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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.

Highlights

► A chemically defined medium (MSMA) was developed to study bacterial interactions. ► *Lactococcus piscium* CNCM I-4031 inhibits the growth of *L. monocytogenes* as in shrimp. ► This interaction requires contact between both strains. ► First report of contact dependant inhibition between a LAB and *L. monocytogenes*.

Keywords : Listeria monocytogenes, Lactococcus piscium, nutrients competition, cellular contact, chemically defined medium, bacterial interaction

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42 **1. Introduction**

43

44 *Listeria monocytogenes* is an opportunistic pathogenic Gram positive bacterium and the
45 etiologic agent of listeriosis, a severe food-borne disease (Vazquez-Boland et al., 2001) with
46 high hospitalization cases (~300 cases/year from 2006 to 2011 in France) and high fatality
47 rates (20 to 30%).The populations at greatest risk are newborn infants, pregnant women,
48 elderly persons, and persons with a weak immune system (Lecuit and Leclercq, 2012). *L.*
49 *monocytogenes* is able to grow in most of the conditions found in the food chain, such as high
50 salt concentrations, presence of CO_2, and low temperatures. The resistance of this bacterium
51 to these environmental factors makes this organism difficult to control in refrigerated food
52 product (Gandhi and Chikindas, 2007). Many studies have been published concerning the
53 inhibition of *L. monocytogenes* using various preservation technologies. Biopreservation
54 technique which consists in using natural, selected protective microorganisms, was
55 demonstrated as an efficient strategy for the control of *L. monocytogenes* in a variety of
56 ready-to-eat seafood or meat products (Ananou et al., 2005; Benkerroum et al., 2005; Pilet
57 and Leroi, 2011). Lactic acid bacteria (LAB) are excellent candidates for preventing the
58 growth of pathogenic bacteria in food products because they show bacteriostatic effect
59 towards many bacterial species through various mechanisms, without causing unacceptable
60 sensory changes in foodstuffs (Stile, 1996; Ghanbari et al., 2013). The growth inhibition of
61 the target bacteria when LAB have reached their maximum level is usually described as
62 Jameson effect (Jameson, 1962)). These inhibitions sometimes involve well known antagonist
63 mechanisms such as production of antimicrobial compounds as bacteriocins, bacteriocin-like
64 inhibitory substance (Richard et al., 2003; Schöbitz et al., 2003 ; Naghmouchi et al.,
65 2006,2007 ; Rihakova et al., 2009), or reuterin (El-Ziney et al., 1999), organic acid (Alomar et
66 al., 2008a) or hydrogen peroxide (Delbes-Paus et al., 2010). However, in some cases, the
67 production of inhibiting metabolites is not evidenced and other hypothesis must be tested. As
68 an example, Nilsson et al. (2005) have demonstrated the involvement of glucose competition
69 in the inhibition of *L. monocytogenes* by a non-bacteriocin producing *Carnobacterium*
70 *piscicola*. In a recent study, a protective strain, *Lactococcus piscium* CNCM I-4031, was
71 isolated from salmon steak stored under modified atmosphere. This strain was able to improve
72 sensory quality of seafood (Matamoros et al., 2009a,b) by preventing the growth of
73 *Brochothrix thermosphacta* (Fall et al., 2010b). *L. piscium* CNCM I-4031 has also shown its
74 ability to limit the growth of *L. monocytogenes* during the storage of cooked shrimp (Fall et
75 al., 2010a). The aim of the present study was to gain insight the mechanism involved in the

76 inhibition of *L. monocytogenes* by *L. piscium* CNCMI-4031 as it is one of the required
77 knowledge on protective flora to ensure their possible acceptability and use for food
78 preservation. For this purpose, a chemical defined medium close to shrimp composition has
79 been first set up to reproduce the inhibition of *L. monocytogenes* by *L. piscium* observed in
80 shrimp matrices. Then different tests to investigate the mechanisms have been developed.
81

82 2. Materials and methods

83

84 *2.1 Bacterial strains, culture media and conditions*

85 *L. piscium* CNCM-I 4031 was isolated from fresh salmon steak packed under modified
86 atmosphere (Matamoros et al., 2009a). The target strain *L. monocytogenes* RF191 was
87 isolated from tropical cooked peeled shrimp by PFI Nouvelles Vagues (Boulogne-sur-mer,
88 France) and used before as target strain in challenge-tests (Fall et al., 2010a). The two strains
89 were stored at -80°C in their culture media with 20% glycerol (Panreac, Barcelona, Spain).
90 For all experiments, *L. piscium* and *L. monocytogenes* were subcultured in Elliker broth and
91 Brain Heart Infusion (BHI) supplemented with 2% NaCl (Biokar Diagnostic, Beauvais,
92 France) respectively for 24 h at 26°C. The cultures were diluted in their culture media, if
93 necessary, to obtain appropriate initial cell concentrations. *L. piscium* was enumerated on
94 spread Elliker agar plates after incubation at 8°C for 5 days under anaerobiosis and *L.*
95 *monocytogenes* on spread Palcam agar plates (Biokar) incubated at 37°C for 24 h.
96

