

## THE INFLUENCE OF THE DENITRIFYING STRAIN OF *STAPHYLOCOCCUS CARNOSUS* NO. 5304 ON THE CONTENT OF NITRATES IN THE TECHNOLOGY OF YOGURT PRODUCTION

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### ABSTRACT

Contamination of food with nitrates is a generally recognized problem. Milk is the basis for the production of many milk mixtures for baby food, and children are considered to be the most vulnerable category to the harmful influence of nitrates. The purpose of the search was to investigate the denitrification of milk with different amounts of nitrates by the denitrifying microorganisms of *Staphylococcus carnosus* in the technology of production of sour-milk products. The denitrification process of *S. carnosus* milk in the amount of 103 CFU.cm<sup>-3</sup> was found to reduce the nitrate content by an average of 88.0 ± 3.9 mg.kg<sup>-1</sup> and in the samples of the first group was 10.3 ± 2.4 mg.kg<sup>-1</sup>, the second 110.7 ± 4.1 and the third 214.5 ± 6.3 mg.kg<sup>-1</sup>, respectively. In the search of the denitrification process of *S. carnosus* milk in the amount of 104 CFU.cm<sup>-3</sup>, was found that in the ready yogurt in the samples of the first group the amount of nitrates was 1.1 ± 0.1 mg.kg<sup>-1</sup>, in the second group 56.4 ± 3.5 mg.kg<sup>-1</sup>, and in the third 159.5 ± 4.1 mg.kg<sup>-1</sup> respectively. In the search of the denitrification process of *S. carnosus* milk in the amount of 105 CFU.cm<sup>-3</sup>, was found that nitrates were practically absent in the samples of the first group, the second group did not exceed 10 mg.kg<sup>-1</sup>, and the third was 107.4 ± 3.9 mg.kg<sup>-1</sup>. Therefore, received data indicate the possibility of using strain *S. carnosus* No. 5304 for denitrification of milk with a high content of nitrates in the technology of production of fermented milk products, in particular yogurt.

**Keywords:** milk nitrates; milk denitrification; yogurt technology

### INTRODUCTION

Contamination of raw materials, water, and food with nitrates is a generally recognized problem (Chamandust et al., 2016; Kukhtyn et al., 2018). Nitrates of food and water in the organism convert hemoglobin into meth and sulfhemoglobin, causing the development of hypoxia (Jiang et al., 2012; Cockburn et al., 2013). Also, food nitrates are considered precursors of highly carcinogenic nitrous compounds (Winter et al., 2007; Hord et al., 2011). The widespread use of nitrogen fertilizers in agriculture causes a large amount of nitrates to be supplied from soil to plants and foodstuffs (Croitoru et al., 2015; Quijano et al., 2017), we often note the significant content of nitrates in drinking water for the population (Kalaruban et al., 2016; Wang and Chu, 2016). Nitrates are also added to foods (cheese, sausage) to inhibit the development of spore-forming microorganisms (Sungur and Atan, 2013).

Children are the most vulnerable and vulnerable to nitrate toxicosis, as the blood enzyme methemoglobin reductase, which restores methemoglobin to hemoglobin, begins to form in humans only at the age of three months (Yeh et al., 2013). Besides, nitrates are poorly utilized in people with gastrointestinal disorders who have impaired intestinal

microbiocenosis (Sindelar and Milkowski, 2012). Therefore, children are virtually unprotected from nitrate toxicosis, and people with dysbacteriosis are more sensitive to the influence of nitrates (Khosrokhavar et al., 2008; Hord et al., 2009). Because cow's milk belongs to the main food products, the determination of nitrate content in milk and dairy products is essential for the prevention of chronic influence of nitrates on children.

Various nitrates in cow's milk have been reported in the literature, so researchers in Italy found in the milk the amount of nitrates up to 140 mg.L<sup>-1</sup> (Licata et al., 2013), while in Iranian cows milk nitrates ranged from 34 to 82.5 mg.L<sup>-1</sup>. (Chamandust et al., 2016). When the content of nitrates is determined in 20 cow's milk samples in Taiwan, it was revealed an average concentration of 14.3 mg.L<sup>-1</sup> (Yeh et al., 2013), and researchers in Romania in the search of 95 milk samples revealed no more than 5 mg.L<sup>-1</sup> of nitrates over a four-year research period (Filip et al., 2019). In Ireland under the research of skimmed milk powder was found nitrate concentration in the range from 5 to 120 mg.L<sup>-1</sup> (Yeh et al., 2013). Thus, literature sources report that milk can be a source of significant input into the organism of nitrate consumers.

