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Diversity of Lactobacillus microflora in homemade raw sausages during the ripening

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Abstract. *The main purpose of our study is to prove the diversity of the Lactobacillus microflora in domestic raw sausages during ripening. Raw sausages from pork meat (bacon from the neck, front pig shoulder, pork belly) with the addition of salt, red chili pepper and mint were prepared for that purpose. They matured in a maturation chamber for 60 days. A total of 6 samples from different stages of natural ripening of raw sausages were taken for testing. The raw sausage samples were diluted in saline solution, homogenized, serially diluted in the same solution and plated on MRS agar; the plates were incubated at 42°C for 24 - 48 h. A total of 24 strains were isolated and identified as Lactobacillus spp. based on their growth, gram-stain activity, catalase and oxidase. Their affiliation to this genus was confirmed by PCR with genus specific primers, 16S ribosomal RNA. The results show different Lactobacillus species: Lactobacillus plantarum - 37%, Lactobacillus sakei - 28%, Lactobacillus brevis - 20% and Lactobacillus curvatus 15%.*

Keywords: *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus curvatus*

Introduction

The types of sausages are separated into two large groups. To the first group belong sausages which are placed on the market after heat treatment while in the second the sausages are placed on the market untreated. Sausages that are thermally untreated are called raw sausages. This type of products are obtained from fine muscle tissue, adipose tissue, salt, sugar and spices (Ranken, 2000; Weiss et al*.,* 2010). According to the amount of water the sausages are divided into two groups: semi-dry sausages with over 35% water and dry ones with less than 35% water. The final product of the last group must not contain more than 30% water (Hughes et al., 2002; Mahachi et al*.,* 2019).

The technology used for the raw sausages preparation is important for their quality. For durable sausages, meat from old animals is used which has solid structure, intense color and more dry matter (Marianski and Mariański, 2009). The meat of young heads has more water and is lighter. The amount of meat in the tenderloin varies from 60-80%. To obtain sausage with high quality the order of additional ingredients is important: the table salt and nitrites are added to the minced meat before adding fat tissue.

Ripening is a process that separates raw sausages from all other groups. It is a sum of physical and chemical changes that give the specific color, appearance, aroma and durability of the sausage. The ripening of the sausages has two phases: In the first phase intensive ripening, stabilization of the consistency, color and taste is achieved. It is a result of bacterial denitrification of Lactobacillus. In the second phase the maturation of the salami with specific molds (Penicillium, Aspergillus) is delayed. Sausage ripening between 12-16°C, moderate ripening between 18-24°C and fast ripening above 25°C.

The study of the microflora of raw dried sausages and its participation in their maturation began in the 1960s (Lerche and Reuter, 1960). Many of the traditional technologies for raw sausages preparation are based on natural fermentation processes caused by natural microbial populations in the substrate (Comi et al., 2005; Baruzzi et al., 2006; Talon et al., 2007; Moura et al., 2007). Such microbial cultures have a wide range of enzymes which can affect a variety of components. Therefore, in these fermentations, a large number of transformations, usually multi-stage, take place at the same time, which are beyond the capabilities of one organism (Tanasupawat and Komagata, 1995). The technological process during the different periods of the sausages maturation cause a number of physicochemical changes under the action of both its own and microbial enzymes (Toldra, 2008). The resulting end product is characterized by high acidity (pH of about 5) and low free water content (Mullins and Daugulis, 2019).

