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RESEARCH ARTICLE

USE OF NOVEL ELECTRO-TRANSFORMED STRAIN OF *LACTOBACILLUS* LMG21688 TO SUPPRESSE *LISTERIA MONOCYTOGENES* GROWTH IN A MILK SYSTEM

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ABSTRACT

Recently our research has allowed developing and using a novel electro-transformed strain of *Lactobacillus* LMG21688 as protective cultures for inhibiting *Listeria monocytogenes* in meat products. The result did not give total satisfaction because of the complexity of the matrix (meat) used. To show the total efficiency of this new strain against *Listeria*, another matrix less complex was tested. Different types of milk (full-cream and skimmed) were used in laboratory fermentations where *Listeria monocytogenes* was co-cultured at 37°C with either electrocompetent LMG21688 or *Lactobacillus curvatus* CWBI-B28 bacteriocin-producing strain. The *Listeria* cfu count reached after 168 hours was 200 times higher in high-fat milk than in skimmed milk.

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INTRODUCTION

Lactic acid bacteria (LAB) play a key role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety (Liu *et al*., 2011). Recent years the interest increased in bacteriocin-like inhibitory substances producing LAB because of their potential use as natural antimicrobial agents to enhance the safety of food products (Hurtado *et al*., 2012). Bacteriocins from LAB are described as "natural" inhibitors, because LAB having a GRAS status. Their role in the preservation and flavor characteristics of foods has been well documented (Kaban 2013; Kargozari *et al*., 2014).

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However, it has been reported that bacteriocins produced by several lactic acid bacteria exert a transitory bactericidal effect against *L. monocytogenes*, often followed by re-growth of *Listeria* cells in bacteriocin-supplemented food models (Kouakou *et al*., 2008 ; Anthoula *et al*., 2013).This growth rebound might be due to factors that severely limit growth of bacteriocin-producing cells (e.g. restricted nutrient availability), to decreased bacteriocin action as a result of adsorption onto food particles, fats, and proteins, to the presence of a curing agent, to the emergence of bacteriocin-resistant cells, and/or to bacteriocin degradation by proteases of food and/or microbial origin (Kouakou *et al*., 2009; Eduardo *et al*., 2013; Schillinger, 2014). To remedy this rebound phenomenon, it would have been interesting to have a multifunctional strain to counteract these many limiting factors. It is in this context that the idea of creating a super strain by introducing Plasmid-Mediated bacteriocin into a technological competent strain to produce more bacteriocins has become necessary. Elsewhere a recent trend exists in the construction by electroporation of the new strains from wild-type strains plasmids isolated from traditional products.

Several Lactobacillus species have been successfully transformed for this purpose by electroporation (Spath *et al*., 2012; Van Pijkeren *et al*., 2012). But in our case, the super electrocompetent LMG21688 strain obtained (Kouakou *et al*., 2010) did not give total satisfaction in the meat matrix singled out to its high complexity. Otherwise, Aasen *et al*. (2003) have shown that the activity of the bacteriocins depends not only on the type of bacteriocin, but also on the physical state of the medium (liquid or solid) and on whether the medium is shaken or not. The study of Aasen *et al*. (2003) and Bani-Jaber *et al*. (2000) further shows that bacteriocins are more effective when applied to a meat surface than in liquid food where fat, water, and bacteriocins are mixed. Other factors, such as adsorption of bacteriocins to proteins in the food matrix, are also reported to interfere with bacteriocin activity (Goff *et al*., 1996; Murray and Richard, 1997).

Here we have studied the ability of our super bacteriocinogenic strain to inhibit growth of *L. monocytogenes* in another matrix such as high-fat and low-fat milk systems in order to highlight the interest of the matrix choice for an efficient activity of our electrocompetent strain.

