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ORIGINAL ARTICLE

The effects of *Lactobacillus reuteri* on the inflammation and periodontal tissue repair in rats: A pilot study

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Abstract *Objective:* The aim of this study was to evaluate the effects of probiotic (PRO) *Lactobacillus reuteri* (DSM17938) as an adjuvant to the treatment of experimental periodontitis (EP).

Material and methods: Fifty-four male adult Wistar rats were included. EP was induced and maintained for 7 days. Subsequently, the ligature was removed and the animals were allocated into three different experimental groups (n = 18/group): EP – no local treatment, the animals received four systemic saline solution (SS) administrations; SRP + SS, the animals underwent SRP treatment, followed by SS administration; and SRP + PRO, the animals received SRP treatment, followed by the systemic administration of PROs (*Lactobacillus reuteri* ; 0.16 ml/day). Six animals from each group were euthanised at 7, 15 and 30 days. Histological and histometric analyses of alveolar bone loss (BL) and immunohistochemical analyses for TRAP, RANKL, OPG, OCN, and PCNA were performed. Shapiro–Wilk, ANOVA, post-hoc Tukey, Kruskal–Wallis, Student–Newman Keuls were performed.

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Results: The SRP + PRO group presented a reduction in inflammation. At 15 days, a lower BL was observed in the SRP + SS and SRP + PRO groups. Greater immunolabeling was noticed for PCNA at 15 days in the SRP + PRO group than in the **SRP** + SS group. The SRP + PRO group demonstrated a higher OCN immunolabeling pattern than the EP group at 15 and 30 days.

Conclusion: The use of *Lactobacillus reuteri* as an adjuvant to SRP for the treatment of EP showed promising results in the control of local inflammatory responses, and enhanced the periodontal tissue repair process according to the employed concentration.

1. Introduction

Periodontitis is a chronic inflammatory disease associated with dysbiotic plaque and is considered one of the major causes of tooth loss, through the progressive destruction of periodontal support tissues (Papapanou et al., 2018). A selection of gram-negative bacteria is observed when the inflammatory process affects the periodontal tissues (Van Dyke et al., 2020). There has been an increasing interest in oral comensal bacteria, since they could impact the inflammatory process. Several studies have reported that the *Lactobacillus* species plays an important role in the maintenance of a balance in oral microflora and could be beneficial in the treatment of periodontal disease (Garcia et al., 2016; Messora et al., 2016; Alok et al., 2017; Butera et al., 2021). Non-surgical periodontal treatment consists of scaling and root planing (SRP), although there are many conditions that can hinder the access of instruments, such as deep pockets, poor dental position and an increase in antibiotic-resistant bacteria (Morales et al., 2018; Ikram et al., 2019). Therefore, adjuvant methods to support SRP treatment and control periodontal diseases are required.

Studies have shown the possibility of the clinical use of probiotics (PROs) of the genus *Lactobacillus* in controlling gingival inflammation (Staab et al., 2009; Iniesta et al., 2012) and in the treatment of periodontitis (Morales et al., 2018; Ikram et al., 2019; Szkaradkiewicz et al., 2014; Pelekos et al., 2019; Vohra et al., 2020). The mechanism of action of PROs in the oral cavity involves competitive exclusion; the ability to interfere with the innate and acquired response through immunomodulation; competitive inhibition of epithelial adhesion; and inhibition of periodontopathogen agents through the production of bacteriostatic substances (Szkaradkiewicz et al., 2014; Garcia et al., 2016; Invernici et al., 2018; Ikram et al., 2019, Vohra et al., 2020).

Animal studies have evaluated the local and systemic effects of PROs on experimental periodontitis (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017; Miessi et al., 2020). These studies have obtained favourable results in bone loss (BL) reduction, recolonization of areas with periodontitis induced by periodontopathogens, and a decrease in the quantity of anaerobic bacteria and pro-inflammatory cytokines (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017; Miessi et al., 2020). In addition, the use of PROs can improve tissue inflammation and SRP effectiveness. Among PROs belonging to the *Lactobacillus* species, *Lactobacillus reuteri* (*L. reuteri*) is important for its capacity to allow the secretion of bacteriocins, reuterine and reutericyclin (Miessi et al., 2020). Furthermore, the anti-inflammatory effects and inhibitory effects on cytokine secretion could be the reason for its beneficial effects on periodontal diseases (Alok et al., 2017).

Recent clinical trials have demonstrated the possibility of including *L. reuteri* as an adjuvant to non-surgical periodontal therapy (Iniesta et al., 2012; Szkaradkiewicz et al., 2014; Morales et al., 2018; Teughels et al., 2020). From the results of these studies, it can be concluded that this species inhibits plaque and that its anti-inflammatory and antimicrobial effects significantly decrease the amount of *Porphyromonas gingivalis* in the subgingival plaque (Iniesta et al., 2012; Szkaradkiewicz et al., 2014; Morales et al., 2018; Teughels et al., 2020). In addition, *L. reuteri* substantially reduces pro-inflammatory cytokines, thereby improving periodontal clinical parameters (Szkaradkiewicz et al., 2014; Invernici et al., 2018). However, there is no report in the literature on the effects of its systemic use as an adjuvant to periodontal treatment in BL control, local immunoinflammatory response and periodontal tissue repair process in systemically healthy animals. Thus, the purpose of the present study was to evaluate the effects of locally applied *L. reuteri* as an adjuvant to the non-surgical periodontal treatment of SRP in experimentally induced periodontitis in rats.

2. Material and methods

2.1. Animals

Fifty-four male adult Wistar rats (*Rattus norvegicus*, *aged* approximately 3 months) were included. The animals were kept in plastic cages in an environment with controlled temperature (21 ± 1 °C), humidity (65–75%) and light cycles (12/12 h), with water and food *ad libitum*. The experimental protocol (#00676-2017) was approved by the Ethics Committee on Animal Use (CEUA) and followed the ARRIVE Guidelines (Percie du Sert et al., 2020).

