Original Research
Endodontic Therapy

Effect of systemic administration of *Bifidobacterium animalis* subsp. *lactis* HN019 on apical periodontitis

Lisa Danielly Curcino ARAUJO[a]
Raquel Assed Bezerra SEGATO[a]
Thaís de Paula Colen REIS[a]
Sérgio Luis de Souza SALVADOR[b]
Flávia Aparecida Chaves FURLANETO[c]
Michel Reis MESSORA[c]
Paulo NELSON-FILHO[a]
Lana Kei Yamamoto de ALMEIDA[a]
Marília Pacífico LUCISANO[a]
Clara Marina Pereira Cavalcanti SILVA[a]
Léa Assed Bezerra da SILVA[a]

[a]Universidade de São Paulo – USP, School of Dentistry of Ribeirão Preto, Department of Pediatric Dentistry, University of São Paulo - USP, Ribeirão Preto, SP, Brazil.

[b]Universidade de São Paulo – USP, School of Pharmaceutical Sciences of Ribeirão Preto, Department of Clinical Analyses, Ribeirão Preto, SP, Brazil.

[c]Universidade de São Paulo – USP, School of Dentistry of Ribeirão Preto, Department of Oral and Maxillofacial Surgery and Periodontology, Ribeirão Preto, SP, Brazil

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Corresponding Author:
Clara Marina Pereira Cavalcanti Silva
E-mail: clarampcavalcanti@gmail.com

https://doi.org/10.1590/1807-3107bor-2023.vol37.0115

Submitted: November 22, 2023
Accepted for publication: July 11, 2023
Last revision: August 7, 2023

Abstract: This study aimed to evaluate the effect of *Bifidobacterium animalis* subsp. *lactis (B. lactis)* HN019 in drinking water on the development of apical periodontitis (AP) in rats. In total 60 animals were divided into a control group (sound teeth); Group I - regular water without AP; Group II - probiotic water without AP; Group III - regular water with AP; Group IV - probiotic water with AP. AP was induced after 3 days in the control groups and after 7, 21, and 42 days in groups III and IV. The animals were euthanized, and the mandibles were subjected to histotechnical processing. Samples were stained with hematoxylin & eosin (H&E) to identify root canal features, apical and periapical regions. Additionally, histoenzymology was performed to detect osteoclasts, immunohistochemistry was used to identify osteoclastogenesis markers, and the Brown & Brenn technique was applied for microbiological analysis. The data were analyzed using GraphPad Prism 8.0.1 with a significance level of 5%. Although no statistical differences were observed, the groups administered with probiotics showed better conditions in terms of histological aspects seen microscopically. Furthermore, there were no differences in the number of osteoclasts ($p > 0.05$). The RANKL marker was not found in the probiotic group at 42 days, unlike in group III.

Keywords: Probiotics; Periapical Periodontitis; Endodontics; Bifidobacterium.

Introduction

Opportunistic pathogens can gain access to the root canal through dental caries, leading to an infectious process that affects the apical and periapical regions.[1] Once microorganisms from caries reach the pulp tissue, facultative bacteria, such as *Streptococcus mutans*, *Lactobacillus acidophilus,* and *Actinomyces viscosus,*[1-4] become predominant and can survive in both aerobic and anaerobic conditions. As the infection progresses, oxygen and nutrition availability decrease, resulting in an anaerobic environment with a prevalence of *Propionibacterium, Eubacteria, Arachnia, Lactobacillus, Bifidobacterium*, and *Actinomyces*.[2,3] These anaerobic microorganisms produce byproducts such as lipopolysaccharide (LPS), which can induce several biological events leading to a chronic inflammatory process and resorption of bone and cementum.[4] In addition, periapical lesions can be

asymptomatic and remain undiagnosed, significantly contributing to the overall inflammatory burden, as some inflammatory markers found are also associated with systemic inflammatory conditions.[5] Thus, the therapeutic use of probiotics for microbial reduction has gained notable attention in recent years, both in the medical and dental fields.[6-8]

The World Health Organization defines probiotics as "living microorganisms which, when administered in adequate amounts, confer a health benefit on the host," recognizing that they are part of the immune defense system.[9] Furthermore, probiotics play a crucial role in maintaining oral health by interacting with the oral microbiome and promoting microbial balance.[10,11] Scientific evidence demonstrates the beneficial effects of probiotics in various conditions within the oral cavity, such as in the treatment of periodontitis,[7,11,12] prevention, and treatment of mucositis and peri-implantitis,[13] impact on bone resorption during orthodontic movement, prevention of white spots in orthodontic patients,[14,15] prophylactic effect against squamous cell carcinoma,[16] and prevention of caries lesions.[17-19] Systematic reviews also highlight the benefits of using *Lactobacillus* and *Bifidobacterium* strains as adjuncts in periodontal treatment.[20,21]

Moreover, these strains have demonstrated inhibitory effects against important endodontic pathogens such as *Enterococcus faecalis* and *Candida* albicans.[9] In the field of endodontics, fewer studies have evaluated the impact of probiotic consumption on the development of apical periodontitis induced in rats. These studies have shown a reduction in inflammation and decreased bone resorption following systemic supplementation with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*[22,23] or a Probiotic Complex.[24] However, Kumar et al.[25] reported methodological heterogeneity in the selection criteria for the strain and dosage in studies relating probiotic therapy to periapical injury. Therefore, they emphasized the need for further research focusing on clinically proven probiotic strains in laboratory and animal studies and clinical trials.

Data from pre-clinical studies and clinical trials support the use of *Bifidobacterium animalis* subsp. *lactis* (B. lactis) HN019 in periodontitis.[26] B. lactis has demonstrated effective and promising results in modulating the immunoinflammatory response and the microbiological profile, showing a protective effect on the alveolar bone.[12,27,28] However, there have been no studies evaluating the effect of *Bifidobacterium animalis* subsp. *lactis* HN019 supplementation on the development of periapical lesions.

The present study aimed to evaluate the in vivo effect of the probiotic *Bifidobacterium animalis* subsp. l*actis* (B. lactis) HN019, consumed through water, on the development of experimentally induced periapical lesions in rats. The null hypothesis was that there would be no difference in the severity of apical periodontitis in rats that received supplementation of *Bifidobacterium animalis* subsp. *lactis* HN019 probiotics.

Methodology

Animals

This study was approved by the Institutional Ethics Committee on Animal Research (CEUA) of the School of Dentistry of Ribeirão Preto, University of São Paulo (protocol 2018.1.782.58.0). The sample size was calculated separately for groups I and II, based on a pilot study with a minimum of 3 animals per group, providing 95% power. To account for potential animal deaths during the experiment, an additional animal was added per group, totaling 4 animals per group (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). For groups III and IV, the sample size calculation was based on previous studies.[27] In summary, power calculations indicated that a minimum of 7 animals per group was necessary to achieve 95% power. Accounting for potential animal deaths during the experiment, an additional animal was added per group, resulting in a total of 8 animals per group and 48 animals for groups III and IV, with a total of 60 animals for the entire investigation.

Sixty adult Wistar male rats (Rattus norvegicus, Albinus), 3 months old and weighing between 170 g and 250 g, were housed in polypropylene cages with 4 animals each. The animals were kept under constant conditions, including a temperature of 22 ± 2ºC, relative air humidity of 55 ± 10%, and a 12/12-hour light-dark cycle. They had *ad libitum* access to food and water.

The animals were divided into five groups as follows: Control Group (filtered water, healthy teeth, without periapical lesion) (n = 4), Group I - filtered water, with coronal access (without periapical lesion - 3 days) (n = 4), Group II - probiotic, with coronal access (3 days) (n = 4), Group III - filtered water, with a periapical lesion (7, 21, 42 days) (n = 24), and Group IV - probiotic, with a periapical lesion (7, 21, 42 days) (n = 24). There were no significant complications during the operative procedures, except for the death of two animals following anesthesia procedures.

