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Protective Effect of a Non-Bacteriocinogenic *Lactococcus piscium* CNCM I-4031 Strain Against *Listeria monocytogenes* in Sterilized Tropical Cooked Peeled Shrimp

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Abstract:

The protective activity of a non-bacteriocinogenic *Lactococcus piscium* CNCM I-4031 strain against *Listeria monocytogenes* was investigated in tropical cooked peeled shrimp stored at 8°C in modified atmosphere packaging (50% N_2-50% CO_2). When inoculated alone (*L. piscium* 10^7 CFU g^{-1} and *L. monocytogenes* 10^4 CFU g^{-1}), protective culture and target strain grew very well on shrimp reaching a maximum cell number of 10^9 CFU g^{-1} after 7 and 14 days, respectively. In the presence of *L. piscium*, growth of *L. monocytogenes* was totally prevented after 3 days of storage. The count was 3.4 log CFU g^{-1} lower than in the control after 10 days and until the end of storage (31 days). Using the Seafood Spoilage and Safety Predictor Software (http://sssp.dtuaqua.dk), it was shown that pH decrease from 6.58 to 5.94 and lactic acid concentration of 89.65 mM measured in the co-inoculated batch did not fully explain the inhibition observed.

Keywords: inhibition; biopreservation; challenge-test; lactic acid; nutritional competition

1. Introduction

Shrimp is one of the most marketed seafood products in the world. In France shrimp is the second post of seafood importation (after salmon) with 105 300 tonnes and 536 million euros in value during the year 2008 (http://www.ofimer.fr/Pages/filiere/bilans_annuels_ofimer). Convenience preparation such as cooked and peeled shrimp packed under modified atmosphere is gaining more and more importance in Europe. Those products are very sensitive to microbial growth due to their neutral pH, weak salinity and presence of nitrogenous compounds with weak molecular weight. Growth of pathogenic microorganisms such as *Listeria monocytogenes* constitutes a major problem in such refrigerated ready-to-eat (RTE) food products. *L. monocytogenes* is a human pathogenic bacteria that is responsible for foodborne listeriosis generally associated with a high mortality rate, 20-40% among the high risk group of people (pregnant woman, immuno-compromised patient…). The presence of *L. monocytogenes* was detected in 26.5% of cooked peeled shrimp analysed by Valdimarsson et al. (1998) and in 11.2% of semi final products according to the survey of Gudmundsdottir et al. (2006), confirming the safety risk if good hygienic practices are not respected. Growth of this undesirable bacteria is usually delayed, but not totally stopped, by the use of classical techniques such as salting, low storage temperature or modification of the atmosphere packaging (Rutherford et al., 2007; Tsigarida et al., 2000). Preservatives, mainly organic acids, that are not always well accepted by the consumer, are often added, with more or less success (Heavin et al., 2009; Mejlholm and Dalgaard, 2002; Tokarskyy and Marshall, 2008; Mejlholm et al., 2008). All these results pointed out that *L. monocytogenes* can constitute a major safety risk for consumers in such products.
Therefore, the research of new natural hurdles to limit the growth of pathogenic bacteria in shrimp is important. Biopreservation, a technology consisting in using protective living microorganisms to inhibit the growth of pathogenic and spoiling bacteria has gained increasing attention to ensure the safety of RTE food. Many studies reported the usefulness of some bacteriocin-producing LAB to limit the growth of *L. monocytogenes* in seafood, especially in cold-smoked salmon (Brillet et al., 2004; Tomé et al., 2008; Vescovo et al., 2006; Weiss and Hammes, 2006). However, the Quality Presumption of Safety status that is a required meadow for a food application in Europe may be more difficult to obtain for bacteriocinogenic bacteria, enhancing the interest for protective cultures which do not produce bacteriocin-like compounds. Selection of such bacteria that inhibits *L. monocytogenes* without changing the delicate flavour of shrimp is still a challenge and has so far been explored only in few studies (Nilsson et al., 1999, 2005; Yamazaki et al., 2003).
In a recent study, a strain of *Lactococcus piscium* has been isolated from raw salmon and has showed capacities to enhance the sensory shelf-life of naturally contaminated cooked/peeled shrimps and cold-smoked salmon (Matamoros et al., 2009a). This strain also showed inhibition of *L. monocytogenes* without bacteriocin production. The aim of this study was to confirm the specific antimicrobial activity of this strain against *L. monocytogenes* in tropical shrimp packed under modified atmosphere and to investigate the inhibitory mechanisms that could be involved in this inhibition. For that purpose, a sterilised shrimp model was used to avoid the background flora. Physicochemical parameters related to the growth of the protective and the target strains were also determined to tentatively explain the inhibition.