2.2. Experimental protocol

For the surgical procedures, the animals were subjected to an intramuscular injection of ketamine hydrochloride (70 mg/kg; Cetamim, Syntec, Santana do Parnaíba, São Paulo, Brazil) and xylazine hydrochloride (6 mg/kg; Xilazin, Syntec, Santana do Parnaíba, São Paulo, Brazil). EP was induced by installing a cotton thread (Chain Cotton No. 24, Coats Corrente, São Paulo, SP, Brazil) around the lower left first molar and was maintained for 7 days in the subgingival position (Longo et al., 2019; Fig. 1). Subsequently, the ligature was removed and the animals were allocated into three different experimental groups (n = 18/group): EP - no local treatment, animals

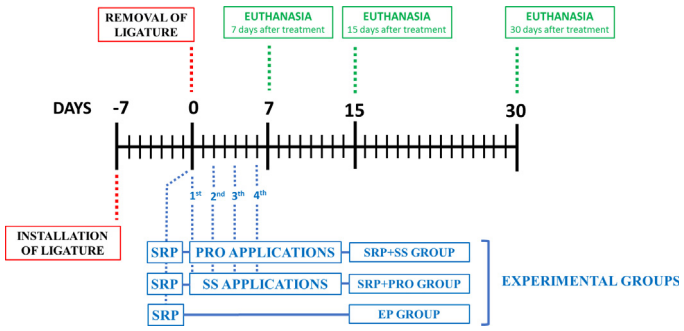

Fig. 1 Schematic illustration of study design. EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group.

received four systemic saline solution (SS) administrations; SRP + SS, animals received SRP treatment, followed by SS administration; and SRP + PRO, the animals were given SRP treatment, followed by four systemic administrations of PROs (Fig. 1).

2.2.1. SRP treatment

SRP was performed in a single session with mini-five 1–2 manual curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) by 10 distomesial traction movements on the buccal and lingual surfaces in groups SRP + SS and SRP + PRO. The interproximal and furcation areas were scraped with the same curettes by cervical-occlusal traction movements. The SRP procedures were performed by a single experienced trained operator, blinded (DMJM) to the experimental groups (Nuernberg et al., 2020).

2.2.2. SS or PRO treatment

EP and SRP + SS group animals received a systemic administration of SS (0.9% isotonic solution of sodium chloride), while SRP + PRO group animals received the PRO *L. reuteri* (*L. reuteri DSM17938*; Colikids, BioGaia, Aché Laboratórios Farmacêuticos S.A., Guarulhos, SP, Brazil) immediately after ligature removal and SRP, by gavage, every 2 days until the 6th day after SRP treatment (4 systemic applications). Each systemic PRO application consisted of 0.16 ml (1×10^8 CFU of *L. reuteri*), which was administered directly into the gastric tube of the animal with an irrigation cannula. The EP and SRP + SS group similarly received 0.16 ml of SS (Miessi et al., 2020).

2.3. Histological and histometric analysis

Six animals from each group were submitted to euthanasia by intramuscular injection of a lethal dose of thiopental (150 mg/kg; Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) and 2% lidocaine (10 mg/kg) at 7-, 15- and 30-days post-treatment. After fixation, the left hemimandibles were carefully handled and subjected to submitted to demineralisation in 10% ethylenediaminetetraacetic acid (EDTA) for 2 months (Ervolino et al., 2019). Subsequently, they were dehydrated by immersion in serial alcohol dilutions. Next, the samples were included in paraffin and sectioned in the sagittal plane, always following the long tooth axis in 4 μm thick slices (Nuernberg et al., 2020). Serial sections of the furcation region of the lower left first molar were collected and mounted on glass slides and stained with Hematoxylin and Eosin (H&E). A certified histologist (EE), blinded to the treatments, performed the histopathological analysis. The following parameters was evaluated using a score system: *intensity of the local inflammatory response*; *extension of the inflammatory process*; *external root resorption – cementum and dentin*; *alveolar bone resorption*; *structural pattern of connective tissue of the furcation region* and *structural pattern of alveolar bone tissue of the furcation region* (Zuza et al., 2018; Miessi et al., 2020). Histomorphometric analysis was performed to measure the BL area in mm^2 in the furcation region (Ervolino et al., 2019; Longo et al., 2019; Nuernberg et al., 2020). After exclusion of the first and last histological sections, in which the furcation region was evident, a trained and blinded examiner (EE) selected three equidistant sections of each specimen for histometric analysis. Another calibrated and blinded examiner performed the histometric analysis (DMJM) (Nuernberg et al., 2020).

2.3.1. Immunohistochemical analysis

Histological sections were deparaffinised in xylene and hydrated in a decreasing series of ethanol and were submitted to indirect immunoperoxidase (Ervolino et al., 2019). Antigen retrieval was performed by immersing the histological slides in 0.1 M citrate buffer (pH 7.4; Diva decloaker®, Biocare Medical, Concord, CA, USA) in a pressurised chamber (Decloaking chamber®, Biocare Medical, Concord, CA, USA) at 95° C for 20 min. The slides containing samples from each experimental group were categorised into five batches. Each batch was incubated with one of the following primary antibodies: (PCNA) (Vector Laboratories Inc., Burlingame, CA, USA), anti-OCN (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TRAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti - RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-OPG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The histological sections were counterstained with fast green for PCNA and Harris haematoxylin for TRAP, OCN, RANKL and OPG (Ervolino et al., 2019). A histologist (EE), blinded to the treatments, performed the immunohistochemical analyses. The cells immunolabeled for TRAP and PCNA were located at the centre of the interradicular septum (Longo et al., 2019). The coronary limit of this area was the alveolar bone crest, from which it extended apically by a distance of 1000 μm (Garcia et al., 2016). For RANKL, OCN and OPG immunolabeling, a semiquantitative

analysis of the immunoreaction was performed throughout the furcation area: score 0, absence of immunolabeling (total absence of immunoreactive cells – IR); score 1, low immunolabeling pattern (1/4 IR cells); score 2, moderate immunolabeling pattern (1/2 IR cells); and score 3, high immunolabeling pattern (3/4 IR cells) (Ervolino et al., 2019; Longo et al., 2019; Nuernberg et al., 2020).