Preparation of probiotic cultures and systemic administration

B. lactis HN019 (HOWARU Bifido, E.I. Dupont® de Nemours and Company, Wilmington, DE, USA) was propagated in MRS Agar (Man, Rogosa, and Sharpe - D™ Lactobacilli MRS Broth, Sparks, USA) for 48 hours at 37°C in an anaerobic environment (GASPAKTM EZ Anaerobe Container System with indicator, Sparks, EUA). After incubation, the bacterial inoculum was transferred to Falcon tubes and homogenized using a vortex mixer (Phoenix AP 65, Araraquara, SP, Brazil). The optical density (O.D) was determined at 625 nm using a spectrophotometer (Micronal - AJX - 1000, São Paulo, Brazil), and the suspension was subjected to serial dilutions up to 10^{-9} in phosphate-buffered saline (PBS buffer) with a pH of 7.0. Duplicate colony-forming unit (CFU/mL) counts were performed, with an O.D. of 2.007 corresponding to the standard inoculum with 2.7×10^9 CFU/mL.[29]

In groups receiving probiotics (groups II and IV), 3 mL of 10^{11} CFU/mL was added to the drinking water (400 mL), resulting in a final proportion of 2.7×10^9 CFU/mL.[27] The solution was orally administered once a day at 10:00 am. The volume ingested per cage was 200 mL/day, considered satisfactory as the effective minimum volume should be 10 mL per animal.[30] To assess the preventive effect, probiotic supplementation began 30 days before the induction of the periapical lesion using a classic model previously employed by the research group.[31,32] Probiotic consumption was continued throughout the development of the lesions, according to the experimental periods.

Induction of periapical lesions

The animals from groups I, II, III, and IV were anesthetized by intraperitoneal injection of 2% xylazine chloridate (Rompum, Bayer Animal Health, São Paulo, Brazil; 10 mg/kg of body weight) and 10% ketamine hydrochloride (Dopalen, Agribands Purina do Brasil Ltda., Paulínia, Brazil; 80 mg/kg of body weight). Subsequently, they were immobilized and positioned on a mandibular retraction table, ensuring that their mouths were open to adequately visualize the lower molars (left and right). The dental crown of the first mandibular molar was accessed using a low-speed electric handpiece with a spherical drill (no. 1/2 - KG Sorensen) mounted on a low-speed motor and contra-angle (Dabi Atlante Equipamentos Odontológicas). The access entry to the root canals was confirmed by visual inspection and verified with a size 10 type K endodontic file (Maillefer S/A, Switzerland). Afterward, the teeth were kept open to facilitate root canal contamination. Groups I and II were euthanized on day 3 to maintain contamination limited to the root canal and prevent the formation of a periapical lesion, which typically occurs within 7 days.[31,33] Groups III and IV had experimental periods of 7, 21, and 42 days to observe the development of periapical lesions.[31,32]

The control group did not undergo dental crown access. The animals were anesthetized and euthanized with a dose of Isoflurane (Isoforine, Cristalia Produtos Químicos Farmacêuticos Ltda., São Paulo, Brazil).

Histopathological and morphometric analysis

The mandibles were excised and fixed in 10% neutral buffered formalin for 24 hours. Subsequently, they were washed for 4 hours in running water and decalcified in a 4,13% EDTA solution (pH 7–7,4) with weekly solution changes until complete decalcification. Afterward, the samples underwent routine laboratory processing[32,33] and were embedded in paraffin. Serial sections of 5µm thickness were made using a microtome (Leica RM2145; Leica Microsystems GmbH, Wetzlar, Germany). Sections were taken at 15 µm intervals in a mesiodistal direction, covering the entire length of the periapical lesion, except in the region of the apical foramen, where the slices

were collected without intervals. For each sample, 15 to 22 slides with 3 sections each were obtained.

The representative sections from each group were stained with hematoxylin and eosin (H&E) and analyzed using conventional optical microscopy with the Axio Imager.M1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with an AxioCam MRc5 camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The distal root of the lower first molars was standardized for all analyses, simultaneously allowing visualization of the root canal, apical foramen, and alveolar bone. A single-blinded evaluator who had no prior knowledge of the group being analyzed conducted the evaluation.

Descriptive analysis was performed on the representative sections from each experimental group and time period, assessing the following parameters: Pulp tissue = Score 0: Normal; Score 1: Mild alteration; Score 2: Moderate alteration; Score 3: Severe alteration (total necrosis); Inflammatory infiltrate = Score 0: Absent; Score 1: Mild infiltrate; Score 2: Moderate infiltrate; Score 3: Severe infiltrate; Apical periodontal ligament = Score 0: Normal aspect; Score 1: Mild enlargement; Score 2: Moderate enlargement; Score 3: Severe enlargement; Alveolar bone = Score 0: Absence of resorption; Score 1: Presence of resorption; Apical cement = Score 0: Absence of resorption; Score 1: Presence of resorption.

Morphometric evaluation of periapical lesions was performed on HE-stained specimens using the Axio Imager.M1 microscope at 5x magnification in fluorescent mode. The microscope was equipped with an Alexa Fluor 488 filter (AF488, Carl Zeiss MicroImaging GmbH, Göttingen, Germany), with the following characteristics: G365 excitation, FT395 reflectors, and LP420 emission. The area of periapical lesions was outlined and measured in mm^2 using specific software for this microscope (AxioVision Rel, version 4.8, Zeiss).[31,32]

Tartrate-resistant acid phosphatase histoenzymology (TRAP)

TRAP activity was performed to label multinucleated cells. The sections were deparaffinized (xylol, 5 minutes twice), hydrated (100% ethanol, 5 minutes twice; 95%, 70%, and 50% ethanol, 2 minutes each; followed by distilled water), immersed in a 50% ethanol/acetone solution (1 minute), and air-dried at room temperature. The TRAP solution was prepared in advance (10 mL acetic acid buffer, 0.1 mL N-N-dimethylformamide, 5 mg Fast Red Violet LB Salt, and 1 mg Naphthol AS-BI phosphoric acid). The solution was pipetted onto the sections and kept in a light-proof environment at 37°C for 40 minutes.[31,32] The sections were then counterstained with hematoxylin (1 minute), mounted on microscopic slides, and examined using the Axio Imager. M1 microscope under conventional light. The quantitative analysis involved counting the multinucleated TRAP-positive cells in direct contact with the alveolar bone surrounding the periapical lesion. The cell count was expressed in absolute numbers, with the area of analysis standardized by tracing a parallel line at the apex of the tooth from left to right.

Immunohistochemistry for identification of osteoclastogenesis markers (RANK and RANKL)

Histological sections were deparaffinized and hydrated. Antigen retrieval was performed using citrate buffer (pH = 6.0) and heating in a microwave oven at maximum power for two cycles of 10 seconds. After reaching room temperature, the slides were washed twice for 10 minutes twice with PBS and 0.5% PBS/Triton solution (Sigma-Aldrich Corp, St. Louis, USA). Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution for 20 minutes under light protection, followed by rinsing in PBS and PBS/Triton solution.[31]

Nonspecific binding was blocked by incubating the slides in a 1% BSA solution (bovine serum albumin)/PBS for 30 minutes. The slides were then incubated overnight under refrigeration with the primary antibodies diluted in 1% BSA: anti-RANK (polyclonal rabbit antibody, Santa Cruz Biotechnology Inc, Santa Cruz, USA; 1:700 dilution) and anti-RANKL (polyclonal goat antibody, Santa Cruz Biotechnology Inc, 1:400 dilution). After reaching room temperature, the slides were washed and incubated with a biotinylated secondary antibody

(polyclonal goat antibody, Santa Cruz Biotechnology Inc, 1:700 dilution) for 1 hour. The slides were treated with the avidin-biotin-peroxidase complex (ABC Kit, Vecstain; Vector Laboratories Inc, Burlingame, USA) for 30 minutes.

Subsequently, the slides were washed again with PBS and PBS/Triton solution, and the reaction was developed using a diaminobenzidine solution (DAB, Sigma-Aldrich Corp, St Louis, USA) and 3% H_2O_2 in PBS. The slides were counterstained with Harris Hematoxylin for 10 seconds, washed in running water, immersed in ammonium water for 30 seconds, washed again in running water, diaphanized, dehydrated, and mounted. The analysis was performed using an Axio Imager.M1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) under conventional light.

The results were qualitatively expressed based on the presence or absence of immunostaining for the markers of osteoclastogenesis.

