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Selection of psychotrophic bacteria active against spoilage and pathogenic micro-organisms relevant for seafood products

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Abstract:

In this study, 51 seafood products were screened to select psychrophilic inhibiting lactic acid bacteria. 5575 colonies were tested for inhibition against four target strains : *Listeria monocytogenes*, *Staphylococcus xylosus, Pseudomonas sp.* and *Serratia liquefaciens*. 456 colonies (8,2%) showed inhibition and 132 (28,9%) of them were picked up. 54 isolates growing at 15°C but not at 30°C were selected. Basic phenotypic characteristics (Gram, catalase and oxidase test) were then tested. Finally, 52 presumptive psychrophilic LAB strains were selected. The inhibition spectrum of these strains was enlarged to 14 spoiling or pathogenic target strains relevant for seafood products. The inhibition profiles were recorded, and clustering was made to separate eight distinct groups.

Keywords: biopreservation, seafood products, psychrophilic lactic acid bacteria, inhibition

1. Introduction

Seafood products, widely marketed throughout the world, are easily spoiled by micro-organisms. Due to its aquatic environment, the microbiology of fish products is quite specific. The main spoilage flora of fresh seafood products stored at low temperature is constituted of Gram negative bacteria like *Shewanella* spp., *Pseudomonas* spp. and *Photobacterium* spp. (Gram and Huss 1996). Vacuum packaging reduces the level of the respiratory bacteria such as *Pseudomonas* spp. However *Shewanella putrefaciens* and *Photobacterium phosphoreum* develop easily under vacuum conditions, due to their capacity to use trimethylamine oxide (TMAO) as an electron acceptor. Reduction of TMAO into trimethylamine (TMA) leads to specific fishy off-odours. Modified atmosphere packaging (MAP) with CO_2 inhibits the development of respiratory organisms such as *Pseudomonas* spp. and *S. putrefaciens*. This result explains the significant extension of the product shelf-life. However *P. phosphoreum* is resistant to CO_2 atmosphere and is still a source of spoiling (Gram and Huss 1996). Lightly preserved fish products (cold-smoked fish, pickled and marinated fish…) are generally distributed at chilled temperature and under vacuum or modified atmosphere packaging. This kind of packaging selects lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Carnobacterium* spp. and *Leuconostoc* spp., which soon become the predominant flora. They are mixed with typical Gram negative spoilage bacteria. They can be part of the spoilage by producing off odours and biogenic amines. *Enterobacteriaceae* (Gram negative) and *Brochothrix thermosphacta* (Gram positive) can also be found in lightly preserved products (Gram and Huss 1996 ; Leroi and others 1998 ; Gram and Dalgaard 2002).

Pathogenic risks in lightly preserved seafood products occur mainly from *Listeria monocytogenes* and biogenic amines production. *L. monocytogenes* is a Gram positive bacterium that can resist salting, drying and cooling (Collins-Thompson 1980). This resistance allows *L. monocytogenes* to grow in preserved products such as smoked salmon (Heinitz and Johnson 1998). Low numbers of *Clostridium botulinum*, *Bacillus* spp. and *Salmonella* spp. have been reported in seafood (Huss 1997). High levels of biogenic amines, particularly histamine in scombroid fish, can lead to food poisoning (ten Brink and others 1990). Their formation is mainly due to microbial activity and can be related to the presence of *P. phosphoreum*, psychrotolerant *Morganella morganii*-like bacteria and LAB, as for example in lightly preserved tuna (Emborg and others 2005).

Biopreservation is a novel and innovative way of reducing microbial risks and/or extending shelf life of lightly preserved products. Biopreservation consists in inoculating in the food matrix bacterial strains selected for their ability to inhibit growth of undesirable bacteria, while not presenting any spoilage capacity (Rodgers, 2001). Many studies have been conducted on the biopreservation of food products (seafood, meat, dairy products). For example Vermeiren and others (2004) investigated the preservative abilities of *Lb. sakei* on cooked meat products. Brillet and others (2004) used *Carnobacterium divergens* to protect cold-smoked salmon from growth of *L. monocytogenes*. According to Helander and others (1997), the control of Gram-negative bacteria is problematic because they are resistant to bacteriocins, which are molecules often produced by LABs and active against related species.

In this study, isolation of bacteria from different seafood products was carried out. The objective was to isolate psychrophilic LAB cultures active against a wide range of Gram negative and Gram positive spoiling or pathogenic target bacteria, to be used in the seafood industry.

