***In* Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation**

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Applications of protective cultures, bacteriocins and bacteriophages in fresh seafood and seafood products

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Chapter content :

Abstract:

Microbial seafood-borne disease represents 10 to 20% of the total food-borne outbreaks. Most of them are from bacterial origin and involve seafood products that have been contaminated by pathogenic *Vibrio, Listeria monocytogenes* and histaminogen bacteria. On the other hand, seafood products are very sensitive to the development of spoiling bacteria producing off-odours. Pathogenic and spoiling microflora are not always reduced or limited by the processing steps that are currently used in these foodstuffs, and the interest for alternative techniques such as bioprotection to improve quality and safety of seafood has increased in the last years. Among the microbial flora of lightly preserved seafood products, lactic acid bacteria usually become dominant during the storage under vacuum or modified atmosphere. In some cases these bacteria are responsible for spoilage but some of them have demonstrated potential for pathogenic or spoiling microflora inhibition. Those bacteria as far as some bacteriocins have been tested, mainly for the control of *Listeria monocytogenes* in cold smoked salmon and in a lesser extend in other products to enhance sensory shelf-life. Many successful results have been obtained at the laboratory scale, nevertheless, the application in seafood industry is still limited.

Keywords: Biopreservation of seafood, seafood safety and spoilage, lactic acid bacteria, sensory quality, *Listeria monocytogenes.*

1. Introduction

Fishery products contribute to a huge source of valuable nutrients such as proteins, vitamins, minerals, omega-3 fatty acid, taurine etc. However, they are also responsible for human intoxication and infection, and 10 to 20 % of the food-borne illnesses are attributed to fish consumption. Aetiology of seafood is not always known but it is clear that indigenous bacteria present in marine environment as well as the result of post contamination during process are responsible for many cases of illnesses.
In the last years, the traditional processes applied to seafood like salting, smoking and canning have decreased in favour of mild technologies involving lower salt content, lower cooking temperature and vacuum (VP) or modified atmosphere packing (MAP). These products designed as lightly preserved fish products (LPFP) are usually produced from fresh seafood and further processing increases risk of cross contamination. The treatments are usually not sufficient to destroy microorganisms and in some cases psychrotolerant pathogenic and spoiling bacteria can develop during the extended shelf-life of LPFP (usually up to 10 days). As several of these products are eaten raw, minimising the presence and preventing growth of microorganisms is essential for the food quality and safety.
The microbial safety and stability of food are based on an application of preservative factors called hurdles. Most of the decontamination technologies such as the oldest one, cooking, and more recent mild technologies i.e. pulsed-light, high pressure, ozone, ultrasound… are not efficient or not compatible with the delicate texture and flavour of seafood. Chemical preservative have also been used but consumers require more natural products with lower chemical treatment. An alternative solution that is gaining more and more attention is the biopreservation technology (Dortu and Thonart, 2009; Calo-Mata *et al.*, 2008; Rodgers, 2001). It consists in inoculating food with microorganisms, or their metabolites, selected for their antibacterial properties. Lactic acid bacteria (LAB) are generally good candidates as some of them show natural capacities to inhibit growth of microorganisms and because they are naturally present in many food products and eaten for years by human without any safety risk. LAB have complex nutritional requirements and in order to obtain a good implantation, it is generally the strategy to use bacteria isolated from the food that has to be preserved. LAB in fish flesh has long been disregarded because they are not currently present in seafood. However, in some environmental conditions, for instance in lightly processed seafood with salt, smoke, vacuum or modified atmosphere packaging, LAB can become dominant. Their occurrence and role vary according to fish and bacterial species. In some cases, they may be responsible for strong off-flavours and degradation of texture that unable their use as food preservative. In many other cases they do not change the organoleptic characteristics of the products and their use as protective culture could offer an alternative to the use of chemical compounds. However, the knowledge of LAB from seafood is still in its infancy, explaining why their use by human for preservation or transformation of marine products is still limited.
After a description of the microbial risks associated with seafood, this chapter presents the particular position of LAB among the microbial flora of processed seafood and the bioprotective solutions that have so far been proposed to ensure quality and safety of fisheries.

2. Microbial risk in seafood

Fish constitutes a major part of protein consumption in many places in the world. In 2006 the total fisheries production, including fish, crustaceans and molluscs, was 143 million tons, about 35% of which was of aquaculture origin (http://www.fao.org/fishery/statistics/en [accessed 25 January 2010]). Only one hundred and ten million tons were for human consumption, out of this 48% were marketed as fresh products, 25% frozen, 15% canned and 11% processed (salted, dried, smoked etc.).

2.1. Microbial seafood-borne disease statistics

The number of outbreaks attributed to seafood consumption is generally high (10-20% of the total food-borne outbreaks), but varies according to the quality of the surveillance system, the level of consumption (from 5–6 kg in some countries to 180 kg/person/year in Maldives) and the consumers' habits (higher risk associated to raw fish and molluscs consumption). During the period between 1988–1992, the percentage of total number of outbreaks was 7.4 % in the USA (Bean *et al.*, 1996) compared with 21.7% in Japan during the years 1981–1990 where people consume a larger amount of raw seafood (Lee *et al.*, 1996). Some of the largest food poisoning outbreaks have been associated with seafood. In 1991 in Shanghai, a hepatitis A outbreak due to consumption of clams involved 300 000 cases with nine death (Tang *et al.*, 1991). Around at the same time, cholera caused more than 400 000 illnesses and more than 4000 deaths in Peru, which primarily source was probably Ceviche, a typical raw lightly marinated fish.
The fish consumption is responsible for higher outbreaks than shellfish (Huss *et al.*, 2000) but the number of cases per outbreak is often much more elevated with shellfish. In finfish, most of the diseases are from bacterial origin, the highest number being attributed to histamine, a biogenic amine from bacterial origin, accounting for 30–40% of fish intoxication. Histamine fish poisoning is principally due to scombroid fish consumption such as tuna and mackerel which contain high level of histidine, the precursor of histamine, although other species have also been involved (Dalgaard and Emborg, 2008). In molluscs, virus generally account for more than 50% of outbreaks but the lack of routine sensitive detection methods cannot allow a precise estimation. *Vibrio parahaemolyticus, V. vulnificus,* and *V. cholerae* are also an important cause of illnesses in molluscan bivalves that concentrate different particles during their filter feeding. Of the 2500 reported cases of illnesses due to bivalves mollusc, 50% were due to *Vibrio*, with 95 deaths during the period 1984–1993 in US (Wittman and Flick, 1995). As biopreservation is not used at the moment for live molluscs, the chapter will focus on microbial risk in finfish.

