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Microbial degradation of seafood

Leroi Françoise*, Joffraud Jean-Jacques

Ifremer, Département of Sciences et Techniques Alimentaires Marines, BP 21105, 44311, Nantes, France

* Corresponding author : Françoise Leroi, email address : fleroi@ifremer.fr

Abstract :

This chapter provides information on the microflora of living fish, contamination and bacterial growth during storage, specific spoilage microorganism concept, bacterial metabolism, spoilage of fresh fish and shelfish depending on storage conditions and spoilage of lightly preserved seafood product such as cold smoked salmon and cooked shrimp packed under modified atmosphere.

ABBREVIATIONS

DGGE: Denaturing gradient gel electrophoresis; **MAP:** modified atmosphere packaged; **TTGE:** temporal temperature gel electrophoresis; **TMAO:** Trimethyl amine oxide; **TMA:** Trymethyl amine ; **TVBN:** total volatile basic nitrogen

INTRODUCTION

The flesh of living fish is sterile. However, the skin, the mucus, the gills and the gastro-intestinal tract contain significant microflora. The composition of the bacterial community is determined to a large extent by the bacteria in the aqueous medium surrounding the fish when it is still in its larval stage (Hansen and Olafsen, 1999) and it thus varies according to a large number of hydrological parameters. The bacterial contents commonly observed vary from 10^2 to 10^5 bacteria/cm² in skin, 10^3 to 10^7 bacteria/cm² in gills and 10^3 to 10^5 bacteria/g in faeces (Abgrall, 1988). At the fish death, there is first a loss of freshness due to autolytic enzyme activity. Then microorganisms present in the fish can contaminate the flesh by moving through the muscle fibres or by spreading during the processing stages (gutting, head cutting, filleting, etc) and their activity leads to spoilage, characterised by off-odours, taste and degradation of texture.

This chapter is devoted to the bacterial degradation of flesh of fish and shrimp, the most consumed shellfish in the world. Molluscs are not dealt with here as the process of bacterial degradation does not usually begin before consumption and molluscs are most frequently eaten alive or immediately after being cooked.

THE MICROFLORA OF LIVING FISH AND SHELLFISH

It is generally agreed that the flora of fish found in temperate waters consist of Gram-negative psychrotolerant bacteria, whose growth is possible at 0°C but optimal around 25°C. Among these, the majority belong to the subclass γ of proteobacteria: *Pseudomonas*, *Shewanella*, *Acinetobacter*, *Aeromonas*, *Vibrio*, *Moraxella*, *Psychrobacter*, *Photobacterium*, etc. and to a lesser extent the CFB *(Cytophaga-Flavobacter-Bacteroides*) group (Huber et al., 2004; Wilson et al., 2008). Nevertheless, Gram-positive bacteria like *Micrococcus, Bacillus, Lactobacillus, Clostridium* or Coryneforms, may also be present in variable proportions (Shewan, 1971; 1977; Hobbs, 1983; Mudarris and Austin, 1988; Gram and Huss, 1996; Gennari et al., 1999; Wilson et al., 2008). Some genera, like *Vibrio, Photobacterium* and *Shewanella*, require the presence of salt to multiply and are thus typically found in seawater while *Aeromonas* is more common in fresh water even though it is often isolated from marine products (Hanninen et al., 1997). In tropical fish, the flora has the same composition overall (Al Harbi and Uddin, 2005; Emborg et al., 2005), but often with a greater proportion of Gram-positive bacteria (*Micrococcus, Bacillus*, Coryneforms) and enterobacteria (Devaraju and Setty, 1985; Liston, 1992; Huss, 1999).

The indigenous microflora of the gastro-intestinal tract of fish have been much more studied than those of the skin or the mucus due to their importance in digestion, nutrition and growth and in disease control in aquaculture (Ringo et al., 1995; Spanggaard et al., 2000). Although this environment is partially anaerobic, most researchers have observed a predominance of aerobic bacteria, also present in the surrounding water, capable of surviving and multiplying in the particular medium of the gastro-intestinal tract (Cahill, 1990). This predominance of aerobic bacteria could be due to the collecting techniques, which are not always suitable for strict anaerobes (Burr et al., 2005). Nevertheless, Huber et al. (2004) have shown by molecular methods that the aerobic flora of rainbow trout intestine usually represents 50 - 90% of the total flora. Gram-negative bacteria dominate the intestinal flora. In general, *Aeromonas*, *Pseudomonas* and members of the *Flavobacterium*/*Cytophaga* group are most often found in the intestine of freshwater fish while *Vibrio*, *Acinetobacter*, and *Enterobacteriaceae* are more common in marine fish (Ringo et al., 1995; Ringo and Birkbeck, 1999). These are fermentative bacteria that develop rapidly in the gastro-intestinal tract due to the low pH, the lack of oxygen and the abundance of nutrients. Staphylococci have also been found to be the dominant flora in the intestine of the Arctic char (Ringo and Olsen, 1999). Although not predominant, lactic acid bacteria *(Lactobacillus, Carnobacterium, Streptococcus, Leuconostoc, Lactococcus, Vagococcus*) have often been isolated from the gastro-intestinal tract of fish (Ringo and Gatesoupe, 1998).

Both the number and diversity of the microflora are probably widely underestimated because the majority of studies carried out so far have used classic microbiological methods involving growth on agar media. The cultivability of bacteria has been estimated to be sometimes less than 2% of intestinal flora rainbow trout (Huber et al., 2004), or even less than 0.01% of the skin flora (Bernadsky and Rosenberg, 1992). (Pond et al., 2006) have identified a strict anaerobe *(Clostridium gasigenes)* in the intestinal flora of rainbow trout. Similarly, Kim et al. (2007) have shown the presence of *Clostridium* in the intestinal mucus. Moreover, molecular methods have enabled a new species belonging to the genus *Mycoplasma* to be detected for the first time in fish. It was found in abundance in the intestine of wild and farmed salmon (Holben et al., 2002).

The worldwide shrimp market is mainly composed of the Nordic shrimp *(Pandalus borealis)*, which is only fished, and the tropical shrimp *(Penaeus* sp.), which can be fished or farmed and whose production has expanded rapidly in recent years. The deep-water tropical shrimp *(Parapenaeus longirostris)* is also found in Europe, particularly on the Spanish and Portuguese markets.

