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Bacterial Risks and Biopreservation of Seafood Products

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Abstract :

The flesh of a healthy, living fish is considered sterile because the immune system prevents the growth of microorganisms. In this chapter, the authors present the current knowledge concerning the study and characterization of seafood microbiota, and then summarize the advances concerning biopreservation, a mild preservation method based on the use of selected protective flora. Biopreservation is a gentle food preservation technology that consists of preventing the development of undesirable microorganisms (pathogens or spoilers) by introducing into the product harmless microorganisms selected for their antimicrobial properties. A better understanding of bacterial interactions will therefore allow better control of its evolution during food preservation and more efficient biopreservation strategies to be proposed. Today, genome analysis of isolated bacteria or complex samples (metagenomics), as well as the numerous bioinformatics tools available, makes it possible to predict the presence of genes coding for molecules involved in bacterial interactions.

Keywords : bacterial interactions, bioinformatics, biopreservation, genome analysis, seafood microbiota

5.1 Introduction

The meat of a healthy, living fish is considered sterile because the immune system prevents the growth of microorganisms. When the fish dies, its immune system stops working and the bacteria present can proliferate freely. The poikilothermic nature of fish leads to the growth of bacteria over a wide temperature range including psychrotolerant bacterial species capable of growing at very low temperatures. It is one of the most perishable foods on the market (Gram and Huss 1996) due to its relatively high post-mortem pH (> 6.0) and the availability of large amounts of non-protein nitrogen as a growth substrate (free amino acids, nucleotides, trimethylamine oxide (TMAO)).

The bacterial ecosystempf seafood products, also called microbiota, is composed of endogenous bacteria and those from the product processing environment. Each manipulation from slaughter to the consumer's table can influence the composition of the final product's microbiota (Sivertsvik *et al.* 2002). This microbiota includes different types of bacterial species that can be classified according to their role in food preservation. There are bacteria pathogenic to humans, bacteria responsible for the organoleptic degradation of the matrix and conversely, beneficial bacteria. A good knowledge of the microbiota and its evolution during processing and storage is therefore necessary to develop strategies to i) reduce the development of pathogens and thus limit the microbiological risk; ii) delay sensory degradation to limit food waste. In this chapter, we will present the current knowledge concerning the study and characterization of seafood microbiota, and then we will summarize the advances concerning biopreservation, a gentle preservation method based on the use of selected protective flora.

5.2. Microbiota, microbial risk and product quality

5.2.1. *Methods for studying the microbiota of seafood*

The microbiota of a given product changes over time and according to storage conditions. In order to follow the dynamics of the microbiota of seafood products and to correlate this information with the quality and spoilage of the product, different types of analyses are used in parallel for a combined or polyphasic approach:

- Classical microbiology, molecular biology and Next Generation Sequencing (NGS) to follow the evolution of different bacterial flora.
- Biochemistry (total volatile basic nitrogen (TVBN), trimethylamine (TMA), biogenic amines, organic acids and volatile compounds) to determine the metabolites produced.

- Sensory analysis, an essential tool to determine the organoleptic quality and the spoilage level of products during storage

Recently, the use of NGS methods has become widespread. This molecular approach is based on a high-throughput sequencing technology of all genes (metagenomics) or the a target gene (metabarcoding). Metabarcoding targeting the 16S rRNA gene has been used in several studies to describe the bacterial genera and microbiota diversity of several seafood products such as salmon, shrimp, cod, yellowfin tuna, red drum, mussels or cuttlefish and blue crab (Table 5.1). Nevertheless, the combination of cultural and acultural methods is essential. Indeed, although molecular methods allow for an increasingly precise description of bacterial diversity, they are not all quantitative. Moreover, only the isolation of strains enables their spoilage potential, biopreservative effect or their other phenotypic characteristics of interest to be tested.

Many bacterial species are present in the microbiota. They co-evolve and interact during storage, impacting the degradation of products. During storage, some bacterial genera become dominant and thus the bacterial diversity of the product tends to decrease at the end of storage compared to the first days of production. Some bacterial genera are common to the microbiota of several products: Gram positive bacteria like *Brochothrix* or lactic acid bacteria (*Aerococcus*, *Carnobacterium*, Lactobacilli, *Lactococcus*, *Leuconostoc*, *Vagococcus*) as well as Gram negative bacteria like *Acinetobacter*, *Photobacterium*, *Pseudomonas*, *Psychrobacter*, *Shewanella*, *Serratia* and *Vibrio*.

In the coming decades, the influence of the microbiome and its impact on health/safety will be characterized along the food chain, in a continuum from the microbiome of the living fish in its environment, through the microbiome of the processed fish as food, to the microbiome of the consumer's gastrointestinal tract.

	Main bacterial genera (or family) identified during storage	Storage (days, conditioning, T°C)	Reference
Cod (*Gadus morhua*)	*Fusobacteriaceae* *Brochothrix* *Photobacterium* *Vagococcus* *Carnobacterium* *Clostridium* *Shewanella* *Acinetobacter*	D7; modified atmosphere; 4°C then 8°C	(Chaillou *et al.* 2015)
Blue crab (*Callinectes sapidus*)	*Rhodobacteraceae* *Vibrionaceae*	D6 to D10; under air (live crabs); 4 or 10°C	(Parlapani *et al.* 2019)
Cooked and peeled shrimp	*Leuconostoc* *Streptococcus* *Vibrio* *Carnobacterium* *Weissela* *Aerococcus* *Trichococcus*	D15 to D49, modified atmosphere; 4°C then 8°C	(Chaillou *et al.* 2015)
Mussels (*Mytilus galloprovincialis*)	*Synechococcus* *Shewanella* *Acidaminococcus* *Psychromonas* *Acinetobacter* *Psychrobacter*	D0 to D15; modified atmosphere, (depurated and not depurated); 4°C	(Odeyemi *et al.* 2019)
Red drum (*Sciaenops ocellatus*)	*Carnobacterium* *Brochothrix* *Lactococcus* *Vagococcus* *Arthrobacter* *Shewanella* *Leuconostoc* *Pseudomonas*	D8 to D29; vacuum, modified atmosphere; 4°C	(Silbande *et al.* 2018a)
Fresh salmon (*Salmo salar*)	*Carnobacterium* lactobacilli *Photobacterium* *Lactococcus* *Brochothrix* *Vagococcus* *Flavobacterium*	D9-J11; modified atmosphere 4°C then 8°C	(Chaillou *et al.* 2015)