97 *2.2 Chemically defined medium set up*

98 To improve the bacteria growth, six different media were prepared by supplementing the MSM with
99 the different compounds listed in Table 1 and recommended by Premaratne et al. (1991), Jensen
100 and Hammer (1993), Lauret et al. (1996) and Fall et al. (2012).
101 The components were prepared as concentrated solution of mix or single solution to avoid
102 medium precipitation. All the amino acids (Sigma Aldrich, Saint-Louis, MO, United-States)
103 were mixed except tyrosine, glutamine and cysteine that were used as single solutions.
104 Vitamins (Sigma Aldrich) were separated in two mix containing riboflavine, thiamine,
105 niacine, vitamin B12 and vitamin D for mix 1 or folic acid, aminobenzoic acid, and piridoxal
106 (vitamin B6) for mix 2. Adenine, guanine, uracil (Sigma Aldrich) and glucose (Merck,
107 Darmstadt, Germany) were prepared as single solutions. All these components were dissolved
108 in distilled water and filter sterilized using Acrodisc 0.45-µm-pore-size membrane (Sartorius
109 Stedim Biotech, Goettingen, Germany) and can be stored at -20°C (mix of amino acids and

110 vitamins) or 4°C up to 15 days. Salts solution containing ammonium citrate, magnesium
111 sulfate or sodium chloride (Merck) were prepared separately in distilled water, sterilized for
112 15 min at 121°C and stored at 4°C up to 15 days. The final media were prepared by mixing
113 the components in the following order : ammonium citrate, magnesium sulfate, sodium
114 chloride, mix of amino acids, glucose, adenine, guanine, uracil, tyrosine, glutamine, cysteine,
115 phosphate buffer [final molarity Na_2HPO_4 $7H_20$ (0.11 M) and KH_2PO_4 (0.05 M)] and finally
116 mix 1 and mix 2 of vitamins. The final pH was 7.0.
117 Fresh MSMA to -F media were then inoculated at 10^4 CFU ml^{-1} with overnight subcultured
118 strains and growth at 26°C was monitored by Petri dish enumeration technique.
119

120 *2.3 Shrimp juice*

121 The shrimp juice was prepared by crushing thawed raw peeled shrimp in a Warring
122 Blender (New Hartford, CT, USA) with distilled water. The mixture was then boiled for 2
123 min and filtered through a filter (no.127, Durieux, Paris, France). NaCl 20 g l^{-1} was added to
124 the clear broth obtained before autoclaving at 100°C for 30 min (Fall et al., 2010b) and
125 growth of strains was performed as in MSM.
126

127 *2.4 Antimicrobial assay*

128 • *Agar spot assay*

129 The presence of antimicrobial compounds in *L. piscium* culture or co-culture with *L.*
130 *monocytogenes* was evaluated using solid BHI (2% NaCl) agar spot assay (Matamoros et al.,
131 2009a). The supernatant of *L. piscium* was obtained after centrifugation of 10 ml of a 24 h
132 culture or co-culture (11600 g for 10 min at 4°C). The supernatant was then filter sterilized
133 (0.45-µm). One milliliter of a suspension containing 10^6 CFU ml^{-1} of *L. monocytogenes* was
134 poured in 15 ml BHI agar plates (2%NaCl, 1% agar) and kept at room temperature for 15-20
135 min. Ten microliters of filtered supernatant were then dropped (as a spot) onto the solidified
136 BHI agar and the plates were incubated 24 to 72 h at 26°C to detect inhibition zones around
137 the spots.
138
139 • *Inhibition test after protein purification*

140 The precipitation of potential antimicrobial peptides was performed by treatment of
141 supernatant using ammonium sulfate precipitation. Ammonium sulfate was added to the
142 filtered supernatant at saturation of 40% and 80% (http://www.encorbio.com/protocols/AM-
143 SO4.htm) for 1 h under stirring. After centrifugation (10000 g, 15 min, 4°C), the supernatant

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144 was removed and the precipitate was resuspended and concentrated 10 fold in distilled water.
145 The pH was adjusted to 6.9 and the suspension was filter sterilized and tested using solid BHI
146 (2% NaCl) spot agar assay as described above.

147

148 - *inhibition test after cells treatments*

149 To remove the putative proteins linked to the *L. piscium* membrane, cultures in MSMA,
150 (24 h at 26°C) were acidified for 2 h at pH 2 with HCl 10 M . The pH was then adjusted to its
151 initial value with NaOH (10 M) and the supernatant was tested after ammonium sulfate
152 precipitation by agar spot assay.

153 The internal content of *L. piscium* cell was tested as follows: cells from *L. piscium* (24 h at
154 26°C in MSMA medium) were pelleted by centrifugation (11600 g, 10 min) and disrupted
155 using 0.2 g of glass beads (150 to 200 µm diameter) and shaking twice for 2 min in a bead
156 beater MM200 (30 Hz) (Fisher Bioblock Scientific, Illkirch-Graffenstaden, France). Cell
157 fragments were then tested against *L. monocytogenes* using agar spot assay.

