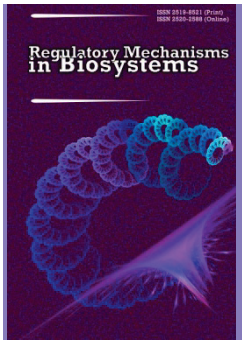

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Morphometric features of enterochromaffin endocrinocytes of the small intestine of piglets receiving prebiotic feed additive

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Enterochromaffin endocrine cells play a key role in stimulating intestinal peristalsis and regulating metabolic disorders by releasing serotonin (5-HT) from their granules. They serve as intermediaries between the intestinal epithelium and specific primary afferent nerve fibers. Histologically, the specificity of enterochromaffin cell characteristics has been established, wherein they possess small secretory granules with pronounced diazo-reaction, located basally or around the nucleus. According to morphometric studies of the small intestine of piglets on day 7, the largest number of enterochromaffin endocrine cells was localized in the duodenum, averaging 7.8 cells per 0.45 mm² area, slightly fewer in the ileum at 5 cells, and the least number in the jejunum at 3.4 cells. On day 14 of the experiment, after feeding the piglets in the experimental group with the prebiotic feed additive "Globigen Jump Start", there was a tendency for their number to increase by 5%, while on day 28, a significant increase of 26.5% was noted, indicating a positive effect on the release of serotonin and improved intestinal peristalsis. Tracking the dynamic change in the number of enterochromaffin cells in the jejunum of the experimental piglets from days 7 to 28 revealed a slight decrease on day 14 (5.9% reduction) and a trend towards an increase of 5.9% on day 28 compared to the value on day 7. Analysis of the quantitative indicators of enterochromaffin cells in the ileum of the control and experimental groups on day 14 also showed a significant difference, with an increase in the experimental group by 36.4% compared to control animals, maintaining this trend on day 28, where the number of enterochromaffin cells in the experimental group exceeded that in control animals by 16.9%. The volume of enterochromaffin cell's nuclei in the duodenum of the experimental group on day 14 was 1% higher than that of the control group, while on day 28, the size of the nuclei tended to increase in both groups. A similar trend was observed in the jejunum; from days 7 to 28, the experimental piglets showed an increase in nuclear volume of 4.3% (day 14) and 6.5% (day 28) compared to the measurement on day 7. The volume of nuclei of enterochromaffin cells in the ileum of experimental piglets was 3.6% greater on day 14 and 3.0% on day 28 compared to the volume on day 7. Thus, the trend of increase in the volume of enterochromaffin endocrine cell nuclei, both on days 14 and 28 of the experiment in all segments of the intestinal tract of the experimental group may indicate active synthetic processes within the cell and stimulation of serotonin production with a positive impact on digestion and intestinal peristalsis.

Keywords: piglets; prebiotic; duodenum; jejunum; ileum; enterochromaffin endocrine cells; nucleus; morphometry.

Introduction

Enterochromaffin cells (EC cells) are the most abundant type of enteroendocrine cells in the gastrointestinal tract (Lund et al., 2018). They play a crucial role in stimulating intestinal motility by releasing the serotonin hormone (5-HT) from their granules. The first descriptions of these cells in the gastrointestinal tract were published more than a century ago. Rudolf Peter Heidenhain, Paul Langerhans, and M. C. Ciacco were among the prominent early researchers in this field. Heidenhain characterized a new class of clear cells (1868) in the gastric mucosa of rabbits and dogs, Langerhans identified pancreatic islets in 1869, and Ciacco (1906) coined the term "enterochromaffin" (Rezzani et al., 2022).

It is worth mentioning the Ukrainian scientist Mykola Kulchytskyi, who also played an important role in enterochromaffin cell research (1887), which later led to the discovery of a diffuse neuroendocrine system in the intestine. He described granular cells in the gastrointestinal crypts of cats and dogs. Kulchytskyi wrote about his discovery as follows: "In the epithelial layer of the intestinal tract, I had the opportunity to study cells that, to my knowledge, have not yet been described by other scientists and that are undoubtedly of great interest in relation to current knowledge of the intestinal tract histology" (Drozdov et al., 2009; Rezzani et al., 2022). At that time, there was still lack of sufficient data on the functions of these cells in the body and their biochemical properties, which generated controversy among the researchers and consequently led to confusion and misinterpretation. The same cells have been described as Kulchytskyi cells, Schmidt yellow cells, Ciacco enterochromaffin cells, Masson argentaffin or silver-reducing cells, and Cordier chromoargentaffin cells – all by the names of researchers who studied them (Simard & Van Campenhout, 1932). Ciacco suggested the term "enterochromaffin" should be used to define the actual characteristics of these cells and their anatomical location only in 1906 (Ciacco, 1906). The studies of all the aforementioned scientists contributed to the description of the diffuse neuroendocrine system by Feyrter (1938), allowing us to understand the syncytial regulatory system, which consisted of both endocrine and neural components (Ahlman & Nilsson, 2001; Drozdov at al., 2009; Koo et al., 2021).

