CHAPTER 9

CURRENT APPROACHES IN MAINTENANCE GUT HEALTH IN FARMING ANIMALS. PART 2. GLYCERIDES BLEND MODULATES INTESTINAL BARRIER FUNCTIONING IN PIGLETS

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INTRODUCTION

The issue of the keeping sustainable piglet intestinal health is becoming increasingly important to both livestock specialists and the scientific community. Sustainable intestinal development and maintaining piglet health are key factors for successful pig breeding. One of the innovative approaches to improve piglet health is to maintain the barrier function of the intestinal system[1]. An effective intestinal barrier consists of a columnar epithelial cell layer that performs two interdependent functions, namely creating a protective barrier against harmful substances and acting as a selective filter for nutrients[2, 3]. Both of these functions are aimed at maintaining homeostatic conditions in the intestine and forming communication pathways between cells and the environment. In addition, the protective property of the intestinal system is the requirement to prevent potentially harmful substances invasion, including pathogens, antigens, and pro-inflammatory factors, from entering the external environment to the intestinal tissue. The intestinal barrier's selective capabilities contribute to the development of the intestinal immune system and the formation of immune tolerance[4, 5].

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A disorder of the intestinal protective mechanisms, namely mucus loss and destruction of epithelial cells contributes to the transport of both beneficial and potentially harmful substances from the intestinal lumen to the internal environment, thus causing an over-activated immune response[6]. Intestinal barrier function depends on the interaction between several barrier components, including the intestinal mucus layer, cytokines and chemokines, antibacterial peptides, and intercellular adhesions[7].

The basic junctions form a continuous and dense branched network between the basolateral cell membranes, which leads to an increase in the compactness of the apical intercellular space. The epithelial ability to maintain barrier function depends on four types of intercellular junctions: desmosomes (DSM), adherens junctions (AJ), tight junctions (TJ), and gap junctions (GJ)[8]. Adhesive contacts and desmosomes are anchor junctions that link actin filaments of the cytoskeleton to the plasma membrane at the sites of intercellular adhesion. Tight junctions create a seal in the plasma membrane to regulate paracellular transport and polarize cells by keeping proteins in their correct compartments[9]. Gap junctions containing connexin electrically connect cells and allow small molecules to diffuse from one cell to another cell.

One of the main representatives of intercellular adhesive contacts is the cadherin subfamily, with E-cadherin being the epithelial-specific protein. This transmembrane protein is involved in cellular processes such as signaling of intercellular contact inhibition, cell proliferation, actin cytoskeleton remodeling, and cell polarity[10]. The functioning of the intestinal epithelial layer requires stable intercellular adhesion to ensure barrier function[11]. A decrease in the content of molecular markers of barrier function can provoke a violation of the epithelial layer integrity and initiate the intestinal barrier destruction[12].

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In spite of the progress in research on the role of adhesive proteins in intestinal barrier function, the important component of barrier function is the extracellular matrix (ECM), which is considered to be a key mediator in many intercellular interactions. There are various components that regulate and activate many cellular processes and ultimately affect the cellular homeostasis. An essential component of the ECM is fibronectin, a glycoprotein involved in tissue integrity through cell-ECM adhesion, proliferation and migration and produced by several types of intestinal epithelial cells[13].

To maintain the protective properties of intestinal system, the biological effects of dietary supplements with short-chain fatty acids and monoglycerides (SCA-M) as the cytoprotector for intestinal barrier have been actively studied recently. Similar supplements contain formic, propionic, citric, and acetic acids, which exhibit antimicrobial properties, confirming the prospects of their usage as supportive agents. Organic acids are used by human as a feed preservative due to their strong antibacterial and antifungal effects. An important point for fruitful application of these compounds is that they have multifactorial effects on the microbiome state, immune response and key intestinal components interactions. Unfortunately, the molecular and cellular mechanisms of SCA-M effect on piglet intestinal health are not well defined. Besides, the growth recent data support the evidence that maintaining the functionality of the intestinal system is a critical requirement for pig breeding to achieve high productive rates and ensure the sustainable development of this field.