MATERIALS AND METHODS

Bacterial strains, strain storage conditions, and broth/plate cultures

Lactobacillus curvatus CWBI-B28, described by Benkerroum *et al*. (2002), is an antilisterial bacteriocin-producing strain. *Lactobacillus* LMG21688 (noted LMGel hereafter) obtained by electroporation of *Lactobacillus* LMG 21688 with a CWBI-B28 derived plasmid (Kouakou *et al*., 2010) is a technologically competent bacteriocin-producing strain. Originally purchased from Gent University, it was included in the THT Company's laboratory culture collection (Gembloux, Belgium) after its electrotransformation. Electroporation is an efficient technique for transferring plasmid DNA isolated from certain bacteriocin producing strain into other technological competent lactic acid bacteria (LAB) strains. Electroporation refers to the process of subjecting living cells to a rapidly changing, high-strength electric field, thereby producing transient pores in their outer membranes facilitating diffusion and exchange of intracellular and extracellular components during the lifespan of the pore (Ersus *et al*., 2010; Puértolas *et al*., 2012; Mahnič-Kalamiza *et al*., 2014).

So the advantage of this technique consisting of varying the intensity of the electrical discharge to obtain adequate electric field permeabilising and facilitating diffusion and exchange of intracellular and extracellular components during the lifespan of the pore was fateful to transform our LMG 21688 strain. *Listeria monocytogenes* M, originally isolated from bacon, was obtained from the collection of the Centre Wallon des Bio-Industries (Gembloux, Belgium). It is sensitive to the bacteriocin produced by CWBI-B28 and was used as an indicator and to artificially contaminate milk samples. It was spread regularly over Palcam agar (Oxoid, Beauvais, France) plates and activated in tryptone soy broth (Biokar, Beauvais, France) at the time of its use.

All strains were grown in the milk system described below (see Milk system and Milk sampling) or on DeMan, Rogosa, and Sharpe medium (MRS, Biokar) (broth or with 1.5% agar, as specified). All strains were stored at -80°C in their respective media with added 40% glycerol (v/v).

Using L. curvatus CWBI-B28 or the electrocompetent bacteriocin-producing strain to control L. monocytogenes growth on milk system

Milk preparation and inoculation

Milk was obtained from the Belgian trade. The listed characteristics of the milk used are:

- For skimmed milk (low-fat): protein (3,6 g/100ml); fat (0,3 g/100ml); carbohydrate (5,1 g/100ml); salts (0,13 g/100ml).
- For full-cream milk (high-fat): protein (3,4 g/100ml); fat (3,6 g/100ml); carbohydrates (4,9 g/100ml); salts (0,13 g/100ml).

For each type of milk *L. monocytogenes* and either CWBI-B28 or LMGel strain were co-inoculated aseptically into flasks containing sterile milk (100 ml from its original packaging) at the final concentration of about 10^6 cfu/ml. A control containing only *L. monocytogenes* at an initial concentration of 10^6 cfu/ml was included. The flasks were incubated for 168 h at 37°C. Each experiment was performed twice and each determination was done in triplicate. Data are presented as means of two independent experiments with SD.

Milk sampling and analysis

At sampling, after 0, 2, 4, 8, 10 up to 168 hours of incubation, 1-ml samples were taken aseptically at regular intervals and homogenized with 9 ml saline in an assay tube.

pH determination

The pH of the milk was measured in the flasks with a portable pH meter (HANNA HI 9025). The pH values reported are means of at least three measurements ± SD.

Microbiological analysis

Growth of the inoculated strains was determined on the basis of viable counts after homogenization in peptone water, as described by Katla *et al*. (2001). A decimal dilution series was prepared and at specific time intervals, samples were taken to perform microbial counts. *L. monocytogenes* was enumerated on Palcam agar (Oxoid, Beauvais, France) after incubation for 48–72 h at 37°C and lactic acid bacteria were enumerated on MRS agar (Oxoid, Beauvais, France) after 24–48 h of incubation at 37°C**.**

Determination of bacteriocin activity and quantification

Bacteriocin production was evaluated in all samples whose treatment had included either CWBIB28 or LMGel. A 1-ml aliquot of milk solution was centrifuged (12,000 rpm/15 min) and the supernatant obtained was filtered (Millipore, 0.2 µl) to eliminate the starter culture.