2.2. Microbial pathogens in seafood

Microbial seafood pathogens can be classified in two categories:

a) Indigenous bacteria that are naturally present in the marine environment i.e. *Vibrio vulnificus, V. parahaemolyticus* and *V. cholerae*, *Listeria monocytogenes* , *Clostridium botulinum* and *Aeromonas hydrophila*. The presence of indigenous microorganisms is normally not a safety concern since they are present at too low level to cause disease. Moreover, adequate cooking eliminates those bacteria or their toxin (toxin of *C. botulinum* is thermolabile). Therefore, the hazard concerns i) products in which the growth of those bacteria is possible during the storage period and which are eaten raw or insufficiently cooked. It can be the case for *Vibrio* in raw fish or tropical shrimp preparation. *Vibrio* are mesophilic bacteria found in tropical water or in temperate water at the end of summer. Their growth is very rapid if the products are kept some hours at room temperature. *L. monocytogenes* is also a problem in lightly preserved fish products (LPFP) such as cold-smoked, lightly marinated fish or insufficiently cooked seafood stored under VP or MAP. During the extending shelf-life of those products, *L. monocytogenes* can still develop and reach unacceptable concentration. Insufficiently salted seafood stored in anaerobic condition or traditional fermented fish can also support growth of *C. botulinum* and production of the botulinic toxin ii) Scombroid and Clupeid fish kept some hours at abuse temperature (> 5°C) with high histamine content. The origin of histamine producing bacteria is not completely well established although there is evidence that some of them are present in the gut, gills and skin of the fish. Most of the histamine producers are mesophilic bacteria (*Morganella morganii, Hafnia alvei, Raoultella planticola*) that produce histamine when fish is stored at abuse temperature, for instance during storage on the vessels or during the thawing step

before processing. More recently, psychrotolerant bacteria (*Photobacterium phosphoreum, Morganella psychrotolerans*) that grow at 2°C have been associated with histamine fish poisoning in cold-smoked tuna (Emborg and Dalgaard, 2006). Once produced, histamine is not destroyed during the canning process and may cause serious problem in those products.
All those indigeneous bacteria can also post contaminate products during the processing step, either by cross-contamination in industry or because some of them (*L. monocytogenes*) are ubiquitous bacteria naturally present in many food industrial environments or in human skin.
b) Exogenous bacteria due to post contamination during fish processing: those bacteria are the same as those that can be found in other food products i.e. *Staphylococcus aureus*, *Salmonella, Shigella*, *Clostridium perfringens*, *Bacillus cereus, Yersinia enterocolitica* or enterohaemorrhagic *Escherichia coli.* Some of those bacteria can also be present in costal and estuarine marine water or in aquaculture ponds, due to human activities. They constitute a serious problem since low dose can cause illness. Normal cooking eliminates the risk but a lot of ready-to-eat food are not or insufficiently cooked (shellfish salads, shrimps, soup etc.). Moreover, the toxin of *S. aureus* is heatstable.
The different pathogenic bacteria, symptoms, minimal infectious doses and seafood responsible for infection are summarized by Feldhusen (2000) and Lee and Rangdale (2008).

2.3. Microbial seafood safety risk assessment

Different qualitative and quantitative risk assessment strategies have been used to categorize risk from seafood. Risk categories and associated microorganisms are described in Table 1. In a semi quantitative seafood safety risk assessment performed on statistics of seafood-borne illnesses during the period 1990–2000 in Australia, Sumner and Ross (2002) have shown that very high risks were due to *V. parahaemolyticus* and *V. cholerae* in cooked prawns, *V. vulnificus* in oysters, *L. monocytogenes* in cold-smoked seafoods, enteric bacteria in imported cooked shrimp eaten by vulnerable consumers and scombrotoxicosis. Almost all the hazard/product pairs in this category have caused the outbreaks of food poisoning in Australasia. In developed countries, changing in consumers' habit has led to an increase of ready-to-eat and convenient food, concept that includes both the easy-to-use aspect and an extended shelf-life of the products. The nutritional aspects are also more and more taken into consideration by the consumers who want natural products, with technological treatment and level of preservatives as low as possible. LPFP, like carpaccio-type marinated fish, cold-smoked fish, peeled and lightly cooked shrimp, desalted cod packed under VP or MAP etc., meet those requirements and their production has increased dramatically those last years. The major safety risk associated with LPFP is *L. monocytogenes* with a prevalence quite elevated, varying from 2 to 60% depending of the studies (Beaufort *et al.*, 2007; Hu *et al.*, 2006; Gudmundsdöttir *et al.*, 2005; Nakamura *et al.*, 2004; Jorgensen and Huss, 1998; Valdimarsson *et al.*, 1998). *L. monocytogenes* may be present in raw material in low number but contamination mainly occurs during processing. A strict hygienic manufacturing practice has been emphasised to reduce the cross contamination with *L. monocytogenes* with daily cleaning and disinfection of the production lines and special attention to hygiene of the employees. However, a production of LPFP consistently free of the bacterium seems impossible as *L. monocytogenes* is not destroyed by the different processing steps. The risk associated with consumption of LPFP is due to the possible growth of *L. monocytogenes* rather than to the initial contamination of freshly processed products, which are commonly inferior to 1 CFU g^{-1}. *L. monocytogenes* can multiply at low temperatures, in a wide range of pH, in aero and anaerobic conditions in the presence of salt or smoke and it can sometimes overpass the European tolerated limit of 100 CFU g^{-1} (Commission Regulation 1441/2007/EC).