The aim of our study was to determine the SCA-M effect on fibronectin content in the small intestine of piglets, total protein content in a blood and average weight gain.

1. Methodological approaches to study the bioeffects of fatty acids blend

The research was carried out in the laboratory of the Research Centre for Biosafety and Environmental Control of Agricultural Resources (Biosafety Centre) of the Dnipro State Agrarian and Economic University (DSAE). The work was carried out on piglets from the 42nd to the 77th day of life in an industrial pig farm. 24 animals were included into the experiment, 2 groups were formed – control and experimental, each consisting of 12 piglets. The experimental group piglets were raised according to the standard protocol of the farm. At the same time, the piglets of the experimental group received SCA-M (C3–C12) from the 42nd to the 77th day of life at a dose of 0.5 kg of the drug per ton of feed. On day 42 of life, control weighing of piglets of

[13] Dhanani K. C. H., Samson W. J., Edkins A. L. Fibronectin is a stress responsive gene regulated by HSF1 in response to geldanamycin. *Scientific Reports*. 2017. Vol. 7. Art. 17617.

both groups was carried out. For weighing, 4 animals from each group were randomly selected. For laboratory studies, 4 pigs from each group were selected on 42nd day of life.

All procedures with piglets were carried out in accordance with the requirements of the Law of Ukraine No. 3447-IV of 21.02.06 "On the Protection of Animals from Brutal Treatment", in accordance with the basic principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Strasbourg, 1986) and approved by the Commission on Bioethics of Animal Experiments of Dnipro State Agrarian and Economic University. After euthanasia, the abdominal cavity was opened and the fragments of the duodenum were isolated.

Protein extract samples were obtained by intestinal tissue homogenizing in 25 mM Tris buffer (pH 7.4) containing 0.2% sodium dodecyl sulfate and a mixture of protease inhibitors. Homogenates were incubated for 60 min at +4°C to extract structural proteins and were centrifuged[14]. The concentration of total protein in each sample was determined by the Bradford method[15]. Samples were mixed in 1:1 ratio with Lemmli buffer containing 0.1 M dithiothreitol and boiled for 5 min. Protein samples were frozen and stored at -20°C for up to two weeks before analysis.

Fibronectin content was determined by immunoblotting as described previously[16]. Anti-fibronectin (Abcam, ab-194586) and anti-GAPDH (Santa-Cruz, sc-69879) antibodies diluted 1/1000 were used in this study. Densitometric analysis of the results was performed using Total Lab TL120 software. The molecular weight of stained polypeptide bands was identified by extrapolating the relative mobility of each polypeptide on the pre-stained proteins with known molecular weights (PageRuler Prestained Protein Ladder, Fermentas, Germany).

Total protein content was measured with Bradford method (Bradford 1976). The results of total protein content in blood serum and tissue extracts were expressed as a percentage relative to the control group. The obtained results are presented as an average ± standard deviation (SD) on histograms. The results were analyzed with using one-way analysis of variance (ANOVA) followed by

[14] Low doses of imidacloprid induce disruption of intercellular adhesion and initiate proinflammatory changes in Caco-2 cells / V. S. Nedzvetsky et al. *Regulatory Mechanisms in Biosystems*. 2021. Vol. 12, No. 3. P. 430–437.

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Bonferroni's post-hoc test. Changes in indicators were considered significant at $P < 0.05$.

2. The results on bioeffects of fatty acids blend

To evaluate the effect of the drug SCA-M on productive parameters, the average daily weight gain of animals in the control and experimental groups was analyzed. The results showed a non-statistically significant increase in the piglet weight of the experimental group compared to that of the control group (Fig. 1).

