Bulgarian Journal of Veterinary Medicine, 2023, **26**, No 4, 616–629
ISSN 1311-1477; DOI: 10.15547/bjvm.2021-0043

Original article

MICROBIOLOGICAL EFFECT OF COMPLETE REPLACEMENT OF NITRITES/NITRATES WITH STARTER CULTURES IN TRADITIONAL RAW-DRIED FERMENTED SAUSAGE "LUKANKA PANAGYURSKA"

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Summary

Krumova-Valcheva, G., E. Gyurova, G. Mateva, M. Milanov, R. Tropcheva, E. Lukach & H. Daskalov, 2023. Microbiological effect of complete replacement of nitrites/nitrates with starter cultures in traditional raw-dried fermented sausage "Lukanka Panagyurska". *Bulg. J. Vet. Med.,* **26**, No 4, 616–629.

The article is focused on microbiological safety of the traditional raw-dried meat product "Lukanka Panagyurska", produced by a starter cultures, containing *Lactiplantibacillus plantarum* GLP3 and *Debaryomyces hansenii* ATCC 36239 instead of using nitrates/nitrites. The aim of the study was to demonstrate that the starter cultures had a similar or better preservative effect as the traditional nitrates transformed into nitrites in the drying-ripening phase. The effect of the same starter cultures, produced by two different technological approaches on the survival of foodborne pathogens (*Salmonella* Typhimurium, *Listeria monocytogenes*, *Escherichia coli* and *Clostridium perfringens*) was examined. The study showed the presence of the lactic acid microorganisms at every stage of the production process of the raw-dried meat product. Zoonotic pathogens as *S.* Typhimurium and *L. monocytogenes* and sanitary indicator bacteria as *E. coli* and *C. perfringens* were combined to control the levels of pathogen inactivation. The preservative effect of the starter cultures resulting from lactic acid fermentation was more effective in comparison to that of nitrites/nitrates. The experiments proved that the microbiological safety of the raw-dried meat product was improved in comparison with the popular practice of adding nitrates/nitrites as a preservative.

Key words: *Lactobacillus*, nitrite-free sausages, pathogenic microorganisms, raw-dried meat product, starter cultures

INTRODUCTION

"Lukanka Panagyurska" is one of the most popular raw-dried sausages consumed in Bulgaria. The main ingredients are beef or pork meat, salt, sugar, nitrate/nitrite, ascorbic acid, and spices – cumin and black pepper. At this moment "Lukanka Panagyurska" is certified as a

product with traditional speciality guaranteed (Anonymous, 2014).

The production of "Lukanka Panagyurska" includes several phases: selection of high-quality fresh meat, slicing, cooling, preparation of the meat, homogenisation, stuffing, ripening (at 30 °C) and drying for 40 days (under specific temperature and humidity).

The addition of nitrate as a preservative is a common practice in the production of raw-dried meat products. As a result of the microbial activity in the filling mass, part of nitrates is reduced to nitrite. When combined with myoglobin, it also has a red colouring effect (Govari *et al*., 2015). The nitrites inhibit the growth of anaerobic bacteria in the products, including *Clostridium botulinum* (MacDougall & Hetherington, 1992; Berardo *et al*., 2015). The accumulation of nitrites after biodegradation of nitrates used in the production of meat sausages was proven (Püssa, 2013). The levels of 150–200 mg/kg of residual nitrites in the final product could lead to serious risks for the consumers. In this regard, scientists seek alternatives to nitrates and their reduction to chemically active nitrites as preservatives in meat products (Abahakoon *et al*., 2015; Govari *et al*., 2015). In the late 1980s, Holly *et al*. (1988) have found that enterococci have the ability to retain the characteristic sensory properties of fermented meat products. In recent years, the researchers are focused on the usage of different lactic acid bacteria as preservative in the production of raw-dried sausages. Some authors (MacDougall & Hetherington, 1992; Nedelcheva *et al*. 2010; Cavalheiro *et al*., 2019) were focused on a double role of inhibiting or controlling the growth of food pathogens or food spoilage microorganisms in meat products proving that the starter cultures of lactic acid microorganisms could inhibit groups of microorganisms in meat products.

