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The effect of *Bacillus* in the composition of the probiotic Natupro® against bacterial pathogenic strains *in vitro*

ABSTRACT

Relevance. The use of probiotics for health benefits is becoming popular because of the quest for safer products with protective and therapeutic effects against diseases and infectious agents. The emergence and spread of antimicrobial resistance among pathogens had prompted restrictions over the non-therapeutic use of antibiotics for prophylaxis and growth promotion, especially in animal husbandry. While single-strain probiotics are beneficial to health, multi-strain probiotics might be more helpful because of synergy and additive effects among the individual isolates.

Methods. In this study, the effectiveness of the multi-strain probiotic Natupro on pathogenic bacterial strains (*Escherichia coli* 320, *Salmonella typhimurium* 415, *Staphylococcus aureus* 12600, *Streptococcus uberis* 700407, *Klebsiella pneumoniae* 13883, *Listeria monocitogenes* 766/20) *in vitro* was studied.

Results. According to the results of the work carried out, it was found that the bacterial strains in Natupro® exhibit lytic activity against pathogenic strains of *Salmonella typhimurium* 415 and *Klebsiella pneumoniae* 13883 and have a bacteriostatic effect on *Escherichia coli* strain 320 and *Salmonella typhimurium* 415.

Key words: bacillus, probiotic, *Salmonella*, antimicrobial property, antimicrobial effect

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Научная статья

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Эффективность применения бактерий рода *Bacillus* в составе пробиотика Natupro® против штаммов патогенных бактерий *in vitro*

РЕЗЮМЕ

Актуальность. Использование пробиотиков с пользой для здоровья становится популярным в связи с поиском более безопасных продуктов, обладающих защитным и терапевтическим действием против болезней и инфекционных агентов. Появление и распространение устойчивости к противомикробным препаратам среди патогенных микроорганизмов привели к введению ограничений на нетерапевтическое применение антибиотиков для профилактики и стимулирования роста, особенно в животноводстве. В то время как пробиотики с одним штаммом полезны для здоровья, пробиотики с несколькими штаммами могут быть более полезными из-за синергизма и аддитивных эффектов отдельных изолятов.

Методы. В данном исследовании была изучена эффективность мультиштаммового пробиотика Natupro в отношении патогенных штаммов бактерий (*Escherichia coli 320*, *Salmonella typhimurium* 415, *Staphylococcus aureus* 12600, *Streptococcus uberis* 700407, *Klebsiella pneumoniae* 13883, *Listeria monocitogenes* 766/20) *in vitro*.

Результаты. По результатам работы было установлено, что штаммы бактерий в составе Natupro® проявляют литическую активность в отношении патогенных штаммов *Salmonella typhimurium* 415 и *Klebsiella pneumoniae* 13883 и оказывают бактериостатическое действие на штамм *Escherichia coli* 320 и *Salmonella typhimurium* 415.

Ключевые слова: bacillus, пробиотик, *Salmonella*, противомикробное действие, антимикробный эффект

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Introduction/Введение

Intensive use of antibiotics has led to an increase in the resistance of pathogenic bacteria to their effects and the accumulation of these drugs in livestock products. The solution to this global problem requires the search for alternative tools to combat pathogens of bacterial infections, which include some strains of bacteria of the genus *Bacillus* [1]. The advantages of spore probiotics are their resistance to the acidic environment of the stomach and remain stable for a long time [2].

A very vital functional property attributed to the *Bacillus* strains is the ability to produce different types of antimicrobial compounds having a broad spectrum of activity against bacteria and fungi [3–6].

Earlier *in vitro* studies have shown high antibacterial activity of 7 different strains of *B. subtilis*, extracted from the soil, against 15 pathogenic strains of *E. coli* [7]. Another study revealed a significant bactericidal effect from the use of *Bacillus subtilis* CP9 from desert camel against *E. coli* (ETEC), *Salmonella typhimurium* and methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* [8].

In addition to *B. subtilis*, *B. amyloliquefaciens* can be successfully used as probiotic strains to suppress the growth of pathogenic *E. coli* [9]. Other studies have revealed the ability of some strains of *B. licheniformis* to inhibit the growth of pathogenic strains of *Prevotella intermedia* and *Streptococcus mutans* [10].

Thus, preparations based on bacteria of the *Bacillus spp*. can be studied for the fight against pathogens of bacterial infections. Such a promising feed additive is Natupro® based on the bacteria *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens*.

In this study, we evaluated the antagonistic activity of the strains of which it consists against pathogenic bacteria *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus uberis*, *Klebsiella pneumoniae* and *Listeria monocitogenes*, which are pathogens of infectious diseases in farm animals.

