Reactivity of mucosa-associated lymphoid tissue (MALT) in pigs that received in food black grapes seed and skin powder

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Abstract
The study was carried out on 20 pigs from the Petrain breed that received powders and skins of black grapes in 1% ratio for 3 months. At the end of the experiment, samples were taken from duodenum, jejunum, ileum, colon, mesenteric ganglia, which were fixed with 10% buffered formalin, included in paraffin, sectioned and stained with HE. Following the examination, there was a diffuse lymphoid infiltration into the lamina propria of the mucous membranes studied, agglomeration of lymph nodes in the submucosa of the jejunum, ileum and colon in pigs from experimental group (EG). lymphoid follicles from mesenteric lymph nodes are larger and more numerous in pigs from EG compared to control group (CG). Polyphenols from black grape powder in this experiment resulted in significant lymphoplasmocyte infiltration into the mucosa, digestive submucosa and mesenteric lymph nodes and increased carcass weight at slaughter by 1.08 Kg compared to LM.
Keywords*: MALT, black grapes powder, polyphenols, pigs*

Introduction

Vine by-products are a rich source of bioactive molecules, polyphenols and have a remarkable potential in the feeding of animals, being part of the class of food supplements [1]. Polyphenols are abundant in nature and extremely diverse. The term polyphenols includes over 8000 molecules which, by the presence of aromatic rings in their structure, carry one or more hydroxyl groups, with a pivotal role in mediating antioxidant properties [2], [3].

Polyphenols are normally produced by plants for their antibiotic and antifungal properties [4], [5]. Some studies have shown that a diet rich in phenolic compounds has numerous beneficial and therapeutic effects in various acute and chronic diseases [6]- [9].

In piglets, diets rich in polyphenols can cause morphological changes in the intestinal tract [10]. In particular, black grape pomace caused an increase in the size of colonic crypts suggesting better nutrient uptake and reduced activation of intestinal lymphoid tissue, with an immune-modulatory role. It has been suggested that the polyphenols from the plant products introduced into the pigs' diet improved the feed utilization ratio by modifying the intestinal microbism and anti-inflammatory effect [11].

The intestinal mucosa is a preferred gateway for the penetration of microorganisms and needs close monitoring by the immune system. The gastrointestinal tract-associated lymphoid tissue (GALT) consists of a diffuse population of lymphocytes and plasmocytes, antigen-presenting cells, present in the epithelium and the lamina propria of the mucosa, as well as agglomerations organized by lymphonodules in the small and large intestine, named Peyer patches (PP) [12]. They cooperate with a large network of lymph nodes, usually located in the mesentery, filtering the drained lymph from the intestinal wall. The protective function of GALT is extremely important for maintaining gastrointestinal tract homeostasis, in inflammatory processes, intestinal infections, ulcerative colitis etc. Experimental studies of the immune system of the intestine are of particular importance for the biomedical sciences and need a suitable model to reproduce the results used in medical applications. It seems that the best animal model for the study of the physiology and pathology of the gastrointestinal system is the pig [13], being omnivorous makes him closer to humans than to other animal species. Studies on the physiology of the immune system of the

intestine in pigs are also important for veterinary medicine, as this species is of particular economic importance, and the disorders of the gastrointestinal tract include a significant proportion of diseases in this specie.

Materials and methods

The study was conducted within a fattening farm on 20 pigs (10 males and 10 females), of which 10 animals represent the control group (LM) and 10 in the experimental group, in which 1% black grapes seed powder was administered for 3 months in the feed ration. Sampling was carried out within the slaughterhouse. After 3 months, the body weight of all pigs was registered and the pigs were slaughtered, after which blood and organs were taken. Samples from the segments of the digestive tract were taken: duodenum, jejunum, ileum and cecum. The fragments of the digestive tract were washed with a 0.9% sodium chloride solution, sectioned longitudinally, transversely, then transferred to the fixative solution (4% formaldehyde), processed by the paraffin inclusion method, sectioned at 5 μm and stained by the hematoxylin-eosin (HE) method.