Cavalheiro *et al*. (2019) affirmed that *L. plantarum* had a strong effect on the microflora in the filling mass when a meat product was made and observed a significant reduction of bacterial counts from the *Enterobacteriaceae* family. At the same time, all sensory product properties remained unchanged. Nedelcheva *et al*. (2010) made an analogous research in Bulgaria. It confirmed the antibacterial features of *Lactiplantibacillus plantarum* GLP3 on several pathogens and toxin-forming microorganisms. The experimental data on raw-dried sausages displayed a reduction of the spoilage/undesirable microflora in the final products. Some new approaches such as using bacteriocins to control pathogenic microorganisms have been developed to increase food safety. The application of a suitable amount of nisin has significantly inhibited the growth of *Listeria monocytigenes* in artificially contaminated fermented sausages (Holly *et al*., 1988). The applied strain of *Debaryomyces hansenii* was chosen among other yeasts due to its ability to produce characteristic aroma compounds after several tests of the ProViotic AD's team on production of traditional Bulgarian raw-dried products like "Lukanka Panagyurska".

The aim of this study was to evaluate the inhibition effect of two types of starter cultures, containing *Lactiplantibacillus plantarum* GLP3 and *Debaryomyces hansenii* ATCC 36239 on foodborne pathogens such as *S.* Typhimurium, *L. monocytogenes*, *E. coli* and *C. perfringens* in experimentally contaminated meat products without addition of nitrates in their formulation.

MATERIALS AND METHODS

ProViotic Meat Starter cultures

The ProViotic Starter Culture™ contains two types of lyophilised (freeze-dried) microorganisms – *Lactiplantibacillus plantarum* GLP3 (property of ProViotic AD) and *D. hansenii* ATCC 36239. During all *in vitro* and *in situ* tests from the ProViotic AD team and in the laboratories of all their collaborators, no inhibition has been detected by either *Debaryomyces hansenii* ATCC 36239 or *Lactiplantibacillus plantarum* GLP3 relative to the other microorganism. Two variants of the ProViotic Starter Culture™ were used in this study. In both variants of the studied starter culture the yeast and lactic acid bacteria were not less than 1×10^9 CFU/g in the starter mix powder. The concentration of live cells of both starter cultures in the ProViotic Starter Culture™ was adjusted during the production of the starters in a way that guaranteed at least 1×10^7 CFU/g for *Lactiplantibacillus plantarum* GLP3 in both variants of the starter and 1×10^5 CFU/g for *D. hansenii* ATCC 36239 in minced meat at the beginning of the fermentation of the sausage samples.

The difference between starter variant 1 and starter variant 2 was only in the way *Lactiplantibacillus plantarum* GLP3 was cultivated. During the production of *L. plantarum* GLP3 for variant 1 ProViotic Starter Culture™, *L. plantarum* GLP3 was cultured in vegan nutrient media at constant optimal for the strain temperature of 37 °C for 24 hours in a bioreactor with no aeration. The fermentation was followed by adjustment of pH to 5.90 and lyophilisation. In the production process of *L. plantarum* GLP3 for the ProViotic Starter Culture™ variant 2, *L. plantarum* GLP3 was cultured in the same vegan nutrient media but in order to increase the amount of synthesised antimicrobial metabolites, especially bacteriocins (plantaricins in the case of GLP3), two temperature regimens were applied with a total duration of the whole fermentation process of 24 hours as well. The fermentation was done in the same bioreactor with no aeration and again, was followed by adjustment of pH to 5.90 at the end of the process and finally, by lyophilisation.

Bacterial cultures

Pure cultures of *S.* Typhimurium ATCC 14028, *L. monocytogenes* NCTC 11994, *E. coli* ATCC 25922 and *C. perfringens* ATCC 13214 were obtained from NCFS's collection of reference strains. The strains were subcultured from fresh agar cultures in Nutrient broth (Merck) before use. The broth culture was ready to use after incubation at 37 ºC for 20±1 hour. One mL of the broth culture was used to innoculate 1 kg of stuffing mass so that a final concentration not less that 1×10^5 CFU/g product was obtained.

Preparation of raw-dried sausages "Lukanka Panagyurska"

The filling mass was produced in an industrial plant by the authentical recipe. It was contaminated with foodborne pathogens on the same day of preparation. The whole filling mass was divided into three equal groups and placed in separate polyethylene bags. The first batch of filling mass (control group) was produced with sodium nitrate according to the traditional recipe for this type of raw-dried meat products. The starter cultures were not added. The second batch of filling mass was made using the first variant of starter culture in the proportion of 1 g/kg of stuffing mass without adding sodium nitrate. In the third batch of filling mass, the second variant of starter culture was used in the proportion of 1 g/kg of stuffing mass. Sodium nitrate was neither added.