158

159 *2.5 Inhibition test in chemically defined medium*

160 *2.5.1 Sequential culture*

161 Bioprotective strain *L. piscium* was inoculated in fresh MSMA medium at 1% (inoculation
162 level: 10^6 CFU ml^{-1}) and incubated at 26°C. After 24 h incubation, the culture was centrifuged
163 at 11600 g for 10 min and the supernatant was filter sterilized on 0.45 µm filter membrane
164 (Sartorius Stedim Biotech) to eliminate *L. piscium* cells. The resultant sterile pre-fermented
165 medium was then inoculated with *L. monocytogenes* at 10^3 CFU ml^{-1} and growth was
166 monitored at 26°C by classical enumeration. A culture of *L. monocytogenes* in non pre-
167 fermented MSMA was performed as control.

168

169 *2.5.2 Mixed culture (co-culture with cellular contact)*

170 *L. piscium* and *L. monocytogenes* were co-inoculated respectively at 10^6 CFU ml^{-1} and
171 10^3 CFU ml^{-1} in MSMA medium. The culture was incubated at 26°C for 48 h and growth of
172 the two strains was monitored as described in 2.1. Controls consisted in monoculture of each
173 strain in MSMA at 26°C.

174

175 *2.5.3 Diffusion chamber culture (co-culture without cellular contact)*

176 A double chambers system separated with a filter size of 0.22 µm (Sartorius Stedim
177 Biotech) was built by a local glassworker. The system was sterilized by autoclaving at 121°C,

178 15 min, before assembling aseptically. Each chamber was filled with 150 ml of MSMA and *L.*
179 *piscium* was inoculated at 10^6 CFU ml^{-1} in the first chamber whereas *L. monocytogenes* was
180 inoculated in the second chamber at 10^3 CFU ml^{-1}. Monoculture of each strain was performed
181 as control (strain in the first compartment and sterile MSMA in the second).
182
183 *2.6 Bacterial interaction mechanism*
184 *2.6.1 Metabolomic profile*
185 Metabolomic profiles were generated from *L. piscium* and *L. monocytogenes* cultures
186 in MSMA after 48 h of incubation at 26°C. Non inoculated MSMA was used as control.
187 Fifteen milliliters of cultures were centrifuged at 8500 g for 10 min and the supernatant was
188 filter sterilized with 0.22 µm membrane filters (Sartorius Stedim Biotech) and stored at -80°C
189 before analysis. Each condition was repeated six times. Metabolomic fingerprints were
190 acquired at LABERCA (Oniris, France) by liquid chromatography coupled to high resolution
191 mass spectrometry (LC-HRMS) operating in the positive electrospray ionization mode (ESI+)
192 and full scan acquisition mode (m/z 50 to 800) at a 30,000 resolution fwhm (Thermo
193 Scientific LTQ© - Orbitrap instrument).
194
195 *2.6.2 Amino acid analysis*
196 The amino acids consumption of *L. piscium* and *L. monocytogenes* in MSMA medium
197 was assessed. The analysis was performed in triplicates on supernatant after 48 h of culture at
198 26°C by High Performance Liquid Chromatography (HPLC, Kontron, Eching am Ammersee,
199 Germany) according to the WATERSAccQ.Tag method. Briefly, 6-aminoquinolyl-N-
200 hydroxysuccinimidyl carbamate (AQC) reacted with amino acids to form a fluorescent
201 complex detected at 395 nm (spectrofluorimetry detector, Shimadzu RF-10XL). The
202 separation was performed on C18 column with acetonitrile gradient from 8% to 30% during
203 23 min at 44.2°C at a flow of 1 ml min^{-1}. Results were analyzed by Drylab software (Molnar-
204 Institute, Berlin, Germany).
205 In this experimentation, a α-aminobutyric acid 2.5 mM was used as a internal standard.
206
207 *2.6.3 Glucose, lactic acid and pH analysis*
208 All the following analysis were performed in co-culture condition (mixed culture) and on
209 control culture of each strain. L-lactic acid was measured on culture supernatant with the
210 enzymatic kit 021 (Biosentec, Toulouse, France) according to supplier recommendation.
211 Glucose was measured by the colorimetric method of Dubois et al (1956). The pH values

