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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*. These 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.

**Keywords:** Lactic acid bacteria; Seafood; Inhibition; Psychrotrophic; *Lactococcus piscium*; *Leuconostoc gelidum*; *Lactobacillus fuchuensis*; *Carnobacterium alterfunditum*

## 1. Introduction

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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*, *V. vulnificus* and *V. 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 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 (*L. monocytogenes*, *St. xylosum*, *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 behaviour. 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

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### 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<sup>-1</sup> tryptone

[Biokar Diagnostics, Beauvais, France], 8.5 g l<sup>-1</sup> 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<sup>-1</sup> Elliker [Biokar Diagnostics], 15 g l<sup>-1</sup> agar [Biokar Diagnostics]). Plates were then incubated in anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) at 8°C for 10 to 15 days. At that time, plates presenting between 10 to 15 colonies were selected for a double-layer inhibition test.

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), *St. xylosus* (DSMZ 20029), *Pseudomonas* group I (EU2189) and *Se. liquefaciens* (EU2196). These strains were pre-cultivated 48 h in Brain Heart Broth (37 g l<sup>-1</sup> BHB, Biokar Diagnostics) as described in Table 2 before being diluted and added to molten soft BHB agar (37 g l<sup>-1</sup> BHB, 10 g l<sup>-1</sup> agar) and then spread on an isolation plate showing growth of 10 to 15 colonies. The target strains were tested separately: one strain per plate. Plates were then incubated for 24 h at the specific target strain growth temperature (Table 2) in order to obtain a regular lawn. The presence of an inhibition zone was checked visually. Based on the colony appearance, approximately one out of three colonies per plate showing an inhibition zone was picked up and cultivated in Elliker broth at 15°C. Selected isolates were isolated twice on Elliker agar plates at 15°C, and frozen in Elliker containing 10 % v/v glycerol (Panreac Quimicia SA, Barcelona, Spain) at -80°C.

## **2.2. Second screening : selection of psychrotrophic lactic acid bacteria among the inhibitory isolates**

The selected isolates were cultivated at 15 and 30°C in Elliker broth in order to check their psychrotrophic characteristic. Growth was monitored visually by checking turbidity after 48 h and one week of culture. Isolates presenting growth at 15°C but no growth at 30°C after one week were selected and tested for three characteristics: Gram, catalase and oxidase tests. Only presumptive LAB i.e. Gram-positive, catalase- and oxidase-negative isolates were selected for further studies.

## **2.3. Third screening: inhibitory spectrum and partial identification of the selected LAB isolates**

Investigations of the inhibitory properties of the selected isolates was enlarged to fourteen spoilage, pathogenic or surrogate target strains relevant to seafood products (listed in Table 2).

Tested LAB isolates were cultivated for 48 h in Elliker broth at 15°C before being spotted onto the surface of Elliker plates (10 µl per spot, six spots per plate) and plates were incubated anaerobically for 10 days at 8°C. A *Lactobacillus curvatus* strain from the IFREMER collection (SF762) that has no inhibitory properties against the fourteen target strains was included in the experiment as a negative control. The 14 target strains were pre-cultivated in culture medium and temperature conditions specified in Table 2. They were then individually added to 15 ml of the same culture medium containing 10 g l<sup>-1</sup> of molten agar. Molten soft agar was then spread onto previously spotted and incubated Elliker plates. After 24 h of aerobic incubation at the target strain appropriate temperature, plates were examined for evidence of inhibition. The size of the inhibition zone was recorded and a score from 0 to 4 was given as follows: 0 for no inhibition, 1, 2, 3 and 4 for an inhibition halo diameter of 1, 2, 3 and 4 cm respectively.

Ward's hierarchical clustering method with squared Euclidian distance was used to separate the 52 LAB isolates into groups according to the size of their inhibition zone for the 14 target strains (Uniwin plus, version 4.01, Sigma plus, Paris, France).

Partial identification of the isolates was based on cell morphology and lactic acid configuration using the Microzym® system (Biosentec, Toulouse, France) after 48 h of growth in Elliker broth at 15°C and 16S-23S intergenic region profile as described by Kabadjova et al. (2002).

