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Inhibition of *Listeria monocytogenes* growth in dry fermented sausages

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ABSTRACT

The growth potential of *Listeria monocytogenes* during the ripening period of dry fermented sausages was investigated. In addition, antilisterial activity of isolated lactic acid bacteria was tested. The initial *Listeria* count in sausage mixture was 10^5 CFU g^{-1}. Samples were taken on day 0, 3, 7, 14 and 28 days after formulation, and total viable count, lactic acid bacteria count, *L. monocytogenes* count and pH values were determined. The growth of *Listeria* was inhibited until day 14, while in the final products it was beneath detection limit (2 $\log_{10}$), indicating the effectiveness of fermentation, ripening and drying in reducing the pathogen population. A total of 32 lactic acid bacteria were isolated and determined, and isolates of *Leuconostoc mesenteroides* showed inhibitory effect towards *Listeria* due to the bacteriocin production.

Key words: dry fermented sausages*, L. monocytogenes,* bacteriocins

Introduction

Food is an important vehicle in epidemiology of human listeriosis. *L. monocytogenes* has been frequently isolated from various types of foods: vegetables (SCHLECH et al., 1983), milk and milk products (LONCAREVIC et al., 1995; KOZAČINSKI and HADŽIOSMANOVIĆ, 2001), fish (LONCAREVIC et al., 1996), meat and meat products (ŽIVKOVIĆ et al., 1998). Despite the hurdles such as low pH, low water activity or high salt content, *Listeria* could survive and even grow in fermented sausages (COLAK et al., 2007). The fate of the pathogen during the ripening of dry fermented sausages is conditioned by a number of different

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factors such as initial contamination, strain adaptation to meat matrix, fermentation process, microclimatic conditions etc. In addition, biochemical profile of predominant lactic acid bacteria play an important role in inhibiting *Listeria* due to the production of metabolites like organic acids or bacteriocins (ZDOLEC et al., 2005a). Bacteriocinogenic lactic acid bacteria are implemented in the production of dry sausages production as a part of the hurdle concept in controlling foodborne listeriosis (HADŽIOSMANOVIĆ et al., 2005; ČAKLOVICA et al., 2005; ZDOLEC et al., 2005b).

With regard to the above mentioned, the aim of our investigation was to monitor changes in *L. monocytogenes* population inoculated into naturally fermented sausages, in order to evaluate the safety of a product contaminated with high number of pathogens. The additional goal of this study was to test the antimicrobial capacity of isolated lactic acid bacteria, in other words the production of bacteriocin active towards *L. monocytogenes.*

Materials and methods

Preparation of inoculum. Listeria monocytogenes NCTC 10527 was obtained from the University of Udine, Food Science Department, Udine, Italy. The pathogen was stored in Brain Hearth Infusion (BHI, BioMerieux, Marcy l`Etoile, France) with 40% glycerol at -20 °C. Before use the culture was subcultivated twice in BHI broth and purified on Oxford agar (BioMerieux). One colony was inoculated into 2 mL BHI and incubated at 37 °C for 24 hours. The culture was then transferred to 10 mL BHI and incubated under the same conditions. Finally, 10 mL of active culture was transferred to 1 liter of broth and incubated again. Next, 10 mL of culture was centrifuged for 5 minutes at 2,000 rpm. Cells were resuspended in 10 mL of sterile saline water, and the cell number was determined on Palcam agar. The suspension was inoculated in sausage mixture the same day it was prepared.

Sausage production. Sausages were manufactured following the standard operative procedure. The sausages had the following composition: pork meat, 62%; beef meat, 10%; pork back fat, 25%; nitrite salt, 2.5%; sugar, 0.3%; spices, 3.0%. The mixture was inoculated with *L. monocytogenes* (10^5 CFU g^{-1} in the final mixture). After filling, sausages were allowed to equilibrate at room temperature (20 °C) and relative humidity of (RH) 95% for 12 h. The sausages were then smoked at 20 ºC, RH 85-90% for 2 days and then hanged in a fermentation chamber till day 28. During this period, the temperature and RH were gradually reduced from 20 °C and 90% to 16-18 °C and 75%.

Sampling schedule and analyses. Three batches of sausages were used for the experiment. Samples were taken on day 0, 3, 7, 14 and 28 days after formulation, transported to laboratory (4 °C) and subjected to microbiological analysis and pH measurement. Each analysis was done in triplicate. For microbiological analysis, the sausage casings were

removed aseptically; 25 g sample was added to 225 mL of sterile saline peptone water and homogenized in a stomacher for 2 min (Stomacher 400 Circulator, Seward, UK). Serial decimal dilutions were prepared and 1 mL or 0.1 mL samples of appropriate dilutions were poured or spread on the non-selective and selective agar plates. Total viable count was determined on the Plate Count Agar (PCA, Biomerieux) and incubated at 30 ºC for 72 hours; Lactic Acid Bacteria (LAB) on the MRS agar (Biomerieux) overlaid with 5 mL of the same medium incubated at 30 °C for 48 hours, and *L. monocytogenes* on the Palcam agar (Merck, Darmstadt, Germany) incubated at 37 °C for 48 hours. The colonies from Palcam were determined using API Listeria kit (Biomerieux). The pH was measured by mixing 10 g of sample with 90 mL of distilled water (pH = 7.0) by means of digital pH-meter 330/SET-1 (WTW, Germany).