2.4. Calibration of the examiner

An examiner was trained and calibrated (DMJM) for the histometric analysis. For this, two BL measurements and TRAP count of 30 samples were performed, with a 1-week interval between them. The measures were analysed to verify the degree of agreement at 5% significance level (Zuza et al., 2018). The Kappa test indicated a high level of agreement (94%) in the intra-examiner measurements of BL and TRAP.

2.5. Statistical analysis

All statistical analyses were performed with the program *BioEstat* (BioEstat; version 5.3; Instituto Mamirauá, Manaus, AM, Brazil). The sample was calculated considering alveolar BL in the furcation region as the primary outcome variable. The secondary outcome was described by the histological characteristics of the furcation area. Considering a minimum difference of 0.1 mm between the treatment means and a standard deviation of 0.01 mm of alveolar BL, the results showed that a sample size of 4 animals ($\alpha = 0.05$) would present a study power of 95%. Taking into consideration a loss of animals and studies with a similar methodology, a sample size of 6 animals per group was chosen (Miessi et al., 2020; Nuernberg et al., 2020). All data were previously submitted to the Shapiro–Wilk normality test ($p < 0.05$). The parametric data of intergroup BL were submitted to the ANOVA test. When ANOVA test detected a statistically significant difference, multiple comparisons were performed using Tukey's post-test ($p < 0.05$). The Kruskal–Wallis test was used for non-parametric data of intragroup BL and immunolabeling of the amount of TRAP-positive and PCNA-positive cells; immunolabeling pattern of OCN, OPG and RANKL; and histopathologic analysis. This test was followed by the Dunn or Student–Newman Keuls tests ($p < 0.05$).

3. Results

3.1. Histological analysis

The distribution of specimens according to each evaluated parameter is shown in Fig. 2. At 7 days, the EP group showed an intense inflammatory infiltrate, extending throughout the connective tissue and bone in the furcation region of the lower left first molar (Fig. 3). The bone tissue of the interradicular septum showed an irregular contour and was composed of thin bone trabeculae covered by active osteoclasts, with a presence of necrotic bone areas (Fig. 3). In the SRP + SS group during the same period the specimens had a moderate inflammatory infiltrate extending throughout the connective tissue of the furcation region (Fig. 3). This connective tissue consisted of few fibroblasts and collagen fibers and a moderate number of inflammatory cells. In the SRP + PRO group, the specimens presented a discrete inflammatory infiltrate that extended to parts of the connective tissue. The connective tissue in the furcation region consisted of few fibroblasts and collagen fibers and few inflammatory cells. The bone tissue of the interradicular septum showed irregular contour and was composed of bony trabeculae, covered by several osteoblasts and active osteoclasts (Fig. 3).

At 15 and 30 days, the histological characteristics of the EP group remained similar to those previously described at 7 days. However, there was an increase in the number of areas with active bone resorption (Fig. 3). A better structural pattern of the alveolar bone tissue was observed in the SRP + SS group at 15 and 30 days, and in group SRP + PRO in all periods, compared to the EP group ($p < 0.05$; Figs. 2 and 3). The histological characteristics of the SRP + SS group at 15 and 30 days remained similar to those previously described, with gradual reduction of the inflammatory infiltrate and a moderate amount of collagen fibers and fibroblasts in the connective tissue (Fig. 3). There was an improvement in the histological characteristics in the specimens of group SRP + PRO at the same intervals. The connective tissue was more organized, with few inflammatory cells, moderate amount of collagen fibers and fibroblasts. The bone tissue presented a less irregular contour and was composed of bone trabeculae covered with many osteoblasts (Fig. 3). The SRP + PRO group demonstrated a better structural pattern of the connective tissue at 15 and 30 days than the EP group in the same period ($p < 0.05$; Figs. 2 and 3). The SRP + PRO group also showed a reduction in inflammatory infiltrate and a decrease in its extension in all periods, compared to the EP group in the same period ($p < 0.05$; Figs. 2 and 3). A larger reduction in external root resorption was observed in group SRP + SS at 30 days and in group SRP + PRO at 15 and 30 days than in group EP over the same period ($p < 0.05$; Figs. 2 and 3).

3.2. Histometric analysis

Histometric analysis revealed no statistically significant difference in BL in the furcation area ($p > 0.05$) in the EP (0.80 ± 0.42 mm^2), SRP + SS (0.69 ± 0.39 mm^2) and SRP + PRO (0.67 ± 0.44 mm^2) groups at 7 days (Table 1). At 15 days, the EP group (0.96 ± 0.29 mm^2) demonstrated higher BL than the SRP + SS (0.58 ± 0.14 mm^2) and SRP + PRO (0.41 ± 0.12 mm^2) groups in the same period ($p < 0.01$; Table 1). In intragroup evaluation, BL was lower in the SRP + SS group at 30 days (0.35 ± 0.07 mm^2) than at 7 (0.69 ± 0.39 mm^2) and 15 days (0.58 ± 0.14 mm^2; $p < 0.05$; Table 1).

