



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

LEOPOLDO COSME SILVA

TESE DE DOUTORADO

**Efeito de probióticos no desenvolvimento de lesão periapical
induzida em ratos**

**Araçatuba, SP
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Tese de Doutorado apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Doutor em Ciência Odontológica - Área de Concentração: Endodontia.

Orientador: Prof. Dr. Titular João Eduardo Gomes Filho

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“Você não sabe o quanto eu camínhei
Pra chegar até aqui
Percorri milhas e milhas antes de dormir
Eu não cochilei
Os mais belos montes escalei”

Resumo Geral

Cosme-Silva L. **Efeito de probióticos no desenvolvimento de lesão periapical induzida em ratos**. 2019. 97 f. [Tese]. Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2019.

Resumo Geral

Objetivo: O objetivo do presente estudo foi avaliar o efeito da administração sistêmica de probióticos nos parâmetros hematológicos, microbiológicos do canal radicular e da saliva e no desenvolvimento da periodontite apical (PA) induzida em ratos.

Material e Métodos: Foram utilizados 24 ratos Wistar machos. PA foi induzida nos primeiros molares inferiores e superiores (lado esquerdo e direito). Os animais foram divididos em 3 grupos: controle, *Lactobacillus rhamnosus* e *Lactobacillus acidophilus*. Os probióticos foram administrados por gavagem (10^9 unidades formadoras de colônias (CFU)) diluídas em 5 mL de água durante o período de desenvolvimento da PA (30 dias). No trigésimo dia, foi realizada punção cardíaca para análise de hemograma, cálcio, fósforo e concentração de fosfatase alcalina no sangue. Além disso, foi realizada análise microbiológica do conteúdo do canal radicular e da saliva. Em seguida, os animais foram eutanasiados e a mandíbula e maxila foram removidas para análise da PA através da microtomografia computadorizada; análises histopatológicas (hematoxilina - H&E) e de imunomarcção para interleucina 10 (IL-10), interleucina 1 beta (IL-1 β), interleucina 6 (IL-6), ligante do recetor ativador do fator nuclear kappa B (RANKL), osteoprotegerina (OPG) e fosfase ácida resistente ao tataráto (TRAP). Os dados foram estatisticamente analisados com nível de significância de 5%.

Resultados: A fosfatase alcalina foi maior nos grupos que consumiram probióticos ($p>0.05$). A contagem total de microrganismos no canal radicular/saliva, o infiltrado inflamatório e a imunomarcção para IL-1 β e IL-6 no PA foram menores nos grupos probióticos quando comparados ao controle ($p<0.05$). Observou-se que a IL-10 foi mais imunomarcada nos grupos probióticos do que no grupo controle ($p<0.05$). Menor volume de reabsorção óssea foi observada nos grupos que consumiram probióticos ($p<0.05$). A imunomarcção para RANKL e TRAP foram menores nos grupos probióticos quando comparados ao controle ($p<0.05$). Observou-se que a OPG foi mais imunomarcada no grupo *Lactobacillus acidophilus* do que no grupo *Lactobacillus rhamnosus* e controle ($p<0.05$). Não houve diferença estatística na contagem de lactobacilos no canal radicular/saliva bem com no perfil hematológico, cálcio e fósforo entre os grupos ($p>0.05$).

Conclusão: A suplementação com probióticos (*Lactobacillus rhamnosus* e *Lactobacillus*

acidophilus) teve um efeito significativo na redução da severidade da PA em ratos, assim como na redução dos microorganismos totais do canal radicular/saliva concomitantemente com o aumento da fosfatase alcalina sanguínea, demonstrando a capacidade dos probióticos na modulação do desenvolvimento da PA.

Palavras chaves: periodontite periapical, endodontia, probiótico.

General abstract

Cosme-Silva L. **Effect of probiotics on the development of induced periapical lesion in rats**. 2019. 97 f. [Thesis, PhD]. School of Dentistry, São Paulo State University, Araçatuba, Brazil. 2019.