212 were recorded with a Mettler pH-meter (Mettler Delta 320, HELSTEAD, UK) at each
213 enumeration time.
214 A lactic acid supplementation was performed using a sodium lactate solution (44.7 g l^{-1})
215 diluted at a required concentration in MSMA.
216
217 *2.7 Effect of nutrients supplementation on L. monocytogenes co-cultured with L. pisicum*
218 Flasks containing 90 ml of MSMA were freshly prepared and inoculated by *L. piscium*
219 and *L. monocytogenes* at 10^6 and 10^3 CFU ml^{-1} respectively before incubation at 26°C. After
220 24 h of growth, when inhibition occurred, the following compounds amino acids mix,
221 nitrogen bases, glucose, vitamins mix, iron citrate and magnesium sulfate were separately
222 added in flasks of mixed cultures, to obtain initial concentration conditions (Table 1). One
223 flask was supplemented with a mixture containing amino acids, nitrogen base and vitamins.
224 The last flask used as control was completed with sterile water. All the samples were re-
225 incubated at 26°C for 24 h and bacterial growth was monitored by plate counts.
226
227 **3. Results and Discussion**
228 *3.1. Bacterial growth in MSMA media*
229 As shown in previous studies, the mechanism of inhibition involved in the interaction
230 between *L. piscium* and *L. monocytogenes* was not due to bacteriocin production, pH decrease
231 or lactic acid production (Matamoros et al., 2009b ; Fall et al. 2010a). The study of other
232 interaction mechanisms like nutrient competition is usually performed through the
233 development of chemically defined media (Nilsson et al., 2005; Nouaille et al., 2009)
234 although it is known that the inhibitory effect is dependent of environmental parameters
235 (Charlier et al., 2009). Using data from shrimp characterization (Fall et al., 2010b),
236 composition different media developed for the growth of *L. monocytogenes* (Premaratne et al.,
237 1991), *Lactococcus lactis* (Jensen and Hammer, 1993) or *Lactobacillus sakei* (Lauret et al.,
238 1996), different media (basal MSM and MSMA to F, Table 1) were tested for their ability to
239 allow the growth at 8°C of the protective and the target bacteria and to reproduce the
240 inhibition observed in shrimp. Results from bacterial growth in monocultures (Table 2)
241 showed a capacity of both strains to grow in all tested chemically defined media. The lowest
242 growth rate was obtained in MSMD without vitamins and nitrogen acid whereas the best
243 growth rates were observed in complete MSMF (0.093 h^{-1}) or MSMA (0.030 h^{-1}) for *L.*
244 *piscium* and *L. monocytogenes* respectively. In comparison to MSMF, the growth of *L.*
245 *piscium* in MSMA was slightly lower (0.058 h^{-1}), which may be explained by the absence of

246 taurine, a compound present in large quantities in shrimp (Fall et al., 2012; Heu et al., 2003).
247 However, the MSMA medium allowing the both strains to grow with close growth rates was
248 chosen to follow bacterial interaction. As the preliminary co-culture tests performed in this
249 medium at 8°C were long, a temperature of 26°C close to *L. piscium* optimal growth
250 temperature was chosen for the next experiments. In these conditions, the growth rates
251 observed in MSMA medium increased 10 fold for both species, reaching 0.256 h^{-1} and 0.572
252 for *L. piscium* and *L. monocytogenes* respectively. Furthermore, in mixed culture performed
253 in MSMA medium when *L. piscium* reached its maximum concentration, the growth of *L.*
254 *monocytogenes* was totally stopped at 10^6 CFU ml^{-1} after 20h culture (Figure 1) as observed in
255 shrimp during the first 4 days (Fall et al., 2010a). Following these results, the growth of *L.*
256 *piscium* and *L. monocytogenes* in MSMA at 26°C was considered as an efficient model to
257 study the interaction mechanism between these bacteria.
258

259 *3.2. Evaluation of culture supernatants for antimicrobial activity*

260 Recently, Matamoros et al. (2009b) have demonstrated that the inhibition of *L.*
261 *monocytogenes* by *L. piscium* was not due to a bacteriocin-like compound in Elliker medium.
262 However as the production of such antimicrobial molecules can be medium dependant, the
263 production of secreted antimicrobial compounds was evaluated in MSMA using the agar spot
264 test. No inhibition zones around the supernatant spot of a 24 or 48 h culture of *L. piscium* was
265 observed.
266 To confirm these results, proteins contained in the supernatant were concentrated ten-fold by
267 precipitation with ammonium sulfate to detect antimicrobial proteins that may be produced at
268 low concentrations by the protective bacteria. Acidification of the medium was also used to
269 release potential antimicrobial proteins or peptides that may adsorb on cell surface of *L.*
270 *piscium*. In a last experiment, the inhibition potential of cell fragments obtained after glass
271 bead disruption was tested. None of these experiments allowed obtaining the inhibition
272 activity. The same results were obtained by repeating these experiments with supernatant or
273 cell extracts of the mixed culture (24 h in MSMA, 26°C) to search for a potential induction of
274 bacteriocin production as shown in the case of *Carnobacterium maltaromaticum*
275 (Himelbloom et al., 2001).
276 All these data suggest that excreted antimicrobial compounds are not clearly involved in
277 the inhibition of *L. monocytogenes* by *L. piscium* unlike what is commonly described for other
278 LAB in seafood (Ghanbari et al., 2013).
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280 *3.3.* Organic acid production