The three batches of the filling mass were mixed separately until complete homogenisation of sodium nitrate or the starter cultures. Each of the batches was divided into four groups, which were individually contaminated with different foodborne pathogens (Table 1). Four equal parts of each batch were put in different polyethylene bags and inoculated with broth culture of single pathogenic or sanitary indicator microorganisms. After contamination and homogenisation, the filling masses were placed at 2 ºC ± 1 ºC for 48 hours for preliminary preparation. After that it was filled into natural casings under aseptic conditions with a filling machine and individual pieces of "Lukanka Panagyurska", each weighing about 200–250 g were prepared. The pieces were placed in the ripening chamber at 30 °C ± 2 °C for 48 hours. The process was controlled by periodical measurement of products' pH. All groups were transferred to chambers with an air temperature of 12 °C ± 2 °C and humidity up to 75% when the pH reached 5.2±0.2. The experimental raw-dried meat products were left for 40 days. They were periodically pressed to obtain the specific flattened shape of "Lukanka Panagyurska".

Sampling procedure

Samples for microbiological examination were taken from the main filling mass at the following stages: at day zero before the contamination (time of application of the starter cultures, sodium nitrate and the subsequent inoculation of the respective pathogenic microorganisms); after the stuffing; at the end of the ripening at 30 °C (after 48 hours) and reaching pH of 5.2 ± 0.2; on the 10^{th}, 20^{th}, 30^{th} and 40^{th} day of drying the product. All microbiological tests were done immediately after the sampling.

Microbiological analyses

All experimental groups were tested by quantitative methods for estimation of lactic acid bacteria (CFU/g), *E. coli*, *L. monocytogenes* and *C. perfringens* (CFU/g) counts. The presence of *S.* Typhimurium was evidenced in 25 g. The most probable number of *S.* Typhimurium (MPN/g) was determined after 20 days of drying.

Enumeration of lactic acid bacteria was conducted by deMan Rogosa and Sharpe agar (MRS medium, Merck) at 37 ^{0}C for 72 hours under anaerobic condi-

Table 1. Experimental design – preparation of the experimental groups

Main filling mass groups	Foodborne pathogens (level of contamination 1×10^5 CFU/g)			
16 kg of filling mass with sodium nitrate	4 kg of filling mass with added *S.* Typhimurium ATCC 14028	4 kg of filling mass with added *L. monocytogenes* NCTC 11994	4 kg of filling mass with added *E. coli* ATCC 25922	4 kg of filling mass with added *C. perfringens* ATCC 13214
16 kg of filling mass with starter culture variant 1	4 kg of filling mass with added *S.* Typhimurium ATCC 14028	4 kg of filling mass with added *L. monocytogenes* NCTC 11994	4 kg of filling mass with added *E. coli* ATCC 25922	4 kg of filling mass with added *C. perfringens* ATCC 13214
16 kg of filling mass with starter culture variant 2	4 kg of filling mass with added *S.*Typhimurium ATCC 14028	4 kg of filling mass with added *L. monocytogenes* NCTC 11994	4 kg of filling mass with added *E. coli* ATCC 25922	4 kg of filling mass with added *C. perfringens* ATCC 13214

tions (ISO 7889:2003). To calculate the number of *E. coli* (CFU/g) the method of incubation at 44 ^{0}C for 24 hours (ISO 16649-2:2001) was used. *L. monocytogenes* (CFU/g) was enumerated by the method of calculation using the Otaviani and Agosti Listeria agar (ALOA, Merck) and culturing for 48 hours at 37 °C (ISO 11290-2:2017). Detection of *S.* Typhimurium was carried out by enrichment in Buffered Peptone Water (Merck), followed by secondary selective enrichment in Rappaport-Vassiliadis medium with soya (RVS broth, Merck) and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn, Merck). Selective solid medium: Xylose Lysine Deoxyholate agar (XLD) and Brilliant green phenol red lactose sucrose agar (BPLS) were used as confirmation medium (ISO/TS 6579-1:2017). The most probable number of *S.* Typhimurium (MPN/g) was determined by using pre-enrichment in a specific amount of non-selective liquid nutrient medium (Buffered Peptone Water (BPW), Merck) in a series of 3 tubes (ISO/TS 6579-2:2012). Inoculation with a double pouring of Sulfite-cycloserine agar (SC), Merck melted and cooled up to 40 °C was used to calculate the amount of *C. perfringens* (CFU/g) (ISO 7937:2004).

Samples prepared by this technology were tested for the same microbiological parameters (presence of *S.* Typhimurium ATCC 14028, *L. monocytogenes* NCTC 11994, *E. coli* ATCC 25922, *C. perfringens* ATCC 13214 and lactic acid bacteria) as well as the acidity of the product at different stages of production in external independent laboratories – Center for Applied Studies and Innovation – Sofia, Bulgaria and ADIV Institute in France. Data from their laboratory protocols proved full compliance with laboratory results reported by us. Statistical analysis of the submitted data was not performed because the replication and additional laboratory tests were performed in the above two external independent laboratories.

