Antilisterial Bacteriocin-producing Strain of *Lactobacillus curvatus* CWBI-B28 as a Preservative Culture in Bacon Meat and Influence of Fat and Nitrites on Bacteriocins Production and Activity

H. Ghalfi,[1] P. Kouakou,[1] M. Duroy,[2] A. Daoudi,[3] N. Benkerroum,[4,*] and P. Thonart[1]

[1]*Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 Passage des Déportés, B-5030 Gembloux*
[2]*THT, 30 rue Phocas Lejeune, B-5030 Gembloux (ISNES), Belgium*
[3]*Département HIDAOA, Institut Agronomique et Vétérinaire Hasssan II, BP 6202, Instituts, 10101-Rabat, Morocco*
[4]*Département des Sciences Alimentaires et Nutritionnelles, Institut Agronomique et Vétérinaire Hasssan II, BP 6202, Instituts, 10101-Rabat, Morocco*

The objectives of this study were to evaluate the effectiveness of a bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 to inhibit the growth of *Listeria monocytogenes* in de Man, Rogosa and Sharp (MRS) broth and in bacon meat. A co-culture of *L. monocytogenes* with the Bac^+ strain in MRS broth, resulted in a reduction of the pathogen counts by 4.2 log cycles after 24 h of incubation at 37 °C. In bacon, the counts of *L. monocytogenes* was reduced to below the detectable limit (<10 cfu/g) in samples inoculated with the Bac^+ strain within 1 or 2 weeks in absence or presence of nitrites (210 mg/kg), respectively. However, a week later, a re-growth of the pathogen has occurred. In contrast, no such reduction in *Listeria cfus* was observed in samples treated with the Bac^- derivative of *Lb. curvatus* CWBI-B28. Nonetheless, the extent of inhibitory effect of the Bac^+ strain against *L. monocytogenes* in bacon was somewhat reduced in the presence of nitrites. A separate study on the influence of nitrites and fats on growth and bacteriocin production by *Lb. curvatus* CWBI-B28 revealed that the curing agent affects the growth of the Bac^+ strain and, thereby bacteriocin production and activity only at concentrations (>5%) far beyond those allowed in the meat industry. Fat content did not affect the bacterial growth even at the highest concentration used (i.e. 50%), however, it interfered significantly with the detection of AUs and the antilisterial activity. Use of the Bac^+ *Lb. curvatus* CWBI-B28 has proven efficient in controlling *L. monocytogenes* in bacon despite the slight antagonistic effect of nitrites, however the efficacy was dramatically reduced upon extended period of storage at 4 °C.

Key Words: Lactobacillus curvatus, *Listeria monocytogenes*, bacteriocin, nitrites, fat, bacon

INTRODUCTION

L. monocytogenes has emerged as an important food borne pathogen in the late twentieth century causing various clinical syndromes and has, therefore, been considered among the pathogens of concern to food safety. Due to its ubiquitous and psychotropic nature, and to its ability to resist stressful conditions, *L. monocytogenes* is widely distributed among foods and is difficult to control during processing or storage. In the pork-meat industry, the pathogen has been detected in every stage along the processing chain (Nesbakken et al., 1996) and the contamination rate ranged between 7 and 33% (Salvat et al., 1995; Okutani et al., 2004). Furthermore, several outbreaks of listeriosis resulting from consumption of pork-meat products have recently been reported in the United States and Europe (Frye et al., 2002; Mayrhofer et al., 2004). In France, pork products including jellied pork

*To whom correspondence should be sent (e-mail: n.benkerroum@gmail.com).
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tongue, 'pâté' and pork 'rillettes' were the main causes of meat-related listeriosis (Goulet et al., 1998; Ryser and Marth, 1999).

Curing of meat with sodium chloride and nitrite salts during processing did not prevent the finished products from *Listeria* contaminations or growth under refrigerated storage (Incze, 1998; Cleveland et al., 2001; Garriga et al., 2002). Moreover, appropriate application of sanitation and hazard analysis and critical control point (HACCP) programmes were insufficient to assure the absence of the pathogen from processed meat products (Bernard and Scott, 1999; Lundén et al., 2002). Therefore, additional or alternative means should be used in the meat industry to effectively control the pathogen, and among these, addition of bacteriocins and/or bacteriocin-producing strains has been strongly recommended during the last years (Leroy and De Vuyst, 1999; Sabia et al., 2003; Dicks et al., 2004). In fact, new industrial starter cultures of lactic acid bacteria (LAB) with such functionality have been recently developed and are attracting increasing interest (O'Sullivan et al., 2002; Lind et al., 2004).