Bacteriocin activity was determined according to an agar well diffusion assay described by Parente and Hill (1992). Molten agar was seeded with an indicator strain (i.e., *L. monocytogenes)* and dispensed into Petri dishes (20 ml per plate). Wells approximately 4.6 mm in diameter were bored in the agar. Two-fold serial dilutions of the bacteriocin homogenate were prepared and 60-µl aliquots of the various dilutions were loaded into separate wells. The plates were incubated overnight at 37°C, and bacteriocin activity was determined in arbitrary units (AU) defined as the reciprocal of the highest dilution showing a definite of inhibition around the well. It was expressed in arbitrary units per milliliter (AU/ml), calculated by multiplying the reciprocal of the critical dilution by 1000/60.

Statistical analysis

Each trial was repeated twice and each determination was done in triplicate. Statistical analysis (analysis of variance α = 0.05% and Student's t-test) of was done with Excel software.

RESULTS

Growth of L. monocytogenes and bacteriocin-producing strain in both types of milk

Figure 1 show that whatever the fat content of the milk, the *L. monocytogenes* cfu count increased from 10^6 to approximately $1.2x10^8$ cfu mL^{-1} over 72 hours of the experiment (no significant difference between sub-batches). Regarding CWBI-B28 and LMGel artificially inoculated (10^6) in milk, Both strains grew exponentially for approximately 72 hours, after which growth slowed down. In fat-rich milk, while the electrocompetent LMGel producing strain reached $2.1x10^{10}$cfu mL^{-1}, CWBI-B28 reached $10^{8.5}$cfu mL^{-1}. Meanwhile in skimmed milk, the LMGel strain reached $4.1x10^9$cfu $mL^{-1}$and $1.1x10^{7.5}$cfu mL^{-1} for CWBI-B28. By the end of the 168-hours experiment, all both bacteriocin-producing trials showed a cfu count near 10^8 and 10^{10}cfu $mL^{-1}$for whole milk and near 10^7 and 10^8cfu mL^{-1} for skimmed milk.

Comparison of antilisterial effects of bacteriocin-producing CWBI-B28 and LMGel strain in skimmed milk system

Fig. 2 shows the growth during storage of the *L. monocytogenes* cfu count in skimmed milk with either CWBI-B28 or LMGel bacteriocin-producing strain added. *Listeria* count initially decreased, whatever the type of bacteriocin-producing strain added, albeit much less markedly and more briefly in the presence of the CWBI-B28 than in the presence of LMGel. In the latter case, *Listeria* was undetectable after 24 hours and remains undetectable until the end of the experience (168 hours). Regarding CWBI-B28, the *Listeria* cfu count decreased more slowly, down to 10^2cfu mL^{-1} at 24 hours followed growth rebound to reach rapidly $8.3x10^3$ cfu mL^{-1} by the end of the experiment (at 168 hours). When the cfu counts in Fig. 1 were examined in relation to the corresponding bacteriocin activities (Fig. 2), the highest cfu counts were found to coincide with peak bacteriocin activity: 2133 and 4266 AU mL^{-1}.These peaks productions also coincide with strong inhibition of *Listeria* for the milk systems seeded, respectively, with CWBI-B28 and LMGel. We noted that the bacteriocin activity decreased quite quickly once the producer strain reached the stationary or decline phase (to below 2000 and 4000 AU mL^{-1} respectively for CWBI-B28 and LMGel (Fig. 2). As expected, no bacteriocin activity was detected in control (data not shown).