According to Regulation (EC) No 1881/2006 (EC, 2006) in foodstuffs (dairy mixtures), the permissible nitrate level is 50 to 200 mg.kg<sup>-1</sup>. In the Ukrainian standard for raw milk, cow's milk with a nitrate content of not more than 10 mg.kg<sup>-1</sup> is authorized for processing (DSTU 3662, 2018). The national standards of the People's Republic of China GB 2761-2017, GB 2762-2017 (GB, 2017) regulate the content of nitrites in raw milk, and they should not exceed 0.4 mg.kg<sup>-1</sup>, and in milk powder 4 mg.kg<sup>-1</sup> (GB, 2017).

Also, no data were found in the literature on the use of denitrifying microorganisms in the technology of dairy products. Researching the influence of microflora on the content of nitrates in the fermentation process of milk is promising since it would allow the production of low-nitrate dairy products.

Consequently, monitoring searches on the determination of nitrates and nitrites content in milk and dairy products will be important to ensure the safety of products and to develop recommendations and technologies for the possible reduction of these harmful substances.

The purpose of the search was to investigate the denitrification of milk with different amounts of nitrates by the denitrifying microorganisms of *Staphylococcus carnosus* in the technology of production of sour-milk products.

### Scientific hypothesis

Is the multiple uses of denitrifying strain *Staphylococcus carnosus* No. 5304 for milk denitrification with excessive content to a safe amount of nitrates in the technology of production of fermented milk products?

## MATERIAL AND METHODOLOGY

### Samples

In the experiments, 15 samples were used of milk with different amounts of nitrates. Milk of the first group (n = 5) had a nitrate content in the range of 100.2 ± 3.4 mg.kg<sup>-1</sup>; milk of the second group (n = 5) was with a nitrate content of 200.1 ± 5.3 mg.kg<sup>-1</sup>; third (n = 5) – 300.1 ± 6.8 mg.kg<sup>-1</sup>.

### Chemicals

To get milk with the amount of nitrates 100, 200 and 300 mg.kg<sup>-1</sup> to it was added KNO<sub>3</sub>. Chemically pure KNO<sub>3</sub> (Khimreaktiv, Ukraine) was used. To quantify *Staphylococcus carnosus* in yogurt was used the medium of yolk-salt agar (Pharmactive, Ukraine). For the production of yogurt was used leaven YoFlex®Acidifixtm 1.0 (Chr. Hansen, Denmark), which contains *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus salivarius subsp. thermophilus*. Avirulent, incapable of invasion strain of *Staphylococcus carnosus* No. 5304 was used in the experiments, which is deposited in the Institute of Microbiology and Virology named after D.K. Zabolotnyj (Kyiv, Ukraine).

### Laboratory Methods

The content of nitrates in milk and yogurt was determined by colorimetric method using a cadmium column, followed by photometric determination of azo compounds, formed by the interaction of nitrites with aromatic amines (GOST

32257, 2013). Herewith a LabAnalyt spectrophotometer (Granum, Ukraine) was used for determination of optical density of solutions, laboratory ionometer I-160MI (Micromed, Russia) to determine the pH. Water bath BN-09.2 LabAnalyt (Granum, Ukraine).

The titrated acidity of milk and yogurt was determined by the classical titrimetric method (GOST 3624, 1992).

### Description of the Experiment

The technological process of making yogurt involved the introduction of a culture of *Staphylococcus carnosus* in an amount of from 10<sup>3</sup> to 10<sup>5</sup> CFU.cm<sup>-3</sup> of milk, denitrification for 2 hours at a temperature of +37 °C, and subsequent application of sourdough cultures of lactic acid bacteria *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus salivarius subsp. thermophilus* followed by fermentation for 6 h at a temperature of 40 – 42 °C to the acidity of the product of 75 – 85 °T. After fermentation, the product was rapidly cooled to 4 °C and stored for 24 hours. After 2, 4, 8 and 24 hours, the nitrate content, the acidity of the product, and the amount of *Staphylococcus carnosus* were determined. All research was performed in triplicate.

