ORIGINAL ARTICLE

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Effect of probiotic therapy on periodontal tissues and intestinal mucosa of rats with ligature-induced periodontitis
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ABSTRACT

Introduction: As periodontitis is caused by dysbiotic biofilm, it is believed that therapy with probiotics can act to control the mechanisms of adhesion and colonization, competing with invading microorganisms. Objective: Evaluate probiotic therapy effect on periodontal tissues and intestinal mucosa of rats with ligature-induced periodontitis. Methods: 32 Wistar rats were divided into four groups (n=8): Control Group (CG); Periodontal disease (PD); Probiotic (PROB); PD + probiotic (PDPRO). PD and PDPRO received a ligature over the first lower molars and PROB and PDPRO the probiotic \textit{Lactobacillus acidophilus} based were given orally for 44 days. The animals were euthanized and the blood was collected for evaluation of triglyceride and cholesterol concentrations. The hemimandibles were collected for histomorphometric and radiographic analysis. The duodenum was removed for morphological evaluation and gingival tissue around the molars was collected for analysis of IL-17. Results: The ANOVA one-way test was used followed by Tukey Test. PDPRO had a significantly lower bone loss than the PD (p<0.05) and a smaller number of osteoclasts on PDPRO when compared to the PD. As for IL-17, there was a decrease in the PDPRO when compared to the PD. The histomorphometry of the duodenum showed that there was a significant increase in the width of the villi in PROB only. Conclusion: The therapy with probiotics was effective to avoid the development of periodontitis by reducing alveolar bone loss and inflammation modulation and increasing the width of the duodenum villi, which may help to restabilize the balance of the gastrointestinal tract.

Keywords: periodontitis; inflammation; probiotics.
INTRODUCTION

Periodontitis is currently considered by the American Academy of Periodontology to be an inflammatory disease initiated by bacteria. The big question is the relationship between inflammation and disease, which comes first, the immune response or change in the homeostatic integrity of the mucosal polymicrobial biofilm. The relationship of the periodontal microbiome with the development of periodontitis is complex and the shift to a dysbiosis microbiota appears to be largely due to large and persistent inflammation, with a pocket formation that alters the environment and bacterial growth\(^1\). Existing evidence suggests that these may contribute to altered gene expression and biological processes that enhance inflammatory tissue destruction\(^2\).

A persistent inflammatory response triggered by pathogenic biofilms, leads to the destruction of the soft and bone tissues of the periodontium, with the consequent loss of teeth. Cytokines are central regulators of the immune inflammatory response that are produced by various cell types including epithelial cells, fibroblasts, dendritic cells, macrophages, and T helper (Th) cells in response to microbes. Accordingly, the net result expression of cytokines within the tissues will generate a specific local milieu that orchestrates the cellular and humoral immune responses necessary to manage oral commensals and pathogens. Among these cytokines, Th17 cytokines have shown the ability to enhance osteoclast differentiation and activation of metalloproteinases (MMPs), critical mediators of soft and hard tissue destruction in periodontitis\(^3\).

New alternatives for periodontitis control have been studied recently to allow treatment with a lower risk of side effects to patients, and in this context, probiotic therapy has emerged as a new way to proceed due to its oral activity being similar to that of the intestinal mucosa\(^4,5\).

Probiotics have been defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host\(^6\). Fermented types of milk produced by microbiological fermentation of regular milk are the most relevant products on the market that
contain probiotics in their composition and are currently indicated to assist control of gastritis, cholesterol, hypertension, and other diseases\textsuperscript{7-9}.

Probiotic organisms normally operate through a huge range of mechanisms, which include exclusion and competition with possible colonizing pathogens by nutrients and adhesion to epithelial cells, synthesis of antimicrobial substances, local and systemic immunomodulation, as well as the development of the function of the mucosal barrier\textsuperscript{10}. The host provides the quantities of nutrients that the intestinal bacteria need, which actively indicate their needs. Thus, the symbiotic relationship prevents an excessive production of nutrients, which favors the establishment of microbial pathogenic potential competitors with the host\textsuperscript{11}.

Probiotic-induced immunological stimulation is also manifested by the increased production of immunoglobulins, enhanced activity of macrophages and lymphocytes, and stimulation of $\gamma$-interferon production. Probiotics may influence the congenital and acquired immunological system through metabolites, components of the cellular wall, and DNA, recognized by specialized cells of the host. The principal host cells that are important in the context of the immune response are intestinal epithelial cells and intestinal immune cells. Components of the cellular wall of lactic acid bacteria stimulate the activity of macrophages. Those, in turn, can destroy microbes rapidly by the increased production of free oxygen radicals and lysosomal enzymes\textsuperscript{12}.

