THE EFFECTS OF A PHYTOGENIC ADDITIVE ON THE HISTOMORPHOMETRIC CHARACTERISTICS OF THE INTESTINES IN WEANED PIGS WITH A SUBCLINICAL NATURAL INFECTION WITH *Lawsonia intracellularis*

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Proliferative enteropathy, also known as proliferative ileitis, is one of the most economically important diseases in pig production worldwide. The estimated losses per affected growing pig usually range from US $1 to $5. The disease is caused by *Lawsonia intracellularis*, a Gram-negative, obligately intracellular bacterium. Control of the disease can be achieved with the use of vaccines or antibiotics. Recently there has been an increase in the efforts in the control of certain pathologies of the digestive system with phytogenic additives. The aim of this work was to assess the effects of a phytogenic additive on the histomorphometric characteristics of the intestines in weaned pigs with a subclinical infection with *L. intracellularis* acquired spontaneously. Histomorphometry analysis showed that crypt depth was significantly shorter (P<0.05), and the villus-height-to-crypt-depth ratio (P<0.05) significantly greater in the treatment group than the control. This improvement in the histological parameters of the intestine, considered to be indicators of its health, proved the positive effect of the tested additive on the digestive system in pigs.

**Key words:** proliferative enteropathy, *Lawsonia intracellularis*, phytogenic feed additive, histomorphometry

**INTRODUCTION**

Proliferative enteropathy (PE), also referred to as proliferative ileitis, is one of the most economically important enteric diseases in pig production all over the world [1,2]. Total losses due to PE are estimated from US $1 to $5 per affected growing pig,
but are probably higher because the estimates are based only on clinical cases of the disease and do not involve subclinical ailments [3-6]. It is characterized by epithelial cell proliferation and hyperplasia leading to thickening of the intestinal mucosa of the ileum, but the jejunum and colon can also be affected [1,7]. The affected animals show clinical signs such as anorexia, diarrhoea, reduced growth, and decreased food conversion, but occasional sudden deaths may occur. It is well known that pigs can also be infected subclinically [1,8,9].

PE is caused by the flagellated Gram-negative obligate intracellular bacterium named *Lawsonia intracellularis* [1]. *L. intracellularis* is transmitted in the pig population by the faecal–oral route, and also through contaminated equipment, insects or rodents [10-12]. The bacteria can remain viable in the faeces at temperatures ranging from 5°C to 15°C for up to 2 weeks [10].

The control of *L. intracellularis* infection includes vaccination and antimicrobial therapy, for example with tiamulin, tylosin, tetracycline, lincomycin or some other antibiotics [1,13]. Subtherapeutic uses of antibiotics in animal feeds have been a part of pig production for more than 70 years [14].

However, the use of antibiotics leads to the potential development of resistant bacteria, leading to risk for animal and human health [15]. Therefore, antibiotic resistance is an important global public health challenge. In recent years, more frequently some alternatives have been opted for the use of phytogenic feed additives for the control of bacterial diarrhoea in animals [16-18].

For all these reasons, the increasing trend in pig production is the use of phytogenic additives which are added to the animal feed. They may improve the production performance of the animals owing to their beneficial effects on the health and weight gain and result in better quality of animal products. These additives are composed of plant parts, spices, aetheric oils and resins. Phytogenic components influence the organoleptic properties of feed, which improve its palatability and consequently may increase feed consumption [19]. They stimulate the production of pancreatic enzymes [20], improve digestion [21], exert antimicrobial effects against various pathogens [22,23], have an antioxidative [24] and anti-inflammatory action [25], and positively influence growth and production performance [26,27].

The present study was conducted to determine the effects of a phytogenic feed additive (PATENTE HERBA® PLUS) on the intestinal histomorphometric characteristics in weaned pigs subclinically naturally infected with *L. intracellularis*.