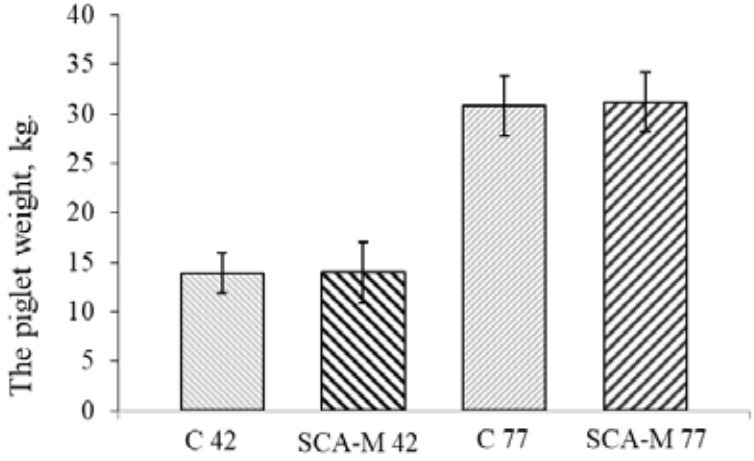

Fig. 1. Average weight gain of animals from 42nd to 77th days in the control group (C) and the group of piglets treated with SCA-M (SCA-M)

The total protein content in the blood serum and small intestine tissue extracts of piglets was determined to control the possible effect of SCA-M on protein metabolism and to normalize the immunoblot results corresponding to individual data. The results of total protein determination did not reveal a significant difference between the control and experimental groups in both blood serum and piglet intestinal tissue extracts (Fig. 2).

The fibronectin content in the small intestine tissue of piglets was determined to identify the SCA-M effect on the state of the extracellular matrix. The obtained results showed a significant increase in fibronectin content in the group of piglets consuming SCA-M compared to the control group (Fig. 3).