Microbiological analysis by Brown & Brenn Staining

The slides were deparaffinized (xylene, 8 minutes three times), hydrated with decreasing concentrations of ethanol (100%, 95%, and 85%) to tap water for 3 minutes, and kept in distilled water. Afterward, the slides were stained using the following sequence of solutions: crystal violet buffered with 5% sodium bicarbonate (30 seconds), distilled water, Gram's iodine solution (1 minute), distilled water, ether-acetone solution (1:1), distilled water, basic fuchsin (1 minute), distilled water, picric acid (1 minute), followed by washing and drying with absorbent paper. The slides were then immersed in acetone solution P.A. and acetone-xylene solution. Finally, the slides were mounted with Entellan® and covered with a coverslip.

Scores ranging from 0 to 5 were assigned for this assessment[31,32] to determine the presence or absence of bacteria and their localization: Score 0 - Absence of bacteria; Score 1 - Presence of bacteria in the crown; Score 2 - Bacteria in the cervical third of the root canal; Score 3 - Bacteria in the middle third of the root canal; Score 4 - Bacteria in the apical third of the root canal; Score 5 - Bacteria in the periapical lesion.

Statistical analysis

Qualitative variables (HE) were analyzed based on the percentage of each score, representing the morphological characteristics of the tissue. For quantitative analysis (TRAP and morphometric evaluation of periapical lesions), groups I and II were compared, and groups III and IV at 7, 21, and 42 days were compared apart, pairwise. The unpaired t-test was used for data with normal distribution, while the Mann-Whitney test was used for data with a non-normal distribution. All analyses were performed, and graphs were created using SAS 9.4 and GraphPad Prism 9.4.1.

Results

Histopathological analysis

After conventional microscopy H&E analysis, the control group (healthy teeth) exhibited normal pulp tissue without inflammatory infiltrates, with regular alveolar bone and cement surface (Figure 1A). Group II did not show an inflammatory infiltrate (Figure 1B) compared to Group I, which displayed a mild reaction (Figure 1C). Between groups III and IV, the systemic probiotic slightly reduced the inflammatory process of the periapical lesion, particularly at 7 and 21 days. Group III (without probiotics) presented a moderate inflammatory infiltrate, while Group IV (with probiotics) had a mild infiltrate. At 42 days, there was no significant difference in the percentage of pulp tissue features between the drinking water and water with probiotic groups. Still, the periodontal ligament was severely altered in group III, whereas in group IV, it was moderately altered (Table 1). Regarding bone resorption, all samples in groups III and IV at 42 days exhibited this alteration, and 48.86% of samples in group IV showed bone resorption (Table 2). Conversely, cemental resorption was observed in group III, with 25% of samples at 7 days, 33% at 21 days, and 75% at 42 days. In group IV, 51.14% of samples exhibited cemental resorption at 21 days, and 100% at 42 days (Table 2). Representative microscopic findings are illustrated in Figure 2.

Morphometric evaluation of periapical lesions

The control group (healthy teeth) showed a normal periodontal ligament without lesion formation. Groups

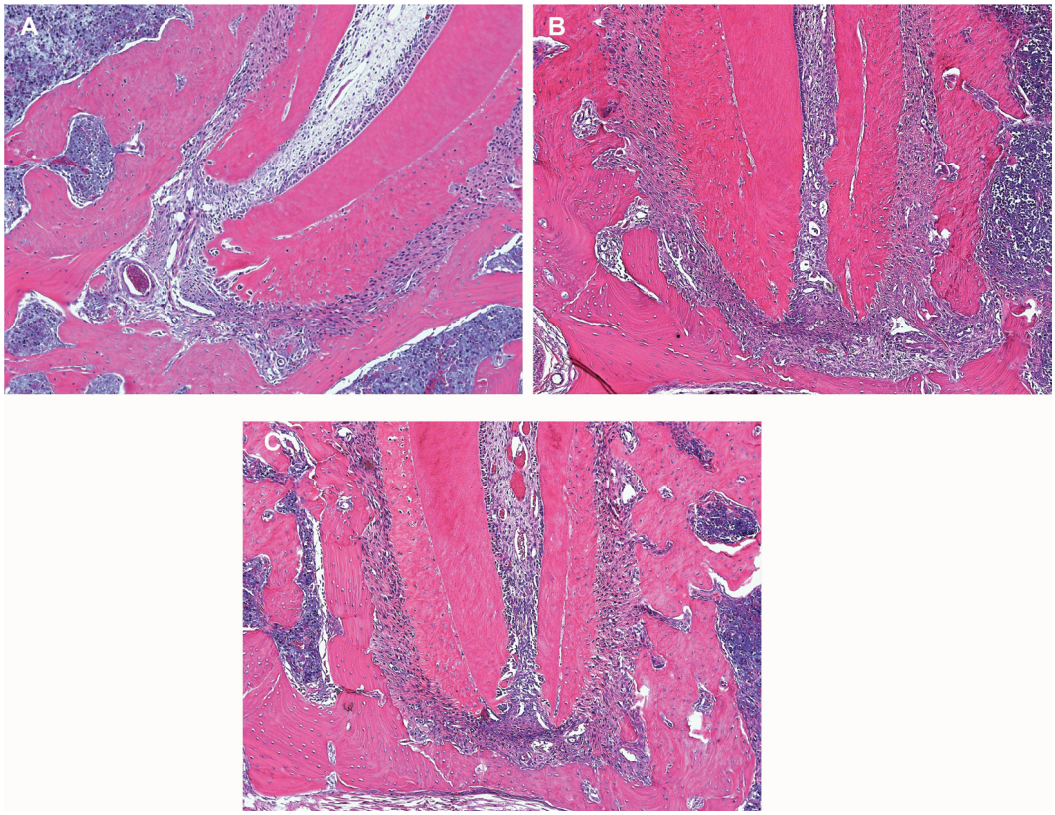

Figure 1. Representative photomicrographs of H&E-stained slides from groups: (A) Group Control; (B) Group I, and Group II(C) (Zeiss, 10x).

Table 1. Table representing H&E analysis of Pulp Tissue, Inflammatory Infiltrate, and Periodontal ligament scores (absent, mild, moderate, and severe) by groups.

Groups	Pulp tissue alteration - %				Inflammatory infiltrate - %				Pulp tissue alteration - %			
	Healthy	Mild	Moderate	Total necrosis	Absent	Mild	Moderate	Severe	Absent	Mild	Moderate	Severe
Control	100	0	0	0	100	0	0	0	100	0	0	0
I	0	75	25	0	75	25	0	0	100	0	0	0
II	33.33	0	66.67	0	100	0	0	0	100	0	0	0
III- 7 days	0	0	75	25	0	25	75	0	25	50	25	0
III- 21 days	0	0	33.33	66.75	0	0	100	0	0	100	0	0
III- 42 days	0	0	0	100	0	0	100	0	0	0	50	50
IV- 7 days	0	71.42	28.85	0	0	75	25	0	0	71.42	28.85	0
IV- 21 days	0	42.86	0	57.14	0	57.14	42.86	0	0	42.86	57.15	0
IV- 42 days	0	0	0	100	0	25	75	0	0	0	100	0

I and II exhibited an increase in the periodontal ligament without the formation of a periapical lesion, with mean values of 0.075 mm² and 0.0525 mm², respectively, and a statistically significant difference between them ($p < 0.05$). Group III, at 7, 21, and 42 days, had mean values of 0.1257 mm², 0.3671 mm², and 1.297 mm², respectively, with no significant difference compared to group IV ($p < 0.05$). In group

IV, the mean values of the periapical lesion were 0.1233 mm² at 7 days, 0.37 mm² at 21 days, and 1.06 mm² at 42 days, also without significant difference compared to group III ($p < 0.05$) (Figures 3 and 4).

Tartrate-resistant acid phosphatase histoenzymology (TRAP)

Generally, the control group (sound teeth) presented the lowest number of osteoclasts. The statistical analysis revealed a significant difference only between healthy teeth and Group I at 2 days ($p < 0.05$). While a visual reduction in positive TRAP cells was observed between groups III and IV, there was no significant difference between them in all experimental groups ($p > 0.05$) (Figures 5 and 6).