Identification of *Leuconostoc* species was performed by RFLP patterns as described by Kim et al. (2003) using endonuclease BsmAI (Biolabs). DNA from *Ln. gelidum* DSM5578 (DSMZ, Darmstadt, Germany) and *Ln. inhae* DSM 15101 were used for comparison.

#### **2.4. Identification of 7 bioprotective lactic acid bacteria**

The sequencing of the 16S rRNA gene of seven selected biopreservative isolates was performed. After 48 h of culture, cells were sedimented by centrifugation, washed and re-suspended in T100E buffer (100 mmol l<sup>-1</sup> Tris-HCl, 10 mmol l<sup>-1</sup> EDTA). Chromosomal DNA was prepared from the suspension using the acetate method (Weisburg et al. 1991).

The primers used for the amplification of the 16S rRNA gene were fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-TAAGGAGGTGATCCAGCC-3') (Weisburg et al. 1991). The PCR amplified DNA fragments with primers fD1 and rD1 contained the complete 16S rRNA gene. PCR reactions were performed in a PTC-100 thermocycler in a total volume of 50 µl containing 1X PCR buffer, 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 1 µg ml<sup>-1</sup> DNA, 0.8 µmol l<sup>-1</sup> each primer, 0.2 mmol l<sup>-1</sup> (each) dNTP and 2U Taq DNA polymerase (New England Biolabs, Hitchin, UK). Amplification consisted of a 60 s denaturation step at 94°C, a 60 s annealing step at 56°C and a 60 s extension step at 72°C. The first cycle was preceded by incubation for 5 min at 94°C. After 35 cycles, there was a final 7 min extension at 72°C. Negative controls containing no DNA template were included in parallel. The nucleotide sequence of the amplified 16S rRNA gene was determined with an ABI 370 automated sequencer using the Taq Dye-Deoxy TM terminator cycle sequencing method (Genome Express company, Meylan, France). Anticipated errors of PCR and sequencing reactions were avoided by sequencing both DNA strands. Sequence treatment was performed using the CAP3 Sequence Assembly Program (<http://pbil.univ-lyon1.fr/cap3.php>) and the invcomp program ([http://www.infobiogen.fr/services/analyse/cgi-bin/invcomp\\_in.pl](http://www.infobiogen.fr/services/analyse/cgi-bin/invcomp_in.pl)). Sequences were then compared to databases using the nucleotide-nucleotide BlastN program (<http://www.ncbi.nlm.nih.gov/BLAST/>, Altschul et al. 1997).

Phenotypic tests were carried out as follow: lactic acid production and configuration as described above, gas production by the modified hot tube method (Dicks and van Vuuren 1987) and polysaccharide production on Elliker Petri dishes.

#### **2.5. Safety characteristics: biogenic amines production and antibiotics resistance**

Histamine and tyramine production was tested for the seven selected isolates. Cultures were grown 72 h at 15°C in Maijala medium (Maijala 1993) supplemented with 2 g l<sup>-1</sup> of tyrosine or 2 g l<sup>-1</sup> of histidine. Standard solutions containing 5 to 1000 µg ml<sup>-1</sup> of pure tyramine or histamine were prepared for calibration curves. 100 µl of each culture supernatant and standard solution were dansylated as described by Eerola et al. (1993). Twenty microliters of dansylated solution were analyzed by HPLC (Waters 600E Multisolvant Delivery System and Waters 2487 Dual λ Absorbance Detector, Waters, France) on a C18 ODS2 Equisorb column (CIL Cluzeau, France). The gradient elution was carried out as described by Connil et al. (2002). Amines were detected by u.v. absorption at 254 nm.

The antibiotic resistance was determined using agar diffusion discs of chloramphenicol, tetracycline, erythromycin, vancomycin, kanamycin, colistin and nalidixic acid as

recommended by the supplier (BioRad, Hercules, CA, USA). Analyses were performed in triplicate.