**MATERIAL AND METHODS**

**Phytogenic feed additive**

The experimental substance used was a commercial phytogenic feed additive PATENTE HERBA® PLUS (Patent Co. DOO, Mišićeva, Serbia) containing the extract of *Castanea sativa*, lysozyme, nicotinamide, and a mixture of an essential oils.
blend (mostly from *Thymus vulgaris*, *Origanum vulgare* and *Coriandrum* sp.). The recipe of the additive is proprietary. An additive was added to the feed in a concentration recommended by the producer - 2 kg/t of feed.

**Animals, housing and treatments**

The study was carried out on a commercial pig farm. A total of 12 seven-week-old weaned pigs (Yorkshire × Landrace × Duroc) from a commercial herd were involved. The farm had a previous record of PE outbreaks, including the clinical and subclinical form of the disease confirmed by real-time PCR assay. The presence of any other disease on the farm was not confirmed by routine laboratory tests.

Two experimental groups of 6 weaned pigs were used in this study. All the animals were naturally infected with *L. intracellularis*, which was confirmed by real-time PCR test as previously described by Richter et al. [28]. One group (6 pigs) was the treatment group and another (6 pigs) was the control group. The treatment group (TG) received the feed supplemented with PATENTE HERBA® PLUS, whilst the control group (CG) was fed with the same feed, but without any additives. The pigs had free access to feed and water. Each individual pig was housed in a separate pan. The accommodation and hygiene conditions were the same for all animals throughout the experiment. The experimental feeding lasted for 28 days.

The treatments and procedures on the animals were approved by the Ethical Committee of the University of Belgrade - Faculty of Veterinary Medicine, and the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (Decision no. 323-07-4087/2016-05/1, issued on 9 May 2016), in compliance with the Serbian Animal Welfare Protection Law and the Directive on the Protection of Animals used for Scientific Purposes (No. 2010/63/EU).

**Sample preparation for histological assessment**

Samples of the small intestine were obtained within 20 minutes after slaughter in an abattoir. A 10 cm long portion of the distal ileum, proximal to the ileocecal valve, was collected from each animal and fixed in 10% neutral formalin for 48–72 h. The tissue samples were processed in an automatic tissue processor (Leica Biosystems, Nussloch, Germany) – dehydrated and embedded in paraffin wax. Sections were cut at a thickness of 4 μm, and stained with haematoxylin and eosin for histopathology. Standard glass microscope slides were used for mounting.

**Histomorphometry**

To evaluate the differences in villous and crypt morphometry in the treatment group and the control intestinal sections the average crypt depth, villous height, and villous width were measured and assessed. At light microscopy (10 × objective lens), intact and 10 well-oriented villi and crypts from each intestinal section of each animal were randomly selected for measurement. The villus length was measured from the
villus-crypt junction to the villus tip and the crypt depth from the crypt base to the villus-crypt junction. The same villus and crypt columns were used to determine the number of goblet cells which was expressed per 100 enterocytes. Villus / crypt ratio was calculated by dividing the villus height by crypt depth. Apparent villus surface area was calculated from the villus height and width [29]. The samples were evaluated and measurements were performed with Olympus BX51 microscope with a colour digital CCD camera (Color View III, Olympus) connected to a computerized image analysis system (Olympus Cell B, Olympus, Japan).

Statistical analysis

The statistical analysis of the obtained results was conducted using the GraphPad Prism version 6.00 software for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Statistical parameters were given as means plus/minus standard deviations (X ± SD). The results obtained for the control and treatment group were compared by using unpaired Student's test to assess the significance of differences. The differences were considered statistically significant if P < 0.05.

RESULTS

Microscopic changes

In the group of infected pigs which were not treated with the phytogenic additive (the control group), in the ileum samples, the architecture of the mucosa was properly organized in the intestinal crypts and villi. On the surface, there was some exudate with low quantities of mucus and desquamated cells (Figure 1a). In the mucosa, the goblet cell division rate was moderate, and a proliferation of epithelium cells of the Lieberkühn crypts with pronounced mitosis was seen (Figure 1b). Moderate hyperplasia of the lymphatic tissues of the Peyer’s patches was present (Figure 1c). The lamina propria mucosae was oedematous in some places, and its blood vessels hyperaemic. In two pigs the lamina propria was slightly infiltrated with lymphocytes and eosinophilic granulocytes (Figure 1d).