As in fish, the bacterial flora of shrimps depends on several factors including the species considered, the geographic location and environment, the temperature and salinity of the water, etc. However, overall, the same species of microorganisms are found in shrimps and in fish from a given geographical zone. In fresh tropical shrimps, the initial bacterial flora consists mainly of *Pseudomonas, Vibrio, Acinetobacter, Moraxella, Flavobacterium* and a high proportion of *Aeromonas* (Vanderzant et al., 1973; Jayaweera and Subasinghe, 1988; Jeyasekaran et al., 2006). In India, Gopal et al. (2005) have detected significant amounts of different species of *Vibrio*, including *V. parahaemolyticus*. Benner et al. (2004), working on Nicaraguan shrimps, reported a predominance of Coryneforms and *Moraxella* followed by lower levels of *Bacillus*, *Lactobacillus*, *Micrococcus, Proteus*, *Shewanella*, *Acinetobacter* and *Pseudomonas*. These results confirm those previously obtained by Matches (1982). Chinivasagam et al. (1996) have shown the influence of the fishing zone on

the nature of the initial flora: mostly Gram-positive bacteria on shrimps fished at low depths and *Pseudomonas* on those caught in deep water. The nature of this initial flora has an effect on the shelf life and the organoleptic properties of shrimps linked to spoilage

MICROBIAL CONTAMINATION OF FLESH AND ITS EVOLUTION DURING PRESERVATION

At fish death, the immune system collapses and bacteria can contaminate the flesh by moving through the muscle fibres. However, bacteria are found in much greater quantities on the skin than in the tissues and it is more likely that the spoilage of the whole fish is mostly due to bacterial enzymes that have spread through the tissues. On the other hand, the various stages of processing (evisceration, head removal, filleting, and trimming) contribute to spreading the bacteria naturally present in fish throughout the muscular tissue, thus accelerating spoilage.

Regarding the bacterial growth, first, there is a lag phase, generally short for fish found in temperate waters but whose length varies depending on the composition and state of the fish, the storage temperature and the species of bacteria. Next, the microorganisms begin a period of exponential growth and can reach level of 10^{6-8} CFU g^{-1}. Clearly, the rate of multiplication depends on the factors mentioned previously. Generally, tropical fish kept in ice have a longer lag phase (1 to 2 weeks) than that of fish found in temperate waters and growth during the exponential phase is slower (Gram et al., 1990; Gram, 1995), probably because the microorganisms cannot adapt well to low storage temperatures (Devaraju and Setty, 1985).

THE CONCEPT OF SPECIFIC SPOILAGE FLORA

Sensory spoilage is not always linked to the total number of microorganisms. In most seafood products, organoleptic rejection occurs well after the total flora has reached its maximum. Although the flora of fish is varied, only certain microorganisms, named “specific spoilage microorganisms” are responsible for the production of unpleasant odours and flavours.

In general, in unprocessed fish, the chemical modifications that lead to sensory rejection are due to only one bacterial species (Hozbor et al., 2006). In the case of processed products, however, the mechanisms are often more complex as several bacterial groups can interact and contribute to the spoilage of the product (Stohr et al., 2001; Joffraud et al., 2006). The specific spoilage flora and associated chemical molecules vary according to the species of fish, the storage temperature, the type of packaging or processing and even the fishing season

Identifying the microorganisms specifically responsible for spoilage is not easy. One method consists in analysing the products during storage (selective microbiological enumeration, sensory and chemical analyses) and establishing correlations between different parameters. However, the enumerations are not always selective enough and it is often more interesting to collect colonies from the total flora at the time of sensory spoilage and then check their capacity to produce bad odours in a pure culture. Tests done directly in the fish matrix always give more relevant results than using a laboratory medium or fish flesh extract (Truelstrup Hansen, 1995; Stohr et al., 2001) but are often more difficult to carry out as the fish must be sterilised without modifying its composition. The collections of fillets in aseptic conditions (Herbert et al., 1971) or low intensity ionisation (Joffraud et al., 1998; Jorgensen et al., 2000b) are the solutions most frequently used. Once the microorganisms potentially involved in spoilage have been identified, it is important to check their

colonisation kinetics on naturally contaminated products and during “real” storage conditions (temperature, packaging). Potential spoilage flora in a model medium may not have the capacity to develop enough to reach levels that could cause perceptible sensory deterioration (Jorgensen et al., 1988). Furthermore, interactions with endogenous flora could modify the spoiling characteristics of microorganisms (Gram and Huss, 1996).

BACTERIAL METABOLISM

Fish is a matrix that particularly favours microbial development. Despite a low percentage of carbohydrates (0.2 to 1.5% depending on the species), fish flesh is rich in non-protein, low molecular weight nitrogenous molecules that are rapidly metabolised by bacteria. These compounds include free amino acids, creatine, nucleotides, urea and trimethylamine oxide (TMAO). The high *post-mortem* pH (>6) and the low acidification during preservation, combined with the small quantity of carbohydrates present, enable the rapid growth of pH-sensitive psychrotrophic spoilage bacteria like *Shewanella putrefaciens*. Lastly, fatty fish are rich in polyunsaturated fatty acids that can be rapidly oxidised by either chemical chain reactions or lipolysis resulting from autolytic or bacterial enzyme activity.

In aerobiosis, the carbohydrates (ribose and lactate) can be metabolised into CO_2 and H_2O. In anaerobiosis, and in the presence of an electron acceptor such as TMAO, some microorganisms are capable of anaerobic respiration that leads to the production of acetic acid. However, spoilage of fresh fish is rarely linked to the production of organic acids due to the low concentration of carbohydrates in flesh. TMAO, whose concentration varies according to the species, plays an extremely important role in spoilage as certain microorganisms such as *Shewanella*, *Photobacterium* and *Aeromonas* reduced it to trymethyl amine (TMA), a pungent molecule responsible for the strong amine odour typical of rotten fish. TMAO is occasionally found in freshwater fish (Anthoni et al., 1990) but is generally associated with marine fish (Seibel and Walsh, 2002).

Urea found in large quantities in selachians can be metabolised into ammonia which also has a strong, unpleasant odour. Deamination of amino acids also leads to the production of ammonia.