### Statistical Analysis

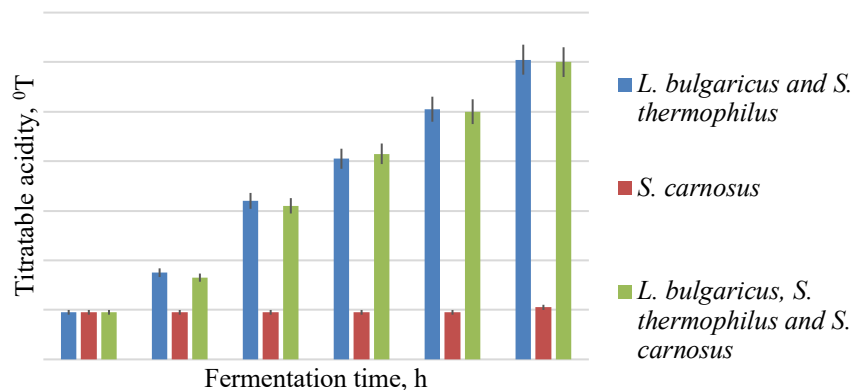
Statistical processing of the results was carried out using methods of variation statistics using the program Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the standard error of the mean (SE) were determined. The difference between the comparable values was considered to be significant for p < 0.05.

## RESULTS AND DISCUSSION

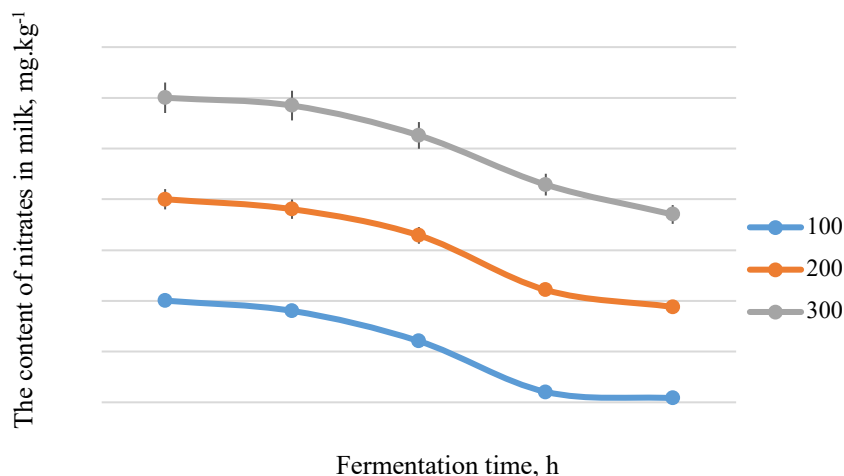
Previous research has revealed that 20.7 to 30.2% of batches of raw milk containing nitrates (more than 100 mg.kg<sup>-1</sup>) are coming to processing plants in the winter-spring period, in summer and autumn, the number of such batches of milk ranges from 2.1 to 7.8%. Therefore, we have researched the use of *S. carnosus* in a consortium of *L. bulgaricus* and *S. thermophilus* in the technology of production of fermented milk products as a possible denitrifying microorganism.

For the production of yogurt, an important technological indicator is the titrated acidity, which characterizes the acid-forming properties of lactic acid bacteria. Whereas, we offer additionally before fermentation for milk denitrification with a high content of nitrates to introduce the bacterium culture of *S. carnosus*, therefore, in the first stage of the search, the influence of this type of bacteria was determined on the change in the titrated acidity of sterilized milk, provided that it is used with classic sourdough yogurt (Figure 1).

It was found that the fermentation of milk by the fermentation of *L.bulgaricus* and *S.thermophilus* was carried out following the requirements of the technological instructions since the acidity did not exceed 85 °T after 6 h of fermentation. At the same time, the denitrifying species of *S. carnosus* practically did not ferment lactose, the acidity of milk did not change within 8 hours of fermentation, and after 24 hours was increased to 21 °T. Under the condition of fermentation of milk by a



**Figure 1** Change of titrated acidity of sterilized milk by fermentation of *L. bulgaricus* and *S. thermophilus* and in consortium with *S. carnosus* at +40 °C.