The morphological features of enterochromaffin cells and their identification by secretory granules has already been studied in more detail using electron microscopic studies (Solcia et al., 1980; Wade & Westfall, 1985; Kuramoto et al., 2007). These granules were available in the cytoplasmic vesicles and had a characteristic size and shape for each cell type (Kuramoto et al., 2007; Prudyus, 2023). Based on the studies, it is now widely recognized that enterochromaffin cells are divided into subtypes,

each of which produces certain hormones. These cells are components of the organism's APUD system. Wade & Westfall (1985) showed that enterochromaffin cells in the duodenum of mice extend from the basal layer of the crypt epithelium to the crypt lumen (Wade & Westfall, 1985).

It is important to note that EC cells are the main enteroendocrine cells (Kim & Camilleri, 2000) able to synthesize and secrete various signaling molecules and hormones, such as serotonin (5-HT), corticotropin-releasing hormone (CRH) (Kawahito et al, 1994), cholecystokinine (Fakhry et al., 2017), glucagon-like peptide-1 (GLP-1) (Lee et al., 2012), YY peptide and substance P (SP) (Nøhr et al., 2013). Due to their endocrine potential, EC cells are involved in altering gastrointestinal motility and regulating metabolic disorders (Martin et al., 2017). In addition, EC cells contain various receptors that are used to perceive metabolites such as glucose, fructose, amino acids, lipid amides (Martin et al., 2017), ketones, niacin, aromatic acids, acylamides, and lactate (Lund et al., 2018), as well as microbial metabolites such as short-chain fatty acids (Martin et al., 2017), and secondary bile acids (Lund et al., 2018).

There is proven evidence that serotonin (5-HT) release occurs in response to the addition of various flavor enhancers (Kim & Camilleri, 2000). Animal studies have demonstrated an interaction between T-lymphocytes and EC cells, with a decrease in EC cells number in mice without a T-cell receptor and a decrease in 5-HT release in mice with severe combined immunodeficiency (SCID) (Rubin et al., 2006; Wang et al., 2007). The results of such studies may indicate that EC cells play an indirect role in the formation of the immune response (Jiang et al., 2024). Several researchers have identified seven families of 5-HT receptor subtypes expressed by different cell types in the gastrointestinal tract, including enteric neurons, smooth muscle myocytes, absorptive enterocytes, and interstitial cells (Tonini, 2005; van Lelyveld et al., 2007; Spohn & Mawe, 2017). This suggests that serotonin (5-HT) released from EC cells acts not only in an endocrine way, but also in an autocrine, paracrine, and neurocritical way.

Scientific studies (Tonini, 2005; van Lelyveld et al., 2007; Hasler, 2009) show that EC cells are able to initiate intestinal smooth muscle contraction by activating excitatory cholinergic neurons responsible for smooth muscle innervation. In response to a stimulus, enterochromaffin cells secrete serotonin, which interacts with 5-HT (4) receptors on the nerve endings of internal primary afferent neurons (IPAN) (Hakanson et al., 1994; Costedio et al., 2007; Jones et al., 2020). In addition, serotonin has a direct effect on serotonin receptor subtypes in intestinal smooth muscle, which predominantly causes relaxation (Björnsson et al., 2002). The simultaneous contraction and relaxation of smooth muscle creates a wave similar to a peristaltic one (Gunawardene et al., 2011).

Thus, enterochromaffin cells can be considered as a kind of influence sensor on the gastrointestinal wall, which become intermediaries between the intestinal epithelium and specific primary afferent nerve fibers (Bellono et al., 2017). This allows one to consider the study of enterochromaffin cells and their functional characteristics a promising method for monitoring and controlling changes caused by a variety of factors in the intestinal lumen, such as the effects of infectious and commensal microorganisms, chemical and physical irritants, and various feed and flavor additives (Jiang et al., 2024). In our opinion, this will improve animal welfare, feeding approaches, and increase animal productivity, which is a top priority in the industry. A clear understanding of the interaction mechanisms between the organism and the intestinal lumen makes it possible to predict and influence further changes in its functioning at critical moments, minimize weaning stress and reduce the use of antimicrobial drugs.