Aim: The objective of the present study was to evaluate the effect of systemic administration of probiotics on haematological, microbiological parameters of the root canal and saliva and on the development of apical periodontitis (AP) induced in rats

Materials and Methods: Twenty-four male Wistar rats were used. PA was induced in the lower and upper first molars (left and right side). The animals were divided into three groups: control, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*. Probiotics were administered by gavage (10⁹ colony forming units (CFU)) diluted in 5 mL of water during the developmental period of PA (30 days). On the thirtieth day, cardiac puncture was performed for hemogram, calcium, phosphorus and alkaline phosphatase concentration in the blood. In addition, a microbiological analysis of the root canal and saliva contents was performed. Afterwards, the animals were euthanized and the mandible and maxilla were removed for analysis of PA by computerized microtomography; interleukin 10 (IL-10), interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG) and tartrate-resistant acid phosphatase (TRAP). The data were statistically analyzed with significance level of 5%.

Results: Alkaline phosphatase was higher in the groups that consumed probiotics ($p > 0.05$). The total count of microorganisms in the root canal/saliva, inflammatory infiltrate and immunolabeling for IL-1 β and IL-6 in BP were lower in the probiotic groups when compared to the control group ($p < 0.05$). It was observed that IL-10 was more immunolabelled in the probiotic groups than in the control group ($p < 0.05$). Lower volume of bone resorption was observed in the groups that consumed probiotics ($p < 0.05$). Immunoblotting for RANKL and TRAP were lower in probiotic groups when compared to control ($p < 0.05$). It was observed that OPG was more immunolabelled in the *Lactobacillus acidophilus* group than in the *Lactobacillus rhamnosus* group and control ($p < 0.05$). There was no statistically significant difference in the lactobacillus count in the root canal / saliva as well as in the hematological profile, calcium and phosphorus between the groups ($p > 0.05$).

Conclusion: Supplementation with probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) had a significant effect on the reduction of PA severity in rats, as well as the reduction of total root canal/saliva microorganisms concomitantly with increased alkaline

phosphatase blood, demonstrating the capacity of probiotics in modulating the development of PA.

Keywords apical periodontitis, endodontics, probiotic.

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Introdução Geral

Introdução Geral

Probióticos são microrganismos vivos que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro podendo interferir no processo saúde-doença (Guarner et al., 2005; Bosch et al., 2012). Os principais microrganismos utilizados como probióticos são bactérias do gênero *Lactobacillus*, *Enterococcus*, *Bacillus* e *Bifidobacterium* (Vivekananda et al., 2010; Bron et al., 2012; Teughels et al., 2013). Os *Lactobacillus* e *Bifidobacterium* são microrganismos encontrados em alguns alimentos, como iogurtes, e são considerados probióticos devido à sua capacidade para modular a composição e atividade metabólica da flora intestinal e melhorar o sistema imunológico em humanos (Spanhaak et al, 1998; Nagao et al, 2000, Messoria et al., 2016). Após o consumo, os probióticos se mantêm estáveis após a passagem pelo trato gastrointestinal devido às suas propriedades acidogênicas (Yuki et al, 1999).

Aparentemente, o mecanismo de ação dos probióticos é baseado na modificação do ambiente bacteriano patogênico através da competição entre patógeno X probiótico, modulando positivamente a resposta imune do hospedeiro (Teughels et al., 2011; Lodi et al., 2015). Os principais efeitos dessa competição são atribuídos à capacidade de aumentar a atividade dos macrófagos, defesa imune, elevar o número de “NK cells” e interferons (Spanhaak et al, 1998; Nagao et al, 2000; Teughels et al., 2011). Foi demonstrado que em modelos animais com sensibilização alérgica os probióticos administrados oralmente diminuíram a produção alergênico-específico de IgE, em parte pela modulação da produção de citocinas sistêmicas (Borchers et al., 2009).

Existem evidências de que a colonização do intestino por probióticos pode causar efeitos benéficos sistêmicos promovendo proteção contra doenças em sítios distantes (Kobayashi et al., 2017). Apesar de ainda não estarem completamente esclarecidos os mecanismos, pensa-se que o efeito imunoestimulador pode estar relacionado com a capacidade dos probióticos interagirem com as placas de Peyer (aglomerados de folículos linfóides situados na parede do intestino delgado), presentes no intestino (Coppola & Turnes, 2004; Bermudez et al., 2012). Desta interação resulta a estimulação dos linfócitos B, a produção de imunoglobulina A (IgA), e a migração de linfócitos T do intestino (Coppola & Turnes, 2004; Bermudez et al., 2012). Sugere-se que quando o probiótico é administrado por via oral seus efeitos benéficos estão relacionados à prevenção da adesão de microrganismos patogênicos nos tecidos do hospedeiro ao passar pela cavidade bucal na ingestão, estímulo e

modulação do sistema imune, regulação da produção de citocinas, como por exemplo a IL-10 e IL-1 β (Coppola & Turnes, 2004; Koduganti et al., 2011; Bermudez et al., 2012). Além disso, o uso de probióticos melhora a integridade da barreira intestinal, aumento da produção de mucinas e eliminação ou inibição do crescimento de patógenos pela produção de bacteriocinas ou outros produtos, como ácidos e peróxidos (Coppola & Turnes, 2004; Koduganti et al., 2011; Bermudez et al., 2012).