2. Materials and Methods

2.1. Strains and culture conditions

The strains of *Lactococcus piscium* CNCM I-4031 and *Listeria monocytogenes* RF191 used in this study were isolated from fresh salmon steak packed under modified atmosphere

(Matamoros et al., 2009b) and from tropical cooked peeled shrimp respectively (CEVPM, Boulogne /mer, France). Bacterial strains were stored at -80°C in their growth medium containing 10% of glycerol. For challenge tests, *L. monocytogenes* RF191 and *L. piscium* CNCM I-4031 were subcultured twice successively in Brain Heart Infusion and Elliker broth (Biokar Diagnostics, Beauvais, France) respectively, for 24 h at 26°C. Cultures were then diluted in physiological solution (0.85% NaCl, 0.1% peptone) to obtain appropriate cell concentrations.

2.2. Shrimp preparation

Farmed tropical shrimp (*Penaeus vannamei*) from Colombia were supplied by MITI (Nantes, France) in frozen, beheaded and peeled conditions, without sulphite added. They were cooked in our laboratory at 100°C during 2.5 min in 3% salted water. After cooking, shrimp were immediately immersed in melting ice and frozen at –80°C. They were transported to IONISOS (Pouzauges, France) for ionisation at 3.75 kGy, under frozen conditions (-80°C).

2.3. Challenge tests

Cooked shrimp were inoculated at 10% (v/w) by spraying diluted cultures to obtain an estimated final level of 7 log CFU g^{-1} and 4 log CFU g^{-1} for *L. piscium* and *L. monocytogenes* respectively.
Four batches were prepared: 1-sterility control, 2-inoculation with *L. piscium* CNCM I-4031, 3-inoculation with *L. monocytogenes* RF191, 4-co-inoculation with *L. piscium* + *L. monocytogenes*. After inoculation, portions of 125 g of shrimp were packed in punnets under modified atmosphere 50% N_2 - 50% CO_2 (Multivac T250, Germany) with a ratio shrimp/gas of approximately 2/1. The samples were stored at 8°C during 31 days and regularly sampled for microbiological and physicochemical analyses. All the analyses were done in triplicate.

2.3.1. Microbial enumeration

Each day of analysis, three punnets per batch were opened and 20 g of shrimp were mixed with 80 ml of physiological solution in sterile plastic bags and blended with a stomacher 400 (Seward Medical, London, UK) for 2 min. Total microbial flora was determined in uninoculated batch by pour plating (26°C, 48-72 h) on Plate Count Agar (Biokar Diagnostics). *L. piscium* was enumerated by spread plating onto Elliker agar plates incubated at 26°C for 48h under anaerobiosis for batch 2 and at 8°C for 7 days for batch 4. *L. monocytogenes* was enumerated on spread plate of Palcam with selective supplement (Biokar Diagnostics) after 24 h at 37°C.

2.3.2. Physicochemical parameters

The pH was measured immediately after microbiological analyses into the stomacher bag by immersing the electrode of the pH-meter (Mettler Delta 320, Halstead, UK). L-lactic and acetic acid determination was performed using enzymatic kits with $NADH_2$ detection at 340 nm (acetic acid 021 and L-lactic acid kit 022, Biosentec, Toulouse, France). D-lactic acid was not monitored as we had previously confirmed that *L. piscium* and *L. monocytogenes* did not produce this isoform. Fifty g of flesh per punnet were homogenised in a Waring Blender (laboratories Humeau, France) and 3 g of homogenate were mixed with 20 ml of water. The mixture was centrifuged at 10000 g for 10 min. The supernatant was transferred in a new

tube and used as sample solution in the assays by following the instruction of the enzymatic test kit supplier. Sugar content was measured by a modified colorimetric method of Dubois et al. (1956). 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 derivated 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. Prior to inoculation, shrimp characteristics (pH, water phase salt content, proteins, lipids and dry mater) were analysed in triplicate from 125 g of crushed flesh. For water content, 8 g of sample were dried at 105°C overnight. The Kjeldahl method (Crooke and Simpson, 1971) was used to determine total proteins. Salt content in shrimp meat was determined using a chlorine analyser (Sherwood MK II analyser 926, Grosseron, Nantes, France). Total fat content was extracted by using the Folch method (Folch et al., 1957).