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

290

291 *3.4. Nutritional competition*

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

314 To confirm these results, supplementations of mixed culture after 24 h at 26°C in MSMA with
315 mix of all amino acids, mix of nitrogen bases, mix of vitamins, magnesium, iron and also a
316 mix of all those compounds was performed. None of the nutrient supplementation tested
317 allowed the re-growth of *L. monocytogenes* (Figure 4) which remained inhibited in all the
318 conditions. These data clearly demonstrated that nutrients listed above were not involved in
319 the interaction between the protective and the target strains. These results are in agreement
320 with those obtained by Alomar et al. (2008b) who did not succeed to prove the amino acids
321 implication in the inhibition of *S. aureus* by *L. garvieae* in microfiltered milk. Similarly,
322 Nilsson et al. (2005) failed to suppress the inhibition of *L. monocytogenes* by *C.*
323 *maltaromaticum* when supplementing the medium with amino acids or vitamins. In this last
324 study, the authors have demonstrated the role of glucose consumption in the inhibition. When
325 *L. monocytogenes* cultures either in diffusion chamber or in medium pre-fermented by *C.*
326 *maltaromaticum* supplemented with glucose, it showed its ability to restart growing and
327 suppress the inhibition effect. On the opposite, in our experiments, the glucose
328 supplementation (7 g l^{-1}) during the co-culture did not restore *L. monocytogenes* growth.
329 Moreover, in these conditions *L. monocytogenes* decreased from 7.1 to 5.3 log CFU ml^{-1} in 7
330 h, and no viable cells were detected after 10 h of culture (Figure 4). This result can probably
331 be explained by the high acidification (pH = 4.23) of the medium due to the growth and lactic
332 acid production by *L. piscium*.
333 The initial glucose present in MSMA medium (7.00 g l^{-1}) was totally consumed in
334 monoculture of *L. monocytogenes*, or*L. piscium* and in mixed culture after 24 h with values of
335 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
336 the hypothesis of competition for glucose, mixed cultures were performed in MSMA
337 containing higher glucose concentration (20.00 g l^{-1}). In that case, the inhibition of *L.*
338 *monocytogenes* was also observed whereas the final glucose concentration was 14.00 g l^{-1} and
339 no pH drop was observed, suggesting that glucose is not a limiting factor which could have
340 explained the inhibition of *L. monocytogenes.*
341 All these results suggested that the inhibition of *L. monocytogenes* by *L. piscium* might
342 involve other mechanisms than antimicrobial production or nutritional competition. It was
343 confirmed by the ability of *L. monocytogenes* to grow in MSMA pre-fermented by *L. piscium*
344 with the same growth rate (0.572 h^{-1}) and same final concentration (9 log CFU ml^{-1}) as in non
345 pre-fermented MSMA (data not shown). The mechanism that is involved in this inhibition can
346 thus not be compared to the one described by Nilsson et al. (2005) for *L. monocytogenes* and

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347 *C. maltaromaticum* for which the same inhibition was observed in sequential culture and co
348 culture.
349

350 *4.4. Cell-to-cell contact inhibition*

351 A co-culture of *L. piscium* and *L. monocytogenes* was performed in diffusion
352 chambers separated by a 0.22 µm membrane. In these conditions, the bacterial cells were
353 physically separated, whereas the diffusion of nutrients and extracellular compounds through
354 the filter was possible. Figure 5 showed that the growth of *L. monocytogenes* was similar to
355 that obtained in monoculture with a maximum population of 9 log CFU ml^{-1} and a growth rate
356 of 0.567 h^{-1} after 30 h of incubation. No bacterial inhibition was observed in this experiment
357 till 48 h of incubation. These results indicate clearly that inhibition of *L. monocytogenes* by *L.*
358 *piscium* requires cell-to-cell contact between the bacteria. Using the same approach with semi
359 permeable membrane between cultures, Woo et al. (2011) led to the same conclusion for *in-*
360 *vitro* detoxification of the pathogenic *Clostridium difficile* by the probiotic *Clostridium*
361 *butyricum* MIYAIRI 588. Bavananthasivam et al. (2012), made the same observation for the
362 inhibition of *Mannheimia haemolytica* by *Pasteurella multocida* that is also contact
363 dependent. To our knowledge, this is the first report on a cell-contact dependent inhibition
364 system within LAB and target bacteria.
365 Contact dependent inhibition mechanism can be explained by exchange of information
366 between bacteria such as conjugation, secretion systems, contact dependent inhibition,
367 allolysis and nanotubes. Conjugation is a horizontal transfer of genetic material between
368 bacterial cells by direct cell-to-cell contact or by a bridge-like connection (Zechner et al.,
369 2012). Secretion systems pathways (type IV and VI) were discovered in Gram-negative
370 bacteria. They facilitate the transport of DNA, proteins or molecules from the bacterial
371 cytoplasm directly into prokaryote cells (Tseng et al., 2009). The type IV secretion system is
372 the unique type secretion system discovered in Gram-positive bacteria (Melville and Craig,
373 2013). In addition to these mechanisms, Aoki et al. (2005) have also demonstrated that some
374 *E. coli* strains may cause contact-depending inhibition (CDI) of other *E. coli* strains. This
375 system is widespread among proteobacteria (Poole et al., 2011), and was *in sillico* identified
376 but not experimentally demonstrated in Gram-positive bacteria (Diner et al., 2012; Holberger
377 et al., 2011). In 2005, Guiral et al. have observed that in nutriment starvation, the competent
378 cells *Streptococcus pneumoniae* produced two cell surface bacteriocins (CibA and CibB). In
379 contact with incompetent cells, these two bacteriocins trigger all hydrolyses and lytic proteins
380 of incompetent bacteria, causing their autolysis. Finally, the presence of nanotubes, recently

381 described by Dubey and Ben-Yehuda (2011) also allow bacteria to exchange their cellular
382 compounds (plasmids, protein, small molecules) with neighboring cells through direct cell
383 contact.