## 2.6. Characterization of the inhibitory activity

The inhibition mechanisms of the seven selected isolates were investigated. The isolates were grown for 48 h at 15°C in 10 ml of Elliker broth, and culture cell-free supernatants were obtained by centrifugation (18000 g for 10 min). Supernatants were then submitted to different successive treatments: heating for 10 min at 80°C (step 1); adjusting the pH to 6.5 with 0.1 or 1 mol l<sup>-1</sup> NaOH (Merck) (step 2); addition of catalase (Merck) to a final concentration of 500 U ml<sup>-1</sup>, 30 min at 25°C (step 3); addition of proteinase K (Boehringer, Mannheim, Germany) to a final concentration of 0.2 mg ml<sup>-1</sup>, one h at 37°C (step 4). After each step, 10µl of the supernatants was spotted onto soft agar inoculated with one ml of pre-cultivated target strains (Table 2). The plates were then incubated for 24 h at target strain temperature (Table 2) and inhibition zones were recorded.

## 2.7. Temperature growth profile

In order to confirm the psychrotrophic behavior of the selected isolates, maximal growth rate as a function of temperature was determined in an automated optical density measurements system Bioscreen C for two isolates: EU2241 and EU2247. Experiments were performed as described by Augustin et al. (1999). Isolates were subcultured ? at 15°C for 24 h (EU2241) or 72 h (EU2247) and then inoculated at 5% (v/v) in modified Elliker medium (20 g/l tryptone, 5 g/l yeast extract, 2.5 g/l gelatin, 7.5 g/l lactose, 7.5 g/l glucose, 1.5 g/l sodium acetate, 0.5 g/l ascorbic acid, 20 g/l sodium chloride). Modified Elliker medium was used to prevent polysaccharide production from the *Leuconostoc* isolates. Moreover the salt enhanced the growth of the tested isolates. Eight half-dilutions were prepared from this dilution, and 200 µl of each of the nine dilutions were placed in the wells of honeycomb sterile plate. The last well of the series was filled with 200 µl of sterile medium as a negative control. The plates were placed in the Bioscreen C (Labsystem, Labsystem France SA, Les Ulis, France) and incubated at constant temperature. Incubation temperatures were 0, 2, 5, 8, 11, 14, 17, 20, 23, 26 and 29°C. OD measurements at 600 nm were performed automatically every 20 min after a 20 s shaking and recorded by the software Research Express v1.05 (Transgalactic Ltd., Helsinki, Finland).

The maximum growth rate was calculated by a linear regression method according to the equation:

$$\ln(N_i) = k - \mu_{\max} * DT_i$$

With  $N_i$  : initial number of cell in the well and  $DT_i$  : absorbance detection time in well in mid-exponential phase.

All calculations were done using Matlab software (The Mathworks, Natick, MA, USA).

## 3. Results and discussion

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### 3.1. Isolation of potentially bioprotective bacteria from seafood products

This study presents the first steps of the selection of inhibitory LAB strains, for a biopreservative strategy of seafood products based on growth at low temperature. A total of 132 colonies were isolated from the first double layer inhibition test. Many presumptive LAB

colonies were obtained from MAP and smoked fish products. This is consistent with previous publications (Leroi et al. 1998; Gonzalez-Rodriguez et al. 2002). With only 15% of the analyzed samples (8 out of 51), the salmon products (fresh MAP salmon and smoked salmon) were the origin of nearly 64 % of the selected isolates (85 out of 132). Cold-smoked salmon has often been found to be a good provider of LAB with antimicrobial properties. Fourteen and 41% of the isolates tested from this matrix respectively by Duffes et al. (1999) and Tomé et al. (2006) have shown anti-listerial properties. From the 132 selected isolates, 54 (41 %) were able to grow at 15°C and showed no growth at 30°C. Fifty-two (96 %) out of the 54 psychrotrophic isolates showed typical LAB characteristics, i.e. Gram-positive, catalase-negative and oxidase-negative. The screening of commercial seafood products allowed the selection of 52 inhibitory isolates, presenting LAB characteristics and unable to grow at 30°C or above. All the results concerning the isolation and characterization of these potentially bioprotective bacteria are presented in Table 1. These 52 LAB isolates were added to the HURDLETECH collection under numbers EU2213 to EU2264.