Comparison of antilisterial effects of bacteriocin-producing CWBI-B28 and LMGel strain in full-cream milk system

Fig. 3 shows the growth of *L. monocytogenes* in our full-cream milk systems co-inoculated with CWBI-B28 or LMGel. In full-cream milk, co-inoculation with strain CWBI-B28 resulted in a slower decrease, down to 10^2 cfu mL^{-1} at 24 hours but much less pronounced than in the skimmed milk. Thereafter, the count increased beyond 10^3cfu mL^{-1} by the end of experiment (Fig. 3). With LMGel, a drastic drop in the *Listeria* cfu count, from 10^6 cfu mL^{-1} at the start of the experiment to an undetectable level by the end of hours 24 was observed. After 72 hours, a slight and very gradual rebound was observed, the count reaching 100 cfu mL^{-1} by the end of the experiment (Fig 3).

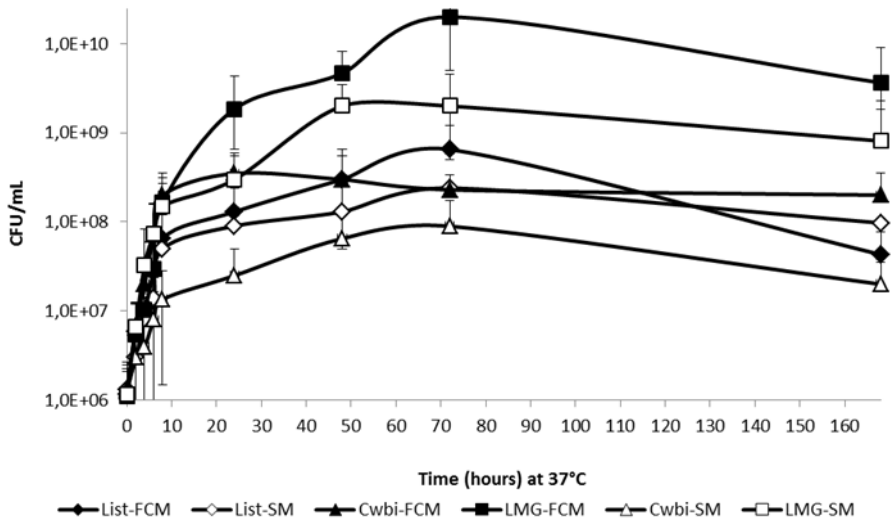

Fig. 1. Evolution of *Listeria monocytogenes* in full-cream milk (FCM) (♦) or in skimmed milk (SM) ( ) and *Lactobacillus curvatus* CWBI-B28 in FCM (▲) or in SM (Δ) and *Lactobacillus* LMGel in FCM (■) or in SM (□)

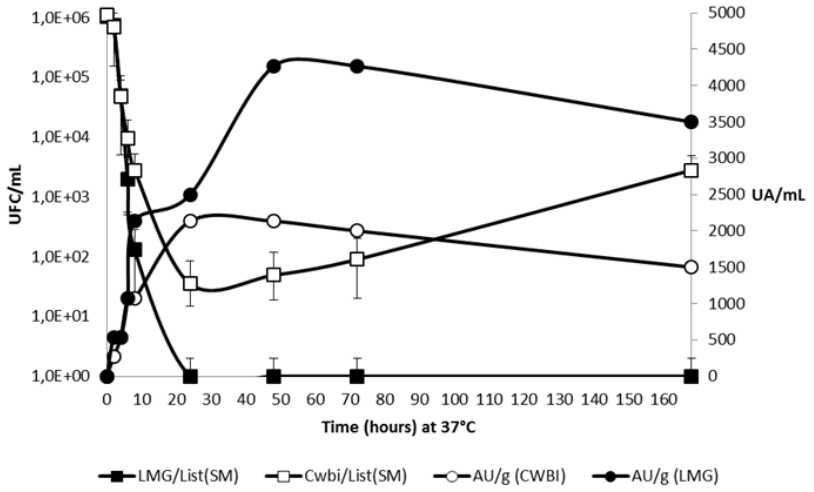

Fig. 2. Evolution of the *Listeria monocytogenes* in the presence of electrocompetent bacteriocin-producing LMGel (■) or bacteriocin-producing CWBI-B28 (□) in skimmed milk. The evolution of bacteriocin activity is also shown (● ; ○). *Listeria monocytogenes* was added initially at 10^6cfu/ml in all series and the counts on day 0 were done 1 h after addition of the bacteriocin-producing strain. Data are presented as averages of two independent trials with SD