Our research is aimed at studying the morphological features of enterochromaffin cells in the intestine of piglets beginning from the neonatal period and continuing into weaning, as well as identifying the influence of feed additive Globigen Jump Start.

Materials and methods

During the research, full compliance with ethical requirements regarding the use of animals in experimental studies was observed. The maintenance, nutrition, care of animals and their withdrawal from the experiment were carried out in accordance with the principles set forth in the "European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes" (Strasbourg, France, March 18, 1986, ETS No. 123) and in Law of Ukraine "On Protection of Animals from Cruel Treatment" (Kyiv, February 21, 2006, No. 3447-IV). The research methodology was approved by the bioethical commission in the Institute of Animal Biology (National Academy of Sciences of Ukraine, Protocol No. 93-01 of June 3, 2021).

The study was conducted at the farm of Barkom LLC (Lviv region, Ukraine) on piglets of the Large White breed, which were formed into two groups of 20 animals each. Piglets of both groups were fed a prestarter feed starting from 5 days of age. From day 7, piglets of the experimental group were fed the prebiotic supplement Globigen Jump Start in the amount of 2 kg per 1t of the main feed. The additive contains dry yeast and egg powder enriched with immunoglobulins (manufactured by EW Nutrition GmbH, Germany). On days 7, 14, and 28 of the experiment, five animals from each group were euthanized for selection of material for histological examination in compliance with the requirements for ethical treatment of animals used in experimental studies (Strasbourg, 1986; Kyiv, 2002).

Histological and morphometric studies were performed in the educational and research laboratory of the Department of Normal and Pathological Morphology and Forensic Veterinary Medicine of Lviv National University of Veterinary Science and Biotechnology named after S. Z. Gzhytskyi. Fragments of the small intestine were fixed in a 10% aqueous solution of neutral formalin and Bouin's solution. After fixation, the tissue was washed and dehydrated in an ascending series of alcohols, followed by embedding in paraffin blocks according to the conventional methods. Histological sections of 7 μm thickness were made from the paraffin blocks on a sled microtome MC-2 (Mulisch & Welsch, 2010). To detect enterochromaffin cell granules, the sections were deparaffinized, rehydrated, and treated for 30 seconds with a dilute solution (1 mg/mL) of stabilized 5-nitroazidine diazotate in 0.1 M of veronal acetate buffer (pH 9.2). The sections were thoroughly washed in running water, and the nuclei were stained with Mayer's hematoxylin for 6 minutes, followed by rinsing in running water. Argentaffin cell granules were stained orange-red (Mulisch & Welsch, 2010).

Determination of morphometric parameters of the intestinal enterochromaffin cells, namely, their number, was made on 0.45 mm^2 (5 fields of view) of the small intestine mucosa, and the size of their nucleus was calculated using a specially adapted morphometric program for Leica DM-2500 microscope (Switzerland) and Leica DFC 450C camera. The obtained digital data of morphometric parameters were processed using the variation statistics method. All the data were analyzed using Statistica 8.0 program (StatSoft Inc., USA). Results in the tables are demonstrated as $x \pm SE$ (mean ± standard error). Differences between the control and experimental groups values were determined by using the Tukey test (with consideration of Bonferroni's correction), where the differences were considered significant at $P < 0.05$.

Results

Histological examination of the piglets' small intestine on days 7, 14 and 28 of the experiment revealed that none of the enterochromaffin cells formed mitotic figures, but were located separately, less often by two cells, mainly in crypts. The availability of secretory granules located basally or around the nucleus is a specific morphological feature of enterochromaffin cells (Fig. 1). The granules were very small with a pronounced diazo reaction, clearly distinguished against the background of the transparent cytoplasm. The EC cell's nucleus was quite large, of a rounded shape, and with a well-defined nucleolus.

According to the morphometric studies results, we identified that on day 7 of the experiment, the largest number of enterochromaffin cells was found in the duodenum – 7.8 cells in average in the field of view on the area of 0.45 mm^2. Slightly fewer EC cells were found in the ileum (5 cells) and the lowest number of them was in the jejunum (3.4 cells in the studied fields of view, Table 1). Comparing the dynamics of changes in the number of enterochromaffin cells in the piglets' duodenum of the control and experimental groups on day 14 of the experiment, we noted their tendency to increase in the animals of the experimental group by 5 %, while on day 28, a significant increase was found, respectively, by 26.5 % (Table 1).