Analysis of results

The amount of different pathogenic bacteria was calculated according to the formula: (ISO 7218:2007/Amd. 1:2013 15):

$$N = \frac{\Sigma a}{V \times 1.1 \times d}$$

where: ∑a: the number of colonies counted in two consecutive dilutions; V: the amount of the inoculated material; d: the smallest dilution at which between 15 and 300 colonies are counted.

The amount of lactic acid microorganisms was calculated according to ISO 7889:2003:

$$N = \frac{\Sigma C}{(n_1 + 0.1 \times n_2) \times d}$$

where: ∑C: the number of colonies listed counted in two consecutive dilutions; n1: the number of Petri dishes used in the first selected dilution; n2: the number of Petri dishes used in the second selected dilution; d: the value of the quantity of sample used in the first dilution.

RESULTS

Data for lactic acid bacteria

The progressive development of *Lactiplantibacillus plantarum* GLP3 in the two variants of starter culture in the experimental (nitrates free) groups is shown on Fig. 1. There was a multiplication of the starter cultures in experimental samples until the end of the ripening process (7.9 $\log_{10}$ CFU/g and 7.1 $\log_{10}$ CFU/g) and decreasing the pH below 5.2. The lactic acid bacteria count decreased by 1 to 1.5 $\log_{10}$ CFU/g after the ripening phase and during the drying process. Nevertheless, it

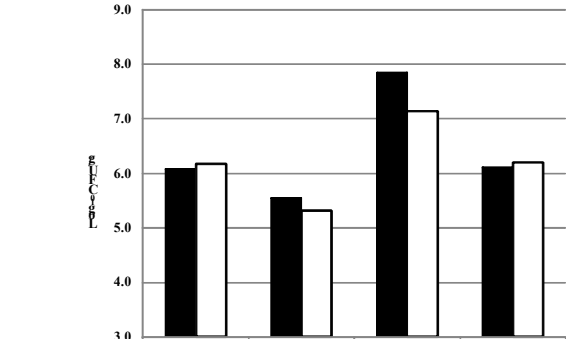


Fig. 1. Dynamics of the growth of lactic acid bacteria used in the production of raw-dried meat product Lukanka Panagyurska during the different stages of production.

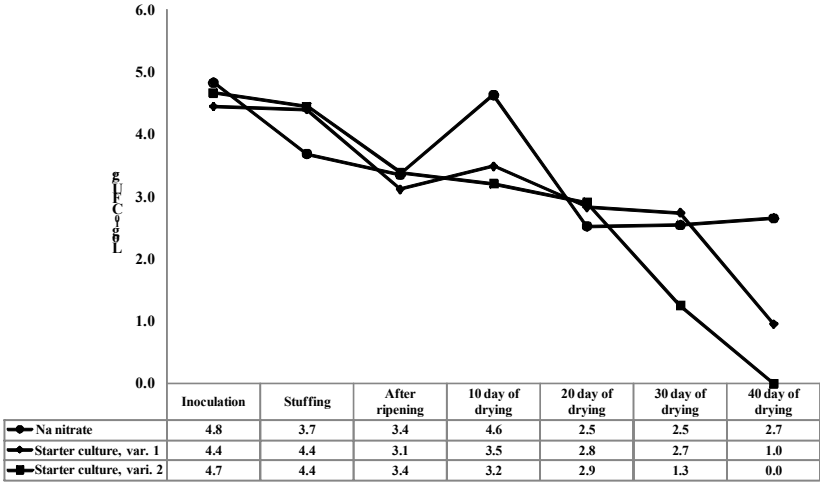


Fig. 2. *E. coli* counts ($\log_{10}$ CFU/g) in the three groups of samples during the different stages of production.

remained at a relatively high level of 6 $\log_{10}$ CFU/g. On the 40th day of the drying stage, the average count of *L. plantarum* GLP3 was 6.1 $\log_{10}$ CFU/g in meat products, processed with starter culture variant 1 and 6.2 $\log_{10}$ CFU/g for the products with variant 2.

Data for E. coli

As shown on Fig. 2, a significant decrease in the number of *E. coli* was observed in the groups prepared with both starter culture variants during the different stages of the experiment. In the samples traditionally prepared by adding sodium nitrate as preservative, the decrease in the amount of *E. coli* was relatively less significant.

At the moment of inoculation of all three batches of the product, the level of *E. coli* reached 4.8 $\log_{10}$ CFU/g for sodium nitrate samples; 4.4 $\log_{10}$ CFU/g for starter culture variant 1 specimens and 4.7 $\log_{10}$ CFU/g for variant 2 samples. During the different stages of the experimental

production of the three groups, a gradual decrease in the number of *E. coli* was noted. In the samples prepared with sodium nitrate, the amount of *E. coli* decreased to 3.4 $\log_{10}$ CFU/g after the ripening stage (at 30 °C for 48 hours). Nevertheless, on the 10th day of the drying stage, a certain multiplication was noticed with increase in the counts of *E. coli* up to 4.6 $\log_{10}$ CFU/g. At the end of the drying period, *E. coli* counts dropped to 2.7 $\log_{10}$ CFU/g.