Results and discussions

Histological preparations of the intestine were examined, the height of the intestinal villi (μm), the depth of the crypts (μm), the diameter of the lymph nodes in the intestinal mucosa and the mesenteric lymph nodes were measured. The results of the measurements are shown in table 1. CG duodenal villi are shorter, with a slightly increased diameter with lymphoid infiltrations in the lamina propria (Fig. 1A). Enterocytes have been surprised detaching from the tip of the villi. The villi originating from pigs of the EG are taller with smaller diameter, presenting a series of small lateral folds, compared to those of CG (Fig. 1B). Both CG and EG were found to have intraepithelial lymphocytes. The lamina propria from the periglandular area only in pigs from EG showed lympho-plasmocytic infiltration (fig. 1C, D).

Table 1. Variation of histological structures in pigs exposed to polyphenols

probes	height of the intestinal villi (μm)		depth of the Lieberkhun crypts (μm)		Lymph nodes (μm)	
	CG	EG	CG	EG	CG	EG
Duodenum	202.2±23.1	356.6±21.2	425±21.8	457,36±29.8		
Jejunum	146.4±25.1	325.3±23.4	380±24.1	445.4±27.9	300/245± 32.1/29.7	793.75/461.25± 29.2/27.2
ileum	125.2±19.1	156.4±19.2	393.4±26.8	420.6±29.1	213/156± 21.5/19.8	954.3/487.3± 32.1/28.9
mesenteric lymph nodes					200/300± 23.5/28.9	423.4/412,4± 25.1/26.7

The jejunum in pigs from EG shows large lymphoepithelial agglomerations at the level of villi (fig. 2A) in the lamina propria (fig. 2B) but also in the submucosa in the form of lymphonodule agglomerations (fig. 2C). The lymph nodes are numerically and larger in size than in the CG (Fig. 2D).

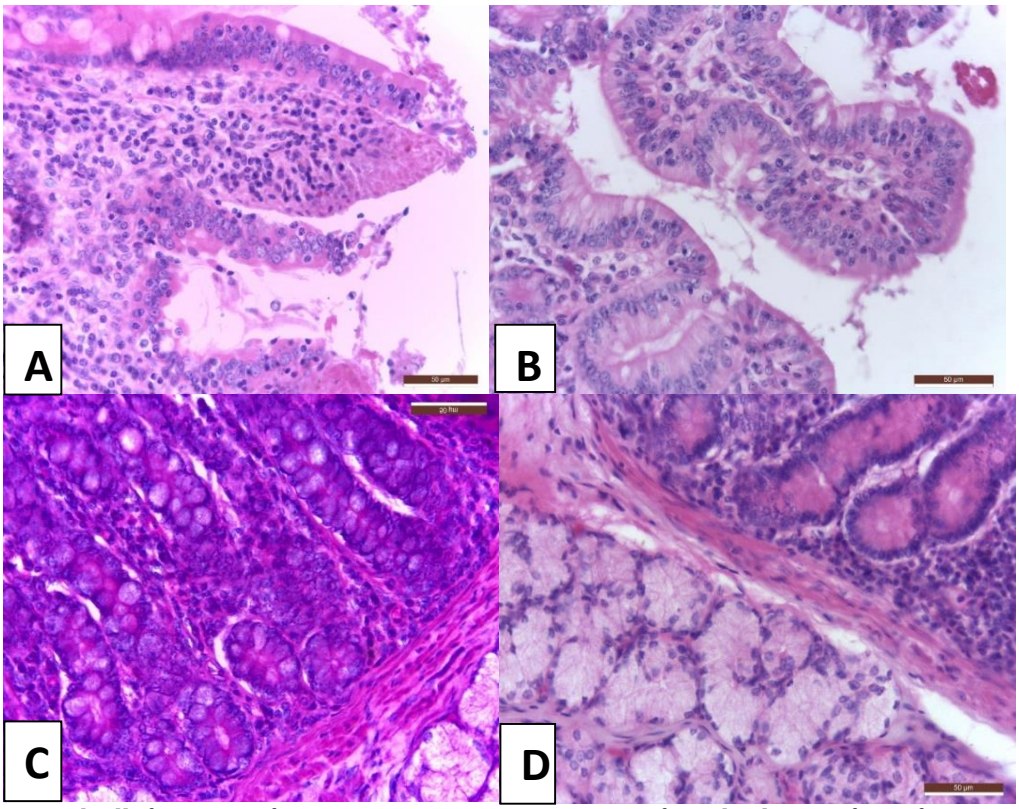
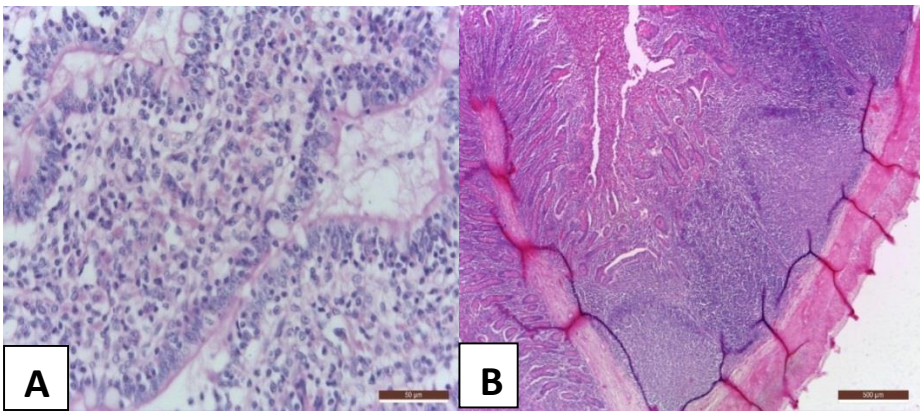