Antilisterial activity of LAB isolates. A total of 32 LAB isolates were collected from one representative high dilution MRS agar plate at every sampling day. The isolates were tested for cell morphology, Gram staining and catalase reaction. All isolates were grown in MRS broth, purified by streaking on MRS agar and maintained in MRS broth plus 30% glycerol at -20 ºC. Sugar fermentation pattern was determined by the API 50 CHL (Biomerieux) and the identification was performed by the APILAB Plus computer program. The isolates were tested for the production of antimicrobial substances (bacteriocins) by the agar well diffusion assay (AWDA; according to SCHILLINGER and LÜCKE, 1989). Strains were grown in MRS broth at 30 °C for 24 hours. Cell-free solutions were obtained by centrifuging the culture (10,000 g, 15 min, 4 ºC). In order to exclude antimicrobial effect of organic acids, pH of supernatants was adjusted to 6.5 by means of NaOH 1 M. Supernatants were also treated with protease (proteinase K; Takara, Bio Inc, Japan) in order to evaluate the proteinaceous nature of the antimicrobial compound. Before performing AWDA, protease treated supernatants were incubated at 37 ºC for 1 hour. The BHI agar plates were overlaid with soft BHI agar (0.7%) inoculated with overnight culture of *L. monocytogenes.* The wells were cut into agar plates, and 50 µl of the culture supernatants were placed into each well. The plates were incubated anaerobically (30 ºC, 24 h) in order to minimize the formation of hydrogen peroxide and examined for inhibition zones. Samples with appearance of inhibition zone using untreated and neutralized supernatants, and absence of inhibition with protease treated supernatants, were considered to be positive for production of antilisterial bacteriocins.

Results and discussion

Microbial succession during the ripening process. As indicated in Table 1, initial lactic acid bacteria count (4 $\log_{10}$ CFU g^{-1}) increased rapidly during first 3 days of fermentation and remained constant towards the end of ripening (>8 $\log_{10}$ CFU g^{-1}), slightly overcome the total viable count. The increase of LAB population was accompanied with pH drop

until day 7 (Table 2). In the second part of ripening, pH slightly increased toward the end of process, and reached the final values between 5.29 and 5.35.

Table 1. Microbial growth* (log_{10} CFU g^{-1} ± SD) during the ripening of dry fermented sausages

Total viable count	Sampling day				
	0	3	7	14	28
Batch 1	6.32 ± 0.10	8.58 ± 0.12	8.32 ± 0.16	8.72 ± 0.12	8.65 ± 0.14
Batch 2	6.23 ± 0.11	8.32 ± 0.10	8.62 ± 0.18	8.61 ± 0.22	8.43 ± 0.12
Batch 3	5.89 ± 0.12	7.30 ± 0.14	8.50 ± 0.14	8.25 ± 0.18	8.20 ± 0.10
Lactic acid bacteria					
Batch 1	4.23 ± 0.12	8.23 ± 0.16	8.43 ± 0.07	8.67 ± 0.11	8.50 ± 0.18
Batch 2	3.83 ± 0.12	7.91 ± 0.21	8.54 ± 0.10	8.77 ± 0.14	8.62 ± 0.15
Batch 3	4.39 ± 0.16	8.27 ± 0.12	8.50 ± 0.16	8.55 ± 0.10	8.51 ± 0.13
L. monocytogenes					
Batch 1	4.97 ± 0.15	5.18 ± 0.07	4.90 ± 0.09	5.40 ± 0.14	<2
Batch 2	5.07 ± 0.17	5.89 ± 0.19	4.66 ± 0.17	4.25 ± 0.12	<2
Batch 3	4.79 ± 0.17	5.04 ± 0.10	4.74 ± 0.12	4.30 ± 0.19	<2

*Each number is the mean of three sausage samples taken from the same batch

Table 2. Changes in pH (mean value ± SD) during the ripening of dry fermented sausages

Batch N°	Sampling day				
	0	3	7	14	28
1	5.80 ± 0.04	5.06 ± 0.02	5.06 ± 0.04	5.14 ± 0.02	5.31 ± 0.01
2	5.75 ± 0.03	5.23 ± 0.03	5.17 ± 0.04	5.24 ± 0.02	5.35 ± 0.01
3	5.81 ± 0.03	5.26 ± 0.01	5.18 ± 0.01	5.27 ± 0.07	5.29 ± 0.07

*Each number is the mean of three sausage samples taken from the same batch

The initial *L. monocytogenes* count in the mixture (5 log_{10} CFU g^{-1}) didn't increase evidently during the manufacturing process. Slight increase in the pathogen number was noticed in all three fermentations in the beginning of fermentation (0.21-0.82 log_{10} increase, batch 1 and 2, respectively), and after that started to decrease slowly until day 14 (0.74 - 1.64 log_{10} decrease batch 3 and 2, respectively). In batch 1, a decrease of *L. monocytogenes* count was observed only between 3rd and 7th day (0.28 log_{10} decrease), and again increased toward the day 14 (0.50 log_{10} increase). However, it can be said that

Listeria was inhibited and did not exceed the normal growth in first part of ripening, probably due to the decrease of pH and antimicrobial mechanisms of the competitive microbial flora. In addition, *Listeria* was significantly reduced in the second part of ripening (<2 $\log_{10}$ CFU g^{-1} in final products).