3.3. Immunohistochemical analysis

A similar immunolabeling pattern of TRAP-positive cells prevailed in all groups and periods with no statistically significant difference ($p > 0.05$; Fig. 4). PCNA immunolabeling revealed more positive cells at 15 days in the SRP + PRO group (64.25 ± 15.19) than in the SRP + SS group (28.75 ± 7.93; $p < 0.05$; Fig. 4). The immunolabeling pattern of OCN was lower in the EP group than the SRP + PRO group at 15 and 30 days ($p < 0.05$; Fig. 5). In intragroup analysis, the SRP + PRO group demonstrated lower immunolabeling pattern at 7 days than at 15 and 30 days ($p < 0.05$; Fig. 5). A similar immunolabeling pattern of RANKL and OPG prevailed in

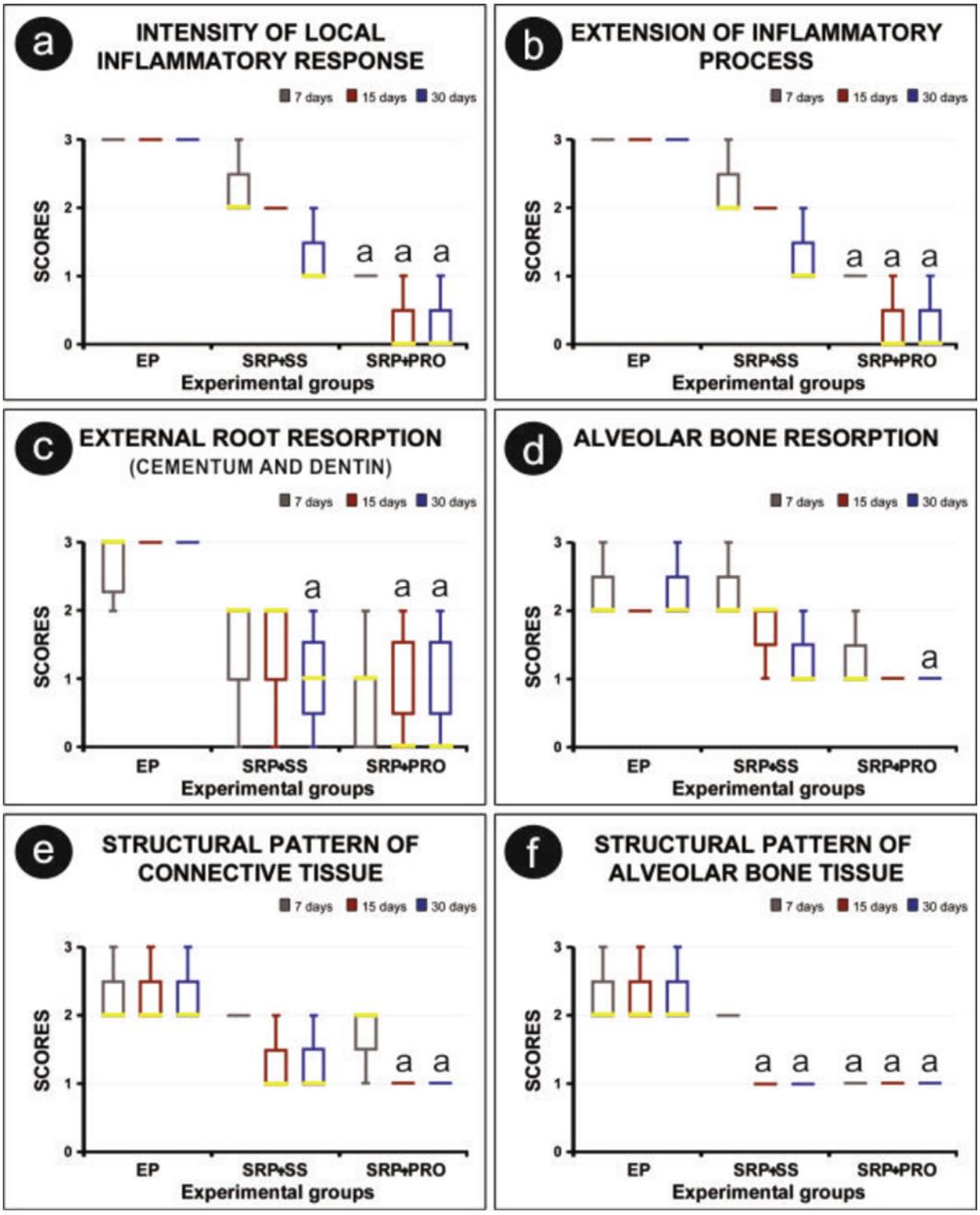

Fig. 2 Graphics indicate the median (horizontal line or yellow horizontal line), first and third quartiles of the Intensity of local inflammatory response (a), Extension of inflammatory process (b), External root resorption (cementum and dentin) (c), Alveolar bone resorption (d), Structural pattern of connective tissue (e) and Structural pattern of alveolar bone tissue scores (f) in the furcation region of the mandibular first molars according to groups and time points. Abbreviations and symbol: EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group; a, significant difference compared with EP group at the same period.

all groups and periods, with no statistically significant difference ($p > 0.05$; Fig. 5).

4. Discussion

Recently studies have demonstrated the effect of other "biotic" compounds that play a role in the categories represented by probiotics. According to the World Health Organization (WHO) probiotics are described as 'live microorganisms which, when administered in adequate amounts confer a health benefit on the host' (WHO, 2002; Varela-López et al., 2018; Vale and Mayer, 2021). Compounds such as paraprobiotics (inactivated probiotic microorganisms) (Butera et al., 2021) and postbiotics (concentrated bacterial active metabolites)