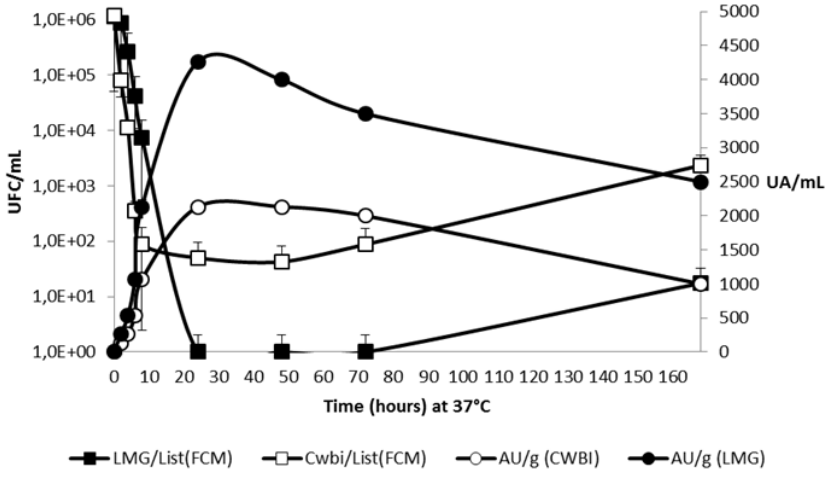

Fig. 3. Evolution of the *Listeria monocytogenes* in the presence of electrocompetent bacteriocin-producing LMGel (■) or bacteriocin-producing CWBI-B28 (□) in full-cream milk. The evolution of bacteriocin activity is also shown (● ; ○). *Listeria monocytogenes* was added initially at 10^6cfu/ml in all series and the counts on day 0 were done 1 h after addition of the bacteriocin-producing strain. Data are presented as averages of two independent trials with SD

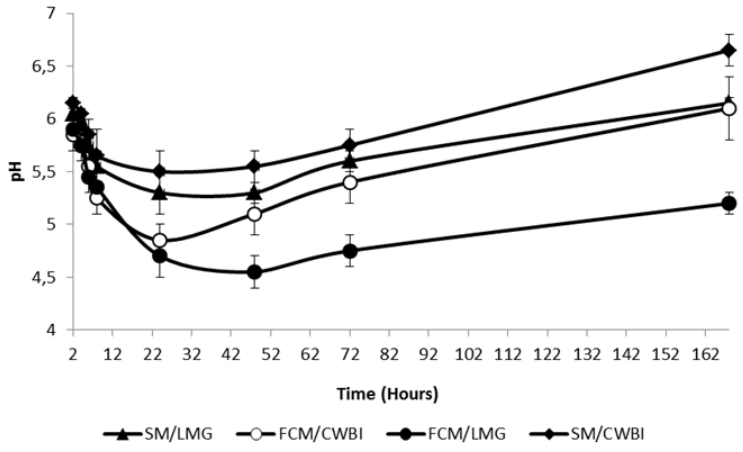

Fig.4. Evolution of pH in skimmed (SM) and full-cream milk (FCM) inoculated respectively with either Lactobacillus LMG (▲ ; ●) or Lactobacillus curvatus CWBI-B28 (♦ ; ○). Error bars show ± the SD.

