COMPARISON OF MICROFLORA OF TRADITIONAL FERMENTED SAUSAGES IDENTIFIED WITH TRADITIONAL AND MOLECULAR METHODS

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Background

Fermentation and drying of meat products are probably the most ancient ways of preservation. Fermentation of sausages in a traditional way has got a long history in the European countries. In view of the need to maintain the traditional sensorial quality of dry sausages and assure its safety, it is important to improve the industrial production of fermented sausages on the basis of development of protective cultures with acceptable technological and sensorial characteristics**,** after selection of strains isolated from naturally fermented products.

Key words: fermented sausage, lactic acid bacteria, Micrococci, identification, bacteriocin

Objectives

The objective of the investigation was the isolation and identification of microflora of naturally fermented sausages in different countries, with traditional and molecular methods (Bosnia-Herzegovina, Croatia, Greece, Italy, Hungary and Serbia-Montenegro) and the selection of lactic acid bacteria**,** which will not have negative effect on the sensorial characteristics and can produce bacteriocins which have antimicrobial effect against pathogenic microbes.

Materials and methods

Sausage preparation: Sausages were manufactured according to each country's standard practice without commercial starter cultures. Three batches of sausages were prepared for the experiments. Samples were taken from each batch for chemical and microbiological analysis at 0, 2, 4, 7, 14, 21and 28 days after formulation.

Isolation and characterization of lactic acid bacteria: A total of 150 (50 per batch) isolates were collected from MRS agar plate. Half of the colonies were isolated from ripening days 0 to 7 and the rest from day 14th to the end. The isolates were tested for cell morphology by phase contrast microscopy, Gram reaction, and catalase formation. Gram-positive and catalase-negative strains were subjected into the following physiological and biochemical tests: gas (CO_2) formation from glucose, arginine hydrolysis, growth in 8 and 10 % NaCl, growth at 4, 10, 15, 37 and 45 ^{o}C, slime formation, hydrolysis of arginine, ammonia, gas and slime formation. Sugar fermentation pattern was determined by the API 50 CHL (BioMerieux) and the identification was performed by the computer program APILAB Plus.

Isolation and characterization of catalase-positive cocci: A total of 150 (50 per batch) isolates were collected from MSA agar plate. The isolates were rapidly checked for cell morphology by phase contrast microscopy, Gram reaction and catalase production to ensure their classification to family *Micrococcaceae*. The isolates were subjected to the following tests: sensitivity to novobiocin, production of ammonia from urea, coagulase production, β-galactosidase activity, oxidase reaction, nitrate reduction and acetoin formation. Micrococci were separated from staphylococci on the basis of fermentation of glucose and growth in the presence of erythromycin and lysozyme. The strains were tested with the API Staph (BioMerieux) and the identification was performed by the computer program APILAB Plus.

Molecular identification of the isolated strains using PCR-based methods: DNA extraction*:* Total DNA was extracted using the method of Daud Khaled et al. (1997) with a modification or by the method described by Andrighetto et al. (2001). RAPD-PCR and electrophoresis of RAPD-PCR products were carried out with the oligonucleotide primer M13 (Andrigetto et al., 2001). 16S rDNA amplification and sequencing**:** After grouping of the strains with RAPD-PCR, one representative strain of each group was selected for

identification by 16S rDNA gene sequencing. The primers used for 16S rDNA amplification and sequencing were P1V1 and P4V3 (Klijn et al., 1991).
Antibacterial activity and assay of the isolated lactic acid bacteria: The antibacterial activity of the isolated lactic acid bacteria was assayed by the agar well diffusion assay (AWDA) described by Schillinger and Lücke (1989). The isolated strains were screened against three pathogens, such as *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli*, in order to evaluate the potential use of the isolates as protective cultures.

