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Inhibition mechanism of *Listeria monocytogenes* by a bioprotective bacteria *Lactococcus piscium* CNCM I-4031

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ABSTRACT

Listeria monocytogenes is a pathogenic Gram positive bacterium and the etiologic agent of listeriosis, a severe food-borne disease. *Lactococcus piscium* CNCM I-4031 has the capacity to prevent the growth of *L. monocytogenes* in contaminated peeled and cooked shrimp. To investigate the inhibititory mechanism, a chemically defined medium (MSMA) based on shrimp composition and reproducing the inhibition observed in shrimp was developed. In co-culture at 26 °C, *L. monocytogenes* was reduced by 3–4 log CFU g^{-1} after 24 h. We have demonstrated that the inhibition was not due to secretion of extracellular antimicrobial compounds as bacteriocins, organic acids and hydrogen peroxide. Global metabolomic fingerprints of these strains in pure culture were assessed by liquid chromatography coupled with high resolution mass spectrometry. Consumption of glucose, amino-acids, vitamins, nitrogen bases, iron and magnesium was measured and competition for some molecules could be hypothesized. However, after 24 h of co-culture, when inhibition of *L. monocytogenes* occurred, supplementation of the medium with these compounds did not restore its growth. The inhibition was observed in co-culture but not in diffusion chamber when species were separated by a filter membrane. Taken together, these data indicate that the inhibition mechanism of *L. monocytogenes* by *L. piscium* is cell-to-cell contact-dependent.

1. Introduction

Listeria monocytogenes is an opportunistic pathogenic Gram positive bacterium and the etiologic agent of listeriosis, a severe food-borne disease (Vazquez-Boland et al., 2001) with high hospitalization cases (~300 cases/year from 2006 to 2011 in France) and high fatality rates (20–30%). The populations at greatest risk are newborn infants, pregnant women, elderly persons, and persons with a weak immune system (Lecuit and Leclercq, 2012). *L. monocytogenes* is able to grow in most of the conditions found in the food chain, such as high salt concentrations, presence of CO_2, and low temperatures. The resistance of this bacterium to these environmental factors makes this organism difficult to control in refrigerated food product (Gandhi and Chikindas, 2007). Many studies have been published concerning the inhibition of *L. monocytogenes* using various preservation technologies. Bio-preservation technique which consists in using natural, selected protective microorganisms, was demonstrated as an efficient strategy for the control of *L. monocytogenes* in a variety of ready-to-eat seafood or meat products (Ananou et al., 2005; Benkerroum et al., 2005; Pilet and Leroi, 2011). Lactic acid bacteria (LAB) are excellent candidates for preventing the growth of pathogenic bacteria in food products because they show bacteriostatic effect towards many bacterial species through various mechanisms, without causing unacceptable sensory changes in foodstuffs (Stile, 1996; Ghanbari et al., 2013). The growth inhibition of the target bacteria when LAB have reached their maximum level is usually described as Jameson effect (Jameson, 1962). These inhibitions sometimes involve well known antagonist mechanisms such as

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production of antimicrobial compounds as bacteriocins, bacteriocin-like inhibitory substance (Richard et al., 2003; Schöbitz et al., 2003; Nilsson et al., 2004; Naghmouchi et al., 2006, 2007; Rihakova et al., 2009), or reuterin (El-Ziney et al., 1999), organic acid (Alomar et al., 2008a) or hydrogen peroxide (Delbes-Paus et al., 2010). However, in some cases, the production of inhibiting metabolites is not evidenced and other hypothesis must be tested. As an example, Nilsson et al. (2005) have demonstrated the involvement of glucose competition in the inhibition of *L. monocytogenes* by a non-bacteriocin producing *Carnobacterium piscicola*. In a recent study, a protective strain, *Lactococcus piscium* CNCM I-4031, was isolated from salmon steak stored under modified atmosphere. This strain was able to improve sensory quality of seafood (Matamoros et al., 2009a,b) by preventing the growth of *Brochothrix thermosphacta* (Fall et al., 2010b). *L. piscium* CNCM I-4031 has also shown its ability to limit the growth of *L. monocytogenes* during the storage of cooked shrimp (Fall et al., 2010a). The aim of the present study was to gain insight the mechanism involved in the inhibition of *L. monocytogenes* by *L. piscium* CNCMI-4031 as it is one of the required knowledge on protective flora to ensure their possible acceptability and use for food preservation. For this purpose, a chemical defined medium close to shrimp composition has been first set up to reproduce the inhibition of *L. monocytogenes* by *L. piscium* observed in shrimp matrices. Then different tests to investigate the mechanisms have been developed.

2. Materials and methods

2.1. Bacterial strains, culture media and conditions

L. piscium CNCM-I 4031 was isolated from fresh salmon steak packed under modified atmosphere (Matamoros et al., 2009a). The target strain *L. monocytogenes* RF191 was isolated from tropical cooked peeled shrimp by PFI Nouvelles Vagues (Boulogne-sur-mer, France) and used before as target strain in challenge-tests (Fall et al., 2010a). The two strains were stored at −80 °C in their culture media with 20% glycerol (Panreac, Barcelona, Spain). For all experiments, *L. piscium* and *L. monocytogenes* were subcultured in Elliker broth and Brain Heart Infusion (BHI) supplemented with 2% NaCl (Biokar Diagnostic, Beauvais, France) respectively, for 24 h at 26 °C. The cultures were diluted in their culture media, if necessary, to obtain appropriate initial cell concentrations. *L. piscium* was enumerated on spread Elliker agar plates after incubation at 8 °C for 5 days under anaerobiosis and *L. monocytogenes* on spread Palcam agar plates (Biokar) incubated at 37 °C for 24 h.