In those kind of products with an extended shelf-life, psychrotrophic LAB have time to develop, therefore, their use as protective culture to prevent *L. monocytogenes* and spoiling microorganisms is a subject of increasing investigations.

3. Lactic acid bacteria in seafood products

3.1. Lactic acid bacteria in living fish

The skin, mucus, gills and gut of fish contain high number of bacteria, whose composition and quantity vary according to the fish species and many environmental parameters. The microbiota of marine fish from temperate waters is usually composed of Gram negative psychrotrophic bacteria from the genera *Pseudomonas*, *Shewanella*, *Acinetobacter*, *Aeromonas*, *Vibrio*, *Moraxella*, *Psychrobacter*, *Photobacterium*. Nevertheless, Gram positive such as *Micrococcus, Corynebacterium*, *Bacillus, Lactobacillus* and *Clostridium* may also be present in variable proportions. In tropical fish the microflora has the same composition overall, but with a predominance of Gram positive bacteria, Enterobacteriaceae and Vibrionaceae.
Although not the most common, it is generally accepted that LAB occur among the normal intestinal microbiota of fish from the first few days and towards. Many genera and species have been reported : *Lactobacillus plantarum, Carnobacterium maltaromaticum* (previously *C. piscicola*)*,C. divergens, C. gallinarum* and *C. inhibens, Streptococcus* spp., *Leuconostoc* spp., *Lactococcus lactis* and *Lc. piscium, Vagococcus salmoninarum, Weissella* spp., etc. (Yang *et al.*, 2007; Huber *et al.*, 2004; Ringo *et al.*, 2001; Jöborn *et al.*, 1999; Ringo and Gatesoupe, 1998). LAB are generally recognized as non-pathogenic for human but virulence of some species such as *Lactococcus garvieae*, *C. maltaromaticum* and *Weissella* sp. has been clearly established for farmed fish (Liu *et al.*, 2009; Eldar *et al.*, 1996; Toranzo *et al.*, 1993).

3.2. Lactic acid bacteria in fresh fish stored in ice or under packaging

At fish death and during evisceration and filleting, microorganisms may contaminate the flesh and this often occur all along the production line as far as in the finished product. LAB are not naturally dominant in the microbiota of fresh fish stored in ice. The low temperature, high *post-mortem* pH (>6), low percentage of sugars (0.2 to 1.5% depending on the species) and high content of non-protein low molecular weight nitrogenous compounds are more favourable for the Gram negative psychrotrophic bacteria naturally present in living fish, like *Pseudomonas* and *Shewanella*. VP does not slow their growth as many of them, notably *Shewanella putrefaciens, Photobacterium phosphoreum* and the Vibrionaceae, are able to use trimethylamine oxide (OTMA), a common marine molecule, as a terminal electron acceptor in anaerobic respiration. These bacteria produce strong off-odours typical of rotten fish due to the reduction of OTMA to trimethylamine, and also sulphurous odours resulting from the breakdown of cysteine and methionine (Gram and Huss, 1996). MAP decreases the number of respiratory microorganisms like *Pseudomonas* and *Shewanella* but *P. phosphoreum* is resistant to CO_2. It therefore multiplies quickly in this type of product and is recognized as the main spoilage bacterium of fresh MAP fish (Dalgaard *et al.*, 1997). This explains why this type of packaging only slightly increases the use-by date of fish compared to meat. However numerous studies carried out on fatty or low-fat fish have shown that more LAB are found in products preserved under MAP than under air (Lalitha *et al.*, 2005; Fletcher *et al.*, 2004). MAP selects both *P. phosphoreum* and LAB but the latter are less competitive and so often play a minor role in the spoilage. When *P. phosphoreum* is eliminated by a frozen step, LAB become the dominant group during the MAP storage of thawed fish (Dalgaard *et al.*, 2006; Emborg *et al.*, 2002).

3.3. Lactic acid bacteria in lightly preserved fish

LPFP are often stored at chilled temperature and under VP or MAP to extend shelf-life and are highly perishable. The initial microbiota depends strongly on the hygiene conditions in the company but is often dominated by Gram negative bacteria typical of fresh fish (Gonzalez-Rodriguez *et al.*, 2002; Leroi *et al.*, 1998; Paludan-Müller *et al.*, 1998). During storage, Gram positive bacteria, particularly LAB, become predominant, sometimes associated with Enterobacteria and *Brochothrix thermosphacta* (Jaffrès *et al.*, 2008; Cardinal *et al.*, 2004). LAB can easily reach 10^{7-8} CFU g^{-1} and such amounts have been found in cold-smoked salmon (CSS) (Leroi *et al.*, 2000; Leroi *et al.*, 1998), smoked trout (Lyhs *et al.*, 1998), smoked herring (Gancel *et al.*, 1997), salted lumpfish roe (Basby *et al.*, 1998), cooked cold-water shrimp (Dalgaard *et al.*, 2003) and warm-water shrimp (Jaffrès *et al.*, 2008; Mejlholm *et al.*, 2005).

The cause of LAB predominance in LPFP has not been extensively studied but it is clear that they are well adapted to the conditions prevailing in those products. Most of the LAB strains isolated from LPFP are psychrotrophic, able to catabolize arginine with low glucose concentration and known to grow with up to 8–10% of salt. VP and MAP are probably other factors promoting LAB development since they are aero-anaerobic bacteria. It has been demonstrated, by challenge tests performed in CSS, that *Lactobacillus sakei, Lb. alimentarius* and *Lb. farciminis* grew faster than *S. putrefaciens*, *P. phophoreum*, *B. thermosphacta* and *S. liquefaciens* (Joffraud *et al.*, 2006).