384 In conclusion, we have demonstrated that *L. piscium* CNCM I-4031 is able to inhibit
385 the growth of *L. monocytogenes* in shrimp matrices and in a chemically modified medium
386 MSMA. This inhibition is not due to the excretion of antimicrobial compounds nor to
387 nutritional competition as frequently described for other interactions between LAB and *L.*
388 *monocytogenes*. The exact mechanisms of inhibition were not identified, however, it was
389 shown that cellular contact is required to obtain the inhibition of the pathogenic bacteria by *L.*
390 *piscium*. This is the first report of contact dependent inhibition for LAB and further studies
391 are in progress to elucidate the specific mechanisms that are involved in this inhibition.
392

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Table

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								
MgSO4,7H2O	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
Tryptophan	0,1		X		X			X
Tyrosine	0,05		X		X			X
Taurine	0,1						X	X
Vitamins								
Riboflavin	$3. 10^{-4}$	X	X	X				
Thiamine	10^{-4}	X	X	X				X
Niacine	0.015	X	X	X				X
Vitamin B12	$1. 10^{-5}$	X	X	X				X
Vitamin D	$5. 10^{-6}$	X	X	X				
Aminobenzoic acid	$4. 10^{-4}$		X	X				X
Folic acid	$3,2. 10^{-4}$		X	X				X
Piridoxal	$5. 10^{-4}$		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

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.

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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

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.

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Amino-acids	Composition of MSMA (g l^{-1})	Consumption of amino acid by *L. piscium* CNCM I-4031 (g l^{-1})	Consumption of amino 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

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)

(± : 95% confidence interval)

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Figures

Figure 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.

Figure 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.

Figure 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 .

Figure 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
—o— :*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

Figure 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.

