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# Effects of curing agents on growth and Sakacin A production by *Lactobacillus sakei* Lb 706 during production of processed meat

**Sahar F. Deraz[1], Ashraf A. Khalil[1*], Sawsan A. Abd-Ellatif[2], Doaa A. Ghreeb[3], Ahmad I. El-Dewany4**

[1]Department of Protein Technology, [2]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Science and Technology, Borg Al-Arab, Post Code 21934, Alexandria, Egypt
[3]Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt
[4]Department of Chemistry of Natural Products, National Research Center, Dokki, Cairo, Egypt

***Corresponding author e – mail**: ashraf_khalil@msn.com

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## ABSTRACT

*Objective:* The effects of ingredients and other technological factors, e.g. salt, nitrite, water activity and fat, on the functionality of *Lactobacillus sakei* Lb 706 were investigated in order to evaluate bacteriocin bioavailability during sausage processing.

*Methodology and results*: A series of *in vitro* fermentation experiments were performed with varying concentrations of NaCl, $NaNO_2$, glycerol and meat fat particles. NaCl and $NaNO_2$ interfered with bacterial cell growth and bacteriocin activity. The growth of *L. sakei* Lb 706 was enhanced in the presence of low concentrations of NaCl (≤ 0.5%), though inconsistently, while high concentration (≥ 4%) of NaCl consistently inhibited growth. $NaNO_2$ had an insignificant effect on bacteriocin activity. At higher sodium nitrite concentration, sakacin A diminished accompanied with lower cell masses

*Conclusions and application of findings*: The use of *L. sakei* Lb 706 as a bacteriocin-producing culture during sausage processing is promising and can be used as a bioprotective culture against *Listeria* spp. The concentration of $NaNO_2$ and NaCl may vary from 20 to 200 ppm and 2.5 to 3%, respectively, depending on the type of sausage and on regulatory requirements. The restrictive and inhibitory effects of nitrite, salt and fat on the growth of bacteria investigated were not very marked, and there were no significant differences between the separate or combined action of salt and nitrite. Although all experiments were conducted in a model system, these findings will aid in industrial implementation of efficient bacteriocin-producing protective cultures of *L. sakei* in meat production. These bacteriocinogenic protective cultures will contribute to meat products that are safer and of more consistent quality.

**Key words**: *Lactobacillus sakei,* sakacin A, cell activity, Sodium chloride, Sodium nitrite

## INTRODUCTION

Consumers increasingly prefer safe, natural food products that are mild and light with low acid, sugar, salt, and fat content (Gould, 1996). This trend has stimulated research on the additives used in meat processing, such as NaCl and curing agents. The quest for more-natural products has also provided an incentive to search for safe food-grade preservatives of biological origin (Stiles, 1996; De Vuyst, 2000). Furthermore, some food-borne pathogenic bacteria, such as *Listeria monocytogenes*, can survive the sausage manufacturing process (Johnson *et al.,* 1988) because of its ability to survive acidic conditions (Gahan *et al.,* 1996) and its tolerance to considerable amounts of sodium chloride and nitrite (Razavilar & Genigeorgis, 1998). In addition, the spread of antibiotic resistance genes among listeriae and other pathogens is raising new concerns (Teuber, 1999).

Bacteriocins produced by lactic acid bacteria (LAB) are antibacterial peptides or proteins that are active against other Gram-positive, mainly closely related bacterial species, including some undesirable spoilage bacteria and food-borne pathogens, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Clostridium botulinum* and *Listeria monocytogenes* (De Vuyst & Vandamme, 1994). The only legally approved bacteriocin in many countries is nisin, approved for use as a preservative in a limited number of food products (Delves-Broughton *et al.,* 1996). Unfortunately, this bacteriocin is not very efficient in a meat environment, because of its low solubility, uneven distribution, and lack of stability (Stiles, 1996).

An alternative and interesting approach for the use of bacteriocins in fermented sausages is the use of bacteriocinogenic LAB adapted to the specific meat environment (Leroy & De Vuyst, 2000). Several bacteriocins produced by strains isolated from fermented sausages are highly active against listeriae (Hugas *et al.,* 1995; Cintas *et al.,* 1997; Herranz *et al*., 2001; Messi *et al.,* 2001; Sabia *et al.,* 2002). Moreover, numerous studies have shown that antilisterial bacteriocin producing lactic acid bacteria are able to suppress growth of *L. monocytogenes* in meat and other foods (Schillinger *et al.,* 1991; Stiles, 1996). Recent approaches in the preservation of cooked meats and minimally processed refrigerated foods are increasingly directed towards biocontrol using a protective microflora, usually LAB, to inhibit growth of *Listeria monocytogenes* and other undesirable micro-organisms (Schillinger *et al.,* 1996; Duffes *et al.,* 1999; Scannell *et al.,* 2000; Bredholt *et al.,* 2001; Katla *et al.,* 2001).