3.4. Spoilage potential of LAB

The use of LAB as protective culture in seafood implies that they do not have any spoiling capacity. LAB have often been thought to play a minor role in the spoilage of marine products. They are not very competitive in refrigerated fresh fish and they produce fewer unpleasant odours compared to Gram negative bacteria like *S. putrefaciens*, *P. phosphoreum* and *Pseudomonas* sp. (Leisner, 1992). Although dominant in LPFP, their role is not very clear. Several authors have found no correlation between LAB and sensory spoilage (Leroi *et al.*, 2001; Hildebrandt and Erol, 1988). However, Paludan-Müller *et al.* (1998) succeeded in increasing the shelf-life of CSS by inhibiting LAB with nisin, suggesting a possible spoiling effect of this bacterial group (for a review of the different characteristics of spoilage, the compounds responsible and their associated precursors, see Huss *et al.,* 1995).

Stohr *et al.* (2001) clearly showed that some *Lactobacillus* species found in CSS were very spoiling (*Lb. sakei*) while others had no effect (*Lb. alimentarius*). *Lb. sakei* generally produces sulphurous and acidic odours (Stohr *et al.*, 2001; Nilsson *et al.*, 1999), associated with the production of H_2S, acetic acid and ethyl and *n*-propyl acetate (Joffraud *et al.*, 2001), but some *Lb. sakei* strains do not affect the organoleptic quality of this product (Weiss and Hammes, 2006). *Lb. alimentarius* which does not spoil CSS has been identified as the bacterium responsible for the sensory deterioration of marinated herring (Lyhs *et al.*, 2001).

Carnobacteria are microorganisms resistant to freezing that grow very well at refrigerated temperatures, in all packaging conditions and in the presence of many preservatives (Laursen *et al.*, 2005; Leroi *et al.*, 2000), explaining why this genus is very often found in refrigerated meat or fish products. The role of this genus is still under discussion (Leisner *et al.*, 2007; Laursen *et al.*, 2005). Many studies show that the inoculation of CSS by various strains of *C. maltaromaticum* and *C. divergens* leads to few or no changes in organoleptic quality (Brillet *et al.*, 2005; Nilsson *et al.*, 1999). When the carnobacteria reach a high enough level, flavours 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 are not sufficient for a trained panel to reject the product (Brillet *et al.*, 2005). In contrast, strains of *C. maltaromaticum* and *C. divergens* inoculated into Arctic shrimp generated strong chlorine, malt, nuts and sour odours and the samples were judged unfit for consumption

(Laursen *et al.*, 2006). Ammonia and numerous alcohols, aldehydes and ketones were produced. Nevertheless, here again, there was variability depending on the strain.
The interaction with other microorganisms should not be disregarded. In a sterile CSS model, Joffraud *et al.* (2006) have shown that the spoilage observed with *Lb. sakei* was weakened in the presence of *S. liquefaciens* even though the latter had also a spoiling effect in monoculture. On the other hand, some associations appear to be much more spoiling than in pure culture (*Carnobacterium* with *Vibrio* or *B. thermosphacta*) due to *de novo* synthesis of total volatile basic nitrogen (Brillet *et al.*, 2005). Similarly, Laursen *et al.* (2006) showed that the unpleasant odours generated in cooked shrimp by an association of *Carnobacterium* sp. and *B. thermosphacta* were different from those due to these two bacteria in pure culture.

4. Bioprotective LAB, bacteriocins and bacteriophages for bacteria control

The application of protective flora, bacteriophages or bacteriocins on seafood products for biopreservation is generally less documented than in dairy or meat product. The main reason is probably that the early stages of biopreservation have occurred mainly in fermented foodstuffs that are not so developed among seafood products. Moreover, the selection of potential protective bacteria in seafood products remains a challenge since they must be adapted to the seafood matrix (poor in sugar) and their metabolic activities should not change the initial characteristic of the product i.e. by acidification and not induce spoilage that could lead to a sensory rejection.
Among the microbial flora identified in fresh or processed seafood, LAB remains the category that offers the higher potential for direct application as bioprotective culture or for bacteriocin production. Most of the studies concern LPFP such as CSS and focus on the inhibition of *L. monocytogenes* (Table 2), considered as explained before as the main bacterial risk associated with the consumption of these products. The increase of knowledge about the microbial spoilage flora of those foodstuffs has also recently highlighted the new interest of bioprotective culture or biopreservatives to extend the sensory shelf-life of several LPFP.

4.1. Control of pathogenic bacteria

Many studies concerning pathogenic bacteria inhibition have been performed in liquid model medium, but the effects are not often confirmed in real products. In the following part of this chapter, we will focus on studies that have given successful results on seafood products.

Control with protective cultures

Among the LAB that were described before, strains belonging to the genus *Carnobacterium* have been particularly studied for their role as protective flora in CSS, probably because they are not acidic bacteria. They belong to the major lactic acid flora of such products at the end of storage and although their presence can sometimes be associated with spoilage activities on seafood products, in many cases they are not directly responsible for undesirable odours or flavours. Moreover, several *Carnobacterium* strains are known to produce anti-listerial bacteriocins (Drider *et al.*, 2006).
Two strains of *C. maltaromaticum* isolated from CSS were evaluated for the inhibition of *L. monocytogenes* in VP CSS. Both strains demonstrated their efficiency to limit the growth of this pathogenic bacteria during 31 days of storage at 5°C (Nilsson, 1999). The growth of the protective *Carnobacterium* strains did not modify the sensory characteristic of the product. One of these strains showing the strongest inhibition activity produces a bacteriocin, named carnobacteriocin B2 that was involved in the anti-listerial activity (Nilsson *et al.*, 2004). Three other strains of bacteriocin producing *Carnobacterium* have been tested with the agar

