Safety and properties of Enzymesporine microencapsulated probiotic product with enzyme and its effect on the physiology of intestinal digestion and weight gain of pigs

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Abstract. The efficiency of the probiotic product Enzymesporine manufactured by Fermlab LLC was increased by the creation of its microencapsulated form with 5% crystalline trypsin, which surpassed its analogues in biological properties. In the microencapsulated probiotic product (MPP), the viability of the microorganisms was maintained at the level of the initial product by using 40% acetone as a precipitant. During in vitro experiments simulating gastric digestion, microcapsules were not destroyed for 120 minutes, which indicated their resistance to the acidic medium of the stomach and the possibility of passing it without loss of viability of the probiotic microflora. Tests in laboratory animals showed that the product did not have mutagenic activity and toxicity. During the use of this product from 48 days of life, reliable results were achieved in the increase of live weight and average daily gain in pigs of Genesus genetics at 98 and 148 days of life. By means of a qualitative caprological analysis, it was found that the product enhances the digesting ability in the intestines of pigs more intensively than its unencapsulated form without a proteolytic enzyme. It is recommended to use this product as a biologically active additive in the diet when feeding pigs of large industrial pig breeding.

1. Introduction

Nowadays in global practice of industrial pig farming there is a consistent trend towards the use of pigs obtained by hybridization. Thus in many foreign countries, a terminal or final hybridization system is adopted, at the beginning of which the specialized lines of mother breeds (the first cross) are crossed: large white, Yorkshire and landrace, and the resulting crossbreeds (F1 hybrids) are crossed with male pigs producing meat breeds or lines. As a rule, they are pigs of Duroc, Hampshire and Pietrain breeds. Thus, in the whole global pig breeding, hybridization is the main method of the increase of the productivity of commercial pig breeding [1]. Therefore, large producers of genetic lines such as Genesus Genetics are characterized by the high productive genetic potential of their animals.

At the same time, in industrial pig farming, the profitability of production directly depends on the cost of feed. In its turn, the choice of feed formulation significantly affects their cost. Thus, usually manufacturers have to find a balance between obtaining good productivity and economic efficiency, which depends on costs. Moreover, the genetic potential of animals is not used to the full extent.
In order to enhance and increase digestibility with high genetic potential, scientists suggest the use of various biologically active substances. They include probiotics in a combination with various auxiliary components that enhance the general biological effect.

Probiotics have the following therapeutic properties: 1) the effect on the intestinal microbiota of the host and pathogenic bacteria, 2) the improvement of specific enzymatic activities, 3) the production of antibacterial substances, 4) the competitive exclusion of pathogenic bacteria, 5) the induction of the production of defensin, 6) the improvement of intestinal barrier function, 7) the modulation of the host's immune functions, 8) the modulation of intestinal carcinogenesis, 9) the modulation of cholesterol absorption [2]. For example, butyrate, a derivative of probiotic microorganisms, plays an important role in the development of intestinal digestion in mammals during ablation and prevents the appearance of pathologies accompanied by diarrhea [3].

According to the literature sources, many probiotic products are quite effective, but as a result of the destruction of a large number of probiotic bacteria in the acidic medium of the stomach, the consumption of these products is quite high. As a result, the effect of the biological impact is not always stable and depends on the individual characteristics of digestion and diet, such as the time spent by the feed mass in stomach, the acidity of the gastric juice and other indicators of the internal medium of the digestive tract aggressively affecting the probiotic microflora.

The problem of bacterial destruction in aggressive medium of the stomach is largely solved by various methods of microencapsulation of probiotics. Microcapsulation is the main modern solution to preserve the viability of probiotics [4]. Microencapsulation is the process of incorporating probiotic bacteria into a specific material or membrane, which has the ability to reduce damage or loss of cells caused by environmental factors, with a controlled release rate under certain conditions [5]. Therefore, this technique has been widely studied over the past decade, since it allows maintaining beneficial properties even for sensitive bacteria during storage and absorption [6].

At the same time, using modern microencapsulation technologies, it is not always possible to maintain the viability of microorganisms [7]. According to experts with experience in microencapsulation of probiotics, an ideal microencapsulated probiotic product can be either a dry powder that can be easily stored for a long time, or a wet gel with long-term stability in a food product [8].

Scientists are studying various methodological approaches to the encapsulation (microencapsulation) of probiotics, including the choice of biomaterials, the selection of suitable technology, and the study of the release of encapsulated probiotics in vitro [9].

The most common biomaterial for the encapsulation of probiotics is sodium alginate [10]. Alginate microencapsulation can be applied to many different probiotic strains. The results show better survival than free cells at a low pH of 2.0, a high concentration of bile salts and moderate heat treatment up to 70 °C. Microcapsulation may act as an important method for increasing the viability of probiotic bacteria in acidic food products and help deliver viable bacteria to the the gastrointestinal tract of a host [11].

