Favourable effects of *Bacillus subtilis* and *Bacillus licheniformis* on experimental periodontitis in rats

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A R T I C L E   I N F O

A B S T R A C T

Objectives: The purposes of this study were to evaluate, in rats: i) the effects of *Bacillus* species on the development of experimental periodontitis (EP) via microtomographic, immunological and hematological assays (Experiment 1—E1); ii) the effects of *Bacillus* species as adjuncts to scaling and root planing (SRP) for the treatment of EP via histomorphometric and immunohistochemical analyses (Experiment 2—E2).

Methods: In E1, 24 rats were divided into groups C1 (control), PROB1, EP1 and EP-PROB1. In groups with EP, the mandibular first molar of each animal received a ligature for 14 days. In groups PROB1, animals received *Bacillus* species for 44 days, starting 30 days before EP induction in Group EP-PROB1. In E2, 24 rats were assigned to groups C2 (control), PROB2, EP-SRP2 and EP-SRP-PROB2. In groups with SRP, EP was induced as described in E1. The ligatures were removed after 14 days and SRP was performed. In groups PROB2, animals received *Bacillus* species for 15 days, starting after SRP in Group EP-SRP-PROB2.

Results: In E1, Group EP1 presented bone loss (BL) and eosinophil numbers greater than Group EP-PROB1 ($P < 0.05$). In Group EP-PROB1, the receptor activator of nuclear factor-kB ligand (RANKL)/osteoprotegerin (OPG) ratio was similar to that of groups without EP. In E2, Group EP-SRP-PROB2 presented fewer TRAP-positive osteoclasts, lower immunolabeling pattern for a proinflammatory cytokine and decreased BL and attachment loss than Group EP-SRP2 ($P < 0.05$).

Conclusions: *Bacillus* species supplementation provided a protective effect against BL and enhanced the effects of SRP in the treatment of EP in rats.

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1. Introduction

In the past few years, the use of probiotics to displace pathogenic microorganisms in order to maintain or restore a natural microbiome has been considered a promising way to combat diverse infectious processes, including those of the oral cavity (Bengmark, 2001; Stamatova & Meurman, 2009a, 2009b; Vuotto, Barbanti, Mastrantonio, & Donelli, 2014). Apparently, this therapy may modify the bacterial environment and modulate the host immune response (Teughels, Loozen, & Quirynen, 2011). The latter effects are appealing for periodontal healthcare because there is evidence demonstrating that the destruction of periodontal tissues is substantially mediated by the host and driven by the bacterial challenge (Sanz and Quirynen, 2005; Sanz & van Winkelhoff, 2011).

Probiotics are defined as live microorganisms that can confer health benefits when administered in adequate amounts (FAO/WHO, 2002). The main microorganisms used as probiotics are bacteria from the genera *Lactobacillus, Enterococcus, Bacillus* and *Bifidobacterium* (Bron, van Baarlen, & Kleerebezem, 2012). Teughels

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et al. (2013) and Vivekananda, Vandana, and Bhat (2010) have demonstrated that bacteria from the *Lactobacillus* genus combined with scaling and root planing (SRP) may improve periodontal clinical parameters and delay recolonization of periodontal pockets with periodontopathogens in patients with chronic periodontitis.

Although promising results have been reported in the scientific literature, it is important to remember that the findings obtained with probiotics cannot be generalized, since they are dependent on the strain, dosage, frequency and route of administration (Teughels et al., 2011). To date, studies that have investigated the effects of probiotics on periodontal diseases used mainly microorganisms from the genus *Lactobacillus* (Teughels et al., 2013; Vivekananda et al., 2010). To the best of the authors' knowledge, there are no studies that have evaluated the effects of the genus *Bacillus* as an adjunct to SRP for the treatment of periodontitis.

Scientific interest in spore-forming *Bacillus* species as probiotics has occurred only in the last few years (Cutting, 2011). Among the species that have been most extensively examined are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans* and *Bacillus licheniformis* (Cutting, 2011). Spore probiotics are being widely used in humans as dietary supplements, in animals as growth promoters and competitive exclusion agents and lastly in aquaculture for enhancing the growth and disease-resistance of cultured shrimps (Cutting, 2011). There are three mechanisms by which *Bacillus* interacts with hosts: stimulation of the immune system, suppression of gastrointestinal pathogens and secretion of antimicrobial compounds (Mongkolthanaruk, 2012).

In previous studies by this group (Foureaux et al., 2014; Messora et al., 2013), the effects of *Bacillus* species on experimental periodontitis (EP) in rats were evaluated. In the first study, (Messora et al., 2013) it was histologically demonstrated that this supplementation reduced both attachment and alveolar bone losses. In the second study, (Foureaux et al., 2014) *Bacillus* species decreased the levels of C-terminal telopeptide and increased the expression of osteoprotegerin (OPG) in periodontal tissues. Tsubura et al. (2009) reported that a mouth rinsing prepared from the supernatant of a cultured medium of *Bacillus subtilis* isolated from Japanese soil improved the gingival index of patients with chronic periodontitis and promoted a marked change in the scores of the benzoyl-DL-arginine-naphthylamide (BANA) test.

However, there is still limited scientific evidence to support the efficacy of *Bacillus* species in periodontitis. Further studies are essential to improve the scientific basis for their use, elucidate the role of these bacteria in the oral cavity and identify their mechanisms of interaction with the host. The purposes of this study were to evaluate, in rats: i) the effects of *Bacillus* species on the development of EP via microtomographic, immunological and hematological assays (Experiment 1–E1); ii) the effects of *Bacillus* species as adjuncts to scaling and root planing (SRP) for the treatment of EP via histomorphometric and immunohistochemical analyses (Experiment 2–E2).

2. Materials and methods

2.1. Sample

This study was conducted in compliance with the ethical principles of animal experimentation, as well as standards for the didactic-scientific practice of vivisection and the Universal Declaration of Animal Rights by UNESCO (United Nations Educational, Scientific and Cultural Organization). The present study was conducted only after review and approval by the Ethics Committees on Animal Experimentation (ECAE) at School of Dentistry of Ribeirao Preto, University of São Paulo—FORP/USP (protocol 2013.1.993.58.6), at Federal University of Lavras—UFLA (protocol 036/2010) and at Lavras University Center—UNILAVRAS (protocol 012/2010). A power calculation was performed to determine the sample size. The animal was considered the study unit. A sample size was desired that would provide 80% power to recognize a significant difference of 20% among groups and a standard deviation of 15% with a 95% confidence interval (α=0.05), considering the change in alveolar bone loss as the primary outcome variable. Therefore, a sample size of six animals per group was required.

Forty-eight adult male Wistar rats (*Rattus norvegicus, albinus*), weighing 170–250 g, were used (Central Animal Facilities-FORP/USP and UNILAVRAS). The rats were kept in a room with a 12 h light/dark cycle and temperatures between 22 and 24 °C. The animals were housed in individual metabolic cages and subjected to a period of 7 days of acclimatization to the environment and team running the project. Throughout the experiment, the animals were fed a selected solid diet and water *ad libitum*. The study was divided into two independent experiments (Fig. 1).