### 3.2. Inhibition spectrum and partial identification of the isolates

The aim of this investigation was to differentiate the 52 LAB isolates upon their inhibitory characteristics against a wide selection of Gram-positive and Gram-negative, pathogenic and spoiling strains relevant to seafood (later referred to as target strains). Most of the target strains used during the third screening to establish the inhibitory spectrum are commonly reported in seafood products.

Clustering was done to separate the 52 isolates into clusters according to the size of their inhibition zone. The mean inhibitory spectrum of each cluster is presented in Table 3. Eight distinct clusters were formed from this analysis. All clusters contained from one to 16 isolates. Cluster 8 grouped together five isolates showing the weakest inhibitory properties, including the negative control *Lb. curvatus* SF762. Isolates from this cluster were not further investigated in this study.

All the tested LAB strains showed at least weak inhibition of *L. monocytogenes*. As demonstrated in many studies, this confirms the ability of LAB strains, isolated from various food products, to inhibit *L. monocytogenes* (Budde et al. 2003; Brillet et al. 2005; Alvarado et al. 2005; Wilson et al. 2005; Weiss and Hammes 2006). As *L. monocytogenes* is commonly found in ready-to-eat foods and lightly preserved seafood products (Ben Embarek 1994; Huss et al. 2000), it is interesting for a bioprotective strain to have inhibitory properties against this particular pathogen.

The 16S-23S intergenic region profile was used for the identification of the isolates at the genus level. According to Kabadjova et al. (2002) one single amplification product is obtained with *Leuconostoc*, *Lactococcus* or *Streptococcus thermophilus* strains, whereas *Lactobacillus* or *Carnobacterium* strains usually give 2 or 3 amplification products. The strains from the clusters 1, 2, 3, 4 and 7 were cocci showing one single PCR product of 750 bp. Considering that strains from clusters 2 and 3 were cocci producing mainly L-lactic acid, they were assigned to the genus *Lactococcus*. The strains from clusters 1, 4 and 7 were cocci producing mainly D-lactic acid and were identified as *Leuconostoc*. A RFLP analysis was conducted according to Kim et al. (2003) and allowed the identification of the 26 isolates as *Leuconostoc gelidum*. The strains from clusters 5, 6 and 8 were rods producing L- or D-lactic acid and showing 2 or 3 amplification products for the 16S-23S intergenic region. They were identified as *Lactobacillus* or *Carnobacterium*.

### 3.3. Selection and characterization of 7 representative strains from the inhibitory clusters

### 3.3.1. Identification at the specie level

For each of the seven interesting clusters, the following isolates showing the highest inhibitory effect were chosen for further analysis: EU2213, EU2229, EU2241, EU2247, EU2255, EU2257 and EU2262 (Table 3).

Sequencing of the 16S rRNA gene and comparison with online databanks enabled a more precise identification of these isolates. Isolates EU2213, EU2247 and EU2262 (from clusters 1, 4 and 7 respectively) were identified as *Ln. gelidum* or *Ln. inhae*, both at 99 % identity. RFLP analysis allowed the separation of 2 restriction fragments of approximately 200 and 700 bp. Comparison of these results with RFLP results from *Ln. gelidum* and *Ln. inhae* (Kim et al. 2003) identified them as part of the *Ln. gelidum* specie. These strains were heterofermentative, produced mainly D(-) lactic acid isomer and had a slimy colony appearance on Elliker agar. Polysaccharide production could be a spoilage characteristic on some products. However, according to the USDA nutrient database (<http://www.nal.usda.gov/fnic/foodcomp/search/>) seafood products have a low sugar content so it is unlikely that polysaccharide production will be a problem. Isolates EU2229 and EU2241 (from clusters 2 and 3 respectively) were identified as *Lactococcus piscium* at 99 % identity. They were homofermentative cocci producing mainly the L(+) isomer of lactic acid. Isolate EU2255 from cluster 5 was identified as *Lactobacillus fuchuensis* at 98 % identity and was heterofermentative. Isolate EU2257 from cluster 6 was identified as *Carnobacterium alterfunditum* at 98 % identity and was homofermentative. The sequencing results and phenotypic characteristics of the seven selected isolates are summarized in Table 4.