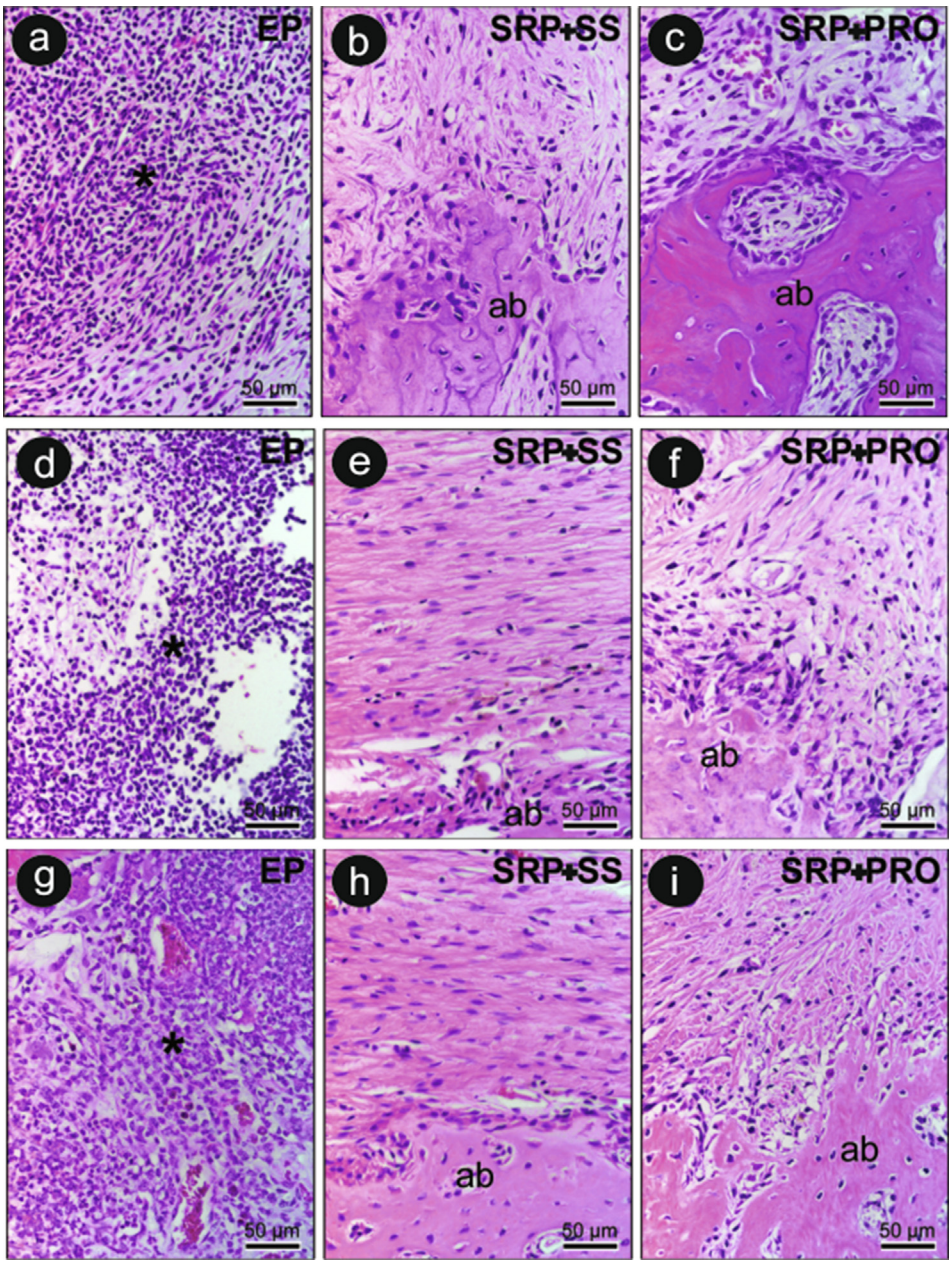

Fig. 3 Photomicrographs of the lower left first molar with experimental periodontitis, demonstrating the magnitude of the local inflammatory response, level of alveolar bone loss and periodontal repair process in EP (a, d, g), SRP + SS (b, e, h) and SRP + PRO (c, f, i) at 7 days (a, b, c), 15 days (d, e, f) and 30 days (g, h, i). Abbreviations and symbols: EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group; asterisks, inflammatory infiltrate; ab, alveolar bone. Original magnification: a-i: 400 ×. Scale bars: a-i: 50 µm. Staining method: hematoxylin and eosin (H&E).

(Vale and Mayer, 2021; Butera et al., 2022) have showed promising results in *in vitro* and clinical studies in the field of dentistry. However, future research is required in order to improve current knowledge about all these treatment possibilities.

For these reasons, the present study evaluated the effects of PROs (*L. reuteri)* as an adjuvant treatment in EP in male adult rats. This design was also applied in other studies conducted by our research group (Garcia et al., 2016; Longo et al., 2019; Miessi et al., 2020; Nuernberg et al., 2020; Zuza et al., 2018). In this study, EP was induced using a ligature around the animals' teeth, as is done in most experimental studies (Garcia et al., 2016; Longo et al., 2019; Miessi et al., 2020; Nuernberg et al., 2020; Zuza et al., 2018). According to some authors, this model allows for a greater accumulation of bacterial plaque and ulceration of the sulcular epithelium, thus facilitating the invasion of the connective tissue by microorganisms and the destruction of supporting tissues (Donos et al., 2018). The results of the current study demonstrated the benefits of using *L. reuteri* for the treatment of experimental periodontal disease in rats. However, the results obtained here should be carefully interpreted, considering the acute characteristic of the model and the differences in tissue metabolism between rodents and humans.

Table 1 Mean and standard deviation of BL (mm^2) in the furcation area data according to the groups, and periods.

Groups	7 days	15 days	30 days
EP	0.80 ± 0.42 mm^2	0.96 ± 0.29 mm^2	0.85 ± 0.86 mm^2
SRP + SS	0.69 ± 0.39 mm^2 #	0.58 ± 0.14 mm^2 *,#	0.35 ± 0.07 mm^2
SRP + PRO	0.67 ± 0.44 mm^2	0.41 ± 0.12 mm^2 *	0.37 ± 0.04 mm^2

EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group.
* Statistically significant difference compared to the group EP ($p < 0.01$), in the same period.
# Statistically significant difference compared to the period of 30 days in the same group ($p < 0.05$).