2.2. Chemically defined medium set up

To improve the bacteria growth, six different media were prepared by supplementing the MSM with the different compounds listed in Table 1 and recommended by Premaratne et al. (1991), Jensen and Hammer (1993), Lauret et al. (1996) and Fall et al. (2012).

The components were prepared as concentrated solution of mix or single solution to avoid medium precipitation. All the amino acids (Sigma Aldrich, Saint-Louis, MO, United-States) were mixed except tyrosine, glutamine and cysteine that were used as single solutions. Vitamins (Sigma Aldrich) were separated in two mix containing riboflavine, thiamine, niacine, vitamin B12 and vitamin D for mix 1 or folic acid, aminobenzoic acid, and piridoxal (vitamin B6) for mix 2. Adenine, guanine, uracil (Sigma Aldrich) and glucose (Merck, Darmstadt, Germany) were prepared as single solutions. All these components were dissolved in distilled water and filter sterilized using Acrodisc 0.45-μm-pore-size membrane (Sartorius Stedim Biotech, Goettingen, Germany) and can be stored at −20 °C (mix of amino acids and vitamins) or 4 °C up to 15 days. Salts solution containing ammonium citrate, magnesium sulfate or sodium chloride (Merck) were prepared separately in distilled water, sterilized for 15 min at 121 °C and stored at 4 °C up to 15 days. The final media were prepared by mixing the components in the following order: ammonium citrate, magnesium sulfate, sodium chloride, mix of amino acids, glucose, adenine, guanine, uracil, tyrosine, glutamine, cysteine, phosphate buffer [final molarity Na_2HPO_4 $7H_20$ (0.11 M) and KH_2PO_4 (0.05 M)] and finally mix 1 and mix 2 of vitamins. The final pH was 7.0.

Fresh MSMA to -F media were then inoculated at 10^4 CFU ml^{-1} with overnight subcultured strains and growth at 26 °C was monitored by Petri dish enumeration technique.

2.3. Shrimp juice

The shrimp juice was prepared by crushing thawed raw peeled shrimp in a Warring Blender (New Hartford, CT, USA) with distilled water. The mixture was then boiled for 2 min and filtered through 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 (Fall et al., 2010b) and growth of strains was performed as in MSM.

2.4. Antimicrobial assay

- Agar spot assay

The presence of antimicrobial compounds in *L. piscium* culture or co-culture with *L. monocytogenes* was evaluated using solid BHI (2% NaCl) agar spot assay (Matamoros et al., 2009a). The supernatant of *L. piscium* was obtained after centrifugation of 10 ml of a 24 h culture or co-culture (11,600 g for 10 min at 4 °C). The supernatant was then filter sterilized (0.45-μm). One milliliter of a suspension containing 10^6 CFU ml^{-1} of *L. monocytogenes* was poured in 15 ml BHI agar plates (2%NaCl, 1% agar) and kept at room temperature for 15–20 min. Ten microliters of filtered supernatant were then dropped (as a spot) onto the solidified BHI agar and the plates were incubated 24–72 h at 26 °C to detect inhibition zones around the spots.

- Inhibition test after protein purification

The precipitation of potential antimicrobial peptides was performed by treatment of supernatant using ammonium sulfate precipitation. Ammonium sulfate was added to the filtered supernatant at saturation of 40% and 80% (http://www.encorbio.com/protocols/AM-SO4.htm) for 1 h under stirring. After centrifugation (10,000 g, 15 min, 4 °C), the supernatant was removed and the precipitate was resuspended and concentrated 10 fold in distilled water. The pH was adjusted to 6.9 and the suspension was filter sterilized and tested using solid BHI (2% NaCl) spot agar assay as described above.

- inhibition test after cells treatments

To remove the putative proteins linked to the *L. piscium* membrane, cultures in MSMA, (24 h at 26 °C) were acidified for 2 h at pH 2 with HCl 10 M. The pH was then adjusted to its initial value with NaOH (10 M) and the supernatant was tested after ammonium sulfate precipitation by agar spot assay.

The internal content of *L. piscium* cell was tested as follows: cells from *L. piscium* (24 h at 26 °C in MSMA medium) were pelleted by centrifugation (11,600 g, 10 min) and disrupted using 0.2 g of glass beads (150–200 μm diameter) and shaking twice for 2 min in a bead beater MM200 (30 Hz) (Fisher Bioblock Scientific, Illkirch-

Table 1
Composition of the seven chemically defined Model Shrimp Medium (MSM and MSM A to F). Concentration is expressed in g l^{-1} of medium.