Microbiological analysis of various Portuguese, Spanish,

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Italian and Greek traditionally prepared raw-dried sausages shows the presence of aerobic mesophilic and psychrotrophic microorganisms, spore-forming bacteria, lactic acid bacteria, halophilic bacteria, coagulase-negative cocci, enterococci. Of these, two groups of microorganisms: lactic acid bacteria and coagulase-negative staphylococci are considered technologically important for the fermentation and maturation of dry sausages (Coventry and Hickey, 1991). Lactic acid bacteria in hygienic quality raw meat are presented with low values (10^{-2}-10^{-3} CFU/g), but under anaerobic conditions and the presence of NaCl, nitrates and nitrites quickly begin to dominate the course of fermentation. Depending on the added sugars, meat proteins and supplements, they produce lactic acid and small amounts of acetic, formic, propionic, valeric, etc. Acids, ethanol, acetone and CO_2, which contribute to the sharp specific taste of sausages (Hammes and Knauf, 1994). They largely determine the sanitary qualities of the final product. In Northern European sausages, lactic acid is the main flavor component, but acetic acid is also involved in the formation of taste. The values of the other acids are extremely low. The values of free amino acids and fats increase during the ripening process and affect the taste of sausages (Dainty and Blom, 1995). Literature data show that in this type of sausages lactic acid bacteria dominate over other groups of microorganisms throughout the fermentation period. Their population is dominated by lactobacilli (especially homofermentative), with Lactobacillus sakei, *L. curvatus, L. casei* and *L. plantarum* being the most commonly identified. *L. alimentarius* has also been found in some Spanish sausages, *L. rhamnosus* in Greek and *L. brevis* and *L. versmoldensis* in Scandinavian sausages. Representatives of genus, Lactococcus, Pediococcus and Leuconostoc.

Homemade raw sausages are typical food for the people in North Macedonia. In this regard, clarifying the factors that affect their quality is of great importance for the protection of human health. Having in mind that the microorganisms are actively involved in the process of sausage maturation, we set ourselves the goal to establish the diversity of the Lactobacillus microflora in domestic raw sausages during ripening.

Material and methods

Preparation of the raw sausages

The sausages for our examination are prepared in a butcher shop in Bitola town according to the following recipe:

- 1st stage: 30 kg pork meat, mixture of bacon from the neck, front pig shoulder and pork belly was cooled to -5°C for 15 hours;
- 2st stage: grinding the cooled meat and adding 300 g salt, 150 g red chili pepper and 60 g mint;
- 3st stage: the prepared mixture is placed in intestines, the last ones are tied, and the finished raw sausages are immersed in hot water, squeezed and hung on wooden sticks for maturation;
- 4th - 10th stages: the hanging raw sausages mature in a maturation chamber from 2nd to 60th days.

Samples collection

The samples (n=6) were taken from the fifth, sixth, seventh, eighth, ninth and tenth stages of the raw sausages preparation (Table 1).

Table 1. Stages of samples collected of raw sausages

Stages of raw sausages maturation					
5th	6th	7th	8th	9th	10th
From 2 to 10 day	From 10 to 14 day	From 14 to 22 day	From 22 to 32 day	From 32 to 40 day	From 40 to 60 day
Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6

Isolation of lactic acid bacteria

For the purpose of the study 25 g of raw sausage samples were diluted in 225 ml saline solution, homogenized, serially diluted in the same solution and plated on MRS agar (De Man, Rogosa and Sharpe Agar, Merck Darmstadt, Germany). The plates were then incubated at 42°C for 24-48 h. The colonies which were morphologically different were picked up and inoculated as stab. A total of 24 strains were isolated.

DNA isolation

Chromosomal DNA from a single-colony inoculated @, GenElute TM, bacterial genomic DNA kit (Sigma Aldrich Co.) was isolated on 24 isolates obtained from raw sausage. From the resulting DNA, 20 μm sections were made and stored at -20°C for several months until needed.

PCR - amplification with primers specific for the genus Lactobacillus

Gene-specific PCR was made with the following primers shown in Table 2. The marker we used for molecular weight was 100 bp. The obtained results are presented on 1% agarose gel.

Table 2. PCR primers specific for the genus Lactobacillus

Lab0677F Lact71R	5-'CTCCATGTGTAGCGGTG-3' 5'-TCAAAACTAAACAAAGTTTC-3	Moura et al., 2007
LactoF LactoR	5'-TGGAAACAGRTGCTAATACCG-3' 5'-GTCCATTGTGGAAGATTCCC-3'	Byun et al., 2004

*Sequencing of the 16S rRNA genes

Sequencing of the 16S rRNA genes.