Em estudo conduzido por Li et al. (2016), foi demonstrado que o uso de probióticos do tipo *Lactobacillus rhamnosus* em ratos com deficiência de esteróides sexuais pode minimizar o processo inflamatório intestinal e também protege contra a perda óssea. Os autores propõem que a administração do *Lactobacillus rhamnosus* pode ser uma estratégia terapêutica para a osteoporose pós-menopausa. Amdekar et al., (2016) também demonstraram que este mesmo probiótico influencia nas vias inflamatórias através da regulação de citocinas pró-inflamatórias. Montazeri-Najafabady et al. (2018) demonstraram que o *Lactobacillus acidophilus* inibiu a perda óssea induzida pela ovariectomia de ratas. Amdekar et al. (2013) e Lee et al. (2018) avaliaram o efeito do *Lactobacillus acidophilus* na osteoartrite induzida em ratos e descobriram que o lactobacilos apresentou propriedades anti-nociceptivas e protegeu contra a destruição da cartilagem.

Uma série de benefícios decorrentes da utilização de probióticos tem sido demonstrada incluindo o aumento a resistência a doenças infecciosas, alívio da intolerância à lactose, prevenção de doenças intestinais como diarreias, prevenção de infecções vaginais e urogenitais, redução de quadros alérgicos e infecções respiratórias, diminuição da concentração de colesterol sérico e aumento da resistência à quimioterapia (Perdigon et al., 1995; Vanderhoof et al., 1999; Arunachalam et al., 2000; Hatakka et al., 2001; Von Bültzingslöwen et al., 2003; Stamatova & Meurman 2005; Vuotto, et al., 2014). Além disso, os efeitos positivos do uso de probióticos também estão relacionados com infecções da cavidade oral (Meurman et al., 2005).

Independentemente da ação que se espera com o uso dos probióticos, a maioria deles coloniza o intestino apenas temporariamente (Collado et al., 2009). Assim, para obter benefícios na saúde, o consumo deve ser diário (Hasslöf et al., 2013). Contudo, os probióticos têm sido historicamente pouco investigados a partir da perspectiva da saúde bucal quando comparado com a saúde geral. Esta tendência foi alterada na última década e estudos foram realizados a fim de avaliar o efeito dos probióticos para a saúde oral (Bosch et al., 2012;

Hasslöf et al., 2013; Lodi et al., 2015; Gruner et al., 2016; Elavarasu et al., 2016; Messora et al., 2016).

Os probióticos podem ser benéficos para prevenir ou tratar doenças orais como cárie, gengivite ou periodontite, que estão associadas a uma alteração na composição e atividade do biofilme bacteriano (Gruner et al., 2016). Existem vários mecanismos pelos quais os probióticos podem influenciar a saúde oral, por exemplo modulação imunológica, impacto sobre o microbioma oral, produção de substâncias antimicrobianas pelo probiótico e exclusão competitiva de bactérias patogênicas dificultando a adesão de patógenos orais (Teughels et al., 2011). Os efeitos dos probióticos nos patógenos causadores da cárie ou doenças periodontais têm sido estudados *in vitro* com bons resultados inibitórios (Chuang et al 2011; Lee et al., 2011; Schwendicke et al., 2014).

Do ponto de vista cariogênico, os estudos sobre os probióticos se concentraram principalmente sobre o seu potencial efeito antagonista nos *Streptococcus mutans* (Cagetti et al., 2013). Os estudos indicam que a suplementação com probióticos levam a uma diminuição nas contagens de *Streptococcus mutans* salivares (Caglar et al., 2008; Toiviainen et al., 2015).

