Evaluating the efficiency of Enzyme-enriched Enzymesporine probiotic feed additive and its impact on the productive properties of pigs in the fattening process

D V Trubnikov¹, A Y Gorobets¹, E V Trubnikova², M I Kartashov³ and A S Belous³

¹ Kursk State Agricultural Academy named after I.I. Ivanov, 70 Karla Marksa Str., Kursk, 305021, Russia
² Scientific and Research Laboratory “Genetika”, Kursk State University, 33, Radishcheva Str., Kursk, 305000, Russia
³ Federal state budgetary research institution "All-Russian Scientific and Research Institute of Phytopathology", Institute Str., Estate 5, Bolshie Vyazemy Village, Odintsovo District, Moscow Region, 143050, Russia

E-mail: denadmiral@mail.ru

Abstract. The microencapsulation method developed for the Enzyme-enriched Enzymesporine probiotic additive (crystalline trypsin) belongs to the sphere of biotechnology and veterinary medicine. The method is used to produce an additive with a high bioaccessibility value. It maintains the viability of the probiotic microorganisms without the loss of the primary CFU value of \(5.5 \times 10^9\) by changing the precipitator (acetone) concentration to 40%. The results, acquired in the in vitro simulation of the digestive process in the stomach, demonstrated the acid-resistance of the microencapsulated additive exposed to hydrochloric acid and pepsin for 120 minutes under pH from 1.5 to 5.5. The introduction of crystalline trypsin at the amount of 5% of the active pharmaceutical ingredient mass caused qualitative improvement in the digestive activity in the pigs’ intestines. The experiments carried out with the pigs of Genesus genetics provided reliable results of the live weight increment and average daily gain within the period from the 49th to 148th days of life to prove the advantage of the encapsulated Enzyme-enriched Enzymesporine probiotic additive compared to the non-encapsulated analogues or the analogues without enzyme.

1. Introduction
As a rule, the genetic potential of the Genesus three-breed hybrid pigs often used in industrial pig farming, when provided with physiologically adequate pig breeding and feeding conditions, increases the productive properties of the livestock [1]. In practice, the maximum exploitation of the potential increases the feeding expenses to the level when the feasibility of production drops below the economically feasible level. Therefore, the farmer usually selects the diet and the feed recipe to achieve a sufficient weight gain, at the same time saving the money spent on feed production, increasing the profitability of the industry. However, quite often the economically balanced approach to the animal feeding causes incomplete utilization of the growth and development potential resulting in underproduction. For this reason, finding new ways of increasing the animal productivity, their safe-
keeping and digestive disorder prevention remains an up-to-date task for the researchers. In this regard, one of the most efficient methods is the introduction of probiotic feed additives.

The microbiota of the gastrointestinal tract of the animals plays a key role in the normal digestive process and the well-being of the animal. The probiotics may make a positive influence on the microbial balance in the animal bowel. When used in the animal husbandry, commercial probiotics increase the productive properties of animals by increasing their daily weight gain [2].

At the same time, there remains a concern that the required quantity of the free bacteria may not survive on their way through the gastrointestinal tract to make their probiotic effect [3].

According to some authors, the technology of microencapsulating the live probiotic bacterial cells is still underdeveloped. Therefore, the delivery of live microencapsulated probiotic bacteria appears to be a relevant problem in the nearest future. To release the encapsulated ingredients, any type of triggers can be used, such as a change in pH, mechanic load, temperature, fermentation activity, time and osmotic force. The task is to select the suitable encapsulation technique and the encapsulation materials [4].

There is a wide variety of microencapsulation techniques, but each of them is designed, first of all, to solve the problem of the probiotic microflora survival. For instance, *Saccharomyces boulardii* and *Enterococcus faecium* were encapsulated with the emulsion and internal gel-formation method, which resulted in a considerable increase of the microorganism survival both in vitro and in vivo [5].

The probiotic release mechanism (in the intestines) plays a special role because, when not comprehended, the quantity of the delivered bacteria may be much lower than that introduced at the beginning. The probiotic release mechanism may also be adjusted depending on the intended effect. The delivery to the small intestine facilitates the immune response, and the large intestine is the traditional destination for the antipathogenic activity. The majority of published data available today do not provide any information on the nature of the release, opening new opportunities for further research. In particular, the digestion of the encapsulation matrix with the intestinal microbiota may be a better method of the direct delivery of the additive to the large intestine than pH [6].

The second important aspect is the selection of biomaterials for the microcapsule formation. The selection of biomaterials for probiotic encapsulation causes the following problems: the physical and chemical properties (chemical composition, morphology, mechanical strength, stability of the stomach and bowel fluids; toxicological analysis; production and sterilization processes [7].