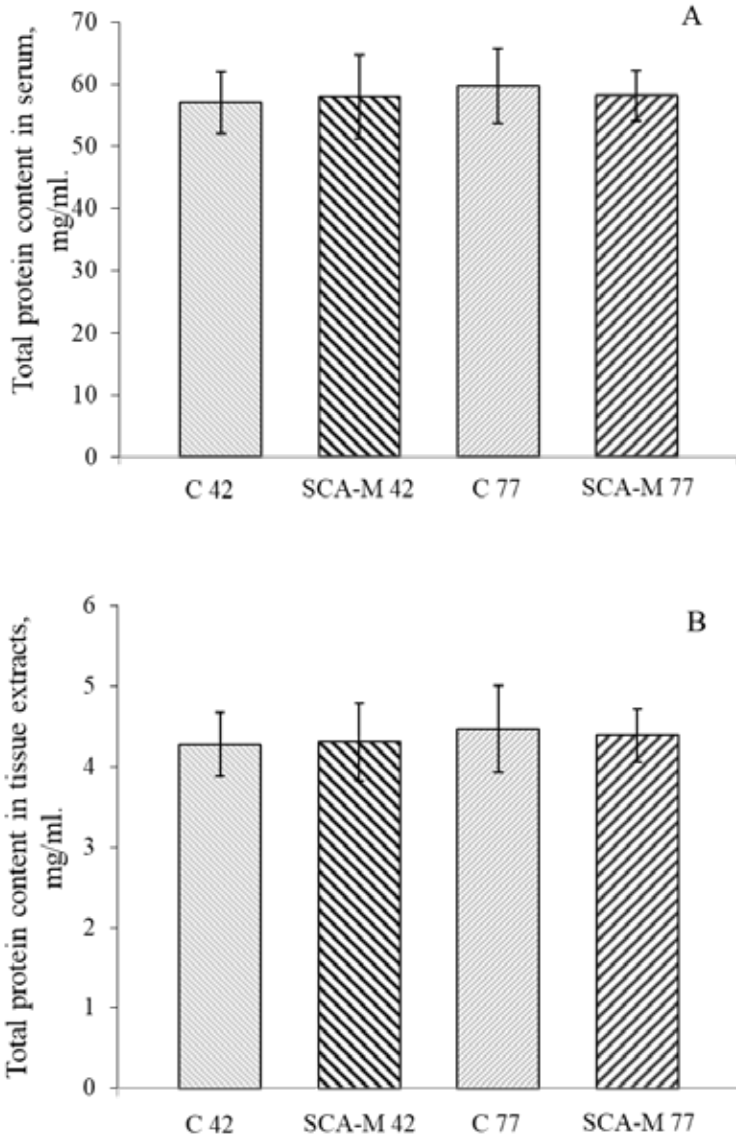

Fig. 2. Total protein content in blood serum (A) and small intestine tissue extracts (B) from control group piglets (C) and piglets treated with SCA-M

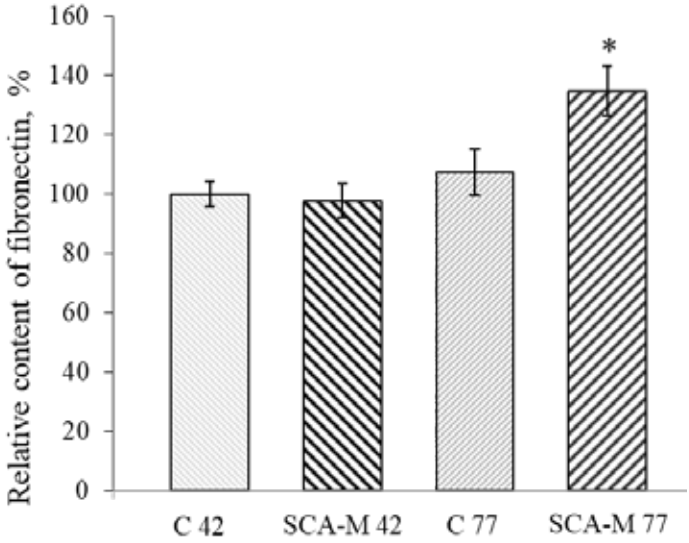

Fig. 3. Fibronectin content in the small intestine of the control group (C) and the group of piglets fed SCA-M. Significance of the differences in compare to 42th day SCA-M group: * – P < 0,05

In general, obtained results indicate that the consumption of SCA-M initiates an increase in the biosynthesis of ECM proteins, in particular, fibronectin.

3. The protective effects of fatty acids blend supplementation

The main tasks in the context of limited antibiotics usage are to ensure human and animal health, food safety, and increase the efficiency and quality of animal production. All these tasks cannot be achieved without understanding the molecular and cellular mechanisms of antibiotic alternatives actions. An equally important issue is the need to integrate research in nutrition, health and veterinary medicine. Both model experiments and field trials of possible antibiotic alternatives in different livestock production systems are considered necessary to address these challenges[17]. Such research should be effective if it is based on the study of fundamental indicators, namely, molecular markers of central pathways for cellular function control and whole organism viability support[18].

[17] Alternatives to antibiotics: a symposium on the challenges and solutions for animal health and production / T. R. Callaway et al. *Antibiotics (Basel).* 2021. Vol. 10, No. 5. Art. 471.

[18] Morphology of inner cell mass: a better predictive biomarker of blastocyst viability / S. Sivanantham et al. *Peer J.* 2022. Vol. 10. Art. e13935.

In recent years, large number research has been conducted to identify functional feed additives with similar beneficial effects as antibiotic growth promoters. The focus has been on prebiotics, probiotics and synbiotics, which are proposed as means of supporting intestinal health in various animals, including pigs[19]. A significant number of studies have shown some beneficial effects of such agents when using these functional feed additives as an alternative to antibiotics. The use of these agents can inhibit the pathogenic microflora growth and promote the commensal microbiota taxa progress. It has been shown that the suppression of *Lawsonia intracellularis* through the feed additive of the probiotic Bacillus pumilus can be one of the ways to maintain piglet health after weaning[20].