Smoked salmon (*Salmo salar*)	*Photobacterium* *Carnobacterium* *Brochothrix Serratia* *Psychrobacter* *Shewanella* Lactobacilli *Lactococcus* *Vibrio* *Salinivibrio* *Aliivibrio* *Pantoea* *Enterococcus* *Staphylococcus* *Pseudomonas*	D0 to D28; D21 to D57; under vacuum; 4°C then 8°C	(Chaillou *et al.* 2015; Maillet *et al.* 2021)
Gravlax salmon (*Salmo salar*)	*Photobacterium* *Serratia/Yersinia* *Vibrio* *Lactococcus* *Carnobacterium* lactobacilli *Aerococcus*	D14 to D21; under vacuum at 8°C	(Wiernasz *et al.* 2020)
Cuttlefish (*Sepia officinalis*)	*Psychrobacter* *Pseudomonas*	D0 to D8 ; under air ;2°C	(Parlapani *et al.* 2018)
Yellowfin tuna (*Thunnus albacares*)	*Brochothrix* *Pseudomonas* *Hafnia*	D6 to D13; on ice; 0°C, vacuum, modified atmosphere; 4°C	(Silbande *et al.* 2016)

Table 5.1: *Microbiota from different seafood products determined by metabarcoding analyses targeting regions of the 16S rRNA gene*

5.2. 2. *Pathogenic flora and microbiological risks*

The identification of bacterial flora provides information on the presence of pathogenic bacteria and the hygienic quality of the product during processing. Food business operators are obliged to withdraw unsafe food from the market. Harmonized safety criteria for the acceptability of foodstuffs, in particular with regard to the presence of certain pathogenic microorganisms, toxins or metabolites, are defined in the European Regulation (EC) No 2073/2005. For seafood products, there are only 4 safety criteria: *Listeria monocytogenes* for ready-to-eat foods, *Salmonella* for cooked crustaceans and cooked or live bivalve molluscs, *Escherichia coli* for live bivalve molluscs, echinoderms, tunicates and gastropods, and histamine for fish with a high

histidine content (Table 5.2). However, other pathogenic bacteria can cause food poisoning and these risks should be known and managed for consumer safety.

5.2.2.1. *Vibrio*

Species of the genus *Vibrio* are Gram-negative, mesophilic bacteria present in marine and estuarine environments. They are also found in the intestine and on the tissues of fish, crustaceans and shellfish. Some species of non-cholera vibrios (*Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* (non-O1/non-O139 serogroups) can cause food poisoning, and *V. cholerae* O1 or O139 can cause cholera. The main cause of transmission is the consumption of fish and seafood, especially shellfish (e.g., oysters) and crustaceans (e.g., tropical shrimp) eaten raw, undercooked, or recontaminated after cooking. The concentration of *Vibrio* varies according to the surface temperature of the water, salinity, turbidity, pH, or chlorophyll A content. Maximum abundance is observed during the warmer months of the year and in warm seawater (Anses 2019a).

5.2.2.2. *Clostridium botulinum* type E

C. botulinum is a gram-positive, spore-forming, strict anaerobic bacillus. It is responsible for severe food poisoning in humans and animals (botulism) by ingestion of preformed toxin in food (intoxination) or by intestinal colonization and toxin production *in situ* (toxi-infection). There are 7 types of botulism toxins, from A to G, according to their immunological properties. Four of these forms (types A, B, E and rarely F) can cause human botulism. The foods most often implicated in outbreaks of type E toxin botulism are canned goods and homemade or artisanal products of salted and dried fish, fish marinades, or vacuum-packed products (Anses 2019b).

5.2.2.3. *Listeria monocytogenes*

Widely present in water, soil, and dust, able to grow at low temperatures (from -2 °C), tolerant of acidic and saline conditions and the absence of oxygen (O_2), this pathogenic species can contaminate processing plants (Fonnesbech Vogel *et al.* 2001). Although cooking is successful in destroying it, it is sometimes found in ready-to-eat foods (Parihar *et al.* 2008; Gambarin *et al.* 2012). *L. monocytogenes* toxi-infections can be very serious, even fatal, in immuno-compromised patients and for the fetus in pregnant women (Todd and Notermans 2011; Anses 2020). It is found in the aquatic environment mainly on the skin, in the gills or digestive tract of fish. As a result, contamination of fish meat can occur during the various successive operations of slaughter, evisceration, filleting, etc. It is necessary to qualify suppliers (farms and slaughterhouses) and to monitor the presence of *L. monocytogenes* at reception and throughout the life of the product. Contamination can also occur in the plant, by humans or by contact with soiled surfaces, especially since *L. monocytogenes* is capable of forming biofilms that are difficult to remove once they have settled. *L.monocytogenes* is a regulatory safety criterion for ready-to-eat foods, described in the European regulation (EC) No 2073/2005. There must be no *L. monocytogenes* in 25 g of product. A threshold of less than 100 CFU/g during the whole shelf life of the product can be accepted provided that the manufacturer is able to demonstrate that this limit will be respected. If these values are exceeded, a product withdrawal or recall is necessary.

5.2.2.4. *Histamine*

Histamine is a biogenic amine derived from histidine. The ingestion of foods with a high histamine content causes histamine intoxication (or scombroid intoxication). It is manifested by allergic-type reactions (facio-cervical redness, itching), neurological disorders (headaches, heart palpitations, dizziness) as well as secondary symptoms of a gastrointestinal nature (vomiting, diarrhea) (Union du mareyage français (UMF) 2010). Histamine is responsible for 30 to 40 % of food-borne illnesses related to the consumption of fish products. It is produced in the muscles of fish species containing histidine. The animal is not ill because it is synthesized after the death of the fish through the action of an enzyme, histidine decarboxylase. This enzyme is produced by mesophilic enterobacteria but also by psychrotolerant species such as *Photobacterium phosphoreum* and *Morganella psychrotolerans* which are able to generate histamine at low temperatures (as low as 2°C). Histamine is a safety criterion of the Regulation (EC) No. 2073/2005 for fishery products made from fish species rich in histidine: an average value of 100 mg/kg must not be exceeded during the conservation of the product. This value increases to 200 mg/kg for products that have undergone enzymatic maturation in brine and to 400 mg/kg for fish sauces produced by fermentation of fish products.

5.2.2.5. *Other bacterial hazards*

Other bacteria introduced *via* the environment contaminated by waste, often domestic or industrial, or *via* human or animal fecal contamination, must also be taken into account as a microbiological hazard.

5.2.2.5.1. *Salmonella*

Salmonella is a gram-negative rod-shape bacteria. Salmonellosis is one of the main gastroenteric syndromes of bacterial origin in industrialized countries. The main reservoir of *Salmonella* is the gastrointestinal tract of mammals (pigs, cattle) and birds (domestic poultry). Some strains can also be isolated from other sources, such as cold-blooded animals (reptiles, turtles) and aquatic animals (mollusks, fish). Salmonella in animal feces can contaminate pastures, soils, and water and survive for several months; thus, the environment can become a source of danger (Anses 2021). Bivalve molluscs have a concentrating role for bacteria in the environment: by filtering large quantities of water to ensure their respiration and nutrition, they retain particles, including bacteria, whether they are free or associated with absorbed food. The safety criterion of the regulation (EC) n°2073/2005 indicates the absence of *Salmonella* in 25 g of product (cooked crustaceans or molluscs as well as live bivalves, echinoderms, tunicates and live gastropods).

