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Selection and evaluation of seafood-borne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria

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ABSTRACT

In this study, inhibitory psychrotrophic lactic acid bacteria were isolated and investigated for future use in biopreservation of seafood products. Screening of 5575 colonies isolated from various seafood products resulted in the selection of 132 colonies presenting inhibitory properties. Among them, 52 isolates had characteristics of LAB and showed growth at 15 °C but not at 30 °C. The inhibition spectrum of these 52 isolates against 14 target strains (Gram-positive and -negative) showed inhibition of typical seafood spoiling and pathogenic bacteria and enabled the formation of seven interesting clusters. Sequencing of the 16S rRNA gene of a representative isolate from each cluster identified three *Leuconostoc gelidum*, two *Lactococcus piscium*, one *Lactobacillus fuchuensis* and one *Carnobacterium alterfunditum*. Theses strains did not produce histamine nor tyramine, and showed no particular antibiotic resistance profile. Growth rate as a function of temperature was tested for one *L. piscium* and one *L. gelidum* isolate and confirmed their psychrotrophic behavior. One out of seven isolates showed bacteriocin-like activity. The inhibition mechanisms of the other isolates are still unknown but may be due to competition for substrate. Absence of a bacteriocin-like component could be a positive point to gain rapid authorization for food application in France. This collection of LAB is now ready for testing on products.

1. Introduction

Biopreservation is an innovative way of extending the shelf-life of food products and reducing microbial risks. Biopreservation consists of the inoculation of food products with selected bacterial strains able to inhibit the growth of undesirable bacteria (for a review see Rodgers, 2001). Lactic acid bacteria (LAB) are particularly interesting candidates for this technique. Indeed, they are frequently naturally present in food products and are often strong competitors, by producing a wide range of antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl and bacteriocins. They are generally recognized as safe microorganisms (Adams, 1999) and benefit from the healthy image of many dairy products.

Although biopreservation is currently applied in fermented food, there are few examples in non-fermented products such as seafood. Some selected microorganisms that have given good results in a model medium are not efficient in seafood because they do not grow in this low-sugar content matrix, are not adapted to a chilled temperature or spoil the product (Wessels and Huss, 1996). Most of the successful studies in marine products have been obtained on the inhibition of *Listeria* spp. by different species of LAB, mainly from the *Carnobacterium* genus (Nilsson et al., 1999; Katla et al., 2001; Yamazaki et al., 2003; Brillet et al., 2004, 2005; Vescovo et al., 2006), which is due to either bacteriocin production (Richard et al., 2003) or competition mechanisms (Nilsson et al., 2005). However, other endogenous pathogenic bacteria, such as *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*, *Clostridium botulinum*, histamine-producing bacteria, and post-contaminating bacteria, such as *Staphylococcus aureus* or *Salmonella* spp., can be associated with seafood safety and require special attention (Feldhusen, 2000; Huss et al., 2000; Sumner and Ross, 2002). Moreover, due to their high content of low-molecular weight nitrogenous compounds, their neutral pH and high water activity value, seafood products are also extremely sensitive to microbial spoilage. *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Aeromonas* spp. and *Pseudomonas* spp. are the main spoilers of fresh fish products stored in air or under vacuum or modified atmosphere packaging (MAP) (Gram and Huss, 1996; Gram and Dalgaard, 2002). In lightly preserved fish products (NaCl $< 6\%$ in water phase, pH > 5) like cold-smoked fish, other microorganisms such as LAB, Enterobacteriaceae, *Brochothrix thermosphacta* and *Vibrio* spp. can contribute to spoilage (Jorgensen et al., 2000; Leroi

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et al., 2001; Stohr et al., 2001; Joffraud et al., 2006). In order to develop the biopreservation technology to improve quality and extend the shelf-life of seafood, the selection of LAB that show inhibitory properties against both pathogenic and spoilage bacteria at chilled temperatures is necessary.

In this study, a large selection of seafood products was screened for the presence of LAB able to grow at 15 °C but not at 30 °C and to inhibit at least one out of four target strains (*Listeria monocytogenes*, *Staphylococcus xylosus*, *Pseudomonas* sp., *Serratia liquefaciens*). The selected isolates were then tested against fourteen new target strains, Gram-positive and -negative, spoiling and pathogenic bacteria, isolated from marine products. The most promising LAB were identified and their inhibition mechanisms tentatively explained. Growth profile as a function of temperature was determined to characterize their psychrotrophic behavior. Biogenic amines production and antibiotic resistance were tested to ensure the safety of the strains for future use in biopreservation of seafood products.

2. Materials and methods

2.1. First screening: isolation of inhibitory bacteria from seafood products

Fifty-one samples of twenty-seven different commercial seafood products listed in Table 1 were obtained from different supermarkets (Nantes, France). They were stored at 4 or 8 °C and opened for analysis between 10 days before and 10 days after the use-by date. Aliquots (30 g) of each product were stomached in physiological water (5-fold dilution) (1 g l^{-1} tryptone [Biokar Diagnostics, Beauvais, France], 8.5 g l^{-1} NaCl), four successive decimal dilutions were prepared and 0.1 ml of the mother suspension and each dilution were spread on Elliker agar plates (four plates per dilution) (48.5 g l^{-1} Elliker [Biokar Diagnostics], 15 g l^{-1} agar [Biokar Diagnostics]). Plates were then incubated in anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) at 8 °C for 10–15 days. At that time, plates presenting between 10 and 15 colonies were selected for a double-layer inhibition test.