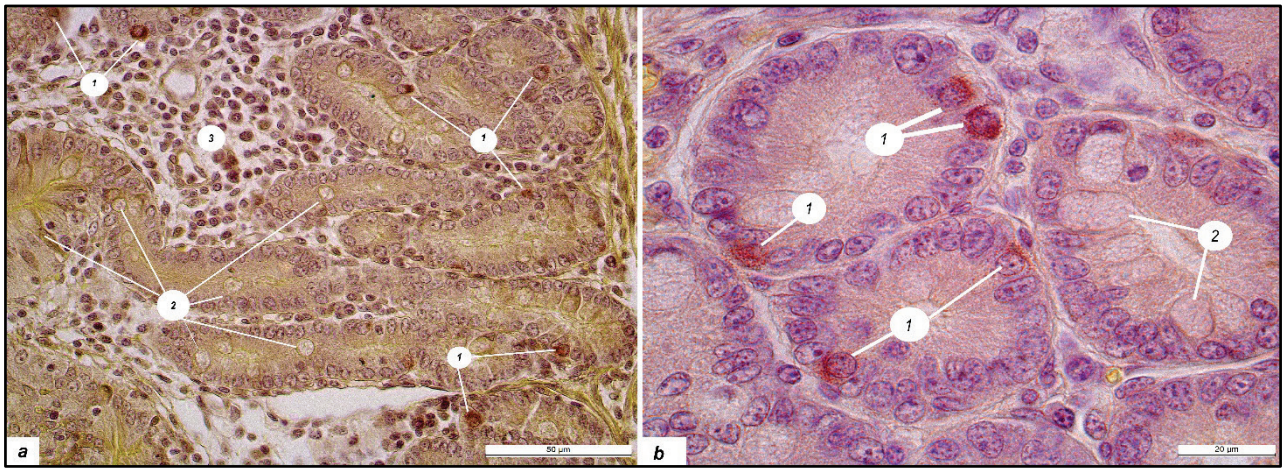

Fig. 1. Piglet's duodenal crypts on day 7 of the experiment: *a* – control group, *b* – experimental group; enterochromaffin cells (*1*) with distinct secretory granules, goblet cells (*2*), lamina propria (*3*); Mayer's hematoxylin + diazo reaction

It may indicate a positive effect of the Globigen Jump Start feed additive on the serotonin release and improvement of intestinal peristalsis (Fig. 2 and 3). Considering the correlation of changes in the number of EC cells in piglets of the control group from day 7 to 28 of the experiment, we noted their tendency to increase by 2.7% on day 14 and 25.6% on day 28 compared to this indicator on day 7. A similar trend was noted in the duodenum of piglets in the experimental group, where the number of EC cells on day 14 and 28 was higher by 7.8% and 58.9% respectively, compared to the number of EC cells on day 7 of the experiment (Table 1).

Morphometric studies revealed a small number of EC cells in the piglets' jejunum on day 7 of the experiment, with an average of 3.4 cells in the defined fields of view. However, on day 14, especially in piglets of the control group, a significant increase of 29.4 % was noted compared to this indicator on day 7 (Table 1).

Comparing the number of EC cells in the small intestine of the control and experimental groups, we noted that on day 14 of the experiment their number was 27.3 % higher in the control group (Fig. 4). Instead, on day 28 of the experiment, an increase in EC cells by 5.9% was recorded in the experimental group compared to the control one. Tracking the dynamics of changes in the number of EC cells in the small intestine of piglets in the experimental group from 7 to 28 days, a slight decrease in the number of these cells on day 14 by 5.9% and a steady increase by 5.9% on day 28 compared to this indicator on day 7 were noted.

As for the ileum of piglets on day 7 of the experiment in terms of switching to another type of feeding from sow's milk to a balanced diet, an average of five EC cells was found in the studied fields of view. When analyzing the quantitative indicators of EC cells of the ileum of piglets in both experimental and control groups on day 14 of the experiment compared to this indicator on day 7, a significant decrease was noted, namely, by 56.0% in control piglets and by 40.0% in experimental piglets. It can probably be explained by the intensive development of the intestine during this period (Fig. 5). However, a significant difference was also found between the quantitative indicators of EC cells in piglets of the control and experimental groups on day 14 of the experiment, namely, their increase in the experimental group by 36.4% compared to the control one and the preservation of this trend on day 28 of the experiment. The number of EC cells in the experimental group was 16.9% higher than the studied indicator in the control group (Table 1).