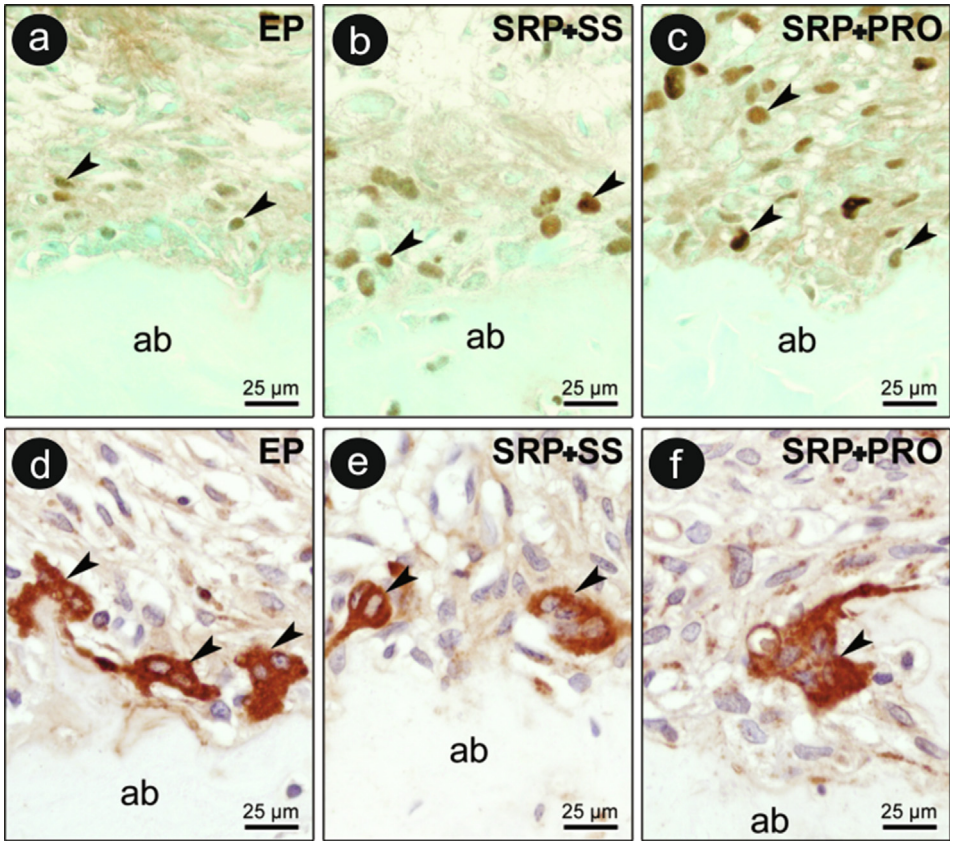

Fig. 4 Immunolabeling pattern for PCNA and TRAP. Photomicrographs evidencing immunolabeling pattern for PCNA (a-c), at 7 days, and TRAP (d-f), at 30 days, in periodontium of lower left first molar with experimental periodontitis in EP (a, d), SRP + SS (b, e) and SRP + PRO (c, f). Abbreviations and symbols: EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group; arrows, immunolabeled cells; ab, alveolar bone. Original magnification: 1000×. Scale bars: 25 µm. Counterstain: a-c, fast green; d-f, Harris hematoxylin.

The histological data of the EP group confirmed the effectiveness of a ligature around the teeth to induce periodontitis (Garcia et al., 2016; Longo et al., 2019; Miessi et al., 2020; Nuernberg et al., 2020; Zuza et al., 2018). In this study, group SRP + PRO demonstrated reduced inflammatory infiltrate and a lower extension on the tissues in all periods compared to the EP group in the same period. The SRP + SS group specimens demonstrated a moderate inflammatory infiltrate at 7 and 15 days, with irregular interradicular septum and several active osteoclasts. At 30 days these parameters improved, with a greater reduction of the inflammatory infiltrate area. These results corroborate those from previous studies (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017; Miessi et al., 2020) which demonstrated that SRP alone is insufficient in controlling the inflammatory process of EP and bone resorption in rats.

Histological events observed in the PRO-treated group specimens were significantly greater or better than in the other two groups. Improvements were also observed in the number of inflammatory cells, collagen and fibroblasts. We also observed that the inflammatory infiltrate was limited to the connective tissues at 7 days. In addition, an inflammatory process was absent, while there was a reduced number of inflammatory cells at 15 and 30 days in most specimens. These observations indicate that the use of PROs provides beneficial and promising results. In this study, PROs contributed to con-