5.2.2.5.2 *Escherichia coli*

Gram-negative, oxidase-negative rod-shaped bacteria, the *E. coli* species is normally present in the intestinal microbiota and feces of humans and warm-blooded animals. Its presence in a food indicates a lack of hygiene during a food preparation step and a potential risk regarding the presence of pathogenic organisms. The actual presence of *Escherichia coli* in live shellfish indicates a lack of control of the quality of the water used by establishments or of the decontamination processes implemented or even an initial contamination in the harvesting area. For live bivalve molluscs and live echinoderms, tunicates and gastropods, the limit for this safety criterion is 230 MPN/100 g of meat or intravalvular fluid (MPN = most probable number). For cooked shellfish products, *E. coli* is also a hygiene criterion with a limit of 1/g at the end of the manufacturing process (Regulation (EC) No 2073/2005).

5.2.2.5.3. *Staphylococcus aureus*

Staphylococcus aureus is a microorganism that is not part of the natural microbiota of fish, but is present on mammalian skin and mucous membranes. Worker handling is the primary source of product contamination (Huss 1995). *S. aureus* produces heat-resistant toxins that can cause severe food poisoning. However, this bacterium does not grow at refrigeration temperatures (0°C-4°C) and cases of poisoning are often related to ready-to-eat products left at too high temperatures. *S. aureus* is a hygiene criterion of Regulation (EC) No. 2073/2005 with a limit of 100 CFU/g at the end of the manufacturing process for shucked and shelled cooked shellfish products.

	Foodstuffs	Limits	Applications
Listeria monocytogenes	Ready-to-eat seafood products that can support the development of *L. monocytogenes* (smoked salmon, cooked peeled shrimp, etc.)	100 CFU/g	Product placed on the market during shelf life
		Absence in 25 g	Before the commodity has left the control of the operator who manufactured it
Salmonella	Cooked crustaceans and mollusks	Absence in 25 g	Product placed on the market during shelf life
	Live bivalve mollusks and echinoderms, tunicates and gastropods	Absence in 25 g	Product placed on the market during shelf life
Escherichia coli	Live bivalve molluscs and echinoderms, tunicates and gastropods	230 MPN/100 g of meat and/or interval fluid	Product placed on the market during shelf life
Histamine	Fish products made from fish species rich in histidine (tuna, mackerel)	100 mg/kg	Product placed on the market during shelf life
	Fish products that have undergone an enzymatic maturation treatment in brine, made from fish species associated with a high amount of histidine (salted anchovies, salted anchovy products, fish sauce)	200 mg/kg	
	Fish sauces produced by fermentation of fish products.	400 mg/kg	

Table 5.2. *Safety criteria for seafood products from Regulation (EC) No 2073/2005*

5.2.3. Seafood spoilage bacteria

Spoilage includes all changes that make a food product unfit for human consumption from a sensory point of view. The mechanisms at the origin of spoilage are autolytic and/or chemical in nature, but are mainly related to bacterial metabolism responsible for bad odors and flavors. The metabolism of nitrogenous compounds in the meat of marine products by bacteria often generates the production of unpleasant odors. For example, the degradation of cysteine and methionine causes sulfurous odors, the degradation of urea causes ammonia odors, and the reduction of TMAO to TMA contributes to the amine odors typical of rotten fish. TMAO is a molecule present in the flesh of marine fish that plays a role in osmoregulation (Huss 1995). In anaerobic conditions, the use of TMAO as a final electron acceptor by some bacteria facilitates their growth in the absence of oxygen.

5.2.3.1 The specific spoilage bacteria

During product storage, some bacteria grow faster depending on various parameters such as temperature, protective atmosphere, microbial interactions, etc. At the time of spoilage, the microbiota of the product is composed of: (i) Specific Spoilage Organisms (SSO) that produce metabolites responsible for unpleasant odors and (ii) bacteria that grow on the product but do not cause unpleasant changes (Gram and Huss 1996; Gram and Dalgaard 2002; Lyhs 2009). The growth of specific spoilage bacteria and the production of their metabolites can be used as indicators for determining the shelf life of a product.

Determining the bacteria responsible for spoilage of a given product is not an easy task and requires an approach combining several types of assays: sensory, chemical and microbiological analyses. The identification of these spoilers is based on the comparison of the spoilage characteristics of the naturally contaminated product with those induced on the product inoculated by spoilage microbiota isolates. These analyses can be performed on sterile media that are as close as possible to the food, such as fish juice, or during challenge tests on sterile or pauci-microbial products (containing as few microorganisms as possible). The last method is the one that comes closest to the naturally contaminated product. It has been shown that within the same species, different strains may not reveal the same spoilage potential. This is why it is preferable to inoculate several strains of the same species during these challenge tests.

5.2.3.2 Influence of conditioning on spoilage bacteria

Food preservation methods can alter the microbiota naturally present on the product and influence the development of specific spoilage bacteria.

Spoilage of fresh marine fish from temperate waters, stored under air (e.g. on an ice), is mainly caused by bacteria belonging to the "Shewanella putrefaciens-like" group, including *S. putrefaciens, S. baltica* or other related species. *Shewanella* produce H_2S and other sulfur compounds as well as hypoxantine and TMA. Spoilage of tropical freshwater fish is often related to *Pseudomonas*. Many species produce typical sensory characteristics such as foul odors with "fruity and rotten" characteristics. *Pseudomonas* produce various spoilage metabolites such as esters, aldehydes, sulfur compounds like methylmercaptan, but no H_2S or TMA. These bacteria are indeed not able to reduce TMAO and therefore their growth is limited in the absence of oxygen. *Shewanella* spp. and *Pseudomonas* are also considered the spoilage bacteria of warm-water marine fish (Gram and Huss 1996; Koutsoumanis and Nychas 1999; Leroi 2014).

For fresh vacuum-packed fish containing TMAO, such as cod, *S. putrefaciens* and *P. phosphoreum* have been identified as the specific spoilage bacteria (Gram and Huss 1996).

The protective atmosphere enriched with CO_2 inhibits the growth of *Pseudomonas* and *S. putrefaciens*, while *P. phosphoreum* and lactic acid bacteria are more resistant to CO_2 (Gram and Dalgaard 2002). For fresh marine fish preserved with this type of conditioning, *P. phosphoreum* thus appears to be the main specific spoilage bacterium. It produces high amount of TMA and has been characterized as a specific spoilage bacterium in cod (Dalgaard *et al.* 1997) and salmon (Emborg *et al.* 2002; Macé *et al.* 2013). For cooked peeled shrimp or warmer water fish packed under modified atmosphere, lactic acid bacteria such as *Carnobacterium maltaromaticum* associated with *Brochothrix thermosphacta* are responsible for spoilage. For cooked whole tropical shrimp, *C. maltaromatcum and S. baltica* have been identified as the main spoilers (Laursen *et al.* 2005, 2006; Mejlholm *et al.* 2005; Leisner *et al.* 2007; Jaffrès *et al.* 2011; Macé *et al.* 2014). Some lactic acid bacteria are responsible for the production of ammonia, H_2S and biogenic amines (Leroi 2010). *B. thermosphacta* is capable of producing ammonia, aldehydes (acetaldehyde, isovaleraldide and), ketones (diacetyl displays cheese-odour) (Dalgaard 2000; Jaffrès *et al.* 2011).