Bacteriocinogenic starter cultures and their bacteriocins could therefore be used in food preservation and safety. However, among the main uncertainties are the levels of production and activity of bacteriocins in situ and the efficacy of the bacteriocin in a given food system. Adsorption to fat and proteins (Chumchalova et al., 1998; Aaesen et al., 2003), protease mediated inactivation (Murray and Richard, 1997; Sarantinopoulos et al., 2002) as well as food matrix (Dicks et al., 2004) are among the limiting factors to bacteriocin activity. Therefore, it is of paramount importance to study the effect of the main environmental factors in the food system where a bacteriocin-producing starter culture or its bacteriocin is to be used (e.g. nitrite and fat concentration) before large scale application. In addition, preliminary experiments on the in situ bacteriocin production and activity are highly recommended.

The purpose of this study was to investigate the ability of a bacteriocin-producing *Lb. curvatus* CWBI-B28, destined to be used in industrial starter cultures for meat fermentations to inhibit the growth of *L. monocytogenes* during storage of bacon at refrigeration temperature (4 °C). Interference of fat or nitrites with the production and the antilisterial activity of the bacteriocin were studied *in vitro*.

MATERIALS AND METHODS

Microorganisms and Media

Lb. curvatus CWBI-B28, an antilisterial bacteriocin-producing strain, previously isolated from meat (Benkerroum et al., 2005) was used in this study. A non-bacteriocin-producing derivative (*Lb. curvatus* M) obtained by mutagenesis trials using ethidium bromide as described previously (Benkerroum et al., 2002) was used as a control where appropriate. *L. monocytogenes*, sensitive to the bacteriocin produced by *Lb. curvatus* CWBI-B28, previously isolated from bacon, was used as an indicator strain and to artificially contaminate bacon samples.

Lb. curvatus CWBI-B28 and its derivative strain were grown in MRS broth (Oxoid, England) and *L. monocytogenes* was maintained on PALCAM agar (Oxoid, England) plates with regular transfers (i.e. weekly). Before use, the strains were activated in MRS broth or in tryptone soya broth (TSB; Oxoid, England) for lactobacillus strains or *L. monocytogenes*, respectively. All strains were maintained at −80 °C in their respective cultivation broths with added glycerol (40% v/v). Incubations were carried out at 37 °C for 48 h, unless otherwise stated.

Effect of *Lb. curvatus* CWBI-B28 Bacteriocin on *L. monocytogenes* in MRS Broth

A series of 200 mL conical flasks (F1, F2 and F3), each containing 100 mL of MRS broth and a fourth (F4) containing 100 mL of neutralised cell-free supernatant (CFS) of an overnight culture of *Lb. curvatus* CWBI-B28 were inoculated with 100 µL of an overnight culture of *L. monocytogenes* to obtain initial inocula of $\sim 10^6$–10^7 cfu/mL. Two of these flasks (F2 and F3) were inoculated with 100 µL of an overnight culture of *Lb. curvatus* CWBI-B28 or *Lb. curvatus* M. Inoculated flasks were incubated at 37 °C and samples were withdrawn at specific time intervals to perform microbial counts. At the same intervals, 1 mL of the culture F2 was centrifuged (8,500 × g, 10 min) and filter-sterilised through a Millipore™ membrane (0.22 µm) for bacteriocin activity determinations. A two-fold serial dilution was aseptically prepared from the filtrate and the bacteriocin activity determined as arbitrary units (AU) per millilitre by the well diffusion assay described previously (Benkerroum et al., 1993). Arbitrary units were defined as the reciprocal of the highest dilution showing a definite zone of inhibition against sensitive strain (i.e. *L. monocytogenes*) per millilitre of tested sample.