*Lactococcus* spp. and *Leuconostoc* spp. have already been isolated from seafood products (Mauguin and Novel 1994; Wilderdyke et al. 2004) although, generally, *Carnobacterium* spp. and *Lactobacillus* spp. were found dominant among the LAB flora (Leroi et al. 1998; Truelstrup-Hansen et al. 1998; Gonzalez-Rodriguez et al. 2002; Rachman et al. 2004).

Although belonging to genera frequently associated with seafood products, the species identified during this study are not often isolated from seafood products. This may be due to the fact that only psychrotrophic isolates were selected during the screening.

### 3.3.2. Safety aspects : Biogenic amines production and resistance to antibiotics

Before considering their use for the biopreservation of food products, some safety aspects of the LAB isolates must be investigated. Knowledge of the antibiotic resistance and sensitivity of the strains is recommended (Rodgers 2001). All of the seven tested strains were found to be sensitive to chloramphenicol, tetracycline and erythromycin except isolate EU2241 which showed intermediate resistance to erythromycin. All isolates were resistant to vancomycin, kanamycin, colistin and nalidixic acid. These resistances are widely described among LAB and are usually considered as intrinsic and non transferable (Mathur and Singh 2005).

Biogenic amines production in food is often related to the presence of bacteria, including LAB (ten Brink et al. 1990). Seafood products have already been related to the presence of high amounts of biogenic amines (Leisner et al. 1994; Emborg et al. 2002) and biogenic amine production by biopreservative LAB strains have already been reported (Connil et al. 2002; Brillet et al. 2004). The histamine and tyramine production for the seven tested strains was below the detection threshold of 5 µg ml<sup>-1</sup> meaning that the isolates characterized in this study could be tested in products without risks of biogenic amine production.

### 3.3.3. Inhibition mechanisms of the seven selected isolates

Investigation of the inhibition mechanisms was carried out. For five out of seven isolates tested (EU2213, EU2229, EU2241, EU2255, EU2262), no inhibition zone was recorded when the untreated supernatant was used. The inhibition recorded in previous screenings seems to be related to the presence of the colony, maybe by strong colony-dependent

acidification or nutritional competition. Tests on products will be carried out to ensure that there is no downshift of the sensorial quality induced by these isolates. The culture supernatant of isolate EU2257 showed activity when spotted against *Lb. farciminis*, *Bx. thermosphacta*, *Sw. putrefaciens*, *L. monocytogenes*, *St. xylosus*, *Pseudomonas* sp, *Se. liquefaciens* and *St. aureus*. Heating stopped the activity, i. e. no activity was detected for spots of step 1, 2, 3 and 4. A thermo sensitive compound may be responsible for this inhibition. More investigation are needed to determine the exact nature of this compound. The culture supernatant of isolate EU2247 identified as a *Ln. gelidum* showed activity against *Lb. farciminis* and *L. monocytogenes*. This inhibition did not disappear after heat treatment, pH adjustment and catalase treatment. Addition of proteinase K stopped the activity, suggesting that this isolate produced a bacteriocin-like component. Bacteriocin-like components have already been identified from *Leuconostoc* spp. (Daba et al. 1991; Hastings et al. 1991; Budde et al. 2003). The inhibition spectrum of this type of component is generally restricted to *Listeria* spp. and closely related Gram-positive strains (Jack et al. 1996; Helander et al. 1997; Millette et al. 2004) and it was also noticed in this study.