Generally, the probiotic encapsulation technology may be divided into two types: 1 – microencapsulation of probiotics in encapsulating solutions and 2 – drying the encapsulating solution for the production of encapsulating cell powders or granules. [8]

The academic literature provides a lot of information about combining the probiotic additives with various auxiliary components to improve digestion metabolism [9, 10]. Together with that, the idea of enhancing the digestion process by applying enzymes in the microcapsule core appears interesting.

Fermentation can increase the production capacity or change the relative diversity of short-chain fatty acids, causing a faecal output increase, moderate pH decrease in the large intestine, reduced fermented output products and faecal enzyme volume, or improve the immune system, which are positive effects [11].

According to the research experience, the combination of probiotic additives with enzymes makes an impact on the gain of live weight of animals and poultry, thereby indicating a synergy effect of the combined probiotic and enzymic treatment [12]. The commercial probiotics used in animal husbandry are said to increase the productive capacity of the animals by increasing their daily weight gain and better efficiency of cattle feeding at the feed yard. In addition, they are to increase the milk production by dairy cows and improve the chicken growth rate [14], and the health and productiveness of the young calves [13].

For this reason, the technical task for this research is increasing the efficiency of the microencapsulated Enzyme-enriched Enzymesporine feed additive by reducing the loss of probiotic bacteria in the microencapsulation process (increasing the CFU/g in the finished product), and increasing the digestive capacity by introducing crystalline trypsin into the additive composition.
2. Materials and research methods

The microencapsulation technology for the Enzyme-enriched Enzymesporine feed additive was developed and the additive was tested on laboratory animals at the biochemical laboratory and vivarium of Kursk State Agricultural Academy, federal state budgetary educational institution of higher education.

Enzymesporine, the primary probiotic additive, is produced by Fermlab, OOO (Moscow, Russia). The additive incorporates the following bacterial strains: *Bacillus subtilis* RNCIM B-314, *Bacillus licheniformis* RNCIM B-8054, *Bacillus subtilis* (*Bacillus natto*) RNCIM B-12079 and crystalline trypsin at the given quantity. According to the regulatory documents, the CFU value varied within the range of 5.0–6.3×10^9 per 1 g of the additive.

For Enzymesporine microencapsulation and enrichment with the enzyme, crystalline trypsin, i.e. for the development of the microcapsule shell, sodium alginate was used. We used the physical and chemical method of non-solvent addition, using 40% acetone solution as a precipitator and 2.2 M calcium chloride solution for stabilization of the capsules. In the process, 0.95 g of enzymesporine and 0.05 g of crystalline trypsin were dispersed in small portions in the sodium alginate suspension, containing 3.0 g of sodium alginate in 100 ml of distilled water under the mixing speed of 800-1000 rpm. Then, 100 ml of 40% acetone solution and 25 ml of 0.2 M calcium chloride solution were slowly introduced. The mix was continuously stirred for 15-20 minutes and then the hardened microcapsules were separated by filtering through the glass filter and drying at 30-35°C. The result was microencapsulated enzymesporine with trypsin in sodium alginate at the temperature of 24-27°C with the microcapsule output. The microcapsules with enzymesporine and crystalline trypsin in their core and sodium alginate shell were produced at the core/polymer proportion of 1:3.

The produced laboratory sample of the microencapsulated Enzymesporine additive underwent bacteriological testing at the Bacteriology Department of Kursk Oblast Veterinary Laboratory. For this purpose, a sodium alginate polymer capsule was destructed with the phosphate buffer of pH 7.8-8.0, the culture was titrated and the probiotic bacteria were plated on peptone-corn agar. As a reference sample, a standard bacterial culture in the corresponding media was used.

For preparation of the plating media, the dehydrated organic glue amylodextrin, dextrodextrine, xylodextrine, sterile corn starch for bacilli cultivation, protein-containing sterile dry cheese whey and dry curd whey were used.

The survival rate of the enzymesporine probiotic bacteria was tested in vitro with the hydrochloric acid and pepsin solution under different pH values, modelling the aggressive impact of the medium on the microflora in the stomach digestion conditions.

The quantity of viable bacteria in one dose of the additive (CFU×ml⁻¹) was counted at the beginning of the experiment and after the incubation in hydrochloric acid with pepsin for 120 minutes (pH 1.5; 2.5; 3.5; 4.5; 5.5).

The biological properties of the microencapsulated enzyme-enriched additive produced by the given method were checked with a reference to the microencapsulated Enzymesporine additive produced by the prototype method.