2. Materials and methods

1) Bacteria isolation from seafood products

Fifty one seafood products were obtained from different supermarket (Table 3). They were stored at 4 or 8 °C, and opened in a range of 10 days before to 10 days after the use by date. A 30 g sample of each product was taken aseptically and diluted in 120 ml of physiological water (1g/l tryptone [Biokar Diagnostics, Beauvais, France], 8,5 g/l NaCl [Merck, Darmstadt, Germany], 1 drop of bromocresol purple). The sample was crushed in a Stomacher 400 (Seward Medical, London, UK) for 2 min. After 30 min for revivification, four decimal dilutions were made in physiological water, and 0.1 ml of each dilution was spread on Elliker agar plates (Elliker, Biokar Diagnostics, with 1.5% agar added). For each sample, 5 plates of each dilution were made (total : 25 plates per sample). Plates were then incubated in anaerobic conditions at 8°C for 10 to 15 days. At that time, plates showing growth of 10 to 15 colonies were selected for the double layer inhibition test.

2) Double layer inhibition test

Four target strains have been selected for the test : *Listeria monocytogenes* (EU2160), *Staphylococcus xylosus* (EU2178), *Pseudomonas* group I (EU2189) and *Serratia liquefaciens* (EU2196) (HURDLETECH collection, stored at IFREMER, Nantes). These strains were incubated twice successively for 72 and 24 h in Brain Heart Broth (Biokar Diagnostics), before being diluted to a previously determined concentration. Information about culture conditions are presented in Table 1. One millilitre of selected dilution was added to 15 ml of soft BHB agar (Brillet and others 2004) and then spread on an isolation plate showing growth of 10 to 15 colonies. The four target strains were spread on different plates of the same set (same product, same dilution). The fifth plate of the set was kept intact as a negative control. Plates were then incubated for 24 h at the specific strain incubation temperature (Table 1). Presence of inhibition zone was checked visually. Based on colony aspect (size, colour, appearance, presence or not of polysaccharide) and product of origin, approximately 1 out of 3 colonies showing an inhibition zone was picked up and cultivated in Elliker broth (Biokar Diagnostics) at 15°C.
Selected strains were isolated twice successively on Elliker agar and then incubated in Elliker broth at 15°C for 4 days before being frozen for conservation at –80°C in Elliker broth containing 10% of glycerol (Panreac Quimicia SA, Barcelona, Spain).

3) Phenotypic tests

The selected strains were pre-cultivated for 72 h in Elliker broth at 15°C. In order to check their psychrotrophic characteristic, each strain was then inoculated in two Elliker broth tubes. One tube was incubated at 15°C, while the other one was incubated at 30°C. Growth was watched visually by checking turbidity after 48 h and one week of culture. Strains presenting growth at 15°C but no growth at 30°C after one week were tested for three characteristics: Gram, catalase and oxidase. Their morphological characteristics were observed by phase contrast microscopy. The Gram test was performed by the KOH method (Gregersen 1978). The Catalase test was performed by dropping 10% H_2O_2 (Merck) on a colony. The Oxidase test was performed on a BBL DrySlide (Becton Dickinson and Company, Le Pont de Claix, France). Only Gram positive, catalase negative and oxidase negative strains were selected for further studies.

4) Inhibition spectrum

Research of the inhibitory capacities of the selected strains was enlarged to 14 spoilage and pathogenic target strains listed in Table 2. The tested LAB strains were incubated for 48 h in Elliker broth at 15°C. Hundred-fold dilutions were made in physiological water. Strains were spotted on Elliker agar plates (10 µl per spot) with 6 spots per plate. Plates were then incubated anaerobically for 10 days at 8°C.
The target strains were cultivated twice successively for 72 h and 24 h in specific medium broth, at their specific temperature (Table 2). They were then diluted in physiological water

(Table 2), and 1 ml of final dilution was added to 15 ml of specific medium (Table 2) containing 10 g/l of agar. Soft agar was then spread on previously spotted and incubated Elliker agar plates. After 24 h incubation at target strain appropriate temperatures (Table 2), the 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 for an inhibition zone's diameter of 1 cm, 2 for a diameter of 2 cm, 3 for a diameter of 3 cm and 4 for a diameter of 4 cm or more (meaning the inhibition zone extended to the next spot on the plate).
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 fourteen target strains (Uniwin plus, version 4.01, Sigma plus, Paris, France).

