Occurrence and role of lactic acid bacteria in seafood products

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Abstract:

Lactic acid bacteria (LAB) in fish flesh has long been disregarded because the high post-mortem pH, the low percentage of sugars, the high content of low molecular weight nitrogenous molecules and the low temperature of temperate waters favor the rapid growth of pH-sensitive psychrotolerant marine Gram-negative bacteria like Pseudomonas, Shewanella and Photobacterium. In seafood packed in both vacuum (VP) and modified atmosphere (MAP) packaging commonly CO₂ enriched, the growth of the Gram-negative aerobic bacteria group (predominantly pseudomonads) is effectively inhibited and the number reached by LAB during storage is higher than that achieved in air but always several log units lower than the trimethylamine oxide (TMA-O) reducing and CO₂-resistant organisms (Shewanella putrefaciens and Photobacterium phosphoreum). Accordingly, LAB are not of much concern in seafood neither aerobically stored nor VP and MAP. However, they may acquire great relevance in lightly preserved fish products (LPFP), including those VP or MAP. Fresh fish presents a very high water activity (aw) value (0.99). However, aw is reduced to about 0.96 when salt (typically 6% WP) is added to the product. As a result, aerobic Gram-negative bacteria are inhibited, which allows the growth of other organisms more resistant to reduced aw, i.e. LAB, and then they may acquire a central role in the microbial events occurring in the product. Changes in consumers’ habits have led to an increase of convenient LPFP with a relative long shelf-life (at least 3 weeks) which, on the other hand, may constitute a serious problem from a safety perspective since Listeria monocytogenes and sometimes Clostridium botulinum (mainly type E) may able to grow. In any case the LAB function in marine products is complex, depending on species, strains, interaction with other bacteria and the food matrix. They may have no particular effect or they may be responsible for spoilage and, in certain cases, they may even exert a bioprotective effect in relation to undesirable bacteria. The bioprotective potential of endogenous LAB in relation to pathogens and spoiling bacteria has often been highlighted. However, the technology is still in its infancy compared with foods dairy and meat products in which either the carbohydrate content (dairy products) or sugar and salt added (meat products) favor the acidification by LAB that enable a natural preservation of the product. Successful studies on LAB as probiotic for fish intensify, but this potential is still to be explored for human. Although not usual, some applications of LAB for fermentation of marine products and by-products are described.

Keywords: Lactic acid bacteria; Fish; Spoilage; Biopreservation; Probiotic; Fermentation
1. Introduction

LAB constitute a large group of non sporulating Gram positive, catalase and oxidize negative rods and cocci that produce lactic acid as the major metabolite of the carbohydrate fermentation. LAB are anaero-aerotolerant and generally have complex nutritional requirements especially for amino acids and vitamins. The genera comprise the LAB are at its core Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus as well as the more peripheral Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus, Weissella and bifidobacterium genus. Phylogenetic studies based on ribosomal DNA sequence comparisons and on signature-sequence analysis present in well-conserved proteins suggests an ancestral position in evolution of Gram positive bacteria with less than 50 GC%, including LAB that belong to the Clostridium phylum. Bifidobacterium spp. with more than 50 GC% belong to the Actinomycetes phylum. By these criteria and on data of paleontologic and metabolism, LAB may have appeared before the photosynthetic cyanobacteria 3 billion years ago (Tailliez, 2001). They are widespread in nature and commonly found in many food products (dairy, meat, fruit, vegetables, etc.), as well as in genital, intestinal and oral cavity of animal and human. Human has empirically used LAB for natural fermentation of milk, meat, vegetables and fruits for thousands of years that led to a new stabilized product. The acidification process due to the lactic acid production as the major end-metabolite of the carbohydrate fermentation is one of the most desirable side effects of their growth, inhibiting micro-organisms including the most common human pathogens. Since the scientific basis of the mechanisms have been set up, the LAB species traditionally used have been selected and produced as lyophilized starters that can be added into the food and allow a better control of the fermentation.

LAB in fish flesh has long been disregarded because they are not currently present in seafood. However, the deep changes in the culinary habits in the last decades of the past century and the current consumer demands have led food industry to produce a great variety of convenient foods, many of them ready-to-eat such as LPFP in which certain ingredients (e.g. salt or sugar) are usually added and a mild processing (e.g. cold-smoked) is frequently applied. These approaches give rise to changes of the normal characteristics of fresh fish (e.g. reduced aw), inhibiting the growth of organisms responsible for the spoilage and enhancing that of other microbiota such as LAB. Given the great increase in the production of these foods, LAB can achieve a great interest in LPFP packaged under different conditions. The occurrence and role of LAB in seafood are discussed in the present review.

2. Microbial ecology of living marine fish

2.1. Typical microbiota

The muscle of living fish is sterile. However, the skin, mucus, gills and gut contain significant bacteria, whose composition and quantity vary according to the fish species, temperature and salinity of the water, level of dissolved oxygen, degree of pollution, feed, stress, etc.

The microbiota of marine fish from temperate waters is usually composed of Gram-negative psychrotrophic bacteria, whose growth is possible at 0°C and optimal at around 25°C. The majority belongs to the class γ of proteobacteria: Pseudomonas, Shewanella, Acinetobacter, Aeromonas, Vibrio, Moraxella, Psychrobacter, Photobacterium, etc., and to a lesser extent to the CFB group (Cytophaga-
Flavobacter-Bacteroides). Nevertheless, Gram-positive bacteria, such as Micrococcus, Corynebacterium, Bacillus, Lactobacillus and Clostridium may also be present in variable proportions (Shewan, 1971; Shewan, 1977; Hobbs, 1983; Mudarris and Austin, 1988; Gram and Huss, 1996; Gennari et al., 1999; Huber et al., 2004; Wilson et al., 2008). The same bacterial genus can be found in tropical marine fish but Gram-positive bacteria, Enterobacteriaceae and Vibrionaceae are often dominant (Liston, 1980). The indigenous microbiota of gastro-intestinal tract have been much more studied than those of the skin or mucus due to their importance in digestion, nutrition and disease 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. This 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 with molecular methods that the aerobic microbiota of rainbow trout intestine usually represents 50 to 90% of the total microbiota. In general, Gram-negative bacteria (Vibrio, Acinetobacter and Enterobacteriaceae) are dominating the microbiota (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. Sometimes, staphylococci have also been found to be the dominant microbiota in the fish intestine. However, both the number and diversity of the microbiota are probably widely underestimated due to the classical microbiological methods involving growth on agar media that so far have been used. The bacterial cultivability has been estimated to be less than 2% of intestinal microbiota rainbow trout (Huber et al., 2004) and less than 0.01% of the skin microbiota (Bernadsky and Rosenberg, 1992).