Thus, the purpose of this work was to study the physicochemical and biological properties of the microencapsulated probiotic product Enzymesporine with an enzyme and its effect on the digestibility and productive qualities of fattening pigs.

2. Materials and methods

The initial probiotic product Enzymesporine is a complex of particles based on the strains: Bacillus subtilis RNCIM B-314, Bacillus licheniformis RNCIM B-8054, Bacillus subtilis (Bacillus natto) RNCIM B-12079. Externally, the product is a homogeneous fine powder, light beige in color, with a slightly pronounced sour-milk smell / Mass moisture content is not more than 8.0% (according to the results of the control check - 4.9%). The number of viable microorganisms (ufc/g) was 5.5x10^9. To enhance the digestibility, the enzyme trypsin crystalline in the amount of 5% by weight of the product was added to the initial Enzymesporine product.
Microencapsulation was carried out according to the method described in the patent of the Russian Federation No. 2689164 from May 24, 2019 and improved in the application for a patent of the Russian Federation No. 2020117491 from May 27, 2020. [12].

During the microencapsulation of a probiotic Enzymesporine, sodium alginate was used as the shell of the microcapsules. Microencapsulation was carried out by the physicochemical non-solvent precipitation method using a 40% acetone solution and a 2.2 M calcium chloride solution to stabilize the capsules. The crystalline trypsin in the amount of 5% of the total amount of the initial product was also introduced. The resulting mixture was constantly stirred for 15-20 minutes, and then the cured microcapsules were separated by filtration on a Schott filter and drying at 30-35 ° C. Thus, the initial probiotic product with the enzyme - the core of the microcapsule, was coated with a polymer shell of sodium alginate in a ratio of 1: 3.

The amounts of viable bacteria in one dose of the product (UFC × ml⁻¹) were determined by counting the grown colonies after microencapsulation and after 120 minutes of interaction with a solution of hydrochloric acid and pepsin (pH 1.5; 2.5; 3.5; 4.5; 5.5).

White laboratory mice in of both sexes weighing 21 ± 2 g were used during testing the product for acute toxicity. Experimental animals were injected with a once intragastrically studied solution of the microencapsulated product Enzymesporine with an enzyme and a comparator drug: non-encapsulated Enzymesporine (without enzyme).

The solutions of both products were presented in the form of a prepared suspension of 1% starch paste, introduced using a metal probe with a smooth round surface at the end in a volume of 0.5 ml, which was maximally acceptable for this type of animal and its body weight. In order to pass the products through the probe and to ensure the maximum concentration of the products, as well as the possibility of passing the suspension of the product, 300 mg of the test substance was dissolved in 1 ml of 1% starch paste. Thus, the experimental mice received a dosage of 7.5 g / kg with an average weight of 20 g (i.e., 0.15 grams of the drug). We used data obtained on intact animals (intragastric administration of an appropriate volume of 1% starch paste) as a control.

The toxic effect of the products was evaluated by the general physiological state of laboratory animals and their survival, as well as by the calculation of LD₅₀. The calculation of surviving and dead animals was carried out on the 3rd day after drug inoculum, followed by monitoring the surviving animals for 2 weeks. In total, 20 mice were used in the experiments.

Testing of the microencapsulated probiotic product Enzymesporine with an enzyme on pigs of Genesus genetics was carried out under conditions of a large livestock complex in comparison with the initial non-encapsulated Enzymesporine. Animals were randomized by weight and gender.

The experimental animals were divided into groups:
• control group No. 1 (n = 20), did not receive probiotic products with food;
• control group No. 2 (n = 20), received the initial (non-encapsulated) Enzymesporine at a dose of 3.0 g per day per 1 animal;
• the study group (n=20) received a microencapsulated product Enzymesporine with an enzyme in a dose of 3.0 g per day per 1 animal.

The total duration of the experiment was 100 days during the period from 48 to 148 days. During the experiment, we observed the experimental animals, took into account the general condition, appetite and determined the absolute weight, average daily weight gain and safety.

The average value and the standard deviation were calculated during the statistical data processing. Differences were considered significant at p <0.05.