diffusion test method against a wide collection of *L. monocytogenes* (51 strains) isolated from seafood. All of the *Listeria* strains were sensitive. The inhibition was confirmed in co-culture with a mix of *L. monocytogenes* strains in sterile CSS (Brillet *et al.*, 2004). One of these strains, *C. divergens* V41 showed its ability to maintain *L. monocytogenes* at the initial inoculating level of 20 CFU g^{-1} during 28 days of storage at 4°C and 8°C. The effect of this strain on sensory characteristics and physico-chemical parameters revealed that it did not spoiled the product (Brillet *et al.*, 2005). In that case also, the inhibitory activity could be linked to the bacteriocin divercin V41, since a bacteriocin negative mutant failed to limit the growth of *L. monocytogenes* in the same conditions (Richard *et al.*, 2003). In the presence of the bacteriocinogenic strain *C. maltaromaticum* CS526 isolated from surimi, the population of *L. monocytogenes* in CSS decreased from 10^3 to 50 CFU g^{-1} after 7 days at 4°C (Yamazaki *et al.*, 2003). This activity could be linked to the production of the bacteriocin piscicocin CS526, since a non-bacteriocin producing strain had a lower effect on the growth of the pathogenic bacteria (Yamazaki *et al.*, 2005; Yamazaki *et al.*, 2003). In another study, the application of *C. divergens* M35 towards *L. monocytogenes* in CSS resulted in a maximal decrease of 3.1 log CFU/g of the pathogenic bacteria after 21 days of storage at 4°C whereas a non-bacteriocinogenic strain had no effect (Tahiri *et al.* 2009).
Among the other LAB, *Lb. sakei* has been also used as protective culture for *L. monocytogenes* inhibition on CSS. The strain Lb790 producing sakacin P was compared to a non bacteriocinogenic strain for the inhibition of *L. monocytogenes*. In both cases, no bactericidal effect was obtained but the growth of the pathogenic bacteria was stopped during 28 days at 10°C (Katla *et al.*, 2001). Another bacteriocinogenic strain of *Lb. sakei* isolated from CSS allowed a 4 log reduction of *Listeria innocua* after 14 days of storage at 4°C. A reduction of 2 log units after 24 h at 5°C was also demonstrated with that strain in CSS juice towards *L. monocytogenes* (Weiss and Hammes, 2006).
Mix of bacteriocin-producing LAB like *Lb. casei , Lb. plantarum* and *C. maltaromaticum* were successfully used to limit the growth of *L. innocua* in CSS (Vescovo *et al.*, 2006). In their study Tomé *et al.* (2008) have also selected a strain of *Enterococcus faecium* among five bacteriocinogenic LAB strains for its ability to induce a decrease of the population of *L. innocua* inoculated in CSS. However in these studies the inhibition activities were not confirmed on *L. monocytogenes .*
Protective cultures have not been applied in many other seafood products except for CSS and *L. monocytogenes* control. Matamoros *et al.* (2009a) have performed challenge-tests in cooked shrimp stored under VP using protective LAB and *L. monocytogenes* and *S. aureus* as target pathogens. Two LAB strains, *Lactococcus piscium* EU2241 and *Leuconostoc gelidum* EU2247 were efficient to limit the growth of both pathogenic bacteria from 2 to 3 log CFU g^{-1} units after 4 weeks at 8°C followed by 1 week at 20°C. The strain of *Leuconostoc* produced a bacteriocin-like compound but its activity was slight lower than the *Lactococcus* strain that was non-bacteriocinogenic.

Control with bacteriocins

Most of the applications of protective LAB for the control of pathogens have been conducted with bacteriocinogenic strains isolated from seafood products. Some of these bacteriocins have been purified and characterized, in particular those produced by carnobacteria. They are listed in Table 3. Divercin V41 is produced by *C. divergens* V41 (Métivier *et al.*, 1998) and piscicocin V1a and V1b are two bacteriocins produced by the same strain, *C. maltaromaticum* V1 (Bhugaloo-Vial *et al.*, 1996). Divercin V41 is closed to divergicin M35, another class IIa bacteriocin that is produced by a *C. divergens* strain isolated from frozen smoked mussels (Tahiri *et al.*, 2004).
Piscicocin V1a, piscicocin V1b and the bacteriocin produced by *C. maltaromaticum* A9b (Nilsson *et al.*, 2004) are peptides that have been also characterized from other *C. maltaromaticum* strains isolated from meat or cheese (Leisner *et al.*, 2007). All these bacteriocins belong to the class IIa of antilisterial bacteriocins that are heat stable peptides