Materials and methods /
Материалы и методы исследования

Probiotic bacterial cultures

The object of the study is Natupro® — feed additive for the normalization of gastrointestinal tract microflora and increasing the natural resistance of the body of farm animals and poultry. This probiotic is developed by Bioproton Europe OY[1] company (Kaarina, Finland) and contains four of *Bacillus* strains: *B. subtilis* BP-0-13-1 — $1.5x10^8$ CFU/g, *B. licheniformis* BP-0-12-1 — $1.5x10^8$ CFU/g, *B. amyloliquefaciens* BP-0-11-1 — $1.5x10^8$ CFU/g and *B. amyloliquefaciens* BP-0-14-1 — $1.5x10^8$ CFU/g.

Pathogenic organisms

Pathogens of farm animals and birds were used to study the antibacterial effect of the additive. Cultures of *Escherichia coli* strain 320, *Salmonella typhimurium* strain 415, *Staphylococcus aureus* strain 12600, *Streptococcus uberis* strain 700407, *Klebsiella pneumoniae* strain 13883, *Listeria monocitogenes* strain 766/20 were obtained from the All-Russian State Collection of strains of microorganisms used in veterinary medicine and animal husbandry (VGNKI[2], Russia).

Determination of total Bacillus strain in probiotic Natupro®

The goal of this stage of the study was to determine the total number of microorganisms included in the feed additive.

Studies were conducted in accordance with the following GOST 31928[3].

For this purpose, 1 g of the feed additive taken from the average sample was placed in a sterile test tube (the feed additive was taken for a single sample), 9 ml of physiological solution was added and thoroughly mixed (initial dilution 1×10^{-1}). The following dilutions were prepared from the resulting suspension (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}: 10^{-7}, 10^{-8}). Seeding was done after sedimentation of suspended particles from the upper layer of the liquid.

For quantitative accounting of bacterial content *B. amyloliquefaciens* strain BP-0-11-1, *B. amyloliquefaciens* strain BP-0-14-1, *B. licheniformis* strain BP-0-12-1, *B. subtilis* strain BP-0-13-1 in 1 ml of probiotic Natupro® of the following dilutions were added to sterile bacteriological dishes 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}. Then 15–20 ml of sterile, melted and cooled to 44–45 °C meat-peptone agar was poured. By carefully shaking the dishes, the seeded material was evenly distributed in the agar. After the medium solidified, the cups were placed (upside down) in a thermostat at 37 °C.

After 24–48-hour thermostatic conditioning, the grown colonies were counted only in the dishes counting were multiplied by the dilutions, summed up, and the number of microorganisms in 1 g of the Natupro® was determined.

Selection of optimal nutrient medium

The studies were conducted according to the following MUK 4.2.2316[4].

In determining the optimal medium, the following parameters were investigated: differentiation properties, sensitivity of the nutrient medium and inhibitory characteristics.

The indicator of growth-supporting characteristics was determined by the minimum incubation time, during which distinct growth of microbial cultures on nutrient medium was observed, visible to the naked eye.

At the same, we determined the optimal seeding dose of each type of test cultures and microorganisms included in the feed additive on selected for work selective nutrient medium. For this purpose, samples of Natupro® were weighed by 1 gram and packed into sterile tubes and then a series of serial dilutions in 0.9% sodium chloride solution was carried out. Simultaneously, we prepared suspensions of daily agar test cultures according to the turbidity standard (L.A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations), which corresponded to 10^9 CFU/ml (the first tube) and made serial dilutions according to a similar scheme. After that, 100 µl of dilutions 10^{-7}, 10^{-8} and 10^{-9} were inoculated on the test medium. The cultures were cultured at 37 °C during 24 hours.

The diagnostic medium was considered suitable if the average number of growing colonies on a Petri dish in a 100-cell culture was at least 50. In addition, the medium must have differentiating characteristics, ensure the growth of morphologically typical colonies and inhibit the growth of microflora not specific for the medium.

[1] https://www.bioproton.com/
[2] https://www.vgnki.ru/
[3] GOST 31928-2013 Probiotics medicine remedies for veterinary use. Methods for determination of the probiotics microorganisms (in Russian).
[4] MUK (Methodological instructions) 4.2.2316-2008 Methods of control of bacteriological nutrient media: Methodological guidelines. Moscow: Federal Center for Hygiene and Epidemiology of Rospotrebnadzor. 2008; 67 (in Russian).