In tissue samples of infected pigs treated with the phytogenic additive a mild lymphatic tissue hyperplasia was noticed in Peyer’s patches, whilst individual lymphocytes were affected by apoptotic processes (Figure 2a). Lamina propria was heavily infiltrated with lymphocytes and a small number of eosinophilic granulocytes (Figure 2b). The mitotic division of the goblet cells (Figure 2c) and the proliferation of the epithelium cells of Lieberkühn crypts (Figure 2d) were less intense in comparison with those in the control group.

Histological morphometry

The results of the morphometric measurements are summarized in Table 1. Hystometric analysis showed that the crypts were significantly shorter (P<0.05), and the villus height-to-crypt depth ratio (P<0.05) significantly higher in the treated group.
of pigs than in the one that did not receive the additive in feed. The treatment did not affect any of the other parameters which were assessed: villus height, width and surface area, and the number of goblet cells per a hundred enterocytes.

Table 1. Effects of feed supplementation with additive on intestinal morphometric parameters

| Item                                | Experimental group | Control       | Treatment      | P value |
|-------------------------------------|--------------------|---------------|----------------|---------|
|                                     |                    | (X ± Sd)      |                |         |
| Villus height (μm)                  |                    | 529.30±115.20 | 571.20±127.20  | 0.0607  |
| Villus width (μm)                   |                    | 187.80±48.69  | 172.60±42.56   | 0.0728  |
| Crypt depth (μm)                    |                    | 216.20±68.95a | 192.10±47.80b  | 0.0284  |
| Villus height/crypt depth ratio     |                    | 2.72±0.84a    | 3.16±0.79b     | 0.0040  |
| Villus surface area (mm²)           |                    | 0.099±0.034   | 0.098±0.030    | 0.7676  |
| Goblet cells/100 enterocytes        |                    | 19.17±3.19    | 23.60±3.93     | 0.0575  |

*a-b Different lower-case superscript letters in the same row indicate significant differences (P <0.05) between the groups

Figure 1. Microscopic changes in the ileum of the pigs in the control group, HE. a) The image of the intestinal villi and the crypts, exudate rich in mucus and desquamated cells on the mucosal surface; b) Proliferation of Lieberkühn crypt cells with noticeable mitoses (arrow); c) Hyperplasia of the lymphatic tissue of the Peyer’s patches (asterisk); d) Lamina propria infiltrated with lymphocytes and eosinophilic granulocytes.
DISCUSSION

The period of weaning and the beginning of non-maternal feeding is considered to be a highly stressogenic time for the piglets owing to the change in diet, separation from the mother and the transport to different ambient conditions [30]. This is also associated with bacterial intestinal infections, such as enteritis caused by *L. intracellularis* [31].

In the current research, supplementation with a phytogenic additive exerted certain effects on the histological morphology of the ileum in weaned pigs infected by *L. intracellularis*. Changes in the intestinal morphology result in disturbances in the nutrient digestion and absorption [30], as it has been proven that a favourable villus / crypt ratio improves the digestibility of feed components [32].

**Figure 2.** Microscopic changes in the ileum of the pigs treated with the phytogenic additive, HE. a) Hyperplasia of the lymphatic tissues of the Payer’s patches with a pronounced halo around individual apoptotic lymphocytes; b) Infiltration of the lamina propria with lymphocytes and eosinophilic granulocytes; c) Proliferation of goblet cells in the intestinal mucosa; d) Proliferation of the epithelium cells of the Lieberkühn crypts (asterisk).
The positive effect of the additive tested in this experiment resulted in higher villus-to-crypt ratio and the decrease in the average intestinal crypt depth, in comparison to the control group pigs which were not given any additive in the feed.