Fig. 1. Intestinal villi from pigs of A- CG; B- EG; C- Lamina propria from duodenum of pigs from CG; D- periglandular lympho-plasmocytic infiltration in EG. Col HE x100.



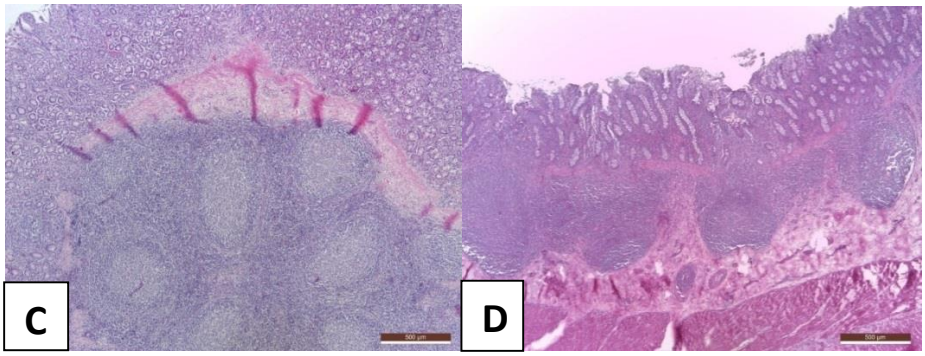


Fig. 2. Jejunum from CG and EG. A- Diffuse lymphoid infiltration into the lamina propria of intestinal villi at EG, HE x100; B- Large lymphoepithelial agglomerations in the mucosa and submucosa of the jejunum in pigs from EG, HE x40; C- Jejunum from pigs from EG with agglomerations of Peyer's patches in submucosa, HEx40; D- Peyer's patches in pigs from CG, HEx40.