Components	Concentration (g l^{-1})	MSM	MSMA	MSMB	MSMC	MSMD	MSME	MSMF
Buffer								
KH_2PO_4	6.56	X	X	X	X	X	X	X
$Na_2HPO_4,7H_2O$	30.96	X	X	X	X	X	X	X
Sugar								
Glucose	7	X	X	X	X	X	X	X
Salts								
$MgSO_4,7H_2O$	0.4	X	X	X	X	X	X	X
NaCl	8	X	X	X	X	X	X	X
Ferric citrate	0.088	X	X	X	X	X	X	X
Amino-acids								
L-alanine	0.05	X	X	X	X	X	X	X
L-arginine	0.1	X	X	X	X	X	X	X
L-cysteine	0.05	X	X	X	X	X	X	X
L-glutamate	0.1	X	X	X	X	X	X	X
L-glutamine	0.05	X	X	X	X	X	X	X
L-glycine	0.1	X	X	X	X	X	X	X
L-histidine	0.05	X	X	X	X	X	X	X
L-isoleucine	0.05	X	X	X	X	X	X	X
L-leucine	0.05	X	X	X	X	X	X	X
L-lysine	0.1	X	X	X	X	X	X	X
L-methionine	0.1	X	X	X	X	X	X	X
L-phenylalanine	0.05	X	X	X	X	X	X	X
L-proline	0.15	X	X	X	X	X	X	X
L-serine	0.15	X	X	X	X	X	X	X
L-threonine	0.1	X	X	X	X	X	X	X
L-valine	0.15	X	X	X	X	X	X	X
L-tryptophan	0.1		X		X			X
L-tyrosine	0.05		X		X			X
Taurine	0.1						X	X
Vitamins								
Riboflavin	$3 \cdot 10^{-4}$	X	X	X				
Thiamine	10^{-4}	X	X	X				X
Niacine	0.015	X	X	X				X
Vitamin B12	$1 \cdot 10^{-5}$	X	X	X				X
Vitamin D	$1 \cdot 10^{-5}$	X	X	X				
Aminobenzoic acid	$4 \cdot 10^{-4}$		X	X				X
Folic acid	$3.2 \cdot 10^{-4}$		X	X				X
Piridoxal	$5 \cdot 10^{-4}$		X	X				X
Trace elements								X
Murashigue (Solution 10X)	X					X		X
Bases								
Adenine	0.005		X					X
Guanine	0.01		X					X
Uracil	0.01		X					X

Graffenstaden, France). Cell fragments were then tested against *L. monocytogenes* using agar spot assay.

2.5. Inhibition test in chemically defined medium

2.5.1. Sequential culture

Bioprotective strain *L. piscium* was inoculated in fresh MSMA medium at 1% (inoculation level: 10^6 CFU ml^{-1}) and incubated at 26 °C. After 24 h incubation, the culture was centrifuged at 11,600 g for 10 min and the supernatant was filter sterilized on 0.45 μm filter membrane (Sartorius Stedim Biotech) to eliminate *L. piscium* cells. The resultant sterile pre-fermented medium was then inoculated with *L. monocytogenes* at 10^3 CFU ml^{-1} and growth was monitored at 26 °C by classical enumeration. A culture of *L. monocytogenes* in non pre-fermented MSMA was performed as control.

2.5.2. Mixed culture (co-culture with cellular contact)

L. piscium and *L. monocytogenes* were co-inoculated respectively at 10^6 CFU ml^{-1} and 10^3 CFU ml^{-1} in MSMA medium. The culture was incubated at 26 °C for 48 h and growth of the two strains was monitored as described in 2.1. Controls consisted in monoculture of each strain in MSMA at 26 °C.

2.5.3. Diffusion chamber culture (co-culture without cellular contact)

A double chambers system separated with a filter size of 0.22 μm (Sartorius Stedim Biotech) was built by a local glassworker. The system was sterilized by autoclaving at 121 °C, 15 min, before assembling aseptically. Each chamber was filled with 150 ml of MSMA and *L. piscium* was inoculated at 10^6 CFU ml^{-1} in the first chamber whereas *L. monocytogenes* was inoculated in the second chamber at 10^3 CFU ml^{-1}. Monoculture of each strain was performed as control (strain in the first compartment and sterile MSMA in the second).

2.6. Bacterial interaction mechanism

2.6.1. Metabolomic profile

Metabolomic profiles were generated from *L. piscium* and *L. monocytogenes* cultures in MSMA after 48 h of incubation at 26 °C. Non inoculated MSMA was used as control. Fifteen milliliters of cultures were centrifuged at 8500 g for 10 min and the supernatant was filter sterilized with 0.22 μm membrane filters (Sartorius Stedim Biotech) and stored at −80 °C before analysis. Each condition was repeated six times. Metabolomic fingerprints were acquired at

LABERCA (Oniris, France) by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) operating in the positive electrospray ionization mode (ESI+) and full scan acquisition mode (m/z 50–800) at a 30,000 resolution fwhm (Thermo Scientific LTQ© – Orbitrap instrument).

2.6.2. Amino acid analysis

The amino acids consumption of *L. piscium* and *L. monocytogenes* in MSMA medium was assessed. The analysis was performed in triplicates on supernatant after 48 h of culture at 26 °C by High Performance Liquid Chromatography (HPLC, Kontron, Eching am Ammersee, Germany) according to the WATERSAccQ.Tag method. Briefly, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reacted with amino acids to form a fluorescent complex detected at 395 nm (spectrofluorimetry detector, Shimadzu RF-10XL). The separation was performed on C18 column with acetonitrile gradient from 8% to 30% during 23 min at 44.2 °C at a flow of 1 ml min^{-1}. Results were analyzed by Drylab software (Molnar-Institute, Berlin, Germany).

In this experimentation, a α-aminobutyric acid 2.5 mM was used as an internal standard.

2.6.3. Glucose, lactic acid and pH analysis

All the following analysis were performed in co-culture condition (mixed culture) and on control culture of each strain. L-lactic acid was measured on culture supernatant with the enzymatic kit 021 (Biosentec, Toulouse, France) according to supplier recommendation. Glucose was measured by the colorimetric method of Dubois et al. (1956). The pH values were recorded with a Mettler pH-meter (Mettler Delta 320, HELSTEAD, UK) at each enumeration time.

A lactic acid supplementation was performed using a sodium lactate solution (44.7 g l^{-1}) diluted at a required concentration in MSMA.