2.2. Experiment 1—Bacillus *species in the development of EP*

In Experiment 1, the effects of *Bacillus* species on the development of EP were evaluated. 24 rats were randomly assigned to one of four experimental groups (n = 6): C1 (control),

Experiment 1 – *Bacillus species in the development of EP*
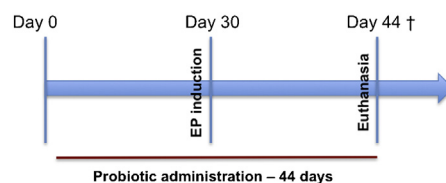
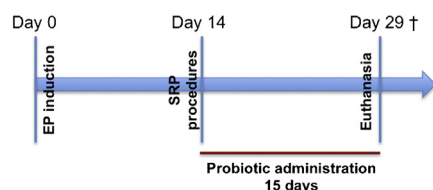**Experiment 2** – *Bacillus species in the treatment of EP*

Fig. 1. Experimental design.

PROB1 (probiotic), EP1 (experimental periodontitis) and EP-PROB1 (experimental periodontitis + probiotic).

2.2.1. Probiotic therapy, induction of EP and euthanasia

The probiotic therapy started in the beginning of the experiment (day 0). In Groups PROB1 and EP-PROB1, the probiotic product (*Bacillus subtilis*–DSM 5750 + *Bacillus licheniformis*–DSM 5749; Bioplus®2B, Chr. Hansen, Hørsholm, Denmark) was administered orally by adding it to the drinking water of the animals in the proportion of 1.5×10^8 colony-forming units (CFU) per milliliter for the duration of the experiment (44 days). Every morning, 10 mL of water containing the probiotics was provided to the animals as the unique hydric source. Only after the complete consumption of this initial hydric source, water *ad libitum* (without probiotics) was given to the animals during the rest of the day. Animals of Groups C1 and EP1 received water without probiotic supplementation.

Thirty days after the beginning of the experiment, all animals were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg body weight; Rompum®, Bayer Animal Health, Sao Paulo, SP, Brazil) and ketamine (80 mg/kg body weight; Dopalen®, Agribands Purina do Brasil Ltda., Paulinia, SP, Brazil). After general anesthesia, animals of Groups EP1 and EP-PROB1 were placed on the operating table, which allowed the rats' mouths to be kept open, facilitating access to posterior teeth of the mandible. A cotton ligature was placed around the right mandibular first molar of each animal. Cotton ligatures were not placed in the animals of Groups C1 or PROB1.

All animals were euthanized 44 days after the beginning of the experiment by decapitation following recommendations of the ECAE. Prior to decapitation, animals were anesthetized by an intraperitoneal injection of xylazine (Rompum®) and ketamine (Dopalen®). 2 mL of blood, stored in tubes with EDTA, was drawn via cardiac puncture for hematological analysis. Hemimandibles were excised and fixed in 4% formaldehyde for 48 h in preparation for microtomographic analysis. Samples of gingival tissues around right mandibular first molars were collected for Western blot analysis.

2.2.2. Microcomputed tomography (micro-CT) analyses

Non-demineralized specimens were scanned by a cone-beam micro-CT system (Skyscan 1172, Bruker, Kontich, Belgium). The X-ray generator was operated at an accelerated potential of 60 kV with a beam current of 165 µA and an exposure time of 650 ms per projection. Images were produced with a voxel size of $6 \times 6 \times 6$ µm. Appropriate software (Data Viewer®, version 1.5.0, Bruker, Kontich, Belgium) was used to generate 3 dimensional models which were then rotated into a standard position according to the following criteria: (1) in the transaxial plane, the right mandibular first molar (M1) had its axis vertically positioned, (2) in the coronal plane, the mandibular bone was vertically orientated, with the mesial root of the M1 in the upper part of the image and (3) in the sagittal plane, the occlusal surface of M1 was horizontally positioned. Linear measurements of alveolar bone level (ABL) were performed at four different sites: buccal, lingual, interproximal and furcation. For buccal and lingual sites, on the transaxial image passing through the distal root of the M1, the linear distances from cementoenamel junction (CEJ) to buccal/lingual alveolar bone crest (ABC) were measured. For interproximal sites, coronal dataset was analyzed using appropriate software (CT-Analyser®, version 1.13.5.1+, Bruker, Kontich, Belgium). The distance between the last image showing the ABC, between mandibular second molar (M2) and M1, and the first image showing the CEJ of M1, was measured. For the furcation site, on the sagittal image passing through both mesial and distal roots of M1, ABL was assessed by measuring the distance between the roof of the furcation and the ABC in the furcation area. The sum of the four linear measurements obtained from each animal were expressed as the ABL value.

For volumetric measurements, a volume of interest (VOI-prismatic section) was outlined from the apexes of all roots of M1 up to the roof of the furcation of M1, touching the roots surfaces, in all images of the coronal dataset, using the same software (CT-Analyser®) applied for the analysis of the interproximal site. The images were binarised so that bone and dental structures could be distinguished according to differences in the density, using a greyscale (inferior limit—65, superior limit—255; greyscale threshold 0–255). This pattern of binarisation was used for all samples. The following parameters were analyzed: i) Bone Volume (BV): percentage of the VOI filled with bone tissue; ii) Mean Bone Porosity (BP): percentage of bone porosity present in the VOI. All micro-CT analyses were performed by one blinded and calibrated examiner (A.J.N.A.).

2.2.3. Western blot analysis

Quantification of receptor activator of nuclear factor-kB ligand (RANKL; SC 7628, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and OPG (SC 11383, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was visualized from total protein extracts of gingival tissues samples. Samples were processed as previously described (Foureaux et al., 2014). Images obtained on radiographic films were scanned by a chemiluminescent documentation system (ECL Prime Western Blotting Detection Reagent—RPN2232, GE Healthcare, Buckinghamshire, UK). Additional analysis of the bands was performed densitometrically using specific software (ImageJ, National Institutes of Health, Washington, DC).

2.2.4. Hematological analysis

A blinded technician conducted the hematological analysis immediately after blood collection. Blood samples were processed and analyzed according to the manufacturer's instructions in a calibrated and automated hematology analyzer (ABX Micros ABC Vet, Horiba ABX Diagnostics, Montpellier, France) to obtain counts ($10^3/µL$) of neutrophils, eosinophils, lymphocytes and monocytes.

2.2.5. Statistical analysis

Data were grouped and presented as means and standard deviations. Normality and homoscedasticity of the data were verified. The significance of differences among groups was assessed by analysis of variance (ANOVA) followed by *post-ho*c Tukey test. The significance level was set at 5% for all tests.

2.3. Experiment 2—Bacillus species in the treatment of EP

In Experiment 2, the effects of *Bacillus* species as adjuncts to SRP for the treatment of EP were analyzed. 24 rats were randomly assigned to one of four experimental groups (n = 6): C2 (control), PROB2 (probiotic), EP-SRP2 (experimental periodontitis + SRP) and EP-SRP-PROB2 (experimental periodontitis + SRP + probiotic).

2.3.1. Induction of EP, SRP, probiotic therapy and euthanasia

In the beginning of the experiment (day 0), the animals of Groups EP-SRP2 and EP-SRP-PROB2 were submitted to EP induction, as described in Experiment 1. After 14 days, all animals were anesthetized as described in the EP induction procedure. Then, in Groups EP-SRP2 and EP-SRP-PROB2, the ligatures were removed and the right mandibular first molars were subjected to SRP with manual Mini Five 1–2 curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) through ten distal-mesial traction movements on the buccal and lingual aspects. The furcation and interproximal areas were scaled with the same curettes through cervico-occlusal traction movements. SRP procedures were performed by the same

experienced and blinded operator (L.F.F.O.). Animals of Groups C2 and PROB2 were not subjected to EP induction.