2.3.3. *Listeria monocytogenes* growth prediction

The seafood spoilage and safety predictor software V3.1 (SSSP V. 3.1) proposed by Mejlholm and Dalgaard (2007b) and developed to predict the growth of *L. monocytogenes* in seafood product was used (http://sssp.dtuaqua.dk, accessed 03 Sept 2009). This software take into account different product characteristics such as pH, water phase NaCl, lactic, acetic/diacetate, benzoic, citric and sorbic acids, phenolique content, storage temperature, % CO_2 in headspace gas at equilibrium and naturally occurring lactic acid bacterial count.

3. Results & discussion

After cooking, shrimp were composed of 73 ± 0.5 % of water, 22.1 ± 0.0 % of proteins, 0.49 ± 0.0 % of total lipid, 0.47 ± 0.04 % of glucose and 1.24 ± 0.02 % of NaCl, and had an initial pH of 6.54 ± 0.02. In uninoculated batches no colony was detected on the Petri dishes within the 31 days of storage, showing that the ionisation method allowed a good decontamination (detection threshold: 5 CFU g^{-1}). Ionisation at low level (2-4 kGy) under frozen conditions to avoid lipid oxidation has already been used to obtain sterile cold-smoked salmon small blocks for bacterial interactions studies (Brillet et al., 2004; Joffraud et al., 2006). Figure 1 shows the growth of *L. piscium* and *L. monocytogenes* in inoculated batches. *L. piscium* increased from 7.0 ± 0.2 log CFU g^{-1} to 9.1 ± 0.1 log CFU g^{-1} in 7 days and remained at its maximum level during all the storage period. *L. monocytogenes* was inoculated at 3.8 ± 0.1 log CFU g^{-1} and reached 9.2 log CFU g^{-1} after 14 days. For both bacteria no lag phase was observed. These results demonstrated the adaptation of *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 to the shrimp matrix. A rapid growth of *L. monocytogenes* at 8°C has also been observed by Matamoros et al. (2009a) in vacuum packed cooked shrimp and in the same products stored at 8°C under modified atmosphere containing 50% CO_2, 30% N_2 and 20% O_2 (Mejlholm et al., 2005). In the presence of *L. piscium* CNCM I-4031, *L. monocytogenes* count increased by approximately 1 log during the first 4 days but growth was then totally stopped. The population was reduced by 3.4 ± 0.2 log CFU g^{-1} compared to the control after 10 days of storage and this reduction lasted during 31 days (Fig. 1). Considering the fact that the natural initial contamination levels of *L. monocytogenes* in shrimp are generally very low (< 1 CFU g^{-1}) (Paranjpye et al., 2008), the protective effect of *L. piscium* CNCM I-4031 demonstrated in this study could be very promising to ensure the