**Figure 2** Dynamics of the process of denitrification of milk with different nitrate content under the influence of *S. carnosus* in the amount of  $10^3$  CFU.cm<sup>-3</sup>.

consortium of microorganisms *L. bulgaricus*, *S. thermophilus* and *S. carnosus*, the dynamics of change of titrated acidity were as if using classical leaven.

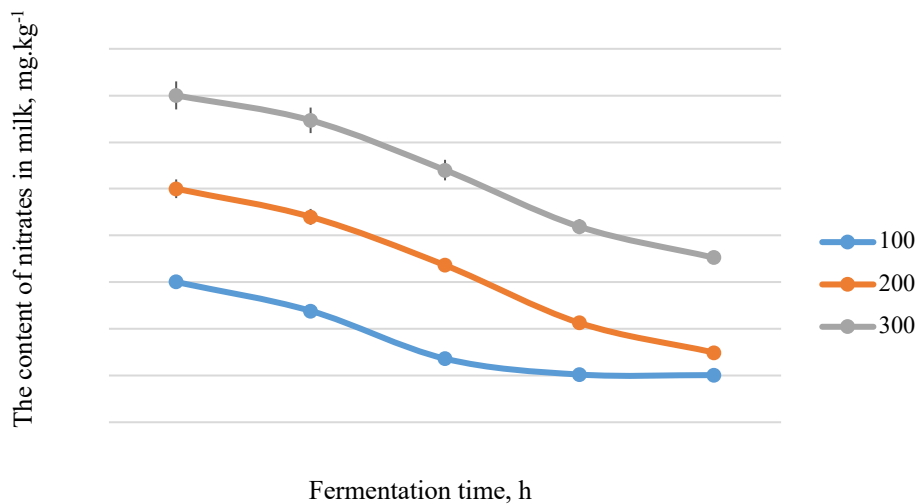
The results of researches on the process of denitrification of milk with different nitrates are given with different nitrate content under the influence of *S. carnosus* in the amount of  $10^3$  CFU.cm<sup>-3</sup> revealed (Figure 2), that for the initial amount of *S. carnosus*  $10^3$  CFU.cm<sup>-3</sup> of milk within two hours of denitrification, the nitrate content was decreased by an average of 10 mg.kg<sup>-1</sup>.

As can be seen from Figure 2, for the initial amount of *S. carnosus*  $10^3$  CFU.cm<sup>-3</sup> of milk within two hours of denitrification, the nitrate content was decreased by an average of 10 mg.kg<sup>-1</sup>. Over the next two days (for the fourth hour of fermentation), the nitrate content was decreased by  $37.4 \pm 3.1$  mg.kg<sup>-1</sup>, and at the end of the yogurt making process, their number was decreased in all samples by  $88.0 \pm 3.9$  mg.kg<sup>-1</sup> and in the samples of the first group was  $10.3 \pm 2.4$  mg.kg<sup>-1</sup>, the second  $110.7 \pm 4.1$  and the third  $214.5 \pm 6.3$  mg.kg<sup>-1</sup>, respectively. It was also found that,

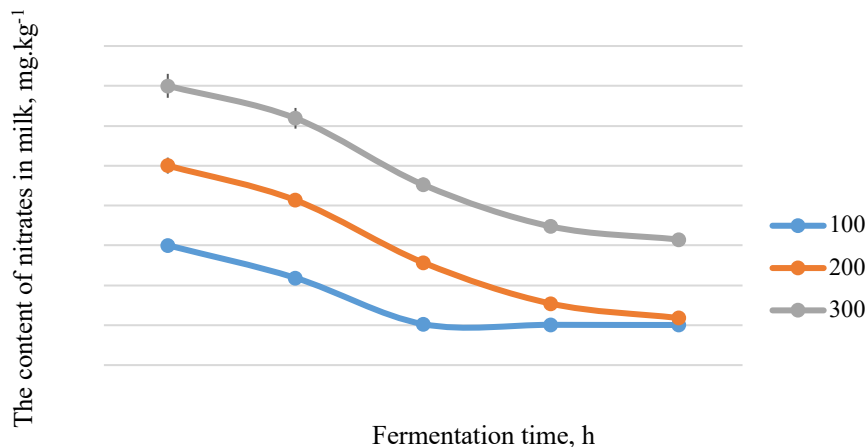
after cooling to +4 °C and endurance for 16 h (24 h), the denitrifying process did not stop, as the nitrate content was decreased by  $6.2 \pm 0.3$ ,  $16.7 \pm 1.2$  and  $29.4 \pm 2.4$  mg.kg<sup>-1</sup>, respectively, possibly related to the nitroductase denitrifying action that accumulated in yogurt during *S. carnosus* development.