For Groups PROB2, the same probiotic therapy described in Experiment 1 was administered for 15 days, starting immediately after SRP in Group EP-SRP-PROB2. Animals of Groups C2 and EP2 received water not supplemented with probiotic.

The animals were euthanized at day 29 with a lethal dose (150 mg/kg body weight) of sodium thiopental (Thiopentax®, Cristalia Produtos Quimicos Farmaceuticos Ltda., Sao Paulo, SP, Brazil). The hemimandibles were excised and fixed in 4% formaldehyde for 48 h in preparation for histomorphometric and immunohistochemical analyses.

2.3.2. Histopathological and histometric analyses

The right hemimandibles were decalcified in 4% EDTA solution. After complete decalcification, they were processed and embedded in paraffin. Serial sections, 4 μm thick, were obtained in a mesio-distal direction. The sections were stained with hematoxylin and eosin for analysis by light microscopy. The histopathological analysis was performed by a blinded and certified histologist (E.E.).

For histometric analysis, two sections representing the most central buccal-lingual portion in the furcation area of the right mandibular first molars were selected. The images of the histologic sections were captured by a digital camera (DFC295, Leica Microsystems, Wetzlar, Germany) connected to a light microscope (DM2000, Leica Microsystems, Wetzlar, Germany). The digital images were saved on a computer and then analyzed using appropriate software (Diracon Bio Informatica Ltda., Vargem Grande do Sul, SP, Brazil). The furcation area not filled with bone or periodontal ligament (Area of No Bone or Periodontal Ligament—ANBL) was measured by outlining the region surrounded by the roof of the furcation (RF), the most coronal portion of the ABC in the furcation, the mesial and the distal roots of the first molar. In order to assess the attachment loss (AL), the linear distance between the CEJ and the epithelial attachment was measured on the distal root of the right mandibular first molar. The values of ANBL and AL for each animal were represented by the mean values of the two histologic sections. Histometric analysis was performed by one calibrated examiner (C.G.S.) who was blinded to the experimental groups and treatments rendered.

2.3.3. Immunohistochemical analyses

Immunohistochemical processing was performed using the indirect immunoperoxidase method, as previously described (Furlaneto et al., 2014). The histological slides containing samples from all experimental groups were incubated with one of the following primary antibodies: rabbit anti-interleukin-1 beta—IL-1β (1:100-Rabbit anti-IL-1β–SC 7884, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-interleukin-10–IL-10 (1:100-Goat anti-IL-10–SC 1783, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-tartrate-resistant acid phosphatase (TRAP; 1:100-Goat anti-TRAP–SC 30833, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

A quantitative immunolabeling analysis was performed for TRAP-positive cells. TRAP-positive cells were counted in an area of 2000 μm x 2000 μm in the central part of furcation septum at 200 × magnification. The coronal limit of the area was the ABC, which extended apically for a distance of 2000 μm.

Semi-quantitative immunolabeling analyses of IL-1β and IL-10 were conducted in the entire furcation region at 400x magnification. The following scores were determined: score 0–no immunolabeling (total absence of immunoreactivity in the area);
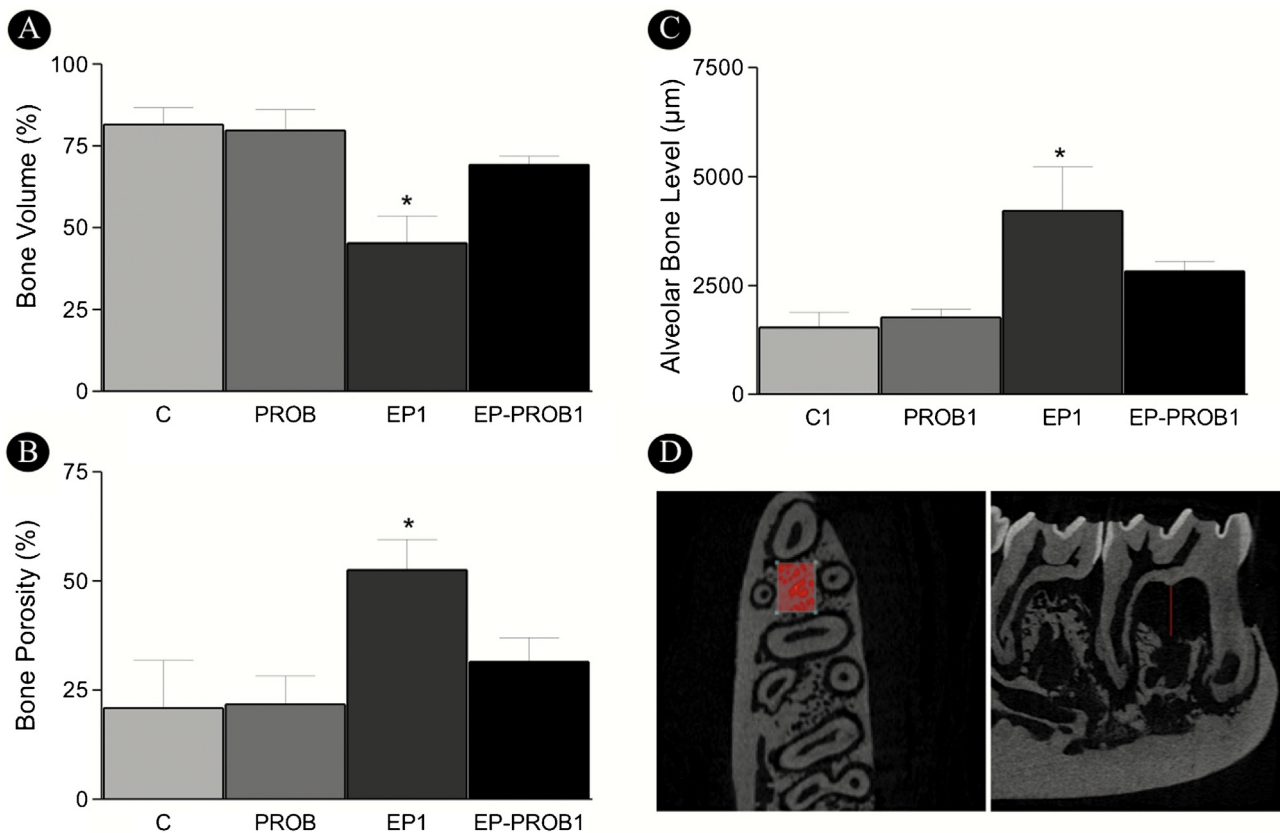

Fig. 2. Micro-CT analyses. Means and standard deviations of (A) BV (%), (B) BP (%) and (C) ABL (mm) in Groups C1, PROB1, EP1 and EP-PROB1, with comparisons among groups. (D) Micro-CT images for volumetric analysis using the coronal dataset (left) and linear measurement in the furcation area using the sagittal plane (right). Abbreviations and symbols: BV = bone volume; BP = bone porosity; ABL = alveolar bone level; * = significant difference when compared with all the other groups (P < 0.05).

score 1–low immunolabeling pattern ($\approx 1/3$ of the area presenting immunoreactivity); score 2–moderate immunolabeling pattern ($\approx 2/3$ of the area presenting immunoreactivity) and score 3–high immunolabeling pattern (almost the entire area presenting immunoreactivity).

