

USE OF A BACTERIOCIN-PRODUCER *LACTOBACILLUS SAKEI* FOR FERMENTED SAUSAGES PRODUCTION

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Abstract The aim of this paper was the technological characterization of a *Lactobacillus sakei* strain able to produce the bacteriocin sakacin P. Experiments were conducted *in vitro*, using MRS-based medium, and *in vivo*, when the strain was inoculated as starter culture in real sausage fermentation. The results obtained underlined that the strain was able to grow in conditions that are commonly used in the production line, and only lactose and high concentrations of NaCl (5% w/v) affected the capability for bacteriocin production. When inoculated in sausages, it showed a good performance, being able to colonize rapidly the ecosystem. A high number of isolates, producing sakacin P, were found already from the third day of fermentation, and this number remained stable throughout the fermentation. The strain inoculated affected also the microbial trends, in fact total bacterial count and fecal enterococci showed a rapid decrease at the end of the fermentation.

Introduction In the last decade, a new approach to food stabilization, based on the antagonism displayed by one microorganism towards another, was established linking the lactic acid bacteria (LAB) and protective cultures with biopreservation. According to Stiles (1996), biopreservation refers to extended storage life and enhanced safety of food using their natural or controlled microflora and (or) their antibacterial products.

The microbial interference caused by LAB is due to the production of organic acids and the pH decrease, the competition for nutrients but it also correlates to the production of bacteriocins. Adding a pure culture of the viable bacteriocin-producing LAB to a food commodity represents an example of biopreservation. This practice offers an indirect way to incorporate bacteriocins in meat products and the success of the operation depends on the capability of the added strain to grow and produce the bacteriocin under the fermentation conditions. The production of a certain bacteriocin in laboratory media does not imply its effectiveness in a food system. When evaluating a bacteriocin-producing culture for sausage fermentation or biopreservation, it is important to consider that meat and meat products are complex systems with a number of factors influencing the microbial growth and the metabolite production. Therefore, the influencing of formula and fermentation technology on the performance of bacteriocin-producing strains needs to be tested (Hugas, 1998).

Within the frame of the European project "Safety of traditional fermented sausages: research on protective cultures and bacteriocins", contract n. ICA4-CT-2002-10037, we isolated a strain of *Lb. sakei* that possessed antimicrobial activity towards *Listeria monocytogenes*. It was determined that the bacteriocin produced was sakacin P (Urso *et al.*, 2004). In this paper, the technological characterization of the strain, in connection with its bacteriocin production, was carried out, as well as the evaluation of its ability to conduct sausage fermentations.

Materials and Methods The growth of *Lb. sakei*, and its capability to produce bacteriocin, was tested in different conditions resembling the fermented sausage production line. Temperatures of 10, 14, 18 and 25°C, pH values of 6.0, 5.7 and 5.4, NaCl concentrations of 2, 3.5 and 5% (w/v), glucose concentrations of 0.5, 1.0, 1.5% (w/v), lactose concentrations of 0.25, 0.5 and 1% (w/v) and sucrose concentrations of 0.5, 1.0 and 1.5% (w/v) were selected. Growth was followed by measuring the optical density (OD) of the cultures at 600 nm with the SmartSpec™ 3000 spectrophotometer (Biorad, Milan, Italy), while quantification of the bacteriocin was performed by the critical dilution method, as suggested by Barefoot and Klaenhammer (1983), using as indicator organism *Listeria monocytogenes*, strain NCTC 10527. The experiments were performed twice and samples were collected in duplicates.

Fermented sausages were prepared in a local meat factory using traditional techniques. The 200 kg batch was inoculated with about 7.5×10^5 cells/g. The ingredients were: pork meat (60 kg), lard (40 kg), a mix of sodium chloride (2.5 kg) and black pepper (70 g), sugars (1.5 kg), and nitrite/nitrate (200 ppm). After amalgamation, stuffing of natural casings produced fresh sausages 25 cm long and 5 cm in diameter. The ripening was performed as follows: the first stage consisted of 2 days drying with the relative humidity (RH) of 85% and a temperature of 22°C that was

then decreased to 12°C, with a rate of 2°C per day with an RH between 60 to 90%. The ripening was then carried out for 38 days at 12°C in storerooms with 65-85% RH. No smoke was applied at any stage. The fermented sausages at 0, 3, 5, 7, 14, 21, 30 and 45 days were analyzed. Three samples were collected and used for the analyses.