Bioprotective cultures may act as starter cultures in the food fermentation process or they may protect foods without any adverse organoleptic changes (De Vuyst & Vandamme, 1994). *Lactobacillus sakei* is a ubiquitous LAB that is commonly associated with the food environment. Although the organism can be isolated from various plant fermentations (Vogel *et al.,* 1993; Hufner *et al.,* 2007), it is mostly isolated from the meat environment (Champomier-Verges *et al.,* 2002; Hammes & Hertel, 2004). *L. sakei* is recognized as one of the most important components of starter cultures used for production of fermented meat products (Hammes & Hertel, 1998; Leroy *et al.,* 2006). Recently, it was shown that this species is also a transient member of the human gastrointestinal microbiota (Dal Bello *et al.,* 2003). In addition to the fact that *L. sakei* occurs ubiquitously, it also displays notable differences in physiological and biochemical properties compared to other lactobacilli (Axelsson & Ahrne, 2000; Chaillou *et al.,* 2005). For example, *L. sakei* is able to proliferate at refrigeration temperatures (4°C) and in the presence of high salt concentrations (up to 9% sodium chloride) (Chaillou *et al.,* 2005). Tolerance for both low temperature and high salt plays a key role in meat processing in many meat processing `environments (Champomier-Verges *et al.,* 2002).

*Lactobacillus sakei* Lb 706, an isolate from fermented sausage, has been shown to produce the antilisterial bacteriocin sakacin A (Holck *et al.,* 1992). Through *in vitro* experiments it has been demonstrated that the temperatures and pH values encountered during the manufacture of fermented sausages are favorable for sakacin A production. Schillinger *et al.* (1991) reported that *Lactobacillus sakei* Lb 706 can be used in fresh meat and cured

meat products as a protective culture. The practical use of these protective cultures will contribute to safer and more uniform end products. However, the choice of an appropriate protective culture for meat products is of utmost importance, since strain competitiveness and bacteriocin production is influenced by specific conditions that prevail in the meat product matrix.

The manufacture of fermented sausages includes mixing of minced meat and fat with salts, curing agents and spices, stuffing into casings and fermentation of the mixture by a well-defined starter culture under controlled conditions. Adding salt (2.5 to 3.0%, wt/wt) to raw sausage is essential; salt decreases water activity ($a_w$) and contributes to flavor and microbial selection (Leistner, 1995). Adding nitrate and nitrite to sausage batter is common. Nitrite is added to produce color, to prevent lipid rancidity, and to inhibit the growth of Salmonellae and Clostridia (Leistner, 1995; Drosinos *et al.,* 2007), and to obtain the typical cured flavor (Dainty & Blom, 1995). The concentration of sodium nitrite may vary from 20 to 200 ppm depending on the type of sausage and on the legislation (Krockel, 1995).

To fulfill industrial requirements, a protective culture should be easy to culture, easy to apply to the meat and give reliable and reproducible results. The culture should also be well adapted to grow in the product and to survive during the storage of the product. Hence, if *L. sakei* Lb 706 or other bacteriocin producing LAB strains are to be applied as novel functional starter or protective cultures for meat processed products, it is necessary to understand how such strains behave under sausage fermentation conditions. To estimate the bacteriocin activity in a sausage environment, many factors such as the presence of typical sausage ingredients, e.g. salt, curing agent, have to be taken into account as well; these factors not only affect cell growth but also interfere with the production of sakacin A.

In this study, the effects of typical sausage ingredients and other technological factors on the functionality of *L. sakei* Lb 706 were investigated. In particular, the effects of salt, nitrite, water activity and fat were quantified. This investigation was performed in order to evaluate bacteriocin bioavailability during sausage processing.

## MATERIAL AND METHODS

**Bacterial strains and media:** *L. sakei* Lb 706 was used as the producer of the antilisterial bacteriocin Sakacin A (Holck *et al.,* 1992). A sakacin A-sensitive indicator organism, *L. sakei* NCDO 2714 was used to determine bacteriocin activity levels. *L.sakei* Lb 706 and *L.sakei* NCDO 2714 were kindly provided by Prof. Ulrich Schillinger (the Institute of Hygiene and Toxicology, BFE Karlsruhe, Germany) and Prof. Ingolf Nes (Laboratory of Microbial Gene Technology, Agriculture University of Norway), respectively. Strains were stored at -80°C in MRS medium, both containing 25% (v/v) glycerol as a cryoprotectant. To produce fresh cultures, the strains were propagated twice at 25 and 30°C for 14-16 h before experimental use, respectively. Solid medium was prepared by adding 1.5% (w/v) agar (Difco Laboratories) to the broth. The overlays used for estimation of bacteriocin titers were prepared with 0.75 % (w/v) agar.

**Fermentation experiments:** A series of *in vitro* fermentation experiments were carried out in MRS broth inoculated with 1% (v/v) of freshly prepared *L. sakei* Lb 706 and incubated under agitation (80 rpm) at 25°C. These fermentations were performed using fermentation liquors with added NaCl and $NaNO_2$ at various concentrations (2, 4 and 6 % [w/v] and 100, 200, and 400 ppm [w/v], respectively, (the latter was sterilized separately by microfiltration [Sartolab; Sartorius, Germany]). An additional fermentation experiment was performed without salt and nitrite. The fermentations containing 0 or 4% (w/v) NaCl were performed in triplicate so as to determine reproducibility of the experiments.