Determination of bactericidal characteristics of Natupro®

The studies were performed according to the MUK 4.2.1890[5]. For this purpose, the test samples of Natupro® were weighed by 1 gram each and into sterile test tubes containing 1 ml of 0.9% sodium chloride solution. Then, 10 µl of the obtained feed additive suspension was injected by "injection" on the surface of Meat-Peptone agar in Petry dishes previously seeded with "lawn" bacterial test cultures of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus uberis*, *Klebsiella pneumoniae* and *Listeria monocitogenes*. The cultures were incubated for 18–24 hours at 37 °C, after which the results of the experiment were recorded by the formation of light zones of growth retardation of test cultures.

Determination of lytic activity of strains included in Natupro®

The studies were carried out according to the following scheme: isolated cultures of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* were isolated from Natupro®. For this purpose, test samples of feed additive were weighed by 1 g each and put into sterile test tubes containing 1 ml of 0,9 sodium chloride solution. Then, 100 µl of the obtained feed additive suspension was added to the MPA surface in Petri dishes and dispersed using the Drigalsky method to obtain individual colonies. Petri dishes were placed in a thermostat and cultured at 37 °C for 24 hours. Individual colonies characteristic of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* strains by their morphological and cultural properties were selected and seeded in test tubes with meat-peptone broth. The cultures were cultured in an incubator at 37 °C for 24 hours. Then, 10 µl of the obtained cultures of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* strains were injected by the "injection" method on the MPA surface into Petri dishes previously seeded with "lawn" bacterial test cultures of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus uberis*, *Klebsiella pneumoniae* and *Listeria monocitogenes*.

Seeds were incubated for 24 hours at 37 °C, after which the results of the experiment were counted by measuring the diameter of the light zones of lysis of test cultures.

Determination of bacteriostatic characteristics of Natupro®

In order to study the bacteriostatic effect, samples of Natupro® were weighed by 1 g and packed into sterile tubes for further introduction of test cultures of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus uberis*, *Klebsiella pneumoniae*, *Listeria monocitogenes* into broth dilutions.

Cultures of test strains were grown on an MPA at 37 °C for 18–20 h, washed with physiological solution, the concentration was increased to 10^9 microbial cells/ml by the turbidity standard 10 U (L.A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations), and 10-fold serial dilutions from 10^{-1} to 10^{-5} were prepared. Afterwards, from the dilution containing 10,000 microbial cells (10^{-5}), the test strains were seeded 100 µl into test tubes with meat-peptone broth and 1 g of the test additive was added for the purpose of joint cultivation. Seeds of test strains without the feed additive were used as a control. The cultures were cultured at 37 °C for 18–20 h. Then ten-fold serial dilutions of 10^{-1} to 10^{-9} obtained broth cultures containing and not containing the Natupro® were prepared using sterile physiological solution. Then, 100 µl of each test strain culture was seeded from dilutions 10–7 and 10–8 into Petri dishes on appropriate dense differential-diagnostic nutrient medium. Petri dishes with cultures were cultured at 37 °C for 24 hours. The colonies of the test strains giving characteristic growth on the corresponding differential diagnostic nutrient medium were then counted.

Results and discussion / Результаты и обсуждение

The results of determining the total number of CFU/g of *B. amyloliquefaciens* (BP-0-11-1), *B. amyloliquefaciens* (BP-0-14-1), *B. licheniformis*, *B. subtilis* in the feed additive Natupro® are shown in table 1 and 2 after 24 and 48 hours.

Table 1. **Total number of bacteria in Natupro®**

Dilution of Natupro®	Total number of bacteria in Natupro®, CFU/g					
	1 repatability		2 repatability		Average value	
	24 h	48 h	24 h	48 h	24 h	48 h
10^{-6}	≥ 300	≥ 300	≥ 300	≥300	–	–-
10^{-7}	68	68	71	71	70	70
10^{-8}	7	7	9	9	8	8

We did not count the grown colonies when the feed additive was diluted 10^{-6} because more than 300 colonies grew on Petri dishes with MPA.

Calculation of the total number of microorganisms in the feed additive: $(7 \times 10^7 + 8 \times 10^8)/2 = 8 \times 10^8$ CFU/g.

The total number of live spore-forming bacteria *B. amyloliquefaciens* (BP-0-11-1), *B. amyloliquefaciens* (BP-0-11-1), *B. licheniformis*, *B. subtilis* was 8×10^8 CFU/g (standard in ND not less than 6×10^8 CFU/g).

The following differential diagnostic nutrient media for cultivation of test strains were selected because of the research:

Chromogenic agar (HiCrome agar) for detecting and counting *E. coli*. Aboundant growth of *E. coli* strain, round, convex colonies are stained blue, and the growth of cultures included in the feed additive is completely suppressed.