Histologic sections were evaluated under light microscopy with an optical microscope (Axiovision 4.8.2, Carl Zeiss Micro-Imaging GmbH, Jena, Germany) by one calibrated examiner (E.E.) who was blinded to the experimental groups and treatments rendered.

2.3.4. Statistical analysis

Data were grouped and presented as means and standard deviations (continuous variables) or medians, interquartiles ranges and maximum and minimum values (ordinal variables). Normality and homoscedasticity of the data were verified. The significance of differences among groups in relation to the immunolabeling pattern of IL-1β and IL-10 was determined by Kruskal-Wallis tests, followed by Dunn's multiple comparison post-test. The data from the histometric analysis were assessed by ANOVA followed by post-hoc Tukey test. The significance level was set at 5% for all tests.

3. Results

3.1. Experiment 1—Bacillus species in the development of EP

3.1.1. Micro-CT analyses

Group EP-PROB1 presented greater BV and decreased ABL and BP when compared with Group EP1 ($P < 0.05$). Means and standard deviations of ABL, BV and BP, as well as intergroup comparisons, are depicted in Fig. 2.

3.1.2. Western blot analysis

Group EP1 presented RANKL/OPG ratio significantly greater than Groups C1 and PROB1 ($P < 0.05$; Fig. 3A). No significant differences regarding RANKL/OPG ratio were observed among Groups C1, PROB1 and EP-PROB1 or between Groups EP1 and EP-PROB1($P > 0.05$; Fig. 3A).
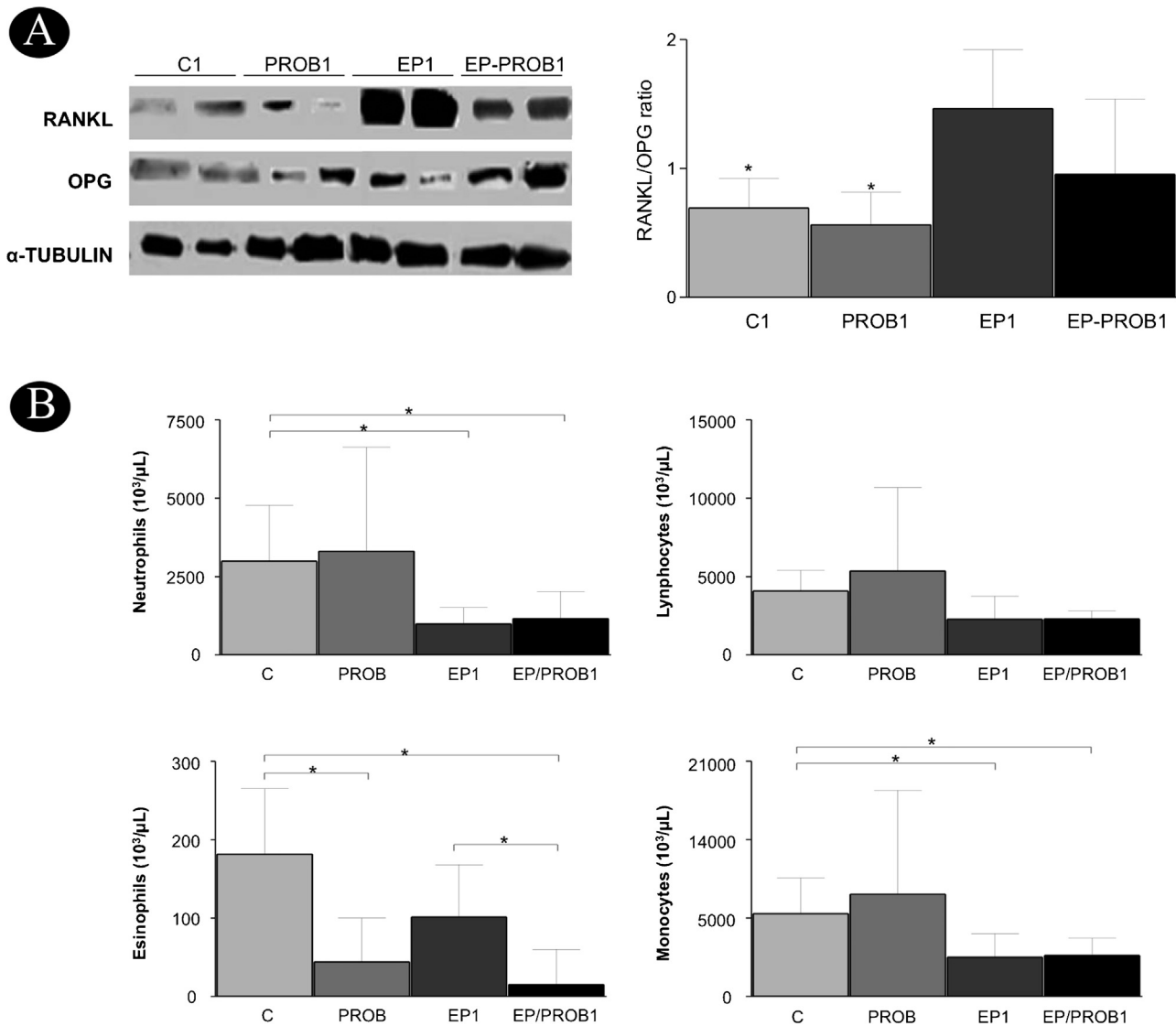

Fig. 3. (A) Western blot analysis. (Left) Representative enhanced chemiluminescence radiographs showing osteoprotegerin (OPG) and receptor activator of nuclear factor-kB ligand (RANKL) expressions from total proteins of gingival tissues. The α-tubulin was used as internal control. (Right) Means and standard deviations of RANKL/OPG ratio in Groups C1, PROB1, EP1 and EP-PROB1, with comparisons among groups. * = significant difference when compared with Group EP1 ($P < 0.05$). (B) Means and standard deviations of neutrophils, eosinophils, lymphocytes and monocytes counts ($10^3/\mu L$) in Groups C1, PROB1, EP1 and EP-PROB1, with comparisons among groups. * = significant difference between groups ($P < 0.05$).

3.1.3. Hematological analysis

Groups EP1 and EP-PROB1 presented a significantly reduced quantity of neutrophils and monocytes compared to Group C1 ($P < 0.05$; Fig. 3B). No significant differences regarding the number of neutrophils, lymphocytes or monocytes were observed between Groups EP1 and EP-PROB1 ($P > 0.05$; Fig. 3B). A decreased number of eosinophils was observed in Groups PROB1 and EP-PROB1 in relation to Group C1 ($P < 0.05$; Fig. 3B). Group EP1 presented a significantly greater quantity of eosinophils than Group EP-PROB1 ($P < 0.05$; Fig. 3B).