2.2. Lactic acid bacteria

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 (Yang et al., 2007; Ringo, 2008). Lactobacilli, notably Lactobacillus plantarum, have been found in Atlantic salmon (Ringo et al., 1997), pollock (Schroder et al., 1980), Arctic char (Ringo et al., 1998) and cod (Strom and Olafsen, 1990). Carnobacteria, including Carnobacterium maltaromaticum (previously piscicola), divergens, gallinarum and inhibens, have been isolated from all these species, as well as from rainbow trout (Jöborn et al., 1999; Ringo et al., 2001; Huber et al., 2004). Carnobacteria have even been quoted as being the dominant genus in the gastro-intestinal tract of juvenile Atlantic salmon (Ringo et al., 1997) and cod (Seppola et al., 2006). Other authors have also reported the presence of Leuconostoc mesenteroides, Lactococcus piscium, Vagococcus salmoninarum, Lactobacillus fuchuensis, Streptococcus spp., and Weissella spp. (Wallbanks et al., 1990; Williams et al., 1990; Ringo and Strom, 1994; Ringo et al., 1998; Liu et al., 2009; Matamoros et al., 2009b). Various factors, like salinity of the water or stress, can affect the presence of LAB (Ringo and Strom, 1994). For instance, the number of lactobacilli in the gastro-intestinal tract of Arctic char was smaller in those reared in sea water than in fresh water, while the number of Leuconostoc and enterococci remained the same (Ringo and Strom, 1994). Atlantic salmon stressed by daily manipulations experienced a drop in the number of Gram-negative bacteria in their intestinal microbiota and an increase of carnobacteria count (Ringo and Gatesoupe, 1998). For a review of LAB in fish and fish farming, see Ringo (2004).

Even thought most LAB are generally recognized as safe by the US Food and Drug administration, their implication in fish disease has been reported. In marine farmed fish, numerous epizootics linked to streptococci have been notified, beginning in Japan and North America then spreading worldwide (Eldar et al., 1996). These bacteria, reclassified as Lactococcus garvieae, are responsible for septicemias,
ophthalmias and hemorrhages. *C. maltaromaticum* has been isolated from different diseased fish and its virulence has been clearly established in rainbow trout and striped bass experimentally infected (Baya et al., 1991; Toranzo et al., 1993). Recently, a novel *Weissella* species has been described as an opportunistic pathogen for rainbow trout (Liu et al., 2009).

3. Occurrence and spoilage of seafood

At fish death and during evisceration and filleting, endogenous micro-organisms from the raw material may contaminate the flesh. The production environment lines and human manipulations are also a source of post-contamination so that micro-organisms can occur in the finished product.

3.1. Fresh fish stored in ice or under different atmospheres

Fish is a matrix that particularly favors 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 metabolized by bacteria. These compounds include free amino acids, creatine, nucleotides, urea and TMA-O. The high post-mortem pH (about 6) combined with the small quantity of carbohydrates, enable the rapid growth of Gram-negative, pH-sensitive psychrotrophic bacteria naturally present in fish, like *Pseudomonas* and *Shewanella*. At chilled storage temperature, those micro-organisms are more competitive than mesophilic LAB and they can easily reach levels of $10^6$ to $10^8$ CFU g$^{-1}$ of flesh in few days (Jorgensen and Huss, 1989). Oppositely, in milk, high carbohydrate concentration (approx. 5%), initial temperature of 37°C, and microaerobic environment are favorable conditions for the development of naturally contaminating LAB such as *Lactococcus* and *Lactobacillus*. In mammalian meat, the glycogen content is higher than in fish, leading to a post rigor acidification (pH<5.5) that prevents growth of the pH-sensitive bacteria like *Shewanella* and allows the implantation of LAB.

In order to increase the shelf-life and also to propose more convenient products for the consumer, fish fillets or steaks are often sailed packed in vacuum or modified atmosphere small containers. VP, which could favor anaero-aerotolerant LAB, does not slow the growth of marine bacteria. Many of them, notably *S. putrefaciens*, *Photobacterium phosphoreum* and the Vibrionaceae, are able to use TMA-O, a common marine molecule, as a terminal electron acceptor for anaerobic respiration. They all require sodium for growth and are typical of marine products. These bacteria produce strong off-odors, especially those typical of rotten fish, which are due to the reduction of TMA-O to trimethylamine, but also sulphurous odors resulting from the breakdown of cysteine and methionine (for a review on spoilage of marine fish, see Gram and Huss (1996), Gram and Dalgaard (2002) and Gram (2009)).