Na periodontia, probióticos tem efeito positivo no controle de placa, alterando a colonização bacteriana e na melhoria dos parâmetros clínicos tais como redução do sangramento gengival, redução da profundidade de bolsas periodontais e diminuição da perda de inserção clínica (Elavarasu et al., 2016, Messora et al., 2016). Isso ocorre, pois os probióticos inibem a adesão e o crescimento de patógenos pela produção de várias substâncias que, em última análise, inibem a formação de biofilme. Bactérias do tipo *Bacillus* combinada com raspagem e alisamento radicular podem melhorar os parâmetros periodontais e atrasar a recolonização de bolsas periodontais (Messora et al., 2016). Os efeitos dos probióticos sobre patógenos periodontais receberam pouco interesse até agora, embora, a administração de alguns probióticos tem sido associada com características saudáveis de gengiva e boas condições periodontais (Teughels et al., 2011; Messora et al., 2016).

Na endodontia, está bem estabelecido que as bactérias fazem parte da etiologia das lesões pulpares e periradiculares e o objetivo da terapia endodôntica é alcançar a redução significativa na carga bacteriana intracanal (Subramanian et al., 2009, Signoretti et al., 2013). A patogênese das lesões periapicais envolve uma série complexa de resposta imune inflamatória à infecção bacteriana do sistema de canais radiculares podendo levar a destruição de tecidos periapicais (Subramanian et al., 2009; Graves et al., 2001). Quando a lesão periapical está instalada, citocinas inflamatórias possuem um papel importante na resposta

imune, iniciando e coordenando eventos celulares e regulando a resposta do hospedeiro às endotoxinas. Além disso, a reabsorção óssea que ocorre nestas patologias aparece como um fator determinante à expansão destas lesões, sendo iniciada pela proliferação de células precursoras de osteoclastos imaturos e diferenciação das mesmas em células osteoclásticas maduras que promovem a degradação dos componentes ósseos orgânicos e inorgânicos. RANKL é uma molécula chave na ativação de osteoclastos e OPG é um receptor para RANKL. O aumento na taxa de RANKL/OPG favorece a reabsorção óssea através de osteoclastogênese e ativação de osteoclastos (Kajiya et al., 2010, Gomes Filho et al., 2015, Martins et al., 2016; Dal-Fabbro et al., 2018).

Até o momento, poucos estudos relacionam probióticos com a endodontia (Bohora et al., 2017; El-Sayed et al., 2019). Bohora et al., (2017) avaliaram a eficácia antibacteriana de probióticos contra patógenos endodônticos como *Enterococcus faecalis* e *Candida albicans* e demonstraram que probióticos das espécies *Lactobacillus* e *Bifidobacterium* foram eficazes na prevenção do crescimento de *E. faecalis* e *C. albicans* (*in vitro*). El-Sayed et al., (2019) avaliaram o efeito inibitório de *Lactobacillus rhamnosus* como um probiótico irrigante no crescimento de *Enterococcus faecalis* e chegaram a conclusão que o lactobacilos poderia ser usado como um novo agente irrigador seguro. Contudo, a possível ação sistêmica dos probióticos no desenvolvimento da PA ainda não foi investigada. A hipótese nula testada neste estudo foi de que não haveria diferença na gravidade da PA em ratos que receberam ou não a administração sistêmica de probióticos.

***Referências da Introdução Geral em Anexo A**

Objetivos

Objetivo geral:

O objetivo geral desse estudo foi avaliar a ação sistêmica dos probióticos (*Lactobacillus Rhamnosus* e *Lactobacillus Acidophilus*) no desenvolvimento da periodontite apical induzida em ratos.

Objetivos específicos:

Os objetivos específicos deste estudo foram avaliar e comparar:

- os parâmetros hematológicos (série branca e vermelha);
- os marcadores do metabolismo ósseo: cálcio, fósforo e fosfatase alcalina no sangue;
- a microbiota dos canais radiculares e da saliva nos diferentes grupos;
- o perfil inflamatório local na periodontite apical nos diferentes grupos;
- o volume de destruição óssea na periodontite apical através de microtomografia computadorizada;
- a expressão das citocinas e IL-1 β , IL-6 e IL-10 como indicadores de inflamação local;
- a expressão de TRAP, OPG e RANKL como indicadores de reabsorção óssea.

Artigo 1

Systemic administration of probiotics reduces the severity of apical periodontitis and counting of total microorganisms in saliva and root canal

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Running title: Probiotics and apical periodontitis

Keywords: apical periodontitis; endodontics; probiotics.

