EFFECTS OF METABIOTIC BACILLUS SUBTILIS FOR MICROBIOCENOSIS FORMATION IN EARLY-WEANED PIGLETS

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Summary

This paper discusses the results of research related to cultivation of the probiotic microorganism B. subtilis on a grain-based nutrient medium of naked oat (Avena nuda) and studies the possibility of using metabolites as a liquid dietary supplement to correct microbiocenosis gastrointestinal tract disorders in early-weaned piglets. The weight fraction of proteinogenic amino acids in the experimental probiotic suspensions was determined by means of capillary electrophoresis. The metabolic activity of Bacillus subtilis DSM-32424 strain was studied on both sprouted as well as non-sprouted naked oat (Avena nuda) ‘Nemchinovsky’ variety grain-based nutrient medium. It was proved that the maximum microbial count reached 4.9×10^7 CFU/cm^3 on the 6th day of cultivation on the medium based on sprouted naked oat. The use of dietary supplement based on Bacillus subtilis DSM-32424 strain in amount of at least 1×10^9 CFU/pig had a positive impact in terms of the formation of microbiocenosis in animals at the time of transferring piglets to plant diet.

Key words: dietary supplement, Enterobacteriaceae, metabiotics, microbiocenosis, nutrient medium, piglets, probiotics

INTRODUCTION
Probiotics are living microorganisms and substances of microbial origin able to provide beneficial effects on physiological functions and biochemical reactions of the body given the natural process of their administration (Shenderov, 2013; Pajarillo et al., 2015; Ardatskaya et al., 2017; Plotnikova, 2018).

Published research (Patel & DuPont, 2015; Bai et al., 2017; Rusaleyev et al., 2019) suggest that the interaction between microorganisms in nature is manifested, among other ways, through their antagonism. Antagonism consists in the fact that bacteria from one species, when developing together with another species, can inhibit its vital activities. This bacterial property is of practical use in veterinary medicine owing to the use of bacteria as probiotics. It is proved that the widespread administration of various probiotic products from living lactobacilli and bif-
Effects of metabiotic Bacillus subtilis for microbiocenosis formation in early-weaned piglets

dobacteria in veterinary and medical practice over the past decades has resulted in decrease of their therapeutic action, prompting scientists to seek novel and more effective microorganisms with probiotic properties (Rusaleyev et al., 2019).

It is noteworthy that alongside the search for more effective probiotics, a scientific justification was developed in support of the administration of metabiotics as an extension of the probiotic concept. Thus, the elaboration of the probiotic concept through the study and use of metabiotics of probiotic microorganisms in order to develop novel dietary supplements and methods for their use in animal husbandry was implemented (Popov et al., 2020a,b).

Metabiotics are a group of medications that contain active metabolites (products of vital functions) of the probiotic cultures, among which lysozyme, bacteriocins, catalases, enzymes, organic and amino acids, polypeptides and other compounds (Neschilyaev et al., 2016; Sánchez et al., 2016; Fisinin et al., 2017). In addition to the bioactive action on the host, they greatly amplify antagonistic action of the producing probiotic strain, thus creating favourable conditions for its vital activity and integration into the microbiome community of gastrointestinal tract (Ushakov et al., 2015; Uyeno et al., 2015; Magomedaliyev et al., 2020).

The clinical performance of dietary supplements based on probiotic products in the formation of microbiocenosis largely depends on biological and chemical composition of gastrointestinal environment, and manifests itself only during the period of administration owing to unfavourable conditions for assimilation. Thus, it is necessary to look for ways to prolong the effect of probiotics’ usage. Still, it compulsory to highlight the essential role of ‘normal microflora’ for the formation of microbiocenosis of gastrointestinal tract in animals (Sánchez et al., 2016). Microflora is directly involved in digestive processes, and produces enzymes necessary for metabolism of proteins, carbohydrates, lipids and nucleic acids. When autoflora is dying out, the body system digests and absorbs it as a source of protein (Pritynchenko et al., 2012; Bekhtereva et al., 2014; Uyeno et al., 2015; Roselli et al., 2017).

Consequently, the production of metabolite-type dietary supplements enriched with cell constituents of bacterial producers is a relevant topic in the field of biotechnology and animal nutrition.

The purpose of this research was to study the influence of B. subtilis metabolites on the formation of microbiocenosis of the gastrointestinal tract in early weaned piglets.

