

Inhibition of *Brochothrix thermosphacta* and sensory improvement of tropical peeled cooked shrimp by *Lactococcus piscium* CNCM I-4031

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Abstract:

Aims: To investigate the antimicrobial spectrum of *Lactococcus piscium* CNCM I-4031 and its protective effect in cooked and peeled shrimp against *Brochothrix thermosphacta*.

Methods and Results: Sixteen pathogenic and spoiling bacteria were inhibited in Elliker, but not in shrimp juice agar plates. In shrimp packed under modified atmosphere and stored at 8°C, *B. thermosphacta* (103 CFU g⁻¹) was inhibited by 4·1 log CFU g⁻¹ when co-inoculated with *L. piscium* (106 CFU g⁻¹). *Brochothrix thermosphacta* spoiled the product after 11 days, with the emission of strong butter/caramel off-odours. In co-culture with *L. piscium*, sensory shelf-life was extended by at least 10 days. The inhibition was partially explained by a drop in pH from 6·6 to 5·6. The physicochemical composition of shrimp and shrimp juice was established to identify the inhibition mechanisms involved.

Conclusion: *Lactococcus piscium* CNCM I-4031 has a wide antimicrobial spectrum. The strain inhibits *B. thermosphacta* in shrimp and significantly prolongs sensory shelf-life.

Significance and Impact of the Study: *Lactococcus piscium* CNCM I-4031 is shown to be a promising agent for improving shrimp quality and may be tested against pathogens and in other food matrices. Knowledge of the physicochemical composition of shrimp and shrimp juice will allow the development of a chemically defined model medium for determining the inhibition mechanisms involved.

Keywords: biopreservation • *Brochothrix thermosphacta* • inhibition spectrum • *Lactococcus piscium* • *Penaeus vanamei* • shrimp composition

1. Introduction

Lightly preserved seafood products like cold-smoked fish, fish carpaccio and cooked shrimp are highly perishable, due to their physicochemical parameters that allow the growth of pathogenic and spoiling bacteria. Preserving food products against unwanted microorganisms by using endogenous and selected protective microorganisms is subject to increasing investigation (Calo-Mata et al. 2008; Dortu and Thonart 2009). In seafood, many studies have underlined the efficiency of lactic acid bacteria (LAB) such as *Carnobacterium maltaromaticum*, *C. divergens*, *Lactobacillus sakei*, *Leuconostoc gelidum* and *Lactococcus piscium* in inhibiting the growth of pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Brillet et al. 2005; Matamoros et al. 2009a). By contrast, the inhibition of spoiling microorganisms has rarely been reported.

In a recent study, the strain of *Lactococcus piscium* CNCM I-4031 (previously named EU2241) showed itself capable of delaying the sensory spoilage of naturally contaminated vacuum-packed cooked and peeled tropical shrimp (Matamoros et al. 2009a). However this sensory improvement could not be correlated to the microbial flora enumerated and the inhibition mechanism was not elucidated, though, interestingly, no bacteriocin was produced. The aim of this study was to bring to light factors capable of explaining the bioprotective effect of *L. piscium* CNCM I-4031. The inhibitory spectrum of *L. piscium* against a wide selection of spoiling and pathogenic bacteria isolated from seafood products was performed *in vitro*. Inhibition activity against one of the most spoiling bacteria, *Brochothrix thermosphacta*, recently identified in shrimp (Laursen et al. 2006), was investigated in peeled and cooked shrimp packed under modified atmosphere (MA). Finally, the physicochemical composition of shrimp was determined to develop a chemically defined model broth that will be useful for studying the inhibition mechanisms involved.

2. Materials and methods

Strains

Lactococcus piscium EU2241 was isolated from commercial (supermarket) fresh salmon steak packed under MA and identified with phenotypic and molecular tests (complete 16S rRNA gene sequencing) by Matamoros et al. (2009b). This strain was deposited in the national collection of microorganisms cultures of Pasteur Institute (Paris, France) with the reference number CNCM I-4031.

Twenty-five strains including pathogenic and spoiling microorganisms isolated from commercial seafood products and two reference strains were tested for their sensitivity to *L. piscium* CNCM I-4031: *Staph. aureus* CIP 76.25 and *Escherichia coli* CIP 76.24 (Pasteur collection, Paris, France), *B. thermosphacta*, *C. divergens*, *C. maltaromaticum*, *C. alterfunditum*, *Carnobacterium* sp., *Vagococcus fluvialis*, *V. carniphilus*, *Enterococcus faecalis*, *Lact. sakei*, *Serratia liquefaciens*, *Serratia* sp., two *Vibrio* sp., *Photobacterium phosphoreum*, one Gram-, oxidase+ cocobacilli and three unidentified Gram-, oxidase+ rods (Ifremer collection, Nantes, France), *L. monocytogenes*, *Salmonella* Weltevreden, *Salm. enterica* serovar Typhimurium, *Vibrio parahaemolyticus* and *V. cholerae* (CEVPM collection, Boulogne, France). All the strains were isolated from raw or cooked shrimp except *P. phosphoreum* and *Lact. sakei* from cold-smoked salmon.