The sequencing of the samples was performed on the DNA genetic analyzer ABI PRISM®310 (PE Applied Biosystems).

Results and discussion

Twenty-four strains isolated from 6 stages of maturation of home-made raw sausages (from 5th to 10th stages) were characterized phenotypically. The species grew anaerobically on

selective MRS agar. The temperature used to cultivate the isolates was 42°C. White glossy colonies were obtained from the samples which were further tested by classical microbiological tests Gram positive. Catalase and oxidase negative rod shaped bacteria indicate that the strains are related to the genera Lactobacillus. For confirmation of genera DNA is isolated and PCR was used with specific primers for the genus Lactobacillus (Heilig et al., 2002).

The results in Figure 1 show that all 24 isolates have been amplified, thus confirming that the isolates belong to the genus Lactobacillus.

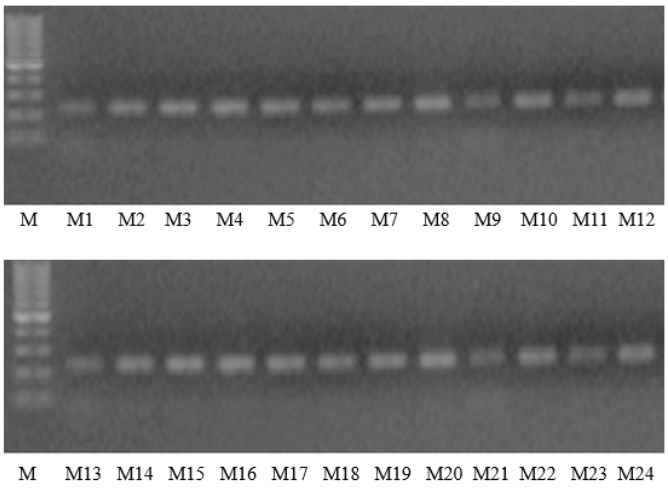

Figure 1. 1% agarose gel determination of Lactobacillus genus belonging to genus-specific PCR primers

The results of the 16S rRNA analysis in Figure 2 show that we have identified four species of lactobacillus. The share of *Lactobacillus plantarum* was the largest - 37%, followed by *Lactobacillus sakei* - 28%, *Lactobacillus brevis* - 20% and *Lactobacillus curvatus* - 15%. The results obtained indicate that from the beginning of ripening of the raw sausages until the end of their ripening in the substrate there are different types of lactobacilli, which according to Polka et al. (2015) help to carry out the process of natural ripening of raw sausages.

Studies by other authors show that in this type of sausages lactic acid bacteria dominate over other groups of microorganisms during the entire fermentation period. Their population is dominated by lactobacilli (especially homofermentative), the most commonly identified being *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus casei* and *Lactobacillus plantarum* (Coppola et al., 2000; Papamanoli et al., 2003; Lee et al., 2006). All the above-cited authors confirm our results of the tests performed.

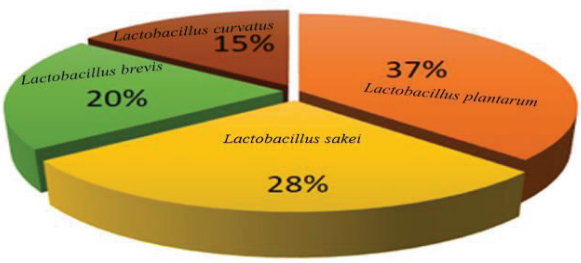

Figure 2. Results of the analysis of the 16S rRNA sequence

Conclusion

It was found that: (i) from fifth to tenth stage (2nd to 60th days) of the raw sausages preparation (maturation) four species of Lactobacillus are isolated: *Lactobacillus plantarum* - 37%, *Lactobacillus sakei* - 28%, *Lactobacillus brevis* - 20% and *Lactobacillus curvatus* - 15%; (ii) the beginning of natural fermentation starts in the fifth stage of the raw sausages preparation; (iii) the obtained isolates from our test can be further used to obtain starter crops that can be used in the industrial production of raw sausages and to contribute in the production of sausages with good quality.

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