The effectiveness of the Enzyme-enriched Enzymesporine microencapsulated probiotic feed additive was tested by assessing its impact on the daily weight gain of the Genesus pigs, regularly used in industrial pig farming, in comparison with the Enzymesporine feed additive and the microencapsulated Enzymesporine additive produced by the method specified in invention patent No. 2689164 [15] registered in the Russian Federation. The assessment was made at a large animal husbandry facility.

The experimental animals were divided into the following groups:

1) intact group (no probiotic additives introduced; n=20);
2) control group No.1 (Enzymesporine additive used in the dose of 3.0 g per day per head, n=20);
3) control group No.2 (microencapsulated Enzymesporine additive produced by the method described in invention patent No. 2689164 used in the dose of 3.0 g per day per head, n=20);
4) group of interest (microencapsulated Enzyme-enriched Enzymesporine additive used in the dose of 3.0 g per day per one head, n=20).

The animals were randomized by weight and gender.

The overall duration of the experiment was 100 days within the period from day 48 to 148. During the experiment, the test animals were carefully observed from the point of view of their general condition, appetite, absolute mass, average daily weight gain and liveability.

At the statistic processing of data, the average value and standard deviation values are calculated. The differences are considered significant at p<0.05.

3. Results and discussions

The most similar to the suggested microencapsulated additive production technology is the enyzmesporine microencapsulation method patented under No. 2689164.

The main drawback of this method is the usage of 50% acetone solution as a precipitator. The bacteriological survey showed that the quantity of viable probiotic bacteria in the produced microencapsulated additive was 5.4*10⁴ CFU/g (colony-forming units), while in the primary Enzymesporine additive the average quantity of sporogenous Bacillus bacteria was (5.0–6.3)*10⁹ CFU/g. It is explained by the negative impact of the acetone on the viability of the encapsulated probiotic microorganisms.

A distinctive feature of the suggested microcapsule production technique is the use of 40% acetone solution as a precipitator, significantly increasing the viability of the probiotic bacteria; at the same time, it does not evoke any reduction in the microcapsule output. Another distinctive feature is the introduction of the crystalline trypsin enzyme into the feed additive.

The microcapsule output of the new production technique for the Enzyme-enriched Enzymesporine microencapsulated additive was 85-90%. The produced microcapsules look like oval-shaped grey-yellow coloured particles of 80-150 μm.

The bacteriological survey of the new microencapsulated Enzyme-enriched Enzymesporine probiotic feed additive showed that the quantity of the viable probiotic bacteria in the produced microencapsulated additive reached 5.5*10⁹ cells per one gram of the microencapsulated substance. Therefore, the developed method allows producing a microencapsulated enzyme-reached probiotic additive featuring a significantly increased viability of the probiotic microorganisms and a considerable increase (by 100,000 times) of the quantity of colony-forming units in the microencapsulated additive.

The in vitro studies aimed at simulating the acid medium of the stomach and bile in the intestines showed that the survival rate of the probiotic microflora in the simulated physiological conditions reaches 100%. The results are shown in table 1.

Table 1. Survival rate of the microencapsulated enzymesporine bacteria in model hydrochloric acid solutions.

| Sample | pH Hydrochloric acid | Total quantity of bacteria in one dose of the additive before experiment CFU×ml⁻¹ | Live bacteria content in the sample per one hour experiment, min. CFU×ml⁻¹ after 120 minutes | Primary non-encapsulated additive | Microencapsulated Enzyme-enriched Enzymesporine probiotic additive |
|--------|----------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|----------------------------------|---------------------------------------------------------------|
| 1      | 1.5                  | 2.9*10⁹                                                                         | 5.5*10⁹                                                                         | 5.5*10⁹                          |                                                               |
| 2      | 2.5                  | 2.1*10⁹                                                                         | 5.5*10⁹                                                                         | 5.5*10⁹                          |                                                               |
| 3      | 3.5                  | 3.2*10⁹                                                                         | 5.5*10⁹                                                                         | 5.5*10⁹                          |                                                               |
| 4      | 4.5                  | 3.7*10⁹                                                                         | 5.5*10⁹                                                                         | 5.5*10⁹                          |                                                               |
| 5      | 5.5                  | 3.8*10⁹                                                                         | 5.5*10⁹                                                                         | 5.5*10⁹                          |                                                               |
In the effectiveness test of the additive produced by the new method, the following results of the live weight and daily weight gain in different experimental groups were acquired, which are presented in table 2.