The breakdown of sulphurous amino acids found naturally in fish leads to the production of hydrogen sulphide (H_2S) (from cysteine), methylmercaptan and dimethyl-disulphide (from methionine). These molecules all play a part in the spoilage process (Shewan, 1974; Lee and Simard, 1984). Certain microorganisms such as *Shewanella*, *Photobacterium* and lactic acid bacteria are capable of this production, but to an extent that varies depending on the strain.

The decarboxylation of amino acids leads to the formation of biogenic amines, which are often linked to spoilage (Veciana-Nogues et al., 1997) even though they have no particular odour in the product (Jorgensen et al., 2000a). Tyrosine is a precursor of tyramine and cadaverine. Arginine can be degraded into putrescine via the agmatine pathway in the presence of arginine decarboxylase, as is the case with *Photobacterium* (Jorgensen et al., 2000b). Putrescine can also be produced by the decarboxylation of ornithine, for example in the case of certain enterobacteria such as *Hafnia alvei* and *Serratia liquefaciens* (Grimont and Grimont, 1992; Sakzaki and Tamura, 1992). Histamine is a biogenic amine formed by the degradation of histidine. It can provoke allergic-like reactions of varying intensity (redness of the skin, swelling, headaches) and fish with high levels of histidine are strictly regulated (Scombridae and Clupeidae, CE regulation n° 2073/2005). In fish, the principal

producers of histidine are mesophilic enterobacteria such as *Morganella morganii*, *Hafnia alvei* and *Klebsiella pneumoniae* (Kim et al., 2002; Kim et al., 2004), but recently psychrotolerant microorganisms such as *Photobacterium phosphoreum* and *Morganella psychrotolerans* have been clearly incriminated in cases of histamine poisoning (Kim et al., 2002; Dalgaard et al., 2006).

SPOILAGE OF FRESH FISH AND SHELLFISH

Fish

Fish spoilage occurs at varying speeds depending on a large number of parameters. Different fish species display a variety of surface characteristics in terms of the resistance of their skin texture and the composition of the mucus, which may contain antibodies and bacteriolytic enzymes. Geographical zone, in particular water temperature, also plays an important role as fish from tropical waters keep much longer in ice than those from temperate or cold waters. Rough handling of the fish can damage its integrity and accelerate spoilage. The storage conditions (temperature and packaging) also play a vital part in fish preservation.

When temperate-water sea fish (like cod for example) are stored at chilled temperature, *Sh. putrefaciens* are the specific spoilage bacteria. Jorgensen et al. (1988) have shown that the number of bacterial cells is inversely correlated to the remaining shelf life, and consider that at approximately 10^8 CFU g^{-1} the sensory deterioration of the product is no longer acceptable. The spoilage is characterised by the production of TMA, H_2S and other sulphurous compounds such as methylsulphide and di-methylsulphide. *Sh. putrefaciens* consists of a heterogeneous group whose taxonomy has greatly changed over the last few years. Vogel et al. (2005) have shown that the large majority of microorganisms that produce H_2S isolated from Baltic Sea fish chilled for several days should now be identified not as *Sh. putrefaciens*, but as *Sh. baltica*. Furthermore, other minor strains have been identified as new species: *Sh. hafniensis*, *Sh. morhuae*, *Sh. glacialipiscicola* and *Sh. Algidipiscicola* (Satomi et al., 2006; Satomi et al., 2007). Tropical sea or freshwater fish are spoiled by *Pseudomonas*. *Sh. putrefaciens* has also been isolated from these products but does not appear to play an important part in spoilage. This could be due to the inability of this microorganism to develop in the presence of a large number of *Pseudomonas* (Gram et al., 1990; Gram and Melchiorsen, 1996). The spoilage caused by this microorganism can be differentiated from *Sh. putrefaciens* by the absence of TMA and sulphurous compounds and the appearance of fruity and rotten odours caused by aldehydes, ketones and esters. When fish is stored at room temperature, *Aeromonas* is more likely to spoil freshwater fish stored in aerobiosis, but it has also been shown that *Sh. putrefaciens* can be involved.

The Effects of Storage Temperature on Spoilage and Shelf life

Storage temperature is the most influential factor on shelf life. Ratkowsky et al. (1982) have described models that express the relation between relative spoilage speed and storage temperature. The relative spoilage speed at a temperature T is defined as the ratio of the shelf life at 0°C to the shelf life at T°C. For example, if the shelf life of a cod is 14 days at 0°C and 6 days at 5°, the relative speed of spoilage will be equal to 14/6=2.3, i.e. the spoilage will be 2.3 times faster at 5°C than at 0°C. Shelf life at 0°C differs depending on the species of fish and the method of preservation, but the effect of temperature on the relative spoilage speed *R* is constant and the following formula has been established: $\sqrt{R} = 1+0.1 * T°C$. This “square-root” model enables the shelf life of a product to be calculated at different temperatures if its shelf life is known at a certain temperature.

The bacteria responsible for the spoilage of fresh fish are different depending on the storage temperature. *Sh. putrefaciens*, *Pseudomonas* sp., *Aeromonas* sp. and *Ph. phosphoreum* are the principal spoilage bacteria found between 0 and 5°C, and their quantity varies depending on the storage atmosphere. At 15-30°C, *Enterobacteriaceae*, *Vibrionaceae* and Gram-positive bacteria are responsible for spoilage. The "square-root" model described previously does not take into account these changes in flora. Nevertheless, the estimations of relative spoilage speed are satisfactory for whole fresh fish, vacuum-packed fish or modified atmosphere packaged (MAP) fish (Gibson and Ogden, 1986; Dalgaard and Huss, 1997). However, for tropical fish, the relative spoilage speeds at 20-30°C are more than double those estimated by the model. A separate model for tropical fish has therefore been developed for temperatures between 0 and 30°C by (Dalgaard and Huss, 1997). It establishes a linear correlation between the Neperian logarithm of the relative spoilage speed and the storage temperature ($\text{Ln}\, R = 0.12 * \text{T°C}$).

Super-chilling consists in storing products at temperatures between 0 and -4°C. The "square-root" model gives satisfactory results for products stored in this way (Dalgaard and Huss, 1997). This technique can greatly extend the shelf life of the product but often lower the product quality (water retention, texture, etc.).