3. Results and discussion

1) Isolation and characterisation

From the 51 seafood products analysed, a total of 5575 colonies have been covered with indicative bacteria. 456 (8,2%) colonies showed an inhibition activity and 132 of these (28,9%) were selected upon their appearance for further tests. All results concerning isolation and characterisation are presented in Table 3. From the 132 selected strains, 54 (40,9%) were able to grow at 15°C and showed no growth at 30°C. According to Shewan and Murray (1979), psychrophilic bacteria have an optimum growth temperature of 15°C and a maximum growth temperature of 18°C, whereas psychrotrophic strains have an optimum growth temperature between 20 and 30°C, and a maximum at 37°C. The selection of strains showing no growth at 30°C orients the selection toward psychrophiles. The selection's main purposes was to select strains that will be capable of growing at chilled storage temperatures. Moreover, psychrophilic strains will not grow at body temperature, meaning no growth will occur in the human tractus. If the product is submitted to abused temperature during storage, the biopreservative strains will not overgrow and spoil the product. Fifty two (96,3%) out of the 54 psychrophilic strains were Gram positive, catalase negative, oxidase negative rods or cocci, which means that they probably belong to the LAB group.
As shown in Table 3, a lot of presumptive LAB colonies were obtained from MAP and smoked fish products. This is coherent with previous publications (Leroi and others 1998 ; Gonzalez-Rodriguez and others 2002). But very few of the colonies coming from smoked fish products showed inhibition, except for the cold-smoked salmon. A significant number of colonies from MAP products showed inhibition and were selected thereafter. In addition, 44 strains out of the 52 selected strains (84,6%) came from salmon : MAP salmon (39 strains) and smoked salmon (5 strains). Moreover, the screening for psychrophilic bacteria eliminated most of the strains isolated from smoked salmon. Of the 37 strains originally selected from smoked salmon, 32 (86,5%) grew at 30°C and were not selected. On the opposite, 40 of 48 (83,3%) of the strains isolated from MAP salmon showed no growth at 30°C and where selected. This is probably the result of the food processing : during cold smoking, temperature rises to 20-25°C, which may inhibit psychrophilic strains. Fish viscera gave a smaller but still important number of colonies. However, none of them showed any interesting anti-microbial potential.

2) Inhibition spectrum

The aim of this experiment was to test the inhibiting capacities of the 52 isolated strains. They were tested against typical pathogenic and spoiling strains (later referred to as target strains). Most of the target strains chosen are commonly found in seafood products (Gram and Huss 1996). Others like *E. coli*, *Salmonella* or *B. subtilis* are not generally associated with seafood products, but can in rare outbreaks be isolated from these products (Heinitz and Johnson 1998 ; Huss 1997). The clustering analysis, based on the size of the inhibition zone

of fourteen target strains, allowed the distinction of eight clusters, containing from one to 16 LAB strains. Mean inhibitory spectrum of each cluster is presented in Table 4. All strains showed inhibiting capacities against at least two of the fourteen target strains. No strain showed inhibition against all of the fourteen target strains. All strains showed inhibition against *L. monocytogenes*. This is interesting because *L. monocytogenes* is a common pathogenic bacterium found in seafood products. This also confirms the ability of LAB strains to easily inhibit *L. monocytogenes*. All strains inhibited at least one Gram positive and one Gram negative strain. However, the mechanism of inhibition needs to be investigated, as it is known that the nature of the antimicrobial compound (acid, bacteriocin, hydrogen peroxide) influence the inhibition of some strains, especially Gram negative (Helander 1997). One of the clusters (group 8) regrouped five strains showing the weakest inhibiting capacities, including negative control *Lb. Curvatus.* Cluster 5 had a high inhibiting potential : although it had weaker inhibition (i.e. smaller inhibiting zone diameter) than what could be seen in other groups, it inhibited all target strains except *Staphylococcus xylosus*. Cluster 7 showed approximately the same inhibiting pattern than cluster 5, but with stronger inhibition of *E. coli*, *Staphylococcus aureus* and *Salmonella enterica*. It is also important to notice that typical seafood product spoiling bacteria are frequently inhibited : all clusters (except cluster 8) presented at least weak inhibition against *Pseudomonas* and *Serratia liquefaciens*. *P. phosphoreum* was inhibited by all clusters except clusters 1 and 4.

Conclusion

Lightly preserved seafood products offer a good range of LAB adapted to biopreservation. MAP and smoked salmon were the products that contained the highest levels of interesting LAB, providing nearly 85% of our final isolates. True psychrophilic bacteria are quite rare and it appeared much easier to isolate psychrophiles from products that have never been heated. Around 8% of the colonies investigated showed an inhibition capacity and less than 40% of the selected colonies finally matched the researched characteristics (psychrophilic presumptive LAB). Some isolates, for example those from cluster 5 (coming from sea bream) and cluster 7 (coming from smoked salmon), exhibited an interesting spectrum against both pathogenic and spoiling microflora. These results are to be confirmed in seafood products. The non spoiling capacity of the selected LAB has also to be checked and a complete identification, using both phenotypic and molecular methods must be provided.