*Lactobacillus acidophilus* in both mono- and co-infection reduced the expression of genes encoding Toll-like receptors (TLR4), which highlights this strain as an interesting immunoregulator, mainly for mucosal diseases associated with increased expression of this receptor. Accordingly, this regulation seems to be important in a future therapeutic perspective in periodontology. It is well-accepted that tight control of the immune system that resolves infection and tissue damage is fundamental to health. Therefore, properties such as adhesion to
gingival epithelial cells and an increase in cell proliferation and immunomodulation can be used as an alternative. Because periodontitis is caused by dysbiosis biofilm, it is believed that probiotic therapy may act to control it due to mechanisms of adhesion and colonization, competing with microorganism invaders, in addition to enabling an improvement in the immune response and also producing antimicrobial substances.

Thus, the objective of this study was to verify the effect of probiotic activity on periodontal tissues and intestinal mucosa of rats submitted or not to experimental periodontitis, analyzing its ability to inhibit periodontitis development by reducing alveolar bone loss and inflammation modulation, as well as the intestinal morphology of these animals.

**METHODS**

**Study population, sample, and groups**

This study was submitted to and approved by the Ethics Committee on Animal Use (CEUA) from UNIOESTE, as ordinance 3730/2016 – GRE.

We used 32 adult male Wistar rats, weighing on average 200 g, coming from UNIOESTE’s Central Vivarium. The animals were kept at the Sectoral Vivarium of Physiology, under controlled temperature (23 ± 2° C) and light conditions (12 hours of light and 12 hours of darkness - 07:00 a.m. up to 07:00 p.m.) and received water and pet food as needed. The animals were randomized and allocated into four groups (n=8) based on preliminary studies and were divided into 1) a Control Group (CG): without periodontal disease and probiotics given; 2) Periodontal disease (PD): with induced periodontal disease and no probiotics given; 3) Probiotic (PRO): without periodontal disease and with probiotics given; 4) Periodontal disease + probiotic (PDPRO): with periodontal disease and with probiotics given. The rats of PD and PDPRO groups received a ligature over the first lower molars which

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acted as a gingival irritating substance for 44 days, favoring the accumulation of dental biofilm and development of PD\textsuperscript{15}, and PROB and PDPRO groups received the \textit{Lactobacillus acidophilus} probiotic (8.0 \times 10^7 CFU/mL) given orally for 44 days 2 ml once a day.

Data collection procedures

On the first day of the experiment, the animals were received and began the acclimatization process. After 7 days, the induction of periodontitis was carried out through ligature and in addition, the probiotic dosages started, which remained for more than 44 days. On the fiftieth day of the experiment, euthanasia was performed on the animals, as well as materials were collected for analysis (Figure 1).

Periodontal disease induction

After seven days of acclimatization, the animals were anesthetized with 0.04 mg/kg xylazine hydrochloride (ANASEDAN Sespo Industry and Commerce, Paulínia-SP) and 0.08 mg/kg ketamine hydrochloride (DOPALEN Sespo, Industry, and Commerce, Paulínia-SP), via peritoneal and positioned in an adapted surgical table, which allowed buccal opening maintenance and facilitated access to the teeth of the posterior region of the mandible. With the aid of modified tongs and an exploratory probe, a cotton yarn number 40 was placed around the left and right first lower molars. This ligature acted to irritate the gum and was maintained for 44 days, favoring plaque accumulation and consequent development of PD\textsuperscript{15}. A 2ml dosage of \textit{Lactobacillus acidophilus} probiotic was given orally for 44 days, once a day, at an 8.0 \times 10^7 CFU/mL concentration.

Euthanasia of the animals

After 44 days of induction of PD and probiotic dosage, the animals were euthanized by decapitation with a guillotine with subsequent collection of their hemimandibles for

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radiographic and histological analysis of the periodontal tissues and blood for analysis of cholesterol and triglycerides.

**Radiographic analysis**

After the euthanasia and removal of gingival tissue around the first molar, dissection of the left side hemimandibles were performed. Afterward, x-rays were taken by using an R-X device Dabi-Atlante brand, Spectro model with 70 Kvp and 8 mA, keeping a 50 cm focus/film distance and 0.3 seconds exposure time. The hemimandibles were placed with the lingual side over the periapical radiographic film and positioned so that the buccal and lingual cusps of the first molars were in the same vertical plane.