Samples prepared with the addition of variant 1 starter culture, demonstrated a gradual decrease in *E. coli* counts from 3.1 $\log_{10}$ CFU/g at the end of the ripening phase to 1 $\log_{10}$ CFU/g at the end of the production period. In this group of experimental products, a slight increase to 3.5 $\log_{10}$ CFU/g was observed on the 10th day of drying followed by a considerable decrease on the 20th day of drying to 2.8 $\log_{10}$ CFU/g.

In the samples prepared with starter culture variant 2, a stronger inhibition of the development of *E. coli* during the different stages of the production process was detected. On the 40th day of drying, the product did not contain any *E. coli*.

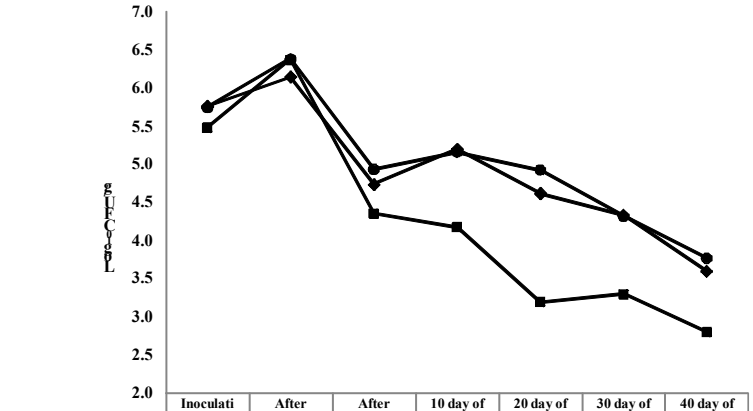
Data for L. monocytogenes

L. monocytogenes was not detected in the filling mass. After the contamination of the samples, *L. monocytogenes* average count ($\log_{10}$ CFU/g) was 5.7 in the samples with sodium nitrate; 5.8 in the samples with starter culture variant 1, and 5.5 in samples with starter culture variant 2.

During the different stages of the experiment, *L. monocytogenes* counts increased before the filling stage up to 6.4 $\log_{10}$ CFU/g in the samples with sodium nitrate and those with variant 1 starter culture. In the samples with variant 2 starter culture, the increase was up to 6.1 $\log_{10}$ CFU/g. After the ripening phase, a significant decrease in *L. monocytogenes* counts up to 1 $\log_{10}$ CFU/g was noticed. *L. monocytogenes* increased from 4.9 $\log_{10}$ CFU/g to 5.2 $\log_{10}$ CFU/g in the group with sodium nitrate after the 10th day of the drying period. The tendency of decrease of *L. monocytogenes* counts was confirmed also on the 40th day of the drying period, when they attained 3.8 $\log_{10}$ CFU/g.

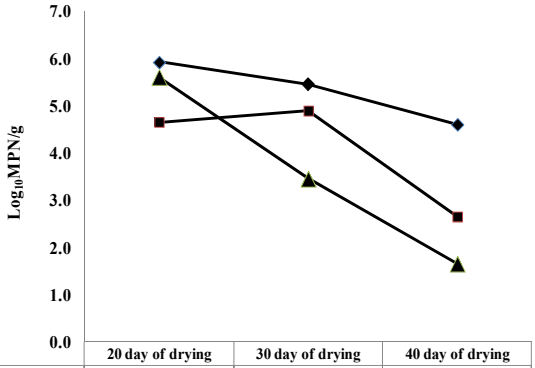
The results from the testing of samples produced with *L. plantarum* GLP3 and *D. hansenii* ATCC 36239 as starter culture variant 1 were similar to those of samples containing sodium nitrate. A significant increase in *L. monocytogenes* counts occurred during the first 48 hours after the contamination – 6.1 $\log_{10}$ CFU/g. A decrease of the amount was noted after the ripening phase – 4.7 $\log_{10}$ CFU/g. On the 10th day of the drying stage, *L. monocytogenes* increased up to 5.2 $\log_{10}$ CFU/g. However, after that period, counts were reduced as followed: on the 20th day – 4.6 $\log_{10}$ CFU/g; on the 30th day – 4.3 $\log_{10}$ CFU/g; on the 40th day – 3.6 $\log_{10}$ CFU/g.