Antilisterial Activity of *Lb. curvatus* CWBI-B28 in Bacon

Preparation of Bacon Trials

Two trials of bacon were prepared to study the antilisterial activity of *Lb. curvatus* CWBI-B28 and the effect of nitrite on the bacteriocin activity against *L. monocytogenes*. In each trial, a bacon block of 1.5 kg

was aseptically sliced into portions of ~350 g each to make four batches (B1 to B4). Portions/batches of bacon were placed individually in sterile aluminium foil under a laminar flow hood (Clean Air, VWR, Belgium) for subsequent inoculations. Three portions of each trial were artificially contaminated with *L. monocytogenes* by spreading evenly, using a sterile bent-glass rod, 3.5 mL of the second decimal dilution of an overnight *Listeria* culture on both sides of a bacon portion. The fourth trial remained uncontaminated to serve as a negative control. After contamination, suspensions (7 mL) of an overnight culture of Bac^+ or Bac^- *Lb. curvatus* were sprayed on both surfaces of B3 and B4 portions. The batch B2 was used as a positive control (non-inoculated sample) and, hence, was not inoculated with any of the LAB strains. Batches of the second trial were prepared in the same manner, except that the bacon blocks used were pre-treated with sodium nitrites to the level of 120 mg/kg of meat. The slices were placed in separate sterile plastic bags, which were then sealed and kept successively at 4 °C for 48 h, 7 °C for 48 h and 35 °C for 2 h, before storage at 4 °C.

Bacon Sampling and Analysis

Duplicates from each treatment were sampled aseptically at selected intervals for bacterial enumerations and for determinations of bacteriocin concentration. For the bacterial enumerations, a 20-g sample was removed, placed in a sterile plastic bag and thoroughly mixed with 180 mL sterile saline solution (0.85% sodium chloride), by squeezing and shaking for 15 min. The suspension was then serially diluted and the counts performed with the standard plate count technique on MRS or PALCAM agars for LAB or *L. monocytogenes*, respectively.

Bacteriocin production was monitored only in batches inoculated with *Lb. curvatus*. A sample of 20 g was crushed in 10 mL of saline solution until a homogeneous paste was obtained. This mixture was then centrifuged at 11,000 × g for 15 min (Avanti J-25I, Beckman, USA) and the supernatant filtered through a Millipore™ membrane (0.22 μm). The bacteriocin activity in AU/mL was determined in the filtrate as above and appropriate conversions were made to express the bacteriocin activity as AU per g of meat samples.

Each trial was repeated twice and the results were means of at least two replicates.

In Vitro Effect of Sodium Nitrites and Fat on Bacteriocin Production and Activity

To study the effect of sodium nitrite or fat on bacteriocin production and activity, two series of flasks containing 100 ml each of sterile MRS broth were prepared. In the first series, sodium nitrite was added to different concentrations ranging from zero (control) to 10%, while in the second series, cod-liver oil was added to different concentrations ranging from zero to 50%. The flasks were then inoculated with 100 μl of each of an overnight culture of *L. monocytogenes* and *Lb. curvatus* CWBI-B28 and incubated at 37 °C. Flasks of the series with added cod-liver oil were incubated under agitation (120 rpm) to ensure a good emulsification. At regular time intervals, samples were taken to determine the cell counts of *L. monocytogenes* and *Lb. curvatus* CWBI-B28, and the level of bacteriocin activity in a CFS as described above.

Each trial was repeated twice and each determination was done in duplicate. Statistical analysis was done by analysis of variance $\alpha = 0.05\%$ and Student t-test.

RESULTS AND DISCUSSION

The results of the effect of the bacteriocin produced by *Lb. curvatus* CWBI-B28 on *L. monocytogenes* in MRS broth or in the CFS of the Bac^+ strain are summarised in Figure 1. The counts of the pathogen were significantly reduced in co-culture with the Bac^+ strain and in its CFS (Figure 1(b)) while *Lb. curvatus* CWBI-B28 grew well (Figure 1(a)) and produced the bacteriocin (Figure 1(b)). In contrast, no inhibition of *L. monocytogenes* was observed when co-cultured with the Bac^- variant of *Lb. curvatus* (Figure 1(b)) in spite of the fact that no significant difference ($p > 0.05$) was observed in the growth of the latter as compared with that of the Bac^+ (Figure 1(a)). In the co-culture with the Bac^+ strain, *Listeria cfus* were reduced by ~4.2 log units after 24 h of incubation and a slight re-growth has occurred thereafter (0.67 log unit increase at 72 h). A similar pattern was observed in the CFS (F4) except that the initial decrease in Listeria counts was faster and a 3.9 log unit reduction was achieved within the first 7 h of incubation. The slower initial reduction rate in *Listeria* counts in presence of the Bac^+ strain than in the CFS could be explained by the lag phase necessary to reach the minimum biomass concentration required to induce bacteriocin production in the Bac^+ strain (Diep et al., 2000; Leroy and De Vuyst, 2005; Verluyten et al., 2004). The results of the bacteriocin activity in MRS broth corroborated such an observation as they showed that arbitrary units had increased steadily during the active growth of the Bac^+ to reach a maximum of 2133 AU/mL at 24 h and then declined to ~530 after 72 h of incubation (Figure 1(a)). Worth mentioning that *L. monocytogenes* was shown to grow normally in MRS spent broth that had supported the growth of the non bacteriocin-producing strain of *Lb. curvatus* (data not shown) suggesting that the inhibition of the pathogen in the CFS of *Lb. curvatus* CWBI-B28 was essentially due to the presence of the bacteriocin rather than the depletion of some