Results and discussion

The results of the microbiological analysis showed that the number of the lactic acid bacteria was generally lower than total viable count in the first days of ripening in all sausages until the 4th day of fermentation. The population of the lactic acid bacteria (LAB) increased rapidly everywhere to about log 8 CFU/g and became the dominant flora of the sausages from this day in all batches in all countries and stayed constant during ripening. The adaptation of lactic acid bacteria to meat environment is well known and this feature made their growth faster. The ripening parameters were different and these are reflected in the composition of LAB. Based on the identification with API 50 CHL and traditional identification keys**,** the dominant microbes were identified as *L. plantarum* in three countries (Bosnia-Herzegovina, Croatia and Greece)*, Lc. lactis* subsp. *lactis* in Italy*, L. sakei* in Hungary and *L. fermentum* in Serbia-Montenegro. Dominant strains were also *L. pentosus* and *L. curvatus* as well as *Leu. mesenteroides* strains. The results of the genetic identification are in a good agreement with the results of other workers. According to the genetic identification**,** the predominant species were *L. sakei, L. plantarum* and *L. curvatus*.
Comparing the results obtained with API 50 CHL and molecular methods**,** it is clear that there were differences between the two identifications (see Table 1 and 3). The database of API 50 CHL identification programme did not contain *L. sakei* strain which has importance in testing of meat industrial strains and was the dominant flora in Italian, Hungarian, Bosnian and Serbian strains by PCR-based methods and second among Greek strains. This strain could be identified only with conventional identification keys and molecular methods. Our results confirm that the API 50 CHL method is not very convenient and may be misleading in the identification of lactic acid bacteria. Molecular genetic techniques showed good reproducibility and gave good results in case of doubtful cases, too**.**
Micrococci population was different among the countries. For example, in the Hungarian sausages the number was very low from the first days (log 2CFU/g) and did not increase further in any of the batches, in fact in batches 1 and 3 it were actually eliminated at the end of ripening process. The average initial micrococci population, was higher in Croatian (log 3 CFU/g), Greek, Bosnian, Italian and Serbian (about log 4 CFU/g) sausages. The tendency was increasing only in Italy, were the final number of population was higher in the end of the ripening process (log 5-6 CFU/g). When comparing staphylococci the dominant strains were *St. xylosus* and *St. saprophyticus* (in 3-3 countries) followed by *St. simulans*, *St. hominis* and *St. capitis*. The API Staph identification gave good results in every country for identification of staphylococci strains; in some cases micrococci needed additional tests.
The bacteriocin producing ability of the isolated strains of all counties were tested against *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* but only 7 strain showed antilisterial activity (Table 4). These strains will be further tested as protective cultures in challenge tests to investigate their inhibition against *Listeria monocytogenes* in sausage models, too.

Conclusions

Based on the API tests, conventional identification keys and molecular methods the dominant *Lactobacillus* and *Staphylococcus* strains isolated from fermented sausages from different countries, the isolated strains were determined and compared. The bacteriocin producing ability of the isolated strains were tested against *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* but only 7 strain showed antilisterial activity. Further investigations are needed to test the inhibition in sausage models.

References

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Table 1. Comparison of the lactic acid bacteria (%) isolated from naturally fermented sausages of different countries and identified with API 50 CHL kit

Bosnia-Herzegovina	Croatia	Greece	Italy	Hungary	Serbia-Montenegro
L. plantarum 40.7 *L. pentosus* 18 *L. curvatus* 16.7 *L. sakei* 8.7 *L. brevis* 7.3 *P. pentosaceus* 4.7 *Leu. lactis* 3.3. *L. salivarius* 0.6	*L. plantarum* (1*) 34 *L. brevis* 20.7 *L. curvatus* 18 *L. pentosus* 6.7 *L. plantarum* (2*) 5.3 *L. fermentum* 4 *P. pentosaceus* 3.3 *Lc. lactis subsp. lactis* 2 *Leu. mesenteroides* subsp. *mesenteroides* 2	L. *plantarum* 43.3 *L. curvatus* 10.7 *L. pentosus* 10.7 *L. brevis* 8.7 *Lc. lactis subsp. lactis* 6.7 *Leu. mesenteroides* subsp. *mesenteroides* 5.3 *L. rhamnosus* 3.3 *L. sakei* 4 *L. lactis* 4 *L. rhamnosus 3.3* *L. paracasei subsp. paracasei* 1.3 *L. salivarius* 0.7 *E. faecium* 0.7	*Lc. lactis* subsp. *lactis* 26 *L. fermentum 14* *L. plantarum* 11.3 *L. curvatus* 8 *L. brevis* 6 *L. mesenteroides* 5.3 *L. paracasei* 4 *P. pentosaceus* 2.7 *L. mesenteroides/ dextrinicus* 2 *L. lactis* 1.3 *L. acidophilus* 0.7 *P. acidilactici* 0.7 *L. cellobiosus* 0.7 Not identified *18*	*L. sakei* 28.7 *Leu. mesenteroides* ssp. *mesenteroides* 6.7 *Leu. mesenteroides dextranicum* 4.7 *L. sanfrancisco* 4.7 *L. plantarum* 3.4 *L. curvatus* 3.4 *L. delbrueckii* 3.4 *L. alimentarius 3.4* *L. amylophilus* 2.7 *L. bavaricus* 2 *W. viridescens* 2 *L. confosus* 1.4 *L. salivarius* 0.7 *L. acidophilus* 0.7 *L. maltoromicus* 0.7 *L. yamanashiensis* 0.7 *L. halotolerans* 0.7 *L. fructivorans* 0.7 *Leu. citreum* 0.7 *Leu. oenos* 0.7 17 % was unidentified	*L. fermentum* 24 *Leu. mesenteroides* ssp. *mesenteroides* 12.6 *L. brevis* 9.3 *L. delbrueckii* ssp. *delbrueckii* 9.3 *L. curvatus* 7.3 *S. faecalis* 6.6 *Lc. lactis* ssp. *lactis* 6.6 *L. plantarum* 6 *L. cellobiosus* 4.6 *L. collinoides* 4.6 *L. delbrueckii* ssp. *bulgaricus* 2.6 *Leu. mesenteroides* ssp. *mesenteroides* 2.6 *S. faecium* 2 *L. acidophilus* 0.6 *L. paracasei* ssp. *paracasei* 0.6