The Effects of Storage Atmosphere on Shelf life: Anaerobiosis and CO_2

Vacuum-packaging or MAP with varying amounts of CO_2 (25-100%) are both widely used to preserve food. Numerous studies have been carried out on seafood products with very different results. Most frequently, high concentrations of CO_2 lead to a 30 to 60% longer shelf life. The efficiency is closely linked to the temperature, which must be as low as possible so that the gas can dissolve in the product (Sivertsvik et al., 2002). However, unlike in meat products, these techniques do not significantly lengthen fish shelf life. In fact, although the number of *Pseudomonas* decreases due to the lack of oxygen in vacuum-packed temperate-water sea fish, *Sh. putrefaciens* can respire in anaerobiosis thanks to TMAO, and can develop up to 10^6-10^8 CFU/g, thus increasing the production of TMA (Gram et al., 1987; Jorgensen et al., 1988; Dalgaard et al., 1993). Nevertheless, it is probable that, below 10^8 CFU g^{-1}, *Sh. putrefaciens* is not the only microorganism responsible for fish spoilage as these authors have also identified the presence of *Ph. phosphoreum* on vacuum-packed cod. This microorganism, which had until recently escaped detection by microbiologists produces 10 to 100 times more TMA per cell than *Sh. putrefaciens*. Consequently, these two microorganisms can provoke the spoilage of vacuum-packed temperate-water sea fish and it is probably the initial quantity of each type that determines which one will dominate the flora. Furthermore, the growth rate of *Ph. phosphoreum* increases in anaerobiosis, which explains why this microorganism plays such an important part in the spoilage of packaged products such as cod (Dalgaard et al., 1993).

In the presence of CO_2, the growth of *Sh. putrefaciens* and *Pseudomonas* is greatly inhibited whereas *Ph. phosphoreum* is relatively resistant (Dalgaard et al., 1993; Dalgaard, 1995). It reduces TMAO to TMA without producing H_2S, which explains why MAP products are characterized by high levels of TMA without the odour of H_2S typical of spoiled fish stored in aerobiosis. *Ph. phosphoreum* can be eliminated if the raw material is first frozen, which increases the shelf life of thawed cod stored in a modified atmosphere at 2°C from 12 to 20 days (Guldager et al., 1998).

Numerous other seafood products have a shelf life similar to that of cod. As *Ph. phosphoreum* is widespread in the marine environment, it seems likely that this

microorganism, or others resistant to CO_2, are responsible for the spoilage of packed seafood products (Van Spreekens, 1974; Dalgaard et al., 1993). Indeed, Emborg et al. (2002) have shown that *Ph. phosphoreum* is the specific spoilage bacteria of MAP salmon. Hovda et al. (2007) have shown that Atlantic halibut *(Hippoglossus hippoglossus)* packed in a CO_2/O_2 (50/50) enriched atmosphere has a microflora dominated by *Ph. phosphoreum* and, due to the presence of oxygen, *Pseudomonas* sp. as well as *Brochothrix thermosphacta* on samples at the end of their shelf life. *Ph. phosphoreum* has also been found on pollock (Rudi et al., 2004) and tuna from the Indian Ocean *(Thunnus albacares)* (Emborg et al., 2005).

Ph. phosphoreum constitutes a heterogenic group whose taxonomy has evolved over the last few years. Some strains isolated from seafood products have characteristics that are not homogenous, notably in luminous and non-luminous strains. For this reason, some of the strains isolated from cod fillets in a modified atmosphere have been reclassified as *Ph. Iliopiscarium* (Ast and Dunlap, 2005).

The efficiency of MAP has also been demonstrated for warm-water fish. (Drosinos et al. (1997) and Drosinos and Nychas (1996) have identified *Br. thermosphacta* as the dominant species on sea bream packed in a CO_2-enriched atmosphere (40%) at the end of their shelf life. This microorganism could play a part in spoilage. Emborg et al. (2005) have demonstrated that, for tropical tuna steaks, a CO_2/O_2 (40/60) modified atmosphere is better than vacuum-packing for inhibiting the growth and histamine production of the psychrotrophic *Morganella*.

The combination of super-chilling and modified atmosphere enabled salmon fillets to retain a high level of quality after 24 days of storage due to the almost total inhibition of psychrotrophic bacterial growth (Sivertsvik et al., 2003). The shelf life is 3 to 4 times longer than that of a refrigerated product under air.

Shellfish

Amongst shellfish, Nordic and tropical shrimps are by far the most consumed products worldwide and this section will be wholly devoted to them.

Tropical shrimps *(Penaeus* sp.) are generally frozen shortly after catch and sold frozen before being processed (usually cooked) on land. Nordic shrimps *(Pandalus borealis)* are generally cooked after catch, sometimes peeled, and then frozen.

The few studies concerning this product have shown that shrimps are very sensitive to spoilage. This sensitivity is due to a relatively high pH (>7) and the fact that the intestinal tract containing enzymes and bacterial flora is not removed immediately after catch. In addition, the muscle contains large quantities of non-protein, water-soluble molecules such as amino acids. These amino acids are used directly by the bacteria for their growth. Shrimps generally contain large amounts of arginine and glycine and small amounts of cysteine and methionine (Chinivasagam et al., 1998).

Shelf life is variable and depends on the nature of the initial flora and the spoilage bacteria, often unknown, that may develop during storage. It can be relatively short: 5 to 8 days for ice-packed *Penaeus merguiensis* (Gonçalves et al., 2003); 6 days at 0°C for *Parapenaeus longirostris* (Mendes et al., 2005). Other authors have found longer shelf lives for tropical shrimps: 13 to 16 days when ice-packed (Cann, 1974; Jayaweera and Subasinghe, 1988; Shamshad et al., 1990); 10 to 17 days when ice-packed or over 20 days when stored in liquid ice for different species of the genus *Penaeus* found in Australian waters (Chinivasagam et al., 1996).

The major cause of early shrimp spoilage is melanosis, which is the formation of black spots on the cephalothorax. This process is biochemical and is not due to bacterial activity, but rather an enzyme complex called polyphenol oxidase. The benzoquinones thus produced interact with amines, amino acids and oxygen to form the melanin responsible for this black coloration.