Table 1

Number of enterochromaffin cells in the small intestine of piglets fed with the Globigen Jump Start feed additive (0.45 mm^2 in 5 fields of view, x ± SD, n = 5)

Day of the experiment	Duodenum		Jejunum		Ileum	
	control group	experimental group	control group	experimental group	control group	experimental group
7^{th} day	7.80 ± 0.86^{a}	7.78 ± 0.78^{a}	3.40 ± 0.40^{a}	3.36 ± 0.56^{a}	5.00 ± 0.89^{c}	5.11 ± 0.76^{c}
14^{th} day	8.01 ± 0.71^{a}	8.41 ± 1.01^{ab}	4.40 ± 0.32^{b}	3.20 ± 0.41^{a}	2.20 ± 0.22^{a}	3.00 ± 0.31^{b}
28^{th} day	9.80 ± 0.73^{b}	12.40 ± 1.09^{c}	3.40 ± 0.24^{a}	3.60 ± 0.21^{a}	7.01 ± 0.53^{d}	8.20 ± 0.72^{e}

Note: different letters in the same indicator indicate samples that are additionally different from each other according to the Tukey test result ($P < 0.05$).

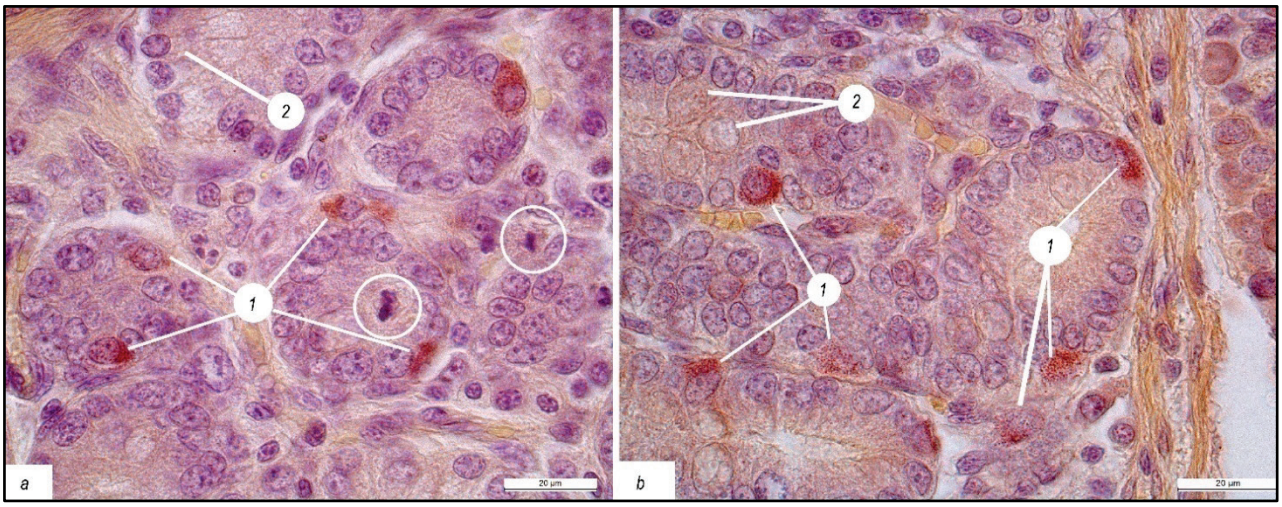

Fig. 2. Piglets' duodenal crypts on day 14 of the experiment: *a* – control group, *b* – experimental group; enterochromaffin cells (1) with expressed secretory granules, goblet cells (*2*), mitosis (in circle); Mayer's hematoxylin + diazo reaction