3.2. Experiment 2—Bacillus species in the treatment of EP

3.2.1. Histopathological analysis

In Groups C2 and PROB2, periodontal ligament (PL) presented a great amount of collagen fibers, fibroblasts and
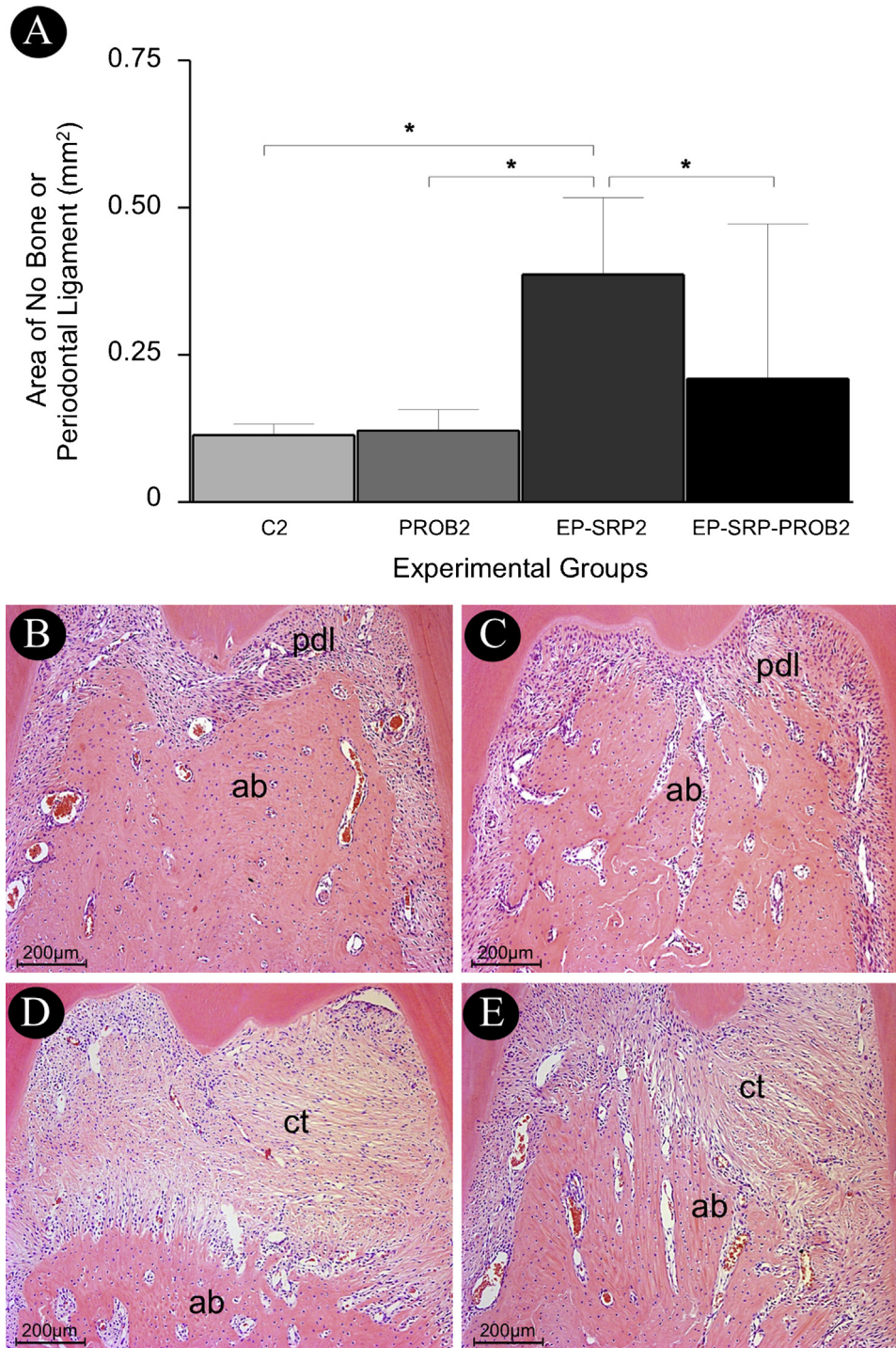

Fig. 4. Histomorphometric analysis of periodontal tissues—alveolar bone loss. Means and standard deviations of ANBL in the furcation area (A), with comparisons among groups. Photomicrographs of periodontal tissues in the furcation area (B–E) of mandibular first molars: Group C2 (B); Group PROB2 (C); Group EP-SRP2 (D) and Group EP-SRP-PROB2 (E). Abbreviations and symbols: ab = alveolar bone; ct = connective tissue; pdl = periodontal ligament; ANBL = area of no bone or periodontal ligament; * = $P < 0.05$. Scale bars: B-E = 200 μm. (Hematoxylin and Eosin stain).

blood vessels. Collagen fibers were inserted both in cementum and in alveolar bone. The cementum surface was totally sound and covered with cementoblasts. The bone tissue of the furcation region presented a few irregularities on its surface and it was coated with osteoblasts or bone lining cells (Fig. 4B and C). In both groups, junctional and sulcular epithelia were completely intact in the interproximal area (Fig. 5B and C).
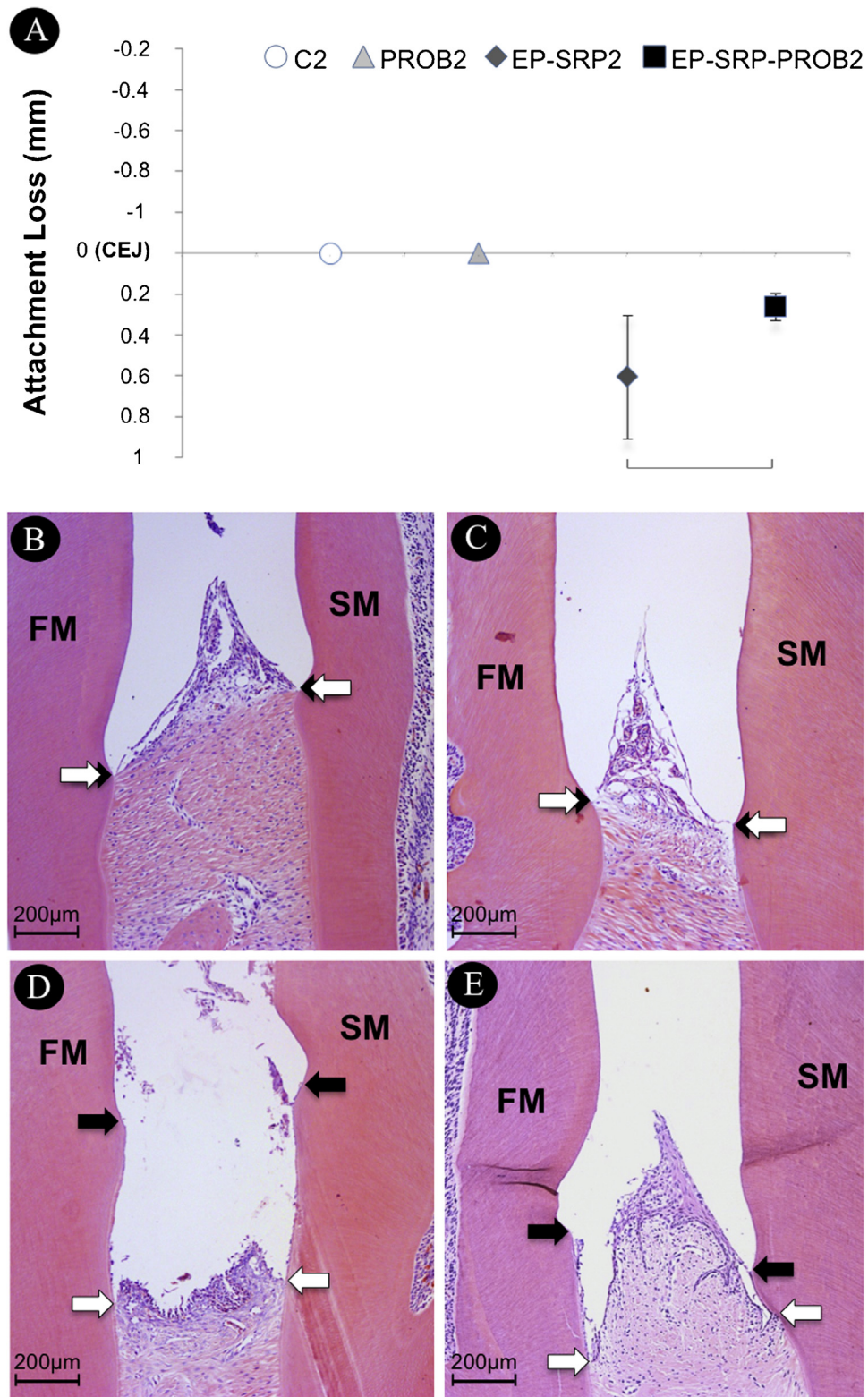