The addition of salt, slight acidification and cold vacuum storage for lightly preserved products, such as smoked salmon, will inhibit aerobic Gram-negative bacteria. Under these conditions, the microbiota is dominated by lactic acid bacteria (lactobacilli and *Carnobacterium*) or other Gram-positive bacteria such as *B. thermosphacta* associated with fermentative Gram-negative bacteria such as *P. phosphoreum* and psychrotrophic enterobacteria (Leroi and Joffraud 2011; Chaillou *et al.* 2015; Wiernasz *et al.* 2021). *Latilactobacillus sakei*, *Companilactobacillus farciminis*, *Latilactobacillus curvatus*, *Serratia liquefaciens*-like, as well as some strains of *B. thermosphacta* and *P. phosphoreum* have been found to be highly

detrimental to smoked salmon (Stohr *et al.* 2001). *L. sakei* was associated with the production of H_2S, acetic acid and esters and *S. liquefaciens* with the production of TMA and sulfur compounds. *P. phosphoreum* has been identified as the most significant producer of biogenic amines in smoked salmon, although enterobacteria and lactic acid bacteria are also capable of producing them.

Increasing the preservative pressure, for example by acidifying with preservatives such as sorbate and benzoate for pickled herring, leads to the growth of lactic acid bacteria, including lactobacilli and yeast (Gram and Dalgaard 2002; Lyhs and Björkroth 2008).

5.2.3.3. Influence of bacterial associations

The observed diversity and dynamics of the microbiota during the storage of products testifies of the numerous bacterial interactions that occur in the product. These interactions influence the spoilage of a given matrix by impacting the nature and quantity of the metabolites synthesized. The interaction of certain species can be beneficial. In smoked salmon, co-inoculation of *L. sakei* and *S. liquefaciens-like*, two species that induce high spoilage when inoculated separately, slows the spoilage process (Joffraud *et al.* 2006). In tuna, the presence of *B. thermosphacta* or *P. phosphoreum* decreases the undesirable odor caused by *Hafnia alvei* in pure culture (sulfur/cabbage, pyrrolidine, fecal), probably due to a hundredfold lower concentration of the latter when co-inoculated (Silbande *et al.* 2018b). Other interactions have little or no impact on the sensory evolution of the matrix. This is the case for *C. maltaromaticum/P. phosphoreum* or *C. maltaromaticum/B. thermosphacta* pairs in smoked salmon. Finally, it has been shown that the presence of two species can give rise to new odors. For example, simultaneous inoculation of *C. maltaromaticum* and *B. thermosphacta* generates an unpleasant "wet dog" odor not identified in the presence of the independently inoculated species on shrimp (Laursen *et al.* 2006). Similarly, two non-spoiling species such as *Vibrio* sp. and *B. thermosphacta* produce unpleasant odors in co-culture in smoked salmon (Joffraud *et al.* 2006).

5.3. Biopreservation of seafood products

Biopreservation is a gentle food preservation technology that consists of preventing the development of undesirable microorganisms (pathogens or spoilers) by introducing into the product harmless microorganisms selected for their antimicrobial properties (Pilet and Leroi 2011). Unlike fermentation where microorganisms have a technological role (milk is transformed into yogurt, cabbage into sauerkraut), biopreservation aims to preserve delicate products without changing their organoleptic and nutritional characteristics. It is a natural method that can replace the addition of additives or allow the scales of technological treatments to be reduced, such as heat, salt, smoke etc.

5.3.1 *Bacterial interactions*

In order to propose effective biopreservation strategies, it is necessary to understand the dynamics of bacterial communities in seafood. This aims to better predict the effect of the addition of bioprotective strains on the microbiota and thus on the sensory profile of the matrix. Bacterial interactions are driven by several mechanisms.

5.3.1.1 *Bacterial competition*

Some scientists suggest that competitions are prevalent in the natural environment (Foster and Bell 2012). Indeed, microorganisms wage a constant battle for access to resources. This is especially true in seafood, where carbon substrates are scarce and undiverse. Glucose, lactate, and free amino acids are predominantly found (Jaaskelainen *et al.* 2019). The preponderance of certain species is determined by the needs of each individual and the access and rate of consumption of available nutrients. Therefore, microorganisms develop active strategies that allow them to be more competitive and reach resources faster. For example, they can increase their mobility, adhere to specific surfaces, or synthesize antimicrobial compounds (Hibbing *et al.* 2010).

Gram-negative bacteria are a rich source of antimicrobial compounds (Hu *et al.* 2015; Ng *et al.* 2015; Masschelein *et al.* 2017; Carroll *et al.* 2020). They belong to different chemical classes such as polyketides, non-ribosomal peptides, lipopeptides, alkaloids, terpenes and quinones (Ng *et al.* 2015). The molecules produced can be associated with a bacterial genus or species, but are often strain-specific. Various examples have been described in the literature among species colonizing seafood. *Shewanella* sp. MTCC 12715 produces macrocyclic polyketides with anti-Staphylococcus and anti-Vibrio activities (Chakraborty *et al.* 2021). *Pseudomonas fluorescens* produces mupirocin, a polyketide active against some gram-positive

bacteria, and *Serratia* sp. produces the broad-spectrum antibiotic andrimide (Masschelein *et al.* 2017). Many bacteria also synthesize siderophores, iron-chelating molecules involved in bacterial competition. Indeed, iron is an essential element for the life of microorganisms and is generally present in low quantities in food matrices. Some bacterial species, such as *Shewanella* sp. or *Pseudomonas* sp. are known to synthesize siderophores in order to internalize the iron necessary for their survival (Gram 1994; Soe *et al.* 2012). Indeed, the competition for iron by these two bacterial genera has been studied in cod tissue (Gram and Melchiorsen 1996).

Lactic acid bacteria isolated from seafood produce numerous bacteriocins (Gómez-Sala *et al.* 2015). These ribosome-synthesized peptides have long been studied for their antimicrobial properties (Arnison *et al.* 2013; Silva *et al.* 2018; Trejo-González *et al.* 2021). There is a wide diversity of bacteriocins in terms of their structure, size, and modes of action. In bacteria isolated from seafood, some strains have been extensively studied for their application in biopreservation (see § 5.3.3). In addition to bacteriocins, lactic acid bacteria synthesize other antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl or carbon dioxide. These molecules and favorable product packaging conditions may explain the preponderance of lactic acid bacteria in certain products. But despite all the knowledge on this bacterial group, some species still hide some mysteries. This is the case of *Lactococcus piscium*, a species often found in the microbiota of seafood. The CNCM I-4031 strain, which does not produce bacteriocins, is currently being studied to understand the mechanism involved in preventing the growth of *L. monocytogenes* (Fall *et al.* 2010; Saraoui *et al.* 2016).