MATERIALS AND METHODS

The research studies took place in the Laboratory of Agrobiotechnology of Federal State Budgetary Scientific Institution Kursk Federal Agrarian Scientific Center (FSBSI Kursk FASC) in Kursk, the Russian Federation. The grains of naked oat (Avena nuda) in its ‘Nemchinnovsky’ variety served as nutrient medium in order to produce metabolites in the culture of Bacillus subtilis DSM-32424 strain; and were provided by the Department of selection and seed farming at FSBSI Kursk FASC. All experiments were conducted in compliance with ethical standards, in accordance with Directive 2010/63/EU of the European Parliament and Council of the European union as of September 22, 2010 – On the protection of animals used for scientific purposes).
The nutrient medium used to produce metabolites of the *Bacillus subtilis* DSM-32424 culture contained sprouted and non-sprouted grains of naked oat (*Avena nuda*), ‘Nemchinovski’ variety at the ratio of 100 g of crushed raw material per 3 L of water. The substance was gradually heated up at temperature from 25 to 90 °C for 6 hours. Then it was left to cool down naturally. Under control through a pH-meter, pH was adjusted to 7.5 with a 20% aqueous solution of NaOH. *B. subtilis* inoculation was done with culture previously standardised in laboratory conditions up to 1×10⁶ CFU/cm³ at a ratio of 4 mL per liter of the prepared nutrient medium.

Cultivation lasted 14 days in a KBCG 100/250 thermostat (Premed, Poland) at 37±1 °C. The number of *B. subtilis* CFU in the culture fluid was monitored on a daily basis on a Levenhuk 740T microscope (Guangzhou Jinghua Optics & Electronics Co., Ltd., China), a Levenhuk M1400 PLUS digital camera (Guangzhou Jinghua Optics & Electronics Co., Ltd., China) and a pH-meter Kelilong pH-013 (Kelilong Instruments, China).

The weight fraction of proteinogenic amino acids in experimental probiotic suspensions (EPS) was determined by means of capillary electrophoresis according the procedure stipulated by GOST R 55569-2013. The calculation of weight fraction of crude protein in g/L was carried out in accordance with GOST 32044.1-2012.

The scientific and economic analysis of experimental probiotic suspension effect on the formation of microflora of piglets’ gastrointestinal tract took place on the premises of the Pig Complex ‘Nadezhda’ in Kursk region during rearing of weaning piglets up to the age of 75 days. Three groups of piglets aged 10 days were formed, with 15 animals in each group. Group 1 received EPS based on sprouted avena; Group 2 received EPS based on whole-grain avena; Group 3 was control. The experimental dietary supplement sample was evaporated in amount of at least 1×10⁹ CFU/mL *Bacillus subtilis*. The analysis of microflora composition in faeces took place on the 10th, 25th (prenursery piglets) and 40th days (early-weaned piglets) of life by means of a quantitative cluster analysis of the large intestine content (Kondrakhina et al., 2004).

Sampling was done directly from the rectum onto sterile dishes. The initial dilution was obtained in the following way: 1 g of the weighed faecal matter was well homogenised with 9 mL sterile buffer solution. A number of subsequent dilutions were produced with a buffer solution from 10⁻³ to 10⁻¹⁰ of the initial dilution, and then inoculated on the corresponding nutrient media. Incubation of all grains was performed at 37–38 °C. The assay content of bifidobacteria was determined using the dilutions of 10⁻⁵–10⁻¹⁰ diluted in Blaurock semi-liquid modified hepatic medium. The *Lactobacillus* count was obtained from dilutions of 10⁻⁵–10⁻⁹ in thin fat-free milk. Enterococci were marked out on the Alkaline polymyxin medium for the isolation of enterococci from dilutions of 10⁻³–10⁻⁷. Staphylococci were detected by inoculation on yolk salt agar (YSA) from dilutions of 10⁻⁴–10⁻⁶. Yeast-like fungi of the genus *Candida* were revealed on the Saburo medium containing levomycetin from dilutions of 10⁻³–10⁻⁶. *Proteus* genus members were detected on slant agar by Shukevich’s method in dilutions of 10⁻¹ to 10⁻⁴ (Kislenko, 2005). Spore-forming anaerobes (clostridia) were identified by inoculation of the suspensions of dilutions...
Effects of metabiotic Bacillus subtilis for microbiocenosis formation in early-weaned piglets

10^{-2}, 10^{-5} and 10^{-6} into a melted column of Wilson-Blair agar medium. The total aerobic plate count and their haemolytic properties were analysed after inoculation of dilutions of 10^{-5}–10^{-7} on 5% blood agar. The potentially pathogenic entero-bacteriaceae were spotted by inoculation of a drop of suspension (0.05 mL) from dilutions of 10^{-3}–10^{-8} into four sections of a plate containing Levine’s medium. The specific media involved in the research studies were purchased from the Federal Budgetary Institution of Science State Scientific Center for Applied Microbiology and Biotechnology (FBIS SSC AMB) in the regional settlement of Oboleinsk in Serpukhov, Moscow Region, Russia. The content of all types of microorganisms in 1 g of faeces was evaluated through the amount of colonies grown on the corresponding medium and taking into account the inoculum and its dilution. Throughout the research period, the animals experienced no harm.