Recent results have shown that the use of probiotics can be beneficial in controlling intestinal infections in post-weaning piglets. It has also been suggested that a mixture of probiotics can be used to increase microbiota diversity, which mitigates the impact of L. intracellularis pathogenic infection[21]. The use of preparations containing Lactobacilli in piglets infected with pathogenic Escherichia coli K88 showed a beneficial effect associated with the villi morphology maintenance and expression of tight junction genes that regulate intestinal barrier function and permeability[22].

A special place among the probiotics suggested as a replacement for antibiotics is occupied by Lactobacillus, which are well known and used by humans to make yoghurts and cheeses[23]. Lactobacillus delbrueckii has been shown to inhibit the proliferation of various pathogenic taxa including Clostridiales[24]. In addition, Lactobacillus delbrueckii improves the morphology of the mucosal layer, the length of villi and increases the content of butyrates, isobutyl and isovaleric acids, which provide antimicrobial effects in the animal intestinal system.

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[24] Effects of dietary supplementation of Lactobacillus delbrueckii on gut microbiome and intestinal morphology in weaned piglets / X. L. Wang et al. *Frontiers in Veterinary Science*. 2021b. Vol. 8. Art. 692389.

Another strategy based on the use of enzymatically processed plant products, in particular, soy has shown beneficial effects on feed transformation, immune response and intestinal barrier function in post-weaning piglets fed an antibiotic-free diet[25]. Consumption of such product improved the small intestinal villi morphometric parameters and the expression of enterocyte adhesion proteins, including Z-occludin 1[26].

Unfortunately, there is currently no experimental evidence that individual antibiotic substitution strategies can be effective in terms of growth promotion and antimicrobial protection. On the other hand, given the individual properties of certain antibiotic candidates, combining individual strategies is a promising direction in developing an effective alternative to antibiotics. For example, a beneficial effect on intestinal health as an alternative to antibiotics has been shown as a result of the probiotic's combined usage with yeast hydrolysate[27].

The multifactorial beneficial effect has also been investigated in the study of the fatty acid mixture effects on the intestinal health of productive animals. The study of the action mechanisms of short- and medium-chain fatty acids on the functions of the animal intestinal system is one of the priority areas of livestock production, as well as poultry production. In recent years, significant progress in improving organic acid regimens for productivity has been shown in many research studies on broiler chickens[28][29][30]. However, the rejection of the antibiotic usage as a growth stimulant in livestock production necessitates the search for alternative agents that simultaneously exhibit antimicrobial properties and provide sufficient animal growth intensity. A wide range of compounds have been suggested and investigated as potential alternatives to antibiotics. In particular, prebiotics, probiotics, organic acids, emulsifiers, enzymes, essential

[25] Enzyme-treated soybean meal enhanced performance via improving immune response, intestinal morphology and barrier function of nursery pigs in antibiotic free diets / S. Long et al. *Animals*. 2021. Vol. 11, No. 9. Art. 2600.

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[29] Organic acid blends improve intestinal integrity, modulate short-chain fatty acids profiles and alter microbiota of broilers under necrotic enteritis challenge / A. Kumar et al. *Animal Nutrition*. 2022. Vol. 8, No. 1. P. 82–90.

[30] Effect of supplemental glycerol monolaurate and oregano essential oil blend on the growth performance, intestinal morphology, and amino acid digestibility of broiler chickens / S. A. Amer et al. *BMC Veterinary Research*. 2021. Vol. 17, No. 1. Art. 312.

oils, medium-chain fatty acids, and monoglycerides have received particular attention in the last decade[31, 32, 33].

Data on the combined effect of natural compounds as an alternative to the antibiotic use in weaned piglets are extremely limited. In particular, the combined use of benzoic acid, Bacillus coagulans, and oregano oil had beneficial effects on the immune status, microbiome, and intestinal barrier function in piglets[34].