To determine whether the effect of the added NaCl was due solely to a reduction in $a_w$, an additional fermentation was carried out in the presence of 9.9 and 21.1 % (w/v) glycerol (sterilized separately) in the absence of salt. Based on extrapolation of data from previous published studies, addition of 9.9 and 21.1% (w/v) glycerol to the basal growth medium should result in an $a_w$ similar to 4 and 8 % (w/v) NaCl, respectively (McMeekin *et al.,* 1987; Chandler & McMeekin 1989).

In another set of fermentation experiments, a modified MRS (mMRS) was used as the fermentation medium for *L. sakei* Lb 706. The concentration of the complex nutrient sources i.e. bacteriological peptone [Oxoid], meat extract [Oxoid], and yeast extract [VWR International] was doubled. This modification to the standard MRS medium was done to investigate potential growth limitation of *L. sakei* Lb 706 due to nutrient depletion. In addition, calculations of the amino nitrogen content of MRS medium (Bridson, 1998) indicated that this composition more closely simulates the actual sausage environment (Dainty & Blom, 1995). All media and solutions were sterilized at 121°C for 20 min.

**Assays:** At regular time intervals, samples were withdrawn aseptically to determine biomass (cell dry mass [CDM] and optical density at 600nm) and the level of soluble bacteriocin activity in a cell-free culture supernatant. Bacterial growth was performed with biomass concentrations obtained from OD 600 measurements to allow comparison with results obtained previously with the same strain (Holck *et al.,* 1992) as well as with other data available from literature.

**Bacteriocin activity assay:** The culture supernatants were assayed for bacteriocin activity by the spot on lawn technique with MRS agar using *L. sakei* NCDO 2714 as indicator strain (Barefoot & Klenhammer 1983; Deraz *et al.,* 2005). Indicator lawns were prepared by adding 0.125 ml of 10 times diluted overnight culture to 5 ml of MRS soft agar (0.75 %). The contents of the tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Bacteriocin samples were sterilized by passage through a 0.45 μm cellulose acetate filter. Serial two-fold dilutions were carried out in the same medium as used for the growth of the indicator strain. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of culture media. The titre of the bacteriocin solution, in AU/ml, was calculated as (1000/d) D, where D is the dilution factor and 'd' is the dose (the amount of bacteriocin solution pipetted on each spot) (Deraz *et al.,* 2005). To avoid errors in bacteriocin activity values, the same person made all observations. Moreover, it was checked to confirm that salt and nitrite did not interfere with the bacteriocin activity assay method.

**Adsorption of bacteriocin to fat and meat:** Bacteriocin-containing cell-free culture supernatant was prepared by inoculating 1 L of MRS broth (Oxoid) with *L. sakei* Lb 706 (1%, v/v), incubation overnight at 25 ˚C and removing the cells by centrifugation ($6{,}500_{xg}$, 20 min). Luncheon meat composed of beef meat, salt, spices, 0.05 % ascorbic acid and 100 ppm sodium nitrite with low fat content, no more than 22%, (the entire product) or beef fat (further referred to as fat) were obtained from a local store and flamed to reduce the superficial contamination. Fat or luncheon particles (100 or 300g $l^{-1}$, ± 2g) were added to the supernatant (50 ml) and stored either at refrigerator or room temperature. Bacteriocin activity in the supernatant was measured over time. A control experiment was performed in the absence of meat and fat.

## RESULTS

**Effect of NaCl:** Cell growth and Sakacin A activity in the presence of various salt concentrations were measured overtime as well as consumption of glucose and production of lactic acid (data not shown). *L. sakei* Lb 706 appeared to be quite salt tolerant. However, as the salt concentration increased, the cells grew more slowly and biomass production became less efficient. The growth of *L. sakei* Lb 706 was affected by as little as 2 % NaCl although considerable growth was still realized. During the fermentations carried out in mMRS with varying concentration of NaCl at 25 ˚C (without pH control and with slow agitation [80 rpm]), *L. sakei* Lb 706 grew exponentially for approximately 12-14 h, after which growth slowed down (Figure 1) and ceased after 17-18 h of fermentation. Addition of 2 and 4 % (w/v) NaCl had no effect on the growth profile of *L. sakei* Lb 706. However, the addition of as much as 6 % NaCl caused the bacteria to enter into the stationary phase earlier, thereby significantly reducing biomass production. The CDM in the presence of 6 % NaCl was only 10 % of the concentration obtained when no salt was added. However, the cell dry weight in the presence of 2 and 4 % (w/v) NaCl was 70 and 40 % respectively, compared to the reference fermentation without salt.