Fig. 5. Histomorphometric analysis of periodontal tissues—attachment loss. Means and standard deviations of AL in the interproximal area (A), with comparisons among groups. Photomicrographs of periodontal tissues in the interproximal area (B–E) of mandibular first molars: Group C2 (B); Group PROB2 (C); Group EP-SRP2 (D) and Group EP-SRP-PROB2 (E). Abbreviations and symbols: ab = alveolar bone; FM = first molar; SM = s molar; AL = attachment loss; black arrows = cementoenamel junction; white arrows = epithelial attachment; * = P < 0.05. Scale bars: B-E = 200 μm. (Hematoxylin and Eosin stain).

In Group EP-SRP2, the bone of the interradicular septum presented irregular outer contours and numerous active osteoclasts. The connective tissue presented a moderate inflammatory infiltrate consisting primarily of neutrophils (Fig. 4D). A severe damage of the junctional and sulcular epithelia was observed in the interproximal area (Fig. 5D). In Group EP-SRP-PROB2, the inflammatory infiltrate in the furcation region was less extensive than the one observed in Group EP-SRP2 and was mainly composed of neutrophils. The connective tissue adjacent to the interradicular septum contained a moderate number of fibroblasts and collagen fibers, a large number of blood vessels and few inflammatory cells. The alveolar bone crest was positioned more coronally when compared with the one of Group EP-SRP2 (Fig. 4E) and few active osteoclasts were observed. The junctional and sulcular epithelia of the interproximal area were less damaged than that of Group EP-SRP2 (Fig. 5E).

3.2.2. Histometric analysis

Group EP-SRP-PROB2 showed decreased ANBL (Fig. 4A) and AL (Fig. 5A; $P < 0.05$) when compared with Group EP-SRP2. No
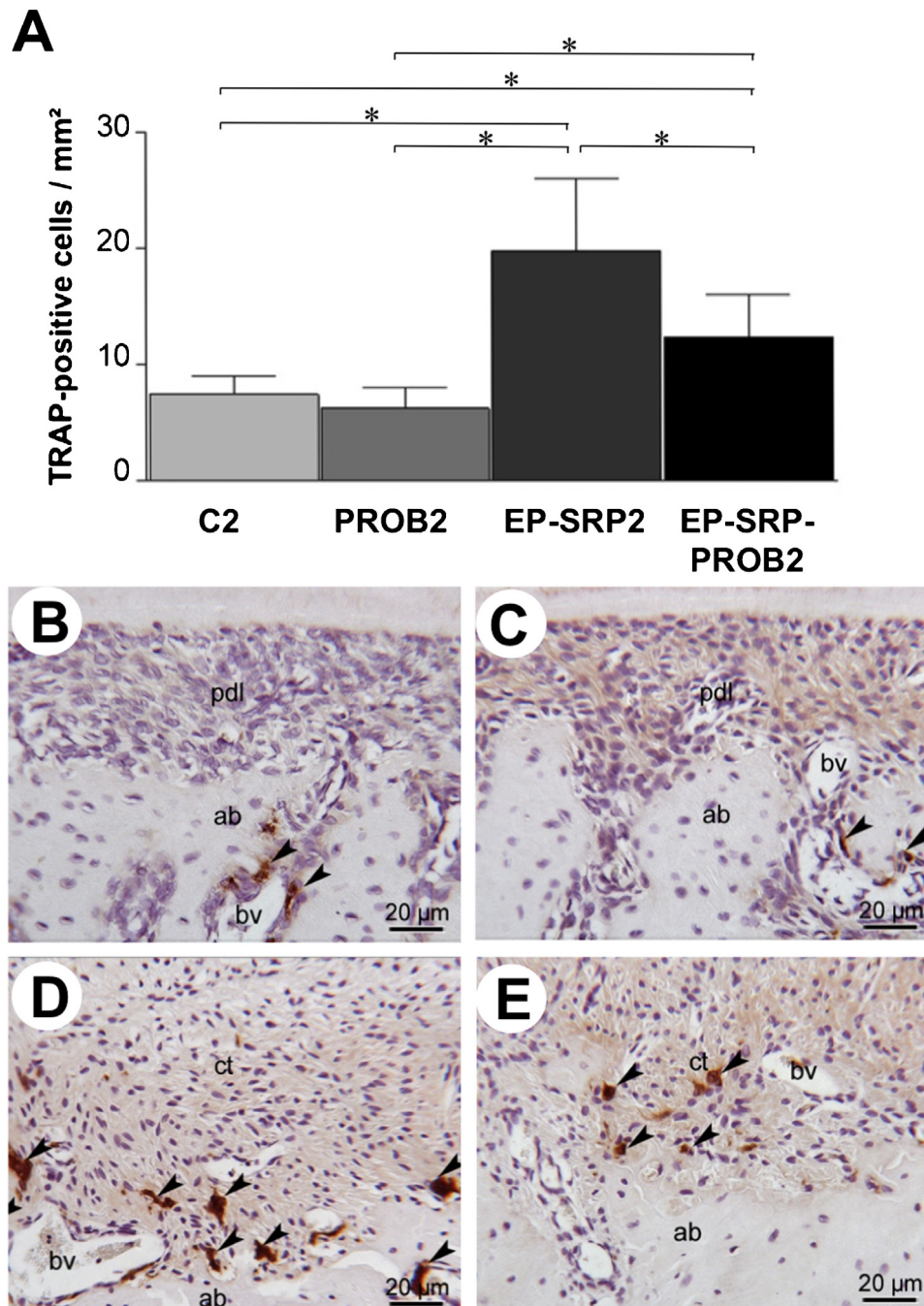

Fig. 6. Immunohistochemical analysis—TRAP. Means and standard deviations of the number of TRAP-positive multinucleated cells (A), with comparisons among groups. Photomicrographs showing immunolabeling for TRAP (B-E) in the furcation areas of mandibular first molars: Group C2 (B); Group PROB2 (C); Group EP-SRP2 (D); Group EP-SRP-PROB2 (E). Abbreviations and symbols: ab = alveolar bone; ct = connective tissue; pdl = periodontal ligament; bv = blood vessel; black arrowhead = TRAP-positive multinucleated cell; * = statistically significant difference (ANOVA, $P < 0.05$). Scale bars: B-E = 20 μm. (Hematoxylin counterstaining).

differences in ANBL were observed among Groups C2, PROB2 and EP-SRP-PROB2. Groups C2 and PROB2 did not present AL.