ingredients of the medium. The rapid increase in bacteriocin activity during the exponential growth phase of the Bac$^+$ strain suggests that the production rate of the bacteriocin is directly related to the production rate of biomass and, hence, the production of bacteriocin by *Lb. curvatus* CWBI-B28 follows primary metabolite kinetic. A similar pattern was described for most known bacteriocins (Leroy and De Vuyst, 1999; Himelbloom et al., 2001; Benkerroum et al., 2002; Onda et al., 2003), though some exceptions have been reported to produce the maximum amount of bacteriocin at the late stationary phase (Yang and Ray, 1994). However, further studies showed that these bacteriocins were, in fact, produced during the exponential growth phase but they undergo post-translational modifications to be active, which results in a delayed detection of AUs by biological techniques. The decrease in bacteriocin activity at the stationary phase of growth is also a common phenomenon among bacteriocins and was mainly explained by their degradation with endogenous proteases produced during the growth phase (Duffes et al., 1999; Callewaert et al., 2000; Onda et al., 2003). It may also be due to the adsorption of bacteriocin on the surface of the producer strain or to specific interactions with the ingredients of the medium, thereby reducing the amount of available bacteriocin. The decrease in arbitrary units could explain, in part, the re-growth of *L. monocytogenes* in the Bac$^+$ strain and in its CFS after 24 h of incubation, observed in this study. Emergence of resistant *Listeria* cells may also account for such a phenomenon, although authors ruled out this alternative for some bacteriocins of LAB (Schillinger et al., 2001; Todorov and Dicks, 2005) especially when used at high concentrations (>3,000 AU/ml).

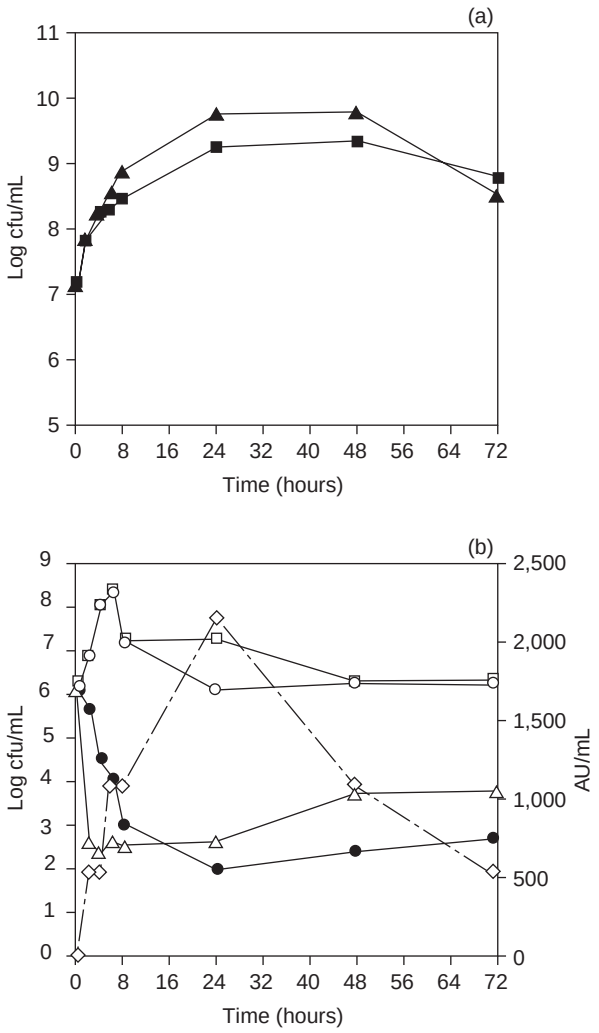

Figure 1. Evolution of cell counts as a function of time: (a) Bac$^+$ *Lb. curvatus* CWBI-B28 (■) and the Bac$^-$ *Lb. curvatus* M. (▲). (b) *L. monocytogenes* in MRS broth inoculated with Bac$^+$ (●) or Bac$^-$ (○) *Lb. curvatus*; (△) Listeria counts in the cell free supernatant of the Bac$^+$ strain and in (□) MRS broth non-inoculated with any of the *Lactobacillus* strains. (◇) Production of bacteriocin.