Media and culture conditions

For all the experiments, the strains were cultivated during two successive periods of 24 h in Elliker broth (Biokar Diagnostics, Beauvais, France) and shrimp juice. The incubation temperatures were 15°C for *P. phosphoreum* and 20°C for the other strains.

Shrimp juice was prepared by crushing 2.3 kg of thawed raw peeled shrimp from Columbia (farmed *Penaeus vanamei*, without sulphite, purchased from industry, Nantes, France) in a Waring Blender (New Hartford, CT, USA) with 4.6 l of distilled water. The mixture was boiled for 2 min and filtered with a filter (no. 127, Durieux Paris, France). NaCl 20 g l⁻¹ was added to the clear broth obtained before autoclaving at 100°C for 30 min. 2.8 l of juice was obtained from 2.3 kg of shrimp. For the shrimp agar and shrimp molten agar preparations, 15 and 10 g l⁻¹ of agar (Biokar diagnostics) respectively were added to the shrimp juice.

Inhibitory spectrum

The inhibitory spectrum of *L. piscium* was obtained by using a modified double layer technique proposed by Matamoros et al. (2009b). Briefly, 10 µl of *L. piscium* precultivated in Elliker and shrimp juice were spotted onto Elliker and shrimp agar plates respectively (three spots/plate) and incubated aerobically at 15°C for 10 d. The 25 target strains were precultivated in Elliker and shrimp juice. One milliliter of a thousand-fold dilution of these cultures was then added individually to 15 ml of Elliker and shrimp molten agar respectively and spread onto previously incubated Elliker and shrimp agar plates. After 24 h of aerobic incubation at the appropriate target strain temperature, the plates were examined for evidence of an inhibition zone.

Challenge tests

Frozen peeled shrimp (the same batch as for the shrimp juice) were cooked in 3% (w/v) of salt water at 100°C for 2.5 min and rapidly cooled to 4°C. The final salt concentration in the meat was 1.2% (w/w). Three batches of cooked shrimp were then inoculated at 2% (v/w) with cultures of the following strains: *L. piscium* (1.8×10^6 CFU g⁻¹), *B. thermosphacta* CD340 alone (2.9×10^3 CFU g⁻¹) and a mixture of *L. piscium* and *B. thermosphacta*. The shrimp were packed (90 g per sample) under MA (50% N₂-50% CO₂) and then stored at 8°C for three weeks. A non-inoculated batch for sterility control was also prepared. Every three days, samples from each batch were analyzed for microbial content, sensory analysis and pH, trymethylamine oxide (TMA-O), trymethylamine (TMA) and total volatile basic nitrogen (TVBN) (Conway and Byrne 1933). For microbial counts, 20 g of each batch were blended with 80 ml of physiological solution (0.85% NaCl, 0.1% peptone) with a stomacher 400 (Seward Medical, London, UK) for 2 min. *L. piscium* was enumerated by spread plating onto Elliker agar plates incubated at 25°C for 48 h under anaerobiosis while *B. thermosphacta* were spread plated on Streptomycin Sulfate Thallous Acetate Agar (STAA) with selective supplement (Oxoid, Hampshire, UK) (20°C, 48 h). In the control, total viable count was enumerated by pour plating in Plate Count Agar (PCA, Biokar) (30°C, 72 h).

Sensory analysis

Shrimp inoculated with *L. piscium* and *B. thermosphacta* in single and mixed cultures were sensory evaluated during storage time by eleven trained judges from Ifremer. Each panelist evaluated three portions (15 g) of shrimp from each batch at each time of analysis. Off-flavors of samples were evaluated by using a scale with four classes (class I: non spoiled, class II: very lightly spoiled, class III: lightly spoiled and class IV: strongly spoiled). The sensory rejection time was estimated when samples were placed in class IV by 50% of the panelists. Odor descriptors, evaluated as described by Matamoros et al. (2009a), were rice/crustacean, butter/caramel, pyrrolidin/sperm, floorcloth, sour/fermented, amine, cheese/foot and cabbage/H₂S.