safety of RTE shrimp towards *L. monocytogenes* risk. Many studies failed to correctly prevent growth of *L. monocytogenes* in food products by using preservatives or non bacteriocin producing LAB. Richard et al. (2003) failed to prevent growth of *L. monocytogenes* with a non bacteriocin mutant of *C. divergens* V41. Yamazaki et al. (2003) also failed to prevent growth of *L. monocytogenes* at 12°C and 20°C in cold-smoked salmon by using a non bacteriocinogenic *C. piscicola* JCM 5348. Paranjpye et al. (2008) demonstrated that ozone and chlorine dioxide were not efficient to inhibit the growth of *L. monocytogenes* in cooked peeled cold-water shrimp. In the study of Mejlholm et al. (2005) an effect of the natural lactic flora on the growth inhibition of *L. monocytogenes* was observed at 2°C but it was hugely reduced at 5°C or 8°C. Some combinations of citric, benzoic and sorbic acids often used in industry were also not totally efficient to prevent growth of *L. monocytogenes* in brined shrimp (Mejlholm et al., 2008). In our experiment, growth of *L. monocytogenes* stopped when *L. piscium* CNCM I-4031 reached its maximum population density. This phenomena is usually described as the Jameson effect (Gimenez and Dalgaard, 2004; Ross et al., 2000). However the mechanisms involved in this effect are still not elucidated. In order to determine if the growth limitation of *L. monocytogenes* could be due to acidification, pH and organic acids production were monitored during the storage (Fig. 2). The pH value remained constant (6.54 - 6.59) in the uninoculated batch during the whole storage time (data not shown) whereas in all inoculated batches, a pH decrease was observed suggesting that acidification can only be linked to the presence and growth of microorganisms. In samples inoculated with *L. piscium* CNCM I-4031, the pH value decreased rapidly from 6.58 ± 0.02 to 5.94 ± 0.13 (Fig. 2) after 7 days of storage and then remained constant during storage. *L. monocytogenes* also reduced the pH from 6.52 ± 0.03 to 5.90 ± 0.01 but only after 17 days of storage. In co-inoculated batch the pH reduction was similar to the one observed in *L. piscium* batch i.e. decrease from 6.62 to 5.91 ± 0.06 after 7 days of storage. No acetic acid was produced by *L. piscium* and *L. monocytogenes.* In sterility control, the naturally occurring water phase L-lactate was constant, between 25.57 – 28.15 mM during the storage time. In all others batches, a L-lactic acid production was recorded. When *L. piscium* CNCM I-4031 was inoculated alone, the maximum concentration (52.81 ± 4.56 mM) was observed at day 7, when the maximum cell count was reached, and coincided with the pH decrease (Fig. 2). *L. monocytogenes* is also a L-lactic acid producer, and quite similar concentrations were noticed at the end of the growth phase (47.64 ± 0.91mM after 17 days). In co-inoculated batch, L-lactic acid concentration increased till 89.65 ± 1.82 mM in 7 days storage corresponding to the sum of lactic acid produced by *L. piscium* and *L. monocytogenes* during this period. Then a decrease till 56.16 ± 1.52 mM was observed. The lactic acid concentration in batch inoculated with *L. piscium* CNCM I-4031 is close to those obtained by Papathomopoulou and Kotzekidou (2009) who demonstrated in their study that 54 mM of lactic acid at pH 4.15 had a great antimicrobial action on *L. monocytogenes* growth in simulated meat medium fermentated with *Lactobacillus sakei*. In another study Juven et al. (1998) showed that 50 mM of lactic acid at pH 5.4 produced by *Lactobacillus alimentarus* were enough to reduce by 2 log the count of *L. monocytogenes* in vacuum packed ground beef. Weak organic acids such as lactic and acetic acids are known to limit microbial growth because of their strong antimicrobial activity (Choi et al., 2009; Mejlholm and Dalgaard, 2007a). However, this antimicrobial effect is more effective when the pH is closed to their pKa value (3.86 and 4.76 for lactic acid and diacetate respectively) as it was shown in the study of Papathomopoulou and Kotzekidou (2009), due to toxicity of the non dissociated fraction of weak organic acids. Comparing our results to these above, the lactic acid produced by *L. piscium* CNCM I-4031 at the pH value of 5.9 may have a limited effect on *L. monocytogenes* growth. In order to confirm this hypothesis, the SSSP V. 3.1 was used. When predicting the growth of *L. monocytogenes* alone, the value of the environmental parameters were: temperature (8°C), % CO_2 in headspace gas at equilibrium (45)%, water phase salt (1.24), pH value (6.54) and water phase lactate (25.5 mM). When predicting growth of *L. monocytogenes* in the presence of lactic acid in the co-culture with *L. piscium* CNCM I-4031, the pH and water phase lactate used were 5.9 and 89.65 mM respectively. The predicted and observed growth of *L. monocytogenes* in shrimp are shown in Figure 3.