Production of Sakacin A appeared to be dependent on the concentration of NaCl used. There was a significant difference in the amount of activity of sakacin A recovered from the samples during production in the presence of various concentration of NaCl. The fermentation conditions allowing measurable bacteriocin production were 2 and 4 %. Higher salt

concentration strongly suppressed bacteriocin production by *L. sakei* Lb 706. When more than 60 g $l^{-1}$ (≥ 6 %) NaCl was added, no bacteriocin activity could be detected throughout the fermentation period. Addition of 2 % NaCl decreased the activity from 6400 to 3200 AU/ml. However, 4 % NaCl decreased the activity to 1600AU/ml.

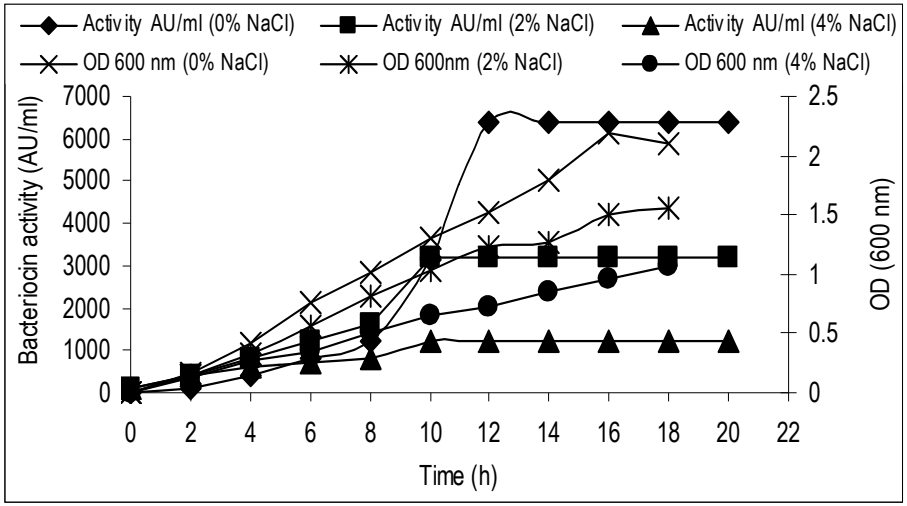
**Effect of $NaNO_2$:** All fermentations at the concentrations of $NaNO_2$ tested affected the growth of *L. sakei* Lb 706. Addition of nitrite decreased the growth rate, the maximum cell yield and the bacteriocin activity (Figure 2). These results indicate that the decrease in sakacin A activity was due to the decrease in cell growth alone, because both specific bacteriocin production and the inactivation rate remained constant. The growth of *L. sakei* Lb 706 was inhibited by as little as 50 ppm and much more severely at 400 ppm, although some growth still occurred (Fig. 2). The CDM in the presence of 100, 200, 400 ppm $NaNO_2$ were 63, 56 and 50 % of the reference fermentation, respectively, where no nitrite salt was added. However, 400 ppm $NaNO_2$ extremely suppressed sakacin A production to as low as 25 % from the original activity when no $NaNO_2$ was added. On the other hand, *L. sakei* Lb 706 was still able to produce considerable amounts of sakacin A in the presence of 100 and 200 ppm $NaNO_2$, which reached to up to 75 and 50 % of the activity obtained in fermentation with no nitrite salt added.

**Combined effect of NaCl and $NaNO_2$:** The combined action of salt and nitrite influenced the growth rate of *L. sakei* Lb 706 to lesser extent compared to its influence on bacteriocin production. The fermentation carried out in the presence of 2 % NaCl and 100 ppm nitrite results in biomass concentration comparable to the fermentation without NaCl or Nitrite, except for a slightly lower maximum attainable biomass concentration (1.9 instead of 2.20 g of CDM $liter^{-1}$, respectively), which results in a slightly higher reduction in bacteriocin production from 6400 to 4800Au/ml, respectively. This means the combined action of 2 % NaCl and 100 ppm $NaNO_2$ led to recovery of 86 and 75% of cell biomass and bacteriocin production of the reference fermentation (Fig 3).

The reduction in bacteriocin activity due to the addition of 2 % NaCl and 100 ppm nitrite was similar to the separate effect of either 2 % NaCl or 100 ppm $NaNO_2$. However, addition of the same concentration of NaCl (2 %) and double concentration of sodium nitrite (200 ppm) led to drastic reduction of bacteriocin production from 6400 to 2400 AU/ml, while biomass was reduced from 2.2 to 1.5 g of CDM $liter^{-1}$, compared to the reference fermentation. The effect of adding 100 ppm $NaNO_2$ combined with double concentration of NaCl (on both bacteriocin production and biomass) were roughly comparable to the putative effect of adding 4 % sodium chloride.