HiCrome improved agar for *Salmonella*. Abundant growth of *Salmonella typhimurium* strain, round, smooth colonies pink-red, while the growth of cultures included in the feed additive is completely suppressed.

Base of HiCrome chromogenic agar for isolation and identification of staphylococci with additive: emulsion of egg yolk with tellurite. Abundant growth of *Staphylococcus aureus* strain, colonies stained brown-black with a transparent zone around them, when seeding strains included in the feed additive inhibited the growth of *Bacillus amyloliquefaciens*.

MacConkey Agar, Modified. Abundant growth of *Klebsiella pneumoniae* strain, round, smooth slimy colonies of crimson color, weak growth of cultures included in the feed additive is observed when the feed additive is seeded on the above medium.

Oxford *Listeria* Oxford Medium Base and Oxford *Listeria* Supplement. Abundant growth of *Listeria monocytogenes* strain, the colonies are small, grayish, surrounded by a black aureole with a transparent area around it. The growth of crops included in the feed additive is observed when the feed additive is seeded on the above medium.

The results of determining the bactericidal characteristics of the feed additive by the method of diffusion on agar show that Natupro® didn't cause visible inhibition of growth of test strain cultures when it was diffused in agar.

The study of the lytic activity of the strains included in Natupro® showed that *B. amyloliquefaciens* strains BP-0-14-1 and BP-0-11-1 and *B. subtilis* strain BP-0-12-1 had lytic activity against cultures of test strains *Salmonella typhimurium* and

Klebsiella pneumoniae, but did not lysis cultures of test strains *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus aureus*, *Streptococcus uber*, *Klebsiella pneumoniae* and *Listeria monocitogenes* (Table 2).

B. licheniformis strain BP-0-12-1 didn't show leading activity to any pathogenic bacteria used in this study.

To study the bacteriostatic properties, Natupro® was added to a nutrient medium, on which test strains of pathogenic bacteria were then cultured in dilutions 10^{-8} and 10^{-9} (Table 3).

Addition of the feed additive to the nutrient medium during cultivation of test cultures caused a tendency to decrease the number of *Escherichia coli* and *Salmonella typhimurium* colonies grown on the nutrient medium, which suggests the presence of Natupro® bacteriostatic characteristics to these bacteria.

The difference in the number of grown cells of *Klebsiella pneumonia* test strain with and without the addition of feed additive is statistically unreliable and doesn't allow to judge about the bacteriostatic characteristics of Natupro with respect to this microorganism.

Studies of the bacteriostatic properties of the Natupro® against *Staphylococcus aureus*, *Streptococcus uber* and *Listeria monocitogenes* were impossible, since the growth of these bacteria and strains of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* was difficult to differentiate on the type of medium used.

Table 2. **Lytic activity of *Bacillus* strains isolated from Natupro®**

Pathogenic bacteria	Diameter of a lytic zone, mm		
	***B. amyloliquefaciens* strain BP-0-14-1 and strain BP-0-11-1**	***B. licheniformis* strain BP-0-12-1**	***B. subtilis* strain BP-0-12-1**
Escherichia coli strain 320	–	–	–
Salmonella typhimurium strain 415	9.0	–	4.0
Staphylococcus aureus strain 12600	–	–	–
Streptococcus uberis strain 700407	–	–	–
Klebsiella pneumoniae strain 13883	5.0	–	3.0
Listeria monocitogenes strain 766/20	–	–	–

Table 3. **Bacteriostatic properties of Natupro®**

Pathogenic bacteria	Number of grown cells, CFU			
	Without Natupro®		With Natupro®	
Dilutions	10^{-8}	10^{-9}	10^{-8}	10^{-9}
Escherichia coli 320	235	10	59	6
Salmonella typhimurium 415	170	13	108	10
Staphylococcus aureus 12600	–	–	–	–
Streptococcus uberis 700407	–	–	–	–
Klebsiella pneumoniae 13883	35	4	26	0
Listeria monocitogenes 766/20	–	–	–	–

Conclusion/Выводы

According to the results of the work carried out, it was found that the bacterial strains in Natupro® exhibit lytic activity against pathogenic strains of *Salmonella typhimurium* 415 and *Klebsiella pneumoniae* 13883 and have a bacteriostatic effect on *Escherichia coli* strain 320 and *Salmonella typhimurium* strain 415.

The results obtained during in vitro experiments can be used for further conducting additional experiments on animals in order to study the preventive and immunological properties of the Natupro® feed additive on laboratory and farm animals.

The author is responsible for the work and the submitted data.
The author is responsible for plagiarism.
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