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Abstract

Aim: The aim of the present investigation was to evaluate the effect of systemic administration of probiotics on the reduction of apical periodontitis (AP) severity.

Methodology: Twenty-four male *Wistar* rats were used. AP was induced in the upper left/right first molars. The animals were arranged into groups: Control, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus*. Probiotics were orally administered for gavage (10^9 colony-forming units (CFU) diluted in 5 mL of water for 30 days) during the development of AP. After 30 days, cardiac puncture was performed to analyze the complete blood count. Moreover, microbiological analysis of the root canal content and the saliva was performed. Then the animals were killed and the jaw removed followed by histological processing for histopathological (inflammatory infiltrate and area of reabsorption through haematoxylin and eosin - H&E) and IL-10, IL-1 β , and IL-6 immunolabeling analyses. The data were statistically analyzed and the level of significance was 5%.

Results: No statistical difference was observed in the blood profile among the groups ($p>0.05$). Total microorganism count in the root canal/saliva, the inflammatory infiltrate, and the immunostaining for IL-1 β and IL-6 in AP were lower in probiotic groups when compared to the control ($p<0.05$). It was observed that IL-10 was more immunolabeled in the probiotic groups than in the Control group ($p<0.05$).

Conclusion: Supplementation with probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) had a significant effect on the severity of AP in rats, demonstrating the beneficial effect of probiotics on the development of AP.

Keywords: apical periodontitis; endodontics; probiotics

Introduction

According to the World Health Organization, probiotics are living microorganisms that when administered in adequate amounts confer benefits to the health of the host and may interfere in the health-disease process (Araya *et al.* 2002). The beneficial effects of consuming probiotics are related to their mechanism of action: production of antimicrobial substances against pathogens; mechanisms of competitive exclusion between pathogen/probiotic; and positive modulation of host defenses through increased macrophage activity, increased number of killer cells and interferons, stimulation of B lymphocytes, and migration of intestinal T lymphocytes (Amdekar *et al.* 2013, George *et al.* 2018).

The use of probiotics for clinical health benefits is an area of great interest in scientific research. Among the major effects of probiotics on human health are anti-inflammatory and antimicrobial activities (George *et al.* 2018). In inflammatory bowel diseases, probiotics offer an alternative or adjunctive approach to conventional therapy (George *et al.* 2018). In addition, antimicrobial activity of probiotics can help to improve the control rate of *Helicobacter pylori*, which is recognized as a major cause of peptic ulcer disease and has also been implicated in the pathogenesis of gastric cancer (Song *et al.* 2018). The main target organ of probiotics is the intestine, but evidence shows that intestinal colonization can cause systemic beneficial effects, promoting protection against diseases at distant sites (Bermudez-Brito *et al.* 2012, Kobayashi *et al.* 2017).

The oral cavity has recently been suggested as an important target for the application of probiotics. Until recently, probiotics were evaluated mainly for the purpose of controlling dental caries (Ahola *et al.* 2002). However, periodontics research has shown that the use of probiotics as a coadjuvant in the treatment of periodontal disease is effective in reducing periodontopathogens and decreasing the levels of proinflammatory cytokines (Teughels *et al.* 2013, Messori *et al.* 2016, Invernici *et al.* 2018).

Apical periodontitis (AP) is an infectious disease mainly caused by the carious process followed by inflammation and necrosis of the dental pulp tissue. AP is characterized by the destruction of periradicular tissues in response to bacterial infection (Kawashima *et al.* 1996). The pathogenesis of AP is complex and mediated by inflammatory cytokines that activate and inhibit inflammation (Lu *et al.* 2015, Cintra *et al.* 2016, Samuel *et al.* 2018). These cytokines (pro- and anti-inflammatory) try to neutralize the pathogens and control the disease (Araujo-Pires *et al.* 2014, Lu *et al.* 2015).

The inflammatory response in AP is mediated by a complex cytokine net. Interleukin-6 (IL-6) has a substantial role in the differentiation of T-helper cells, which are cells that have the potential to potentiate inflammation (Diehl & Rincón 2002, Araujo-Pires *et al.* 2014). The IL-1 family, including IL-1 β , regulates and initiates inflammatory responses through the expression of integrins in leukocytes and endothelial cells (Dinarello 2011). IL-1 β also acts on bone resorption in AP through the activation of osteoclasts (Tani-Ishii *et al.* 1994, Martinho *et al.* 2012). IL-10 is an anti-inflammatory cytokine secreted by macrophages and acts on the balance of the immune system to modulate the inflammatory response against pathogens (Correa *et al.* 2010, Martinho *et al.* 2012).