The in situ antilisterial activity of *Lb. curvatus* CWBI-B28 against *L. monocytogenes* was studied in bacon with or without added nitrites. The results are summarised in Figure 2. In the batches fermented with the non bacteriocin-producing *Lactobacillus* strain, the evolution of *Listeria* counts paralleled that observed in the non-inoculated samples (B2; without added starter culture) regardless of nitrite addition. In these samples, the counts of *L. monocytogenes* increased during the first week of storage at 4 °C and then decreased to 1.2 log cfu/g and 1.82 log cfu/g in the absence or presence of sodium nitrites, respectively (Figures 2(a) and (b)). These results suggest that the pathogen has a potential to grow on bacon at refrigeration temperature or, at least, to remain viable. In contrast, in the batches fermented with the bacteriocin-producing *Lb. curvatus* CWBI-B28, *L. monocytogenes* was completely inactivated within 1 and 2 weeks in the absence or presence of nitrites, respectively. However, a significant re-growth occurred the following week. In the mean time, *Lb. curvatus* grew equally well in samples fermented with the Bac$^+$ or Bac$^-$ strain irrespective of presence or absence of nitrites as suggested by the steady increase of the counts of the total LAB on MRS agar to reach high levels which were maintained throughout the whole period of storage (Figure 3). Concomitantly, in situ bacteriocin production by *Lb. curvatus* CWBI B-28 was monitored in bacon samples with or without added nitrites. Figure 2 shows that the bacteriocin activity has increased to a maximum of ~267 and 330 AU/g within 2 and 1 weeks in the presence or absence of nitrites, respectively. Thereafter, the arbitrary units declined regularly to an undetectable level after 6 weeks of storage at 4 °C (Figure 2(a) and (b)). In addition, the cell counts of *L. monocytogenes* in both batches indicated that in situ bacteriocin production is more effect-

ive in controlling *L. monocytogenes* in the absence than in presence of nitrites suggesting that the curing agent would antagonise with the in situ bacteriocin activity or production. In this regard, Verluyten et al. (2003) have shown that addition of sodium nitrites to the fermentation medium of *Lb. curvatus* LTH 1174 inhibited biomass formation thereby decreasing both volumetric and specific curvacin A production. However, these results show that nitrite would not induce a significant inhibition of the *Lb. curvatus* at concentration levels used in the meat industry (Figure 3). Yet, such an effect may be strain dependant as it is well established that nitrites have a potent antimicrobial activity mainly directed towards microorganisms using sulphur–iron enzymes (e.g. ferrodoxins) to generate energy from sugar metabolism. Nonetheless, interference of nitrites with the antimicrobial activity of bacteriocins has been reported in meat systems (Chumchalová et al., 1998; Benkerroum et al., 2003; Hornbæk et al., 2004) and was explained by their effect on the growth of the producer strain (Verluyten et al., 2003). Therefore, the ecological factors prevailing in meat systems would act synergistically with nitrites to enhance their inhibitory effect against LAB.

The results of the study on the influence of nitrites or fat content on the antilisterial effectiveness of *Lb. curvatus* CWBI-B28 in a laboratory medium (MRS broth) are summarised in Figures 4 and 5, respectively. Figure 4 shows that the addition of nitrites to MRS broth at concentrations lower than 3% had no effect on growth, bacteriocin production or antilisterial activity of *Lb. curvatus* CWBI-B28 (Figures 4(a), (b) and (c)). However, at a concentration of 5% or higher, nitrites have significantly ($p<0.05$) inhibited the growth of the Bac$^+$ strain (Figure 4(a)) and reduced bacteriocin production (Figure 4(b)). Consequently, the inhibition of *L. monocytogenes* was significantly reduced ($p<0.05$) and the pathogen even grew at 10% nitrites concentration (Figure 4(c)). Such concentrations are far beyond those allowed in the meat industry worldwide (i.e. maximum of 120 mg/kg of meat).