Table 2. Table representing HE analysis of Bone resorption and Cementum resorption scores by group.

Groups	Bone resorption - %		Cementum resorption - %	
	Absent	Present	Absent	Present
Control	100	0	100	0
I	100	0	100	0
II	100	0	100	0
III- 7 days	100	0	100	0
III- 21 days	100	0	75	25
III- 42 days	0	100	66.67	33.33
IV- 7 days	100	0	25	75
IV- 21 days	51.14	48.86	100	0
IV- 42 days	0	100	48.86	51.14

Immunohistochemistry for identification of osteoclastogenesis markers (RANK and RANKL)

In the Control Group (healthy teeth), the RANK antibody marking was observed only in structures such as blood vessels and alveolar bone, with no marking in osteoclast cells. Groups I and II showed no marking for both markers, similar to the control group.

Group III at 7 days exhibited RANK and RANKL antibody marking only on the tooth crown and in some specimens inside the root canal, indicating no staining in the periapical lesion. At 21 and 42 days, immunoreactivity of the RANK antibody was observed at the apical level. The RANKL antibody was observed only at 42 days, in the apical foramen and inside the lesion.

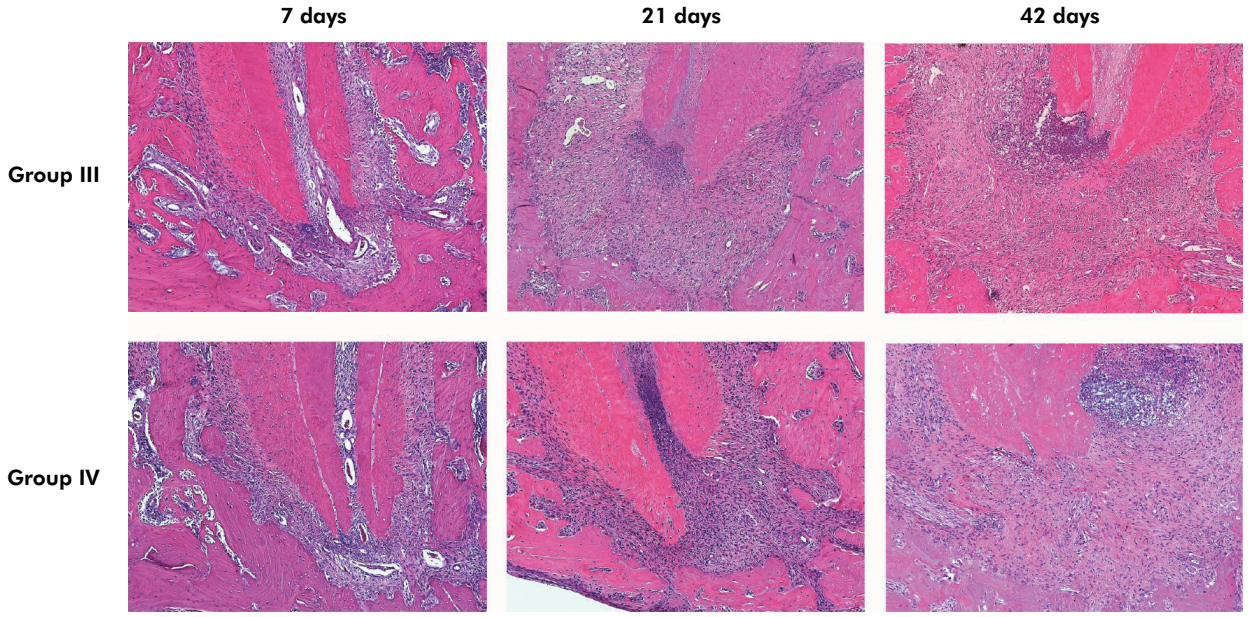

Figure 2. Representative photomicrographs of HE-stained slides from groups: III – 7 d; III – 21 d; III – 42 d; IV – 7 d; IV – 21 d; IV – 42 d (Zeiss, 10x).

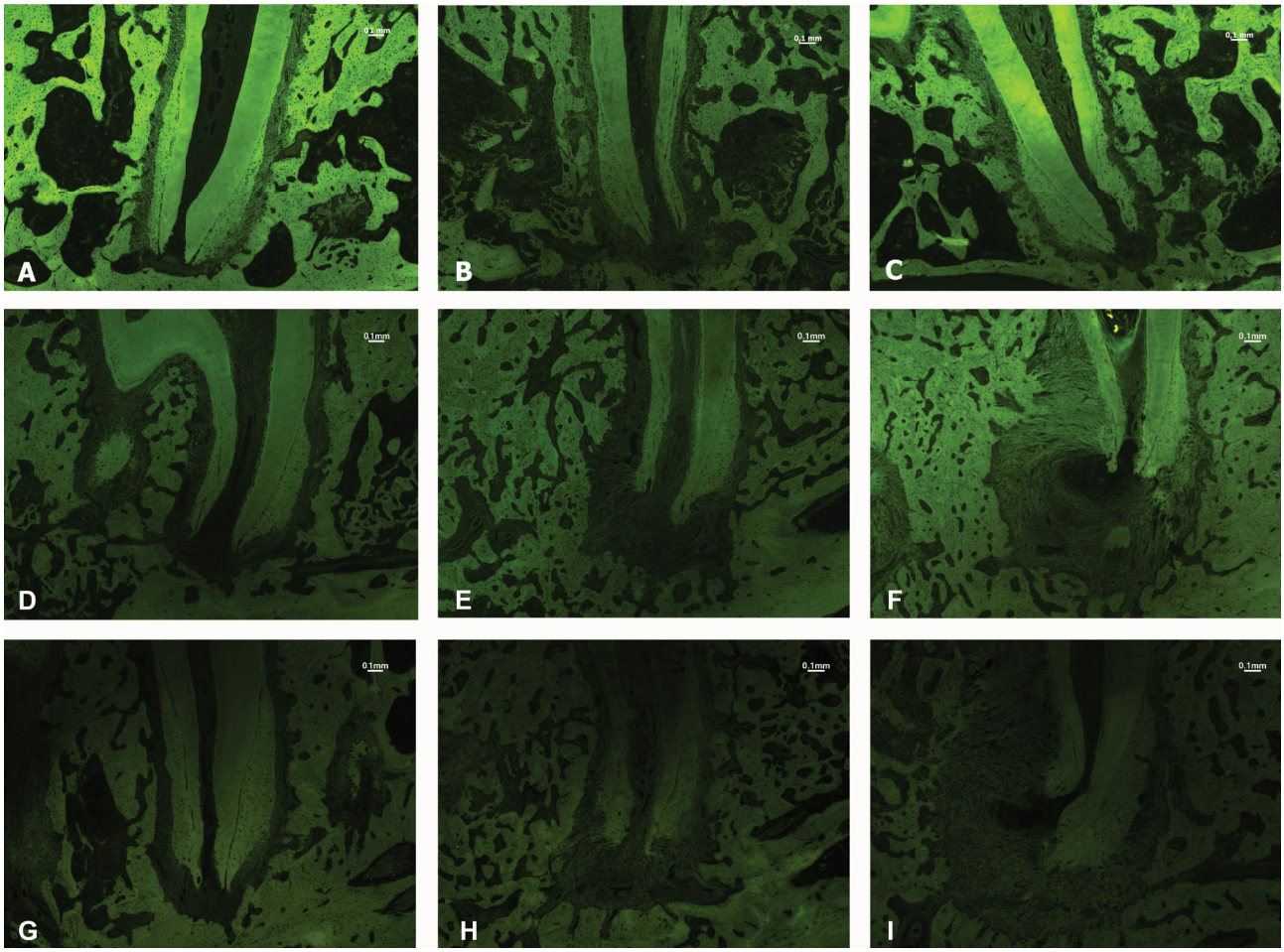

Figure 3. Representative images of fluorescence microscopy from: (A) Group Control; (B) Group I; (C) Group II; (D) Group III – 7 days; (E) Group III – 21 days; (F) Group III – 42 days; (G) Group IV – 7 days; (H) Group IV – 21 days; (I) Group IV – 42 (Zeiss, 10X).