The results of searches of the process of milk denitrification are presented with different nitrate contents under the influence of *S. carnosus* in the amount of  $10^4$  CFU.cm<sup>-3</sup> revealed (Figure 3), indicates the similar dynamics of nitrate reduction in milk as in Figure 2.

However, the intensity of the denitrification process at the initial amount of *S. carnosus*  $10^4$  CFU.cm<sup>-3</sup> of milk was faster than its content of  $10^3$  CFU.cm<sup>-3</sup>. Thus, during the first two hours of denitrification, the amount of nitrates was decreased by  $29.1 \pm 3.2$  mg.kg<sup>-1</sup>, and during the four hours by  $81.3 \pm 2.1$  mg.kg<sup>-1</sup>. In the yogurt samples with an initial nitrate content of  $100.2 \pm 3.4$  mg.kg<sup>-1</sup> milk, their amount was  $18.1 \pm 1.4$  mg.kg<sup>-1</sup>, and in the second group samples  $118.2 \pm 3.7$  mg.kg<sup>-1</sup>, respectively.



**Figure 3** Dynamics of milk denitrification process with native nitrate content under the influence of *S. carnosus* in the amount of  $10^4$  CFU.cm<sup>-3</sup>.



**Figure 4** Dynamics of denitrification of milk with native nitrate content under the influence of *S. carnosus* in the amount of  $10^5$  CFU.cm<sup>-3</sup>.

After 8 hours of fermentation the denitrification in the samples of the first group was revealed by  $1.1 \pm 0.1$  mg.kg<sup>-1</sup>, in the second group by  $56.4 \pm 3.5$  mg.kg<sup>-1</sup>, and in the third group by  $159.5 \pm 4.1$  mg.kg<sup>-1</sup>. After 24 hours from the start of fermentation, the amount of nitrates in the samples of the second group was  $24.7 \pm 2.1$  mg.kg<sup>-1</sup>, and  $126.3 \pm 4.6$  mg.kg<sup>-1</sup> in the third group.

The results of researches on milk denitrification process with different nitrate content under the influence of *S. carnosus* in the amount of  $10^5$  CFU.cm<sup>-3</sup> (Figure 3), revealed the preservation of the general trend of denitrification of *S. carnosus* nitrates, which are shown in Figures 2 and 3.

Data of Figure 4 indicates the retained general tendencies for denitrification of *S. carnosus* nitrates, which are shown in Figures 2 and 3. However, with the initial amount of *S. carnosus*  $10^5$  CFU.cm<sup>-3</sup> of milk, nitrate content in the samples of the first group was almost undetectable after 8 hours of denitrification, and in the samples of the second group the nitrate content was  $27.1 \pm 2.3$  mg.kg<sup>-1</sup> of product.

In the finished chilled yogurt, nitrates were practically absent in the samples of the first group, in the second group their amount did not exceed 10 mg.kg<sup>-1</sup>, and in the third was  $107.4 \pm 3.9$  mg.kg<sup>-1</sup>.

In the second stage, our searches were directed to study the activity (reproduction) of *S. carnosus* No. 5304 in sterilized milk in a consortium with lactic acid bacteria *L. bulgaricus*, *S. thermophilus*, which are used in yogurt production technology (Figures 5 and 6).

It was found that *S. carnosus* is developing well in sterilized milk, during the eight hours of incubation the number of cells is increased by more than 2.5 orders of magnitude. In this case, the growth curves of *S. carnosus* with different initial numbers have almost the same nature of increase. At the same time, the data of Fig. 6 indicate the onset of the antagonistic action of lactic acid microorganisms used in sourdough for yogurt (*L. bulgaricus*, *S. thermophilus*) on the development of *S. carnosus* No. 5304.