Bacterial viruses also affect the dynamics of microbial communities. Phages can both lie dormant in a bacterial host (then called a prophage) and lyse it under conditions of stress in order to infect other targets. A recent study found over 109 phages per gram of cheese matrix (Dugat-Bony *et al.* 2020). The prevalence of phages in seafood is not known, yet several bacterial genera represented host them. This is the case of lactic acid bacteria or the genera *Listeria* or *Bacillus* (Canchaya *et al.* 2003). The isolation of phages from seafood processing plant samples (Arachchi *et al.* 2013) and the activation of prophage gene expression in *L. piscium* in coculture with other lactic acid bacteria (Andreevskaya *et al.* 2018) also point in this direction. The use of phage therapy as a means of biopreservation is proving to be an interesting strategy. Virulent phages have been used to target *Shewanella* in catfish fillets (Yang *et al.* 2019).

5.3.1.2. *Bacterial cooperation*

In food, a particular mechanism called the "Jameson effect" was described in 1962 (Jameson 1962). It is defined as the cessation of population growth when a species has reached its maximum concentration. Several studies describe this phenomenon in seafood (Ross 2000). These observations could be explained today by quorum sensing, a bacterial communication system that is the subject of numerous studies (Waters and Bassler 2005). Quorum sensing induces the production of autoinducers in response to cell density. These autoinducers can regulate the expression of specific genes such as those involved in the synthesis of antimicrobial compounds, bioluminescence, virulence or biofilm formation... In Gram-positive bacteria, small peptides called AIPs (Autoinducing Peptides) serve as signal molecules. In Gram-negative bacteria, the best known are the acyl homoserine lactones (AHLs) encoded by the lux genes. The presence of AHLs of different classes has been detected in seafood (Gram *et al.* 2002). Enterobacteria strains isolated from fishes and *Pseudomonas*, *Photobacterium* and *Aeromonas* strains have also shown the ability to produce these types of molecules (Ravn *et al.* 2001; Gram *et al.* 2002). The knowledge gained on quorum sensing molecules in seafood allows the development of new biopreservation strategies. Recently, the use of two strains of *Lactiplanctibacillus plantarum* and *Lacticaseibacillus casei* communicating through the LuxS/AI-2 system allowed for the biopreservation of chilled shrimp and decreased the concentration of ABVT (Li *et al.* 2019). Another strategy was to treat diced salmon with selected quorum sensing inhibitors called 5'-AMP (5′-adenylic acid) and 5'-CMP (Cytidine-5′-monophosphate) to combat *S. baltica* and *P. fluorescens-related* spoilage (Wang *et al.* 2021).

Another process of bacterial cooperation called metabiosis has been described in seafood since the 2000s. It corresponds to interspecies sequential metabolic interactions (Gram and Dalgaard 2002; Jørgensen *et al.* 2000), i.e. the development of certain microorganisms through the use of substrates derived from the metabolism of other microorganisms. This phenomenon has helped explain the accumulation of high concentrations of biogenic amines (putrescine and agmatine) in smoked salmon. These are believed to be produced from arginine through the combined activity of enzymes synthesized by *P. phosphoreum*, *S. liquefaciens*, *H. alvei*, *Carnobacterium* sp. or *L. sakei* (Jørgensen *et al.* 2000). This phenomenon is considered harmful, but from a biological point of view, it allows microorganisms to survive in a rather hostile environment.

5.3.2. *Selection of bioprotective microorganisms*

The selection of biopreservation microorganisms, also called protective cultures (PCs), requires collections to be screened. The microorganisms, often bacteria for seafood applications, can come from various biotopes. However, it is interesting to have germs isolated from the food to be biopreserved, which guarantees a good re-implantation in the product. Therefore, knowledge of microbial ecosystems is a prerequisite for successful biopreservation. It is important to prioritize the many criteria for selecting PCs in order to minimize the number of tests.

5.3.2.1. *Antimicrobial activity*

The first step in selecting a PC is to screen for antimicrobial activity against target strains. Since not all strains of the same species are equally susceptible to PCs (Brillet *et al.* 2004), it is important to test multiple strains when possible.

There are many strains of pathogens that can be used as targets, however, laboratories that are not equipped to work with class 2 pathogens often perform an initial screen on surrogate species, such as *Listeria innocua* instead of *L. monocytogenes.* A final validation on the pathogen species is always necessary. The target bacteria for spoilage are rather specific to each product and must be carefully selected according to the matrix.

The tests are generally completed under model conditions, in liquid or agar culture media, sometimes in fish juice. Different methods exist. One can mention the spot on lawn method which consist of spotting the deposition of culture spots of culture drops of the strains on agar plates previously inoculated with a target strain. After incubation at the growth temperature of the target (or at the storage temperature of the fish to be as close as possible to the real conditions), the presence of clear halos around the spots indicates an inhibition whose intensity is stronger as the diameter of the halo increases (Matamoros *et al.* 2009a). Inhibitions specifically related to the excretion of antimicrobial compounds can be observed by depositing sterilized culture supernatants. In case of activity, different rapid tests are implemented for a pre-characterization of active molecules, including organic acids, hydrogen peroxide or bacteriocins. All these methods can be miniaturized to allow rapid screening of several hundred bacteria under various conditions. If no collections of PCs are available for screening, another strategy is to enumerate the bacteria present in the food to be biopreserved by the classical Petri dish technique, and then to apply a thin overlay of agar inoculated with the target strain on the surface of the dish. The use of selective enumeration media and their incubation conditions (temperature, anaerobiosis) then allows the screening to be directed towards more adapted PCs. A specific medium for lactic bacteria can be used for example. Lactic acid bacteria are indeed good

candidates for biopreservation because they are often present in foods, carry little risk for health and have a wide range of antimicrobial activities.

Omics tools are now also used for pre-selection of PCs. Biosynthetic clusters, often involved in the production of non-ribosomal antimicrobial peptides, terpenes and polyketides or bacteriocins can be searched *in silico* in available genomes. The presence of such gene clusters can predict potential antimicrobial activity. The genomic comparison and de-replication process can then determine whether the molecules for which they code are novel. For example, Begrem *et al* (2020) showed the high potential diversity of antimicrobial metabolites in several *Carnobacterium* species.

5.3.2.2. *Sensory acceptability*

One of the big challenges of biopreservation is to find bacteria that have antimicrobial capabilities but that do not degrade the sensory quality of the product. Unfortunately, many bacteria are responsible for bad odors, flavors or textures. Some strains belonging to Lactic acid bacteria group, in particular produce organic acids which, although necessary in fermentation, are not very compatible with a biopreservation application. Some produce bitter flavors, CO_2 responsible for the swelling of the packaging, H_2S and other malodorous molecules. The low acidification and the absence of bad odors can be tested in a first step in a model liquid medium. To best mimic the feed, some use fish juices enriched with malodorous metabolite precursor molecules, such as TMAO and sulfur amino acids (Wiernasz *et al.* 2017). The more efficient tool to define sensory profiles remains human? perception. Without involving a huge sample group, a few people trained in sensory analysis can sniff out different cultures to select the most neutral candidates possible. It is important to know that the ability of a bacterial species to spoil a food is strain dependent, although broad signatures can emerge.