The post-weaning piglet's intestinal system is extremely vulnerable to enteropathogens due to antibody deficiencies, lack of immune response and intestinal barrier function. Therefore, immune response maintenance, intestinal morphology and barrier function are considered to be the main issues for maintaining piglet health after weaning[35, 36]. Unfortunately, the number of studies on the effects of short-chain fatty acids and monoglycerides as an alternative strategy to antibiotics in pig production is extremely limited. In particular, it has been shown that folic acid feed supplementation can improve growth performance and intestinal morphology of weaned piglets by maintaining the epithelial cell renewal balance[37].

Intestinal epithelial cells provide the most critical functions of the intestinal system, namely nutrient transport and barrier properties to toxins and pathogens[38]. Given this fact, maintaining and rehabilitating damaged epithelial cells is a promising area for ensuring intestinal health. Recent results have demonstrated the beneficial effect of a monoglyceride blend on the transmission and spread of porcine epidemic diarrhea virus (PEDV) in piglets during early weaning[39]. It should be noted that the monoglycerides mixture in this study

[31] Role of yeast and yeast-derived products as feed additives in broiler nutrition / R. M. Bilal et al. *Animal Biotechnology.* 2023. Vol. 34, No. 2. P. 392–401.

[32] Organic acid blends improve intestinal integrity, modulate short-chain fatty acids profiles and alter microbiota of broilers under necrotic enteritis challenge / A. Kumar et al. *Animal Nutrition*. 2022. Vol. 8, No. 1. P. 82–90.

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[36] Bacteriophage as an alternative to antibiotics promotes growth performance by regulating intestinal inflammation, Intestinal barrier function and gut microbiota in weaned piglets / Y. Zeng et al. *Frontiers in Veterinary Science*. 2021. Vol. 8. Art. 623899.

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[38] Beneficial effects of phytogenic feed additives on epithelial barrier integrity in an in vitro co-culture model of the piglet gut / D. Wendner et al. *Molecules*. 2023. Vol. 28, No. 3. Art. 1026.

[39] Monoglyceride reduces viability of porcine epidemic diarrhoea virus in feed and prevents disease transmission to post-weaned piglets / F. C. Phillips et al. *Transboundary and Emerging Diseases*. 2022. Vol. 69, No. 1. P. 121–127.

was constructed with using an integrated analysis of the individual properties of the individual components, which ensured a sufficiently high efficiency in preventing viral disease transmission to piglets from contaminated feed. Thus, the combination of different monoglycerides may be a promising direction for finding alternative strategies to antibiotics.

Another useful property of monoglycerides is their soft antimicrobial effect. Mixtures of free fatty acids and monoglycerides can destabilize bacterial cell membranes, causing a wide range of direct and indirect inhibitory effects[40]. Recent studies have shown that the mechanisms of fatty acids and monoglycerides impact are aimed at remodeling bacterial phospholipid membranes and can cause irreversible damage that inhibits the growth of various microorganisms[41]. Recent studies have shown that the activity of fatty acids and their derivatives, including monoglycerides, is directed against various bacteria types[42].

At the same time, the antibacterial activity is largely dependent on the composition of fatty acids and remains not fully understood. The variation of monoglyceride compositions in feed additives is an important factor to find out optimal bioeffect. Observed in our study results demonstrated beneficial effect on fibronectin content in intestinal tissue that could be initiated by SCA-M. In this line SCA-M activates cell metabolism via indirect mechanism related to multiple regulatory pathways as well as cell reactivity. In spite of recent reports, the chronic effect of various fatty acids and monoglycerides on the piglet intestinal system cells, in particular, on epithelial cells as the main target of enteropathogens and toxins, remains unexplored. Also, the possible toxic effects of these compounds on the intestinal epithelial barrier function remain unclear. Given the fact that epithelial cells form intercellular connections with the help of specialized tight junction proteins, adhesion proteins make a major contribution to the intestinal barrier function. The interaction of epithelial cells with ECM also plays a significant role in the intestinal barrier formation[43]. In our study, the fibronectin content was determined to assess the state of ECM as an indicator of the cell-ECM adhesion strength. The results of the present study indicate a stimulating effect of the SCA-M mixture on the fibronectin content