Shrimp meat and shrimp juice composition

Water, protein, salt, carbohydrate and total amino acids were determined in triplicate in fresh shrimp and shrimp juice. For water content, 8 g of sample were dried at 105°C overnight. Mineral content was determined by heating dried samples in a muffle furnace at 550°C until they became white. The Kjeldahl method (Crooke and Simpson 1971) was used to determine total proteins. Salt content was measured with a chlorine analyser (Sherwood MK II analyser 926, Cambridge, UK) according to the manufacturer instruction. Total amino acid composition was measured on a lyophilized sample by digestion of 15 mg in 6 mol l⁻¹ HCl at 110°C overnight in a sealed hydrolysis vial purged with nitrogen. Samples were then derivatized with EZ, using a fast kit (Phenomenex, Le Pecq), and analyzed by gas chromatography (Perkin ELMER Autosystem XL, Norwalk, USA) with a Zebron ZB-AAA column (10 m x 0.25 mm), a flame ionization detector and helium as gas vector. The column temperature was increased from 110°C to 320°C at 32°C min⁻¹. Sugar content was measured by the colorimetric method of Dubois (1956) modified by Chaplin (1986). The nature of the sugar was identified by gas chromatography using the method of Kamerling et al. (1975) modified by Montreuil et al. (1986). The injection of compounds derived from glycoside residues was performed on an AT 6890N chromatograph (Agilent Technologies, USA) equipped with an automatic sample loader, a CP-Sil-5CB column of molten silica (Chrompack) with helium as carrier gas and a flame ionization detector. The temperature profile was programmed as follows: 50°C for 1 min, from 50°C to 120°C at 20°C min⁻¹, from 120°C to 240°C at 2°C min⁻¹, from 240°C to 280°C at 10°C min⁻¹ and 280°C for 10 min.

3. Results

L. piscium demonstrated antagonistic activity against 16 of the 25 strains tested on Elliker agar plates but no inhibition was recorded in shrimp agar (data not shown). All the Gram-positive bacteria were inhibited except for one *Carnobacterium* sp. strain. As for the Gram-negative bacteria, *E. coli*, *Salm. Weltevreden*, *Salm. enterica* serovar Typhimurium and *Serratia* sp. were inhibited whereas the different *Vibrio*, *P. phosphoreum* and three unidentified Gram-, oxidase+ rods were not.

In the control no colony was enumerated on PCA (threshold 5 CFU g⁻¹). When inoculated alone in the shrimp matrix, *L. piscium* and *B. thermosphacta* grew very well, reaching 8.8 and 8.9 log CFU g⁻¹ after 4 and 14 d of storage respectively (Fig. 1). In batches co-inoculated with *L. piscium*, the growth rate of *B. thermosphacta* was lowered and the maximum cell number was 4.8 log CFU g⁻¹ corresponding to a 4.1 log CFU g⁻¹ decrease. This inhibition lasted for 21 d of storage.

Sensory analysis indicated that the control and the batch inoculated with *L. piscium* remained unspoiled throughout the storage time, whereas the products inoculated with *B. thermosphacta* CD340 alone were characterized by a strong butter/caramel off-odor from 11 d onwards. In the latter batch, the product was classified as strongly spoiled by 40%, 50% and 80% of the panelists after 11, 18 and 21 d respectively. On the contrary, when *B. thermosphacta* was co-inoculated with *L. piscium*, no off-odor was detected and none of the judges classified the products as strongly spoiled until after 21 d. *L. piscium* and *B. thermosphacta* did not produce TVBN in either pure or co-culture. Initial TMAO content was 8.4 mg-N 100 g⁻¹ and no TMA was produced. Initial pH was 6.6. A fall to 5.6 and 5.9 was observed respectively in samples with *L. piscium* (pure and mixed culture) after 7 d and *B. thermosphacta* alone after 14 d (Fig. 1).

Water, protein, sugar and amino acid content were compared between shrimp meat in which inhibition was observed, and shrimp juice where no inhibition was recorded. Shrimp meat was constituted by 78.3% water and 18.9% protein, whereas only 1.9% protein was found in the shrimp juice. Shrimp meat and juice contained 2.4 g kg⁻¹ and 2 g l⁻¹ carbohydrates

respectively, identified as glucose at 99%. Table 1 summarizes the total amino acid concentration in shrimp meat and juice. Arginine, asparagine and glutamine could not be measured with the method used in our study.

4. Discussion

According to Jaffrès et al. (2008), *Carnobacterium* spp., *Vagococcus* spp., *B. thermosphacta* and *S. liquefaciens* make up the majority of bacteria in spoiled cooked and peeled shrimp packed under MA and stored at chilled temperature. All of these species were inhibited in a model medium, indicating that *L. piscium* is a promising strain for sensory shelf-life extension. Potential pathogens, excepting *Vibrio* spp., were also inhibited, which is important for ensuring product safety, particularly ready-to-eat products with extended shelf-life. Its wide spectrum of activity, especially against Gram-negative bacteria, which is not current for LAB, provides a particular advantage to this bioprotective strain. The absence of a bacteriocin-like component is also a positive point for gaining rapid authorization for food applications. Although pH decreased from 6.6 to 5.6 in 7 d, no odors were perceived by the panelists. Four of them ate the products and could not distinguish them from the sterile control.