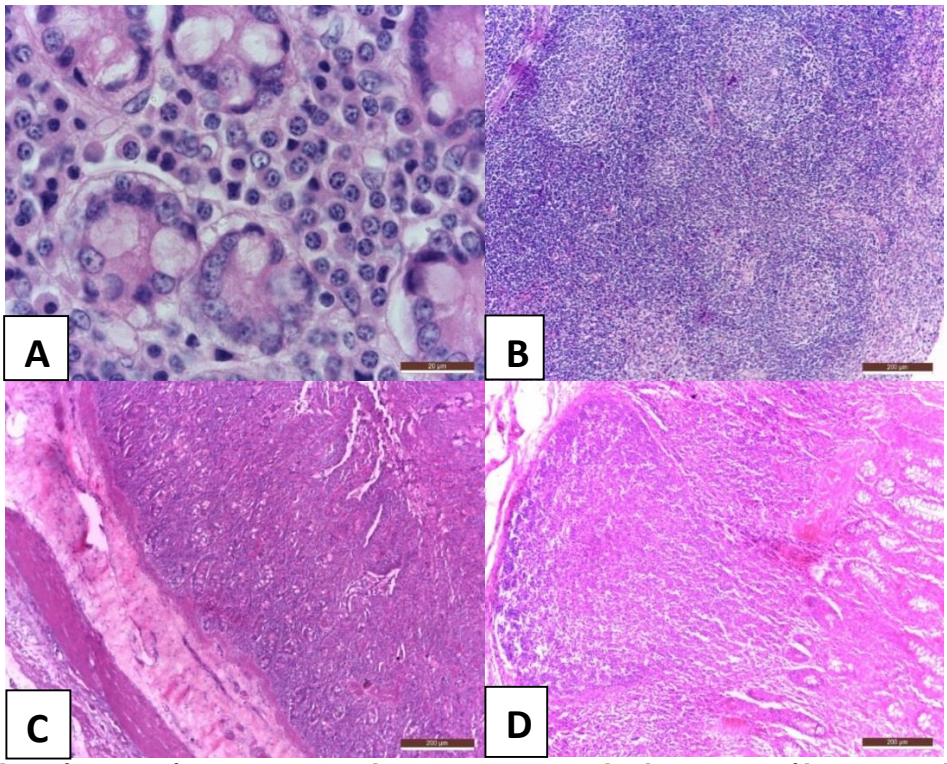


Fig. 3 Ileum from pigs from EG (A-B) and CG (C-D). A- Lymphoplasmocytic infiltration into the lamina propria, periglandular, HE x1000; B- Agglomerations of lymphatic follicles in the submucosa, HEx40; Ileum from pigs from CG. C- Mucosa, muscularis and serous submucosa, HE x40; D- Lymphoepithelial agglomeration in ileum at CG, HEx40.

The ileum from EG shows lymphoplasmocytic infiltration into the lamina propria, periglandular (Fig. 3A) and lymphatic follicles in the submucosa (Fig. 3B). At CG, smaller lymphoepithelial agglomerations were detected (Fig. 3C, 3D).

Histological examination of the colon in pigs from EG revealed a lymphoplasmocyte infiltrate appearance of the mucosa and large lymphoid agglomerations in the submucosa (Fig.

4A). In the pigs from the CG the colon in the examined sections did not presented such changes (fig. 4B).

In the mesenteric lymph nodes in the pigs from EG were surprised more lymphatic follicles and of larger dimensions (fig. 5A) compared to those from CG (fig. 5B).

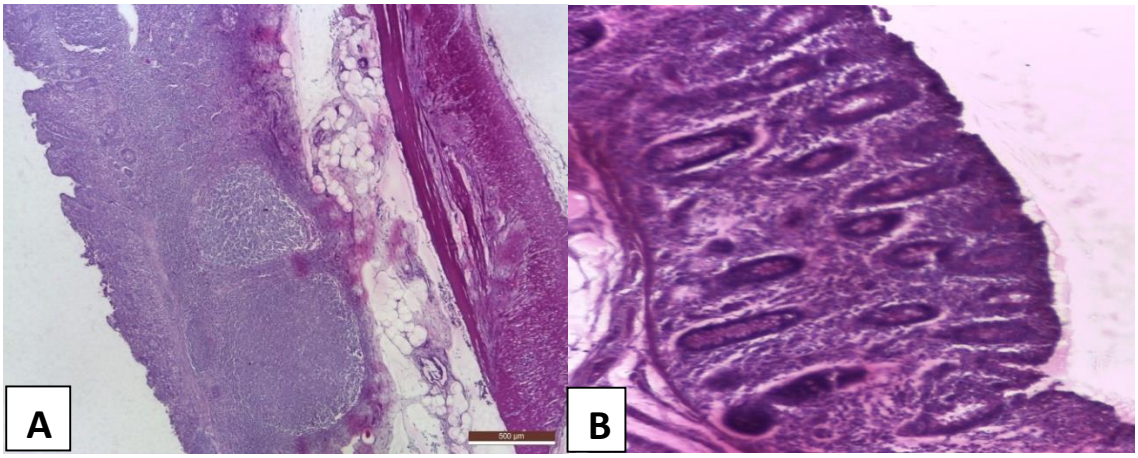


Fig. 4 Colon from pigs from LE (A) and CG (B). A- Lymphoepithelial agglomerations of the mucosa and agglomerations of lymph nodes in the submucosa, HEx40; B- Colon of pigs from CG without lymphoid agglomerations. Col HEx100.