As in the previous case, the corresponding bacteriocin activities 2133 and 4266 AU mL^{-1}, were found to coincide with the strong inhibition of *Listeria*. As CWBI-B28 and LMGel failed to affect listerial growth in this milk system, the negative effect of these strains could be attributed to bacteriocin. Fat clearly antagonised this effect, since the *Listeria* cfu count was significantly reduced in the low-fat containing milk system.

pH metering in the milk system

To assess the possible contribution of pH to the bactericidal effect of our bacteriocin-producing *Lactobacillus* strains, we examined the ability of these strains to generate an acidic pH. Fig. 4 shows the pH of the full-cream or skimmed milk as a function of storage time after inoculation with CWBI-B28 or LMGel strains (10^6cfu mL^{-1}). A non-inoculated control was also included. The starting pH (recorded 2 h post-inoculation) ranged from 5.8 to 6.5, and the differences observed were not significant ($P > 0.05$). This result remained the same for the control throughout the experiment.

After 24 hours yet, LMGel or CWBI-B28-inoculated samples showed a significant pH decrease ($P < 0.05$). Among them, two groups were distinguishable: (1) the skimmed milk samples and (2) the full-cream milk samples. Within the first group, no significant difference between samples was observed at any time during the experiment. The minimum pH attained, observed at the end of 24 hours, was 5.3 ± 0.1 (average for the three samples). However the full-cream milk sample showed a significantly greater pH decrease, down to pH 4.3 ± 0.1 and to pH 5 ± 0.1 by the end of 48 and 24 hours respectively for LMGel and CWBI. After these times, samples with CWBI-inoculated showed a significant pH increase, to 6.5 ± 0.1 for skimmed milk and to 5.7 ± 0.1 for the full-cream milk sample, whereas with low-protease *Lactobacillus* LMGel strain, no significant difference between samples was observed. The difference between the two types of strain (LMGel and CWBI) remained significant throughout the experiment.

DISCUSSION

Transient inhibition of Listeria monocytogenes by Lactobacillus curvatus CWBI-B28 has been observed previously in the meat system (kouakou *et al*., 2008). It is due mostly to the bacteriocin produced by the strain, since a bacteriocin-negative derivative of this strain is unable to inhibit Listeria growth (kouakou *et al*., 2008; 2009). The results presented here demonstrate that bacteriocin production by *Lactobacillus* LMGel promotes inhibition of Milk's *Listeria* growth when these bacteria are inocultured in Milk. That the observed inhibition is due mainly to bacteriocin rather than to nutritional competition between the strains, organic acids, or hydrogen peroxide is supported by the fact that a low bacteriocin producing with *Lactobacillus curvatus* CWBI-B28 has only a minor effect on *Listeria* growth in both milk system (Fig. 2 & 3). Like most published, bacteriocins (Leroy & DeVuyst, 1999; Himelbloom, Nilsson, & Gram, 2001; Benkerroum *et al*., 2002; Onda *et al*., 2003), that produced by *L. curvatus* CWBI-B28 is secreted during the exponential and early stationary growth phases of fermentation.

Throughout the exponential phase the bacteriocin activity increases with the cfu count, suggesting that bacteriocin production follows primary metabolite kinetics. A drastic decrease is observed upon further incubation. Duffes *et al.* (1999) observed a similar decrease during the stationary phase for *Carnobacterium divergens* and *Carnobacterium piscicola* cultures in a simulated cold smoked fish system and suggested that it might be due to degradation by endogenous proteases (Duffes *et al*., 1999; Onda *et al*., 2003) induced during the growth phase and/or to adsorption of bacteriocin on the surface of producer cells (Onda *et al*., 2003). It has been shown in one of our past work (kouakou *et al*. 2008) that more than one type of protease contributes to bacteriocin degradation. Results of our experiments have clearly indicated the presence of proteolytic activity, notably against bacteriocin, in both *L. curvatus* and food. This was in accordance with various studies showing that proteolytic activity can be due to proteases occurring naturally in food or to those produced by microbial contaminants such as spoilage bacteria, *Pseudomonas, Proteus*, and other genera of the Enterobacteriaceae family generally recognized as highly proteolytic and as principally responsible for food product putrification (Silvina *et al*., 1997). But the use of electrocompetent strain LMGel (weakly proteolytic) in this present work, cancels the hypothesis that proteases from bacteriocin-producing strain would be responsible for the inefficiency of the bacteriocin. - A high-fat content antagonises the antilisterial effect of bacteriocinogenic strains. As mentioned above, Lactobacillus grew significantly better in high-fat than in low-fat milk.