2.7. Effect of nutrients supplementation on L. monocytogenes co-cultured with L. piscium

Flasks containing 90 ml of MSMA were freshly prepared and inoculated by *L. piscium* and *L. monocytogenes* at 10^6 and 10^3 CFU ml^{-1} respectively before incubation at 26 °C. After 24 h of growth, when inhibition occurred, the following compounds amino acids mix, nitrogen bases, glucose, vitamins mix, iron citrate and magnesium sulfate were separately added in flasks of mixed cultures, to obtain initial concentration conditions (Table 1). One flask was supplemented with a mixture containing amino acids, nitrogen base and vitamins. The last flask used as control was completed with sterile water. All the samples were re-incubated at 26 °C for 24 h and bacterial growth was monitored by plate counts.

3. Results and discussion

3.1. Bacterial growth in MSMA media

As shown in previous studies, the mechanism of inhibition involved in the interaction between *L. piscium* and *L. monocytogenes* was not due to bacteriocin production, pH decrease or lactic acid production (Matamoros et al., 2009b; Fall et al., 2010a). The study of other interaction mechanisms like nutrient competition is usually performed through the development of chemically defined media (Nilsson et al., 2005; Nouaille et al., 2009) although it is known that the inhibitory effect is dependent of environmental parameters (Charlier et al., 2009). Using data from shrimp characterization (Fall et al., 2010b), composition of different media developed for the growth of *L. monocytogenes* (Premaratne et al., 1991), *Lactococcus lactis* (Jensen and Hammer, 1993) or *Lactobacillus sakei* (Lauret et al., 1996), different media (basal MSM and MSMA to F, Table 1) were tested for their ability to allow the growth at 8 °C of the protective and the target bacteria and to reproduce the inhibition observed in shrimp. Results from bacterial growth in monocultures (Table 2) showed a capacity of both strains to grow in all tested chemically defined media. The lowest growth rate was obtained in MSMD without vitamins and nitrogen acid whereas the best growth rates were observed in complete MSMF (0.093 h^{-1}) or MSMA (0.030 h^{-1}) for *L. piscium* and *L. monocytogenes* respectively. In comparison to MSMF, the growth of *L. piscium* in MSMA was slightly lower (0.058 h^{-1}), which may be explained by the absence of taurine, a compound present in large quantities in shrimp (Fall et al., 2012; Heu et al., 2003). However, the MSMA medium allowing the both strains to grow with close growth rates was chosen to follow bacterial interaction. As the preliminary co-culture tests performed in this medium at 8 °C were long, a temperature of 26 °C close to *L. piscium* optimal growth temperature was chosen for the next experiments. In these conditions, the growth rates observed in MSMA medium increased 10 fold for both species, reaching 0.256 h^{-1} and 0.572 for *L. piscium* and *L. monocytogenes* respectively. Furthermore, in mixed culture performed in MSMA medium when *L. piscium* reached its maximum concentration, the growth of *L. monocytogenes* was totally stopped at 10^6 CFU ml^{-1} after 20 h culture (Fig. 1) as observed in shrimp during the first 4 days (Fall et al., 2010a). Following these results, the growth of *L. piscium* and *L. monocytogenes* in MSMA at 26 °C was considered as an efficient model to study the interaction mechanism between these bacteria.

3.2. Evaluation of culture supernatants for antimicrobial activity

Recently, Matamoros et al. (2009b) have demonstrated that the inhibition of *L. monocytogenes* by *L. piscium* was not due to a bacteriocin-like compound in Elliker medium. However as the production of such antimicrobial molecules can be medium dependant, the production of secreted antimicrobial compounds was evaluated in MSMA using the agar spot test. No inhibition zones around the supernatant spot of a 24 or 48 h culture of *L. piscium* was observed.

To confirm these results, proteins contained in the supernatant were concentrated ten-fold by precipitation with ammonium sulfate to detect antimicrobial proteins that may be produced at low concentrations by the protective bacteria. Acidification of the medium was also used to release potential antimicrobial proteins or peptides that may adsorb on cell surface of *L. piscium*. In a last experiment, the inhibition potential of cell fragments obtained after glass bead disruption was tested. None of these experiments

Table 2
Maximum growth rate (μ_{max}) (h^{-1}) of *Lactococcus piscium* CNCM I-4031 and *Listeria monocytogenes* RF191 in the different developed Model shrimp Medium (MSM and MSMA to F), Elliker, BHI and shrimp juice at 8 °C.

Medium	μ_{max} of *L. piscium* CNCM I-4031 (h^{-1})	μ_{max} of *L. monocytogenes* RF191 (h^{-1})
MSM	0.029	0.018
MSMA	0.058	0.030
MSMB	0.043	0.028
MSMC	0.033	0.020
MCMD	0.018	0.014
MSME	0.043	0.023
MSMF	0.093	0.018
Elliker	0.050	0.037
BHI	0.066	0.035
Shrimp juice	0.044	0.033

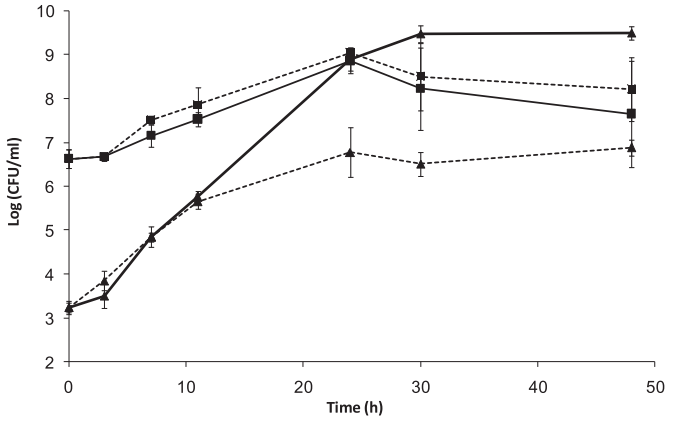

Fig. 1. Growth of *Listeria monocytogenes* RF191 (▲) and *Lactococcus piscium* CNCM I-4031 (■) in pure culture (full line) and in co-culture (dotted line) in MSMA at 26 °C.

allowed obtaining the inhibition activity. The same results were obtained by repeating these experiments with supernatant or cell extracts of the mixed culture (24 h in MSMA, 26 °C) to search for a potential induction of bacteriocin production as shown in the case of *Carnobacterium maltaromaticum* (Himelbloom et al., 2001).