with low molecular weight (< 10 KDa) (Drider *et al.*, 2006). Piscicocin CS526 produced by *C. maltaromaticum* CS526 isolated from surimi is also considered as a class IIa bacteriocin although it possesses an alternate residue in the N-terminal consensus motif shared by these peptides (Yamazaki *et al.*, 2005). Carnocin UI49 is the only class I bacteriocin that have been characterized from LAB isolated from seafood products (Stoffels *et al.*, 1992).
Recently, Pinto *et al.* (2009) have described two bacteriocins produced by strains of *Enterococcus faecium* and *Pediococcus pentosaceus* isolated from non fermented shellfish. These peptides were similar to the well-known class II bacteriocins enterocin B and pediocin PA-1.
These last studies suggest that the specificity of bacteriocins is not linked to LAB origin but more likely connected to the bacterial species. A new peptide showing no similarity with other known bacteriocins has been lately characterized. It is called Weissellicin 110, produced by a strain of *Weissella cibaria* that is coming from a traditional fermented fish product from Thailand, however its amino acid sequence was not totally determined yet (Srionnual *et al.*, 2007).
Among the peptides that have been described above, very few have been applied directly on seafood products for pathogenic bacteria control. Since bacteriocin purification techniques allowing the recovery of high amounts of peptides in water or salt solution are usually not available, most of the studies use bacteriocin containing supernatant or semi-purified fractions. Crude extract of culture supernatant containing piscicocins or divercin V41 were added on CSS to limit the growth of *L. monocytogenes* during 21 days of storage at 4°C and 8°C (Duffes *et al.*, 1999). In both cases, the bacteriocins showed a rapid bactericidal effect after 3 days and during the first week of storage. However this effect tended to disappear after 10 days on the opposite with the constant bacteriostatic effect obtained with the protective strain. (Nilsson *et al.*, 1999) made the same observations with semi-purified carnobacteriocin A9b in CSS inoculated with *L. monocytogenes*. Recently, Tahiri *et al.* (2009) have also shown that the application of non-purified or purified divergicin M35 on CSS inoculated with *L. monocytogenes* resulted in more rapid but less pronounced reduction of the pathogenic bacteria counts comparing to the producing strain. In the case of *Lb. sakei* Lb790, addition of nearly pure sakacin P to the protective culture carried out an immediate bactericidal effect with no re-growth of the pathogenic bacteria during storage, leading to a more efficient effect than with the protective culture alone (Katla *et al.*, 2001).
Some attempts with commercial bacteriocins like nisin were made to limit the development of *L. monocytogenes* in CSS (Nilsson *et al.*, 1997). However the growth was only delayed and the final population was similar to that obtained in the control at the end of the storage. In the same way, the results obtained with nisin or pediocin ACCEL to control the growth of *L. monocytogenes* on cooked fish showed a limited and short effect (Yin *et al.*, 2007). It is likely that high and buffered pH usually encountered in fish products is not suitable for nisin solubility and activity.

Control with bacteriophages

Although bacteriophages were proposed for several applications in food safety to control the major pathogenic bacteria (Garcia *et al.*, 2008) the only application in seafood is reported by Guenther *et al.* (2009) in smoked salmon and mixed seafood contaminated with two different *L. monocytogenes* strains at a level of 10^3 CFU g^{-1}. The best results were an inhibition by 2 log CFU g^{-1} during 6 days of storage in mixed seafood but this effect was variable considering the product and the *L. monocytogenes* strain used (Guenther *et al.*, 2009).

4.2. Control of spoiling microorganism

Control with protective culture

Less information is available in this field since the microflora involved in the spoilage activity of seafood product is complex and in most of the cases has not been characterized. The activity of protective culture or bacteriocin is thus directed on the increase of sensory shelf-life, or the inhibition of common microbial indicators such as total viable count or LAB.
The *Carnobacterium* species that were described above for the inhibition of *L. monocytogenes* do not seem to offer a great potential in extending the shelf-life of seafood products. Leroi *et al.* (1996) succeeded in increasing the sensory use-by-date of CSS slices by inoculating them with strains of *Carnobacterium* sp. However the results varied depending on the batch treated. For Brillet *et al.* (2004) no effect of *C. divergens* V41 was recorded on the spoilage flora of CSS and this strain did not improve its organoleptic properties. Using a strain of *C. maltaromaticum* Paludan-Müller *et al.* (1998) only slightly extended the shelf-life of smoked salmon. The application of the protective strain *C. divergens* M35 had no significant effects on the total flora of CSS during storage at 4°C for 21 days (Tahiri *et al.*, 2009). Similarly, *C. maltaromaticum* had no effect on the inhibition of the Gram positive spoilage bacteria *B. thermosphacta* in cooked shrimps (Laursen *et al.*, 2006).
These studies suggest that selection of protective strains to improve the sensory quality of seafood products should focus on specific spoilage microorganism's inhibition. This approach was chosen by Matamoros *et al.* (2009b) who have isolated seven strains from various marine products on the basis of their activity against many spoiling and pathogenic, Gram positive and Gram negative marine bacteria. Among those strains, two *Le. gelidum,* and two *Lc. piscium* demonstrated promising effect in delaying the spoilage of tropical shrimp and of VP CSS. A recent study demonstrated that this protective effect could be due to the inhibition of *B. thermosphacta* identified as one of the major spoiler organisms in cooked shrimp stored under MAP (Fall *et al.*, 2010). One of the strain of *Lc. piscium* was able to limit by 4.1 log CFU g^{-1} the growth of this target bacteria and thus to avoid the apparition of undesirable odours in the products. Altieri *et al.* (2005) also succeeded in inhibiting *Pseudomonas* sp. and *P. phosphoreum* in VP fresh plaice fillets at low temperatures with a strain of *Bifidobacterium bifidum.*
In the same field, some attempts have been made to select microorganisms that are able to limit the growth of histaminogen microflora or to induce biogenic amines degradations. In seafood, biogenic amines are usually produced by spoiling microorganisms like *P. phosphoreum* or enterobacteria like *Morganella morganii* or *M. psychrotolerans* (Emborg and Dalgaard, 2006). They are often used as indirect spoilage indicators, signalling the presence of these bacteria and have thus been included in some models proposed to predict the spoilage of marine products (Jorgensen *et al.*, 2000; Veciana-Nogues *et al.*, 1997). Moreover, histamine is also responsible for food poisoning often linked to seafood consumption (Emborg and Dalgaard, 2006). The studies concerning biogenic amine degrading bacteria mainly focus on fermented fish. A strain of *Staphylococcus xylosus* showed its ability to degrade histamine and tyramine in salted and fermented anchovy (Mah and Hwang, 2009). In the same way, mixed starter cultures of *Lb. plantarum, Lb. casei, Pediococcus pentosaceus* and *S. xylosus* were efficient to limit accumulation of histamine, tyramine, cadaverine, putrescine and tryptamine in silver carp sausages (Yongjin *et al.*, 2007).

