SAFETY OF TRADITIONAL FERMENTED SAUSAGES: RESEARCH ON PROTECTIVE CULTURES AND BACTERIOCINS

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Introduction

The main objective of the project was to isolate bacteriocin producing starter cultures from the endogenous lactic acid microflora of naturally fermented sausages and to select protective starter cultures which are active against pathogenic microbes in order to improve product safety and without any adverse effect on the sensorial characteristics of the sausage.

Materials and Methods

Traditional microbiological methods, API 50 CHL, API Staph kits and PCR-based methods were used for identification of isolated strains. AWDA method was applied for screening bacteriocin production and critical dilution method for determination of bacteriocin activity. Indicator strains for testing bacteriocin production were *Listeria monocytogenes* (NCTC 10527), *Staphylococcus aureus* (NCBF 1499) and *Escherichia coli* O157:H7 (NCTC 1207). For challenge tests *Listeria monocytogenes* was inoculated into sausage batter under laboratory conditions. The inoculated sausages were fermented and ripened at factories as usual.

Results and Discussion

For the purposes of the project, local factories were asked to produce a typical traditional sausage without starter cultures. Variations exist in the composition of the mix, the dimensions, as well as the fermentation-ripening process and time. The sausages were considered ready for consumption on the 28th day. Physicochemical and microbiological analyses of the raw materials, intermediate and final products (days 0, 2, 4, 7, 14 and 28) were carried out, in order to determine the hygienic parameters and the changes occurring as a result of the fermentation and ripening. The most important result, from the point of view of product safety, is that no *Listeria monocytogenes*, *Salmonella* or *Staphylococcus aureus* were detected in the final product in any of the countries. Enterobacteria, *Escherichia coli* and *Pseudomonas*, when present, were progressively eliminated regardless of their initial population. As expected, the main populations detected throughout the experiment were Lactic Acid Bacteria (LAB) and Micrococci. The physicochemical changes can be summarized as follows: decrease of the pH, decrease of a_w, decrease of nitrates and nitrites and increase of NaCl. Finally, sensorial analysis of the final products resulted in an overall acceptability of the product above 70% for all partners except for Croatia, possibly due to the extension of the regular ripening time for project harmonization purposes.

One hundred and fifty strains of LAB and 150 strains of catalase-positive cocci from the sausage fermentations were isolated in each country. These strains were characterized biochemically (API Systems) and genetically (sequencing of partial 16S rDNA). It was shown that the API system is insufficient for the identification of LAB, while it gives better results for staphylococci (Gasparik-Reichardt *et al.*, 2004). The main population of catalase-positive cocci belonged to the *Staphylococcus xylosus* and *S. saprophyticus* species and the main populations of LAB belonged to the *Lactobacillus sakei* and the *L. curvatus* species. The screening of all the isolated strains for the production of bacteriocins resulted in only 6 strains of *Lactobacillus sakei* (isolated by the Italian partners and designated as I151, I152, I153, I154, I155 and I156) and one strain of *Leuconoctoc mesenteroides* (isolated by the Greek partners and designated as E131), showing antibacterial activity towards *L. monocytogenes* only and not against *S. aureus* or *E. coli*. Based on the technological characteristics as well as the ability for bacteriocin production, three of the six *L. sakei* strains (I151, I154 and I155) were suggested as potential bio-protective cultures. *Lc. mesenteroides* E131, as an obligate heterofermentative strain produces carbon dioxide and slime, which are undesirable during the fermentation of sausages. Subsequently, it could be used only for the production of bacteriocin.

RAPD-PCR using three different primers was performed for the *L. sakei* strains and the results showed a coefficient of similarity higher than 86% suggesting that the strains could be considered as the same from a genetic point of view (Rantsiou *et al.*, 2005). The identification and the sequencing of the genes responsible for the bacteriocin production revealed that *L. sakei* I151 was producing sakacin P, since at both the DNA and protein levels, a 100% homology to the

sppA gene and the SakP protein was observed, while *Lc. mesenteroides* E131 showed 100% similarity at both DNA and protein level to the *mesY* gene and MesY protein, respectively, thereby revealing the production of mesenterocin Y.

An ammonium precipitation protocol was developed for the production of semi-purified bacteriocins from *Lc. mesenteroides* E131 and *L. sakei* I154 (Drosinos *et al.*, 2005). Purified bacteriocin of *L. sakei* I154 was stable after 1 h at 40-100 °C, but after 15 min of heat treatment, it retained only 40% of the initial activity and was completely inactivated after 30 min. It retained activity for only two weeks at the temperature of -20 °C, while the activity was retained only 1 week at 30°C. Purified bacteriocin of *Lc. mesenteroides* E131 lost 40% of activity after incubation at high temperatures (90 and 100°C). Storage at temperatures ≤4°C did not affect the activity even after 4 weeks. At 30°C, however, activity was decreased after 2 weeks. The addition of Tween 80 had no effect on the activity of *Lc. mesenteroides* E131, while on the activity of *L. sakei* I154 it had a beneficial effect. At 30 °C, however, it retained activity for 3 weeks (20% of activity was retained).

The laboratory experiments to determine the optimal conditions (pH and temperature) for the highest antimicrobial activity against *L. monocytogenes* have shown that: the bacteriocins have greater effect at lower temperatures and pH values, the highest antilisterial activity was detected with bacteriocin of *Lc. mesenteroides* at pH 5.0 at all (12, 18 and 24 °C) temperatures. In spite of the inhibition or reduction of *L. monocytogenes*, the applied treatments did not kill the bacteria totally.

Using the protective cultures (*L. sakei* I151, I154 and I155) and semi purified bacteriocins, reduction of *L. monocytogenes* was measured in challenge tests during the production of fermented sausages (Caklovica *et al.*, 2005). The best results were obtained by *L. sakei* I151 and the bacteriocin of *Lc. mesenteroides* E131. The *in situ* validation of the selected culture and bacteriocins, suggests that *L. sakei* I151 and *Lc. mesenteroides* E131 bacteriocin are effective against *L. monocytogenes* and could be used as protective starter culture and bioprotective agent, respectively, in the production of naturally fermented sausages.

The evaluation of the effect of the addition of the selected protective culture or the bacteriocin, alone or in combination, on the technological and traditional quality characteristics of the products, was checked in a pilot production. The results of both microbiological and physicochemical analyses, showed a similar trend to that of the naturally fermented sausages with no additions. Generally, no essential differences in the organoleptic parameters among the samples in all fermentations were observed. All the samples showed acceptable flavour and taste without off-odours, but in some cases (Serbia, Croatia), the inoculated samples were graded better than the control. In order to determine the critical technological parameters, industrial scale production of sausages with the addition of *L. sakei* I151 as starter/protective culture and the bacteriocin of *Lc. mesenteroides* E131 as protective agent, was undertalen. The production of sliced and packaged products under modified atmosphere or vacuum, was studied in order to evaluate the influence of packaging on the microbial flora. It was revealed that both MAP and vacuum-packaging may be considered as effective packaging techniques with regard to the preservation of desirable traditional technological, sensorial and safety properties of the final product. Standard Operating Procedures (SOPs) were developed for the industrial scale production in each country.

Conclusions

The innovative features of the project are as follows: isolation of new protective cultures, purification of bacteriocins and their experimental implementation in fermented sausages, genetic characterization of genes and SOP's development for each country.

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