### 3.4. Study of the psychrotrophic behavior of *Leuconostoc gelidum* EU2247 and *Lactococcus piscium* EU2241

Different strategies for the biopreservation of seafood products have already been conducted (Brillet et al. 2004; Altieri et al. 2005) but none of them focused on the cold adaptation of protective strains. *Lactococcus* or *Leuconostoc* species are not usually described as psychrotrophic lactic acid bacteria. For that reason, the growth rate as a function of temperature was tested for two isolates: *Lc. piscium* EU2241 and *Ln. gelidum* EU2247. According to optical density measurements, optimum growth temperature of isolate EU2241 was 26°C with a maximal growth rate of:  $\mu_{\max 26} = 0.5921 \text{ h}^{-1}$  ( $T_{g26} = 1 \text{ h } 11 \text{ min}$ ). Growth was recorded at 0°C, with a maximal growth rate of:  $\mu_{\max 0} = 0.0243 \text{ h}^{-1}$  ( $T_{g0} = 28 \text{ h } 31 \text{ min}$ ). The optimum growth temperature of isolate EU2247 was 20°C, with a maximal growth rate of  $\mu_{\max 20} = 0.4399 \text{ h}^{-1}$  ( $T_{g20} = 1 \text{ h } 34 \text{ min}$ ). Growth was also recorded at 0°C, with a maximal growth rate  $\mu_{\max 0} = 0.0278 \text{ h}^{-1}$  ( $T_g = 24 \text{ h } 56 \text{ min}$ ). No growth was recorded at 29°C or above. Maximal growth rate as a function of temperature is presented in Fig. 1.

LAB are generally mesophilic organisms, with an optimal growth temperature around 30°C and a maximum between 35 and 45°C. Some LAB have been described as psychrotrophic organisms which means that they are capable of growing at 0°C (van de Guchte et al. 2002). The isolates presented in this study grow well at low temperature and have a maximum growth temperature below 30°C, which is very uncommon among psychrotrophic LAB. In fact, only one LAB isolated from vacuum-packed refrigerated beef and presenting the same characteristic has been identified, *Lb. algidus* (Kato et al. 2000). The interest of selecting bioprotective LAB isolates possessing this characteristic is that they will have an inhibitory effect at chilled storage temperature, making them more competitive against the spoiling flora which generally develops at this temperature. They will not grow at the body temperature (meaning that no growth will occur in the human digestive system). Moreover they will not grow in the conditions used to enumerate total mesophilic bacteria (PCA, 30°C), a count used by many industries as a hygienic criterion, i.e. inoculation with these bioprotective strains will not mask any hygienic problem. Their growth characteristics make them very promising for the biopreservation of refrigerated food products.

## 4. Conclusions and future work

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The creation of a collection of lactic acid bacteria strains from seafood products has been successfully carried out. All these 52 isolates share similar characteristics: inhibition of

spoiling and pathogenic/surrogate bacteria and psychrotrophic characteristics with absence of growth at 30°C. The identification of seven representative isolates revealed different genera and species such as *Ln. gelidum*, *Lc. piscium*, *Lb. fuchuensis* and *Cb. alterfunditum*. To our knowledge, no work has been conducted on their potential application as bioprotective agents of those species in seafood products. Most of the tested strains do not produce bacteriocins, which can be an advantage in obtaining authorization for use for a human food application. In France (and in Europe), there is no regulation concerning the use of new strains in food; the authorization is delivered case by case by the veterinary or the fraud services (DSV- Direction des Services Vétérinaires or DGCCRF-Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes), based on clear identification of the strains and proof of their efficacy and safety following recommendations from AFSSA (AFSSA 2002). The production of bacteriocin often implies extensive work on presumptive resistance mechanisms before the legal authorizations can be obtained. In this study, potentially bioprotective but non-bacteriocin producing LAB strains were investigated. This way, if these strains are to be used at industrial scale, authorization will be easier to obtain. Inhibitory isolates from group 5 or 7, identified as *Lb. fuchuensis* and *Ln. gelidum* respectively, seems to be particularly interesting as they inhibit most of the pathogenic/surrogate and spoiling strains tested. However, more work is needed to investigate their *in situ* antimicrobial activity, as well as their safety and sensory acceptability for a food application. A combination of different bioprotective LAB could also be a strategy to improve the quality and safety of seafood products. Finally, their growth characteristics as a function of temperature is rather uncommon and make them very promising for the biopreservation of chilled seafood products.