5.3.2.3. *Validation of antimicrobial and organoleptic effects in foodstuffs in a pauci-microbial matrix*

The results observed in liquid medium must be validated in food. The microbiota, the composition of the product (sugars, proteins, vitamins, etc.) as well as the technological parameters, such as salt, smoke, atmosphere and storage temperature, have an impact on the implantation of PCs and their metabolism.

The growth and antimicrobial activity of PCs are verified by carrying out challenge tests. These consist of co-inoculating the PC and the target strain (pathogen, spoilage) in the food, containing if possible a very low quantity of endogenous

bacteria. This is particularly important when one wishes to test the effect of PCs on target spoilage bacteria, whose natural presence in food could make interpretation difficult. Sterile or *pauci*-microbial matrices can be obtained either by processing the product under excellent hygienic conditions or by applying a non-destructive decontamination process such as ionization. The ISO 20976-1 (2019) standard for performing challenge tests specifies the importance of using cocktails of several target strains from the food rather than collection strains, and of performing pre-cultures of the targets under stress conditions similar to those they might encounter in the matrix (low temperatures, salt etc). In addition, the AFNOR standard (NF V01-003, 2018) specifies the protocol for validating the shelf lives of refrigerated products. The scenario is a conservation for 1/3 of the time of the total shelf life at 4°C and 2/3 at 8°C, mimicking the temperature profile observed from distribution to conservation at the consumer.

The use of a *pauci*-microbial matrix also makes it possible to control that the PC, even if inoculated at high concentration, does not cause organoleptic changes in the product until the best before date (BBD) of the product. For example, a strain of *L. sakei*, very promising to prevent the development of histaminogenic bacteria such as *Photobacterium* sp. and *Morganella* sp., has been found acceptable in tuna but responsible for strong sulfurous odors in other products (salmon, shrimp, cod). Acidification of a PC in a model liquid medium is often considered a rejection criterion, whereas some strains may be quite acceptable when introduced into a low-sugar fish meat with a high buffering capacity. For example, a bioprotective strain of *L. gelidum* performed poorly in gravlax because the presence of sugar in the recipe led to acidification and production of sticky exopolysaccharides on the surface of the product, whereas it was very effective in extending the SLED (Shelf Life Expiration Date) of fresh salmon and cooked peeled shrimp (Brillet 2011).

5.3.2.4 *Safety of protective bacterial culture*

Biopreservation bacteria must not represent a risk to human health. The first criterion is the detailed taxonomic knowledge of the strain. New sequencing tools enable excellent identification of bacteria and total genome sequencing is now easily accessible, allowing some important metabolic pathways to be specified in order to guarantee the safety of bacteria. Although not mandatory, the membership of the species to the QPS list (Quality Presumption of Safety) of the European Food Safety Agency (EFSA) facilitates its acceptance (EFSA Panel on Biological Hazards (BIOHAZ) 2022). Numerous lactic acid bacteria are listed, which often directs the selection towards this taxonomic group. Phenotypic and physiological knowledge is

also important, as well as data regarding their origin, their ability to colonize certain niches and their traditional use, or not, in food processing.

The antibiotic resistance profile is sought to avoid selecting PCs that would allow horizontal transfer of resistance genes, one of the critical public health issues identified by the World Health Organization. The physiological tests recommended by EFSA include the determination of minimum inhibitory concentrations (MICs) of several antibiotics which can be performed according to ISO 10932 (2010). However, acceptable MIC thresholds are not defined for all bacterial species. The study of a large number of strains for a given species may define an average resistance threshold, specific to the species. The *in silico* study of genes involved in antibiotic resistance completes the phenotypic tests and specifies if they are located on mobile elements (plasmids), generating a higher risk of transmission.

The production of toxic molecules such as biogenic amines is also a sought-after criterion. Histamine is a molecule responsible for allergic type syndromes and is regulated in some seafood products. The assays can be performed in liquid medium rich in histidine or directly in product. The presence of gene coding for histidine decarboxylase, detected by genome analysis or by specific PCR, now completes these analyses. The production of tyramine, which is not regulated, is sometimes a rejection criterion for some ferment producers because this molecule can be responsible for migraines, at very high concentrations (> 5000 mg/kg).

Although few toxicity studies of PCs are performed on animals, cell culture tests are sometimes performed. A first screening can be achieved by applying bacterial cultures or sterile filtered culture supernatant to Vero or CaCo2 cells. Lactate dehydrogenase (LDH) released from the cytosol of cells is an indicator of necrotic cell death when released. In addition, the integrity of the epithelial barrier in the presence of PCs can be measured by transepithelial electrical resistance on Caco-2 cell monolayers. Hemolytic activity is investigated by phenotypic tests on blood agar plates, the results of which depend on the conditions and the blood used. These tests can be complemented by genomic analysis of the presence of genes coding for hemolysins but also for other endo or exotoxins or virulence factors involved in adhesion, invasion or resistance to phagocytosis by lymphocytes.

5.3.2.5. *Technological skills*

To consider an industrial application, it is important that PCs can be easily produced in a fermenter and at a reasonable cost. Bacteria that grow well on simple culture media and at temperatures that do not require energy-intensive heating or refrigeration are obviously more interesting. The ability to grow on media with

vegetable rather than animal proteins can also be important for vegan productions for example. Moreover, some bacteria require a salt content that can damage the fermenters. The ability of PCs to withstand freeze-drying is also important. The strains are usually marketed as freeze-dried powder to be rehydrated and must be easily revived.

5.3.2.6. *Validation of results in naturally contaminated products*

The final step in the selection of PCs is the application in naturally contaminated foods. We have just seen that the effects of a PC can vary depending on the products to be treated, their composition and their endogenous flora. However, each plant has its own specificities (origin of the raw material, technological parameters of transformation, cleaning/disinfection process) and its own signature in terms of environmental microbiota. To validate the effect of PCs on spoilage, PCs are introduced into products recovered in the factory just after manufacturing, and the control is simply a non-biopreserved batch. During preservation, classical analyses are performed (counts, sensory fingerprints, physico-chemical measurements), and completed by omic methods to study finely the microbiota (metabarcoding, metagenomics) and the volatilome. Specific data processing tools are developed to link all these results.

The validation of the effect of a PC on pathogenic bacteria is more complicated to show. Their prevalence in seafood products is (fortunately) rarely sufficient to acquire statistically exploitable data. This is why the effect of PCs against pathogens, in the presence of endogenous flora, is assess in the laboratory co-inoculating the PC and the target in foods from the factory.