3.2.3. Immunohistochemical analyses

Group EP-SRP-PROB2 presented less TRAP-positive multinucleated cells with decreased immunolabeling for IL-1β and increased immunolabeling for IL-10 than Group EP-SRP2 ($P < 0.05$; Figs. 6 and 7).

4. Discussion

The use of *Bacillus* species as probiotic dietary supplements is expanding rapidly with an increasing number of studies demonstrating immune stimulation, antimicrobial activities and competitive exclusion (Cutting, 2011). In this study, two independent experiments, consisting of different analyses, were performed in order to investigate the effects of *Bacillus* species on the development and treatment of EP. In Experiment 1, probiotic therapy reduced alveolar bone loss and decreased the number of peripheral blood eosinophils. Furthermore, in Experiment 2, the therapy provided additional benefits to SRP in the treatment of periodontitis, reducing the number of osteoclasts, as well as the loss of attachment and alveolar bone.

In Experiment 1, EP promoted bone resorption in Groups EP1 and EP-PROB1. However, the damage was minimized by the treatment with probiotic. In Group EP-PROB1, the oral administration of *B. subtilis* and *B. licheniformis* led to an increase of 52.74% in BV and to decreases of 40.08% in BP and 66.97% in ABL when compared with Group EP1. These changes were observed using microtomographic analyses. These results may be explained, in part, by the effects of the probiotic therapy on the hematologic parameters and the RANKL/OPG ratio in periodontal tissues.

Group EP1 presented a significantly greater number of peripheral blood eosinophils than Group EP-PROB1. Eosinophils play a role as antigen-presenting cells that modulate immune responses by amplifying T-helper cell type 2 (Th2) responses (Padigel, Lee, Nolan, Schad, & Abraham, 2006; Shi, 2004; Spencer & Weller, 2010) therefore perpetuating the inflammatory process (Svensson & Wenneras, 2005). In this context, it can be suggested that the probiotic therapy inhibited Th2 response in Group EP-PROB1, decreasing the severity and progression of EP in these animals. This hypothesis is supported by some studies that have
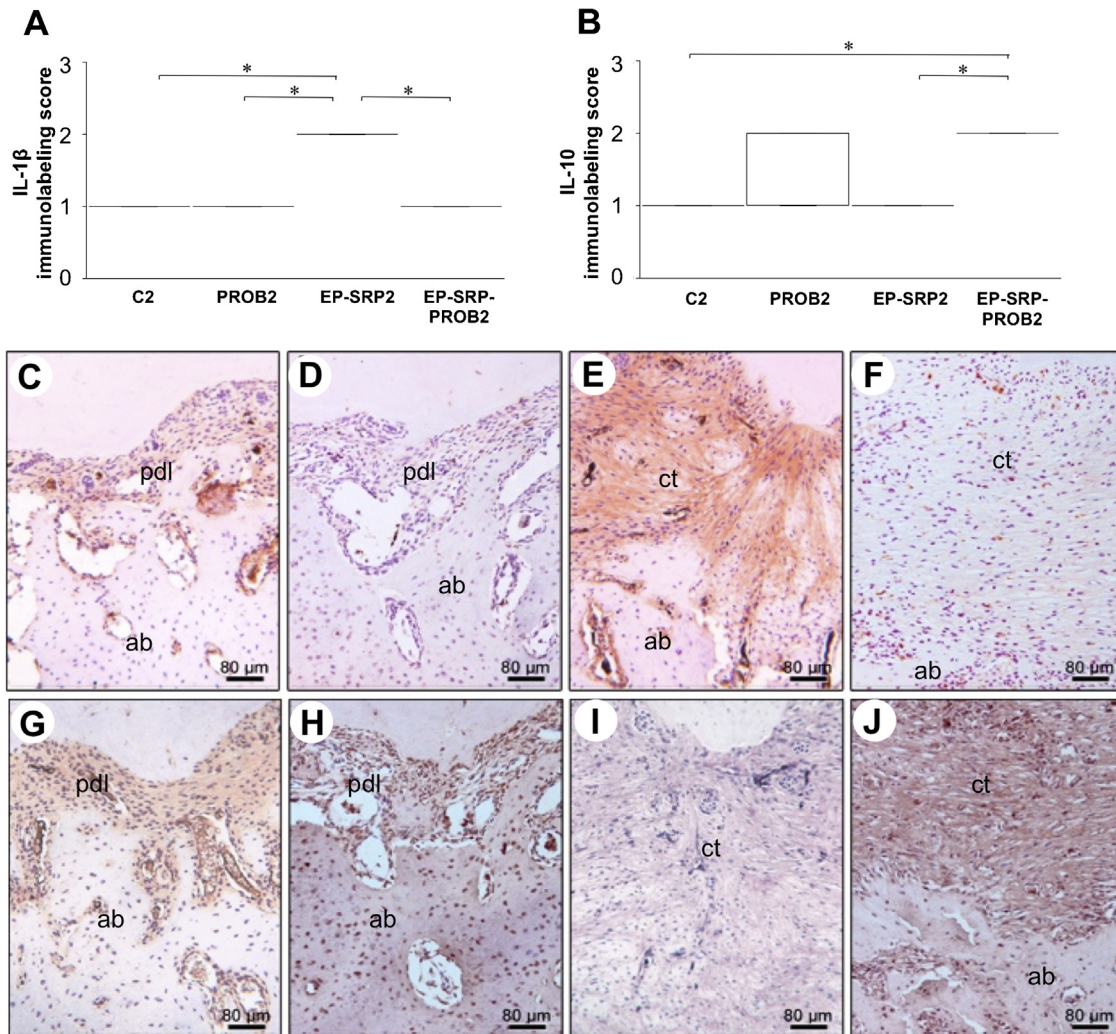

Fig. 7. Immunohistochemical analysis—IL-1β and IL-10. Medians, interquartile range and maximum and minimum values of the immunolabeling scores for IL-1β (A) and IL-10 (B), with comparisons among groups. Photomicrographs showing immunolabeling for IL-1β (C–F) and IL-10 (G–J) in the furcation areas of mandibular first molars: Group C2 (C,G); Group PROB2 (D,H); Group EP-SRP2 (E,I); Group EP-SRP-PROB2 (F,J). Abbreviations and symbols: ab = alveolar bone; ct = connective tissue; pdl = periodontal ligament; * = statistically significant difference (Kruskal-Wallis, $P < 0.05$). Scale bars: C-J = 80 μm. (Hematoxylin counterstaining).

already demonstrated that *B. subtilis*-derived poly-c-glutamic acid (cPGA) is capable of inhibiting Th2 Lee, Park, Park, and Hong (2013) and Th17 responses (Lee, Kim, Won, Lee, Sung, & Poo, 2014). cPGA-treated mice exhibited reductions in both frequencies and absolute numbers of splenic mast cells, basophils and eosinophils (Lee et al., 2013). In the present study, it seems that EP did not promote a significant increase in the number of eosinophils in Group EP1 when compared with Group C1. A possible explanation for that would be the release of small but consistent amounts of chemotactic factors into the circulation in patients with periodontitis and consequent eosinophil accumulation at tissue sites (e.g. lymph nodes), reducing the number of circulating eosinophils (Lopez, Loos, & Baelum, 2012). It is also important to emphasize that, although some studies have documented differences in the hematological parameters for subjects with chronic periodontitis compared to healthy subjects, the findings are not always conclusive (Lopez et al., 2012). The variation found in the blood parameters investigated in adult study populations frequently appears to be modest and the values frequently do not exceed the normal reference values (Loos, 2005).