Fat content is another factor that has been reported to interfere with the bacteriocin activity in food

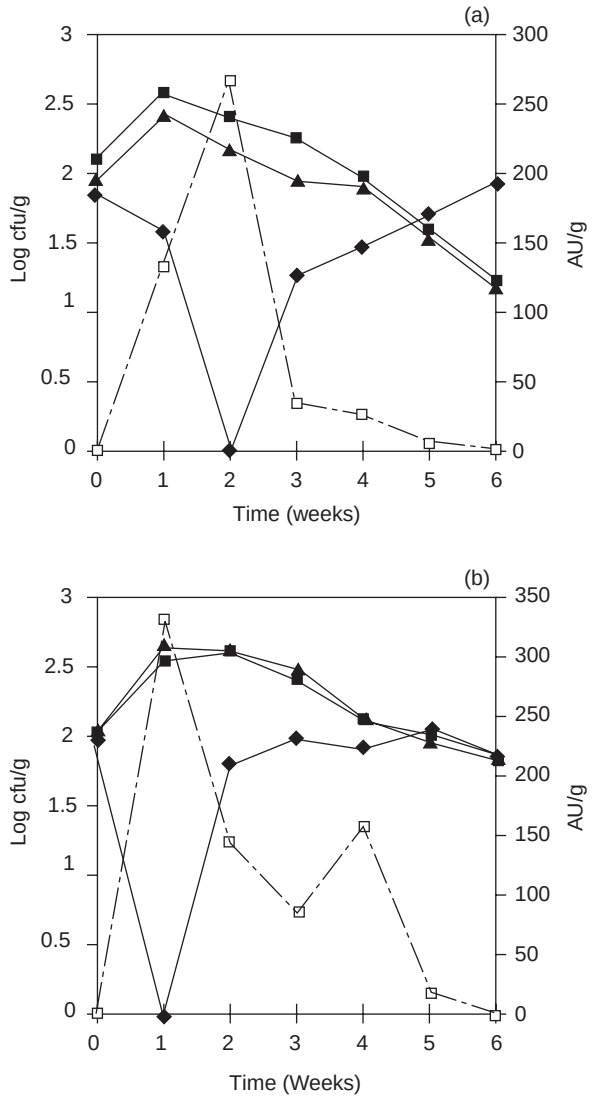

Figure 2. Evolution of the counts of *L. monocytogenes* in bacon in presence (a) or absence (b) of nitrites and fermented with a bacteriocin producing (◆) or with a non-bacteriocin-producing (▲) strain. Growth of *L. monocytogenes* in a control without added starter culture (■) was also monitored. (□) Production of bacteriocin was presented for *Lb. curvatus* CWBI-B28.

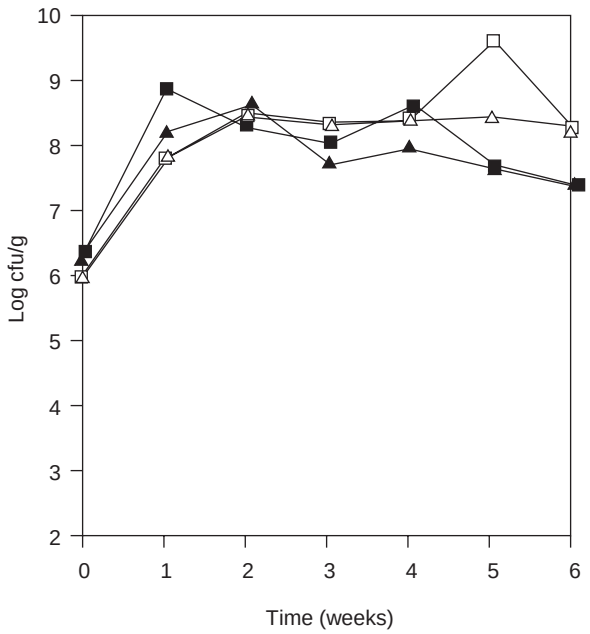

Figure 3. Evolution of cell counts of lactic acid bacteria in bacon meat fermented with the Bac$^+$ (■, □) or Bac$^-$ (▲, △) strain in presence (■, ▲) or in absence (□, △) of nitrites (120 mg/kg).