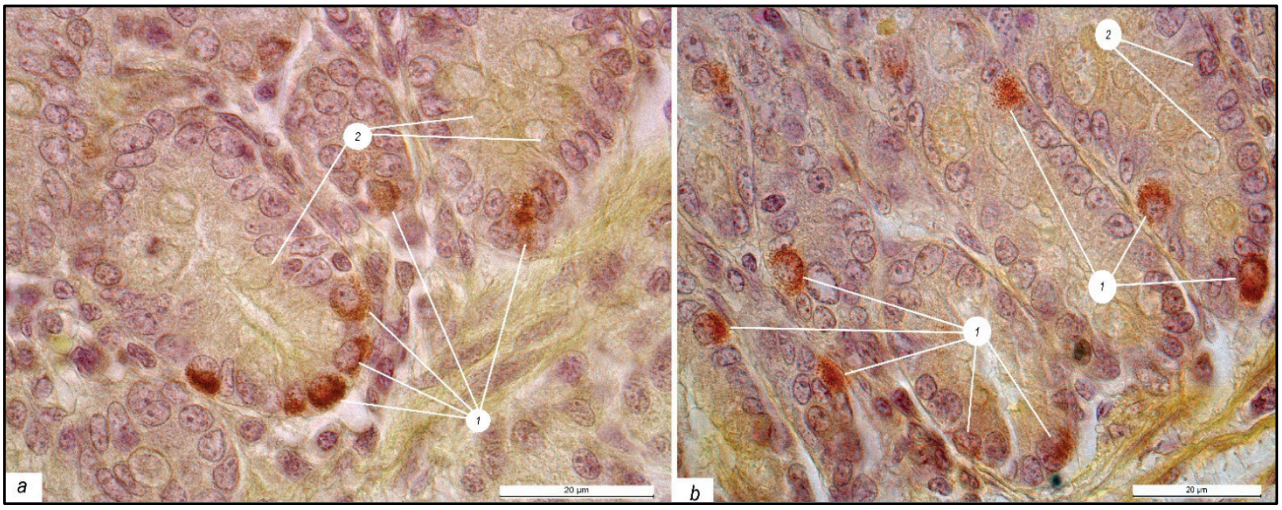

Fig. 3. Piglets' duodenal crypts on day 28 of the experiment: *a* – control group, *b* – experimental group; enterochromaffin cells (*1*) with expressed secretory granules, goblet cells (*2*); Mayer's hematoxylin + diazo reaction

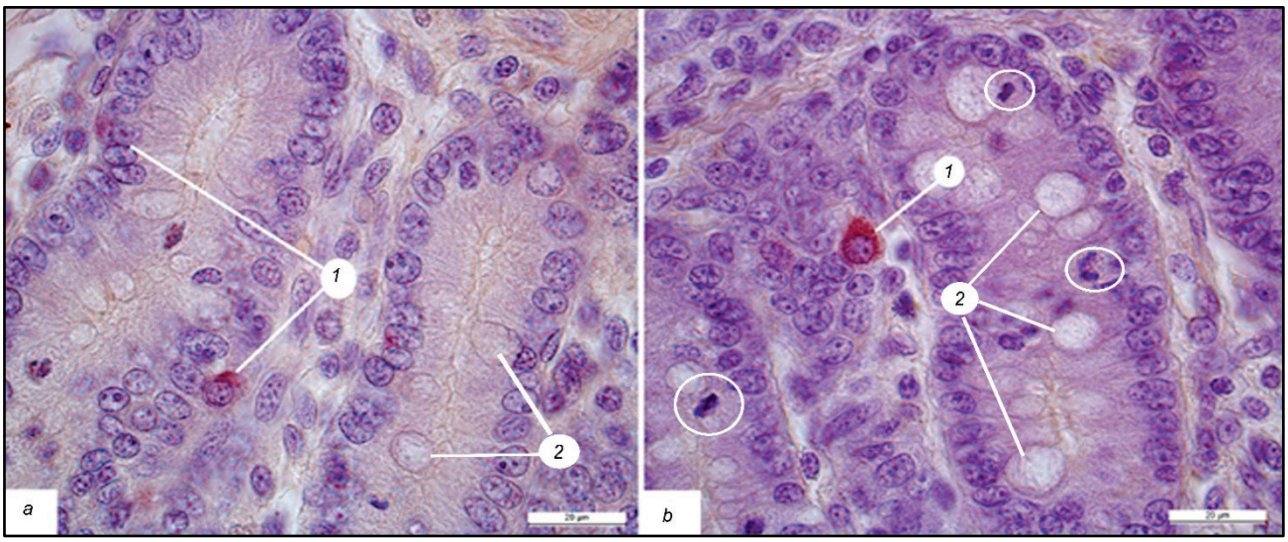

Fig. 4. Crypts of the piglets' ileum on day 14 of the experiment: *a* – control group, *b* – experimental group; enterochromaffin cells (*1*) with expressed secretory granules, goblet cells (*2*), mitosis (in circle); Mayer's hematoxylin + diazo reaction