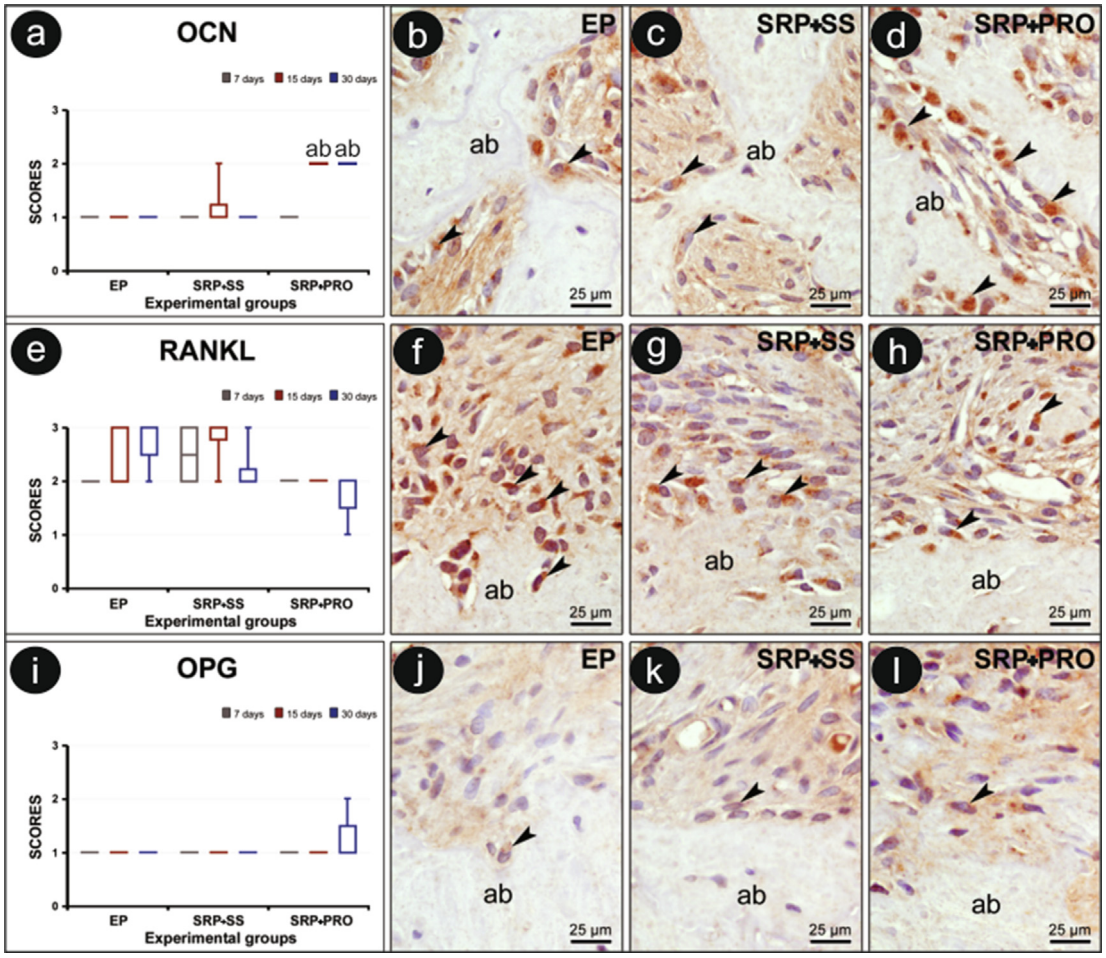

Fig. 5 Graphics indicate the median, first and third quartiles of the OCN (a), RANKL (e) and OPG scores (i) in the furcation region of the mandibular first molars, according to groups and time points. Photomicrographs evidencing immunolabeling pattern for OCN (b-d), at 30 days, RANKL (f-h), at 15 days and OPG (j-l), at 30 days, in the periodontium of the lower left first molar with experimental periodontitis in EP groups (b, f, j), SRP + SS (c, g, k) and SRP + PRO (d, h, i). Abbreviations and symbols: EP, Experimental Periodontitis group; SRP + SS, scaling and root planing group; SRP + PRO, scaling and root planing plus probiotic group; arrows, immunolabeled cells; ab, alveolar bone; a, significant difference compared with EP group at the same period; b, significant difference compared with day 7 in the same experimental group. Original magnification: 1000 ×. Scale bars: 25 µm. Counterstain: Harris hematoxylin.

trolling the inflammatory process and have not caused any adverse effects on the periodontal tissues, in line with the results of previous studies (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017; Miessi et al., 2020).

Concerning the immunohistochemical evaluation, it was observed that TRAP-positive cells exhibited a similar pattern in all groups and periods, with no statistically significant differences. A greater number of PCNA-positive cells at 15 days in the SRP + PRO group specimens was observed, which indicates a contribution of *L. reuteri* to increasing the activation of cell proliferation. A similar immunolabeling pattern of RANKL and OPG prevailed in all groups and periods, with no statistically significant differences noted. The SRP + PRO group demonstrated a higher OCN immunolabeling pattern than the EP group at 15 and 30 days. In intragroup analysis, the SRP + PRO group demonstrated a lower immunolabeling pattern of OCN at 7 days than at 15 and 30 days. These data support the assumption that PROs are beneficial in the bone repair process. These benefits in the tissue repair process can be justified by a greater control of the inflammatory process in PRO-treated animals. PROs have the ability to interfere with the innate and acquired response through immunomodulation, by controlling the activity of metalloproteinases responsible for the release of pro-inflammatory agents, in addition to the possibility of promoting competitive inhibition with periodontopathogenic agents (Szkaradkiewicz et al., 2014; Garcia et al., 2016; Alok et al., 2017; Invernici et al., 2018).

Histometric data analysis has also confirmed the benefits of SRP and *L. reuteri* in reducing BL at 15 days compared to EP, but without differences between the treated groups. A recent study using *L. reuteri* as an adjuvant treatment of EP in immunosuppressed rats demonstrated no benefits in BL control (Alok et al., 2017). However, other studies (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017; Miessi et al., 2020) have demonstrated a significant reduction in BL when compared to the conventional

treatment or the untreated group in rats using *Bacillus subtilis* (Oliveira et al., 2017), *Bifidobacterium Lactis* (Ricoldi et al., 2017), *Sacharomyces cerevisiae* (Garcia et al., 2016). The ambiguity in these results may be due to the high level of heterogeneity between the studies concerning doses, the duration of treatments, different species of PROs used, and different administration routes adopted. Another fact that must be considered is that the total amount (dose) of *L. reuteri* administered after 4 treatment sessions (400 million live *L. reuteri*) proved insufficient to generate an effect that would cause a significant reduction in alveolar BL, despite promoting a reduction in the inflammatory process and an accelerated repair, as observed in the histological analysis.