Other organoleptic changes are generated by the action of bacteria. Chinivasagam et al. (1996) have shown that when tropical Australian shrimps are ice-packed in an oxygen-rich environment, the growth of *Pseudomonas fragi* increases, whereas using liquid ice where oxygen is limited leads to the development of *Sh. putrefaciens*. The sensory characteristics of contaminated products are very different depending on the predominance of one or the other of these bacteria. To confirm these observations, Chinivasagam et al. (1998) have demonstrated experimentally that strains of *Ps. fragi* inoculated into shrimp juice produce odours of fruit and onion, whereas strains of *Sh. putrefaciens* give off a sulphurous odour. Moreover, these two bacteria display different profiles of volatile compounds.

Packaging fresh shrimps *(Parapenaeus longirostris)* in a modified atmosphere combining 40- 45% CO_2 with 30 or 5% O_2, respectively, delays microbial growth and the production of TVBN and TMA compared to air-packed or iced stored shrimp, especially at the end of storage (López-Caballero et al., 2002). Amine production and the low level of H_2S-producing microorganisms and enterobacteria suggest that *P. phosphoreum* could be involved in the spoilage of shrimps both in air and MAP. The production of biogenic amines (tyramine, putrescine, cadaverine, agmatine) during storage is higher for atmospheres with modified O_2 concentrations than for air. (Gonçalves et al., 2003) have shown that, for the same species of shrimp and using the same gas compositions, shelf life can be prolonged at least by 2 days (9 days instead of 4 to7 days with the ice storage). This type of packaging can also extend the shelf

life of cooked whole shrimps *(Pandalus borealis)* by 200% compared to air-packed products (Sivertsvik et al., 1997).

SPOILAGE FLORA OF LIGHTLY PRESERVED PRODUCTS

Semi-preserved seafood products have undergone a very light preservation process, such as salting, drying, smoking or marinating, leading to a final pH greater than 5 and a salt content below 6% in the aqueous phase. Examples include smoked fish, carpaccio of salmon or trout, gravelax, marinated anchovy fillets, cooked products like peeled shrimps, preserved in brine or in a modified atmosphere etc. In terms of microbiology, these products are very fragile. Because the raw material follows a long preservation process with a good deal of handling, there is a significant risk of recontamination. Moreover, no step of the process involves the total elimination of microorganisms and the final product is often preserved for several weeks at low temperature before being eaten. This allows some psychrotrophic bacteria, which can spoil organoleptic qualities or present a risk to human health, to multiply.

We shall deal with two examples representative of this product category, cold-smoked salmon and cooked peeled shrimps, due to their great economic importance in the European market.

Cold-smoked Salmon

In France, there is a long tradition of salting, drying and cold-smoking seafood products, mainly salmon and herring. Nowadays, in the industrialised countries, the aim of smoking is not so much to ensure a long shelf life for the product but to give it a particular colour and taste. With more than 20,000 tonnes produced each year, France has always been the world leader in the production and consumption of smoked salmon. It was originally a luxury product, eaten only on special occasions and made by traditional methods. In recent decades, however, smoked salmon has become an everyday food, available in supermarkets all year round and produced on an industrial scale.

The production of smoked salmon involves the different steps of filleting, salting, drying and smoking at low temperatures (< 25°C) but each plant has developed its own technology: salting by dry salt or by injection, smoking with different wood essences and different smoke generators etc (Duffes, 1999; Sérot et al., 2004; Knockaert, 2005; Cardinal et al., 2006). The final salt concentration is usually between 2.5 and 3.5% in the flesh while the smoke content, estimated by the quantity of total phenols, is between 2 and 20 ppm. These products are generally sold sliced and vacuum-packed. The producer is responsible for the use-by date, which is usually three to six weeks at 4°C in Europe. However, it is not uncommon to notice deterioration in taste from the end of the second week of preservation (Leroi et al., 2001; Cardinal et al., 2004).

It has been clearly shown that this organoleptic spoilage is mainly due to microbial activity, with autolytic or chemical reactions of lipid oxidation playing a lesser role (Joffraud et al., 1998). For a very long time, however, the mechanisms of microbial spoilage of smoked fish were poorly understood. Although lactic flora have always been found to be dominant in this product (Magnusson and Traustadottir, 1982; Hildebrandt and Erol, 1988; Civera et al., 1995), their role in spoilage was not clear because no correlation between their number, nor even that of the total flora, and the sensory deterioration could be identified (Rakow, 1977; Cann et al., 1984; Hildebrandt and Erol, 1988; Dodds et al., 1992; Huss et al., 1995; Truelstrup Hansen,

1995; Gram and Huss, 1996). More recent studies have specified the composition of the flora of these products and their link with spoilage.

On leaving the factory, product contamination can range from 10^2 to 10^6 bacteria/g, the initial level varying mainly according to the factory and its hygiene standards and independently of the origin and state (fresh or frozen) of the raw material (Truelstrup Hansen et al., 1998; Leroi et al., 2001). Despite the presence of inhibitory factors like salt, smoke, preservation at low temperature and vacuum-packaging, bacterial growth in these products can be quite rapid. It is not unusual to observe total flora concentrations of 10^6 CFU/g from the first week of storage, which can regularly reach 10^{7-9} CFU/g before the use-by date (Cardinal et al., 2004; Dondero et al., 2004; Espe et al., 2004).

The initial flora of smoked salmon is often dominated by Gram-negative bacteria typical of fresh fish flora, such as *Shewanella, Photobacterium,* and *Aeromonas*, later identified as *Serratia* (Leroi et al., 1998). Nevertheless, as well as *Photobacterium*, Olofsson et al. (2007) have also found *Brochothrix*, *Yersinia* and *Carnobacterium.* During vacuum-packed preservation at low temperatures, the diversity of genera decreases and Gram-positive bacteria, especially lactic acid bacteria, very often become predominant. Variations between factories (Leroi et al., 2001) and even between batches from the same factory (Truelstrup Hansen et al., 1998) have often been observed but the lactic flora always seems to dominate, regardless of the geographical provenance of the products analysed (processed in Europe). According to the authors, 50 to 90% of the colonies taken from the total flora of samples at the end of preservation are lactic acid bacteria (Leroi et al., 1998; Truelstrup Hansen et al., 1998). The technological parameters of salting and smoking can influence the final proportion of this group of bacteria (Leroi and Joffraud, 2000) so it should be possible to control the level of lactic flora by varying these parameters (Tomé et al., 2007).