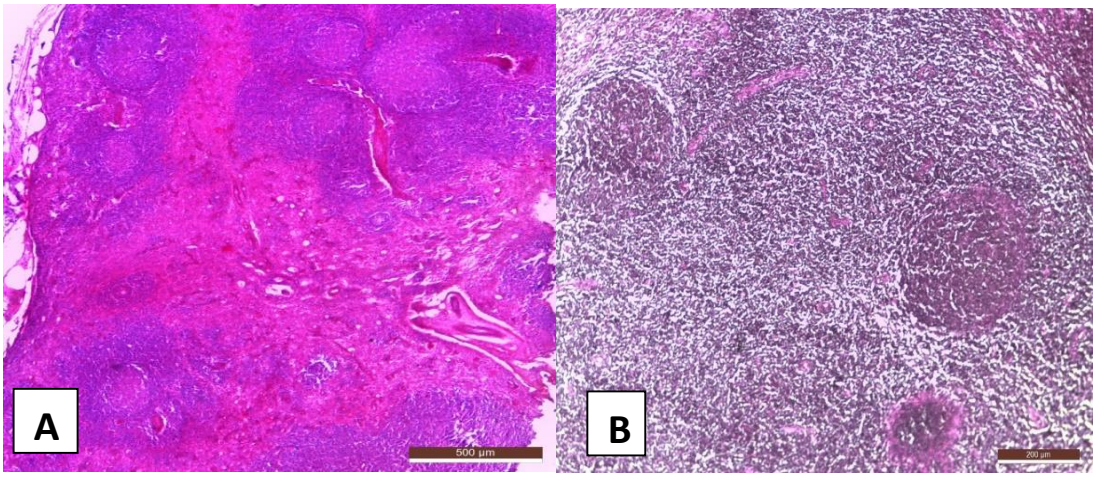


Fig. 5 Mesenteric lymph node in pigs from EG (A) and CG (B), HE x40 (A) x100 (B).

We mention that throughout the experiment the pig had no digestive and respiratory conditions. In addition the yield at slaughter was 1.08kg / individual higher at LE compared to LM.

Discusions

In this study, the inclusion of polyphenols in the diet resulted in an increase in the number of leukocytes in the lamina propria and intraepithelial in the EG. During intestinal inflammation, leukocytes are recruited to the site of infection or inflammation where, through complex interaction, contribute to attracting other immune cells and facilitates the healing of mucous membranes by releasing chemical mediators necessary for the inflammatory response. However,

reactivity can be beneficial in the case of the present experiment, although excessive recruitment and accumulation of leukocytes in the intestine under pathological conditions can be harmful [14]. It is commonly accepted that leukocytes contribute directly to the pathology of the disease when they are over-recruited and activated, leading to the release of toxic substances, massive trans epithelial migration, morphological changes of the villi and crypts and extensive mucosal lesions [14]. It is obvious that leukocytes can act as a double-edged sword that contributes to homeostasis by eliminating pathogens and participating in harmful inflammatory processes, processing and exacerbating inflammation by releasing pro-inflammatory mediators. Thus, the presence of numerous leukocytes in the lamina propria suggests that it is possible that polyphenols from grapes powder as well as those from olive mills [15] may not play a role in promoting inflammation in adult pigs. Our results are in agreement with previous research in the J774 monocyte / macrophage cell line, where different doses of hydroxyprosol have been proved capable of preventing the activation of macrophages [16]. Polyphenols exert a modulatory effect on the inflammatory response also in leukocyte [17], [18]. In fact, they are capable of deregulating the inflammatory response, keeping the tissues free of free radicals and thus preventing inflammatory cascade [19].

In pigs' jejunum, Peyer's patches are organized in the form of associated lymphoid follicles, while the ileum contains a continuous lymphoid follicle (lymphoid plaque) that extends from the distal ileum to the proximal colon [21]. The functional significance of such a lymphoid organization is not known, but it is assumed to play the barrier role of the small intestine (jejunum and ileum), in which the number of bacteria is moderate and of the large intestine where there is abundant microflora, which also contains potential pathogen microorganisms.

The functions of immune cells in the intestine are coordinated by a large network of regulatory substances, interleukins and chemokines, but they are also modulated by the enteric nervous system, which is involved in regulating inflammation and immunity during pathological processes [21-22]. Many lymphatic organs have innervation from cholinergic and adrenergic neurons [23]. Adrenergic and cholinergic nerve fibers also release neuropeptides as co-transmitters and neuromodulators that influence immune cells [24]. GALT cells express receptors for catecholamines, somatostatin, substance P, vasoactive intestinal polypeptide, galanin and neuropeptide Y, which modulate immunoglobulin activation, proliferation and / or synthesis and release [25].