Different modified atmospheres have been tested, mainly mixtures of O$_2$/CO$_2$/N$_2$ at different concentrations and their effect in fishery products has been reviewed by Sivertsvik et al. (2002). Carbon dioxide decreases the number of Gram-negative bacteria, particularly the respiratory micro-organisms like *Pseudomonas* and *Shewanella* (Banks et al., 1980; Gill and Tan, 1980; Stammen et al., 1990). However, *P. phosphoreum*, a marine micro-organism that is not present in meat and dairy products, is resistant to CO$_2$ (Dalgaard et al., 1993). It therefore multiplies well in this type of product and is recognized as the spoiling bacterium of fresh MAP fish (Dalgaard, 1995; Dalgaard et al., 1997). This explains why MAP only slightly increases the use-by date of fish compared to meat. However, MAP equally favors the development of LAB in fresh fish. Research carried out on herring fillets showed that preservation under 100% CO$_2$ clearly leads to a predominance of *Lactobacillus*
in the microbiota, while under air Alteromonas and Pseudomonas are more common (Molin et al., 1983; Molin and Stenström, 1984), however high CO₂ levels result in poor sensory characteristic due to the carbonated flavor. Numerous further studies carried out on other species of fatty or low-fat fish have shown that total LAB counts were higher in fresh MAP fish than under air: Banks et al. (1980) and Lannelongue et al. (1982) in ocean perch, Oberlender et al. (1983) in swordfish, Stenström (1985) in cod, Wang and Ogrydziak (1986) in redfish, Ordonez et al. (2000) in hake, Fletcher et al. (2004) and Rudi et al. (2004) in salmon, Lalitha et al. (2005) in tropical perch. In some cases, however, MAP does not seem to increase significantly the LAB number (Debevere and Boskou, 1996). Emborg et al. (2002) showed that P. phosphoreum was the dominant bacterial species in fresh salmon MAP fillets (60% CO₂, 40% N₂). When the fillets were frozen four weeks at –20°C and thawed before packaging, P. phosphoreum, being a very cold-sensitive species, was eliminated so that C. maltaromaticum became dominant. This study illustrates that, in fish, MAP selects both P. phosphoreum and LAB, but the latter are less competitive so often play a minor role in the spoilage. Research corroborating this thesis has also been carried out on garfish by Dalgaard et al. (2006).

3.2. Lightly preserved fish
LPFP which are uncooked or mildly cooked products, with low level of preservatives (NaCl< 6% WP corresponding to approx. aw < 0.96 , pH >5), include carpaccio-type marinated fish, gravads, pickled fish, seafood in brine, cold-smoked fish, peeled shrimp stored in MAP or in brine, etc. LPFP are usually produced from fresh seafood and further processing involves one or a few additional steps that increase risk of cross contamination. The treatments reduce the microbiota load but are not sufficient to eliminate them totally. These highly perishable products are usually stored at chilled temperature under VP or MAP to extend shelf-life. The initial microbiota depends strongly on the hygiene conditions within the company (Gancel et al., 1997; Truelstrup Hansen and Huss, 1998; Leroi et al., 2001) but is often dominated by Gram-negative bacteria typical of fresh fish (Leroi et al., 1998; Paludan-Müller et al., 1998; Gonzalez-Rodriguez et al., 2002b). The addition of NaCl at a concentration of about 5.5 - 6.5% WP, equivalent to an aw about 0.96, which is an inhibitory value for Gram-negative bacteria (e.g. Pseudomonas spp.), allows the growth of other organisms more resistant, i.e. the LAB. This phenomenon is similar to that occurring in dry fermented sausages since the addition of curing salts (salt, sugar, nitrate, nitrite and ascorbate) reduces aw to 0.96. As a result, the Gram-negative bacteria are inhibited while the typical microbiota (LAB and Micrococcaceae) of these products is boosted and it is soon and spontaneously established. At the end of the storage, Gram-positive bacteria, particularly LAB, become predominant, sometimes associated with Enterobacteria and Brochothrix thermosphacta (Truelstrup Hansen et al., 1996; Leroi et al., 2001; Cardinal et al., 2004; Lyhs et al., 2007; Jaffrès et al., 2008). In LPSP, LAB can easily reach 10⁷-⁸ CFU g⁻¹ and such amounts have been found in CSS (Truelstrup Hansen, 1995; Leroi et al., 1998; Leroi et al., 2000), cold-smoked rainbow trout (Lyhs et al., 1998), smoked herring (Gancel et al., 1997), maatjes herring (Lyhs et al., 2007), salted lumpfish roe (Basby et al., 1998), seafood salad (Andrighetto et al., 2009), cooked cold-water shrimp (Dalgaard and Jorgensen, 2000; Dalgaard et al., 2003) and warm-water shrimp (Mejlholm et al., 2005; Jaffrès et al., 2008). In contrast, in gravad rainbow trout, LAB are present but not predominant (Lyhs et al., 2001b; Paarup et al., 2001). More detailed taxonomic studies show that Lactobacillus, mainly sakei and curvatus, and Carnobacterium, mainly maltaromaticum, are most frequently dominant, although Leuconostoc and Lactococcus are also sometimes found. Table 1 summarizes the main species isolated from different LPFP.
LAB have probably long been disregarded in seafood as some genus, especially *Carnobacterium*, grow poorly in the presence of acetate contained in the MRS medium (De Man et al., 1960) classically used to enumerate lactic microbiota. Since less selective media such as Nitrite Actidione Polymixine agar (Davidson and Cronin, 1973) or Elliker (Elliker et al., 1956) have been used, presence of LAB in processed fishery products has been highlighted. 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% WP of salt (Dellaglio et al., 1994; Mauguin and Novel, 1994; Samelis et al., 1994, Seafood Spoilage and Safety Predictor (SSSP) software, v. 3.1 [http://sssp.dtuaqua.dk/]). Conversely, Gram-negative bacteria are more sensitive to NaCl concentration. Although *P. phosphoreum* requires salt for its development (approx. 2.5 %) (Leroi et al., 1998), higher concentration (5% in liquid culture medium) greatly lower their growth (Morii and Kasama, 2004) and no growth occurred at concentration higher than 6% (unpublished data from our laboratory). Same results were observed for *Aeromonas* spp. (Gram, 1991) and *S. putrefaciens* (Le Den, 1995). VP and MAP are probably other factors promoting LAB development since they are anaero-aerotolerant bacteria. Carnobacteria are micro-organisms resistant to freezing that grow very well at refrigerated temperatures, in all packaging conditions and in the presence of many preservatives (Leroi et al., 2000; Laursen et al., 2005), explaining why this genus is very often found in refrigerated VP and MAP meat, poultry or fish products (Holzapfel and Gerber, 1983; Collins et al., 1987; Schillinger and Lüke, 1987; Montel et al., 1991; Millière and Lefebvre, 1994) and LPFP (see table 1). It has been demonstrated, by challenge tests performed in CSS, that *Lactobacillus sakei, alimentarius* and *farciminis* grew faster than *S. putrefaciens, P. phosphoreum, B. thermosphacta* and *Serratia liquefaciens* (Joffraud et al., 2006). On naturally contaminated products, Tomé et al. (2007) have shown that it was possible to select the LAB as the dominant microbiota at the end of the storage period by varying the salting-drying-smoking conditions. The salting and smoking parameters also influence the final LAB composition: with classical salt (5% WP, aw 0.97) and smoke level (10 ppm), the ratio *Carnobacterium/Lactobacillus* was 30/70, and turned to 60/40 with lower smoke concentration (3 ppm) (Leroi and Joffraud, 2000).