The role of *B. thermosphacta* as spoiling bacteria in shrimp demonstrated by Mejilholm et al. (2005) was confirmed in this study but no production of TVBN was detected. The butter/caramel off-odors have already been described in shrimp by Laursen et al. (2006) and may be linked to the production of 2-3 butanedione (diacetyl). The rapid inhibitory effect of *L. piscium* against *B. thermosphacta* without changing the product's characteristics is remarkable. Laursen et al. (2006) failed to prevent *B. thermosphacta* growth with a *C. maltaromaticum* strain previously selected for its antimicrobial properties and did not extend the sensory shelf-life of shrimp. Inhibition of *B. thermosphacta* by *Lact. sakei* in vacuum-packed beef meat slices stored at 4°C, was observed by Katikou et al. (2005) but did not exceed 1.5 log. In cooked meat packed under MA at 7°C, a decrease of 3 log was recorded with a strain of *Lact. sakei* subsp. *carnosus*, but only after 30 days storage (Vermeiren et al. 2006). In our study, *L. piscium* lowered pH from 6.8 to 4.3 in Elliker broth, which might explain the inhibitory activity observed with the double layer method in agar plates. However, in shrimp juice, pH decreased in the same range and no inhibition zone was observed in shrimp agar, anticipating other inhibition mechanisms. The decrease of pH in shrimp meat due to *L. piscium* may have contributed to the inhibition of *B. thermosphacta* but cannot totally explain it. Indeed, the model developed by Mc Clure et al. (1993), which includes the combined effect of pH, sodium chloride and storage temperature, accurately predicted the maximum cell number (N_{max}) of *B. thermosphacta* in shrimp: Predicted and observed $\text{Log}_{10}(N_{max})$ were respectively 9.3 and 8.9 in our experimental conditions (pH = 6.6, T°C = 8°C and NaCl = 1.2 % w/w). In the presence of *L. piscium*, pH dropped to 5.6 and the model under-estimated the inhibition of *B. thermosphacta* (predicted and observed $\text{Log}_{10}(N_{max})$ were 8.9 and 5.6 respectively) indicating that pH alone is unlikely to be responsible for all the inhibition. The absence of inhibition in shrimp juice agar suggests that compounds such as proteins, peptides and free amino-acids present in shrimp meat but not in shrimp juice may be implicated in this competition. The results of shrimp characterization will be useful for setting up a chemically defined model medium to determine the nature of the interaction between these strains.

The protective effect of *L. piscium* CNCM I-4031 found by Matamoros et al. (2009a) in naturally contaminated shrimp may be explained by the inhibition of *B. thermosphacta*, a microorganism that was not enumerated in that study. *L. piscium* CNCM I-4031 is a promising strain for improving the quality of lightly preserved seafood products and more knowledge concerning the inhibition mechanism of this strain is necessary to optimize its use as a protective culture under different environmental conditions and to extend it to other food matrices such as meat.

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Tables

Table 1: Total amino acid content in fresh shrimp meat and shrimp juice. Values are averages of three analyses \pm standard deviation.

	Shrimp meat (g kg ⁻¹)*	Shrimp juice (g l ⁻¹)*
Asparagine	nd*	nd*
Aspartic acid	12.6 \pm 3.6	0.9 \pm 0.0.7
Arginine	nd*	nd*
Alanine	11.1 \pm 0.7	1.2 \pm 0.3
Cysteine	nd*	nd*
Glycine	14.2 \pm 0.2	3.1 \pm 0.6
Glutamic acid	20.3 \pm 8.5	1.8 \pm 1.2
Glutamine	nd*	nd*
Histidine	0.6 \pm 0	0
Isoleucine	6.2 \pm 0.9	0.3 \pm 0.1
Leucine	13.4 \pm 1	0.7 \pm 0.1
Lysine	6.6 \pm 1.1	0.3 \pm 0.1
Methionine	5.3 \pm 0.4	0.2 \pm 0.0
Proline	8.5 \pm 0.7	2 \pm 0.8
Phenylalanine	7.8 \pm 1.6	0.2 \pm 0.0
Serine	5.2 \pm 0.9	0.3 \pm 0.1
Tyrosine	4.1 \pm 0.7	0.3 \pm 0.0
Threonine	6.2 \pm 0.9	0.3 \pm 0.1
Valine	5.8 \pm 0.8	0.4 \pm 0.1

*nd, not determined

Figures

Figure 1: Growth (solid line) and pH (dotted line) of *Lactococcus* sp. CNCM I-4031 (■), *Brochothrix thermosphacta* CD340 alone (x) and *B.thermosphacta* co-inoculated with *Lactococcus* sp. CNCM I-4031 (▲) in peeled and cooked shrimp packed under modified atmosphere and stored at 8°C.