The average depth of the intestinal crypts in the ileum was significantly reduced in the pigs treated with the supplement in comparison to the control. Shallow intestinal crypts and high villi are the indicators of the gut health [33,34]. In this research, the villus-to-crypt ratio was higher in pigs which were provided with the phytobiotic supplement, which is a result in line with some other reported previously [35-37].

It is shown that antibiotics and various feed additives (prebiotics and probiotics) increase villus length with a concomitant decrease of the crypt depth [38] which is in accordance with the results of the present study. Besides having some other functions, the crypts are the source of multipotent stem cells that give rise to the cells of the villi. High villus length-to-crypt depth ratio as a result of shallower crypt depths implies to decreased cellular turnover and redirection of energy to be utilized for growth [38].

The length of the villi did not differ significantly between the pig groups, which is in line with the findings published by Namkung et al. [39] and Nofrarias et al. [37]. However, in the research conducted by Kroismair et al. [40], there was a tendency of shortening of the villi in the jejunum and ileum influenced by the addition of essential oils.

The numbers of goblet cells estimated in relation to a hundred epithelial cells were similar in both the treated and control pigs, and similar to those reported by Nofrarias et al. [37]. By contrast, it is characteristic of the advanced stages of proliferative enteropathy that there is a reduction in the number and even the absolute absence of goblet cells [41].

The villus surface area did not differ significantly between the two groups of pigs, which is in compliance with the results previously published by other authors [42].

Previous research conducted by Papatsiros et al. [16] and Draskovic et al. [17] suggested that phytogenic additives may be a sound alternative solution to the control of the bacterial infection in pigs caused by *L. intracellularis*. In addition, the results of the present study indicate that the supplementation of feed additives containing a mixture of different plant extracts and essential oils could improve the histomorphometric parameters of the ileum and consequently enhance the absorption and growth performance of the pigs. Having in mind all the threats posed by antimicrobial resistance and the beneficial effects of the tested phytogenic additive in this study, it can be considered that this supplementation gives the possibility of the replacement of antibiotics used in livestock production.

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Authors’ contributions
DV and SZ designed the investigation, interpreted the results and wrote the manuscript. GM performed the statistical analysis and analyzed the data. BNJ helped performed analysis and co-wrote the paper. TR and TV made substantial contributions to the interpretation of data and writing the manuscript. KV carried out a histomorphometry analysis and evaluated histological preparations. All authors have approved the final version of the manuscript.

Declaration of conflicting interests
Patent Co. partly funded this study and one of the co-authors (Jasna Bosnjak-Neumuller) is employee of Patent Co. Other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Proliferativna enteropatija poznata kao i proliferativni ileitis smatra se jednom od ekonomski najznačajnih bolesti u svinjarskoj proizvodnji širom sveta. Procenjeni gubici po obolelom tovljeniku obično se kreću od 1 do 5 američkih dolara. Uzrok ovog oboljenja je *Lawsonia intracellularis*, gram negativna obligatna intracelularna bakterija. Kontrola ovog oboljenja ostvaruje se primenom vakcinacije ili antibiotika. U poslednje vreme sve je češća primena fitogenih aditiva u cilju kontrolisanja određenih patoloških stanja digestivnog trakta. Cilj ovog rada bio je da se ustanove efekti fitogenog aditiva na histomorfometrijske karakteristike creva kod odlučene prirodno inficirane bakterijom *Lawsonia intracellularis*. Histomorfometrijska analiza pokazala je da su kripte bile značajno (P<0,05) pliće, a količnik visine vilusa i dubine kripti značajno veći (P<0,05) u tretiranoj grupi prasadi nego u kontroli. Ovo poboljšanje histoloških parametara creva, koje se smatraju indikatorima njegovog zdravlja, dokaz je pozitivnog efekta ispitanog aditiva na digestivni sistem svinja.