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## Tables

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Table 1: Number of colonies screened and selected per product

\*: MAP : modified atmosphere packaging

†: 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

Product name	Colonies covered	Colonies showing inhibition† (%)	Isolated colonies	Selected isolates based on growth temperature‡	Selected isolates based on LAB characteristics§
MAP* salmon	718	151 (21%)	48	39	39
MAP* sea-bream	240	11 (4%)	13	7	5
Cold-smoked salmon	554	221 (40%)	37	5	5
MAP* rough head grenadier	520	24 (4%)	7	3	3
MAP* 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* 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
<b>Total</b>	<b>5575</b>	<b>456 (8%)</b>	<b>132</b>	<b>54</b>	<b>52</b>

‡: isolates able to grow at 15°C but not at 30°C

§: Gram-positive, catalase- and oxidase-negative isolates, able to grow at 15°C but not at 30°C

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

Species	Code*	Medium†	Temperature
<i>Bacillus subtilis</i>	DSMZ10	BHB	37°C
<i>Brochothrix thermosphacta</i>	EU2206	BHB	20°C
<i>Clostridium sporogenes</i>	ENITIAA	RCM	37°C
<i>Escherichia coli</i>	CIP76.24	BHB	37°C
<i>Lactobacillus farciminis</i>	EU2204	BHB	20°C
<i>Listeria monocytogenes</i>	EU2160	BHB	20°C
<i>Photobacterium phosphoreum</i>	EU2183	BHB + 15 g l <sup>-1</sup> NaCl	15°C
<i>Pseudomonas</i> group I	EU2189	BHB	20°C
<i>Psychrobacter</i> spp.	CCUG 42949	BHB	20°C
<i>Salmonella enterica</i>	CIP81.3	BHB	37°C
<i>Serratia liquefaciens</i>	EU2196	BHB	20°C
<i>Shewanella putrefaciens</i>	EU2187	BHB	20°C
<i>Staphylococcus aureus</i>	CIP76.25	BHB	37°C
<i>Staphylococcus xylosum</i>	DSMZ 20029	BHB	37°C

\*: 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.

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

Table 3: Mean inhibitory spectrum of eight clusters of psychrotrophic LAB against fourteen target strains (the number represent the mean inhibition halo diameter of the group, in cm). Groups are obtained by the Ward's hierarchical clustering method.

Cluster*	Representative isolatet	Target strain													
		Gram +							Gram -						
		<i>Cl. sporogenes</i>	<i>L. monocytogenes</i>	<i>Lact. farciminis</i>	<i>Bx. thermosphacta</i>	<i>Staph. aureus</i>	<i>B. subtilis</i>	<i>Statph. xylosum</i>	<i>Psychrobacter</i> sp.	<i>Sw. putrefaciens</i>	<i>Pseudomonas</i> sp.	<i>Ser. liquefaciens</i>	<i>Ph. phosphoreum</i>	<i>Salm. enterica</i>	<i>E. coli</i>
1 (16)	EU2213	4.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	2.0	0.0	0.0	0.0
2 (12)	EU2229	4.0	2.0	1.0	1.0	1.0	0.0	0.0	1.3	1.0	3.0	2.0	1.0	0.0	0.0
3 (6)	EU2241	4.0	2.0	1.0	2.0	1.0	0.0	0.0	4.0	4.0	2.0	2.0	3.0	1.0	0.0

4 (9)	EU2247	4.0	1.7	3.0	1.0	0.0	0.0	0.0	4.0	4.0	2.0	2.0	0.0	0.0	0.0
5 (2)	EU2255	3.0	2.0	1.0	1.0	1.0	1.0	0.0	2.0	2.0	2.0	2.0	2.0	1.0	1.0
6 (1)	EU2257	2.0	2.0	1.0	1.0	0.0	0.0	1.0	3.0	2.0	1.0	1.0	2.0	0.0	0.0
7 (2)	EU2262	4.0	1.0	1.0	1.0	3.0	0.0	0.0	3.0	4.0	1.0	1.0	1.0	3.0	3.0
8 (4)	N/A	0.0	1.0	0.8	0.0	0.0	0.0	0.0	1.0	0.4	0.2	0.2	0.2	0.0	0.0

\*: in brackets : number of isolates per cluster.