The images were scanned and analyzed by a blind and calibrated examiner in three measures by the Sigma-Scan 2.0 program and a mean was calculated, forming a linear measure which distance extends from the cement-enamel junction up to the alveolar bone crest of the mesial side of the first lower molar of the rat, with the measurements in centimeters\textsuperscript{16}.

**Histomorphometry analysis of alveolar bone**

The right-side hemimandibles obtained were placed in a 10\% formaldehyde solution for 24 hours. After this period, they were washed in running water for one hour and then immersed in a 5\% trichloroacetic acid (TCA) solution, at 10°C for 30 days. The parts were evaluated to verify the decalcification degree expected, with the renewal of the TCA solution every five days. After decalcification, the tissues were immersed in 5\% sodium sulfate for approximately two hours to neutralize the TCA, washed in running water for two hours, and kept in 70\% alcohol until the histological processing to include in paraffin (Purified Paraffin, code 1228, lot 1008459, Vetec Química Fina, Rio de Janeiro, Brazil). The hemimandibles fragments were dehydrated in ascending alcohol series, cleared in xylene, and embedded in paraffin. The
paraffin blocks were cut with a manual microtome (Olympus, CUT 4055 - Charleston, South Carolina, USA) to obtain the thickness of 7 µm sections buccolingually, which were assembled in histological slides and stained through the Hematoxylin and Eosin (HE).

After obtaining the histological slides, microscopic analysis was performed by a blind and calibrated examiner through the evaluation of stained histological sections. The slides were analyzed with a commonly transmitted light microscope (Leica Microsystems, Switzerland) for observations of the alveolar process and counting of osteoblasts, osteocytes, and osteoclasts of the animals’ hemimandibles, as well as the distance from the cementoenamel junction to the alveolar bone crest of the mesial region of the first molars.

The quantification of osteoblasts, osteocytes, and osteoclasts was performed in five consecutive fields of vestibular alveolar bone crest starting from the highest point of the crest. For the observation, a 100 times increase in immersion in the microscope was used, two observations per field, and then the mean values were calculated for each animal and each group.

A measurement of the smallest distance between the apex of the vestibular alveolar bone crest and the cementoenamel junction was repeated once a day, on three different days, and the mean among the values was calculated. To do so, we used a device attached to a computer, which allowed us to capture the images through the LazEz® software.

**Analysis of IL-17 expression (ELISA)**

A portion of the gum tissue around the teeth from the left side hemimandible, subjected or not to ligature placement was removed and used for analysis by enzyme-linked immunosorbent assay (ELISA) of IL-17 cytokine. For the dosage, previously sensitized plates with monoclonal antibodies (Biosource, INVITROGEN ®, California, USA) were used according to the manufacturer's instructions. Plates were incubated with gingival tissue
supernatants or with different cytokine recombinant IL-17 concentrations, as indicated by the manufacturer. A detection antibody conjugated to peroxidase-specific cytokine was added to the plates and, after incubation time, have been washed and reactivity has been revealed by adding the revelation solution, according to the manufacturer's instructions. The reaction was blocked with a stop solution and reading was carried out at 450 nm in a microplate reader. Cytokine concentration was calculated by using a linear regression curve, from the standard curve performed for the respective cytokine. The results were expressed in pg/mL.

**Collection of blood samples for total cholesterol and triglycerides**

At the time of euthanasia, the blood was collected into tubes containing heparin. Blood samples were centrifuged at 3000 rpm and the plasma was separated and stored at -20°C for dosing concentrations of total cholesterol and triglycerides, which were performed by radioimmunoassay with *specific kits* (Cholesterol Liquiform – Labtest and Triglycerides Liquiform – Labtest).

**Histomorphometry analysis of the duodenum**

The duodenum is located on the first portion of the small intestine, is directly connected to the stomach’s pylorus, and is responsible for to finish absorption of nutrients. It has been removed after euthanasia in the animal.

Immediately after collecting the duodenum, tissue samples were opened in the mesenteric edge and placed in Bouin (750 ml picric acid saturated solution 2.50 ml formaldehyde, and 50 ml glacial acetic acid). Placing aims to stop cell metabolism to stabilize structures and intra and extracellular biochemical components, preserving and conserving the tissue elements, as well as allowing penetration of other substances after placing. After the
placement, the samples still went through dehydration in a series of increasing concentrations of alcohol.