A clear logarithmical decrease in *L. monocytogenes* counts ($\log_{10}$ CFU/g) was found in the third experimental group of samples during the whole production period. At the end of the process (40 days – 12 °C and humidity up to 75%), the amount of *L. monocytogenes* was reduced up to 2.8 $\log_{10}$ CFU/g (Fig. 3).



	Inoculation	After stuffing	After ripening	10 day of drying	20 day of drying	30 day of drying	40 day of drying
Na nitrate	5.7	6.4	4.9	5.2	4.9	4.3	3.8
Starter culture, var. 1	5.8	6.1	4.7	5.2	4.6	4.3	3.6
Starter culture, var. 2	5.5	6.4	4.3	4.2	3.2	3.3	2.8

Fig. 3. *L. monocytogenes* counts ($\log_{10}$ CFU/g) in the three groups of samples during the different stages of production.



	20 day of drying	30 day of drying	40 day of drying
S. Typhimurim (Na nitrate)	5.9	5.5	4.6
S. Typhimurim (starter culture, var. 1)	4.7	4.9	2.7
S. Typhimurim (starter culture, var. 2)	5.6	3.5	1.7

Fig. 4. Most probable number of *Salmonella* Typhimurium ($\log_{10}$ MPN/g) in samples during the different stages of production.

Data for Salmonella Typhimurium

Salmonella Typhimurium was found in all three groups of experimental samples during the period after the contamination, the filling stage and after the first 10 days of drying. Most probable number of *S.* Typhimurium in 1 g (MPN/g) of the samples was calculated during all other phases of the drying period.

As shown on Fig. 4, the reduction of *S.* Typhimurium was insignificant – from 8.5×10^5 MPN/g to 4.1×10^4 MPN/g in the samples with sodium nitrate for whole process of drying. There was an increase in *S.* Typhimurium counts during the drying phase in the samples with starter culture variant 1: on the 20^{th} day – 4.7 $\log_{10}$ MPN/g; on the 30^{th} day – 3.5 $\log_{10}$

MPN/g. A decrease in the most probable number – 2.7 $\log_{10}$ MPN/g was noticed during the last 10 days of the ripening stage.

The highest level of reduction in the counts of salmonellae was observed in the samples produced with the starter culture variant 2. The amount of *S.* Typhimurium on the 20th day of drying was 5.6 $\log_{10}$ MPN/g and on the 40th day of drying: 1.7 $\log_{10}$ MPN/g (a decrease by up to 4 $\log_{10}$ MPN/g).

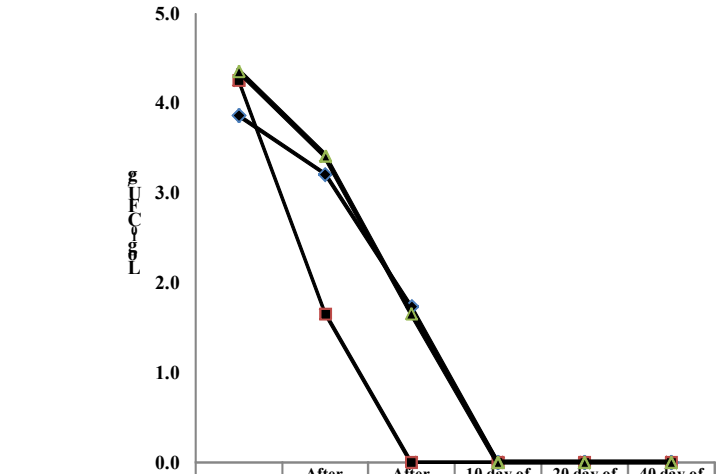
This experiment demonstrated a better antimicrobial activity against *S.* Typhimurium in the samples containing starter cultures with *L. plantarum* GLP3 and *D. hansenii* (variants 1 and 2) compared to the traditional method for production of raw-dried meat products with sodium nitrate.

The experiment proved that *Salmonella* bacteria did not influence the multiplication of lactic acid microorganisms in both experimental groups of product. Similar to other experiments with products contaminated with *L. monocytogenes* and *E. coli*, a good growth of the lactic acid bacteria was demonstrated. At the end of the production, their number reached 7.2 $\log_{10}$ CFU/g in the sample containing starter culture 1 and 7.8 $\log_{10}$ CFU/g in the sample containing starter culture 2 (Fig. 4).

Data for C. perfringens

The *C. perfringens* count ($\log_{10}$ CFU/g) at the moment of inoculation of the filling mass was 3.9 $\log_{10}$ CFU/g for the samples with sodium nitrate; 4.3 $\log_{10}$ CFU/g for the samples with starter culture variant 1 and 4.4 $\log_{10}$ CFU/g in samples produced with starter culture variant 2 (Fig. 5).