One might thus expect bacteriocin production to be high under these conditions. But to our amazement the level of antilisterial protection conferred by the bacteriocinogenic strain was much lower in high-fat milk than in low-fat milk, as indicated by 100- to 1000-times-higher *Listeria* cfu counts (Fig 2 vs 3). Several authors (Chumchalova´ *et al*., 1998; Davies *et al*., 1999; Blom *et al*., 2001) think that the unsatisfactory effect of bacteriocin-producing strains in situ is due to hydrophobic interactions of the bacteriocins with fat. Several factors in the food model system may interfere with bacteriocin activity. Sakacin A may adsorb to food and fat particles and this may result in its inactivation (Schllllnger *et al*., 1991). Some bacteriocins, like nisin, have a stabilising effect on the fat-water interface (Bani-Jaber *et al*., 2000); their association with fat is readily reversible and does not prevent their antilisterial action.

Other bacteriocins, like sakacin P, bind tightly to lipids in the food matrix (Aasen *et al*., 2003). They may remain trapped, unable to interact with the target pathogen. This could also make them hard to recover from the matrix. It has been shown that the bacteriocin produced by *L. curvatus* is sakacin P (Dortu *et al*., 2008) and Kouakou *et al*., 2010 have confirmed that the bactériocin production was plasmid-borne, and this plasmid was transfer into the *Lactobacillus* LMG21688 nonbacteriocinogenic strain, but technologically competent with low proteolytic activity. This may explain both the lesser antilisterial protection conferred by bacteriocinogenic LMGel in our high-fat milk model and the abnormally low bacteriocin activities measured in this model (Fig. 2 vs. Fig. 3).

The pH can influence bacteriocin activity in other ways as well, notably by affecting bacteriocin solubility. Nisin, for example, is 228 times more soluble at pH 2 than at pH 8 (Liu & Hansen, 1990). Acid production can also influence bacteriocin production (Buncic *et al*., 1997; Leroy & De Vuyst, 1999; Onda *et al*., 2003). For example, Buncic *et al*. (1997) found bacteriocin production by *L. sake* and *L. casei* strains to be maximal at maximal acid production. In our system, the bacteriocin activity was highest from the end of 24 hours to the end of 72 hours, period during which the pH was lowest. The lesser bacteriocin activity observed at the end of the 168-hours storage period was likely due to multiple causes, such as reduced production (there being fewer LAB cfus) and proteolytic degradation of bacteriocin (Kouakou *et al*., 2008). We have recently demonstrated the superior antilisterial performance of a low-protease *Lactobacillus* LMGel strain expressing the sakacin P of *L. curvatus* CWBI-B28 (Kouakou *et al*., 2010). It seems likely that the strong pH increase after 24 hours with CWBI added, was due to the observed protease (from milk and strain) activity increase at this time which should favour the production of basic nitrogen-containing compounds (Kok, 1990; Kouakou *et al*., 2008).

Conclusion

The results presented here demonstrate effectively that the activity of the bacteriocins depends on the physical state of the medium (liquid or solid) inasmuch contrary to the meat matrix (kouakou *et al*., 2008), a novel bacteriocin production method developed with a technologically competent strain is capable of producing higher levels of potent bacteriocins much more efficiently in milk (liquid medium) so as to overcome the adverse effects on bacteriocin effectiveness that may occur in some situations. however, it should be noted that fat efficiently antagonises this action. it would be interesting therefore in the future to try to work with a strain capable of protecting its bacteriocin not only against proteases as is the case of LMGel but also against lipids in order to overcome this other obstacle. Such a method should contribute to improving greatly both the safety and quality of some of the food that we eat.

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