After dehydration, alcohol has been removed by xylol, preparing the tissue for paraffin. With the paraffin previously heated at 60ºC, the tissue elements of the sample were infiltrated by paraffin, and, when at regular temperature, paraffin solidifies which facilitates histological slides with semi-seriated with 6 mm thickness, which were stained by two methods: hematoxylin and eosin (HE) and placing periodic acid-Schiff (PAS).

The morphometric analysis was performed by a sampling of images captured at 20x lens under an Olympus optical microscope with a Plus Zeiss Axioscop 2 set to capture images. After being scanned, the images were analyzed with the Sigma-Scan 2.0 program, and the villi height was measured through the vertical distance between the villus tip and the junction level of 10 villi of villi in five different fields of each sample. Subsequently, the depth of the crypt's intestinal disorders has been analyzed, by measuring the vertical distance between the villi-crypt junction and the lower limit of the same 10 crypts in five different fields of each sample. Besides, the width of 10 random villi was measured, in the mid-point region of the villi height, in three fields per animal. These measurements were repeated for three days, then the mean of these values was calculated to estimate the width variation of the villi.

**Statistical analysis**

The results were analyzed by using the Bioestat® - Version 5.3 (Mamirauá Sustainable Development Institute, Brazil, AM). In all variables, normal distribution tests and homogeneous variance were carried out (Shapiro-Wilk Test). As distribution was considered normal and variance was homogeneous, the parametric tests ANOVA oneway followed by Tukey multiple comparison tests were used. The differences were considered significant when p<0.05.
RESULTS

Radiographic and histomorphometry analysis of cementoenamel junction measurement to alveolar bone crest and analysis for quantification of osteocytes, osteoblasts, and osteoclasts

Through the radiographic and histomorphometry evaluations described in Table 1, illustrated in Figure 2, it has been noticed that the animals in PD and PDPRO groups showed high alveolar bone loss when compared to the CG and PROB (p<0.05), and the PDPRO group had a significant reduction in bone loss when compared to the PD group.

Still, in Table 1, it was observed that CG and PROB groups had no significant differences, while PD and PDPRO groups were significantly different from CG and PROB and between themselves (p<0.05) in the count of osteocytes, osteoblasts, and osteoclasts. However, the PDPRO group showed lower osteoclastic activity compared to the PD group.

Analysis of IL-17 Expression (ELISA)

In Table 2, a significant increase in the expression of IL-17 was observed in the PD and PDPROB groups about the CG and PRO groups, which did not differ between them. A statistically significant difference could be verified between the groups PD and PDPRO (p<0.05), where it was significantly lower in the PDPRO group.

Total cholesterol and triglycerides analysis

Still, in Table 2, it was found that for both total cholesterol and triglyceride dosages, there was no statistically significant difference among the groups.

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Histomorphometry analysis of the duodenum

Table 3 and Figure 3 show that there was no statistically significant difference among the groups regarding the heights of the villi and crypt measurements. However, regarding the width of the villi, a significant increase could be noticed in the group that received supplementation with the probiotic.

DISCUSSION

Probiotics have been introduced in periodontal healthcare lately. The reason why this is happening is related to the current view of periodontal inflammation etiology associated with the dental biofilm\textsuperscript{19}. Management of the disease by attempting to remove bacteria (debridement) is partially effective for periodontitis and fails in high-risk individuals. Detailed consideration of host response, genetic and environmental factors, as well as microbiological factors, are needed. Identifying a true infectious pathogen for periodontitis has frustrated researchers for decades. More recently, concepts focused on controlling inflammation to control polymicrobial biofilm dysbiosis have emerged for periodontitis, as well as a range of other polymicrobial inflammatory diseases\textsuperscript{1}. However, there is not sufficient scientific evidence yet to support the effectiveness of probiotics in the treatment of periodontal disease\textsuperscript{19}.

Two aspects are important in this study regarding the use mode of probiotics. The first refers to the experimental period established for probiotic use. In some studies, the use of probiotics was assessed in a model that presented a microbial environment already mature\textsuperscript{20,21}. On the other hand, Gatej et al.\textsuperscript{22} observed that the treatment with \textit{Lactobacillus rhamnosus GG} was conducted before the initiation of the disease to assess its ability to prevent alveolar bone loss. In the present study, probiotic therapy started together with the induction of periodontitis was useful to control the development of periodontitis, in the control of bacterial biofilm. The second aspect refers to the type of probiotic used. In this study, the \textit{Lactobacillus acidophilus}
probiotic was effective to inhibit periodontitis by reducing bone loss in the PDPRO group, both radiographically and histologically (Table 1), also demonstrating effectiveness in improving bone pattern with significant osteoclasts decrease and osteoblasts and osteocytes increase in the PDPRO group when compared to the PD group (Table 1). Albuquerque-Souza et al.13 found that L. acidophilus LA-5 emerges as an important therapeutic alternative for periodontitis due to its immunomodulatory potential, while the study of Zhao et al.23 demonstrates that L. acidophilus may offset the secretion of IL1B, IL6, and IL8 by gingival epithelial cells in the presence of P. gingivalis and could therefore be used to prevent periodontal infections caused by this microorganism.