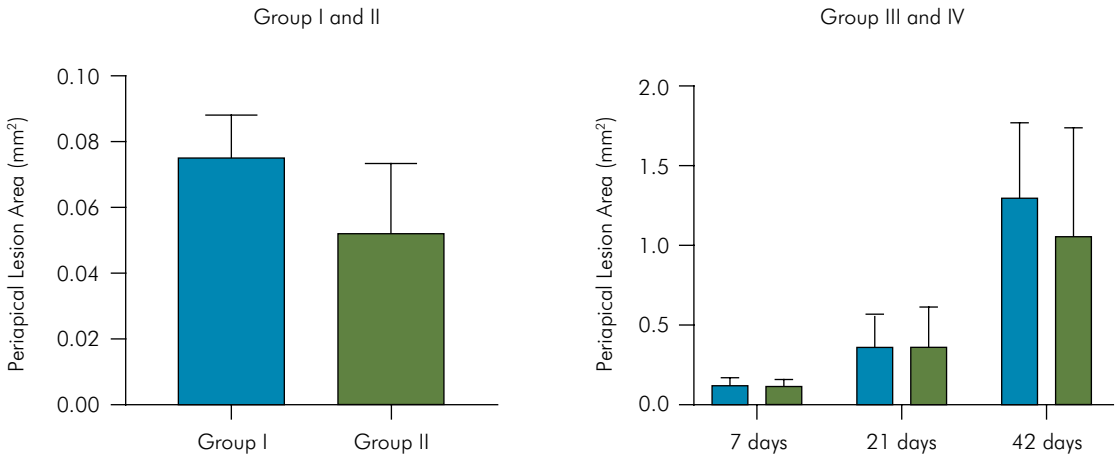

Figure 4. Graph with statistical analysis of periapical lesion area in fluorescence microscopy;(*) represents statistical difference.

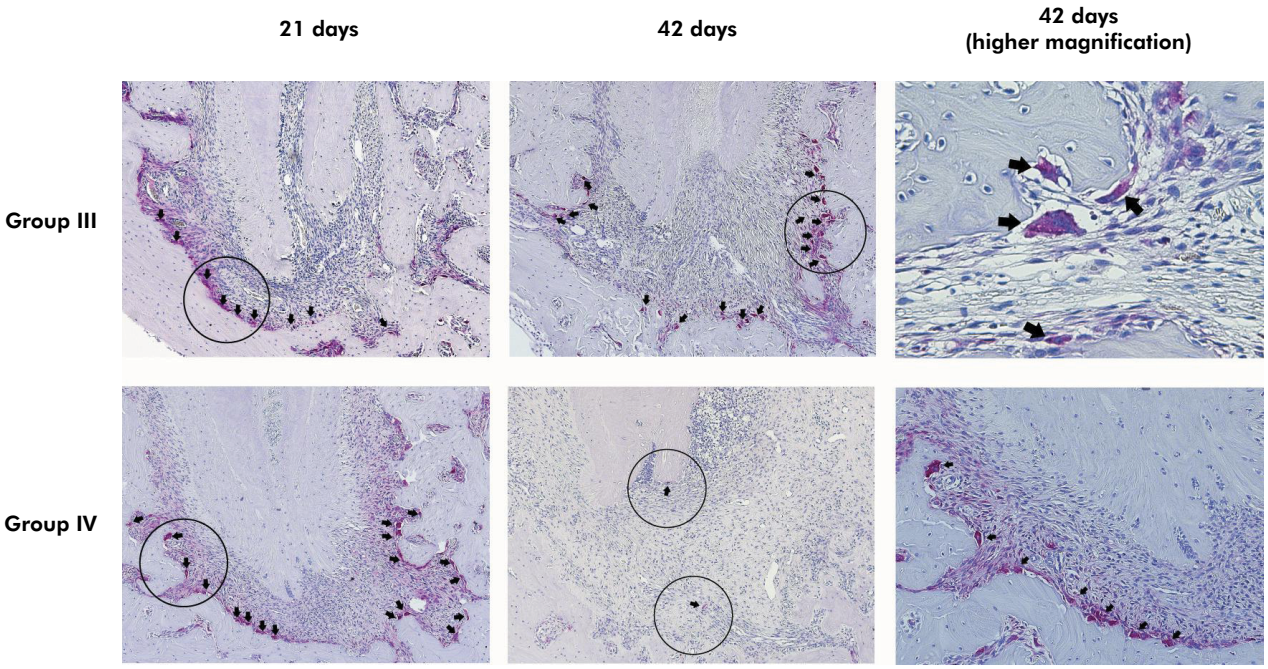

Figure 5. Representative photomicrographs of TRAP-positive cells in group III – 21 days (Zeiss 10x); III – 42 days (Zeiss 10x); III – 42 days (Zeiss 40x); IV – 21 days (Zeiss 10x); IV – 42 days (Zeiss 10x); IV – 42 days (Zeiss 20x). Arrowheads indicate osteoclast.

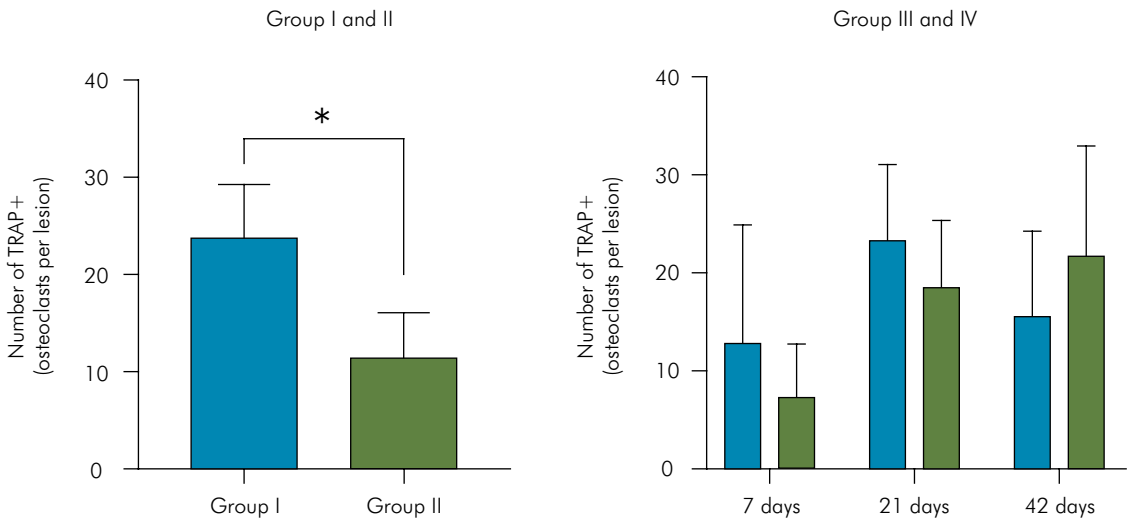

Figure 6. Graph with statistical analysis of the number of osteoclasts per lesion;(*) represents statistical difference.

Group IV at 7 days exhibited RANK and RANKL antibody marking only on the crown. At 21 days, immunoreactivity was observed in the crown and root canal of both the RANK and RANKL antibodies. However, at 42 days, only immunoreactivity of the RANK antibody was observed inside the lesion, whereas no marking was observed for the RANKL antibody, indicating a decrease in these markers during this period (Figure 7).