The ripening phase of the products at 30 °C and the decrease in pH to 5.2 resulted in a significant reduction in the viable *C. perfringens* cells: 1.7 $\log_{10}$ CFU/g in products containing sodium nitrate and starter culture variant 2. In products containing starter culture variant 1, viable *C. perfringens* cells were completely inhibi-



	Inoculation	After stuffing	After ripening	10 day of drying	20 day of drying	40 day of drying
Na nitrate	3.9	3.2	1.7	0.0	0.0	0.0
Starter culture, var. 1	4.3	1.7	0.0	0.0	0.0	0.0
Starter culture, var. 2	4.4	3.4	1.7	0.0	0.0	0.0

Fig. 5. *C. perfringens* counts ($\log_{10}$ CFU/g) in the three groups of samples during the different stages of production.

ted during the ripening phase (Fig. 5). In this experimental group, the tendency towards increased input of lactic acid microorganisms and maintainance of their level at 8 $\log_{10}$ CFU/g until the end of the experimental production process was preserved.

DISCUSSION

The growth of *E. coli*, *L. monocytogenes*, *S.* Typhimurium and *C. perfringens* in the raw-dried product "Lukanka Panagyurska" without addition of sodium nitrate and containing the tested two starter culture variants was not completely inhibited under the specific conditions of ripening and drying. The pH values reached 5.2–5.4 at the end of the ripening stage.

The decrease in the number of the added foodborne pathogens or sanitary indicator microorganisms in products produced with both starter cultures was observed after the end of the ripening stage. The most significant changes in the amount of *E. coli* was in the sample containing starter culture variant 2 – from 4.7 log10 CFU/g to complete inhibition on the 40th day of the drying period. The products with sodium nitrate maintained the survival of *E. coli*, albeit at low levels (2.7 $\log_{10}$ CFU/g at the end of the experimental production process). Similar results were reported by Wang *et al*. (2013) who used starter cultures containing *Lactobacillus sakei* for the production of Chinese fermented sausages. The reported results showed that the lactic acid bacteria dominated the general microflora, including *E. coli*. During the fermentation of the sausages, the pH decreased up to 4.5 on the 15th day of the ripening phase. This decrease in the acidity of the medium and the antimicrobial action of the GLP3's bacteriocins are the reasons for the inhibition of *E. coli* at the end of the experimental period although *E. coli* is often found in sausages that ferment spontaneously. Other scientists argued that the lactic acid bacteria were not highly active against Gram-negative bacteria because of their protective lipopolysaccharide coating (Kęska *et al*., 2017).

The tendency for inhibition of the growth was shown in the samples contaminated with *C. perfringens* for the two variants of starter cultures. In the samples containing starter culture variant 1, full inhibition during the ripening stage was observed whereas in the group containing starter culture variant 2, the full inhibition of *C. perfringens* occurred at the end of the ripening stage.

The sodium nitrate added in control group of product inhibited the development of *C. perfringens*. However, that did not lead to its complete absence at the end of the ripening process. Similar data were reported from the experimental production of pork fermented sausages with *L. plantarum* where the amount of *C. perfringens* decreased by 2.0 $\log_{10}$ CFU/g after 9 days of fermentation (DiGioia *et al*., 2016).

The same results can be seen in the samples contaminated with *L. monocytogenes* and *S.* Typhimurium, although the presence of both zoonotic pathogens was observed until the end of the drying process. However, it was noticed that the growth of the pathogens in samples with starter culture variant 1 and 2 was significantly inhibited at the end of the production process. In the samples prepared with sodium nitrate (traditional recipe), the inhibition process was not so significant. These results are related to the aknowledged and described sensitivity of these pathogens to the lactic acid bacteria and nitrate (Okuyam *et al.* 1999). Similar results were reported by Muhammad *et al.* (2019) who conducted an *in vitro* study of

the antimicrobial potential of *L. plantarum* against various pathogens (*S.* Typhimurium, *E. coli, L. monocytogenes* and *S. aureus*) and reported relative antimicrobial activity of *L. plantarum* against *L. monocytogenes* and *S. aureus*, as well as against *E. coli* and *S.* Typhimurium. Other authors (Ito *et al*., 2003; Son *et al*., 2017) also detected a similar bactericidal effect on Gram-negative pathogens. A comparable study model presented results in which the calculated amount of the four groups of pathogens decreased by 2.0 to 5.0 $\log_{10}$ CFU/g (Atassi *et al*., 2016; AlKalbani *et al*., 2019), which is in agreement with our results. However, in contrast to our experimental setup, the authors used lactobacilli isolated from fermented meat products and tested their antibacterial properties in a laboratory environment. The statistical model for the relationship between lactobacilli and their antimicrobial activity showed 90% inhibition of *L. monocytogenes* and up to 96% inhibition of *S.* Enteritidis in the presence of *L. plantarum* (Arena *et al*., 2016). Some authors noted that the decrease of pH in similar products had a little effect on inhibition of *L. monocytogenes*, yet adding nisin from *Lactococcus lactis* to the filling mass significantly inhibited the growth of *L. monocytogenes* in fermented sausages (Hampikyan & Ugur, 2007).