All these data suggest that excreted antimicrobial compounds are not clearly involved in the inhibition of *L. monocytogenes* by *L. piscium* unlike what is commonly described for other LAB in seafood (Ghanbari et al., 2013).

3.3. Organic acid production

The L-lactic acid concentrations produced by each strain after 24 h of culture in MSMA were similar (3.71 ± 0.01 g l^{-1} for *L. piscium* and 3.73 ± 0.09 g l^{-1} for *L. monocytogenes*). In mixed culture the production reached 4.28 ± 0.06 g l^{-1}. No acetic acid nor D-lactic acid was produced. A monoculture of *L. monocytogenes* was performed in buffered MSMA supplemented with 4.28 g l^{-1} of lactic acid and compared to the monoculture in MSMA. The pH of the medium remained constant and no difference between both conditions was observed (data not shown). These observations indicated that the mechanism of inhibition is not due to production of lactic acid as demonstrated for other inhibition of pathogenic bacteria by LAB (Alomar et al., 2008a).

3.4. Nutritional competition

To determine the role of nutritional competition for some components of MSMA in the inhibition, the metabolomic fingerprints of *L. piscium* and *L. monocytogenes* cultured 48 h in MSMA were compared and sterile MSMA was added as control. Each test was performed in 6 independent cultures, allowing statistical analysis. Principal component analysis revealed a good discrimination of each strain on the basis of their global metabolic profiles (Fig. 2), suggesting that the two strains have clearly different metabolisms on MSMA. The consumption of nutrients like nitrogen bases and vitamins was particularly investigated since they are difficult to measure using classical methods. Diagnostic signal of adenine, guanine and uracil represented in Fig. 3A, B and C revealed that these bases were completely consumed by *L. piscium* and partially by *L. monocytogenes*. The rapid growth of *L. piscium* and its favorable initial ratio may lead to a quicker uptake and thus competition for those bases may be partially responsible for the inhibition. Riboflavin was the only vitamin totally consumed by both strains (Fig. 3D) and could thus be involved in the competition. Amino acids concentrations were measured by HPLC, after 48 h of cultures at 26 °C and are presented in Table 3. Cysteine, histidine and glycine were the major amino-acids consumed by *L. piscium* whereas *L. monocytogenes* also metabolized leucine, isoleucine and in few quantities the other amino-acids. These results are in agreement with those obtained in shrimp matrices where a weak amino-acids consumption was observed for *L. piscium* (Fall et al., 2012). The sums of the most amino acids uptake by *L. piscium* and *L. monocytogenes* were inferior to their initial concentration in MSMA. Competition for one of those nutrients is thus unlikely, except for cysteine which was almost totally consumed by the two strains, and in a lesser extends for histidine and glycine.

To confirm these results, supplementations of mixed culture after 24 h at 26 °C in MSMA with mix of all amino acids, mix of nitrogen bases, mix of vitamins, magnesium, iron and also a mix of

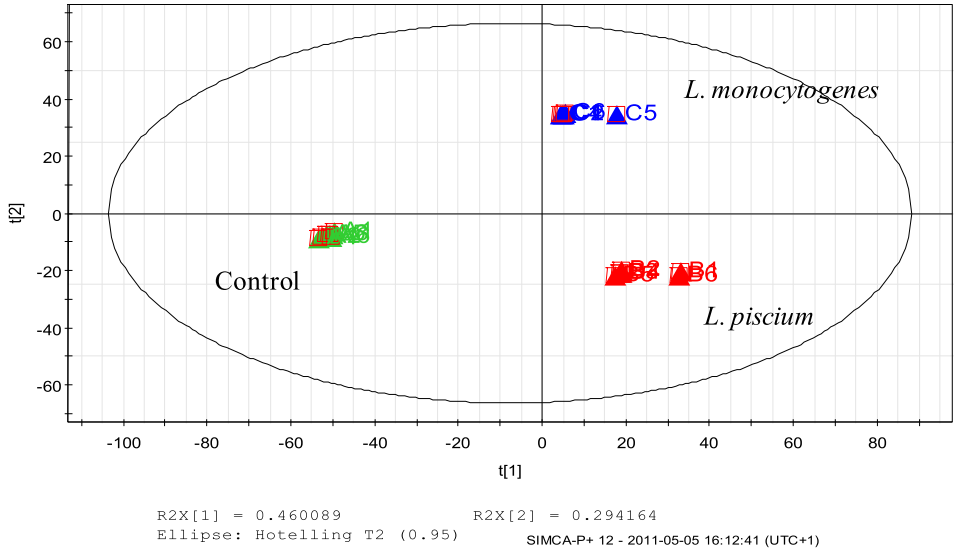

Fig. 2. Representation of Principal Component Analysis of metabolic fingerprint obtained for pure MSMA (control) and MSMA inoculated with *L. piscium* CNCM I-4031 or *L. monocytogenes* RF191 after 48 h of incubation at 26 °C.