The digestibility of fodder masses was examined microscopically, while fecal samples of experimental animals of each group were also taken on the 98th (n = 5) and 148th (n = 5) days of life. The fecal masses were examined for the content of detritus, muscle fibers, connective tissue, mucus, neutral fat, soap, starch grains, helminths, protozoa and fungi. The studies on the content of helminths, protozoa, and fungi were carried out in order to exclude the negative effect of these factors on the animal organism.
3. Results and Discussion

The resulting microencapsulated probiotic product, Enzymesporine with trypsin, is a gray-yellow powder consisting of microcapsules coated with a shell of sodium alginate with an incorporated core, which includes the probiotic product Enzymesporine with crystalline trypsin in a ratio of core: shell of 1:3. The output of finished microcapsules was 85-90%. The size of the microcapsules varies from 80 to 150 microns. It is necessary to note that crystalline trypsin, which is a part of the microencapsulated probiotic product, is thermally stable in its properties up to 90-100 °C heating and is resistant to acids. During in vitro experiments, after heating and returning the medium from acidic to alkaline, this enzyme restores its proteolytic properties. Therefore, when planning the experiment, we supposed that the heat treatment of animal feed and passing through the acidic medium of the stomach of animals will not cause further inactivation of this component of the probiotic product.

The results of microbiological studies on the determination of UFC showed that the number of viable probiotic bacteria in the produced microencapsulated drug was $5.5 \times 10^9$ cells per 1 g of microencapsulated product. This indicator did not decrease even when it was exposed to a mixture of hydrochloric acid and pepsin for 120 minutes at different pH values from 1.5 to 5.5, which indicated a high acid resistance of the microcapsule shell and the ability to withstand the acidic medium of the stomach. At the same time, microcapsules had the ability to dissolve in the alkaline medium of the intestine at a pH of 7.0-8.0.

During the determination of acute toxicity, the studies in mice showed that after intragastric administration to the laboratory animals of the microencapsulated Enzymesporine with an enzyme, no toxic effects and mortality of mice were observed in comparison with the analogue at a dose of 7.5 g/kg. At the same time, there were no changes in behavior during intragastric administration of drugs, no manifestations of intoxication were noted both with the use of Enzymesporine MPP and with the comparator drug.

The test results of the MPP “Enzymesporine with an enzyme” on pigs from the 48th to 148th days were as follows. From the 48th to 98th days, the average daily weight gain of the control group No. 1 (intact) was 0.61 kg, in the control group No. 2 with the Enzymesporine it was 0.656 kg, and when feeding the Enzymesporine MPP with the enzyme it was 0.712 kg, which was significantly higher ($p < 0.05$) than the values of control groups. From the 98th day to 148th, the average daily weight gain in the group with Enzymesporine with an enzyme was 0.824 kg, which was significantly higher ($p < 0.05$) than in the intact group of control No. 1 - 0.736 kg and control groups No. 2 - 0.802 kg, respectively. Survivability in all groups was 100%. The results are shown in Figure 1.

The analysis of the fecal masses of experimental animals showed that piglets on days 98 and 148 of the experimental group showed a high content of detritus, consisting of the smallest residues of nutrients, microorganisms, rejected dead intestinal epithelium, which lost their structure. In general, the fecal masses of the experimental animals have a lot of detritus, which indicates a good mechanical and chemical processing of food substances. Moreover, in the control groups of pigs, a moderate amount of detritus was noted, which is not pathology, but only indicates a lesser degree of digestion of the feed.

Muscle fibers and connective tissue were absent in all groups of experimental animals, which indicated normal digestion processes. The absence of mucus in the fecal masses of animals indicates the absence of inflammatory processes in the upper and lower intestines, because normally a small amount of mucus is noted only in newborn animals.

Normal fecal mass usually contains a small amount of magnesia and calcium salts of fatty acids (soaps) and, along with them, little fatty acids. Indeed, in pigs on the 98th days and 148th days, the content of a small amount of substances of this group of soaps was observed. Moreover, in pigs of the group at 45 days their number was little. Neutral fat is normally not found in small quantities. In our experience, all the experimental animals showed a lack of neutral fat.

Starch grains in experimental animals in a single or small amount may indicate a slight acceleration of evacuation function, which in turn is a physiological feature of this animal species.
The content of indigestible fiber in experimental animals of the studied group varied from a small amount in pigs of the experimental group, both on the 98th and 148th day, to a large number in piglets of the control groups at the same age. The absence of digestible fiber in all animals of the experimental group indicates a good degree of digestion in comparison with control animals, where digested fiber is found in large quantities. Helminths, protozoa and fungi were not found in the fecal masses of experimental animals.

Figure 1. Live weight of pigs of scientific and economic experiment from the 48th to 148th days (M ± m) in kg

4. Conclusion

According to the results of the study, we conclude that microorganisms do not lose their viability when they pass through the acidic medium of the stomach. The process of digestion and assimilation of nutrients in the body of animals of the experimental group proceeded more intensively as a result of the use of the enzyme crystalline trypsin in Enzymesporeine MPP formulation, which ultimately had a positive effect on the increase in live weight and average daily weight gain of animals. Based on the results of studies on mice with injected the doses used in the experiment, we can conclude that the studied product is not toxic. All these results open up prospects for the use of this drug in industrial pig farming in order to increase feed performance in animals and improve the quality and safety of meat products for humans.

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