The maximum population density of *L. monocytogenes* RF191 observed in shrimp when inoculated alone was 9.4 log CFU g^{-1} whereas predicted was 8.5 log CFU g^{-1}. The model sensibly under estimated the growth of *L. monocytogenes*. In the presence of 89.65 mM of lactic acid at pH (5.9) the predicted μ_{max} and $N_{31\ days}$ were 0.029 h^{-1} and 8.5 log CFU g^{-1} whereas observed μ_{max} and $N_{31\ days}$ were 0.009 h^{-1} and 6.1 ± 0.1 log CFU g^{-1} respectively. These results showed that in shrimp meat the lactic acid produced by *L. piscium* CNCM I-4031 combined to the limited decrease of pH (5.9) cannot totally explain the antilisterial activity. When introducing the lactic acid bacteria count in the model, no growth of *L. monocytogenes* was predicted after 4 days, as observed in our study. However, the reason of the inhibition remained unknown. Matamoros et al. (2009b) have shown that the antimicrobial activity of this strain could not be linked to bacteriocin-like compounds nor hydrogen peroxyde. Other mechanisms like nutritional competition or synergic effects between lactic acid production and depletion for specific nutrients may be involved in this bacterial interaction. In the batch inoculated with *L. piscium* CNCM I-4031 alone, glucose concentration decreased from 0.36 ± 0.00 % till 0.16 ± 0.01 % during the first 3 days of storage and remained almost constant during all the storage period. The same evolution was noticed in the co-inoculated batch (date not shown) whereas in the batch inoculated with *L. monocytogenes* alone, the glucose concentration decreased slowly from 0.43± 0.01 % till 0.17± 0.01 % in 17 days of storage. The rapid consumption of glucose by the *L. piscium* strain could partially explains its inhibition activity towards *L. monocytogenes* as it has been shown previously with a strain of *Carnobacterium maltaromaticum* (*piscicola)* (Nilsson et al., 2005). To elucidate the implication of glucose or other nutrient compounds competition in the inhibition of *L. monocytogenes* by *L. piscium* CNCM I-4031, a liquid medium simulating shrimp composition is under development.

4. Conclusion

The inhibitory effect of *L. piscium* CNCM I-4031 described in this study suggests that this strain may be efficient to control *L. monocytogenes* growth in cooked and peeled shrimp stored in modified atmosphere at abuse temperature.

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Figures

Figure 1

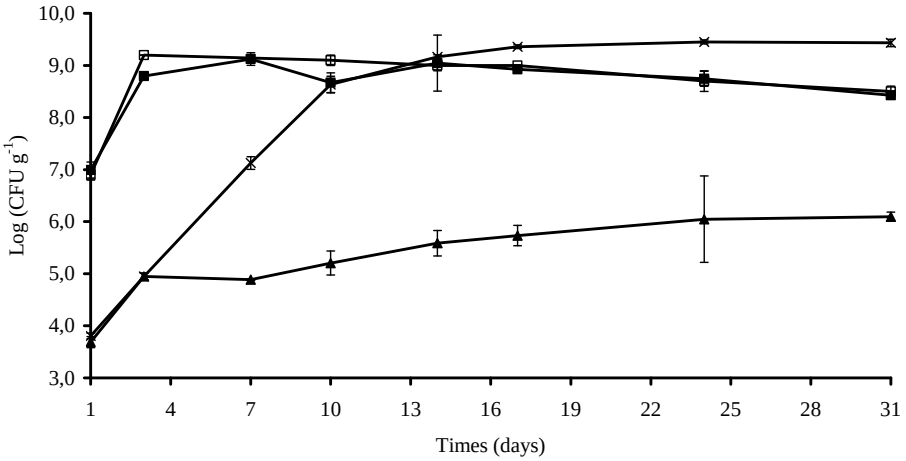

Figure 1: Growth of *Lactococcus piscium* EU2241 and *Listeria monocytogenes* RF191 in peeled and cooked shrimp packed under modified atmosphere and stored at 8°C. (■) *L. piscium* EU2241 alone; (□) *L. piscium* EU2241 co-inoculated with *L. monocytogenes* RF191; (x) *L. monocytogenes* RF191 alone and (▲) *L. monocytogenes* co-inoculated with *L. piscium* EU2241. Values are averages of three analyses ± standard deviation.