Microbiological analysis by the Brown & Brenn technique

Bacteria were not observed in the control group (healthy teeth), with a score of 0 for all specimens. In

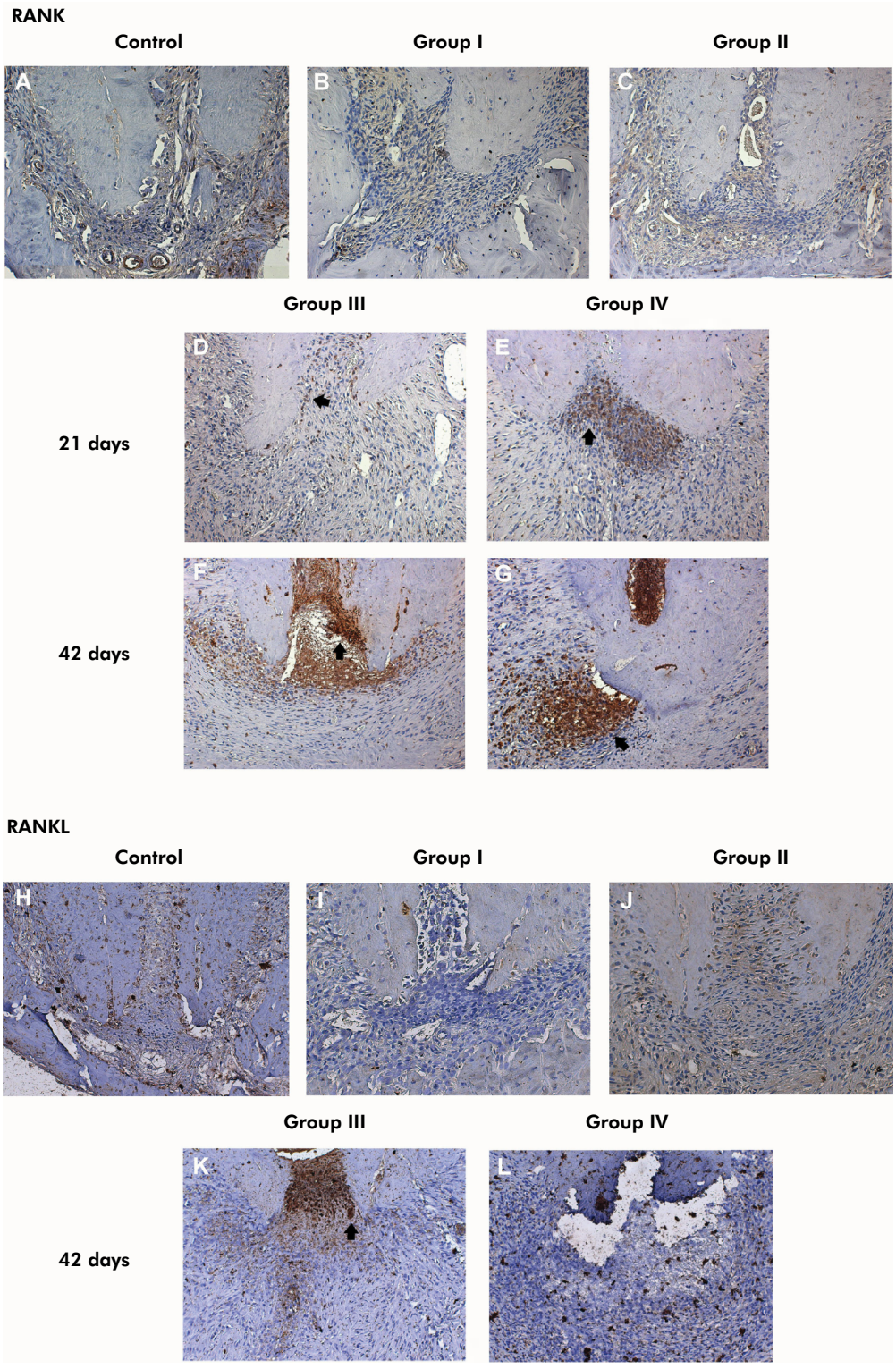

Figure 7. Representative photomicrographs of immunohistochemistry in group (A) control, (B) group I, (C) II, (D) III – 21 days ; (E) III - 42 days; (F) IV – 21 days; (G) IV – 42 days for qualitative analysis of osteoclastogenesis marker RANK. For analysis of osteoclastogenesis marker RANK-L (A) control, (B) group I, (C) II, III – 42 days and group IV – 42 days (Zeiss, 10x). Arrowheads indicate immunoreactivity.

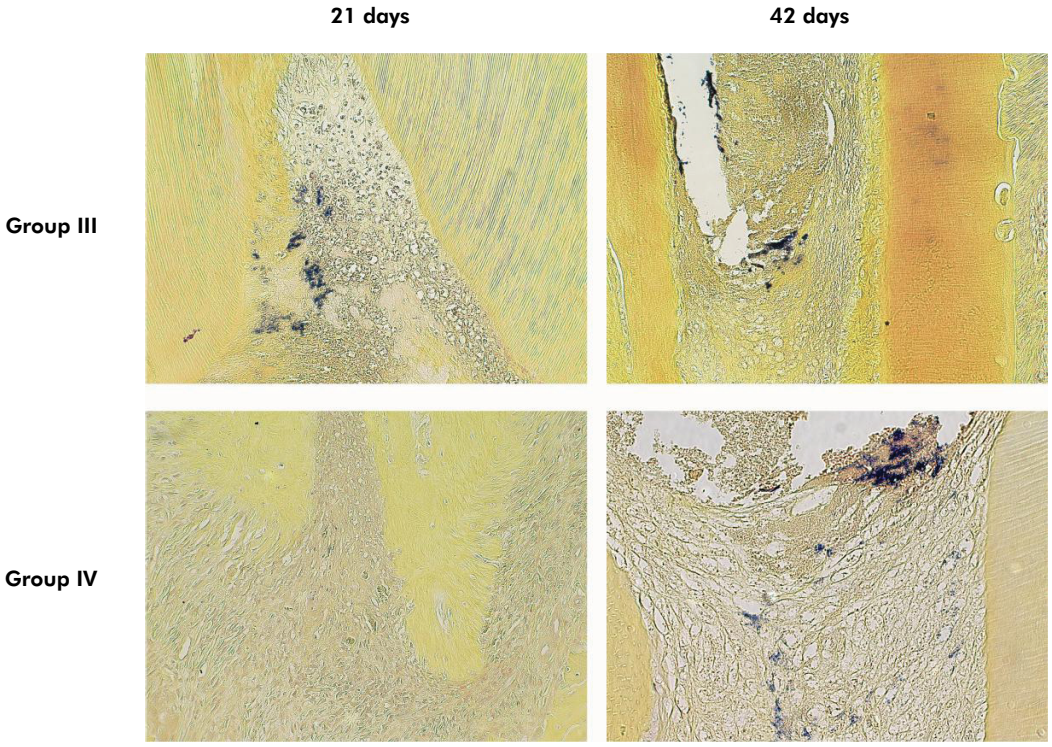

Figure 8. Representative photomicrographs of different areas of the apical and periapical root canal system, using the Brown and Brenn technique. Group III – 21 d - crown (20x); group III – 42 d – middle third (20x); group IV – 21 d – apex region (20x) and group IV – 42 d – crown (40x). Arrowheads indicate the presence of bacteria.

Groups I and II at 3 days, bacteria were noticeable only in the tooth crown, indicating a score of 1 for all samples.

In Group III at 7 days, a score of 1 was detected with the presence of bacteria only in the tooth crown. At 21 days, bacteria were observed in the tooth crown and the cervical third of the root canal, resulting in a score of 2. Finally, after 42 days, bacteria were present in the middle third of the canal, classifying it as a score 3. In Group IV at 7 days, bacteria were present only at the tooth crown, scoring 1. At 21 and 42 days, bacteria were found only in the cervical third of the root canal, resulting in a score of 2 (Figure 8).

Discussion

The present study is the first to evaluate the impact of systemic consumption of the probiotic strain *B. lactis* HN019 through drinking water in apical periodontitis. Apical periodontitis is an immune defense response to microbial factors that cause inflammation, bone resorption, and cementum resorption.[2,3] The *B. lactis* HN019 strain was chosen for its ability to modulate the immune system, antimicrobial properties,[26,34] and potential for bone tissue repair.[12,30] Moreover, it has been used in several preclinical and clinical studies related to gut and oral health.[12,27,30] The concentration of 10^9 CFU/mL, which has been used in previous studies,[12,32,35] was shown to be effective in improving immunoinflammatory parameters in periodontitis. In a clinical study, *B. lactis* HN019 was administered at a concentration of 10^9 CFU/mL, which was considered safe.[36] Therefore, this study aimed to investigate the influence of *B. lactis* on inflammatory, microbiological, and bone markers (RANK, RANKL, OPG, and TRAP) in the periapical lesion.