Potentiometric measurements of pH were made using a pin electrode of a pH-meter (Radiometer Copenhagen pH M82, Cecchinato, Italy). Means and standard deviations were calculated.

The fermented sausages were subjected to microbiological analysis to monitor the dynamic changes in the populations responsible for the ripening of fermented sausages and their hygienic quality. In particular, 25 g of each sample were transferred into a sterile stomacher bag and 225 ml of saline/peptone water (8 g/l NaCl, 1 g/l bacteriological peptone, Oxoid, Milan, Italy) were added and mixed for 1 min and 30 s in a Stomacher machine (PBI, Milan, Italy). Further decimal dilutions were made and the following analyses were carried out on duplicate agar plates:

a) total bacterial count (TBC) on Peptone Agar (8 g/l bacteriological peptone, 15 g/l bacteriological agar, Oxoid) incubated for 48 – 72 h at 30°C; b) LAB on MRS agar (Oxoid) incubated with a double layer at 30°C for 48 h; c) Coagulase negative cocci (CNC) on Mannitol Salt Agar (Oxoid) incubated at 30°C for 48 h; d) total enterobacteria and *Escherichia coli* on Coli-ID medium (Bio Merieux, Rome, Italy) incubated with a double layer at 37°C for 24-48 h; e) fecal enterococci on Kanamycin Aesculin agar (Oxoid) incubated at 42°C for 24 h; f) *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37°C for 24-48 h; g) yeasts and moulds on Malt Extract Agar (Oxoid) supplemented with tetracycline (1 mg/ml, Sigma, Milan, Italy) incubated at 25°C for 48-72h. For *L. monocytogenes*, the ISO/DIS method (1990) was used, while for *Salmonella spp.* the ISO/DIS method (1991) was applied. After counting, means and standard deviations were calculated. At each sampling point, 30 LAB strains, from MRS plates, were randomly selected, streaked on MRS agar and after an overnight at 30°C in MRS broth, stored at -20°C with 30% (v/v) glycerol.

The capability of the isolated strains to produce sakacin P was assessed by both physiological and molecular methods. The agar well diffusion assay (AWDA) using *L. monocytogenes* NCTC 10527 as indicator strain, was performed as described by Schillinger and Lücke, 1987. Moreover, the *sppA* gene, encoding for the SakP protein, was targeted in the isolated strains using specific PCR primers, sakP_F and sakP_R, as previously described by Reminger *et al.* (1996).

Results As reported in the materials and methods, growth was monitored by measuring the OD at 600 nm, considering that a value of 0.5 was equal to about 108 cells/ml, as determined from plate counts (data not shown). The best growth was observed at 25°C, were already at 20h the bacteriocin quantity reached the maximum values measured. The acidification was deep, in fact the strain was able to decrease the pH at values of 4. At 18 and 14°C the cells reached values higher than 10⁸/ml and the acidification was good. Only at 10°C the growth and acidification was not satisfactory, although the bacteriocin production reached levels comparable with the other temperatures tested. Based on the results obtained and considering its use in the production line, the temperature of 18°C was chosen to perform the other characterization experiments.