5.3.3. Examples of the application of protective cultures in seafood

The bacteria most used in food processing are generally lactic acid bacteria, which are difficult to implant in the fresh fish flesh. Because of the increase of lightly processed products consumption s such as salted, smoked, acidified, lightly cooked or preserved fish and shellfish, under protective atmosphere, importance of lactic acid bacteria has been highlighted and thereby most of the biopreservation examples of concern these products. Studies on the biopreservation of seafood products began in the 1990s, with a particular focus on the control of pathogenic bacteria, especially *L. monocytogenes.* It is only twenty years later that the first trials to combat organoleptic spoilage took place. There are many studies on the selection of PCs with antimicrobial activities, but we will only present here those that have led to product applications,

and more particularly those for which the use of live PCs is used (culture supernatants and bacteriocins pose a regulatory problem).

5.3.3.1. *Control of the Listeria monocytogenes risk*

The genus *Carnobacterium* probably has the most examples of application against *Listeria* sp**.** in seafood. This genus includes twelve species, two of which, *C. divergens* and *C. maltaromaticum,* are very frequently isolated from marine matrices. The percentage of strains with anti-listeria activity is much higher than in other lactic acid bacteria (Passerini *et al.* 2021). Beyond their antimicrobial properties, *Carnobacteria* are particularly interesting because they multiply well at low temperatures and resist the pH, salinity and packaging conditions (vacuum or modified atmosphere CO_2/N_2) encountered in seafood products, giving them an important ecological advantage. Their sensory acceptability depends on the strain and the food but *C. divergens* and *C. maltaromaticum* species are often quite neutral, not very acidifying and do not produce histamine.

Several authors have isolated *C. divergens* strains (V41, M35, etc.) that slow down or completely prevent the growth of *L. monocytogenes* in vacuum-packed smoked salmon or in cooked peeled shrimp tails packaged in a protective atmosphere throughout the product's shelf life. This effect has been validated on a large number of *L. monocytogenes* strains (Brillet *et al.* 2004, 2005; Tahiri *et al.* 2009; Saraoui *et al.* 2017). We find other numerous examples of *L. monocytogenes* control in smoked salmon and salmon gravlax by *C. maltaromaticum* strains (Nilsson *et al.* 1999; Vescovo *et al.* 2006; Wiernasz *et al.* 2020). The activity is often due to the production of class IIa bacteriocins. Also, the culture supernatant, even if it has an interesting listericidal activity just after its introduction into the food, often loses its efficacy over time. However, some carnobacteria also inhibit *L. monocytogenes* without any evidence of bacteriocin production. Nutritional competition for glucose could partly explain this phenomenon (Nilsson *et al.* 2005). As with all lactic acid bacteria species, the effect of the strains is matrix dependent. *C. maltaromaticum* CTC1741, for example, shows an inhibitory effect on *L. monocytogenes* in only one of the 3 batches of smoked salmon tested, with no explanation given (Aymerich *et al.* 2019).

While the anti-listeria effect of *Carnobacteria* is often proven, studies do not always take into account their impact on food quality and safety. Many strains of *Carnobacterium* sp. do not appear to affect the organoleptic properties of seafood, although they may produce mild buttery/caramel odors. However, other strains result in more unpleasant, chlorinated, malty, chemical or sour odors, enhanced in the presence of other species such as *B. thermosphacta* or *Vibrio* sp. (Joffraud *et al.* 2006; Leisner *et al.* 2007; Jaffrès *et al.* 2011). *C. divergens* V41, *C. maltaromaticum* V1,

SF668 and SF1944 selected for their anti-listeria activity do not degrade the quality of smoked salmon and salmon gravlax. They also do not produce histamine and tyramine concentrations, although varying between studies, rarely exceed 350 mg/kg (Brillet *et al.* 2005). Given the evidence of their benefits for seafood biopreservation and the minimal risk associated with tyramine production, some European and Canadian producers of ferments are already commercializing *Carnobacterium* ssp. strains and other producers are considering marketing them in the very near future.

Lactobacilli have also been extensively studied. Strains of *L. sakei* isolated from seafood have been shown to inhibit *L. monocytogenes* in smoked salmon (Katla *et al.* 2001; Weiss and Hammes 2006;. Lacumin *et al.* 2021). The bacteriocin-producing strain CTC494 has been robustly studied in numerous batches of vacuum-packed cold-smoked salmon, hot-smoked sea bass, and sea bream under protective atmosphere (Aymerich *et al.* 2019; Bolivar *et al.* 2021). The anti-listeria effect varies with the storage temperature profile and efficacy is optimal with an inoculum 10^4 *L. sakei/g*. Beyond that, the *L. sakei* strain is no longer sensorially acceptable. Other lactobacillus species (*L. casei*, *L. curvatus*, *L. plantarum*, *Lactiplantibacillus pentosus*, *Lactobacillus delbruecki*) show efficacy in various fresh or processed products.

Lactococci are widely used in the food industry, particularly *L. lactis* in dairy products. However, its mesophilic nature does not give it an ecological advantage in seafood, although a successful trial was conducted against *L. innocua* on cooked shelled shrimp (Hwanhlem *et al.* 2015). The species *L. piscium/carnosum*, on the other hand, is quite often found in processed fish. Two strains have been particularly studied and are still the subject of numerous works by INRAE/Oniris and Ifremer laboratories which have notably shown that the species does not present a risk *a priori* for human consumption. *L. piscium* is able to inhibit the growth of *L. monocytogenes* by 2 to 4 log (CFU/g) in cooked peeled shrimp stored under vacuum or protective atmosphere (Fall *et al.* 2010; Matamoros *et al.* 2009b). All *L. monocytogenes* strains tested are susceptible to *L. piscium* CNCM I-4031 and this strain also decreases the virulence of *L. monocytogenes* (Saraoui *et al.* 2018). Regardless of the PC/target ratios, *Listeria* growth arrest is only expressed when *L. piscium* has reached its early stationary phase (Saraoui *et al.* 2018). The mechanism of inhibition is not related to the production of antimicrobial peptides, organic acids, hydrogen peroxide, or other molecules excreted massively into the medium, nor to nutritional competition. Contact between the bioprotective strain and the pathogen is required to observe inhibition (Saraoui *et al.* 2016).

Other strains of *Leuconostoc gelidum*, *Pediococcus acidilactici*, and *Streptococcus phocae* species have also been shown to reduce the growth of *L.*

monocytogenes or *L. innocua* in shrimp, Indian sardinella, and smoked salmon, but the work is less complete.

5.3.3.2. *Control of other pathogens*

There is little work on the control of *Salmonella* or *S. aureus.* These pathogens are less often implicated in seafood-related illnesses. In addition, biopreservation is less suitable for the need of bacterial decontamination or complete elimination? than for a simple inhibition of growth. This is the requirement for *Salmonella* in some products, and even *L. monocytogenes* in the United States (zero tolerance). However, a study by Tasaku *et al.* (2017) presents the reduction of *Salmonella typhymurium* in Tilapia by-products by *Pediococcus pentosaceus* and of *S. aureus* in fresh whole shrimp by *L. rhamnosus* or *L. plantarum* and by *L. piscium* and *L. gelidum in* cooked peeled shrimp.