**Adsorption of bacteriocin to fat and meat particles:** Measurable bacteriocin activity disappeared from the bacteriocin-containing culture supernatant in the presence of beef fat (Fig. 5 a,b). The depletion of sakacin A in culture supernatant was dependent on the amount of fat added, with the apparent inactivation being higher at 300 than at 100 g fat $l^{-1}$. On the other hand, bacteriocin activity in culture supernatant with luncheon meat particles was rather stable and comparable to bacteriocin activity in a control experiment. The decrease of bacteriocin activity over time was showing exponential kinetics. When fat was used, the activity lose was more pronounced than with equal amounts of luncheon. Specific bacteriocin production and the specific apparent bacteriocin inactivation rate were comparable to those with standard MRS (unpublished data).

**Influence of $a_w$:** To determine whether a reduction in $a_w$ was solely responsible for the inhibitory effect of NaCl on bacterial growth and sakacin A production, or other (ionic) effects were responsible, two fermentation experiments were performed with glycerol added as an $a_w$–lowering agent. The presence of glycerol in the growth medium had similar effects on the growth rate and specific bacteriocin production as was the case for salt. In this study, the values obtained for fermentation in the presence of 9.9 and 21.1% glycerol were roughly comparable to the putative effects of 2.0 and 6 % sodium chloride, respectively. The biomass and bacteriocin production values obtained for fermentations in the presence of 20 and 60 g $1^{-1}$ of NaCl were comparable to the corresponding values obtained after the addition of 99 and 211 g $1^{-1}$ of glycerol, respectively. Both biomass and bacteriocin production were lower when glycerol was used as $a_w$–lowering agent instead of NaCl (Fig 4).



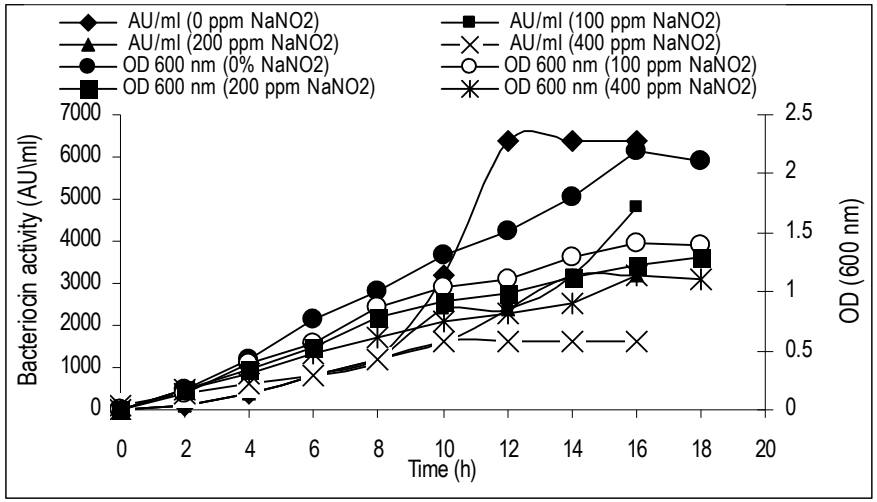
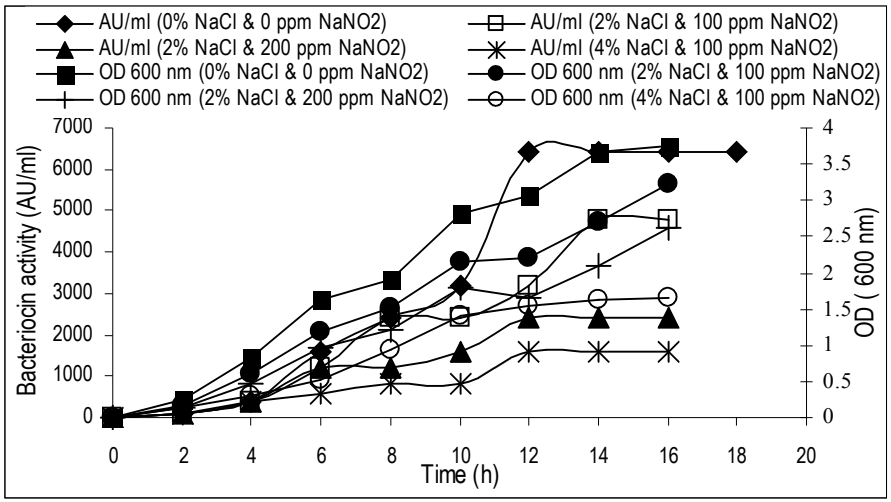




**Figure 1**: Influence of different concentrations of NaCl on the growth of *Lactobacillus*. sakei Lb 706, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. NaCl was added at 20 g $l^{-1}$ (2%), 40 g $l^{-1}$ (4%). **Figure 2:** Influence of different concentrations of sodium nitrite on the growth of *L*. sakei Lb 706, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. Sodium nitrite was added at 0 ppm, 100 ppm, 200 ppm and 400 ppm.