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Figure 1

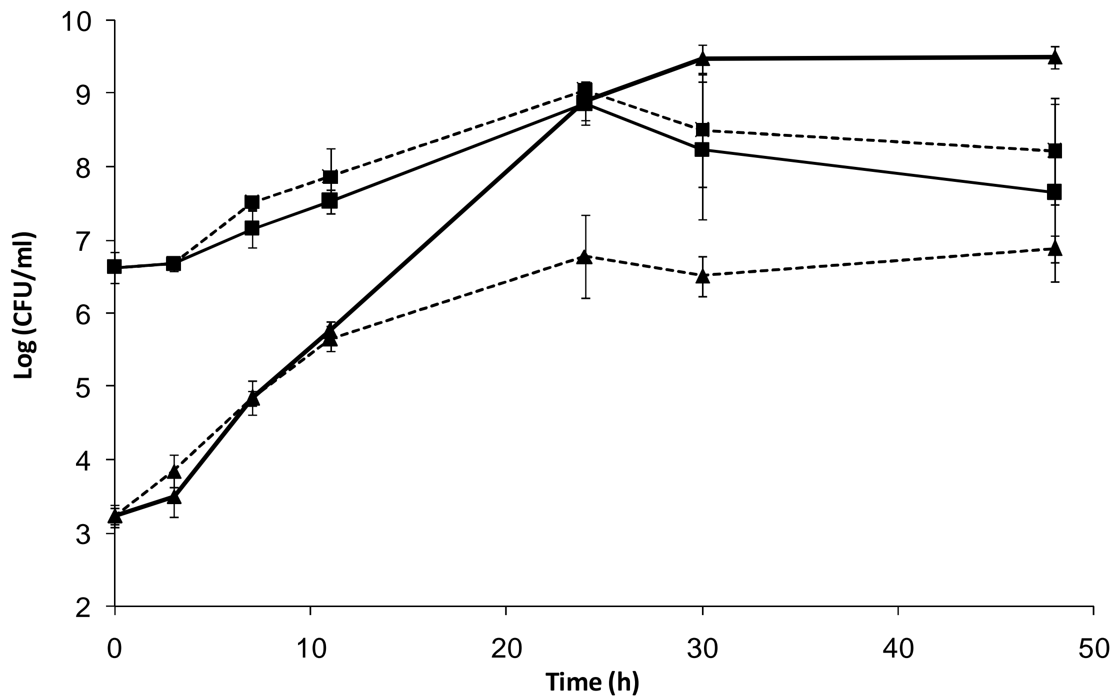

Figure 2

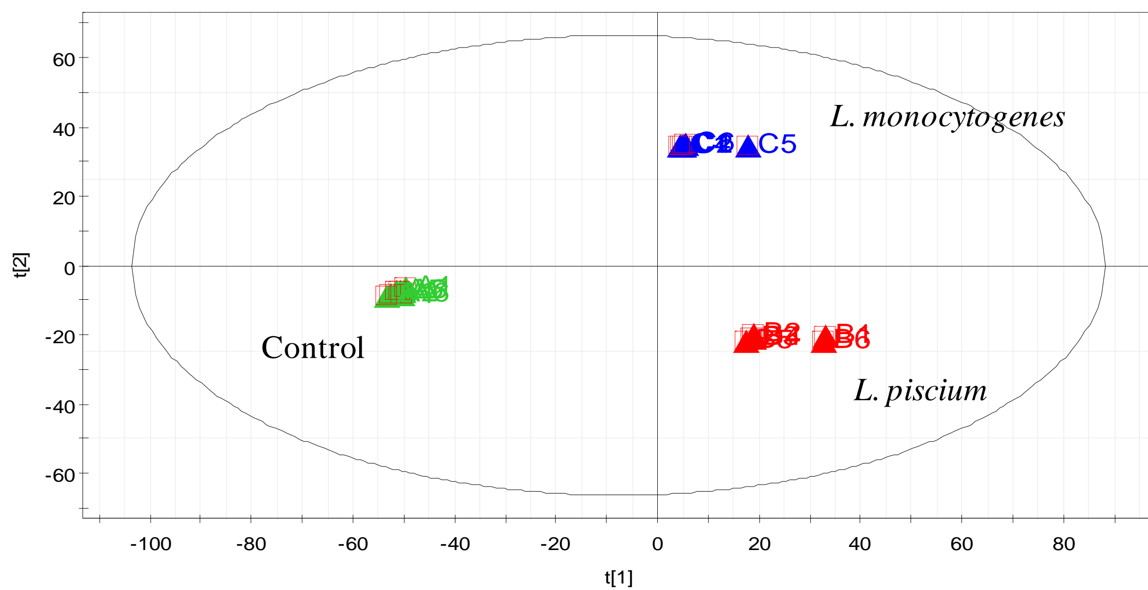

Figure 3

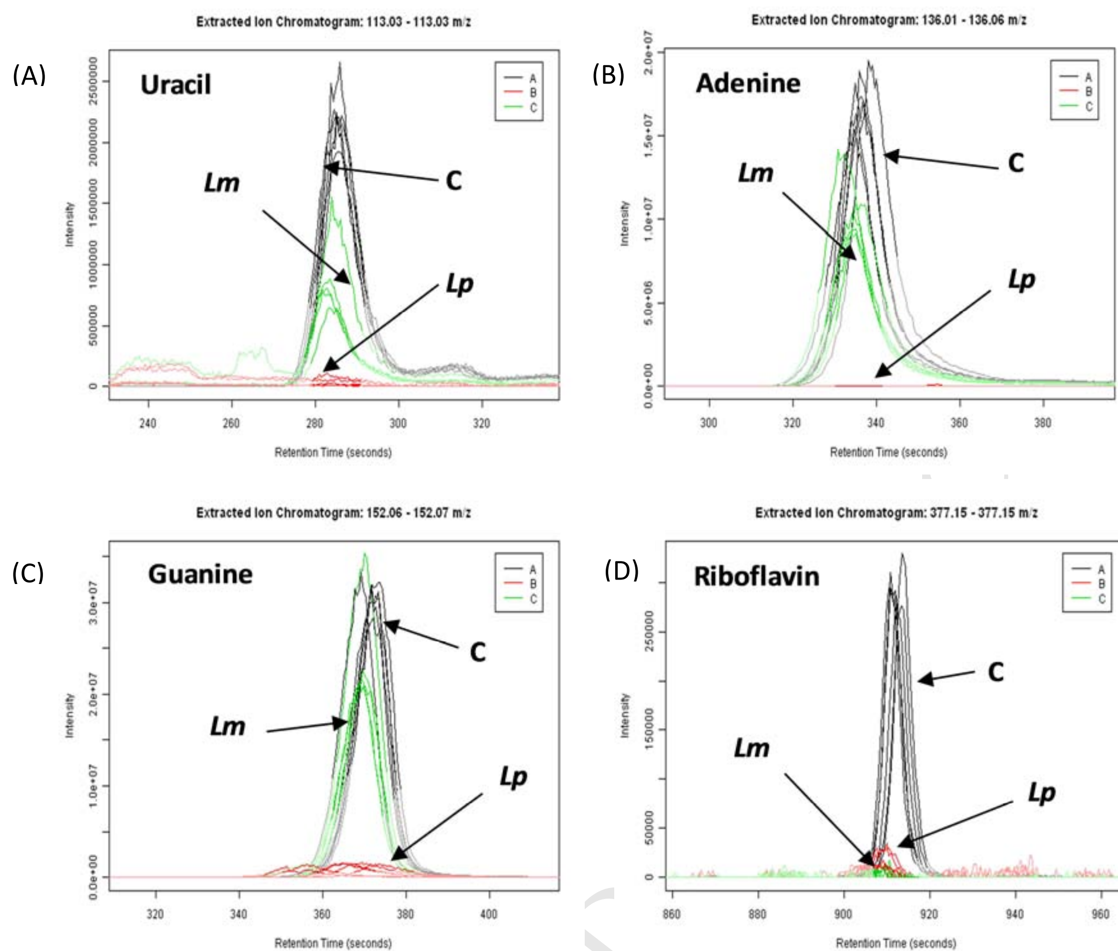