Oyster decontamination trials have also been conducted. The use of *L. casei, L. delbrueckii* and *Lactobacillus gasseri* in the purification baths resulted in a 2-log reduction in *V. parahaemolyticus* contamination. This pathogen was also reduced by 1 log when bacteriocin from a *L. lactis* subps. *lactis* strain was applied on Nile perch paté.

We have seen that histamine poisoning is linked to the production of histamine by bacteria, particularly *Enterobacteria*. Some authors have tried to select PCs capable of degrading histamine. However, waiting until the molecule is formed and then degrading it is not the most desirable strategy. It is better to prevent its production, for example by limiting the growth of the producing bacteria. There is a patent on the use of a strain of *L. sakei* that prevents the growth of *Morganella morganii* and *P. phosphoreum* in fresh vacuum-packed tuna, and very significantly decreases the concentration of histamine in the event of a cold chain break (Podeur *et al.* 2016).

5.3.3.3. *Improvement of the sensory quality*

As early as 1995, it was shown that lactic acid bacteria strains could increase the (sensory) shelf life of certain products (Kim *et al.* 1995; Leroi *et al.* 1996). However, the microbiota and metabolic interactions involved in spoilage are complex, so explanations and reproducibility of results are quite rare. A clear example is a strain of *Vagococcus pennaei* that significantly decreases organoleptic spoilage of gravlax salmon during preservation while it does not persist well ? on the product and little changes are observed on the composition of the endogenous microbiota (Wiernasz *et al.* 2020).

Many species (*L. casei, L. paracasei, L. curvatus, L. plantarum, L. lactis,* etc.) have been used to delay spoilage of vacuum-packed fresh salmon fillets (+15 days), hake and megrim on ice, swordfish fillets and tuna burgers (Passerini *et al.* 2021). The most extensive work is on shrimp. *C. divergens* V41, known for its anti-listeria activity, increases the use-by date of cooked peeled tropical shrimp tails packaged in a protective atmosphere by at least 10 days. One explanation is the inhibition of *Enterobacteria* and *Shewanella* sp. (Saraoui *et al.* 2017). *L. piscium* also increases the shelf life of cooked peeled shrimp by at least 15 days (Matamoros *et al.* 2009b). The remarkable effect could be explained by the inhibition of *B. thermosphacta* (Fall *et al.* 2010, 2012). Moreover, the combination of *C. divergens* V41 and *L. piscium* CNCM I-4030 both inhibited *L. monocytogenes* growth and increased the use-by date of cooked peeled shrimp (Saraoui *et al.* 2017). To our knowledge, there is at least one industrial application in France involving the use of a *L. gelidum* strain (LLO ferment, Biocéane company) for the preservation of peeled cooked tropical shrimp.

5.3.4. *Regulatory aspects*

The use of microbial food cultures (ferments) in food manufacturing is not specifically regulated in Europe. There is no legal definition of ferments, so the EFFCA (European Food and Feed Culture Association) has proposed the following: "Microbial food cultures are live bacteria, yeasts and moulds used for food production". Currently, traditionally used ferments are considered ingredients and must be clearly listed as such on the packaging. The precise name of the species does not necessarily have to be listed, and the terms "ferments" or "lactic ferments" are often used. The status of PCs used for preservation purposes is currently not clearly defined (ingredients or additives) but their use must be mentioned on the label.

Although there is no specific regulation, a food in which biopreservatives strains are added must meet, as any food, the conditions of the hygiene package and not be harmful to health or unfit for human consumption (Regulation (EC) No. 178/2002, 2002). The added bacteria must therefore meet the safety criteria listed in the previous paragraph. In 2007, EFSA introduced the concept of "Qualified Presumption of Safety" (QPS) of microorganisms used in food and feed. This system allows the safety assessment procedures of microorganisms to be harmonized within the different sample groups of EFSA but is not intended to evaluate all microorganisms. The evaluation is done on species for which sufficient knowledge is available to affirm that all strains are assumed to be safe. A list of species has been established and is regularly updated. However, strains that are not on the list can be used, either because they have not been the subject of any application, or because work at the strain level has proven its safety, even if knowledge at the species level is considered insufficient to put it on the QPS list. In addition, the International Dairy Federation (IDF) has

compiled an inventory of microorganisms with technological benefits in dairy products, now extended to other foods, based on extensive knowledge and bibliographic studies, and publishes a regularly updated list used worldwide.

The use of a PC for the biopreservation of food is therefore essentially based on its proven efficacy, its safety and the history of traditional use of this species in food without adverse health consequences, followed over several generations and on a wide range of populations. If this is the case, no application for pre-marketing authorization is required. If, on the other hand, the product is considered to be novel on the European market (i.e. not consumed to any significant degree in the Community before 14 May 1997), then the food must comply with Novel Food Regulation (EC) No 258/97. A novel food must undergo a pre-market assessment and an authorisation procedure including a risk assessment by EFSA and a risk management by the European Commission before it can be placed on the market.

In addition to the regulations, certain codes of practice and the specifications of products with a quality sign must be taken into account. In addition, for some foods, specifications may limit the use of PCs. In France, the AFNOR V45-065 standard for smoked salmon stipulates, for example, that only salt and sugar are authorized as ingredients. On the other hand, the use of PCs can lead to non-compliance with microbiological criteria relating to aerobic mesophilic flora and lactic flora (e.g. criteria of the *Fédération du Commerce et de la Distribution*, French Commerce, Services and Distribution Federation).

The United States also has no specific regulations for the use of ferments in food. The FDA (Food and Drug Administration) has introduced the GRAS (Generally Recognised as Safe) status which concerns microbial food cultures but also ingredients in the broad sense. Unlike QPS, GRAS status is granted for the use of a microorganism (a strain in a food) and not for a microbial species. Many ferments have been used in products for centuries and those with documented use prior to 1958 automatically have GRAS status. In other cases, a substantiated notification can be made to the FDA by a person or industry demonstrating GRAS status, and a receipt from the FDA stating that they have no objection is sufficient.

5.4. Conclusion

Biopreservation is a natural food preservation technique that meets the growing demand for safe, convenient and authentic food without chemical preservatives. It is consistent with the European objectives of promoting the "Clean label". Although industrial applications already exist for seafood products, certain regulatory and technological barriers remain. Biopreservation is indeed quite complex to implement since the selected microorganisms must meet the dual challenge of limiting

undesirable bacteria (pathogens, spoilers) and not degrading the quality of the product. A better understanding of bacterial interactions will therefore allow better control of its evolution during food preservation and more efficient biopreservation strategies to be proposed. Today, genome analysis of isolated bacteria or complex samples (metagenomics), as well as the numerous bioinformatics tools available, make it possible to predict the presence of genes coding for molecules involved in bacterial interactions. However, the predicted molecules largely exceed those expressed under laboratory conditions and probably active in the matrix. Therefore, accessing the *in situ* metabolic functionalities of microbial communities and studying the role of individual organisms in the microbiota remains a major challenge. New strategies such as metatranscriptomics should in the coming years lead to a better understanding of the dynamics of seafood microbiota.

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