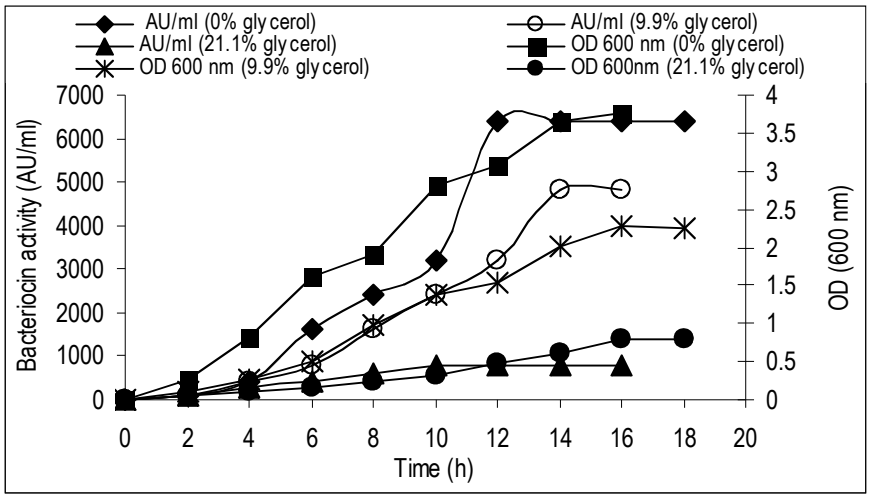




**Figure 3:** Influence of different concentrations of sodium chloride combined with different concentration of sodium nitrite on the growth of *L*. sakei Lb 706, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. Sodium chloride and sodium nitrite was added at 0g $l^{-1}$ (0%) + 0ppm, 20g $l^{-1}$ (2%) +100 ppm, 20g $l^{-1}$ (2%) + 200 ppm and 40g $l^{-1}$ (4%) + 100 ppm, respectively; **Figure 4:** Influence of different concentrations of glycerol on the growth of *L*. sakei Lb 706, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. Glycerol was added at 9.9% and 21.1%.

## DISCUSSION

The application of antimicrobial peptides against food-borne pathogenic bacteria has received great attention especially in products such as fermented sausages (Drosinos *et al.,* 2005). In the past years, many LAB isolated from fermented sausages have been screened for the production of antimicrobial compounds. Several lactobacilli, mainly belonging to the species *L. sakei* (Hugas *et al.,* 1995), *L. curvatus* (Mataragas *et al.,* 2002), *L. brevis* (Benoit *et al.,* 1994), *L. plantarum* (Messi *et al.,* 2001) and *L. casei* (Vignolo *et al.,* 1993) have been found to be bacteriocinogenic. However, the production of a bacteriocin under laboratory conditions does not necessarily mean that sufficient and effective bacteriocin production and bioavailability will occur in the food ecosystem. Severe limiting factors include, for instance, restricted nutrient availability for the bacteriocin-producing cells and adsorption of the bacteriocin onto meat particles, fats, and proteins (Stiles, 1996; Verluyten *et al.,* 2004). In addition, meat processing technology may interfere with the bacteriocin production capacity of the starter cultures or co-cultures used as protective culture. Therefore, it is important to know both the effects of environmental factors, such as temperature and acidity, and the influence of specific sausage ingredients, such as salt and nitrite, on the growth characteristics and production of bacteriocins by the starter cultures and/or protective cultures used in processed meat products (Leroy & De Vuyst, 1999a: Leroy & Vuyst. 1999b: Messens *et al.,* 2002).

The most important ingredient used in the production of processed meat is nitrite curing salt (NCS). Nitrite is normally supplied in the form of NCS, a mixture of sodium chloride and sodium nitrite. The environmental factors studied in this study (salt, nitrite, fat) had significant influences on the functionality of *L. sakei* Lb 706. Adding salt and/or curing agents has consequences for both growth and bacteriocin production of *L. sakei* Lb 706. Salt affects the growth and bacteriocin production of lactic acid bacteria (Rozes & Peres 1996; Uguen *et al.,* 1999; Verluyten *et al.,* 2004), e.g. the production of sakacin K by *L. sakei* CTC 494 (Leroy & De Vuyst, 1999b) and production of carnobacteriocin B2 by *Carnobacterium piscicola* A9b (Himelbloom *et al.,* 2001).

Although *enterococci* are more salt resistant, production of the enterocins A and B by *Enterococcus faecium* CTC 492 is also inhibited in the presence of NaCl (Aymerich *et al.,* 2000). In our study, NaCl negatively affects the production of sakacin A by *L. sakei* Lb 706. Production of sakacin A may decrease because the amount of biomass formed decreases since bacteriocin production generally exhibits primary metabolic kinetics (Leroy & Vuyst, 1999a). Therefore, Sakacin A production is growth related, and lower growth rate results in formation of less biomass and thus in lower sakacin A production. Bacterial metabolism is sensitive to salt, because salt exhibits specific ionic and water binding properties (Korkeala *et al.,* 1992). The latter effect is of utmost importance because the addition of salt to the fermentation liquor leads to a decrease in $a_w$. Decreases in $a_w$ below the optimum values for growth often result in a linear decrease of the growth rate (McMeekin *et al.,* 1987). Indeed, the growth rate often decreases linearly at $a_w$ values below the optimum $a_w$ (McMeekin *et al.,* 1987; Passos *et al.,* 1993).