In addition, due to its potential to fight against pathogenic bacteria, many studies are aimed at precisely understanding the probiotic activity. Reddy and Babu24 suggest in their study that, to probiotic species act over periodontal disease, at least 12 months are required. In this study, probiotic was given for 44 days, based on the study of Foureaux et al.5, whose study emphasizes that probiotics given orally can produce benefits, both by going through the oral cavity and modulating the oral mucosa immunity systemically. Also, according to this author, it is suggested that the beneficial effects of probiotics may include preventing the adhesion of pathogens to host tissues while passing through the oral cavity. Additional effects may include stimulating and modulating the immune system; reducing the production of proinflammatory cytokines and increasing the production of anti-inflammatory cytokines25; enhancing the integrity of the intestinal barrier26.

Maekawa and Hajishengallis27, observed inhibition of periodontal inflammation, bone loss, and oral microbiota modulation, to favor the growth of aerobic bacteria at the expense of anaerobic bacteria which are strongly associated with periodontitis. In this study, mice with ligature-induced periodontitis and treated with L. brevis CD2 exhibited significantly reduced expression of all pro-inflammatory cytokines tested (TNF, IL-1β, IL-6, and IL-17A), which are

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involved in inflammatory periodontal bone loss. Altogether, these data show that the topical treatment with *L. brevis* CD2 probiotic obstructs inflammation and periodontal bone loss. These data corroborate the findings of our study, which showed a statistically significant decrease of IL-17 in the PDPRO group when compared to the PD group (Table 2), therefore suggesting inhibition of periodontal inflammation. Th17 cells have been implicated in the pathogenesis of periodontitis, mainly due to their involvement as specialized lymphocytes that can activate bone resorption.

Several studies have been demonstrating the beneficial effect of probiotics over blood lipids\(^{28-30}\) and triglycerides\(^{30-32}\). However, in this study, there was no statistically significant difference among the groups regarding these parameters (Table 2). Therefore, although the use of probiotics has been recommended for various diseases such as diabetes, metabolic syndrome, and cardiovascular diseases, its potential for reducing cholesterol and triglycerides, must be evaluated using more clinical studies to be more properly applied and thus achieve better results\(^{32}\).

Finally, evidence suggests that the periodontitis effect may not be limited to the oral cavity, but also have systemic consequences. Changes in intestinal morphology, such as shorter villi, have been associated with the presence of toxins and an increase in counts of pathogenic bacteria in the gastrointestinal tract\(^{19}\). In this study, histomorphometry analysis of the animals’ duodenum showed no changes in the villi height and crypt height among the groups, but there was a significant increase in the villi width in the group that received supplementation with a probiotic (Table 3). Foureaux et al.\(^{5}\) found statistically significant interactions between PD and probiotics in both duodenum and jejunum. However, there are no studies associating PD and PROB use that have evaluated the villi width. Galeano et al.\(^{33}\) noticed that animals with a diet supplemented with probiotics not only showed an increase in the villi height and width, but also
a reduction in the crypts width and depth, in comparison with pigs with a regular diet with and without antibiotics.

The Lactobacillus rhamnosus GG (LGG) can survive the low pH of the stomach and the bile acids of the duodenum and exerts anti-inflammatory properties in vivo. LGG has been shown to mediate the effects of pro-inflammatory cytokines interferon -γ and TNF -α on the epithelial barrier integrity and to improve intestinal epithelial barrier function via tight junction changes. These findings imply that LGG acts on the host’s immune response by producing factors capable of modulating inflammation, an idea consistent with the proven fact that probiotics engage with the innate and acquired immune response\textsuperscript{22}.

There is a lot of heterogeneity among the studies aimed at probiotics effects, possibly because different doses, treatment periods, bacterial species, and application forms were used, which may justify the results of our study\textsuperscript{19}. A limitation of this study was the impossibility of analyzing the microbiota in the tissues studied. However, considering the positive results obtained with the use of this probiotic, it is believed that it can contribute positively to the modulation of inflammation in patients with periodontal disease. Further studies with the same probiotic are needed.