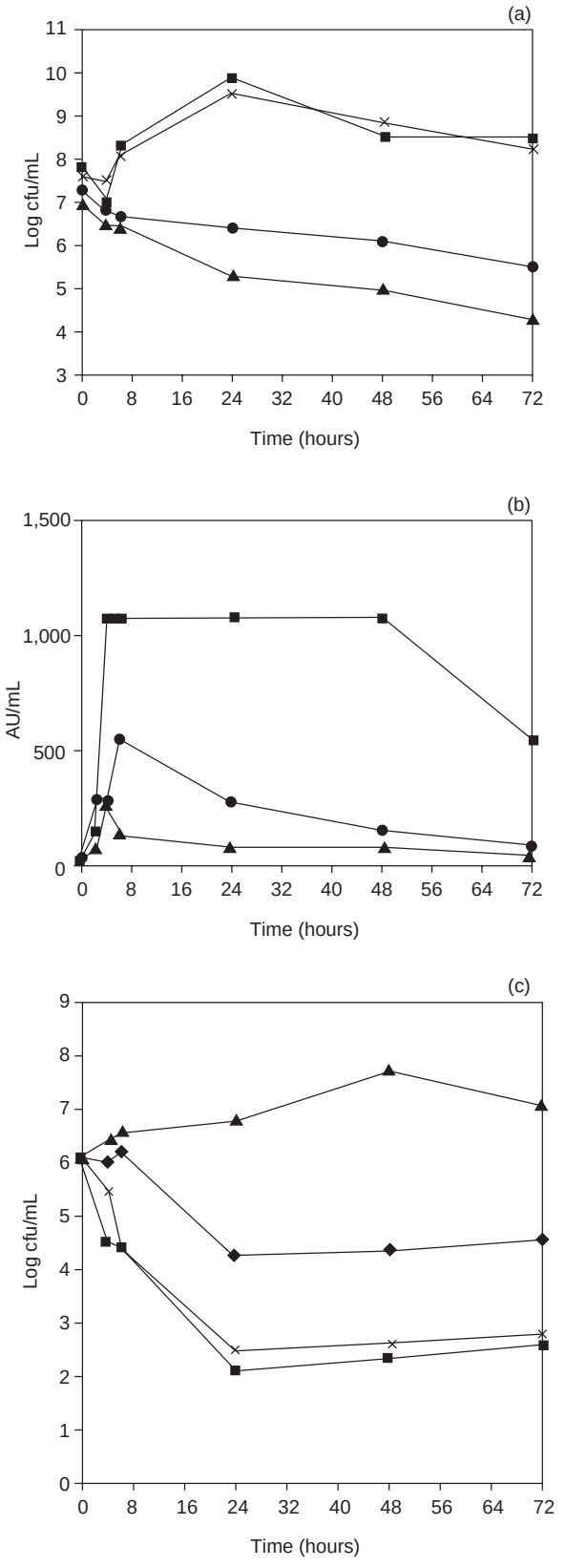

Figure 4. Effect of different concentration of sodium nitrite; 0% (■), 3% (×), 5% (●), 10% (▲) on: (a) the growth of *Lb. curvatus* CWBI-B28; (b) production of bacteriocin, (c) growth of *L. monocytogenes* in liquid medium.

systems (Bani-Jaber et al., 2000, Cleveland et al., 2001). The results of Figure 5 show that the growth of *Lb. curvatus* CWBI-B28 was not affected by fat concentration as it grew well at all concentrations tested to exceed 8 log cfu/g in all samples. However, the production of bacteriocin was significantly reduced at the high fat concentrations (15, 20 and 50%) as compared to the control without added cod-liver oil (Figure 5(b)). As for the antilisterial activity of *Lb. curvatus* CWBI-B28,