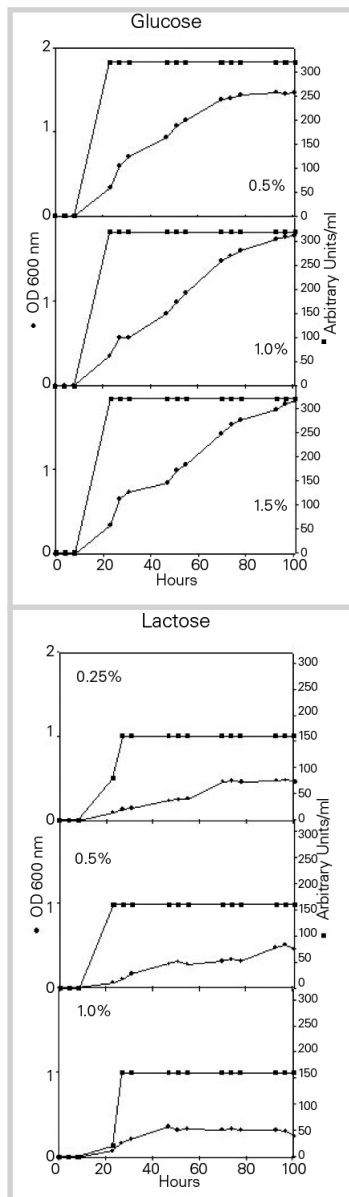
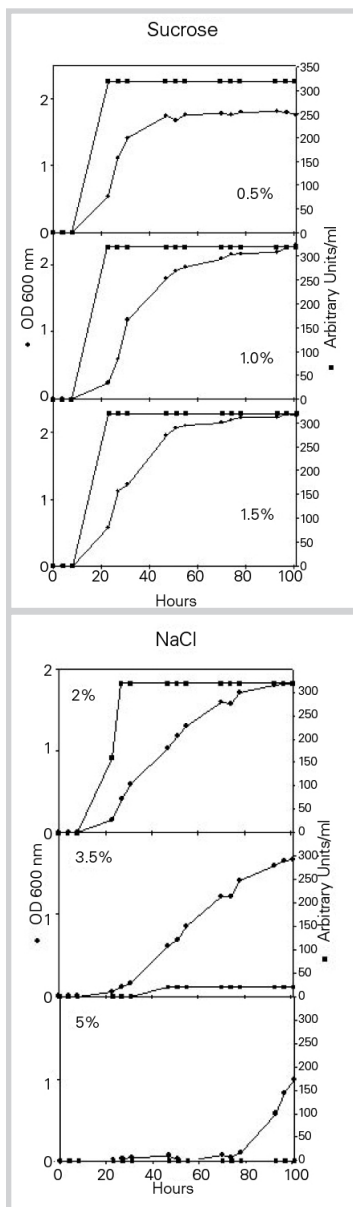


Figure 1: Growth curves of the bacteriocin producing strain in MRS medium with different concentrations of sugars, NaCl and diverse pH. Arbitrary units are reported as well. The results reported are the mean of duplicate samples. (results continue on next page)



5.9 decreased to 5.3. After that time, it started to increase reaching a final value of about 6.

Discussion In the meat fermentations, the use of starter cultures is a commonly used procedure. It assures safety of the product and stability of the aromatic and sensory profile of the sausages produced by a specific industry. Despite these advantages introduced by the use of commercial starters, meat processors, often are not completely satisfied with the starter-mediated fermentations.

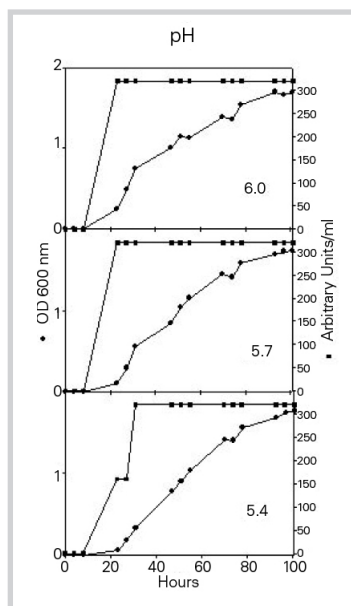
The aim of this paper was to characterize by the technological point of view a "wild" strain of *Lb. sakei*, isolated from naturally fermented sausages, that exhibits antimicrobial activity towards *L. monocytogenes*.

Lb. sakei showed a different growth behavior when differ-

The main parameters affecting the growth and the production of bacteriocins (Figure 1) were determined to be the concentration of NaCl and lactose. As shown, with the increase of the concentration of salt, from 2 to 3.5% already a difference in the behavior of the bacteriocin production was observed.

Lb. sakei was able to grow, but the quantity of bacteriocin was very low. When the concentration of NaCl increased to 5%, the strain showed little growth and no bacteriocin production. Concerning the lactose, in all the conditions tested, it did not support significant growth of the strain, and also the production of bacteriocin was not satisfactory. For the other conditions tested, no significant differences were observed. Different pHs did not affect the growth and the production of the bacteriocin, nor did different concentrations of glucose and sucrose, although sucrose was the sugar that allowed maximum values of OD.