However, a low concentration of NaCl (1%, w/v) showed no effect on bacterial growth or sakacin A production by *L. sakei* Lb 706, while an inhibition that increased linearly was evident with higher salt concentration. Bacterial growth enhancing effect of low concentrations of salt (1 to 2%, w/v) are not common but a few cases have been reported previously (Korkeala *et al.,* 1992; Vignolo *et al.,* 1995; Ganzle *et al.,* 1998; Uguen *et al.,* 1999) . Uguen *et al.,* (1999) reported an increased lacticin 481 production when the osmolarity of the growth medium increased due to addition of NaCl. Also, for plantaricin S, the highest production was observed at a sodium chloride concentration of 2.5 % (w/v) (Leal-Sanchez *et al.,* 2002).

On the other hand, homofermentative LAB are more resistant to NaCl than heterofermentative LAB are, and strains resembling *L. sakei* have been shown to be more resistant than strains resembling *Lactobacillus curvatus* (Korkeala *et al.,* 1992) or *Lactobacillus pentosus* (Doßmann *et al.,* 1996). It appears that cultivation of LAB in environments with a lot of salt (NaCl >30 g $l^{-1}$ ) hampers bacterial growth, while lower amounts from 10 to 20 g $l^{-1}$ can exhibit a positive effect (Korkeala *et al.,* 1992; Passos *et al.*, 1993; Ganzle *et al.,* 1998). Another possible explanation for the inhibition of bacteriocin production by NaCl is interference with the binding of the induction factor (IF) to its receptor (Nilsen *et al.,* 1998). The IF is excreted by the producing strain, and when it reaches a critical concentration, the binding to its receptor initiates bacteriocin production. For *Carnobacterium piscicola* A9b, the induction capacities are negatively affected by

the addition of NaCl (Nilsson *et al.,* 2002). Moreover, for *Enterococcus faecium* CTC 492, addition of IF can overcome the inhibition of enterocin production (Nilsen *et al.,* 1998; Aymerich *et al.,* 2000; Aymerich *et al.,* 2002). Even at NaCl concentrations that do not affect growth, the induction of bacteriocin production decreases, indicating that higher concentrations of the inducer are necessary to sustain bacteriocin production (Nilsen *et al.,* 1998).

However, in the case of *L. sakei* Lb 706, it appears that the water binding effect of salt molecules is the major factor responsible for the decrease in bacteriocin production since using glycerol as an agent to decrease $a_w$ instead of salt has a comparable effect. Based on extrapolation of data from previous studies (Chandler & McMeekin 1989; McMeekin *et al.,* 1987), addition of 9.9 and 21.1% glycerol to the basal growth medium should have resulted in the same decreases in the $a_w$ as approximately 4 and 8 % NaCl, respectively. The fact that our values were slightly low may be explained by differences in growth medium, experimental error, or unknown effects. Hence, because salt decreases $a_w$, the presence of relatively high salt concentrations in sausage batter may be one of the predominant factors that reduce the efficacy of bacteriocin-producing starter or protective cultures. During the fermentation stage, a salt concentration of 2 to 2.5 % in the water phase of the sausage batter does indeed decrease sakacin A production considerably. However, the activity probably is sufficient to have a significant antilisterial effect in the sausage environment, as demonstrated by Schillinger *et al.* (1992), *L. sakei* Lb 706 reduced viable counts of listerias by about one log cycle.

Nitrite is known mainly for its antimicrobial activity against spore formers; it has a limited effect on the growth of lactic acid bacteria at concentrations less than 200 mg liter $^{-1}$ (Korkeala *et al.,* 1992; Vignolo *et al.,* 1995; Doßmann *et al.,* 1996), but at 400 mg liter$^{-1}$ inhibition is more pronounced (Korkeala *et al.,* 1992). It has been shown that biomass formation and bacteriocin production by *L. sakei* Lb 706 decrease as the concentration of sodium nitrite increases. Nitrite has little effect on sakacin A production but decreases the bacteriocin titer indirectly because of its effect on cell growth. Since sakacin A production is growth related, formation of a small amount of biomass results in a low sakacin A yield. Different explanations have been suggested that the presence of un-dissociated nitrous acid molecules, enhancing the toxic effect of lactic acid, as a result of intracellular accumulation (Leroy & De Vuyst, 1999 a & b). Furthermore, it has been mentioned previously that nitrite might interfere with active transport mechanisms (Davidson, 1997), which could explain the surprisingly low biomass obtained when 0.04 % sodium nitrite is used.