The RANK/RANKL/OPG system, which is part of the TNF receptor superfamily, plays a role in bone remodeling,[37] and its regulation can be influenced by alterations in the microbiota.[38] RANK is produced by osteoclast precursor cells, while RANKL is expressed by osteoblasts. The binding

of RANK to RANKL leads to the differentiation and activation of osteoclasts, resulting in increased bone resorption.[37] In this study, we observed a decrease in RANKL expression in the group that received oral probiotic supplementation. Cosme-Silva et al.[24] demonstrated a decrease in RANKL expression after supplementation with a multi-strain formula (*Lactobacillus acidophilus, Lactobacillus salivaris, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium bifidum, Bifidobacterium animalis* subs. *lactis,* and *Streptococcus thermofilus*). The authors associated these findings with a reduced number of TRAP-positive cells following probiotic intervention.

Although our results showed no statistically significant difference in the number of osteoclasts (TRAP-positive cells), it is important to consider that the method of probiotic administration and concentration differed from previous studies, which may explain the discrepancy. In a previous study that evaluated the non-surgical treatment of periodontitis with probiotics, the positive multinucleated cells were smaller in animals that consumed *B.* lactis.[12] However, the better microscopic characteristics observed in terms of inflammatory infiltrate and periodontal ligament in the groups with probiotic intervention support the potential of *B. lactis* in apical periodontitis. Similarly, a multi-strain formula improved histological features such as inflammatory infiltrate and cytokine expression;[24] and using Lactobacillus rhamnosus and L. acidophilus in rats with apical periodontitis led to a decrease in inflammation and bone resorption.[22,23]

There are few studies investigating the effect of probiotics on apical periodontitis. Some studies have examined the use of probiotics as an *in vitro* irrigating solution and have suggested their potential effectiveness in preventing the growth of endodontic pathogens.[9] Since endodontic pathogens play a crucial role in the development of apical periodontitis[2], the antimicrobial properties of probiotics could be significant in influencing its progression. Therefore, in this study, we analyzed the presence and localization of bacteria using Brown and Brenn staining. The group receiving probiotics had a lower score at 42 days, with bacteria found only in the cervical third of the root canal (score 2), whereas the corresponding group without probiotics showed bacteria in the middle third (score 3). In other experimental periods, no difference was observed between the groups. This result can be attributed to the ability of probiotics to co-aggregate with pathogenic bacteria, thereby reducing the severity of inflammatory infiltrates and preventing the spread to the apex and periapical regions.[10,11]

Several studies have demonstrated the efficacy and benefits of B. lactis, which can be safely administered. However, the outcomes depend on various factors that limit its potential, including the specific strain, frequency, administration method, and dosage[25,26]. The present study administered the probiotic through drinking water, similar to studies conducted by Gatej et al.[39] and Foureaux et al.,[40] which yielded favorable results in periodontal disease. This method more closely resembles clinical trials and provides a more realistic effect. This may explain the differences found in studies that employed the gavage method.[22,23,24] Furthermore, we were the first to test the *B. lactis* strain in apical periodontitis, a strain already widely used and proven to be safe in periodontitis, thus contributing a new aspect to the discussion regarding the strains used and the vehicles of administration. Therefore, future research should be conducted to gain a better understanding of these promising effects on periapical lesions.

Conclusion

Although the probiotic group exhibited improved microscopic features in terms of inflammatory infiltrate, periodontal ligament, and bacterial presence after inducing periapical lesions, no statistical difference was observed compared to the group that received only water. However, further studies employing different methodologies and strains should be conducted.

Acknowledgment

The authors would like to express their gratitude to the São Paulo Research Foundation (Fapesp) for providing the scholarship to LDCA – grant number 2018/22038-3). The authors would like to thank Marina Del Arco (Laboratory Assistant, Department Clinical Analyses, School of Pharmaceutical Sciences of

Ribeirao Preto, University of São Paulo USP, Ribeirão-Preto / SP, Brazil) for her technical support during the microbiological procedures. The authors also extend their appreciation to Nilza Letícia Magalhães and Marco Antonio dos Santos (Laboratory Assistant, Department of Pediatric Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo USP, Ribeirão- Preto / SP, Brazil) for their technical support during the analysis and histological process. The authors declare no conflicts of interest.

References

1. Samaranayake L, Matsubara VH. Normal oral flora and the oral ecosystem. Dent Clin North Am. 2017 Apr;61(2):199-215. https://doi.org/10.1016/j.cden.2016.11.002
2. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004 Nov;15(6):348-81. https://doi.org/10.1177/154411130401500604
3. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 2: Redefining the endodontic microbiota. J Endod. 2005 Jul;31(7):488-98. https://doi.org/10.1097/01.don.0000157990.86638.49
4. Silva LA, Leonardo MR, Assed S, Tanomaru Filho M. Histological study of the effect of some irrigating solutions on bacterial endotoxin in dogs. Braz Dent J. 2004;15(2):109-14. https://doi.org/10.1590/S0103-64402004000200005
5. Georgiou AC, Crielaard W, Armenis I, de Vries R, van der Waal SV. Apical periodontitis is associated with elevated concentrations of inflammatory mediators in peripheral blood: a systematic review and meta-analysis. J Endod. 2019 Nov;45(11):1279-1295.e3. https://doi.org/10.1016/j.joen.2019.07.017
6. McCabe LR, Parameswaran N. Advances in probiotic regulation of bone and mineral metabolism. Calcif Tissue Int. 2018 Apr;102(4):480-8. https://doi.org/10.1007/s00223-018-0403-7
7. Morales A, Gandolfo A, Bravo J, Carvajal P, Silva N, Godoy C, et al. Microbiological and clinical effects of probiotics and antibiotics on nonsurgical treatment of chronic periodontitis: a randomized placebo- controlled trial with 9-month follow-up. J Appl Oral Sci. 2018 Jan;26(0):e20170075. https://doi.org/10.1590/1678-7757-2017-0075
8. Pudgar P, Povšič K, Čuk K, Seme K, Petelin M, Gašperšič R. Probiotic strains of Lactobacillus brevis and Lactobacillus plantarum as adjunct to non-surgical periodontal therapy: 3-month results of a randomized controlled clinical trial. Clin Oral Investig. 2021 Mar;25(3):1411-22. https://doi.org/10.1007/s00784-020-03449-4
9. Bohora AA, Kokate SR, Khedkar S, Vankudre A. Antimicrobial activity of probiotics against endodontic pathogens: a preliminary study. Indian J Med Microbiol. 2019;37(1):5-11. https://doi.org/10.4103/ijmm.IJMM_18_333
10. Meurman JH, Stamatova IV. Probiotics: evidence of oral health implications. Folia Med (Plovdiv). 2018 Mar;60(1):21-9. https://doi.org/10.1515/folmed-2017-0080
11. Stanisic D, Jovanovic M, George AK, Homme RP, Tyagi N, Singh M, et al. Gut microbiota and the periodontal disease: role of hyperhomocysteinemia. Can J Physiol Pharmacol. 2021 Jan;99(1):9-17. https://doi.org/10.1139/cjpp-2020-0215
12. Ricoldi MS, Furlaneto FA, Oliveira LF, Teixeira GC, Pischiotini JP, Moreira AL, et al. Effects of the probiotic Bifidobacterium animalis subsp. lactis on the non-surgical treatment of periodontitis: a histomorphometric, microtomographic and immunohistochemical study in rats. PLoS One. 2017 Jun;12(6):e0179946. https://doi.org/10.1371/journal.pone.0179946
13. Galofré M, Palao D, Vicario M, Nart J, Violant D. Clinical and microbiological evaluation of the effect of Lactobacillus reuteri in the treatment of mucositis and peri-implantitis: A triple-blind randomized clinical trial. J Periodontal Res. 2018 Jun;53(3):378-90. https://doi.org/10.1111/jre.12523
14. Pazzini CA, Pereira LJ, da Silva TA, Montalvany-Antonucci CC, Macari S, Marques LS, et al. Probiotic consumption decreases the number of osteoclasts during orthodontic movement in mice. Arch Oral Biol. 2017 Jul;79:30-4. https://doi.org/10.1016/j.archoralbio.2017.02.017
15. Khoroushi M, Kachuie M. Prevention and treatment of white spot lesions in orthodontic patients. Contemp Clin Dent. 2017;8(1):11-9. https://doi.org/10.4103/ccd.ccd_216_17
16. La Rosa GR, Gattuso G, Pedullà E, Rapisarda E, Nicolosi D, Salmeri M. Association of oral dysbiosis with oral cancer development. Oncol Lett. 2020 Apr;19(4):3045-58. https://doi.org/10.3892/ol.2020.11441
17. Angarita-Díaz MP, Forero-Escobar D, Cerón-Bastidas XA, Cisneros-Hidalgo CA, Dávila-Narvaez F, Bedoya-Correa CM, et al. Effects of a functional food supplemented with probiotics on biological factors related to dental caries in children: a pilot study. Eur Arch Paediatr Dent. 2020 Feb;21(1):161-9. https://doi.org/10.1007/s40368-019-00468-y
18. Kumar VN, Krishnamurthy M, Poorni S, Patil S, Raj AT. Probiotics in caries prevention. J Contemp Dent Pract. 2018 Feb;19(2):123-4. https://doi.org/10.5005/jp-journals-10024-2224