Figure 2

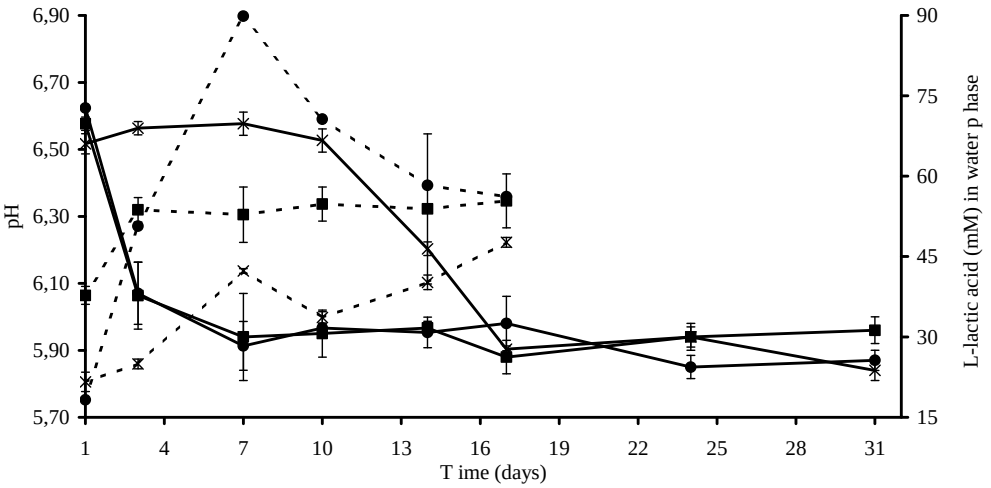

Figure 2: L-lactic acid (dotted line) and pH (solid line) evolution in peeled and cooked shrimp packed under modified atmosphere, stored at 8°C and (■) inoculated with *Lactococcus piscium* EU2241 alone, (**x**) *L. monocytogenes* RF191 alone and (●) co-inoculated with *L. piscium* EU2241 and *L. monocytogenes* RF191. Values are averages of three analyses ± standard deviation.

Figure 3

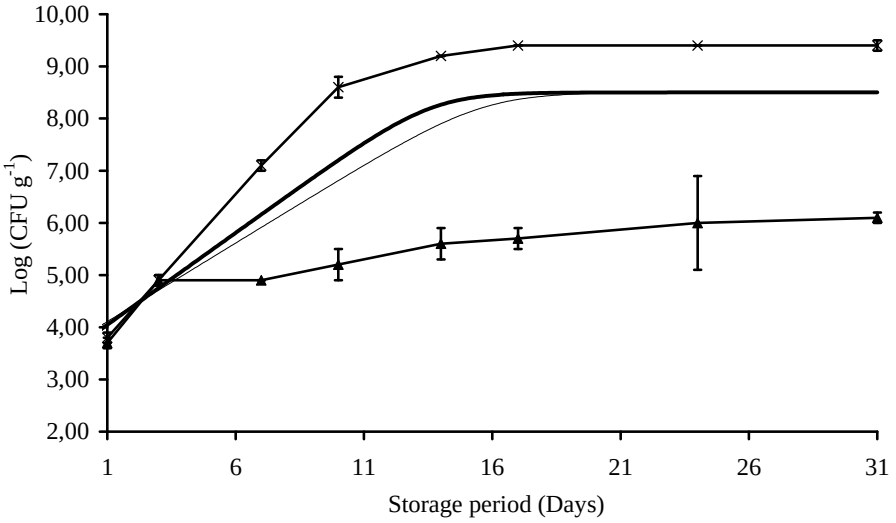

Figure 3: Observed and predicted growth by the Seafod Spoilage and Safety Predictor V.3 software of *Listeria monocytogenes* in peeled and cooked shrimp packed under modified atmosphere and stored at 8°C. (**x**) observed *L. monocytogenes* RF191 alone; (▲) observed *L. monocytogenes* RF191 co-inoculated with *Lactococcus piscium* EU2241; (thick line) predicted *L. monocytogenes* alone; (thin line) predicted *L. monocytogenes* co-inoculated with *L. piscium* EU2241.