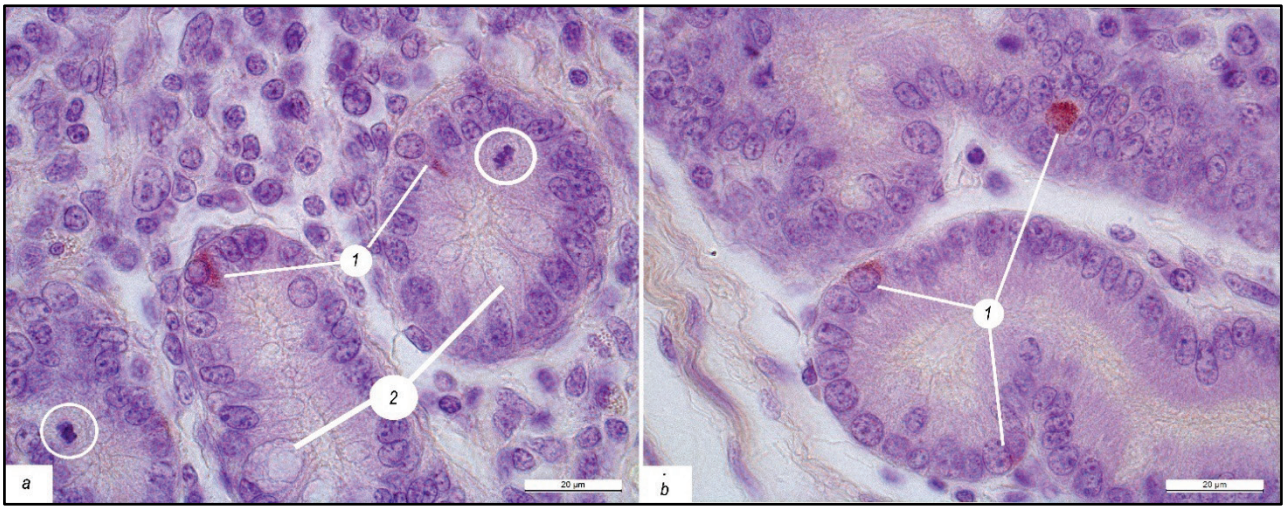

Fig. 5. Crypts of the ileum on day 14 of the experiment: *a* – control group, *b* – experimental group; enterochromaffin cells (*1*) with expressed secretory granules, goblet cells (*2*), mitosis (in circle); Mayer's hematoxylin + diazo reaction

Thus, according to morphometric studies results, we noted that the number of EC cells varies in all parts of the small intestine, which probably depends on the intestine's physiological function. The largest number of EC cells was found in the duodenum, which is associated with the digestion function and the production of numerous enzymes.

Calculating the nucleus volume of the small intestine's enterochromaffin cells on day 7 of the experiment, it was found their size in the duo-

denum was on average 108.1 ± 3.8 μm^3, in the jejunum – 105.4 ± 3.8 μm^3, in the ileum – 109.5 ± 2.8 μm^3 (Table 2). The volume of the EC cells' nucleus in the duodenum of the experimental piglets on day 14 of the experiment was 1% higher compared to the same indicator in piglets of the control group. Meanwhile, on day 28, the nucleus size increased at the level of a trend in both groups and was equal to 111.3 μm^3 in the control group and 112.1 μm^3 in the experimental one, which is 0.7 % more in piglets of the experimental group receiving the prebiotic feed additive Globigen Jump Start (Table 2).

Table 2

Morphometric parameters of enterochromaffin cells' nucleus volume in different parts of piglets' small intestine (μm^3, x ± SD, n = 5)

Day	Duodenum		Jejunum		Ileum	
	control group	experimental group	control group	experimental group	control group	experimental group
7th day	108.1 ± 3.8	107.6 ± 2.3	105.4 ± 5.8	106.0 ± 3.8	109.5 ± 2.8	108.7 ± 2.8
14th day	109.7 ± 3.5	110.8 ± 2.1	106.9 ± 3.7	110.0 ± 3.2	109.0 ± 3.1	113.5 ± 2.2
28th day	111.3 ± 3.4	112.1 ± 1.7	109.2 ± 3.6	112.3 ± 3.5	109.9 ± 3.4	112.9 ± 4.4

In the jejunum, the difference between the volume of the EC cells' nucleus of the control and experimental groups on day 14 of the experiment was 2.9%, while on day it was 28 – 2.8% respectively. In the ileum of piglets, the EC cells' nucleus volume on day 7 was 109.5 ± 2.8 μm^3. During the experimental period from 7 to 28 days under the conditions of adding Globigen Jump Start to the common diet, an increase in the nucleus volume by 4.3% (14th day) and 6.5% (28th day) was noted compared to this indicator on day 7. In piglets of the control group, a similar trend was recorded, i.e. the EC cells' nucleus volume during the experiment was slightly smaller but steadily increased both on day 14 and 28 by 1.4% and 3.6% respectively, compared to this indicator on day 7.