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Figure 4

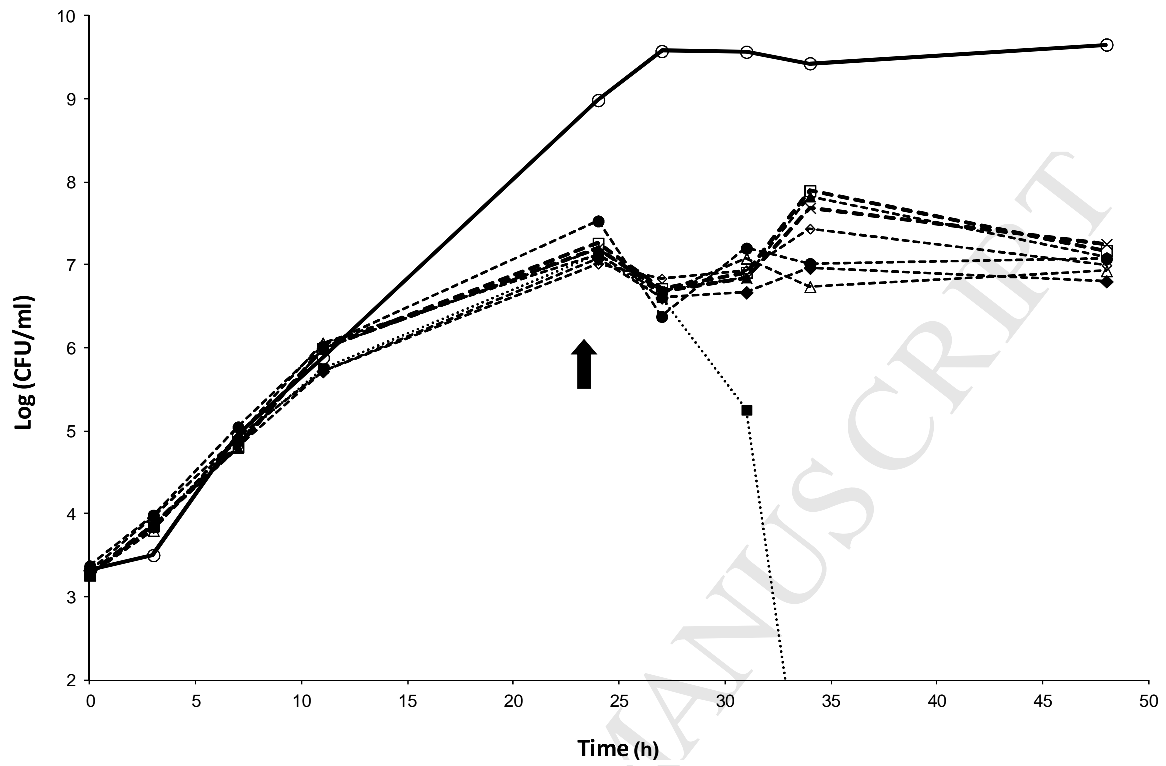
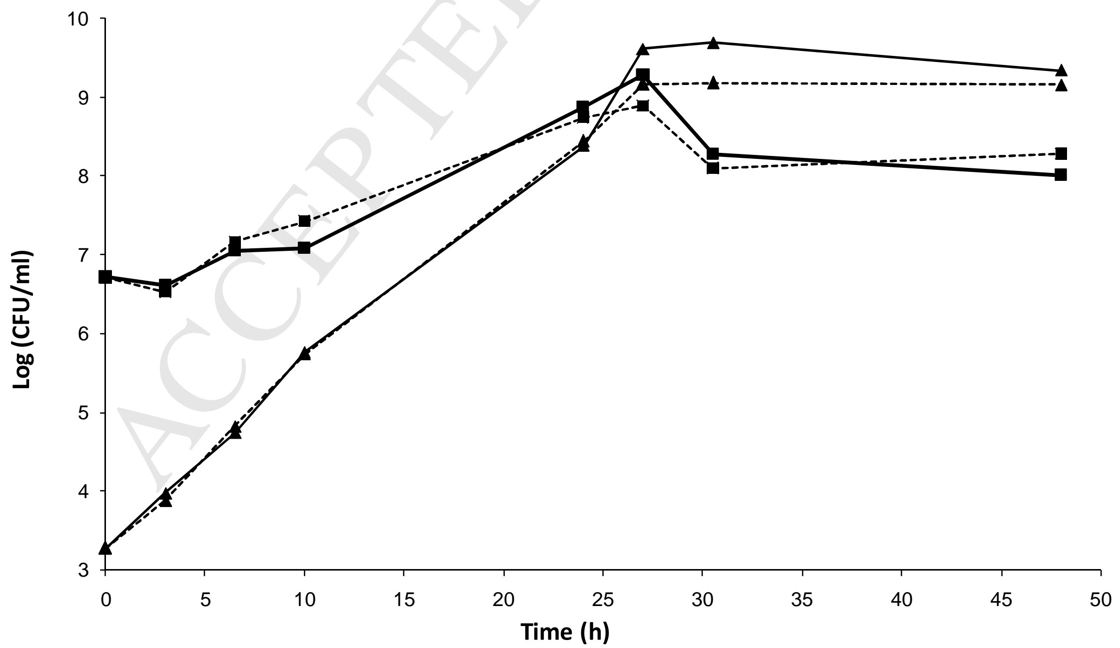


Figure 5



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