19. Bustamante M, Oomah BD, Mosi-Roa Y, Rubilar M, Burgos-Díaz C. Probiotics as an adjunct therapy for the treatment of halitosis, dental caries and periodontitis. Probiotics Antimicrob Proteins. 2020 Jun;12(2):325-34. https://doi.org/10.1007/s12602-019-9521-4
20. Martin-Cabezas R, Davideau JL, Tenenbaum H, Huck O. Clinical efficacy of probiotics as an adjunctive therapy to non-surgical periodontal treatment of chronic periodontitis: a systematic review and meta-analysis. J Clin Periodontol. 2016 Jun;43(6):520-30. https://doi.org/10.1111/jcpe.12545
21. Vives-Soler A, Chimenos-Küstner E. Effect of probiotics as a complement to non-surgical periodontal therapy in chronic periodontitis: a systematic review. Med Oral Patol Oral Cir Bucal. 2020 Mar;25(2):e161-7. https://doi.org/10.4317/medoral.23147
22. Cosme-Silva L, Dal-Fabbro R, Cintra LT, Santos VR, Duque C, Ervolino E, et al. Systemic administration of probiotics reduces the severity of apical periodontitis. Int Endod J. 2019 Dec;52(12):1738-49. https://doi.org/10.1111/iej.13192
23. Cosme-Silva L, Dal-Fabbro R, Cintra LT, Ervolino E, Plazza F, Mogami Bomfim S, et al. Reduced bone resorption and inflammation in apical periodontitis evoked by dietary supplementation with probiotics in rats. Int Endod J. 2020 Aug;53(8):1084-92. https://doi.org/10.1111/iej.13311
24. Cosme-Silva L, Dal-Fabbro R, Cintra LT, Ervolino E, Prado AS, Oliveira DP, et al. Dietary supplementation with multi-strain formula of probiotics modulates inflammatory and immunological markers in apical periodontitis. J Appl Oral Sci. 2021 Jan;29:e20210483. https://doi.org/10.1590/1678-7757-2020-0483
25. Kumar G, Tewari S, Tagg J, Chikindas ML, Popov IV, Tiwari SK. Can Probiotics Emerge as Effective Therapeutic Agents in Apical Periodontitis? A Review. Probiotics Antimicrob Proteins. 2021 Apr;13(2):299-314. https://doi.org/10.1007/s12602-021-09750-2
26. Araujo LD, Furlaneto FA, Silva LA, Kapila YL. Use of the Probiotic Bifidobacterium animalis subsp. lactis HN019 in Oral Diseases. Int J Mol Sci. 2022 Aug;23(16):9334. https://doi.org/10.3390/ijms23169334
27. Oliveira LF, Salvador SL, Silva PH, Furlaneto FA, Figueiredo L, Casarin R, et al. Benefits of Bifidobacterium animalis subsp. lactis Probiotic in Experimental Periodontitis. J Periodontol. 2017 Feb;88(2):197-208. https://doi.org/10.1902/jop.2016.160217
28. Invernici MM, Furlaneto FA, Salvador SL, Ouwehand AC, Salminen S, Mantziari A, et al. Bifidobacterium animalis subsp lactis HN019 presents antimicrobial potential against periodontopathogens and modulates the immunological response of oral mucosa in periodontitis patients. PLoS One. 2020 Sep;15(9):e0238425. https://doi.org/10.1371/journal.pone.0238425
29. Chitprasert P, Sudsai P, Rodklongtan A. Aluminum carboxymethyl cellulose-rice bran microcapsules: enhancing survival of Lactobacillus reuteri KUB-AC5. Carbohydr Polym. 2012 Sep;90(1):78-86. https://doi.org/10.1016/j.carbpol.2012.04.065
30. Messora MR, Oliveira LF, Foureaux RC, Taba M Jr, Zangerônimo MG, Furlaneto FA, et al. Probiotic therapy reduces periodontal tissue destruction and improves the intestinal morphology in rats with ligature-induced periodontitis. J Periodontol. 2013 Dec;84(12):1818-26. https://doi.org/10.1902/jop.2013.120644
31. Bezerra da Silva RA, Nelson-Filho P, Lucisano MP, De Rossi A, Queiroz AM, Bezerra da Silva LA. MyD88 knockout mice develop initial enlarged periapical lesions with increased numbers of neutrophils. Int Endod J. 2014 Jul;47(7):675-86. https://doi.org/10.1111/iej.12204
32. de Oliveira KM, da Silva RA, De Rossi A, Fukada SY, Feres M, Nelson-Filho P, et al. Absence of interleukin 22 affects the oral microbiota and the progression of induced periapical lesions in murine teeth. Int Endod J. 2015 Jan;48(1):46-59. https://doi.org/10.1111/iej.12274
33. Pucinelli CM, Lima RB, Almeida LK, Lucisano MP, Córdoba AZ, Marchesan JT, et al. Interferon-gamma inducible protein 16 and type I interferon receptors expression in experimental apical periodontitis induced in wild-type mice. Int Endod J. 2022 Oct;55(10):1042-52. https://doi.org/10.1111/iej.13802
34. Miller LE, Lehtoranta L, Lehtinen MJ. The Effect of Bifidobacterium animalis ssp. lactis HN019 on cellular immune function in healthy elderly subjects: systematic review and meta-analysis. Nutrients. 2017 Feb;9(3):191. https://doi.org/10.3390/nu9030191
35. Maia LP, Levi YLAS, Silva PHF, Wons CL, Pizzo Pitelli L, Castro JG, et al. Impact of Bifidobacterium animalis subsp. lactis HN019 probiotic in the prevention of periodontitis associated with immunosuppression. J Periodontol. 2023 Mar;94(3):389-404. https://doi.org/10.1002/JPER.22-0146
36. Ibarra A, Latreille-Barbier M, Donazzolo Y, Pelletier X, Ouwehand AC. Effects of 28-day Bifidobacterium animalis subsp. lactis HN019 supplementation on colonic transit time and gastrointestinal symptoms in adults with functional constipation: A double-blind, randomized, placebo-controlled, and dose-ranging trial. Gut Microbes. 2018;9(3):236-51. https://doi.org/10.1080/19490976.2017.1412908
37. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys. 2008 May;473(2):139-46. https://doi.org/10.1016/j.abb.2008.03.018
38. de Sire A, de Sire R, Curci C, Castiglione F, Wahli W. Role of dietary supplements and probiotics in modulating microbiota and bone health: the gut-bone axis. Cells. 2022 Feb;11(4):743. https://doi.org/10.3390/cells11040743
39. Gatej SM, Marino V, Bright R, Fitzsimmons TR, Gully N, Zilm P, et al. Probiotic Lactobacillus rhamnosus GG prevents alveolar bone loss in a mouse model of experimental periodontitis. J Clin Periodontol. 2018 Feb;45(2):204-12. https://doi.org/10.1111/jcpe.12838
40. Foureaux RC, Messora MR, de Oliveira LF, Napimoga MH, Pereira AN, Ferreira MS, et al. Effects of probiotic therapy on metabolic and inflammatory parameters of rats with ligature-induced periodontitis associated with restraint stress. J Periodontol. 2014 Jul;85(7):975-83. https://doi.org/10.1902/jop.2013.130356