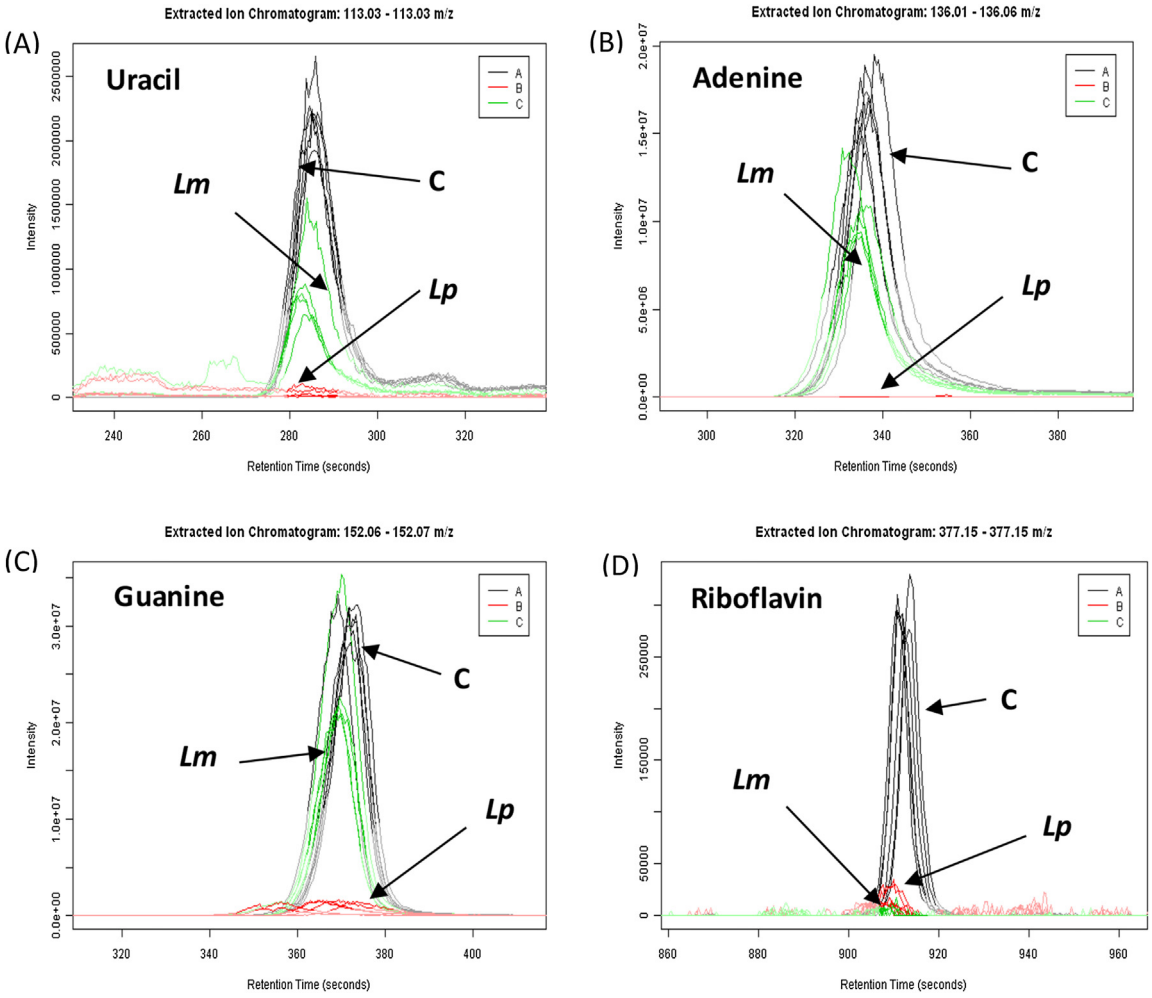

Fig. 3. Chromatograms peaks of ionized forms of uracil (A), adenine (B), guanine (C) and riboflavin (D) in pure MSMA (C) and MSMA inoculated with *L. piscium* CNCM I-4031 (Lp) or *L. monocytogenes* RF191 (Lm) after 48 h of incubation at 26 °C.

all those compounds was performed. None of the nutrient supplementation tested allowed the re-growth of *L. monocytogenes* (Fig. 4) which remained inhibited in all the conditions. These data clearly demonstrated that nutrients listed above were not involved in the interaction between the protective and the target strains. These results are in agreement with those obtained by Alomar et al. (2008b) who did not succeed to prove the amino acids implication in the inhibition of *Staphylococcus aureus* by *Lactococcus garvieae* in microfiltered milk. Similarly, Nilsson et al. (2005) failed to suppress the inhibition of *L. monocytogenes* by *C. maltaromaticum* when supplementing the medium with amino acids or vitamins. In this last study, the authors have demonstrated the role of glucose consumption in the inhibition. When *L. monocytogenes* cultures either in diffusion chamber or in medium pre-fermented by *C. maltaromaticum* supplemented with glucose, it showed its ability to restart growing and suppress the inhibition effect. On the opposite, in our experiments, the glucose supplementation (7 g l^{-1}) during the co-culture did not restore *L. monocytogenes* growth. Moreover, in these conditions *L. monocytogenes* decreased from 7.1 to 5.3 log CFU ml^{-1} in 7 h, and no viable cells were detected after 10 h of culture (Fig. 4). This result can probably be explained by the high acidification (pH = 4.23) of the medium due to the growth and lactic acid production by *L. piscium*.

The initial glucose present in MSMA medium (7.00 g l^{-1}) was totally consumed in monoculture of *L. monocytogenes*, or *L. piscium* and in mixed culture after 24 h with values of 0.26 ± 0.02, 0.54 ± 0.00 g l^{-1} and 0.25 ± 0.00 g l^{-1} respectively. In order to further investigate the hypothesis of competition for glucose, mixed cultures were performed in MSMA containing higher

Table 3
Concentration of amino acid (g l^{-1}) in sterile MSMA and consumption after 48 h at 26 °C by *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 (Glutamate, threonine, arginine and glutamine could not be measured by this method).