Table 2
Target strains, culture medium and growth temperature for two successive pre-cultures (72 and 24 h) for double-layer realization.

Species	Code[a]	Medium[b]	Temperature
Bacillus subtilis	DSMZ10	BHB	37 °C
Brochothrix thermosphacta	EU2206	BHB	20 °C
Clostridium sporogenes	ENITIAA	RCM	37 °C
Escherichia coli	CIP76.24	BHB	37 °C
Lactobacillus farciminis	EU2204	BHB	20 °C
Listeria monocytogenes	EU2160	BHB	20 °C
Photobacterium phosphoreum	EU2183	BHB + 15 g l^{-1} NaCl	15 °C
Pseudomonas group I	EU2189	BHB	20 °C
Psychrobacter spp.	CCUG 42949	BHB	20 °C
Salmonella enterica	CIP81.3	BHB	37 °C
Serratia liquefaciens	EU2196	BHB	20 °C
Shewanella putrefaciens	EU2187	BHB	20 °C
Staphylococcus aureus	CIP76.25	BHB	37 °C
Staphylococcus xylosus	DSMZ 20029	BHB	37 °C

[a] CCUG: Culture Collection, University of Göteborg, Sweden; EU: HURDLETECH collection established during the European SEAFOODplus project, stored at IFREMER, Nantes, France; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; CIP: Collection de l'Institut Pasteur, Paris, France; ENITIAA: collection from ENITIAA, Nantes, France.

[b] BHB: Brain–Heart Broth (Biokar Diagnostics, Beauvais, France); RCM (Reinforced *Clostridium* medium, prepared according to Oxoid recommendations): 3 g l^{-1} yeast extract (Biokar Diagnostics), 10 g l^{-1} meat extract (Oxoid Ltd, Basingstoke, Hampshire, England), 10 g l^{-1} peptone (Oxoid Ltd), 5 g l^{-1} glucose (Merck), 1 g l^{-1} potato starch (La Bovida, Nanterre, France), 5 g l^{-1} NaCl (Merck), 3 g l^{-1} sodium acetate (Merck), 0.5 g l^{-1} cysteine hydrochloride (Sigma–Aldrich, St Quentin Fallavier, France), 0.5 g l^{-1} agar (Biokar Diagnostics). The pH was adjusted at 6.9 before autoclaving.

Four target strains from the HURDLETECH collection (collection established during the Integrated Project (IP) SEAFOODplus contract No. FOOD-CT-2004-506359) stored at IFREMER (Nantes, France) were selected for the first inhibition test: *L. monocytogenes* (EU2160), *S. xylosus* (DSMZ 20029), *Pseudomonas* group I (EU2189) and *S. liquefaciens* (EU2196). These strains were pre-cultivated 48 h in Brain–Heart Broth (37 g l^{-1} BHB, Biokar Diagnostics) as described in Table 2 before being diluted and added to molten soft BHB agar (37 g l^{-1} BHB, 10 g l^{-1} agar) and then spread on an isolation plate

Table 1
Number of colonies screened and selected per product.

Product name	Colonies covered	Colonies showing inhibition[b] (%)	Isolated colonies	Selected isolates based on growth temperature[c]	Selected isolates based on LAB characteristics[d]
MAP[a] salmon	718	151 (21%)	48	39	39
MAP[a] sea-bream	240	11 (4%)	13	7	5
Cold-smoked salmon	554	221 (40%)	37	5	5
MAP[a] rough-head grenadier	520	24 (4%)	7	3	3
MAP[a] shrimp	157	26 (16%)	11	0	0
Smoked haddock	217	4 (2%)	4	0	0
Roe cod (tarama)	174	0 (0%)	4	0	0
MAP[a] whiting	456	14 (3%)	3	0	0
Smoked herring	300	2 (1%)	2	0	0
Smoked shark	476	2 (0.5%)	2	0	0
Sea-bream viscera	184	1 (0.5%)	1	0	0
Red mullet viscera	371	0 (0%)	0	0	0
Smoked tuna fish	368	0 (0%)	0	0	0
Smoked trout	354	0 (0%)	0	0	0
Mackerel viscera	198	0 (0%)	0	0	0
Salmon carpaccio	128	0 (0%)	0	0	0
Smoked mackerel	121	0 (0%)	0	0	0
Herring viscera	39	0 (0%)	0	0	0
Salted cod, shrimp (fresh), roe lumpfish, roe salmon, marinated sardines, anchovy, mussel, pickled shell fish, pickled herring	0	0 (0%)	0	0	0
Total	5575	456 (8%)	132	54	52

[a] MAP: modified atmosphere packaging.

[b] Number of colonies showing inhibition of at least one target strain among *Listeria monocytogenes* (EU2160), *Staphylococcus xylosus* (DSMZ 20029), *Pseudomonas* group I (EU2189) and *Serratia liquefaciens* (EU2196). In bracket, percentage of colonies showing an inhibition.

[c] Isolates able to grow at 15 °C but not at 30 °C.

[d] Gram-positive, catalase- and oxidase-negative isolates, able to grow at 15 °C but not at 30 °C.