In smoked salmon, the major species are *Carnobacterium maltaromaticum* and *Lactobacillus curvatus* or *Lb. sakei*. Other species such as *Carnobacterium divergens, Lactobacillus farciminis, Lb. alimentarius, Lb. plantarum, Lb.homohiochii, delbrueckii, Lb. casei, Lb. coryneformys, Leuconostoc mesenteroides, Enterococcus faecalis* and *Weisella kandleri* are more rarely isolated (Leroi et al., 1998; Truelstrup Hansen et al., 1998; Jorgensen et al., 2000b; Gonzales-Fandos et al., 2004; Rachman et al., 2004). Despite this predominance, quite often the number of enterobacteria (*Ph. phosphoreum* and *Br. thermosphacta*) is also fairly high (Truelstrup Hansen et al., 1998; Jorgensen et al., 2000a; Jorgensen et al., 2000b; Rachman et al., 2004). In contrast, yeasts are rarely present, except sometimes when the product contains a high concentration of salt or phenol (Leroi and Joffraud, 2000). Nevertheless, they do not reach high enough population levels to contribute to spoilage. To illustrate the complexity of the flora of smoked salmon, Gonzales-Fandos et al. (2004) have shown that of 96 isolates coming from 30 batches of smoked salmon in Spain and preserved for 3 weeks at 2°C, 49% were lactic acid bacteria, 20% enterobacteria, 16% micrococci (mostly coagulase-negative staphylococci), 5% *Br. thermosphacta*, 5% Gram-negative *(Moraxella, Acinetobacter and Pseudomonas),* 2,5% mobile *Aeromonadaceae* and 2,5% *Bacillus.*

Recently, temporal temperature or denaturating gradient gel electrophoresis (TTGE, DGGE) molecular methods have been applied to smoked salmon. In some cases, these techniques have given similar results to the culture method

(predominance of *Lactobacilli*, *Ph. phosphoreum* and *Ph. iliopiscarium* at the end of preservation (Olofsson et al., 2007). In contrast, (Cambon-Bonavita et al., 2001) only found clones corresponding to Gram-negative bacteria *(Vibrio*, *Photobacterium*, *Enterobacteriaceae*, *Alteromonas)* and assumed that this technique is biased because it does not allow a good amplification of the DNA of Gram-positive bacteria. Nevertheless, in both studies, clones no doubt corresponding to new species of *Photobacterium* and *Vibrio* were identified by the culture-independent technique, underlining how these tools can complement classic culture methods.

The role of lactic acid bacteria in the sensory deterioration of smoked salmon is still not very clear. Several authors have shown that there is no correlation between the total lactic flora and sensory spoilage. Paludan.Müller et al. (1998), however, were able to prolong the shelf life of smoked salmon by inhibiting lactic acid bacteria with nisine (bacteriocin), suggesting a possible spoilage effect by this bacterial group. By inoculating cubes of smoked salmon sterilised by ionisation, Stohr et al. (2001) clearly demonstrated that certain species of lactic acid bacteria were very spoiling (i.e. *Lb. sakei)* while others had no effect at all (i.e. *Lb. alimentarius).* However, the potential for spoilage seems to vary according to the strain tested. *Lb. sakei* generally produces sulphurous and acidic odours (Truelstrup Hansen et al., 1995; Nilsson et al., 1999; Stohr et al., 2001), associated with the production of H_2S, acetic acid, ethyl and *n*-propyl-acetate (Joffraud et al., 2001), but some *Lb. sakei* do not spoil the organoleptic qualities of this product (Weiss and Hammes, 2006). Similarly, *Lb. alimentarius,* which does not spoil smoked salmon, has been identified as the bacteria responsible for the sensory deterioration of marinated herrings (Lyhs et al., 2001). The role of Carnobacteria is still under discussion (Laursen et al., 2005; Leisner et al., 2007). Many studies have shown that they are probably not very spoiling in smoked salmon. Inoculation by different strains of *Cb. maltaromaticum* and *Cb. divergens* results in no or few changes in organoleptic qualities (Leroi et al., 1996; Paludan.Müller et al., 1998; Duffes, 1999; Nilsson et al., 1999). When the Carnobacteria reach a sufficient level, odours of butter and plastic may be detected, associated with the production of 2, 3-butanedione (diacetyl) and 2,3-pentanedione (Joffraud et al., 2001; Stohr et al., 2001), but these odours/flavours do not lead the product to be rejected by a specialist jury (Brillet et al., 2005) and thus even less so by a jury of consumers.

Among the other bacteria frequently found in smoked salmon, Stohr et al. ()2001) showed that *Se. liquefaciens* was very spoiling, releasing odours of amines, cheese, acid or rubber, associated with the molecules TMA, dimethyldisulphur, 2,3 butanediol and 2-pentanol (Joffraud et al., 2001). However, *Se. liquefaciens* is considered less spoiling than *Lb. sakei* as the unpleasant odours are perceived much later (Joffraud et al., 2006). *Br. thermosphacta* also leads to the sensory rejection of the product due to the odours of blue cheese and plastic, well correlated with 2-heptanone and 2-hexanone. Nevertheless, it is quite rare for these bacteria to reach sufficiently high levels in naturally contaminated products to be the sole explanation for sensory rejection. Although strongly spoiling in fresh fish packed in a modified atmosphere, *Ph. phosphoreum* seems to play a more moderate role in the deterioration of smoked salmon. Weak odours of “acid”, “amine” and “feet” result in the product being judged as moderately spoiled (Joffraud et al., 2006). Moreover, there is a great variability according to the strain (Leroi et al., 1998; Stohr et al., 2001). Jorgensen et al. (2000b) give much greater weight to the spoiling action of this species. They have shown a good correlation between the sensory quality of smoked salmon and the

production of tyramine and histamine (Jorgensen et al., 2000a), two potential chemical indicators for spoilage (see below). *Sh. putrefaciens,* the most common spoilage bacteria in fresh fish, and *Vibrio* sp. have never been implicated in spoilage phenomena in smoked salmon, even when inoculated at high concentrations.