The statistical data processing included descriptive statistics employing the statistical analysis package for Microsoft Excel. The significance of differences between mean values was evaluated by Student’s t-test. The differences were considered statistically significant at P<0.05.

RESULTS

Cultivation of the probiotic microorganism B. subtilis on a grain-based nutrient medium of naked oat (Avena nuda) allowed defining the specific growing microorganism. The evaluation of the metabolic activity of a probiotic microorganism revealed that the maximum count reached 4.9×10^7 CFU/cm^3 on the 6th day of cultivation on the medium based on sprouted avena. On the 10–11th day the number of CFUs has stabilised around 1.9-2.0×10^7 CFU/mL.

The experimental liquid probiotic suspension was a bioactive product. Its physical properties were revealed through its specific odour and colour. The dynamics of proteinogenic amino acids correlated with CFU values of probiotic microorganism B. subtilis. The analysis of proteinogenic amino acids (Table 1) outlined the culture liquid as an experimental probiotic suspension. It is noteworthy that the weight fraction of crude protein was slightly different in the experimental samples.

It is reasonable to assume that the reduction of the crude protein was possible owing to fermentation of the naked oat grains during germination. The qualitative and quantitative composition of the probiotic suspension featured the complete protein content of the suspension enriched with organic acids. Along with that, the analysis of indicators (Table 1) demonstrated increase in the activity of B. subtilis metabolism after cultivation on a non-sprouted avena-based nutrient medium. The synthesis of lysine was higher when compared to the sprouted grain control. In the culture fluid, the amount of amino acids was within the following ranges: lysine from 7.85 to 10.53 g/L, methionine from 2.03 to 2.35 g/L, leucine + isoleucine from 5.79 to 9.7 g/L. A similar trend was observed for other proteinogenic amino acids (P≤ 0.05).

When B. subtilis was cultivated on a grain-based nutrient medium of nude oat (Avena nuda) both non-sprouted and sprouted, EPS (experimental probiotic suspension) was a dietary supplement with...
The experiment with pre-weaning piglets provided background parameters of gastrointestinal microbiocenosis on the 10th day of life. The microbial background in faeces reached 96%, and was made of bifidobacteria and lactobacilli at parity, i.e. averaging about 0.2×10^8 CFU/g. The total Escherichia count reached 1.87% or 0.27×10^6 CFU/g (Table 2). Other microorganisms on a standalone basis reached less than 1%. Besides no Salmonella or Candida yeasts were detected.

On the 25th day an increase in B. bifidum was observed in Groups 1 and 2 (Table 2). In Group 2 the count of bifidobacteria in faeces of piglets reached 0.25×10^9 CFU/g, which was by 8% higher than that in Group 1, and by 89.2% higher than in control group. The count of lactobacilli in faeces of piglets from Group 2 reached 0.82×10^8 CFU/g, which was by 14% higher than that in Group 1, and by 36% higher than in Group 3 (control).

Increased count of Proteus was also observed, with maximum value of 0.12×10^5 CFU/g recorded in control group: by 16.7% higher vs Group 1, and by 21.7% higher vs Group 2. A similar shift in proportions was observed for other microorganism types: in Groups 1 and 2 their count was significantly lower, which can be attributed to the prominent antago-