The dosage used and the administration time of PROs in other studies (Garcia et al., 2016; Messora et al., 2016; Oliveira et al., 2017; Ricoldi et al., 2017) were different from the present study. The PROs bacteria were administered in drinking water for 44 days in some studies (Messora et al., 2016), whereas it was administered in milk or water for 15 days in other study (Oliveira et al., 2017). Furthermore, some studies assessed the effect of the local use of PROs in areas with EP (Garcia et al., 2016; Miessi et al., 2020). In the present study, we proposed the use of *L. reuteri* for a shorter period of time (four applications within 7 days) at a lower dosage (1×10^8 CFU/day), deposited directly through the animals' gastric tube. It is believed that increasing the number of daily applications, treatment duration, or the use of dual-strain *L. reuteri* probiotic could be effective in the treatment of periodontal disease, and thus also promote a reduction in alveolar bone loss in animals. A recent review that evaluated five placebo controlled randomised clinical trials concluded that preparations containing *L. reuteri* showed a better performance trend than other *Lactobacillus* strains (*Lactobacillus ramnosus* SP1, *L. reuteri* or the combination of *Streptococcus oralis KJ3*, *Streptococcus uberis KJ2* and *Streptococcus rattus JH145*), in relation to the reduction of probing depth after non-surgical periodontal treatment (Donos et al., 2020).

A previous clinical study with *L. reuteri* demonstrated its effectiveness when administered once daily for plaque control in patients with moderate and severe gingivitis (Staab et al., 2009; Iniesta et al., 2012). Additionally, the prescription of the same PRO, twice daily, generated significant results in the reduction of periodontal clinical parameters such as bleeding on probing (BOP), probing depth and PI, with a reduction of periodontopathogens like *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Ikram et al., 2019). The use of PRO tablets, twice daily, had bacterial plaque inhibitory, anti-inflammatory and antimicrobial effects in the SRP + PRO group, as well as a reduction in the evaluated periodontal parameters of gingival index (GI) and BOP (Ikram et al., 2019; Pelekos et al., 2019). Another study investigated the clinical effects of chewing gum containing the same PRO bacteria twice a day on inflammation control and levels of inflammatory mediators in the gingival crevicular fluid of patients with gingivitis (Donos et al., 2020). The authors observed a reduction in the clinical parameters of GI and gingival bleeding (Donos et al., 2020). Such clinical findings strengthen the antiplaque and antimicrobial properties in inhibiting the colonization of pathogens in the host tissue by the competitive mechanism and anti-inflammatory effect. Moreover, *L. reuteri* has a strong ability to adhere to host tissues and, therefore, compete with pathogenic bacteria. It also has a capacity to allow for the secretion of bacteriocins, reuterine and reutericyclin (Alok et al., 2017). Furthermore, the beneficial effects of *L. reuteri* on periodontal diseases can be attributed to its anti-inflammatory effects and inhibitory effects on cytokine secretion (Szkaradkiewicz et al., 2014; Garcia et al., 2016; Invernici et al., 2018). In addition, other recent randomized clinical trials in humans have concluded that the adjuvant use of *L. reuteri* in the treatment of periodontitis in smokers was effective in controlling gingival inflammation and reducing deep pockets (Theodoro et al., 2019).

Systemic absorption of the probiotic may contribute to the protection of the gastrointestinal microbiota and increase the patient's immune-inflammatory response. It is also noteworthy this therapy that can be applied at any time, especially in patients with periodontal disease who are, for some reason, unable to perform a good control of dental biofilm, which facilitates the installation of inflammatory periodontal diseases. Also, it is a therapy of great importance for patients unable to go to the dental clinic for periodontal treatment, such as the sick and bedridden, including those admitted to the hospital and intensive care units. A selection of gram-negative bacteria is observed when the inflammatory process affects the periodontal tissues. This selection is justified by soft tissue swelling, increased pocket depth, and the accumulation of tissue breakdown products, plasma proteins and haemoglobin, which are used as an energy source (Van Dyke et al., 2020). This study has demonstrated promising histological results in inflammation reduction and osteoblastic activity enhancement, reinforcing the anti-inflammatory effects of these beneficial bacteria as an adjuvant in the treatment of periodontitis.

The definition of the most effective local PRO *L. reuteri* concentration, 0.16 ml (1×10^8 CFU of *Lactobacillus reuteri*) will serve as a starting point for future investigations in animals and humans. The absence of microbiological analyses of the treated areas in the animals, due to the great difficulty in collecting sufficient bacterial samples from the periodontal pockets of rats can be pointed out as a limitation of this study. Additional *in vivo* analysis of the antimicrobial action of PRO *L. reuteri* will generate important evidence and will help to explain the benefits in reducing BL and favoring the reduction of inflammatory infiltrate in the furcation area, thus promoting an acceleration of tissue and bone repair not observed in this present study.

5. Conclusion

The use of *Lactobacillus reuteri,* in the concentration employed as an adjuvant to SRP for the treatment of EP showed promising results in the control of local inflammatory responses, and enhanced the periodontal tissue repair process.

Ethical Statement

The experimental protocol (#00676-2017) was approved by the Ethics Committee on Animal Use (CEUA) and followed the ARRIVE Guidelines.

CRediT authorship contribution statement

Valdir Gouveia Garcia: Conceptualization, Methodology, Investigation, Funding acquisition. **Daniela Maria Janjácomo Miessi:** Methodology, Formal analysis, Data curation. **Tiago**

Esgalha da Rocha: Validation, Formal analysis, Investigation, Writing – original draft. **Natália Amanda Gomes:** Formal analysis, Visualization, Data curation, Writing – original draft. **Marta Aparecida Alberton Nuernberg:** Methodology. **Jânderson de Medeiros Cardoso:** Methodology. **Edilson Ervolino:** Methodology, Formal analysis, Resources. **Letícia Helena Theodoro:** Resources, Visualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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