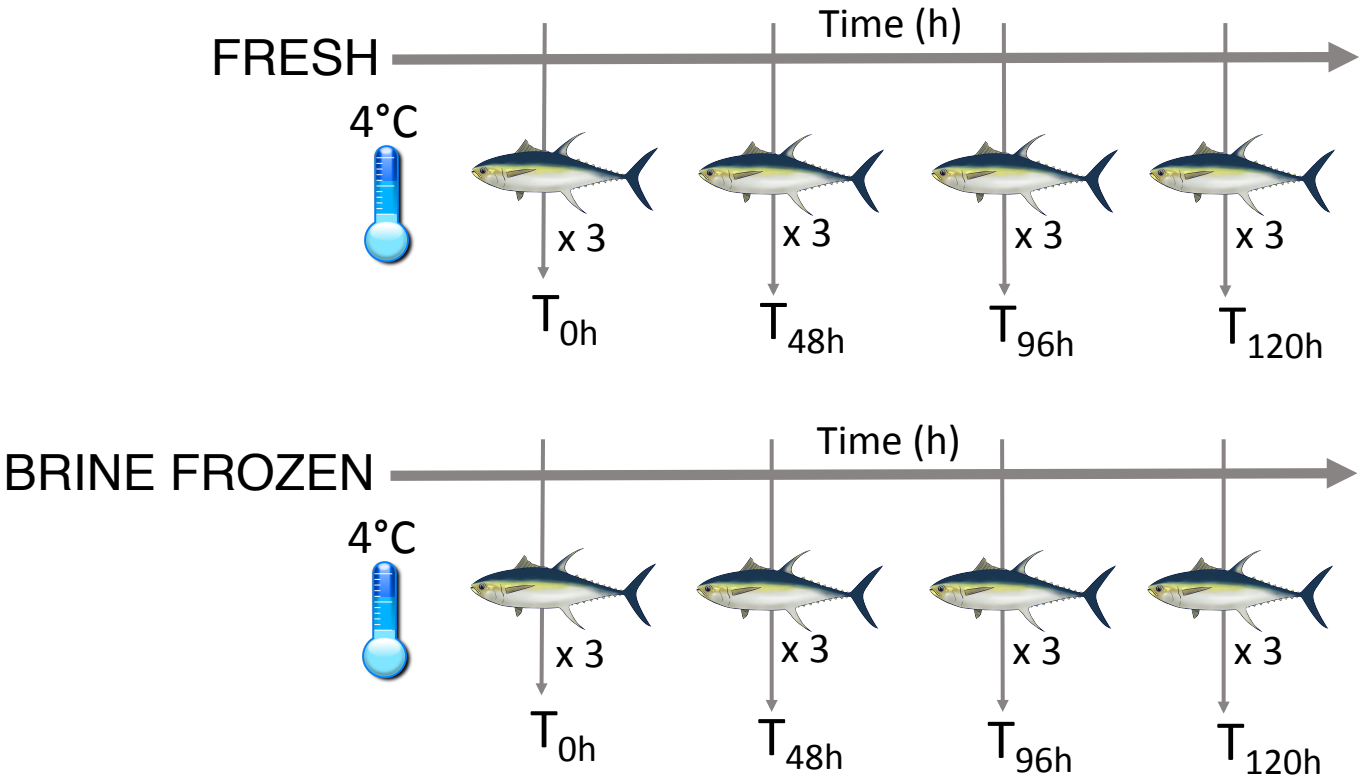

Figure 1

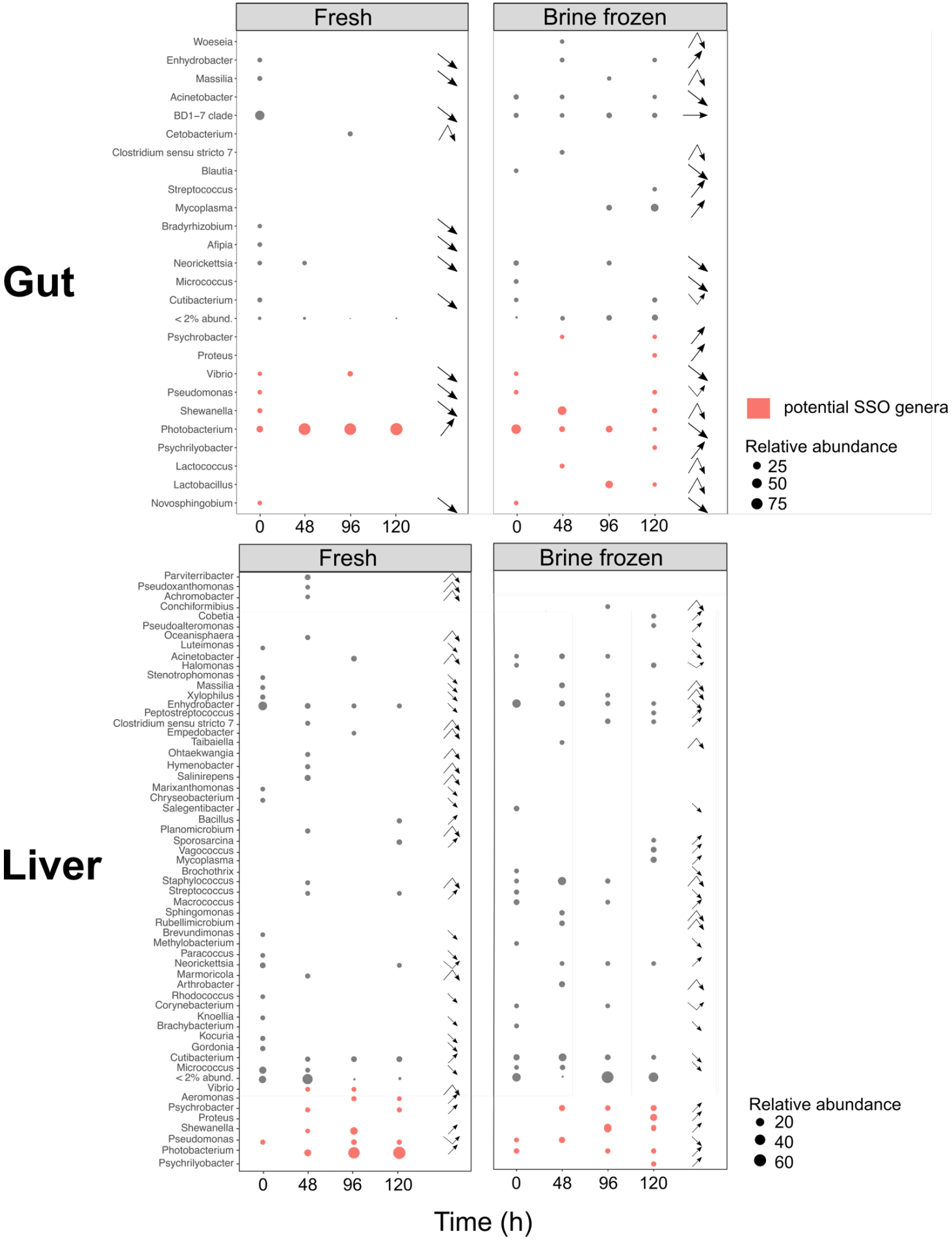

Figure 2

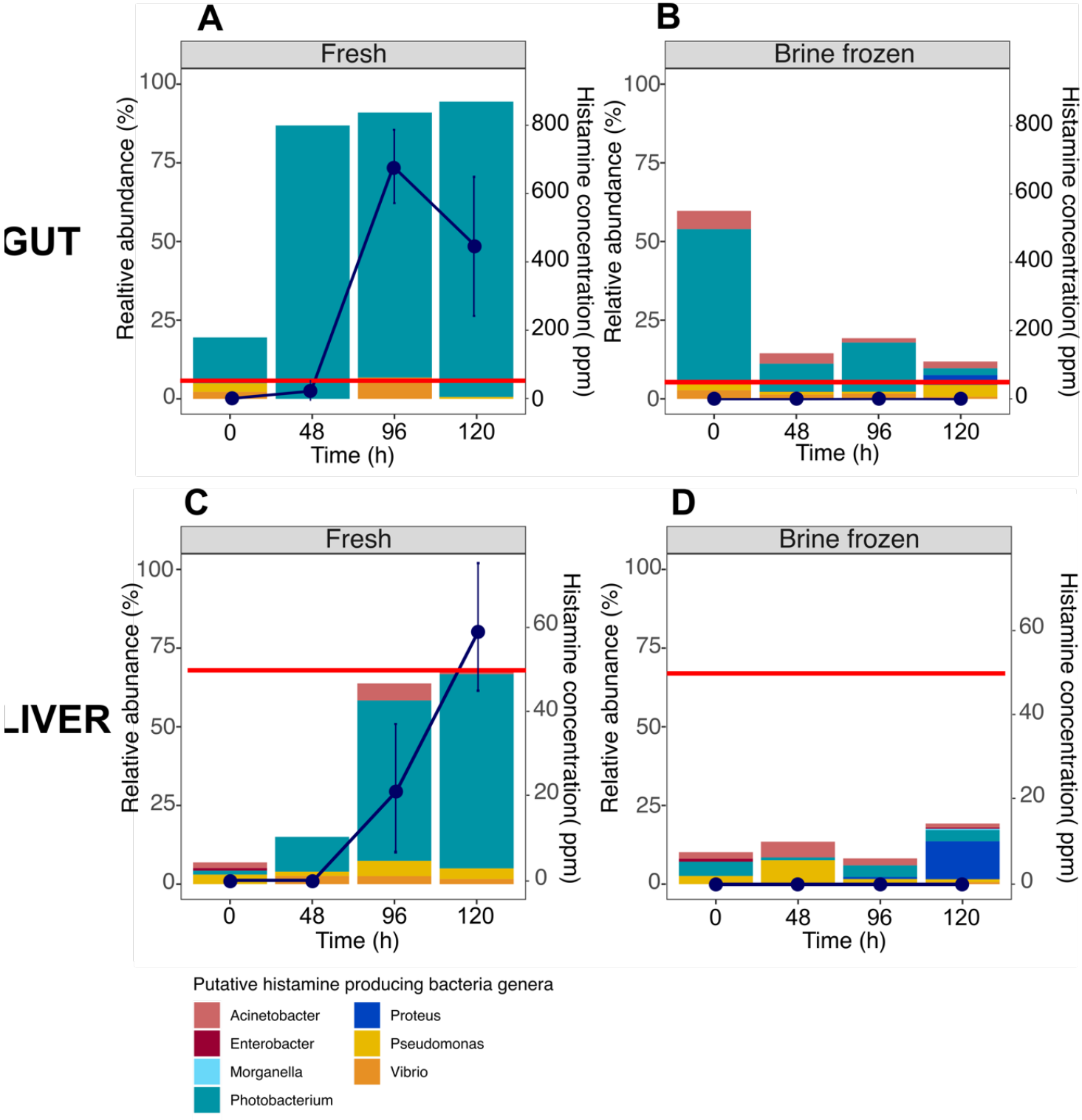

Figure 3

Table 1: 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 conservation conditions in gut and liver samples. Bold values indicate a significant effect of the tested factor ($p < 0.05$).

	Community dissimilarity					
	Time			Conservation		
	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