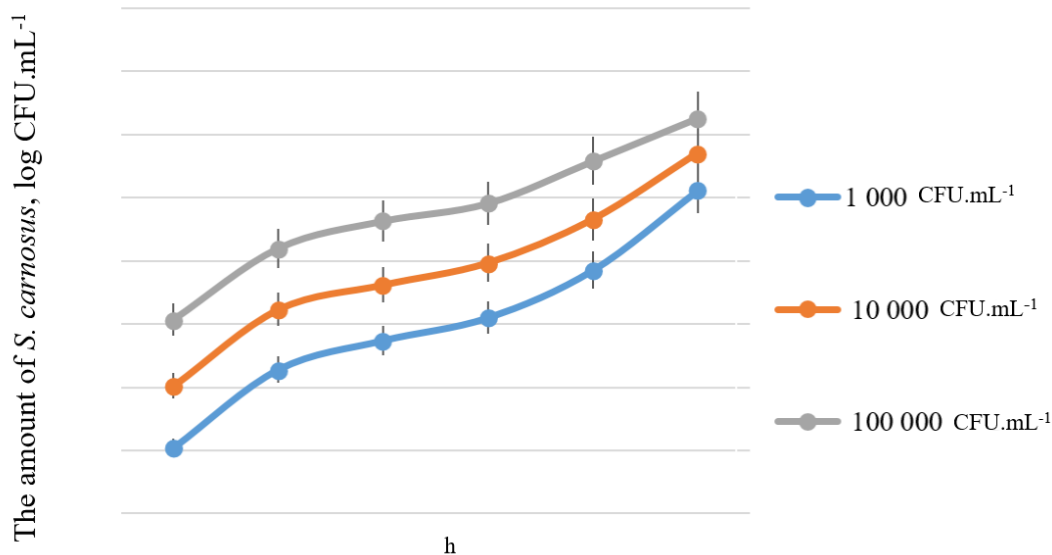


Figure 5 Reproduction of *S. carnosus* No. 5304 in sterilized milk with different initial amounts at +40 °C.

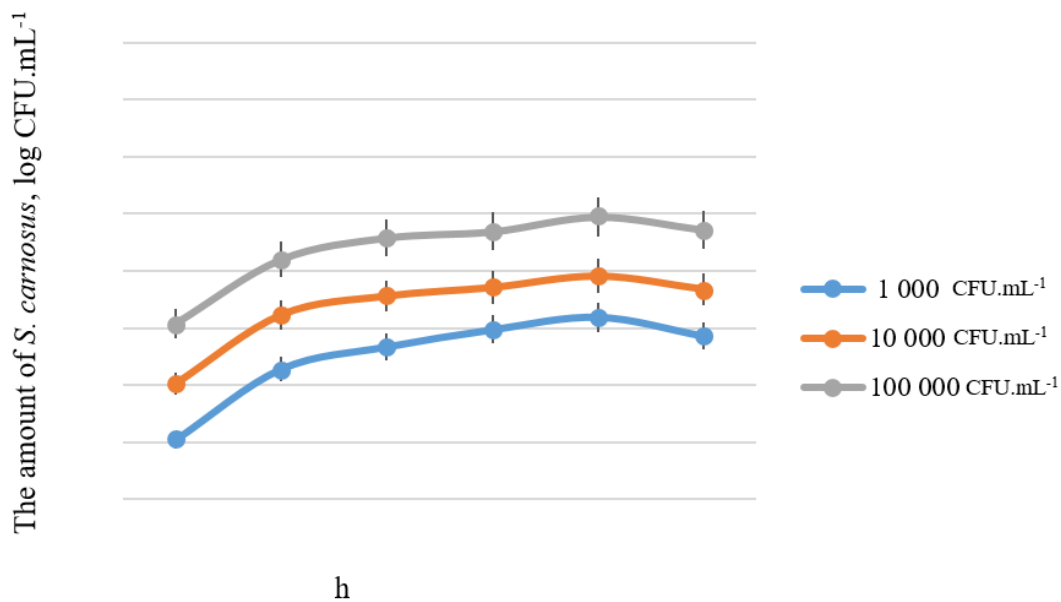


Figure 6 The influence of fermentation of lactic acid bacteria (*L. bulgaricus*, *S. thermophilus*) on the reproduction of *S. carnosus* in sterilized milk with different initial amounts at +40 °C (Ferment of lactic acid bacteria introduced for 3 h of incubation).