Although the bacteria responsible for sensory deterioration are now quite well identified, spoilage remains a complex phenomenon because the interactions between all these bacterial groups change their metabolism. For example, when *Lb. sakei* is co-inoculated with *Se. liquefaciens*, spoilage is clearly delayed. Conversely, when *Cb. maltaromaticum* and *Vibrio* sp. are both present (two non-spoiling bacteria in pure culture) spoilage is increased significantly (Joffraud et al., 2006). (Brillet et al., 2005) noted that *Cb. divergens* did not produce TVBN in pure culture in sterile smoked salmon but some TVBN was detected when the bacterium was added to commercial samples containing a natural endogenous flora. Some phenomena of metabolic interaction between lactic acid bacteria and enterobacteria have been explained by Jorgensen et al. (2000b). For example, a high production of putrescine (>200 µg/g) cannot be due to the simple degradation of ornithine, whose concentration does not exceed 10 µg/g in salmon flesh. Arginine deaminase-positive lactic acid bacteria like *Cb. divergens* and *Lb. sakei*, metabolise arginine, found in greater quantity in salmon, into ornithine, which is then converted to putrescine by ornithine decarboxylase-positive enterobacteria *(Se. liquefaciens* and *Hafnia alvei).*

All these results show that it is over-optimistic to expect to predict the quality of lightly preserved seafood products using only one microbiological or biochemical parameter. Not only is the microflora very complex but also the organoleptic properties of spoilage are very varied, with odours that can be described as amine, sulphur, acidic, sour or “cabbage”. Some authors have succeeded in improving the organoleptic quality of smoked salmon by modifying the technological parameters, like the salt, the smoke or the addition of bioprotective bacteria, but have not demonstrated the inhibition of a particular group of bacteria (Leroi et al., 1996; Leroi et al., 2000). The situation is not the same in fresh fish, preserved in air or in a modified atmosphere or vacuum packed, where one or two well identified species dominate the flora and for which a predictive model of sensory spoilage has been suggested (Dalgaard et al., 2002). However, in the case of smoked salmon, a multiple approach has enabled some authors to correlate quality to several microbiological and biochemical measurements. For example, Leroi et al. (2001) has put forward a predictive model for the remaining shelf life of smoked salmon, based on TVBN content and enumeration of the flora on Rogosa medium at pH 5.5 (quite selective for lactobacilli). If the TVBN content is less than 35 mg N/100 g of flesh, the product is not spoiled. If it rises above this level, the number of bacteria on Rogosa medium must be considered. A product with 50 mg N/100 g of TVBN will only be spoiled if the number of colonies on Rogosa is higher than 10^5 CFU/g. Another model, based on pH and the quantity histamine and tyramine, has been developed by Jorgensen et al. (2000a).

Cooked Shrimps Packed in a Modified Atmosphere

France is the second biggest importer of shrimps in Europe, and this product corresponds to the highest value of all seafood imported. Tropical shrimps account for 80% of the shrimps imported. Refrigerated cooked ready-to-eat shrimps represent the largest shrimp consumption in France. They may or may not be peeled, are often packed under a CO_2-enriched protective atmosphere, and can also pass through brine containing salt and different organic acids (citric, ascorbic, benzoic, etc.). Thanks to

increasing consumption, the shrimp market has been steadily expanding over the last ten years.

In the literature to date, knowledge of this product's microbiology has focused on the Nordic shrimp *(Pandalus borealis)*. The flora of fresh shrimps is greatly reduced during the cooking stage of processing (70 – 80°C throughout) and the spoilage flora is seemingly the result of a recontamination by Gram-positive bacteria before or during packaging. The microorganisms are then selected according to the different characteristics of the product and the storage conditions. More specifically, the shelf life and development of the microflora depend on the initial contamination, the characteristics of the product (brined or not for example) and the storage conditions (modified atmosphere, temperature). The combined effect of these different parameters could create a barrier effect against microbial development.

Brined Shrimps

From and Huss (1990) carried out one of the first studies on brined Nordic shrimps. The spoilage microflora was dominated at 5°C by yeasts and lactic acid bacteria *(Streptococcus* sp., *Lactobacillus* sp.). Another study of brined Nordic shrimps by Einarsson and Lauzon (1995) demonstrated the importance of benzoate and sorbate in preventing the development of flora at 4.5°C. When the flora is fully developed, it is dominated by coryneform bacteria and *Moraxella* sp.

Dalgaard and Jorgensen (2000) studied Nordic and tropical shrimps *(Penaeus* sp.) that had been brined, drained, packed in a modified atmosphere and stored at different temperatures between 0 and 25°C. They showed that the effect of temperature on shelf life is even greater than with other seafood products. For example, batches at 8°C had a shelf life 15 to 33 times longer than those stored at 25°C. Certain variations in the composition of the product, no matter how small, can have a large effect on this shelf life. Thus, shelf life at 5°C is over three months for tropical shrimps containing 2.3% salt in an aqueous solution and over 6 months for Nordic shrimps with 3.3% salt. The initial pH of these products is between 5.7 and 5.9. TVBN levels are relatively low when the products are spoiled (20 mg/100g at 15 and 25°C and 10 mg/100g at the end of shelf life for tropical shrimps stored at lower temperatures). Most of the spoilage flora has not been identified, but lactic acid bacteria make up an important part of the total microflora, whatever the storage temperature. Amongst these bacteria, several strains isolated from products stored at 15 and 25°C have been identified as *Enterococcus faecalis*. This microorganism is probably responsible for spoilage at these temperatures, but does not appear to develop much, if at all, at temperatures between 0 and 8°C. The authors also observed the presence of *Lb. curvatus* on tropical shrimps stored at 0°C.

In continuing their studies on the flora isolated from these products, Dalgaard et al. (2003) observed an evolution of the microflora from bacilli to cocci between 5 and 8°C for Nordic shrimps and between 8 and 15°C for tropical shrimps. At 25°C, the flora is dominated by cocci. Most of the strains isolated at different temperatures have been identified as *Carnobacterium (Cb. divergens)* and *Enterococcus (En. faecalis)*. *En. faecalis* is present on products stored at 15°C and higher whereas *Cb. divergens* and *Lb. curvatus* are found on products stored at 8°C and lower. Three of the isolated strains correspond to an unknown *Carnobacterium* species. At high concentrations, *En. faecalis* and *En. durans* could represent a health risk for consumers. In this study, tyramine may represent a chemical indicator of spoilage, but is at levels low enough not to present a risk. The authors recommend storing the shrimp at low temperature and with at least 3% salt (water phase) so as to avoid spoilage problems and health risks.