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 odors compared to Gram-negative bacteria like *S. putrefaciens, P. phosphoreum* and *Pseudomonas* spp. (Leisner, 1992). Although dominant in LPFP, their role is not very clear. Several authors have found no correlation between total LAB counts and sensory spoilage (Hildebrandt and Erol, 1988; Truelstrup Hansen, 1995; Leroi et al., 2001). 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. The sensory spoilage characteristics due to LAB activity, the compounds responsible and their associated precursors have been summarized by Huss et al. (1995). The carbohydrates fermentation leads to sour and marinated off-odors and flavors due to organic acid production. The amino acids are catabolized by carnobacteria into aldehydes and alcohols and give malty notes. Degradation of proteins by this genus may also conduct to bitter taste. Cysteine and arginine can be metabolized into H₂S and NH₃ respectively, responsible for sulphurous and ammonia off-odors. Citrate is a precursor of diacetyl responsible for dairy and butter-like odors, often produced by *Leuconostoc* and *Lactococcus* species. Some visible changes may also be observed like blowing due to CO₂ as a result of carbohydrate, amino acids and organic acids fermentation by heterofermentative LAB.
The damages caused by lactobacilli are species and strain dependant. Inoculation of bacterial strains in CSS sterilized by ionization and sensory evaluation by a trained panel at the end of storage showed that *L. sakei* was high spoiler while *Lactobacillus alimentarius* had no effect (Stohr et al., 2001). *L. sakei* generally produces sulphurous and acidic odors (Truelstrup Hansen, 1995; Nilsson et al., 1999; Stohr et al., 2001), associated with the production of H$_2$S, acetic acid and ethyl and n-propylacetate (Joffraud et al., 2001). The spoiling capacity depends also on the strain. Indeed, Weiss and Hammes (2006) found *L. sakei* strains that do not affect the organoleptic quality of CSS. In the same way, *L. alimentarius* which does not spoil CSS has been identified as the bacterium responsible for the sensory deterioration of marinated herring (Lyhs et al., 2001a). The role of carnobacteria has been reviewed by Laursen et al. (2005) and Leisner et al. (2007) and is still under discussion. Many studies show that the inoculation of CSS by various strains of *C. maltaromaticum* and *divergens* leads to few or no changes in organoleptic quality (Leroi et al., 1996; Paludan-Müller et al., 1998; Duffes et al., 1999a; Nilsson et al., 1999; Brillet et al., 2005). When the carnobacteria reach a high enough level, flavors of butter and of 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, it has been anticipated that carnobacteria play a role in the spoilage of cooked MAP shrimp as they are part of the dominant microbiota at the time of sensory rejection (Dalgard et al., 2003). This hypothesis has been confirmed by Laursen et al. (2006) who showed that strains of *C. maltaromaticum* and *divergens* inoculated into Arctic shrimp generated strong chlorine, malt and nuts, sour and sickly sweet-off-odors, associated to ammonia and numerous alcohols, aldehydes and ketones. The samples were judged unfit for consumption. However, another *C. maltaromaticum* strain tested in their study did not cause sensory spoilage.

Among the other bacteria frequently found in LPFP, *S. liquefaciens* is very spoiling, releasing odors of amines, cheese, acid or rubber, associated with TMA, dimethyldisulphur, 2,3 butanediol and 2-pentanol (Joffraud et al., 2001). However, *S. liquefaciens* is considered less spoiling than *L. sakei*, as the unpleasant odors are perceived much latter (Joffraud et al., 2006). *B. thermosphacta* also leads to the sensory rejection of the product due to blue cheese and plastic odors, well correlated with 2-heptanone and 2-hexanone. Although strongly spoiling in fresh MAP fish, *P. phosphoreum* seems to play a more moderate role in the deterioration of smoked salmon. Weak acid, amine and feet odors 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) gave much greater weight to the spoiling action of this species. *S. putrefaciens*, the most common spoilage bacteria in fresh fish, and *Vibrio* spp. have never been implicated in spoilage phenomena in CSS, 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. In a sterile CSS model, Joffraud et al. (2006) have shown that the spoilage observed with *L. sakei* was weakened in the presence *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 odors generated in cooked shrimp by an association of *Carnobacterium* spp. and *B. thermosphacta* were different from those due to these two bacteria in pure culture.

These results show that it is over-optimistic to expect to predict the quality of LPSP using only one microbiological or biochemical parameter. However, in the case of CSS, a multiple approach has enabled some authors to correlate quality to several
microbiological and biochemical measurements. Leroi et al. (2001) have put forward a predictive model for the remaining shelf-life of CSS, based on TVBN content and enumeration of the flora on Rogosa medium at pH 5.5 (quite selective for lactobacilli). Another model based on pH and the histamine and tyramine concentrations has been developed by Jorgensen et al. (2000a).