### Table 1. Indicators of amino acid composition of the probiotic suspension. Values are presented as mean±SD, n=3

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Control</th>
<th>Avena with B. subtilis</th>
<th>Sprouted Avena</th>
<th>Sprouted Avena with B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avena</td>
<td>Experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight fraction of crude protein, g/L</td>
<td>0.80±0.23</td>
<td>0.73±0.31</td>
<td>0.79±0.29</td>
<td>0.70±0.37</td>
</tr>
<tr>
<td>Arginine</td>
<td>14.61±0.40</td>
<td>14.40±1.50*</td>
<td>6.55±0.11</td>
<td>3.87±1.25</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.91±0.52</td>
<td>9.80±1.80</td>
<td>5.18±0.25</td>
<td>7.67±1.97</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.31±1.10</td>
<td>12.00±0.90*</td>
<td>6.71±1.12</td>
<td>8.93±1.87</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.70±0.90</td>
<td>9.12±0.70*</td>
<td>4.38±1.35</td>
<td>5.60±1.21</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.09±1.30</td>
<td>4.30±1.90</td>
<td>3.45±1.21</td>
<td>2.49±1.37</td>
</tr>
<tr>
<td>Leucine + isoleucine</td>
<td>24.94±0.90</td>
<td>25.19±0.30*</td>
<td>19.04±0.90</td>
<td>15.49±0.50</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.65±1.70</td>
<td>5.81±1.90</td>
<td>3.78±1.25</td>
<td>3.46±1.37</td>
</tr>
<tr>
<td>Valine</td>
<td>11.65±0.70</td>
<td>12.68±0.80*</td>
<td>13.40±0.70</td>
<td>8.72±1.29</td>
</tr>
<tr>
<td>Proline</td>
<td>19.27±0.10</td>
<td>22.37±0.30</td>
<td>22.34±0.90</td>
<td>19.97±0.30</td>
</tr>
<tr>
<td>Threonine</td>
<td>14.97±0.90</td>
<td>10.06±1.70*</td>
<td>15.59±0.20</td>
<td>7.89±1.33</td>
</tr>
<tr>
<td>Serine</td>
<td>20.61±0.90</td>
<td>17.68±1.20*</td>
<td>19.81±0.80</td>
<td>10.83±0.10</td>
</tr>
<tr>
<td>Alanine</td>
<td>15.13±0.50</td>
<td>20.50±1.40</td>
<td>23.13±0.40</td>
<td>20.49±0.80</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.32±1.50</td>
<td>18.20±1.60*</td>
<td>7.84±1.58</td>
<td>8.66±1.33</td>
</tr>
</tbody>
</table>

Note: * statistically significant difference at P<0.05 between Avena with B. subtilis and sprouted Avena with B. subtilis on the 14th day of the experiment.
Table 2. Dynamics of microbiocenosis formation in piglets (mean ± SD, n=5)

<table>
<thead>
<tr>
<th>Microorganism type (CFU/g in faecalis)</th>
<th>Group 1 B. subtilis in medium of non-sprouted Avena</th>
<th>Group 2 B. subtilis in medium of sprouted Avena</th>
<th>Group 3 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.2×10^9±</td>
<td>0.23×10^9±</td>
<td>0.31×10^9±</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>0.23×10^9±</td>
<td>0.71×10^9±</td>
<td>0.8×10^9±</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>0.1×10^4±</td>
<td>0.4×10^4±</td>
<td>0.1×10^4±</td>
</tr>
<tr>
<td>Proteus</td>
<td>0.3×10^4±</td>
<td>0.1×10^4±</td>
<td>0.47×10^4±</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>0.27×10^7±</td>
<td>0.37×10^6±</td>
<td>0.93×10^6±</td>
</tr>
<tr>
<td>Clostridium</td>
<td>0.3×10^5±</td>
<td>0.28×10^5±</td>
<td>0.21×10^5±</td>
</tr>
<tr>
<td>Escherichia</td>
<td>0.2×10^6±</td>
<td>0.31×10^6±</td>
<td>0.50×10^6±</td>
</tr>
<tr>
<td>Salmonella</td>
<td>negative</td>
<td>0.2×10^2±</td>
<td>0.3×10^2±</td>
</tr>
<tr>
<td>Candida</td>
<td>negative</td>
<td>0.2×10^2±</td>
<td>0.3×10^2±</td>
</tr>
</tbody>
</table>

Note: * statistically significant at P≤0.05.
nistic effect of *B. bifidum*. It is noteworthy that on the 25th day, *Salmonella* were found in feces of piglets from all three groups. Their largest count, i.e. $0.11 \times 10^5$ CFU/g, was registered in controls, whereas the lowest one – in Group 2, $0.2 \times 10^2$ CFU/g, which was by 26% lower than that in Group 1.

On the 40th day in Groups 1 and 2, the largest share of total microbial counts was that of *B. bifidum*: 79% and 80.73% respectively. Lactobacilli reached 0.4% in feces of Group 1, and 18.83% – in Group 2. Other types of microorganisms reached 0.52% and 0.43% respectively.

Staphylococci predominated in the control group: 44.28% of total count. The share of lactobacilli was 38.38%, bifidobacteria were only third with 14.76%. The share of *Proteus* was 1.47%, and that of other types of microorganisms – 1.1% of the total count. When compared to Group 2, the count of bifidobacteria in feces in piglets from Group 1 was lower by 13.9%. In control group 3, the count of bifidobacteria was lower by 91.7% than in Group 2.