Mejlholm et al. (2008) have studied Nordic shrimps that have been either brined or brined and drained then packed in a modified atmosphere. They focused on the effect of hygiene (during industrial or manual processing), the composition of the brine and the storage conditions (atmosphere and temperature) on the overall evolution of the microflora and the shelf life. Different groups of organic acids were tested: benzoic, citric and ascorbic or acetic, citric and lactic, including the effect of diacetate. The pH of brined shrimps is between 5.6 and 5.8. The shelf life depends on the nature of the organic acids, their concentration, the temperature and also the initial contamination, which seems to have a significant effect. In fact, industrial products are more contaminated than products processed manually, which leads to shorter shelf lives and a more diverse spoilage flora. This is especially true for brined and drained MAP shrimps, which have a shelf life of over 75 days at 7°C for manually processed products, but only 28-35 days for industrial products. Similarly, shrimps in brine composed of acetic, citric and lactic acid have shelf lives of 69-84 days and 42-49 days for manual and industrial products, respectively, at the same temperature. The modified atmosphere seems to prolong the shelf life of shrimps from 53-60 days for brined shrimps to over 75 days for the same shrimps that have been drained and packed in a modified atmosphere, although these results were not confirmed by subsequent tests.

The composition of the brine influences the nature of the flora that will develop on the shrimps. When it contains benzoic, citric and sorbic acid, the microflora is dominated by *En. faecalis*-like, *Leuconostoc pseudomesenteroides*, coagulase-negative *Staphylococcus*, yeasts, *Ps. fluorescens, En. malodoratus*, *Cb. maltaromaticum* and *Lb. sakei.* Adding diacetate to the brine can inhibit the growth of *Ps. fluorescens,* coagulase-negative *Staphylococcus* sp. and *Enterococcus* sp. When the brine contains acetic, citric and lactic acid, the microflora is composed exclusively of yeasts and lactic acid bacteria, such as *Lb. sakei,* which are responsible for unpleasant sour and buttery odours. When the yeasts dominate the flora, 10^6 CFU/g is enough to spoil the product.

Whatever the composition of the brine at the start of the process, the spoilage microflora of brined and drained and MAP shrimps is dominated by *Lb. sakei*. Spoilage of the industrial product leads to sour flavours and a release of gas in the packaging, probably produced by *Lb. sakei*.

The spoilage of some brined shrimp samples with a reduced microflora is due to oxidation rather than microbial activity.

Shrimps without Brine

Cooked, peeled, frozen or unfrozen Nordic shrimps without brine and packed in a modified atmosphere (50% CO_2) have an initial pH of 7.7, a salt content of 1.9% in aqueous solution and a TVBN of 10mg/100mg (Mejlholm et al., 2005). If the product is frozen for 4 months preceding refrigerated storage then the initial concentration of microorganisms will be reduced, but the rate of growth, the maximum level of flora and the shelf life remain the same. The latter is 26 days at 2°C, 16 days at 5°C and 10 days at 8°C. A low production of TVBN (lower than 20mg/100g) and no production of TMA, organic acids or biogenic amines were observed. From 116 isolates, 60% were identified as *Cb. maltaromaticum*, 27% were identified as *Br. thermosphacta* and 13% were identified as *Psychrobacter* sp (Mejlholm et al., 2005). When inoculated together, *Cb. maltaromaticum* and *Br. thermosphacta* produced the same unpleasant odours as spoiled products that have been contaminated naturally. This is not the case when the two products are inoculated separately. The mechanisms of this interaction have not yet been explained. The presence of *Br. thermosphacta* has

already been observed in MAP fish. Furthermore, this microorganism has already been described as producing odours of butter, blue cheese and feet. *Cb. maltaromaticum* produces an unpleasant chlorine odour whereas in previous studies this bacterium was described as non-spoiling only producing a buttery odour. *Psychrobacter* sp. does not seem to play a part in the spoilage of cooked shrimps.

The potential for spoilage by *Carnobacterium* on cooked, peeled, MAP Nordic shrimps without brine varies depending on the species and the strain (Laursen et al., 2005). *Cb. divergens* and some *Cb. maltaromaticum* produce unpleasant odours whereas another group of *Cb. maltaromaticum* is not spoiling. Spoilage bacteria produce ammonia, tyramine, different alcohols, aldehydes and ketones. These authors have confirmed that *Br. thermosphacta* and *Cb. maltaromaticum* cultured together lead to some bad odours (wet dog) that are not produced when these species are grown separately. However, this association does not produce new metabolites and consequently the "wet dog" odour does not come from a metabiotic phenomenon in which a bacterium produces a metabolite from a substrate formed by another bacterium. On the other hand, *Cb. maltaromaticum* decreases the formation of diacetyl by *Br. thermosphacta* and the latter reduces the activity of *Cb. maltaromaticum*. The "wet dog" smell may be due to the interaction between metabolites formed by *Cb. maltaromaticum*, *Cb. divergens*, and partly by *Cb. mobile*, and those formed by *Br. thermosphacta*. The presence of oxygen increases the potential for spoilage and the number of metabolites produced by *Br. thermosphacta*. In fact, *Br. thermosphacta* can be aerobiotic or anaerobiotic depending on the respective percentages of O_2 and CO_2. To reduce the potential for spoilage in shrimps, it is therefore recommended to use an atmosphere with less O_2 and more CO_2.

In contrast to cooked, peeled, MAP shrimps without brine, in which the specific spoilage bacteria seem well defined (Mejlholm et al., 2005; Laursen et al., 2006), the characteristics of the spoilage flora of brined or brined/drained and MAP shrimps are not as clearly established. The composition of this spoilage flora depends on a large number of parameters: the initial contamination, the preservation parameters (organic acids and salt) and the storage conditions (atmosphere, temperature).

CONCLUSION

The susceptibility of fish and shrimp flesh to spoilage can be explained by physico-chemical characteristics (neutral pH, high concentration of low molecular weight nitrogenous compounds, etc.) which favour microbial growth. For most unprocessed products, the specific microorganisms responsible for sensory degradation have been identified and the influence of storage parameters such as the temperature and the storage atmosphere are well documented. Predictive models for spoilage and some other indicators are available (Seafood Spoilage and Safety Predictor: www.dfu.min.dk/micro/sssp/). The spoilage mechanisms for lightly preserved products are much more complex as they must take into account several groups of interacting bacteria. It is therefore necessary to continue research in this field to better understand these phenomena with the objective of providing relevant quality indicators, predictive models of storage and effective methods of preservation.

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