Within 6 hours after the introduction of the leaven, we notice a slowdown in the reproduction of *S. carnosus*, compared with the development of pure culture. At 24 hours the search for staphylococcus reproduction was completely stopped their number even decreased due to the high acidity of the product.

The data obtained indicate that the process of denitrification takes place in the first hours of fermentation, and subsequently the enzymatic activity of staphylococci is suppressed by lactic acid microorganisms.

Nitrates and nitro replacement have a detrimental influence on human health and can accumulate and spread in an alarming amount in the “soil-plant-animal-human” trophic chain (Bahadoran et al., 2016; Chamandust et al., 2016; Kukhtyn et al., 2018a; Hou et al., 2020). Therefore, the production of safe food products is an urgent issue, which is exacerbated by increased consumption and

reduced food raw materials. Searches show that there is now a problem of nitrates in raw milk and dairy products (Licata et al., 2013; Sungur and Atan, 2013; Chamandust et al., 2016). Thus, researchers (Sungur and Atan, 2013) found that 50% of cheese samples in Turkey had more nitrate levels than allowed, which is specified in the EU Regulation No. 1881/2006 (EC, 2006). Our research also found samples of raw milk with a nitrate content of more than 100 mg.kg<sup>-1</sup>.

At the same time, milk is the basis for the production of many milk mixtures for baby food, and children are considered to be the most vulnerable category to the harmful influence of nitrates. (Thomson, Nokes and Cressey, 2007; Chetty and Prasad, 2016; Merino et al., 2017). In addition, scientists report that in people with chronic diseases of the stomach and intestines, the utilization of nitrates by the microbiome of the large

intestine is slowed down. Therefore, the amount of nitrates required for chronic nitrate poisoning will be less (Winter et al., 2007; Sindelar et al., 2012; Kalaycioglu and Erim, 2019).

Therefore, we support the opinion of other researchers (Hord et al., 2011; Jiang et al., 2012) about the need to reduce the maximum amount of nitrates in dairy products, especially for baby food. Besides, according to the national standards of the Republic of China, the amount of nitrites is regulated in raw milk, and in the European regulation this rate is absent (EC, 2006; GB, 2017).

Taking into account the above data, the influence of denitrifying bacteria *S. carnosus* on the dynamics of changes in the amount of nitrates in the technology of production of fermented milk products were investigated. Eventually, non-pathogenic denitrifying strains of bacteria can be actively developed along with lactic acid bacteria without changing the organoleptic and physicochemical properties of the product. In particular, researchers (Bal-Prylypko and Leonova, 2015) used strains of *S. carnosus* and *S. carnosus* spp. utilis in the technology of production of cooked sausages for utilization of sodium nitrite to safe quantities. Our research found that the denitrifying species of *S. carnosus* does not ferment lactose, as the titrated acidity of milk within 8 hours of fermentation did not change. Therefore, we believe that the development of milk denitrifying species of *S. carnosus* in milk does not influence the activity of the fermenting cultures of lactic acid bacteria *L. bulgaricus* and *S. thermophilus*.

In the search of the process of denitrification of milk with different content of nitrates of *S. carnosus* in the amount of  $10^3$  CFU.cm<sup>-3</sup>, it was found that at the end of the technological process of making yogurt (8 h) the amount of nitrates was decreased, on average by  $88.0 \pm 3.9$  mg.kg<sup>-1</sup> and in the samples of the first group was  $10.3 \pm 2.4$  mg.kg<sup>-1</sup>, the second  $110.7 \pm 4.1$  and the third  $214.5 \pm 6.3$  mg.kg<sup>-1</sup> respectively.

In the search of the denitrification process of *S. carnosus* milk in the amount of  $10^4$  CFU.cm<sup>-3</sup>, it was found that  $1.1 \pm 0.1$  mg.kg<sup>-1</sup> of nitrates were found in the prepared yogurt in the samples of the first group, in the second group  $56.4 \pm 3.5$  mg.kg<sup>-1</sup>, and in the third  $159.5 \pm 4.1$  mg.kg<sup>-1</sup>, respectively. Thus, the received data indicate that an increase in the initial amount of *S. carnosus* denitrifying bacteria in milk before fermentation influences the denitrification intensity.