4. Beneficial role of lactic acid bacteria in marine products

4.1. Biopreservation and lactic antagonism

A mixed population of micro-organisms that interact together generally colonizes food. The interactions have been classified on the basis of effects like competition, commensalism, mutualism, ammensalism or neutralism (Viljoen, 2001). In food more than one type of interaction may occur simultaneously leading to the specificity of the final product. Microbial antagonism has currently been observed and people thousands years ago used naturally occurring yeast, moulds and bacteria cultured foods with improved preservation properties. In 1962, Jameson, in studies concerning growth of *Salmonella*, reported the suppression of growth of all micro-organisms on the food when total microbial population achieved the maximum population density characteristic of the food. The same "Jameson effect" has been reported for *Staphylococcus aureus* in seafood (Ross and McMeekin, 1991) and *L. monocytogenes* in CSS (Gimenez and Dalgaard, 2004). Among the different micro-organisms naturally present in food and responsible for pathogens inhibition, LAB are currently listed. The acidification process due to the lactic acid production is one of the most described effect. However, other mechanisms may be involved such as production of inhibitory molecules, redox modification, competition for substrat etc. In CSS, Mejlholm and Dalgaard (2007) have modeled the antagonism effect of naturally occurring LAB against *L. monocytogenes*. The model is entirely empirical and it does not include assumptions about mechanisms of the microbial interaction that take place. Since the nineties, many studies have been conducted on the selection of bacteria with antimicrobial properties that could be inoculated at high level in seafood in order to inhibit the growth of undesirable micro-organisms. This technology is termed biopreservation.

LAB are good candidates for this technology as they produce a wide range of inhibitory compounds (organic acids, hydrogen peroxide, diacetyl and bacteriocins). In addition, they often have the GRAS status granted by the US-FDA and benefit from the healthy image associated with dairy products (Rodgers, 2001). Although a great deal of work has been done on the selection of bacteria exhibiting antimicrobial properties in liquid medium and the number of bacteriocins characterized is increasing every day, very few commercial applications have appeared in seafood products. A major hurdle is that these products are not fermented and the selected LAB strain should not change their organoleptic and nutritional qualities. Many bacteria that gave promising results in liquid medium proved to be ineffective in products, either because they were poorly established in the environmental conditions (Wessels and Huss, 1996), or because they produced unpleasant odors (Nilsson et al., 1999). Nevertheless, since the importance of LAB in semi-preserved fish has been highlighted, research into this subject has intensified to prevent growth of pathogenic and spoiling bacteria.

4.1.1. Safety risk

The major microbial risks associated with LPFP are *Clostridium botulinum* type E and *L. monocytogenes* (Beldsoe et al., 2001). The growth of *C. botulinum* type E is
adequately controlled by the combination of salt (3.5% WP) and low temperature (less than 5°C). Contrastingly, *L. monocytogenes* can grow at chilled temperature (0°C) and support low pH (4.5) and aw (0.92). *L. monocytogenes* is a pathogenic bacteria responsible for listeriosis, which is primarily a food-borne disease. The prevalence of *L. monocytogenes* in LPFP such as CSS, cold-smoked trout and shrimp is highly variable but quite elevated, varying from 2 to 60% depending of the studies (Jorgensen and Huss, 1998; Valdimarsson et al., 1998; Nakamura et al., 2004; Gudmundsdöttr et al., 2005; Hu et al., 2006; Beaufort et al., 2007). *L. monocytogenes* may be present in raw material in low number but contamination mainly occurs during processing (Eklund et al., 1995; Rorvick et al., 1995). A strict hygienic manufacturing practice has been emphasized 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 (Ribeiro Neunlist et al., 2005). 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 is 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 (Cornu et al., 2006) and it can sometimes overpass the European tolerated limit of 100 CFU g⁻¹ (CE 2073/2005). Therefore, the use of protective culture may be a useful hurdle to prevent *L. monocytogenes* development in the food matrix and thus control the safety risk. Many studies have been performed in liquid model medium but results have not been validated in real products. In this review, we will only consider succesful data obtained in seafood products. Carnobacteria, communly found in seafood, are non aciduric bacteria with low spoiling potential, explaining why most of the promising results have been obtain with this genus. Nilsson (1999) have isolated two strains of *C. maltaromaticum* able to limit the growth of *L. monocytogenes* for 31 days at 5°C without modification of the sensory quality. At the same time, Duffes et al. (1999b) have isolated three carnobacteria with anti-listerial properties. In CSS, *C. piscicola* V1 was bactericidal against *L. monocytogenes* at 4 and 8°C (*L. monocytogenes* count decreased from 6.3 x 10² CFU g⁻¹ to less than 10 CFU g⁻¹ whereas *L. monocytogenes* alone reached 6.3 x 10⁴ and 1.2 x 10⁸ CFU g⁻¹, respectively at 4 and 8°C). The inhibition lasted for 28 days. *C. divergens* V41 presented a bacteriostatic effect. *C. piscicola* SF668 delayed *L. monocytogenes* growth at 8°C and had a bacteriostatic effect at 4°C (Duffes et al., 1999a). In the presence of the strain *C. maltaromaticum* CS526 isolated from surimi, the population of *L. monocytogenes* in CSS decreased from 10³ to 50 CFU g⁻¹ after 7 days at 4°C (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⁻¹ of the pathogenic bacteria after 21 days of storage at 4°C (Tahiri et al. 2009). However, all those tests are rarely repeated on different strains of *L. monocytogenes* and the consequences of adding a protective culture on the microbial ecosystem and on the sensory characteristics of the final product are not always taken into account. To our knowledge, the most promising results have been achieved by Brillet et al. (2004, 2005). The three strains *C. divergens* V41, *C. piscicola* V1 and *C. piscicola* SF668 previously listed were screened for their anti-listerial activity against a collection of 57 *L. monocytogenes* strains selected from the French smoked salmon industry, using an agar spot test. All the Listeria strains were inhibited but three different groups could be distinguished differing in sensitivity to the three *Carnobacterium* strains. However, *C. divergens* V41 always had the highest inhibitory effect. The anti-listerial capacity was then tested in sterile CSS blocks co-inoculated with the three *Carnobacterium* and mixtures of *L. monocytogenes* strains. *C. divergens* V41 was the most efficient strain, maintaining the level of *L. monocytogenes* at <50 CFU g⁻¹ during the 4 weeks of VP storage at 4
and 8°C, whatever the sensitivity of the set of *L. monocytogenes* strains. In addition, these authors clearly showed that *C. divergens* V41 did not acidify the product nor change its organoleptic properties. This strain is actually tested by a company for a commercial application. Among the other LAB genus, most of the tests have been performed against *Listeria innocua*, excepted Katla et al. (2001) who have shown that *L. sakei* Lb790 was able to stopped *L. monocytogenes* growth in CSS for 28 days at 10°C. Another strain of *L. sakei* allowed a 4 log reduction of *L. innocua* after 14 days at 4°C (Weiss and Hammes, 2006) but confirmation with *L. monocytogenes* has been done only in CSS juice. Vescovo et al. (2006) reduced *L. innocua* count by 3.3 and 2.8 log respectively with strains of *Lactobacillus casei* and *L. plantarum* without affecting negatively the sensory quality of the product. Tomé et al. (2008) have done test in CSS with LAB inoculated before processing. Interestingly, a great inhibitory effect of *Enterococcus faecium* ET05 against *L. innocua* was observed just after smoking and lasted, or still increased, during 21 days at 5°C. Although less effective, *Lactobacillus curvatus* ET30 and *Pediococcus acidilactici* ET34 showed a bacteriostatic effect on *L. innocua*.