Control with bacteriocins

As most of the bacteriocins produced by LAB isolated form seafood products are active against Gram positive flora only, their relevance in preventing spoilage activities that are usually linked to various Gram negative and Gram positive flora is limited. The effect of nisin and pediocin ACCEL was observed on total viable counts of fresh fish fillets at 0°C and 4°C (Yin *et al.*, 2007). The growth of the total flora was slightly delayed when the bacteriocin were present in the samples stored at 0°C but the bacterial counts reached the same level than in the non-treated control after 5 days of storage. In brined shrimp stored at 4.5°C, nisin Z was more efficient to extend the microbial shelf-life than other tested bacteriocins (Einarsson and

Lauzon, 1995) however this effect was limited comparing to the use of food additives such as benzoic or sorbic acids that are usually found in such products.

5. Industrial application

Unlike meat or dairy products, seafood products are mainly non-fermented. Therefore the addition of bacterial cultures concept, even with protective effects, is new and probably not totally yet accepted by seafood producers, for which the main goal is to avoid bacterial contamination by the use of good hygienic practices. However, in LPFP, the use of protective culture is gradually considered as an alternative to the use of food additives and it is gaining interest in the seafood industry. Industrial starters like SafePro® (CHR Hansen, DK), Bovamine Meat Culture™ (NPC, US), HOLDBAC™ (Danisco, DK) have been developed for *L. monocytogenes* control for the meat industry, but to our knowledge they are not used in seafood products. Some patents claimed the usefulness of LAB for the treatment of food without seafood specification (Nauth and Zheng, 2006; Stiles *et al.*, 2005; Fliss *et al.*, 2004). However one of them mentions the application of protective culture for seafood (Daniel and Lorre, 2001). This starter named LLO is applied in France for extending the shelf-life of cooked shrimp stored under MAP (Meyer, 2005) and has also showed limitation of histamine production in Tuna stored at 5°C (http://www.bioceane.com/uk/pdf/ferment_histamine.pdf [Accessed 25 January 2010]).
Concerning bacteriophages, the preparation LISTEX™ P100 was approved by the FDA for all food products (http://www.ebifoodsafety.com/en/news-2007.aspx [Accessed 25 January 2010]) but no studies are available concerning its efficiency in seafood.

6. Future trends

The presence of LAB in many processed seafood product is now well established and although some strains are sometimes involved in spoilage, the bioprotective potential of many strains has been highlighted in the last years. The results obtained with protective culture, bacteriocins and bacteriophages for improving safety and quality of seafood products are at this moment in favor of the use of live cultures that seem to be more efficient than bacteriocin during the long storage period on these foodstuffs. Nevertheless some fields as bacteriophages application or control of spoilage flora still have to be more explored in marine products.
Control of pathogenic bacteria has widely focused on *L. monocytogenes* considered as the main risk in ready-to-eat seafood. However, in these minimally processed products, the new combination of hurdles like low salt content, coupled to MAP can give selective advantages to other pathogenic bacteria like clostridia, vibrio or staphylococci that should also be addressed in biopreservation studies. It is also important to note that most of the studies are done in challenge-test where sometimes only one strain of the pathogenic bacteria is used as target. The applications of protective cultures, bacteriocin or bacteriophages in naturally contaminated products or using mixed strains of target pathogenic microorganisms isolated form seafood should improve the guaranty to reproduce the results in real industrial products.
Concerning control of sensory quality, identification of the specific spoilage flora for the different products is required to select appropriate bioprotective solutions. LPFP spoilage is often due to a complex association of Gram positive and negative bacteria that still need to be explored. The use of combination of protective cultures with different antimicrobial spectrum to master both pathogenic and spoiling bacteria is an exciting challenge for the next years.
Although many protective strains have shown their efficiency to control *L. monocytogenes* in CSS without any sensory modification, industrial applications delay developing. A brake on

expansion is that CSS is a traditional product that benefit from a high quality image by the consumers, so producers are not yet ready to communicate on other ingredient than salt and smokewood.
Moreover, the European regulation concerning addition of protective cultures in unfermented ready-to-eat food is still under discussion. In 2007, the European Food Safety Authority (EFSA) adopted guidelines for Qualified Presumption of Safety (QPS) that can be referred to as the European equivalent of the American GRAS status in terms of risk assessment (EFSA, 2007). Any microorganisms used in the food chain should be suitable for the QPS status. A list of 47 LAB species has been published in the EFSA journal (EFSA, 2008). LAB species that gave promissing results in seafood are not included in this list, which is supposed to be annually updated. Proof of their beneficial effect, precise taxonomic data and strong evidence of safety are needed for obtaining the QPS status if notified to EFSA. However, with the increasing market of LPFP, the request for alternative preservation solution and the intensification of research, there is no doubt that biopreservation of fisheries products will expand in the future.
Additional research work is therefore needed in the selection of appropriate strain and their combination to limit the growth of both pathogenic and spoilage flora, understanding of the inhibition mechanism to optimise their activity in the products and characterization and safety aspects of the cultures or their metabolites to obtain the QPS status.

7. Source of further information and advice

Borresen, T. (Ed.) 2008. *Improving seafood products for the consumer.* Cambridge, Woodhead publishing limited.
Informations on QPS status :
http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620763335.htm
[Accessed 25 January 2010]
Informations on French application of protective culture for seafood products :
http://www.bioceane.com/uk/index.htm

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Tables

Table 1 : Risk categories for seafood products and associated microorganisms

Risk	Seafood products	Agent
High	Mollusc (fresh or frozen)	Virus, bacteria, toxin from microalgae (heatstable)
	Raw fish : Ceviche, Suchi etc.	Indigenous bacteria (*Vibrio*)
	Lightly preserved fish (NaCl < 6% WP, pH > 5): carpaccio, cold-smoked fish, marinated products, gravads etc.	Growth of indigenous bacteria (*Listeria monocytogenes*, production of toxin from *Clostridium botulinum*)
	Mildly heat processed: cooked and peeled shrimp, salads, soup etc.	Recontamination with enteric bacteria, growth of *Listeria monocytogenes*, *Vibrio*
	Scombroid fish	Histamine production
Low	Cooked fish and crustacean	Ciguatera in tropical area
	Semi preserved fish (NaCl > 6% WP, pH < 5): salted, dried, marinated, hot smoked fish etc.	Recontamination with enteric bacteria
	Heat processed: sterilised, canned etc.	*Clostridium botulinum* spore