Considering that the etiopathogenesis of periodontal disease and AP are similar and that probiotics can modulate the inflammatory response, it is possible that AP can also be affected by probiotics consumption. However, no information is available concerning the effect of probiotics on AP. The aim of this study was to evaluate the systemic effect of probiotics *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* on the severity of AP. To this end, we evaluated the blood profile, microbiota of root canal/saliva, and severity of inflammation in the AP of rats that received a systemic probiotic administration or not. Thus, the null hypothesis tested in this study was that no difference would be observed in the severity of AP in rats receiving a systemic probiotic administration or not.

Material and Methods

Animals

Twenty-four male *Wistar* rats (*Rattus norvegicus*), each weighing 200-250 g, were used in this study. The animals were housed in a temperature-controlled environment (22°C \pm 1°C, 70% humidity) with a 12 h light-dark cycle and ad libitum access to water and food. The experimental protocol was performed in compliance with the relevant laws and institutional guidelines in accordance with the ethical standards of the Declaration of Helsinki and approved by the Institutional Ethics Committee on Animal Use (516-2017) of Universidade Estadual Paulista, São Paulo, Brazil. The general health condition of the animals was observed throughout the experimental period. Body weight, food and water was daily monitored.

Sample size calculation

The sample size estimate was based on data from a previous study (Dal-Fabbro *et al.* 2019). Considering an alpha error of 0.05 and 95% power to recognize a significant difference, a minimum of seven animals per group was considered necessary. Taking into account possible animal deaths, one more animal was added in each group, resulting in eight rats per group, totaling 24 animals.

Induction of AP

The rats were then administered anesthesia via intramuscular injections of ketamine (87 mg/kg, Francotar; Virbac do Brazil Ind e Com Ltda, Roseira, São Paulo, Brazil) and xylazine (13 mg/kg, Rompum, Bayer AS, São Paulo, Brazil). Coronal pulp tissue was exposed and disorganized in the animals through the access opening of the coronary surface of the right upper first molar and left upper first molars using a spherical carbide bur with 0.5 mm diameter (Jet Carbide ¼, Kavo Kerr Group, Orange California, USA). The coronal pulp tissue was exposed and left open to the oral cavity in this way until killed (Cintra *et al.* 2016, Dal-Fabbro *et al.* 2019).

Probiotic therapy

The animals were randomly assigned into three groups ($n = 8$): rats with AP and a regular diet (Control); rats with AP, a regular diet, and supplemented with *Lactobacillus rhamnosus* LR 04 (DSM 16605) (Coana Importação e Exportação Ltda, Florianópolis, Brazil); and rats with AP, a regular diet, and supplemented with *Lactobacillus acidophilus* LA 14 (Aché Laboratórios Farmacêuticos S.A. Guarulhos, Brazil).

Starting from the first day of AP induction, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were orally administered to the animals once a day (at 09:00) for 30 days for gavage (10^9 colony-forming units (CFU)) diluted in 5 mL of water) during the period of development of AP (Ricoldi *et al.* 2017). The Control group received 5 mL of water (without probiotics)

Blood Sample Collection

At 30 days, the subjects were again anesthetized with the same protocol as previously described, and a cardiac puncture was performed to collect 5 mL blood from each of the subjects. The samples were placed in EDTA, homogenized, and immediately transferred to a technician, who was blinded to the case status, for processing. The following parameters of

the complete blood count were analyzed using an automatic analyzer (ABX Micros ABC Vet; Horiba ABX Diagnostics, Montpellier, France): hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), fibrinogen, total leukocytes, neutrophils, lymphocytes, eosinophils, and monocytes (Samuel *et al.* 2018).