From day 48 to 98, the average daily weight gain in the intact group constituted 0.61 kg; in the group with the Enzymesporine additive, it was 0.656 kg, with the additional introduction of microencapsulated Enzymesporine additive, it was 0.682 kg, and with the additional introduction of the studied additive, it reached 0.712, which is obviously higher than the values achieved in the intact and the control groups. From day 98 to 148, the average daily weight gain in the group fed with Enzyme-enriched Enzymesporine was 0.824, which is obviously higher than 0.736 in the intact group and 0.782 and 0.802 kg in the control groups 1 and 2 respectively. The survival rate in all the groups was 100%.

Table 2. Dynamics of the live weight of pigs in the scientific and economic experiment, from day 48 to 148 (M±m), kg

|                  | day 48   | day 98   | day 148  |
|------------------|----------|----------|----------|
| Intact           | 16.2±0.42| 46.7±0.43| 83.5±0.5 |
| Enzymesporine    | 16.2±0.31| 49±0.44  | 88.1±0.56|
| Microencapsulated Enzymesporine | 16.2±0.38| 50.3±0.42 | 90.4±0.61 |
| Microencapsulated Enzyme-enriched Enzymesporine | 16.2±0.31| 51.8±0.58 | 93±0.68  |

\[ a \] \( p<0.05 \) compared to the intact group of animals;  
\[ b \] \( p<0.05 \) compared to the control group 1;  
\[ c \] \( p<0.05 \) compared to the control group 2.

4. Conclusion

Therefore, the acquired results allow assuming that the suggested method increases the viability of the probiotic microorganisms and the effectiveness of the Enzymesporine additive in the microencapsulation process. This is related both with the increase of the CFU/g content in the finished product and the introduction of the crystalline trypsin ferment, enhancing the digestive capacity in the pig bowel. The microencapsulated Trypsin-enriched Enzymesporine probiotic additive makes a more prominent impact on the pig metabolism, compared to a non-encapsulated probiotic additive.

Therefore, this microencapsulated additive may be recommended for use in the animal husbandry as the most promising one for the productivity increase.

Acknowledgments

The reported study was funded by RFBR, project number 19-316-90011.

We would like to express our sincere gratitude to Kharchenko Ekaterina Vladimirovna and Sein Oleg Borisovich from Kursk State Agricultural Academy and to Alexander Nikolaevich Khudin from Kursk State University for the scientific and research laboratories provided for the experimental part of the research.

References

[1] Chistyakov VT 2018 Vestnik of Voronezh state agricultural university 4(59) 71-8  
[2] Danfeng S, Salam I and Saeed H 2012 IntechOpen 10 36  
[3] Călinoiu L-F, Stefănescu B E, Pop I D, Muntean L and Vodnar DC 2019 Coatings 9(3) 194  
[4] Solanki H K, Pawar D D, Shah D A, Prajapati V D, Jani G K, Mulla A M and Thakar P M 2013 BioMed Research International 6 21  
[5] Wentao Q, Xinxiao L, Tingting Y and Weiquan G 2019 Journal of Food Science Technology 56(3) 1398-404
[6] Cook M T, Tzortzis G, Charalampopoulos D, Khutoryanskiy V V 2012 *Journal of Controlled Release* **162**(1) 56-67
[7] Gbassi G K and Vandamme T 2012 *Pharmaceutics* **4** 149-63
[8] Mortazavian A, Razavi S H, Ehsani M R and Sohrabvandi S 2007 *Iranian journal of biotechnology* **5**(1) 21
[9] Gamko L N and Chernyuk Y N 2010 *Zootecniiya* **10** 26-8
[10] Gebru E, Lee J S, Son J C, Yang S Y, Shin S A, Kim B, Kim M K and Park S C 2010 *Journal of animal science* **88**(12) 3880-6
[11] Crittenden R, Playne M J 2009 *Prebiotics. In Handbook of Probiotics and Prebiotics* eds Y K Lee and S Salminen (Hoboken, NJ, USA: John Wiley & Sons Inc.) pp 535–61
[12] Rahman M S, Mustari A, Salauddin M and Rahman M M 2011 *Journal of the Bangladesh Agricultural University* **11**(1) 111–8
[13] Kalavathy R, Abdullah N, Jalaludin S and Ho YW 2003 *Br. poult. sci.* **44**(1) 139–44
[14] Krehbiel C R, Rust S R, Zhang G and Gilliland S E 2003 *J. anim. sci.* **81**(14_suppl_2) E120-32
[15] Trubnikov D V, Sein O B, Gorobets A Y and Trubnikova E A 2019 *Enzymesporine microencapsulation method*. Patent RF, no. 2689164