The present research studies contributed to the justification of a reasonable opportunity to use *Avena nuda* as a nutrient medium to produce metabolites during cultivation of the probiotic microorganism *B. subtilis*. Cultivation of the probiotic microorganism *B. subtilis* allowed obtaining a metabolite composition by way of proteinogenic amino acids. The experimental probiotic suspension when *B. subtilis* was cultivated on the grain-based nutrient medium, both non-sprouted and sprouted, was a dietary supplement containing probiotics in an amount of at least $2.0 \times 10^7$ CFU/mL.

Research studies involving animals proved that the use of the probiotic suspension of *B. subtilis* with a grain-based nutrient of naked oat (*Avena nuda*) in the nutrition of piglets had a significant influence on the proportion of microorganisms in gastrointestinal tract microbiocenosis.

**DISCUSSION**

The production of dietary supplements based on probiotic microorganisms is based on their metabolites obtained during culturing on different nutrient media. Grain-based nutrient media are the best choice for obtaining biologically active substances from probiotic microorganisms. The outcome of studies in view of *B. subtilis* cultivation are consistent with the focus of the research and with the findings of other authors (Parker, 1974; Nguyen et al., 2016).

However, the production of medicines and dietary supplements based on *B. subtilis* was done in Russia and Germany only in medical practice (Bactistatin and Hylak Forte products). The research studies related to the production and administration of dietary supplements based on metabolites in animal husbandry and veterinary medicine are at their initial stage of research (Shenderov, 2013; Lebeer et al., 2018).

Several recent research papers (Plotnikova, 2018; Singh et al., 2018) discuss an analogue of the capsulated metabolite and prove that it can be used to correct microbiocenosis gastrointestinal tract disorders in animals.

In our research studies, *B. bifidum* prevails in formation of microbiocenosis in piglets through metabolites. The use of probiotic metabolites, according to some authors, not only stimulates the activity of enzyme systems, producing beneficial effect on metabolism in body systems of animals, but also improves the ecological environment of intestine from physiological point of view in order to create and
Effects of metabiotic Bacillus subtilis for microbiocenosis formation in early-weaned piglets

...maintain own microflora, as well as regulates symbiotic relationships with the host (Wang, 2014; Patel & DuPont, 2015; Nechilyaev et al., 2016; Bai et al., 2017).

Published research on B. subtilis (Huang, 2013; Orel, 2014; Patel & DuPont, 2015; Ardatskaya et al., 2017; Strekalovskikh & Belousov, 2018) revealed a significant correlation between the content of Bifidobacterium and Lactobacillus, as well as significant negative correlation between Enterobacteriaceae and Clostridium perfingens. The results from intestinal microflora studies makes it possible to affirm that the use of probiotics influences positively microbial composition: the count of lactobacilli and bifidobacteria increases. There is an established tendency towards reduction in the number of Escherichia and staphylococci, and absence of lactose negative Escherichia.

The experimental data obtained in these studies are consistent with research data (Pajarillo et al., 2015; Sánchez et al., 2016), confirming the favourable effect of EPS on formation of microbiocenosis in early-weaned piglets. Throughout the research, it was shown that B. bifidum was integrated better into microbiome of gastrointestinal tract in piglets of Group 2, which received suspension enriched with probiotic microorganisms grown on sprouted avena medium (Nguyen et al., 2016).

It was demonstrated that the faecal count of bifidobacteria in piglets from this Group was by 13.9% higher than that in Group 1. In controls, bifidobacteria count was by 91.7% lower compared to Group 2, proving the active formation of gastric intestinal microbiocenosis in piglets through direct cultivation of B. bifidum in the intestinal tract. The experimental probiotic suspension on the basis of the probiotic microorganism B. subtilis helps colonisation of microbiome in normal intestinal microflora, consistent with other studies (LeBlanc, 2014; Ardatskaya, 2015; Fedorova et al., 2016; Sánchez et al., 2016).

CONCLUSION

The administration of grain-based B. subtilis probiotic suspension from Avena nuda to piglets greatly influenced the proportion of microorganisms in the microbiocenosis composition of the gastrointestinal tract. In piglets treated with an experimental probiotic suspension, the content of B. bifidum in faeces was significantly higher than in controls. The microbiome was predominantly represented by bifidobacteria and lactobacilli, with the remaining microflora being less than 1%. A more prominent antagonistic effect was revealed through B. subtilis on the medium based on the Avena sprouts.

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Effects of metabiotic Bacillus subtilis for microbiocenosis formation in early-weaned piglets


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