Table 2: Applications of protective cultures for pathogenic bacteria control in seafood

Products	Protective strains (inoculum level)	Bacteriocinogenic Y/N (bacteriocin name)	Target microorganisms (inoculation level)	Storage conditions	Effect	Reference
Cold smoked salmon	*Carnobacterium maltaromaticum* A9b (10^6 CFU/g)	Y (carnobacteriocin B2)	*L. monocytogenes* ($2\ 10^2$ CFU/g)	Vacuum packed 32 d at 5°C	Initial level till 25 d and decrease below 1 CFU/g	Nilsson *et al.* 1999, 2004
	Carnobacterium maltaromaticum A10a (10^6 CFU/g)	N			10^3 CFU/g maintained during 31 d	Nilsson *et al.* 1999
	Carnobacterium divergens V41 (10^5 CFU/g)	Y (divercin V41)	*L. monocytogenes* (20 CFU/g)	Vacuum packed 9 d at 4°C and 19 d at 8°C	20 CFU/g maintained during 28 d	Brillet *et al.* 2004
	Carnobacterium divergens V1 (10^5 CFU/g)	Y (piscicocin V1a and V1b)			$<10^2$ CFU/g maintained during 28 d	
	Carnobacterium divergens SF668 (10^5 CFU/g)	Y (unknown)			Growth limitation of 1 to 3 log CFU/g at the end of storage period	
	Carnobacterium maltaromaticum CS526 (10^4 or 10^6 CFU/g)	Y (piscicocin CS526)	*L. monocytogenes* (10^3 CFU/g)	Vacuum packed 21 d at 4°C	Decrease till 10^2 CFU/g after 12 d maintained during 24 d	Yamazaki *et al.* 2003
	Carnobacterium maltaromaticum JCM5348 (10^4 or 10^6 CFU/g)	N			Initial level maintained during 24 d	
	Carnobacterium divergens M35 (10^6 CFU/g)	Y (divergicin M35)	*L. monocytogenes* (10^2 CFU/g)	Vacuum packed 21 d at 4°C	Growth limitation of 3 log CFU/g after 21 d	Tahiri *et al.* 2009
	Lactobacillus sakei Lb790 (10^3 CFU/g)	Y (sakacin P)	*L. monocytogenes* (10^3 CFU/g)	Vacuum packed 28 d at 10°C	Initial level maintained during 28 d	Katla *et al.* 2001
	Lactobacillus sakei Lb790 (10^3 CFU/g) + sakacine P (1,1 µg/g)				Decrease till 10^2 CFU/g in 28 d	
	Lactobacillus sakei 5754 (10^7 UFC/g)	Y (unknown)	*L. inocua* (10^4 CFU/g)	Vacuum packed 14 d at 4°C	Growth limitation of 4 log CFU/g after 14 d	Weiss and Hammes 2006
	Lactobacillus casei T3 and *Lactobacillus plantarum* Pe2 (10^6 CFU/g)	Y (unknown)	*L. innocua* (10^4 CFU/g)	Vacuum packed 30 d at 4°C	Growth limitation of 1,5 log CFU/g after 30 d	Vescovo *et al.* 2006
	Enterococcus faecium ET05 (nd)	Y (unknown)	*L. innocua* (10^4 CFU/g)	Vacuum packed 21 d at 5°C	Growth limitation of 2,5 log CFU/g after 21 days	Tomé *et al.* 2008
Cooked shrimp	*Lactococcus piscium* EU2241 (10^5 UFC/g)	N	*L. monocytogenes* (10^3 CFU/g)	Vacuum packed 28 d at 8°C, then 7 d at 20°C	Growth limitation of 2 log CFU/g after 7 days and during the storage period	Matamoros *et al.* 2009
	Leuconostoc gelidum EU2247 (10^5 UFC/g)	Y (unknown)	*S. aureus* (10^3 CFU/g)		Growth limitation of 1,5 log CFU/g after 21 days	

Table 3: Bacteriocins from lactic acid bacteria isolated from seafood

Bacteriocin name	**Producing strain**	**Strain origin**	**Synonyms**	**Reference**
Piscicocin CS526	*C. maltaromaticum CS526*	Surimi		Yamazaki *et al.* 2003, 2005
Piscicocin V1a	*C. maltaromaticum* V1	Fish viscera	Piscicolin 126 (Leisner *et al.* 2007)	Bhugaloo-Vial *et al.*, 1996
Bacteriocin A9b	*C. maltaromaticum* A9b	Smoked salmon	Carnobacteriocin B2 (Leisner *et al.* 2007)	Nilsson *et al.* 1999, 2004
Piscicocin V1b	*C. maltaromaticum* V1	Fish viscera	Carnobacteriocin BM1 (Leisner *et al.* 2007)	Bhugaloo-Vial *et al.*, 1996
Divercin V41	*C. divergens* V41	Fish viscera		Metivier *et al.*, 1997
Divergicine M35	*C. divergens* M35	Frozen smoked mussels		Tahiri *et al.* 2004
Carnocin UI49	*Carnobacterium* sp.	Fish		Stoffels *et al.* 1992
Weissellicin 110	*Weissella cibaria* 110	Plaa-som, fermented fish product		Srionnual *et al.* 2007
Bac ALP7	*Enterococcus faecium* ALP7	Shellfish	Enterocin B	Pinto *et al* 2009
Bac ALP57	*Pediococcus pentosaceus* ALP57	Shellfish	Pediocin PA-1	Pinto *et al.* 2009