Amino-acids	Composition of MSMA (g l^{-1})	Consumption of amino acid by *L. piscium* CNCM I-4031 (g l^{-1})	Consumption of mino acid by *L. monocytogenes* RF191 (g l^{-1})
Cysteine	0.052 ± 0.006	0.049 ± 0.005	0.040 ± 0.002
Glycine	0.101 ± 0.005	0.032 ± 0.006	0.088 ± 0.001
Histidine	0.079 ± 0.004	0.042 ± 0.002	0.047 ± 0.001
Isoleucine	0.053 ± 0.002	0.012 ± 0.003	0.037 ± 0.001
Leucine	0.053 ± 0.002	0.005 ± 0.003	0.032 ± 0.001
Lysine	0.072 ± 0.001	0.011 ± 0.004	0.019 ± 0.002
Methionine	0.091 ± 0.007	0.004 ± 0.006	0.015 ± 0.003
Phenylalanine	0.052 ± 0.003	−0.002 ± 0.004	0.016 ± 0.001
Proline	0.112 ± 0.010	0.004 ± 0.014	−0.004 ± 0.008
Serine	0.141 ± 0.005	0.002 ± 0.006	0.010 ± 0.005
Tryptophan	0.026 ± 0.002	0.004 ± 0.002	0.007 ± 0.000
Tyrosine	0.055 ± 0.003	−0.001 ± 0.003	0.010 ± 0.002

(±: 95% confidence interval).

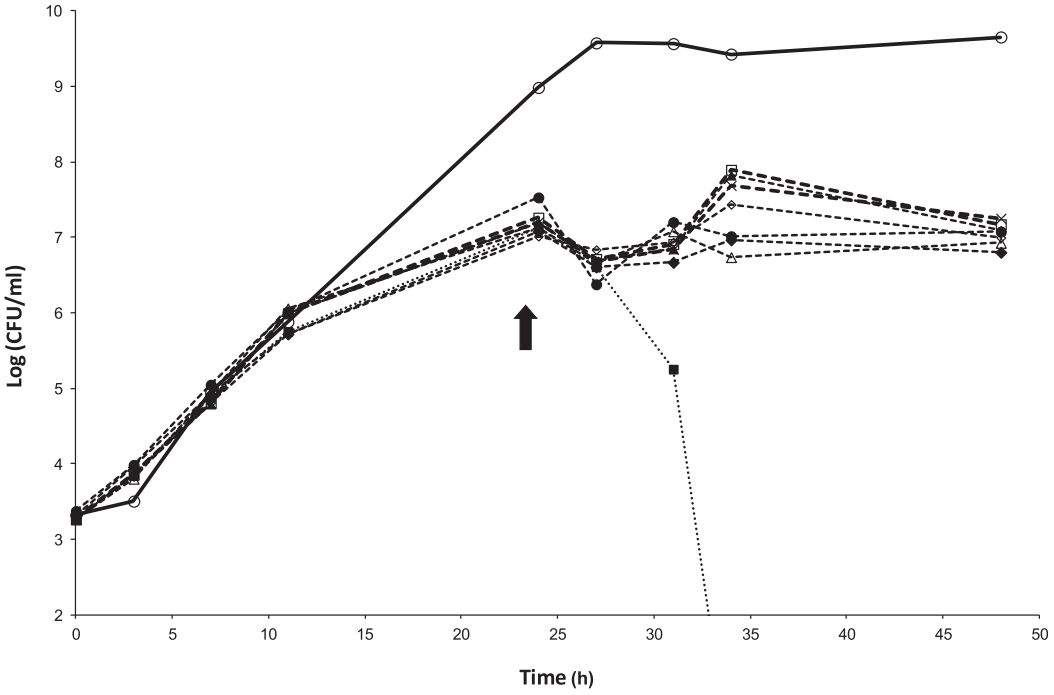

Fig. 4. Growth of *L. monocytogenes* RF191 in co-culture at 26 °C with *L. piscium* CNCM I-4031 in MSMA medium supplemented after 24 h with nitrogen bases, vitamins, amino-acids, iron citrate, magnesium sulfate, glucose and the mix of amino acids, vitamins and nitrogen bases. ⬆: Indicate the time of supplementation with nutriments; —○—: *L. monocytogenes* in pure culture; ··◆··: *L. monocytogenes* in co-culture; ··×··: *L. monocytogenes* in co-culture + iron citrate; ··□··: *L. monocytogenes* in co-culture + magnesium sulfate; ··◇··: *L. monocytogenes* in co-culture + amino acids; ··△··: *L. monocytogenes* in co-culture + vitamins; ··▲··: *L. monocytogenes* in co-culture + nitrogen bases; ··●··: *L. monocytogenes* in co-culture + vitamins + amino acids + nitrogen bases; ··■··: *L. monocytogenes* in co-culture + glucose.

glucose concentration (20.00 g l^{-1}). In that case, the inhibition of *L. monocytogenes* was also observed whereas the final glucose concentration was 14.00 g l^{-1} and no pH drop was observed, suggesting that glucose is not a limiting factor which could have explained the inhibition of *L. monocytogenes*.

All these results suggested that the inhibition of *L. monocytogenes* by *L. piscium* might involve other mechanisms than antimicrobial production or nutritional competition. It was confirmed by the ability of *L. monocytogenes* to grow in MSMA pre-fermented by *L. piscium* with the same growth rate (0.572 h^{-1}) and same final concentration (9 log CFU ml^{-1}) as in non pre-fermented MSMA (data not shown). The mechanism that is involved in this inhibition can thus not be compared to the one described by Nilsson et al. (2005) for *L. monocytogenes* and *C. maltaromaticum* for which the same inhibition was observed in sequential culture and co culture.