The outgrowth and survival of *Listeria* after inoculation in various kinds of fermented sausages is well documented (SCHILLINGER et al., 1991). Many factors influence the ability of *L. monocytogenes* to survive in fermented matrices, e.g. the initial number of pathogen, pH, salt and water content, microclimate conditions of ripening, competitiveness of natural microbial flora (LÜCKE, 2000). ADAMS and NICOLAIDES (1997) and LÜCKE (2000) reported that lowering the pH is one of the most important factors of sausage ripening and destroying of undesirable microbial flora. In our study the lowest pH value was 5.06 (day 3) which isn't the guarantee of microbiological quality, since *Listeria* is able to survive even in more acid media (SEELIGER and JONES, 1986). Additional hurdle in the growth inhibition of *Listeria* are certainly antimicrobial compounds (bacteriocins) of the autochthonous microbial flora (LAB), the production and activity of which are at their highest during the exponential phase of bacterial growth (COCOLIN et al., 2005). On the other hand, the effectiveness of bacteriocins in foods can be limited by a range of factors, such as a limited diffusion in solid matrices, binding to lipids, inhibition through natural proteolitic enzymes and other inhibitors (ZDOLEC et al., 2005a). However, in our study, despite the high level of contamination which isn't usual in a regular dry sausage production, *L. monocytogenes* was efficiently reduced as a result of common protective factors; fermentation, drying and antimicrobial mechanisms.

Antilisterial capacity of LAB isolates. Biochemical identification of 32 LAB isolates showed that 17 isolates (53.13%) belonged to the genus *Leuconostoc* and 15 isolates (46.87%) to the genus *Lactobacillus.* According to API identification system, Leuconostocs were identified as *Leuconostoc mesenteroides*, while 10 isolates (66.66%) of lactobacilli were identified as *Lactobacillus curvatus*, and 5 isolates (33.33%) as *Lactobacillus brevis.* The most common isolated LAB in fermented sausages are lactobacilli: *L. sakei*, *L. plantarum* and *L. curvatus* (HAMMES, 1990). In addition to the technologically desirable homo-fermentative lactobacilli, the presence of hetero-fermentative LAB in fermented sausages has been also reported (GASPARIK-REICHARDT et al., 2005). In our experiment hetero-fermentative *L. brevis* and *L. mesenteroides* were isolated in high content, indicating possible appearance of sensorily undesirable compounds in dry sausages (carbon dioxide).

Using the Agar Well Diffusion Assay (AWDA), inhibition of *Listeria* growth was found by *L. mesenteroides* strains. *L. monocytogenes* was inhibited with untreated and neutralized supernatant, while the inhibition was absent indicating the proteinaceus structure of inhibitor. A relatively high content of bacteriocinogenic *Ln. mesenteroides*

in total population of LAB isolates could be the indicator of additional protective role of indigenous microbial flora against the inoculated pathogen. The antilisterial activity of *Leuconostocs* species was also noted in several studies (HARRIS et al., 1989; HARDING and SHAW, 1990; HÉCHARD et al., 1992; MATARAGAS et al., 2003).

In conclusion, the implementation of bacteriocin produced by *Ln. mesenteroides* in dry sausages and other meat products could be an important factor of improving food safety. For these purpose further investigations are essential, in order to develop extraction procedures, stability measures, as well as a test of growth kinetics, bacteriocin production and the influence to pathogens in controlled conditions.

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SAŽETAK

Istražena je sposobnost rasta bakterije *Listeria monocytogenes* tijekom zrenja fermentiranih kobasica, te antimikrobno djelovanje izolata bakterija mliječne kiseline. Početni broj *L. monocytogenes* u nadjevu bio je 10^5 CFU g^{-1}. Kobasice su uzorkovane 0., 3., 7., 14. i 28. dana zrenja, a određivan je ukupni broj bakterija, broj bakterija mliječne kiseline, broj *L. monocytogenes*, te pH nadjeva. Rast *L. monocytogenes* bio je zaustavljen do 14. dana, dok je u gotovom proizvodu bila ispod praga detekcije (<2 $\log_{10}$ CFU g^{-1}). Ukupno su bila izdvojena i determinirana 32 izolata bakterija mliječne kiseline među kojima je *Leuconostoc mesenteroides* pokazivao inhibicijsko djelovanje prema *L. monocytogenes* putem proizvodnje bakteriocina.

Ključne riječi: fermentirane kobasice, *L. monocytogenes,* bakteriocini