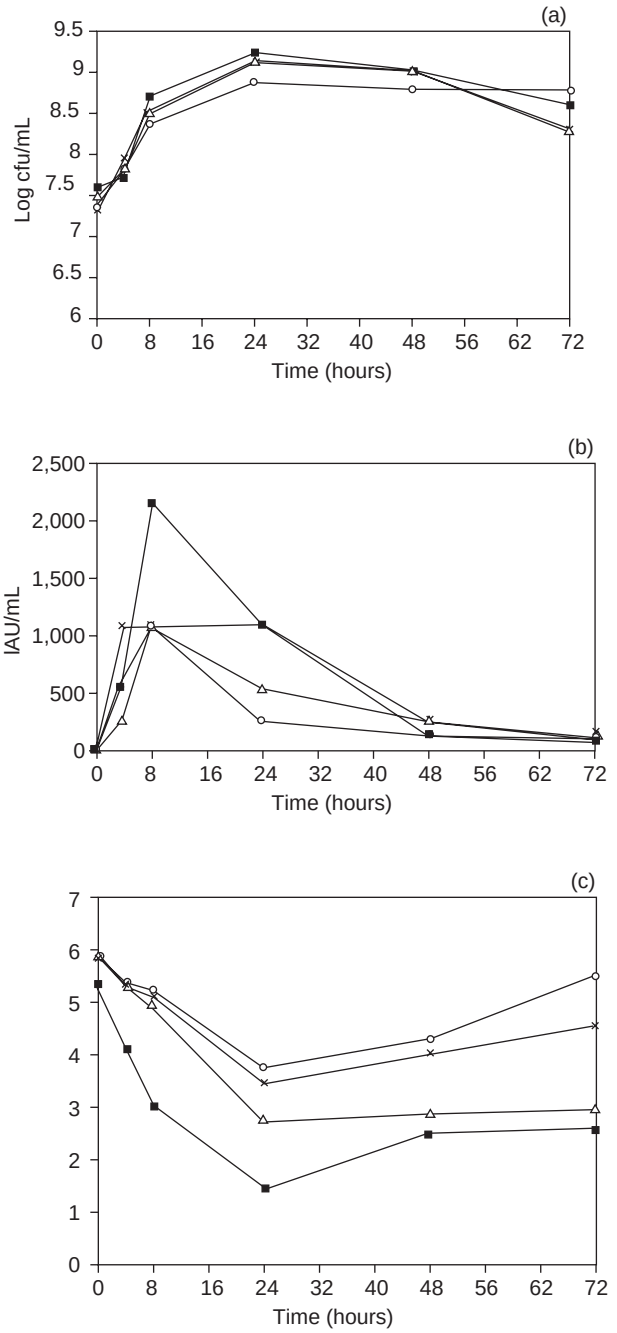

Figure 5. Effect of different concentrations of fat 0% (■), 15% (△), 20% (×), 50% (○) on: (a) the growth of *Lb. curvatus* CWBI-B28; (b) the production of bacteriocin; (c) the growth of *L. monocytogenes*.

Figure 5(c) shows that the inhibition of *L. monocytogenes* was reduced in presence of high fat content either because the fat provides a good protection to *L. monocytogenes* against bacteriocin activity or due to the decrease in bacteriocin production with the increase in fat concentration. It should be noted, however, that at fat concentrations less than 7%, no adverse effect has been observed on bacteriocin production or activity against *L. monocytogenes* (data not shown). In this regard, several authors explained the reduced antimicrobial activity of bacteriocins in high-fat systems by the hydrophobic interactions of these antimicrobials with fat and the hindrance of their active sites (Chumchalová et al., 1998; Davies et al., 1999; Blom et al., 2001). The authors' results are in agreement with such findings despite the decrease in the arbitrary units in the presence of increased fat concentrations (Figure 5(b)) that may be due to the decrease in available active bacteriocin upon hydrophobic interactions with fat, and hence its low detection by the biological method used to determine the AU. Nonetheless, the results obtained revealed that the activity of bacteriocins in a given medium does not depend solely on the fat content. Other parameters including the nature of the bacteriocin itself, the physical state of the medium (liquid or solid) and the culture conditions (e.g. pH, temperature, agitation and the medium) are of importance to the effect of the fat on bacteriocin activity (Goff et al., 1996; Murray and Richard, 1997; Bani-Jaber et al., 2000; Aasen et al. 2003).

CONCLUSIONS

This study demonstrated that nitrites and fat content interfere with the bacteriocin activity against *L. monocytogenes* in culture media. However, such interferences were significant at high concentrations usually exceeding those used in the meat industry. In effect, regardless of nitrites addition to the bacon (a high fat product), the use of a bacteriocinogenic starter culture has proven efficient in controlling *L. monocytogenes*, especially during early stages of fermentation and storage. However, such a control may be less effective in late storage and may be limited in food systems with a long shelf life. Several authors have, indeed, demonstrated that the bacteriocin activity is reduced upon extended storage to eventually vanish providing an opportunity to the pathogen to re-grow either because of the low bacteriocin concentration or because of emergence of resistant mutants (Rodríguez et al., 1994; Buncic et al., 1997; Winkowski and Montville, 1992; Song and Richard, 1997; Mattila et al., 2003; Scannell et al., 2001).

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