The inhibition mechanism varies according to the protective cultures. Many strains isolated and tested in seafood products produce bacteriocins with anti-listerial activity (Drider et al., 2006; Tomé et al., 2008; Pinto et al., 2009; Tomé et al., 2009). In the case of *C. divergens* V41, Richard et al. (2003), using a chemical mutant divercin -, clearly showed that the inhibition in CSS was due to the production in situ of divercin V41. Comparing their results with non-bacteriocin producing strains, Yamazaki et al. (2003) and Tahiri et al. (2009) also concluded that the inhibitory effect of *C. maltaromaticum* CS526 and *C. divergens* M35 respectively was due to the production of pisciococin CS526 and divergicin M35, two class IIa bacteriocins caracterised by Yamazaki et al. (2005) and Tahiri et al. (2004). In contrast, Nilsson et al. (1999) isolated a strain of *C. maltaromaticum* that inhibited *L. monocytogenes* without producing bacteriocin. Competition with respect to glucose contributed to the anti-listerial activity (Nilsson et al., 2004; Nilsson et al., 2005). Similarly, conclusive effects can be found in the literature for *Lactobacillus* strains that either do or do not produce bacteriocin (Katla et al., 2001). Using the bacteriocin directly in the food can also be considered as a biopreservation strategy (Galvez et al., 2007). In CSS, Duffes et al. (1999a) showed an immediate reduction with divercin V41 that did not last during storage at 8°C. The bacteriocin may be destroyed by endogenous fish proteases so other ways of incorporating them, either in capsule form or attached to the packaging, are currently being investigated (Calo-Mata et al., 2008).

4.1.2. Shelf-life

Another important challenge is to reduce food loss which one very often due to development of spoiling micro-organisms. However, there have been very few conclusive attempts to control the spoilage microbiota with protective culture. Leroi et al. (1996) significantly prolonged the use-by date of commercial CSS by inoculating strains of *Carnobacterium* sp. but the results varied depending on the batch treated. *C. divergens* V41 with antilisterial properties had no effect on the other microbiota and did not delay the spoilage of CSS (Brillet et al., 2005). Similarly, no improvement was found in cooked shrimp sprayed by a *Carnobacterium* sp. previously selected in model conditions for its capacities to inhibit *B. thermosphacta*, a major spoiling bacteria of shrimp (Laursen et al., 2006). Improving the quality of marine products with LAB is probably more difficult than controlling the development of a pathogen because spoilage is the consequence of a complex ecosystem composed of different species that vary from one product to another. Consequently, current work is focusing on the screening for other LAB species with wide antimicrobial spectrum. Recently, Matamoros et al. (2009b) have isolated seven strains from various marine products, which are active against many spoiling, pathogenic, Gram-positive and -
negative marine bacteria. Some of them (Leuconostoc gelidum and L. piscium) have shown a very promising effect in delaying the spoilage of naturally contaminated tropical shrimp and VP CSS (Matamoros et al., 2009a). However, no correlation with the classical quality indices measured was evidenced. Recently, Fall et al. (2010a) evidenced the in situ inhibition of B. thermosphacta, a major spoiling bacteria, by L. piscium that could explain the protective effect observed in shrimp. Additionally, those strains also showed an inhibitory effect on L. monocytogenes (Fall et al., 2010b) and S. aureus. Altieri et al. (2005) succeeded in inhibiting Pseudomonas spp. and P. phosphoreum in VP fresh plaice fillets at low temperatures by using a Bifidobacterium bifidum starter, and extending the shelf-life, especially under MAP. Some safety aspects for the bioprotective bacteria have also to be taken into consideration for a food application. The production of histamine must be checked, as it is a regulated compound in fish rich in histidine, leading to allergic-like syndromes. In seafood products, the production of histamine linked to lactobacilli and carnobacteria (Jorgensen et al., 2000b; Emborg et al., 2002; Brillet et al., 2005) or L. gelidum and L. piscium isolated by (Matamoros et al., 2009b) has never been reported.