**Conclusion**

Therapy with *Lactobacillus acidophilus* probiotic was effective to inhibit periodontitis development by reducing alveolar bone loss and inflammation modulation and increasing the width villi of the duodenum, which can help to re-stabilize the gastrointestinal tract balance.
ACKNOWLEDGMENTS

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Figures

Figure 1: Diagram of procedures for data collection and representation of the timeline of the experiment.

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Figure 2: Representative radiographic and histometric photomicrographs of the animals' jaws. (A) CG group. (B) PD group (C) PROB group. (D) PDPROB group. Hematoxylin and Eosin (HE). Increase 400x.
Figure 3: Representative photomicrograph of the duodenal section. (A) Control Group; (B) PD Group; (C) PROB Group; (D) PROB + PD Group. Hematoxylin and Eosin. Increase 4x.
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Tables

**Table 1**: Measures of distance from the cementoenamel junction up to the alveolar crest and Osteocytes, osteoblasts, and osteoclasts quantities in all groups. The values represent mean ± SD

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Radiographic Evaluation (cm)</th>
<th>Histomorphometry Evaluation (cm)</th>
<th>Osteoblasts (UNITS)</th>
<th>Osteoclasts (UNITS)</th>
<th>Osteocytes (UNITS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>0.28 ± 0.03 A</td>
<td>22.16 ± 3.18 A</td>
<td>0.83 ± 0.40 A</td>
<td>135.0 ± 14.79 A</td>
<td>0.25 ± 0.006 A</td>
</tr>
<tr>
<td>PD</td>
<td>0.41 ± 0.06 B</td>
<td>9.33 ± 2.58 B</td>
<td>4.50 ± 1.90 B</td>
<td>82.66 ± 7.20 B</td>
<td>0.61 ± 0.01 B</td>
</tr>
<tr>
<td>PROB</td>
<td>0.27 ± 0.02 A</td>
<td>21.60 ± 3.13 A</td>
<td>0.80 ± 0.44 A</td>
<td>120.80 ± 14.72 A</td>
<td>0.28 ± 0.008 A</td>
</tr>
<tr>
<td>PDPROB</td>
<td>0.35 ± 0.04 C</td>
<td>16.16 ± 3.92 C</td>
<td>1.83 ± 0.75 C</td>
<td>90.00 ± 3.52 C</td>
<td>0.46 ± 0.007 C</td>
</tr>
</tbody>
</table>

Different letters - Statistically difference (p<0.05) among groups. Anova Test and Tukey post-test.
Table 2: Values of the IL-17 expression and cholesterol and triglycerides dosages, in all groups. Values represent mean ± SD.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>IL-17 (pg/mL)</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>61.97 ± 8.63 A</td>
<td>203.33 ± 30.56</td>
<td>210.00 ± 38.05</td>
</tr>
<tr>
<td>PD</td>
<td>96.11 ± 6.86 B</td>
<td>224.33 ± 27.95</td>
<td>227.67 ± 34.85</td>
</tr>
<tr>
<td>PROB</td>
<td>71.77 ± 6.56 A</td>
<td>215.67 ± 22.26</td>
<td>214.50 ± 43.00</td>
</tr>
<tr>
<td>PDPROB</td>
<td>80.76 ± 4.36 C</td>
<td>209.50 ± 42.56</td>
<td>179.00 ± 45.90</td>
</tr>
</tbody>
</table>

Different letters - Statistically difference (p<0.05) among groups. ANOVA and Tukey Tests.

Table 3: Measures of villi heights (VH) and widths (VW) and crypts heights (CH) in all groups.

The values represent mean ± SD and are expressed in mm.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>VH</th>
<th>VW</th>
<th>CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>0.117 ± 0.02</td>
<td>0.0405 ± 0.0007 A</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>PD</td>
<td>0.106 ± 0.01</td>
<td>0.0378 ± 0.0005 A</td>
<td>0.101 ± 0.01</td>
</tr>
<tr>
<td>PROB</td>
<td>0.111 ± 0.02</td>
<td>0.0457 ± 0.0005 B</td>
<td>0.103 ± 0.02</td>
</tr>
<tr>
<td>PDPROB</td>
<td>0.119 ± 0.01</td>
<td>0.0389 ± 0.0005 A</td>
<td>0.105 ± 0.01</td>
</tr>
</tbody>
</table>

Different letters - Statistically difference (p<0.05) among groups. Anova and Tukey Tests