The EC cells' nucleus volume in the ileum on day 7 was 109.5 μm^3. In the experimental group of piglets on day 14, the EC cells' nucleus was 4.2% greater, and 2.7% greater on day 28, compared to the same indicator in the control group. In addition, it should be noted that during the entire experimental period from 7 to 28 days in the control group of piglets, the nuclei of the EC cells remained practically unchanged. Whereas in the experimental group, a significant increase in the volume of the EC cells' nuclei by 3.6% on day 14 and 3.0% on day 28 was noted compared to the volume of the EC cells' nuclei on day 7.

Thus, comparing the enterochromaffin cells' nucleus volume in the two groups of piglets, we found that in the experimental group, both on day 14 and 28, the nuclei were larger in all parts of the intestinal tract, which may indicate active synthetic processes inside the cell and serotonin production stimulation.

Discussion

Some functions of the gastrointestinal tract, like motility, secretion, absorption, microcirculation, local immune defense, cell proliferation, and feed absorption, are regulated by the interaction of gastrointestinal endocrine cells with each other and the enteric nervous system, independently of the central nervous system. Understanding the role of the EC cells in the regulation of biological functions of the animal organism related directly to feeding, as well as the use of prebiotic or probiotic supplements, can be crucial in determining animal feeding strategies. Understanding the complex interactions between individual feed components, intestine microbiota, and intestinal tract cells makes it possible to develop patterns for the prevention or treatment of certain metabolic animal diseases.

It is known that changes in the distribution and quantity of EC cells may depend on the feeding type and the intestinal microflora condition (Turnbaugh et al., 2009; Clarke et al., 2013; Bayer et al., 2021; Szklany et al., 2021). These authors studied the health status of pigs fed rye grain and winter rye hybrid (helltop) and showed that neither diet altered the homeostasis and somatostatin production by neuroendocrine cells. The results were important for the development of a broader piglet feeding strategy (Szklany et al., 2021). The same authors evaluated the number of EC cells in the small intestine of suckling piglets stimulated with red bean lectin (Turnbaugh et al., 2009). The results showed an increase in the number of the serotonin-producing cells in the duodenum, so the authors suggested this could be used as a stimulant for intestinal development in piglets.

It is known that about 95% of the organism's serotonin (5-HT) is located in the gastrointestinal tract, and 90% is contained in the secretory granules of the EC cells' basal and apical parts (Gershon, 2004; Spohn & Mawe, 2017). After intestinal metabolism stimulation, EC cells promote the release of 5-HT from their granules. It is an important mediator that stimulates visceral sensitivity, peristalsis, and intestinal permeability (Martin et al., 2017).

It is well known that the interaction between serotonin (5-HT) and the intestine is highly complex and bidirectional. Such bidirectional relationship demonstrates the mechanism by which 5-HT released from the EC cells can modulate microbiota species, glucose, and lipid metabolism (Yano et al., 2015; Fung et al., 2019). Interestingly, Jones et al. (2020) indicated that apical 5-HT release positively activates the microbiota and, conversely, induces EC cells proliferation (Jones et al., 2020). On the contrary, the release of basal 5-HT can activate afferent fibers of the vagus nerve, causing intestinal peristalsis and promoting gastric emptying through the transmission of intestine-brain axis signals (Bertrand & Bertrand, 2010; Martin et al., 2020). In addition, when 5-HT is released into the bloodstream, it can also stimulate glucose and lipid metabolism. If some duodenal mucosa receptors, such as free fatty acid receptors, are activated, EC cells protect the small intestinal mucosa by regulating the 5-HT biosynthesis and release, but if receptor stimulation is excessive, it can cause abnormal 5-HT release.

The purpose of our study was to determine the number of EC cells in the small intestine of piglets and to establish their distribution in the intestinal tract depending on changes in feeding patterns and the use of prebiotic feed additives.

Conclusion

We found the use of Globigen Jump Start prebiotic supplement in the experimental group of piglets had a positive effect on the small intestine's morphometric parameters, namely, an increase in the number of enterochromaffin cells, especially in the duodenum, and their functional activity, which was manifested by an increase in the volume of the cell nucleus. Such a functionally active state may indicate the stimulation of serotonin production by EC cells with a positive effect on digestion, intestinal motility, and possibly the immune system of the piglet's intestinal tract. In addition, the animals become more resistant to the various kinds of technological stresses (weaning stress, nutritional, and iatrogenic stress).

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