In an *in vitro* study of the antimicrobial activity of *L. plantarum* strains against *Salmonella* spp., a Bulgarian scientific team demonstrated a significant reduction in the concentration of *Salmonella* spp. as early as 60 hours after cocultivation with *L. plantarum*. They explained the high antimicrobial activity of probiotic strains with the decrease in the acidity of the environment due to the production and accumulation of lactic and other organic acids (Teneva *et al*., 2017).

The two varieties of starter culture described in our study kept their growth potential throughout the production process of raw-dried sausages. The inhibition of pathogenic microorganisms was due to bacteriocins produced by *L. plantarum*. Two decades ago Okuyma *et al*. (1999) proved the presence of these enzymes. Studies have also been conducted by several Bulgarian teams, which also demonstrated the ability of *L. plantarum* to inhibit the growth of pathogenic microorganisms (Denkova & Nedelcheva, 2009; Nedelcheva *et al*., 2010). Their studies proved the inhibitory properties of *L. plantarum* against *E. coli*, *Pseudomonas vulgaris*, *Salmonella* spp., *S. aureus* and *L. monocytogenes*. They applied different technologies for growth of *L. plantarum*, in all cases preserving its growth abilities. Data also confirmed the antimicrobial properties of *L. plantarum*, included in starter cultures for the production of meat products.

Some strains of *L. plantarum* were shown to grow even at low pH of the medium (Pennacchia *et al*., 2004). The main antibacterial properties of bacteriocins produced by *L. plantarum* against *S.* Typhimurium, *E. coli*, and *L. monocytogenes* when the pH was about 7 were confirmed by the studies of Muhammad *et al*. (2019). In contrast to the data reported by them, the amount of lactic acid bacteria in our study decreased at the stage of drying in the process of adaptation to the new environment. Recovery was observed during the ripening stage when the pH reached 5.2–5.4.

The use of starter culture variant 2 showed better inhibitory properties against pathogenic bacteria. In all contaminated groups of raw-dried sausages, a significant reduction in the number of pathogens was observed, even complete inhibition of *C. perfringens* and *E. coli*.

The activity against several microorganisms of lactic acid bacteria producing bacteriocins in the production of raw-dried and fermented sausages was proved. A significant number of studies have shown that lactic acid microorganisms can be used to reduce the foodborne pathogens. All this increased their use as preservatives in the production of meat products– an alternative to the chemical compounds used in traditional production (Kęska *et al*., 2017).

In conclusion, the use of starter cultures in the production of raw-dried meat products showed several advantages in terms of the microbiological properties of the products. During the individual stages of production of raw-dried sausages: preparation, filling, ripening, and drying, the amount of lactic acid microorganisms was maintained at a satisfactorily high level. The minimal reduction in the number of living cells in the filling mass was due to the stressful state in which they fall during the adaptation to the new living environment (the filling mass). During the next stages of production of raw-dried sausages, the counts were restored and maintained at a relatively constant level. The metabolites secreted by *Lactiplantibacillus plantarum* GLP3 during its replication, the main of which was lactic acid, changed the pH, and at the same time had an inhibitory effect on the pathogenic and sanitary indicator microorganisms in the tested meat product "Lukanka Panagyurska".

The use of sodium nitrate, reduced to sodium nitrite in the tested samples of "Lukanka Panagyurska", also killed some of the pathogens and sanitaryindicator microorganisms. In experimental groups of product (free of nitrates/nitrites), produced with the two variants of the starter culture, complete inhibition of the microbial agents was observed.

All this gave us reasons to conclude that variant 2 of the ProViotic starter culture containing *L. plantarum* and *D. hansenii* was a promising alternative in the production of raw-dried meat product "Lukanka Panagyurska" for obtaining microbiologically safe foods free of chemicals such as nitrites and nitrates.

ACKNOWLEDGEMENTS

This study was performed and financed with the support of Mr Kiril Petkov and his company ProViotic AD. Presented data showing only the scientific part of the project were categorically confirmed in another Bulgarian and in a French laboratory before the meat product was offered in retail stores**.**

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Paper received 12.04.2021; accepted for publication 24.08.2021

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