L. : Lactobacillus, Lc. : Lactococus , Leu. : Leuconostoc, E. : Enterococcus, P. : Pediococcus S. :Streptococcus and in Table 2 St.: Staphylococcus and * means serotype

Table 2. Comparison of the *staphylococci* and *micrococci* (%) isolated from naturally fermented sausages of different countries and identified with API Staph kit

Bosnia-Hercegovina	Croatia	Greece	Italy	Hungary	Serbia-Montenegro
St. saprophyticus 30.7 *St. simulans* 22 *St. xylosus* 16 *St. epidermidis* 10.6 *St. caprae* 10.6 *St. capitis* 6 *St.aureus /intermedius* 2.7 *St. auricularis* 0.7 *St. sciuri* 0.7	*St.xylosus* 29.2 *St. capitis* 25 *St. carnosus* 25 *St. saprophyticus* 20.8	*St. saprophyticus* 34.7 *St. xylosus* 14.7 *St. simulans* 11.3 *St. haemolyticus* 11.3 *St. haemolyticus* 11.3 *St. caprae* 8 *St. capitis* 6 *St. aureus/intermedius* 5.3 *St. sciuri* 3.3 *St. hominis* 2 *St. auricularis* 0.7 *St. warneri* 0.7 *St. cohnii subsp. cohnii* 0.7 *St. cohnii subsp. urealyticum* 0.7 *St. epidermidis* 0.7	*St. xylosus* 74 *St. hominis* 10 *St.warneri* 8 *St. saprophyticus* 3.3 *St. lentus* 2 *St. apidermidis* 1.3 *St. simulans* 0.7 Not identified 0.7	*St. xylosus* 43 *Micrococcus* spp. 16 *St. hominis* 15 *St. lentus* 10 *St. warneri* 6 *St. capitis* 4 *St. epidermidis* 2 *St. haemoliticus* 1 *St. auricularis* 1 *St saprophyticus* 1 *St. cohnii* 1	*S. saprophyticus* 21.1 *St. simulans* 14.4 *St. xylosus* 21 *St. auricularis* 12.2 *St. warneri* 6.7 *St. aureus* 6.7 *St. hominis* 4.4 *St. cohnii* 1.1 *M. varians* 35 *M. nishinomiyaensis* 23.3 *M. lylae* 10 *M. luteus* 6.7 *M. roseus* 10 *Micrococccus* spp. 15

Table 3. Comparison of the lactic acid bacteria (%) isolated from naturally fermented sausages of different countries and identified with PCR method

Bosnia-Herzegovina	Croatia	Greece	Italy	Hungary	Serbia-Montenegro
L. sakei 39.3 *L. curvatus* *L. plantarum* 16.7 *L. brevis* 7.3 *P. acidilactici* 6 *P. pentosaceus* 2.7 *L. alimentarius* 2.7 *Lb. Farciminis* 1.3	*L. plantarum* (1) 51.3 *L. curvatus* 21.3 *L. plantarum* (2) 6.6 *L. brevis* 16 *L. fermentum* 6 *L. pentosus* 4 *Pediococcus pentosaceus* 2 *Lc. lactis* subsp. *lactis* 2	*L. curvatus* 43.3 *L. sakei* 23.3 *L. plantarum* 18 *L. paraplantarum* 4 *E. faecium/durans* 3.3 *L. casei/paracasei* 2.7 *L. farciminis* 2 *Leu. mesenteroides* 2 *L. alimentarius* 1.3	*L. sakei* 42.7 *L. curvatus* 36 *L. plantarum* 6 *L. paraplantarum* 4.7 *L. paraplantarum/ pentosus* 2.7 *Leu. mesenteroides* 2.7 *W. paramesenteroides/ hellenica* 2.7 *Leu. citreum* 0.7 *L. brevis* 0.7 *Lc. lactis subsp. lactis* 0.7 *Enterococcus pseudoavium* 0.7	*L. sakei* 64 *L. curvatus* 7.3 *Leu. paramesenteroides/ W. hellenica* 6.7 *W. viridescens* 6 *Leu. mesenteroides* 4.7 *Ln. kimchii* 2.7 *L. plantarum* 2 *L. plantarum/ paraplantarum* 2 *Leu. citreum* 2 *E. infantar* 0.7 *Enterococcus spp.* 0.7 *St.saprophyticus* 0.7	*L.sakei* 52.7 *L. curvatus ssp. curvatus* 16.7 *L. brevis* 9.3 *L. plantarum/ paraplantarum* 6 *L. parakasei ssp. parakasei* 5.3 *E. fecium/durans* 4.6 *L. johansoni* 2.7 *L. casei MCRF* 2.7

Table 4. Number of isolated LAB which were active against the indicator strains

Indicator microbes	Bosnia-Hercegovina	Croatia	Greece	Italy	Hungary	Serbia-Montenegro
Listeria monocytogenes	0/150 LAB	0/150 LAB	**1/150 LAB**	**6/150 LAB**	0/150 LAB	0/150 LAB
Staphylococcus aureus	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB
Escerichia coli	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB	0/150 LAB

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