In the search of the denitrification process of *S. carnosus* milk in the amount of  $10^5$  CFU.cm<sup>-3</sup>, it was found that nitrates were practically absent in the samples of the first group, in the second group did not exceed  $10$  mg.kg<sup>-1</sup>, and the third group was  $107.4 \pm 3.9$  mg.kg<sup>-1</sup>. Therefore, received data indicate the possibility of using strain *S. carnosus* No. 5304 for denitrification of milk with a high content of nitrates in the technology of production of fermented milk products, in particular yogurt.

Scientists have mostly investigated the influence of denitrifying bacteria of the genus *Pseudomonas* on the reduction of nitrate content in milk (Samigullin and Ezhkova, 2004), which found no implementation due to organoleptic changes in the product during the development of these bacteria. The influence of technological processing (separation) of milk on the reduction of nitrates in products was also investigated

(Lima et al., 2006). In addition, there are literature data on that heat treatment of vegetables and fruits does not significantly affect on reduction of nitrates in finished products (Min et al., 2012; Habermeyer et al., 2015; Inoue-Choi et al., 2016; Bahadoran et al., 2016). However, technological process of vegetable fermentation significantly reduced nitrate content practically to the level of  $1 - 5$  mg.kg<sup>-1</sup>. (Kukhtyn et al., 2018b). In this search, it was found that *S. carnosus* develops well in milk and exhibits denitrifying properties without altering organoleptic properties. Lactic acid bacteria (*L. bulgaricus*, *S. thermophilus*) inhibit the development of *S. carnosus*, and in the ready product, its amount is reduced, which is probably due to the high acidity of the product. Therefore, we consider the possibility of using *S. carnosus* strain No. 5304 in the content of the leaven, which could be used for denitrification of milk with a high content of nitrates. The received data are of practical interest in terms of enabling the use of a safe method for processing milk with a nitrate content greater than the maximum amount.

So, as a result of the search, we note that, given the negative influence of nitrates of water r, raw materials and food nitrates on the organism of consumers, monitoring searches of milk content should be carried out. Based on the received data, it is possible to develop a ferment for denitrification of milk with a high content of nitrates.

## CONCLUSION

The denitrification process of *S. carnosus* milk in the amount of  $10^3$  CFU.cm<sup>-3</sup> was found to reduce the nitrate content by an average of  $88.0 \pm 3.9$  mg.kg<sup>-1</sup> and in the samples of the first group was  $10.3 \pm 2.4$  mg.kg<sup>-1</sup>, the second  $110.7 \pm 4.1$  and the third  $214.5 \pm 6.3$  mg.kg<sup>-1</sup>, respectively. In the search of denitrification of milk *S. carnosus* in the amount of  $10^4$  CFU.cm<sup>-3</sup>, it was found that in the ready yogurt in the samples of the first group the amount of nitrates was  $1.1 \pm 0.1$  mg.kg<sup>-1</sup>, in the second group  $56.4 \pm 3.5$  mg.kg<sup>-1</sup>, and in the third  $159.5 \pm 4.1$  mg.kg<sup>-1</sup>, respectively.

In the search of the process of denitrification of *S. carnosus* milk in the amount of  $10^5$  CFU.cm<sup>-3</sup>, it was found that nitrates were practically absent in the samples of the first group, the second group did not exceed  $10$  mg.kg<sup>-1</sup>, and in the third was  $107.4 \pm 3.9$  mg.kg<sup>-1</sup>.

The antagonistic influence of lactic acid microorganisms, leaven (*L. bulgaricus*, *S. thermophilus*) on the development of *S. carnosus* No. 5304 has been revealed. 6 hours after the introduction of the leaven, we notice a slowdown in the reproduction of *S. carnosus*, and for 24 hours the reproduction of staphylococci has completely stopped.

Therefore, the received data indicate the possible use of *S. carnosus* strain No. 5304 in the content of the leaven for denitrification of milk with a high content of nitrates.

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