[40] Antibacterial free fatty acids and monoglycerides: Biological activities, experimental testing, and therapeutic applications / B. K. Yoon et al. *International Journal of Molecular Sciences*. 2018. Vol. 19, No. 4. Art. 1114.

[41] Competing interactions of fatty acids and monoglycerides trigger synergistic phospholipid membrane remodeling / B. K. Yoon et al. *The Journal of Physical Chemistry Letters*. 2020. Vol. 11, No. 13. P. 4951–4957.

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[43] Beneficial effects of phytogenic feed additives on epithelial barrier integrity in an in vitro co-culture model of the piglet gut / D. Wendner et al. *Molecules.* 2023. Vol. 28, No. 3. Art. 1026.

and, accordingly, on the cell-ECM adhesion. The strength of such adhesion is a component of piglet health after weaning because this period is characterized as a trigger for the mature organism functions formation. Stimulation of ECM protein synthesis can promote cell fixation and activation of their specialized functions[44]. Thus, an increase in fibronectin content can stimulate the cells of the intestinal system directly through interaction with the ECM, as well as modulate intercellular interaction of epithelial cells. The increase in fibronectin content determined in our study may reflect an increase in the epithelial cell-ECM adhesion strength, which enhances the integrative properties of the intestinal barrier. At the same time, the consumption of SCA-M had a slight effect on the productive parameters, which may be the result of a short-term exposure to the drug.

CONCLUSIONS

Consumption of SCA-M by piglets during 5 weeks contributes to an increase in fibronectin content in the small intestine tissue. Upregulation in fibronectin content could be a part of ECM strengthening and intestinal barrier maintaining. The further study with respect to molecular biomarkers of intestinal barrier modulation with organic acids is required.

SUMMARY

Searching for modern strategies to ensure productive parameters in the context of modern requirements for the relatively limited use of antibiotics as growth stimulants is relevant for the pig breeding industry. First and foremost, maintaining intestinal health is the main goal of feed additive application that have beneficial effects on the productive animals' health. Monitoring the intestinal system state and timely responding to the development of enteropathogens is essential in the context of limited antibiotics usage. Molecular markers of the intestinal system are promising tool for monitoring the basic functions of the intestine, including the intestinal barrier. Given the fact that protection against enteropathogens is provided by specialized cell-cell and cell-extracellular matrix adhesions, changes in the content of intercellular adhesion proteins may reflect the intestinal barrier density. Fatty acid mixtures are considered to be one of the promising alternatives to the antibiotics use in animal farming. In order to determine the intestinal barrier function status, the modulation of fibronectin content as the extracellular matrix marker in piglets was determined after consumption of a short-chain

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fatty acid-monoglyceride mixture (SCA-M). Control group of animals consumed a standard diet. The experimental group exposed to a mixture of SCA-M at a dose of 5 g/kg for 4 weeks. The fibronectin content was determined by immunoblotting in the small intestine tissue of the control and experimental groups piglets. It was shown that the fibronectin content in piglets of the experimental group was significantly higher ($P < 0.05$) compared to the control group. The determination of total protein in the blood serum and intestinal tissue extracts of piglets did not showed a significant difference between the control and experimental groups. Comparative analysis of the average daily weight gain found out a tendency to a more progressive increase in piglet weight. In general, the obtained results indicate a beneficial SCA-M effect on the fibronectin content modulation and intestinal barrier density maintenance in piglets. Comprehensive molecular markers studies of the piglet intestinal system will be useful for the advance of antibiotic-free strategies in accordance with modern pig breeding requirements.

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