As shown in Figure 2, LAB populations, potentially represented by the inoculated strain, showed a rapid increase in the counts, and already at three days they reached values between 10^8 - 10^9 colony forming unit (cfu)/g, that remained stable throughout the fermentation. Sausages were characterized by a low impact of the CNC, while an increase of the TBC and fecal enterococci was observed within the first 20 days of fermentation. TBC started to decrease after that time, whereas the fecal enterococci remained at high counts until the last day of fermentation where they showed a steep drop from 10^5 to 10^2 cfu/g. Yeasts and *E. coli* showed low counts, and total enterobacteria decrease gradually during fermentation. *S. aureus* was always below 50 cfu/g, as well as the moulds. *Salmonella spp.* were always absent in 25 g of product and *L. monocytogenes* isolated at zero days, was absent in the following stages of fermentation. On the 30 strains isolated at each sampling point at zero days we found a very low number of producing strains, while for the rest of the fermentation this number increased. The same results were obtained when the strains were subjected to sakacin P specific amplification. The pH showed the classical trend typical of the sausage fermentation: within the first 7 days of fermentation a deep acidification was observed. The values from



ent temperatures were tested. Passing from 25 to 10°C a progressive extension of the lag phase was observed, and at 10°C the growth was pretty weak. The same trends, in connection with the increase of the cell numbers, were observed for the acidification.

To carry out the other tests selected for the technological characterization, the temperature of 18°C was selected. The main effect on the growth and bacteriocin production was observed when lactose was added to the medium. No influence was observed for the different values of pH, while a strong impact was determined when using different concentrations of salt.

Finally, the strain characterized was used as a starter culture in real sausage fermentation. It was able to establish itself during fermentation and this is supported by both the LAB counts reached (10^9 cfu/ml) and by the evidence that at each sampling point, for a large number of isolates it was possible to amplify the sakacin P gene by specific PCR. Concerning the influence of the starter used on the microbial ecology of the fermented sausages, as determined by traditional plating, it is important to highlight that especially the counts of TBC and fecal enterococci, showed a significant decrease starting from day 21 and day 30, respectively. Moreover, *L. monocytogenes* detected at 0 days, as reported in the results, was not isolated at later stages. The production of bacteriocin may have a role in the inactivation of *L. monocytogenes* present on the fresh sausages.

Conclusions Selection of strains, autochthonous to fermented meat products, that have evolved in order to perform the fermentation of traditional products can lead to the production of product-specific starter cultures. It can be argued that this procedure is helping in the effort to maintain and preserve the organoleptic characteristics of traditional products and is promoting a standardized production. In this work the technological characterization of such a strain was described. In addition, the potential use of strain as a biopreservation agent, through the production of a bacteriocin, was evaluated.

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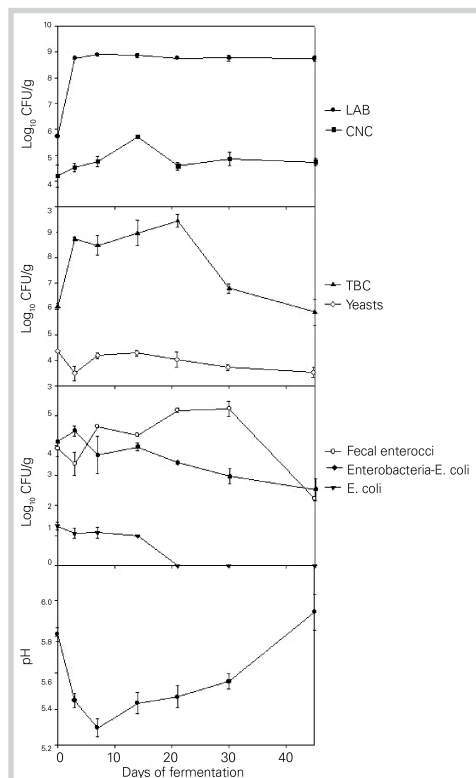


Figure 2: Trends of the populations monitored during the fermentation of the sausages inoculated with *Lb. sakei*. Abbreviations: LAB, lactic acid bacteria; CNC, coagulase negative cocci; TBC, total bacterial count.