†: name of the selected representative isolate in the HURDLTECH collection

Table 4: Phenotypic and molecular characteristics of the seven representative LAB isolates

Cluster	Representative isolate	Product	Lactic acid configuration	Polysaccharide production	Fermentation	Identification by sequencing the complete 16S rRNA gene
1	EU2213	MAP salmon	D(-)	Yes	Hetero-fermentation	<i>Leuconostoc inhae/gelidum</i>
2	EU2229	MAP salmon	L(+)	No	Homo-fermentation	<i>Lactococcus piscium</i>
3	EU2241	MAP salmon	L(+)	No	Homo-fermentation	<i>Lactococcus piscium</i>
4	EU2247	MAP salmon	D(-)	Yes	Hetero-fermentation	<i>Leuconostoc inhae/gelidum</i>
5	EU2255	MAP sea-bream	L(+)	No	Hetero-fermentation	<i>Lactobacillus fuchuensis</i>
6	EU2257	MAP rough-head grenadier	L(+)	No	Homo-fermentation	<i>Carnobacterium alterfunditum</i>
7	EU2262	cold smoked salmon	D(-)	Yes	Hetero-fermentation	<i>Leuconostoc inhae/gelidum</i>

Figures

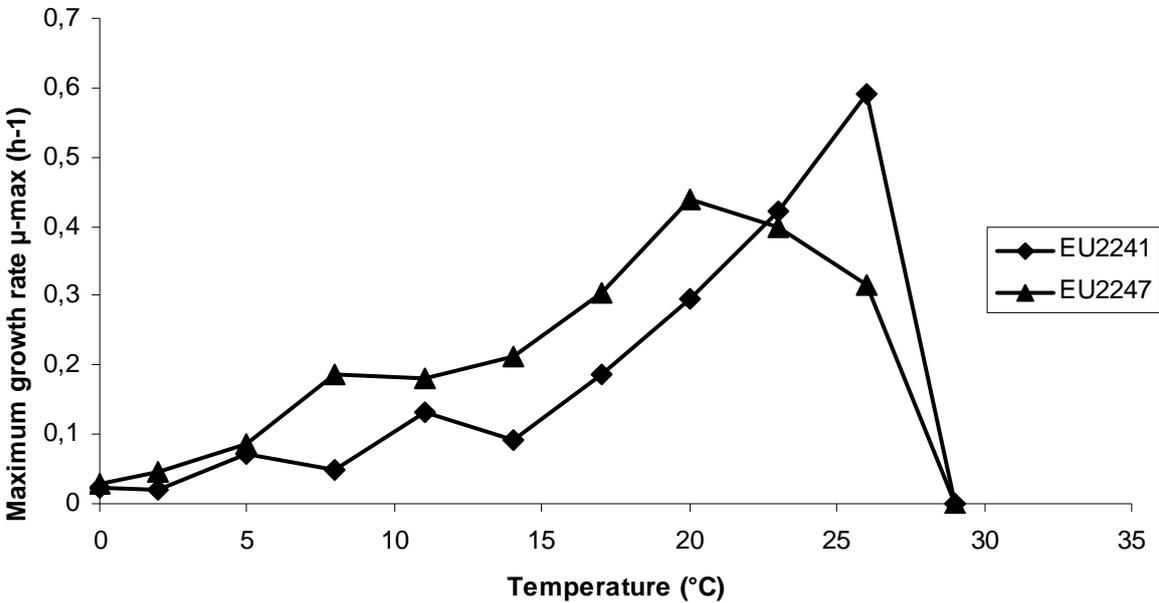


Figure 1: Maximum growth rate  $\mu_{max}$  as a function of temperature for two LAB isolates, *Lactococcus piscium* EU2241 (♦) and *Leuconostoc gelidum* EU2247 (▲).