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Tables

Table 1 : target strains used in first screening and appropriate cultures conditions for double layer realisation

Strain	Code	Incubation temperature	Used dilution
4. L. monocytogenes	EU2160	20°C	1/100
S. xylosus	EU2178	37°C	1/1000
Pseudomonas sp.	EU2189	20°C	1/1000
S. liquefaciens	EU2196	20°C	1/1000

Table 2 : target strains used in the inhibition spectrum and appropriate cultures conditions for double layer realisation

Code*	Specie	Medium**	Temperature	Dilution
EU2199	**5. Psychrobacter *sp.***	BHB	20°C	10^{-2}
EU2183	*Photobacterium phosphoreum*	BHB + 15g/l NaCl	15°C	0
EU2204	*Lactobacillus farciminis*	BHB	20°C	10^{-2}
EU2206	*Brochotrix thermosphacta*	BHB	20°C	10^{-3}
EU2187	*Shewanella putrefaciens*	BHB	20°C	10^{-2}
EU2211	*Bacillus subtilis*	BHB	37°C	10^{-3}
EU2160	*Listeria monocytogenes*	BHB	20°C	10^{-2}
EU2178	*Staphylococcus xylosus*	BHB	37°C	10^{-3}
EU2189	*Pseudomonas* group 1	BHB	20°C	10^{-3}
EU2196	*Serratia liquefaciens*	BHB	20°C	10^{-3}
CIP81.3	*Salmonella enterica*	BHB	37°C	10^{-2}
CIP76.25	*Staphylococcus aureus*	BHB	37°C	10^{-1}
CIP76.24	*Escherischia coli*	BHB	37°C	10^{-2}
ENITIAA	*Clostridium sporogenes*	RCM***	37°C	10^{-2}

* : EU : HURDLETECH collection, stored in IFREMER, Nantes, France ; CIP : Institut Pasteur Collection, Paris, France ; ENITIAA : ENITIAA collection, Nantes, France

** : BHB : Brain Heart Broth, Biokar Diagnostic ; RCM medium was prepared as follow : 3 g/l yeast extract (Biokar Diagnostics), 10 g/l meat extract (Oxoid LTD., Basingstoke, Hampshire, England), 10 g/l peptone (Oxoid LTD.), 5 g/l glucose (Merck), 1 g/l potato starch (La Bovida, Nanterre, France), 5 g/l NaCl (Merck), 3 g/l Sodium Acetate (Merck), 0.5 g/l cystein hydrochloride (Sigma-Aldrich, St Quentin Fallavier, France), 0.5 g/l agar (Biokar Diagnostics). The pH was adjusted at 6.9 and medium was then autoclaved

Table 3 : Number of colonies screened and selected per product

Product name	Number of colonies covered	Number of colonies showing inhibition	Number of isolated colonies	Number of selected potential LAB
MAP shrimp	157	26	11	0
MAP rough head grenadier	520	24	7	3
MAP salmon	718	151	48	39
MAP sea-bream	240	11	13	5
MAP whiting	456	14	3	0
Smoked haddock	217	4	4	0
Smoked mackerel	121	0	0	0
Smoked herring	300	2	2	0
Smoked trout	354	0	0	0
Smoked tuna fish	368	0	0	0
Smoked shark	476	2	2	0
Smoked salmon	554	221	37	5
Red mullet viscera	371	0	0	0
Mackerel viscera	198	0	0	0
Herring viscera	39	0	0	0
Sea-bream viscera	184	1	1	0
Shrimp (fresh)	0	0	0	0
Salmon carpaccio	128	0	0	0
Salted cod	0	0	0	0
Roe cod (tarama)	174	0	4	0
Roe lumpfish, roe salmon	0	0	0	0
Marinated sardines, anchovy, mussel, pickled shell fish and pickled herring	0	0	0	0
Total	5575	456	132	52

Table 4 : Mean inhibitory spectrum of eight groups of psychrophilic lactic acid bacteria againts fourteen target strains. (the number represent the mean inhibition halo diameter of the group, in cm). Groups are obtained by the Ward's hierarchichal clustering method. Brackets : number of isolates/group.

	6. Target strain													
	7. Gram +							***8. Gram -***						
Group	*L. monocytogenes*	***8.1. S. xylosus***	*S. aureus*	*C. sporogenes*	*B. subtilis*	*L. farciminis*	*B. thermosphacta*	*E. coli*	*S. enterica*	*Pseudomonas*	*P. phosphoreum*	*S. putrfaciens*	*S. liquefaciens*	*Psychrobacter*
1 (16)	1	0	0	4	0	0	0	0	0	2,2	0	0	2	0
2 (12)	2	0	1	4	0	1	1	0	0	3	1	1	2	1,3
3 (6)	2	0	1	4	0	1	2	0	1	2	3	4	2	4
4 (9)	1,7	0	0	4	0	3	1	0	0	2	0	4	2	4
5 (2)	2	0	1	3	1	1	1	1	1	2	2	2	2	2
6 (1)	2	1	0	2	0	1	1	0	0	1	2	2	1	3
7 (2)	1	0	3	4	0	1	1	3	3	1	1	4	1	3
8 (5)	1	0	0	0	0	0,8	0	0	0	0,2	0,2	0,4	0,2	1