Contact with invading microorganisms is crucial for the development and maturation of the immune system associated with the gut [26]. This system develops gradually in the first 6 weeks of life in several stages, depending on the diet and the environmental [27-29]. In newborns, the intestinal mucosa is very thin with rare lymphoid cells that present antigen for T cells that trigger a proper immune reaction in adults [28]. Their small intestine contains rudimentary Peyer patches (PPs), which non-specifically expand by rapid intrafollicular proliferation of B cells at two postpartum weeks [30]. Simultaneously with the significant numerical growth of B cells, the number of T cells inside the lamina propria and of the interfollicular areas is reduced [31]. The gradual emergence of conventional activated T cells is influenced by an influx of antigens that are presented by dendritic cells, together with major histocompatibility complex class II (MHC II +) and membrane markers CD45 and CD16 with which it coexpresses [28]. Between the second and fourth week of life, there is an increase in the number of mature CD4 + cells in the lamina propria, while CD8 + T cells grow significantly from 4 to 6 weeks of life [28]. Similarly, significant numbers of IgA + plasmocytes were detected after the fourth week of life [26], [30], although the occurrence of IgA-producing B cells in the lamina propria was reported on the sixth day life [30]. IgM + immunoblasts appear earlier, but exceed the number of IgA + B cells after 3 weeks [26], [32]. The maturation of immunity in the weaned pigs, as a result of the interaction with

environmental antigens and the attainment of the adult-like immunocompetence is installed from 7 to 9 weeks of life, determined either by the distribution of immune cells in the small intestine [33] or by functional in vitro analyzes [32] and by defining the role of the lamina propria as a mucosal effector site for bone vaccines [34]. Lymphocyte types vary by area and age. Thus, CD4 + lymphocytes were rare in the follicles and moderately numerous in the interfollicular area. CD8 + lymphocytes were sporadically seen only in the lymph follicles, but were numerous in the interfollicular area. CD21 + (LB) lymphocytes were very numerous in the follicles. No age-related differences were observed between the experimental groups from 3 days to 4 months [22].

To date, there is sufficient evidence to support the anti-inflammatory effect and immune stimulation activity of polyphenols [35], [36], [37]. The ability of polyphenols to act as antioxidants or free radical scavengers, as well as their ability to inhibit some enzymes involved in the generation of free radicals, such as cytochrome oxidase P450, lipoxygenases, COX, are due to the hydroxyl groups that are good hydrogen donors [38]. Polyphenols exert their multiple anti-inflammatory properties by modulating mitogen activation protein kinase and nuclear factor-j. Another way is to inhibit inflammatory cytokines and chemokines, suppressing the activity of inducible cytokine oxidase synthetase and COX [39], [40], [41]. COX-2 is an inducible enzyme that converts arachidonic acid into prostaglandins and is generally induced at the site of inflammation in response to inflammatory stimuli including pro-inflammatory cytokines such as interleukin-1 αβ / b, interferon-c, and tumor necrosis factor by inflammatory cells and tumor promoters such as tetradecanoyl phorbol acetate and Ras [42]. In the study of Varricchio et al. [15], COX-2 immunoreactivity was detected in leukocytes infiltrated into the mucosa, Peyer's patches and solitary follicles of the cecum and colon in pigs fed the control diet. In pigs fed a diet rich in polyphenol, COX-2 immunoreactivity was quite weak. The low level of COX-2 expression in intestinal immunoreactive cells in polyphenol-treated pigs may suggest a protective role of polyphenols, modulating and reducing the inflammatory response. This is in agreement with the research by Willenberg et al. [43], who reported the reduction of COX-2 expression induced by polyphenols both in vitro (HCA-7 cancer cell line and primary monocytes) and in vivo (C57BL / 6N mice). In vivo the effect of polyphenols on the morphology of intestinal villi is controversial. Fiesel et al. [11], report the absence of changes in the height of the villi and the depth of the crypts in the small intestine of the weaned pigs to which grape seeds and pomace flour extract were introduced into the feed, while Sehm et al. [10] report that in the piglets the red grape seeds had an inhibitor effect.

Conclusions

Polyphenols in seeds and black seeds powder cause increased diffuse lymphoid infiltration into the lamina propria of the intestinal mucosa. Also lymphatic follicles larger in volume and more numerous in the mesenteric lymph nodes of the pigs from EG were noted. There was also an increase in the height of intestinal villi, which explains the slightly increased sacrifice efficiency at EG compared to CG.

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