Furthermore, the most likely explanation for the apparent inactivation of bacteriocin in the presence of fat could be that bacteriocin molecules adsorbed to the fat became undetectable. Fat is an ingredient of sausage batter. Moreover, a common characteristic of bacteriocins by lactic acid bacteria is their hydrophobic nature, which is likely to provoke unspecific binding of the bacteriocin molecules to the hydrophobic surfaces of fat particles (Holzapfel *et al.,* 1995). Likewise, fat is responsible for a large loss of bacteriocin activity in *Lb. sakei* CTC 494 culture supernatant. However, this does not necessarily imply that the bacteriocin adsorbed to the fat particles of a sausage batter will loose its antibacterial activity (Leroy & De-Vuyst, 2005).

In this work, we examined the effects of sodium chloride and sodium nitrite on growth and bacteriocin production by *L. sakei* Lb 706, a potential protective culture for sausage fermentation. Whereas nitrite affected bacteriocin production only slightly because it decreased cell growth, salt had a more drastic effect because it decreased both cell growth and specific bacteriocin production. Addition of salt may be one of the major causes of the reduced efficacy of bacteriocin-producing starter cultures in food environments. Work is in progress to examine the roles of other compounds in sausage batter, such as spices, proteins, and fat particles.

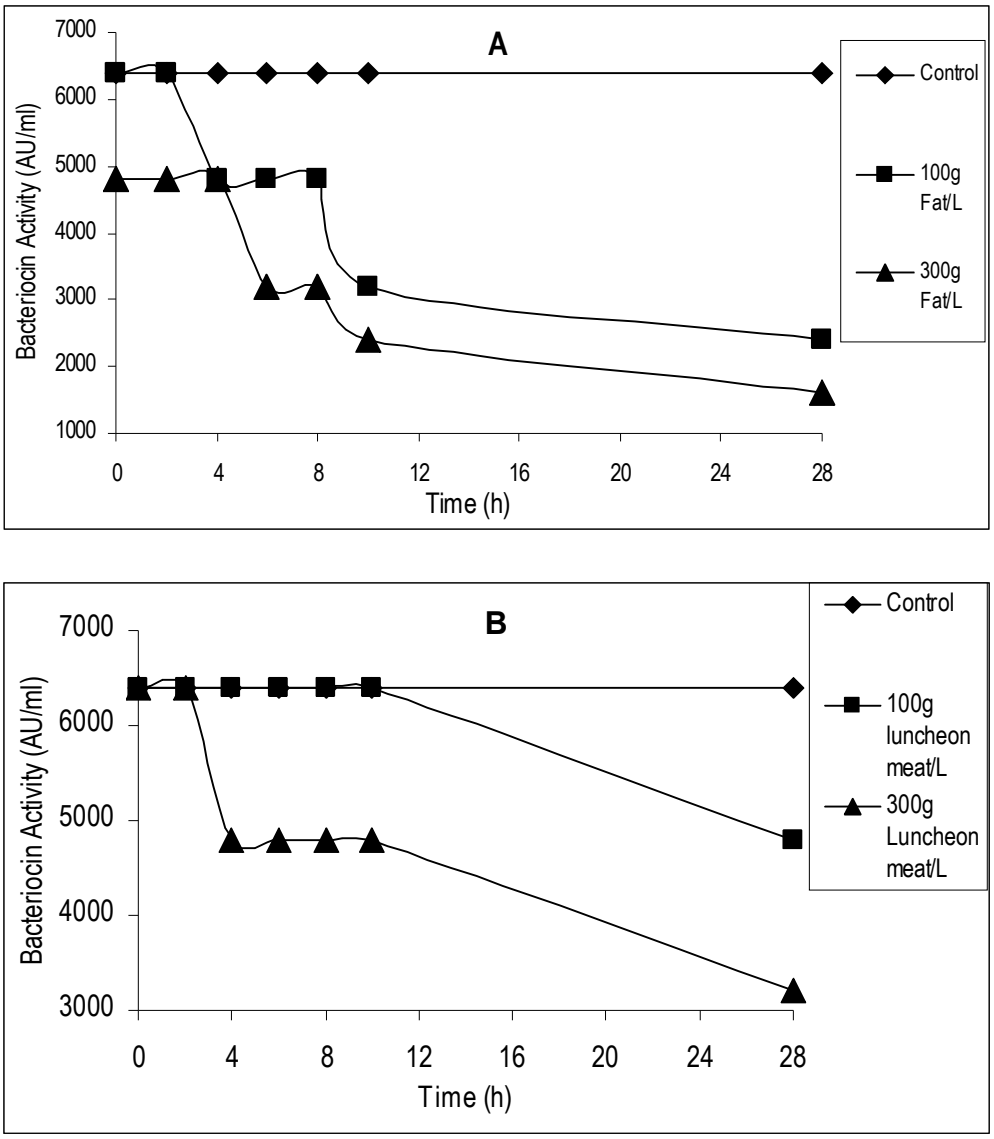



**Figure 5:** Bacteriocin activity in MRS culture supernatant in the presence of 100g (■) or 300g (▲ ) of fat $l^{-1}$ (A) and in the presence of 100g (■) or 300g (▲ ) of luncheon meat $l^{-1}$. (♦) symbol represents control experiments in the absence of fat (A) or luncheon meat (B).

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