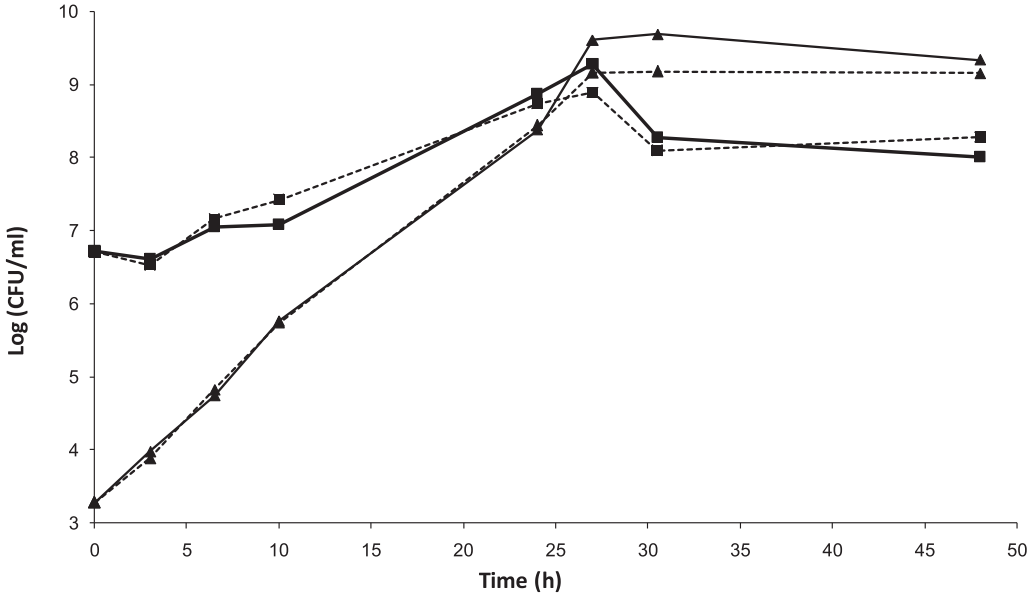

Fig. 5. Growth of *Listeria monocytogenes* RF191 (▲) and *Lactococcus piscium* CNCM I-4031 (■) alone (against sterile MSMA) (full line) and in co-culture (dotted line) in MSMA at 26 °C using a diffusion chamber.

3.5. Cell-to-cell contact inhibition

A co-culture of *L. piscium* and *L. monocytogenes* was performed in diffusion chambers separated by a 0.22 μm membrane. In these conditions, the bacterial cells were physically separated, whereas the diffusion of nutrients and extracellular compounds through the filter was possible. Fig. 5 showed that the growth of *L. monocytogenes* was similar to that obtained in monoculture with a maximum population of 9 log CFU ml^{-1} and a growth rate of 0.567 h^{-1} after 30 h of incubation. No bacterial inhibition was observed in this experiment till 48 h of incubation. These results indicate clearly that inhibition of *L. monocytogenes* by *L. piscium* requires cell-to-cell contact between the bacteria. Using the same approach with semi permeable membrane between cultures, Woo et al. (2011) led to the same conclusion for *in-vitro* detoxification of the pathogenic *Clostridium difficile* by the probiotic *Clostridium butyricum* MIYAIRI 588. Bavananthasivam et al. (2012), made the same observation for the inhibition of *Mannheimia haemolytica* by *Pasteurella multocida* that is also contact dependent. To our knowledge, this is the first report on a cell-contact dependent inhibition system within LAB and target bacteria.

Contact dependent inhibition mechanism can be explained by exchange of information between bacteria such as conjugation, secretion systems, contact dependent inhibition, allolysis and nanotubes. Conjugation is a horizontal transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection (Zechner et al., 2012). Secretion systems pathways (type IV and VI) were discovered in Gram-negative bacteria. They facilitate the transport of DNA, proteins or molecules from the bacterial cytoplasm directly into prokaryote cells (Tseng et al., 2009). The type IV secretion system is the unique type secretion system discovered in Gram-positive bacteria (Melville and Craig, 2013). In addition to these mechanisms, Aoki et al. (2005) have also demonstrated that some *Escherichia coli* strains may cause contact-depending inhibition (CDI) of other *E. coli* strains. This system is widespread among proteobacteria (Poole et al., 2011), and was *in sillico* identified but not experimentally demonstrated in Gram-positive bacteria (Diner et al., 2012; Holberger et al., 2012). In 2005, Guiral et al. have observed that in nutriment starvation, the competent cells *Streptococcus pneumoniae* produced two cell surface bacteriocins (CibA and CibB). In contact with incompetent cells, these two bacteriocins trigger all hydrolyses and lytic proteins of incompetent bacteria, causing their autolysis. Finally, the presence of nanotubes, recently described by Dubey and Ben-Yehuda (2011) also allow bacteria to exchange their cellular compounds (plasmids, protein, small molecules) with neighboring cells through direct cell contact.

In conclusion, we have demonstrated that *L. piscium* CNCM I-4031 is able to inhibit the growth of *L. monocytogenes* in shrimp matrices and in a chemically modified medium MSMA. This inhibition is not due to the excretion of antimicrobial compounds nor to nutritional competition as frequently described for other interactions between LAB and *L. monocytogenes*. The exact mechanisms of inhibition were not identified, however, it was shown that cellular contact is required to obtain the inhibition of the pathogenic bacteria by *L. piscium*. This is the first report of contact dependent inhibition for LAB and further studies are in progress to elucidate the specific mechanisms that are involved in this inhibition.

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