Group EP1 was the only one to present a RANKL/OPG ratio, which demonstrates the effects of EP on alveolar bone resorption, significantly greater than that of Groups C1 and PROB1. RANKL is a key molecule in osteoclast activation and OPG is a decoy receptor for RANKL. Therefore, the increase in RANKL/OPG ratio favors bone resorption through osteoclastogenesis and activation of osteoclasts (Kajiya et al., 2010). In contrast to what was observed in Group EP1, no significant differences in RANKL/OPG ratio were observed when Group EP-PROB1 was compared with Groups C1 and PROB1. This finding may be explained in part by the possible effects of *Bacillus* species products on the reduction of interleukin (IL)-17 levels (Lee et al., 2014). IL-17A can play a role in the pathogenesis of periodontitis by modulating bone remodeling through induction of RANKL (Awang et al., 2014; Kotake, Yago, Kawamoto, & Nanke, 2012). Besides, it has been shown that serum levels of IL-17A correlate with clinical attachment loss in patients with aggressive periodontitis (Schenkein et al., 2010). It is also important to consider that *Bacillus* species increased the expression of OPG in periodontal tissues in a previous study by this group (Foureaux et al., 2014).

In Experiment 2, probiotic administration started immediately after SRP in an attempt to improve the impact of the adjunctive therapy. According to Teughels et al. (2011) probiotics will have difficulty colonizing the mouth and exerting beneficial clinical effects in an oral microbiological environment that has already matured. It is important to consider that the efficacy of SRP in this experimental model has been clearly demonstrated (Garcia et al., 2015; Theodoro et al., 2015). In these studies, the animals treated with SRP after EP induction had a significant reduction in alveolar bone loss compared to untreated animals (Garcia et al., 2015; Theodoro et al., 2015). Significant reductions in ANBL and AL (45.80% and 56.69%, respectively) were observed in Group EP-SRP-PROB2 when compared with Group EP-SRP2. These results demonstrate the potential of *Bacillus* species as adjuncts to SRP for the treatment of EP. It is possible that they can reduce the necessity for retreatment of periodontal sites in non-surgical approaches, as demonstrated by Teughels et al. (2013) when the effects of *Lactobacillus* bacteria as an adjunct to SRP were evaluated. According to Sbordone, Ramaglia, Gulletta, and Iacono, (1990) SRP presents short-term effects and the recolonization of treated pockets by potential periodontal pathogens can be observed 3 weeks post-SRP. In this context, a hypothesis to explain the results of the present study is that *Bacillus* species reduced the recolonization of treated periodontal pockets by periodontopathogenic microorganisms. Furthermore, the *Bacillus* species might have modulated the immunoinflammatory response of the animals, improving tissue repair after SRP procedures.

Regarding immunomodulatory properties, it has been demonstrated that *B. subtilis* can reduce pro-inflammatory cytokines (IL-1, tumor necrosis fator-alpha [TNF-α], IL-6 and interferon-gamma) and increase anti-inflammatory cytokines (IL-10 and transforming growth factor-ß) in the plasma of patients with inflammatory bowel disease (Selvam et al., 2009). To the authors' knowledge, there are no studies evaluating the immunoinflammatory properties of *Bacillus* species in periodontal tissues. In the present study, it was observed that *Bacillus* species influenced the balance of pro-inflammatory and anti-inflammatory cytokines. A lower expression of IL-1β and a greater expression of IL-10 were observed in Group EP-SRP-PROB2 when compared with Group EP-SRP2. IL-10 exerts inhibitory effects on IL-1β and TNF-α, whose synergistic actions on inflammatory processes amplify the host response (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). The elevated ratio IL-1β/IL-10 in gingival crevicular fluid may be related to progression of aggressive periodontitis (Teles et al., 2010). Considering the antimicrobial properties, compounds of *Bacillus* species have been used in agriculture for the inhibition of phytopathogens, for the stimulation of plant growth and in the food industry as probiotics (Mongkolthanaruk, 2012). There is only one study that reported the effects of *Bacillus* species on oral biofilm. Tsubura et al. (2009) verified that a mouth rinse prepared from the supernatant of a cultured medium of *B. subtilis* promoted a marked change in the scores of BANA test in patients with chronic periodontitis. In this study, neither the microbiologic profile of the oral biofilm of the animals, nor the colonization of their oral cavity by the probiotic bacteria administered were evaluated. Nevertheless, it was observed that *Bacillus* species present antimicrobial activity against some periodontal pathogens in *in vitro* assays performed in the laboratory of this group (data not shown). Regarding the colonization of the oral cavity, it is important to consider that many of the gastro-intestinal probiotics exert their effect without colonizing or with only a temporary colonization of the host (Teughels et al., 2011). Furthermore, probiotics may influence the biofilm not only quantitatively but also qualitatively. For example, Nissen, Sgorbati, Biavati, and Belibasakis (2014) demonstrated that lactobacilli can reduce the virulence of putative opportunistic oral pathogens. In this study, *L. salivarius* and *L. gasseri* down-regulated *Aggregatibacter actinomycetemcomitans* exotoxins expression (Nissen et al., 2014).

A decreased quantity of TRAP-positive cells was observed in Group EP-SRP-PROB2 when compared with Group EP-SRP2. It can be explained by the possible effects of *Baccilus* species and their subproducts on osteoclastic differentiation and activation paths, such as nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) pathways. Park, Kim, Kim, Ryu, and Lee (2010) demonstrated that surfactin produced by *B. subtilis* significantly reduces the production of TNF-α, IL-1ß, IL-6 and IL-12 through suppression of NF-kB activity in *Porphyromonas gingivalis* lipopolysaccharides-stimulated THP-1 human macrophage cells. In a previous study by this group (Foureaux et al., 2014), it was demonstrated that *Bacillus* species can decrease the expression of p38 MAPK in the periodontal tissues of stressed rats. According to Matsumoto, Sudo, Saito, Osada, & Tsujimoto (2000) the activation of p38 MAPK pathway plays an important role in RANKL-induced osteoclastic differentiation of precursor bone marrow cells.

Most probiotic strains with reported beneficial effects in either animal or clinical studies are lactobacilli or bifidobacteria. However, many other bacterial genera have demonstrated probiotic potential, including spore-forming *Bacillus* species (Foligne et al., 2012; Hong, Duc le, & Cutting, 2005; Sorokulova et al., 2008). The present study provided a proof-of-concept that

B. subtilis and *B. licheniformis* present therapeutic potential in the prevention and treatment of periodontitis. Whether this probiotic preparation can find application as an adjunctive therapy in human periodontitis is yet to be investigated in clinical trials.

5. Conclusions

It can be concluded that the supplementation with *Bacillus* species provided a protective effect against bone loss and enhanced the effects of SRP in the treatment of EP in rats.

Conflict of interest

The authors declare that they do not have a conflict of interest.

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Ethical approval

The present study was conducted only after review and approval by the Ethics Committees on Animal Experimentation (ECAE) at School of Dentistry of Ribeirao Preto, University of São Paulo–FORP/USP (protocol 2013.1.993.58.6), at Federal University of Lavras–UFLA (protocol 036/2010) and at Lavras University Center–UNILAVRAS (protocol 012/2010).

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