Despite the promising results obtained on L. monocytogenes reduction and shelf-life extension, the use of protective culture in fish industry is not usual compare to dairy and meat products. A small French company commercializes a strain of patented Lactococcus lactis (patent n° PCT/FR02/03180, 2001) for an application in shrimp, but most of the biggest companies producing microbial starters do not sail LAB for a specific seafood application. The strains that could be used in seafood, such as the previously described Carnobacterium spp., Lactobacillus spp., Lactococcus spp. or Leuconostoc spp., have frequently been found in marine products at high level, but human has not traditionally used them for preservation of fish. Their presence in seafood and their potential advantages for management of the quality and safety have been discovered only in the last 20 years. The low carbohydrate concentration in fish and the fact that the naturally dominating carnobacteria are not aciduric probably explains why those bacteria have long been disregarded. However, those characteristics may be considered as an advantage to improve quality and safety of LPFP without changing their delicate flavor and texture.

With the exception of those encompassed by the Novel Food Regulation (1997), micro-organisms used for fermentation of food were not subject to regulation in Europe. On 19 November 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. A list of micro-organisms judged suitable for QPS status has been published in the EFSA journal (2008). It contains 73 species of micro-organisms, among them 47 LAB species belonging to genus Bifidobacterium, Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus and Propionibacterium. Additionally, microbial food cultures with a long history of safe use are considered as traditional food ingredients and are legally permitted for use in human food in the EU without pre-market authorization. C. maltaromaticum and divergens, L. piscium or L. gelidum pointed out as potential bioprotective bacteria for seafood products are not included in this list. Exclusion from this list does not necessarily imply any risk associated with its use. Many micro-organisms commonly encountered in food production were not considered because they are not presently the subject of pre-market authorizations and so would not be notified to EFSA. The QPS list is supposed to be annually updated. With proof of their beneficial effect, precise taxonomic data and strong evidence of safety, it is reasonably conceivable that new bioprotective strains for marine products obtain the QPS status if notified to EFSA.
4.2. Probiotics

Probiotics are living micro-organisms, generally LAB, which, when they are ingested in sufficient quantities, exert a positive effect on health such as regulation of transit (diarrhea or constipation), stimulation of the immune system and reduction of the digestive inflammatory diseases. Most of the studies concerning probiotics for human have been performed on LAB isolated from dairy products (Ebringer et al., 2008), probably because they have been ingested for thousands of years by human through traditional products like yogurt, cheese, koumiss, kefir etc. Few examples of probiotics available in the form of fermented milk products, dietary supplements or medicinal products are strains of *Bifidobacterium lactis*, *B. animalis*, *Lactobacillus casei*, *L. acidofilus*, *L. rhamnosus*, *L. johnsonii*, *L. reuteri* and *Enterococcus faecium*. The yeast *Saccharomyces boulardii* is also available as commercial preparation on the probiotic market.

LPFP, which are the main category of marine products containing viable LAB, have never been considered as probiotic for human, because they are not eaten in sufficient quantity. For this reason, incorporating new strains into seafood does not seem a realistic approach. However, the efficacy of some strains isolated from those products could be investigated to develop new dietary supplement. In case of strong evidence of beneficial effect for human, evaluation as to whether those strains share the safety status of traditional food-grade organisms should be carefully assessed. These last years some work has been carried out on the use of LAB as probiotic for living fish. Many studies still remain at an *in vitro* level (Ringo, 2008; Ma et al., 2009) and some promising strains did not confirmed their effect in living fish (Spanggaard et al., 2001). However, some authors have demonstrated the *in vivo* potential of LAB, and Lauzon et al. (2008) have emphasized the importance and potential of LAB in aquaculture. A strain of *Carnobacterium* sp. isolated from the intestines of Atlantic salmon has been successfully implanted in salmonids and reduced the diseases due to *Aeromonas salmonicida*, *Vibrio ordalli* and *Yersinia ruckeri* (Robertson et al., 2000). *Lactobacillus fructivorans* isolated from seabream intestinal microbiota significantly improved survival of seabream larvae and fry, and stimulated the immune system (Picchietti et al., 2007). Merrifield et al. (2010) and Ringo et al. (2010) have reviewed the application of favorable LAB for salmonids as pre and probiotic. Despite the successful proof of these concepts, it is still difficult to find a strategy applicable at the industrial level farming. The science of probiotic in aquaculture is in its infancy (Azad and Al-Marzouk, 2008) and there is a real issue in developing feed with beneficial effect for fish.

4.3. Fermentation

Unlike in dairy, meat, cereal and plant products, LAB are not often used for fermenting marine products. There are some traditional fermented products, like anchovies placed in a barrel with salt and sugar for a curing process, and fish sauces being very popular in Asiatic countries. Recently in Japan, fish sauce production has increased dramatically (quadruple the amount produced five years ago) as the fishing industry tries to reduce waste by making full use of fish materials. Many fish sauces are made of small seawater fish with long term fermentation of more than a year. Halophilic LAB, mainly *Staphylococcus* spp. and *Tetragenococcus* spp., were isolated as dominant bacteria during fermentation (Taira et al., 2007) but their real role is quite controversial. Much of the degradation is essentially due to the presence of endogenous enzymes in fish. However, it is clear that certain salt tolerant strains of *Tetragenococcus halophilus* and *T. muriaticus* contribute to lower the pH value and reduce the risk of putrefaction of the fermenting sauce, especially when an extra source of carbohydrate is added, like soy sauce *koji* (Uchida et al., 2005). On the other hand, this positive effect can be balanced by the fact that *T. halophilus* is an
histamine producer, and levels of 1000 ppm have been recorded in some fish sauces (Satomi et al., 2008). Traditional lightly salted fermented fish products are widespread in South-East Asia. They are typically composed of freshwater fish species, salt (2–7%), a carbohydrate source (rice, millet, sugar or fruit) and spices (garlic, ginger, chilli, pepper). The mixture is tightly packed in banana leaves or plastic bags and left to ferment for two to five days at 30°C before being consumed as main course or as a snack. In those kind of products Paludan-Müller et al. (1999) have shown than LAB isolated from fish raw material were dominated by L. lactis subsp. lactis. However, the LAB responsible of the fermentation were mostly of vegetable origin, dominated by aciduric heterofermentative Lactobacillus spp. and homofermentative Lactobacillus plantarum/pentosus. There have also been some attempts to develop new fermented marine products, like salted salmon fillets inoculated with different commercial lactobacilli starters and with sugar added (Morzel et al., 1997). Nevertheless, this research is still at the laboratory stage and the products, to our knowledge, have not been commercialized.

Fish is a source of high quality proteins, essential minerals, vitamins and polyunsaturated fatty acids. Some recent works, therefore, are concerned with the development of new products that ideally should retain all the nutritional properties of fish but not its typical odor so that they can be included in meat-based preparations. Glatman et al. (2000) and Gelman et al. (2001) have inoculated yellowfin tuna with high level of L. plantarum, Pediococcus pentosaceus and L. mesenteroides and they showed that a strain of L. mesenteroides could effectively ferment tuna flesh, making it loose its character and develop new odors and texture close to those of meat, and additionaly reduced the level of histamine. Best results were obtained after 5 weeks of fermentation at 8°C.

The silage of fish has been investigated since the 1950s and should have provided a commercial alternative for some of the by-products of the fish industry. Different strains of L. plantarum, L. acidophilus, Pediococcus halophilus and P. acidilactici have been tested, with some success, for their ability to lower the pH of fish flesh rapidly. However, the low level of sugar in the matrix requires a carbohydrate supplement, such as starch, malt or molasses to be added. Nevertheless, in the anaerobic conditions favoring the LAB fermentation, the risk of contamination by Bacillus and Clostridium cannot be avoided. Paradoxically in presence of air, yeasts and moulds may then degrade the product. An avenue of research for developing these products is to look for psychrotrophic strains suitable for the very rapid fermentation of fish. Yoon et al. (1997) described the characteristics of LAB for the preparation of silage from tuna viscera. Successful studies have been performed on biotransformation of salmon (Bower and Hietala, 2008) and tuna waste (Vijayan et al., 2009) as novel aquafeed ingredient for fish.

5. Conclusion

The characteristics of fish flesh favor the growth of psychrotrophic Gram-negative bacteria that are much more competitive than LAB in this matrix. 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 VP or MAP fish fillets, carpaccio, cold-smoked fish, peeled and mildly cooked shrimp, etc., meet those requirements and their production has increased dramatically those last years. In those products, the environmental conditions favor the development LAB, explaining the high interest for
this bacterial group in the last decade. Whether the presence of LAB in the final product is from fish origin or post-contamination during processing is not yet established and more detailed studies, as it has been done for L. monocytogenes in some fish industry, should be done to better understand and control the route of contaminations. The role of LAB in marine products is complex, depending on the fish species, treatment and storage conditions, bacterial species and strains, and interaction between the bacteria. Sometimes, LAB have no particular negative effect, but in certain cases they are responsible for strong sensory degradation, leading to rejection of the products. The use of LAB in the fish industry is not extensively developed, except in Asia for preparation of fish sauces and traditional food with fermented mixture of fish and vegetable. In most cases, fermentation is due to LAB naturally present in the fish or in the carbohydrate added sources (vegetables, garlic etc.), and no selected starter is added to control the fermentation. Those last years, the bioprotective potential of endogenous LAB in relation to pathogens has been highlighted. Increasing numbers of studies are aiming to exploit this ability to control the quality and the safety of marine products and some industry producing LAB starters are currently testing some bacteria for a fish application. However, this technology is still in its infancy compared to dairy products. The LAB have not been traditionally used in seafood for technological applications so the strains now available have not still received the QPS status. Moreover, combining bioprotection of seafood with no modification of the sensory characteristics of the product still remains a challenge. An application of marine LAB as probiotic for human does not seem realistic in the next few years as many strains are psychrotrophic bacteria that do not support temperature higher that 30°C. Moreover, seafood products are not consumed in a high quantity enough to naturally observe a positive effect. However, some Lactobacillus and Carnobacterium strains easy to cultivate and resistant to various conditions could be studied. Their production on fish protein hydrolysates and ingestion as dietary supplement may combine the beneficial effect of fish and LAB. Finally, very encouraging results have been obtained with the use of marine LAB as probiotic for fish, and this is probably a way of rapid development for the marine LAB market.

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Table 1
Principal lactic acid bacteria species isolated from lightly preserved seafood products.

<table>
<thead>
<tr>
<th>Lactic acid bacteria</th>
<th>Products</th>
<th>References</th>
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<tbody>
<tr>
<td>Carnobacterium sp.</td>
<td>Salted lumfish</td>
<td>Basby et al. (1998)</td>
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<td></td>
<td>Cold-smoked salmon</td>
<td>Leroy et al. (1998)</td>
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<td></td>
<td>Gravad rainbow trout</td>
<td>Leroi et al. (1998)</td>
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<td></td>
<td>Brine shrimp (preservatives)</td>
<td>Paludan-Müller et al. (1998)</td>
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<td>Cold-smoked salmon</td>
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<td>Carnobacterium divergens</td>
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<td>Paludan-Müller et al. (1998)</td>
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<td>Gravad rainbow trout</td>
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<td>Brine shrimp preservatives</td>
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<td>Seafood salad</td>
<td>Mejholm et al. (2005)</td>
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<td></td>
<td>Cooked MAP shrimp (preservatives)</td>
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<td></td>
<td>MAP rough head grenadier</td>
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<td>Seafood salad</td>
<td>Lyys et al. (1999)</td>
</tr>
<tr>
<td>Leuconostoc pseudomesenteroides</td>
<td>Cold-smoked salmon</td>
<td>Andrighetto et al. (2009)</td>
</tr>
<tr>
<td>Leuconostoc gelidum</td>
<td>MAP salmon steak</td>
<td>Truestrup Hansen and Huss, (1998)</td>
</tr>
</tbody>
</table>

(continued on next page)