Microbiological analysis of the root canal and saliva

After the 30-day period of induction of the AP, the microbiota of the root canal and saliva were collected. At the time of sampling, every effort was made to ensure aseptic techniques. For the collection of the root canal sample, the teeth were then cleansed with 30% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite solution for 30 s each, followed by neutralization of the solution with 5% sodium thiosulphate (Jacinto *et al.* 2006). Then, a Kerr file (#10) (Dentsply Maillefer, Ballaigues, Suisse) at a depth of 4 mm was inserted into the distal conduit of the right upper first molar (Iwama *et al.* 2006). The files were cut at 4 mm, corresponding to the depth inserted in the root canal, and transferred to microtubes containing Reduced Transport Fluid (Syed & Loesche 1972) and kept on ice for transportation to the laboratory. A sterile swab was used for collection of the saliva sample along the jugal mucosa, which was also inserted into microtubes containing RTF and transported on ice to the laboratory. In the laboratory, the saliva and root canal samples were serially diluted (10^{-1} a 10^{-6}) and plated in two different cultures: Brain Heart Infusion Agar containing 5 mg/ml hemin and 10 mg/ml menadione, yeast extract, and 5% defibrinated sheep blood for counting of total microorganisms (MT) and Rogosa Agar culture (Merck™, Darmstadt, Germany) for total lactobacilli (LT) count. Plates of the saliva samples for MT and LT were incubated in an oven containing 5% CO₂, and plates from the root canal samples were incubated in jars containing the anaerobic system (Oxoid AnaeroGen, Thermo Scientific Loughborough, Leicestershire). All plates were maintained at 37°C for 48 h. After incubation, the total number of colony-forming units per milliliter (CFU/ml) was counted from a representative area of each agar plate, yielding 50-300 colonies, using a magnifying glass (Duque *et al.* 2009).

Histological and histometric analyses

After blood sample collection, the animals were killed by an overdose of anesthetic solution. The maxilla was removed, fixed in a solution of 4% buffered formaldehyde for 24 h, and then decalcified in buffered (pH = 8) 17% EDTA (Sigma Chemical Co, St Louis, MO).

Semi-serial 6 µm cuts were made in the left upper first molars in the laterolateral direction, allowing sectioning in their longitudinal axes, and stained with haematoxylin and eosin (HE). Slides without staining were reserved for immunohistochemistry. Histopathologic analyses were performed by a single certified histologist (E.E.), who was blinded to the experimental groups, using a light microscope (DM 4000 B; Leica) and a color camera (DFC 500; Leica, Wetzlar, Germany). The distal root was standardized for all analyses (Dal-Fabbro *et al.* 2019). Histopathological analysis was conducted using the quality of inflammation and the cellularity pattern of dental and periodontal tissues as guidelines to score the inflammatory infiltrate as follows: absent (0 to few inflammatory cells, score = 1), mild (< 25 cells, score = 2), moderate (25–125 cells, score = 3), and severe (> 125 cells, score = 4) (Azuma *et al.* 2017). Presence or absence of necrosis was also recorded (Gomes-Filho *et al.* 2015, Dal-Fabbro *et al.* 2019).

For histometric analysis, the periapical lesion area associated with the distal root was histometrically measured. The area of the periapical lesion was calculated by rounding up the boundary lesion (considering the outer external surface of the cementum, periodontal ligament, and the outer surface of the alveolar bone) and was expressed in square micrometers (Azuma *et al.* 2017). The measurement was conducted in five equidistant sections to the root canal. For this, an image-processing system was used that consisted of a light microscope (DM 4000 B; Leica), a color camera (DFC 500; Leica, Wetzlar, Germany), a color image processor (Leica QWin V3, Leica Microsystems, Wetzlar, Germany), and a personal computer (Intel Core i5, Intel Corp, Santa Clara, CA; Windows 10, Microsoft Corp, Redmond, WA) (Gomes-Filho *et al.* 2015).

Immunohistochemical Analysis

Immunolabeling in the histological sections was performed using an indirect immunoperoxidase technique (Gomes-Filho *et al.* 2015, Cosme-Silva *et al.* 2018). Histological sections were divided into three batches, and each batch was incubated with one of the following primary antibodies (1:100): IL-10 (Rabbit - orb221323, Biorbyt, San Francisco, CA, USA); IL-1β (Rabbit - orb101745 Biorbyt), and IL 6 (Rabbit - orb6210 Biorbyt). The sections were incubated with biotinylated secondary antibody for 2 h and subsequently treated with conjugated streptavidin to the - HRP for 1 h (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, Carpinteria, CA, USA). The disclosure was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB chromogen

Kit - Dako Laboratories) as a chromogen. As a negative control, the specimens were subjected to the procedures described above by suppressing the primary antibody use.

Semiquantitative immunolabeling analyses of IL-10, IL-1 β , and IL-6 were performed by a certified histologist (E.E.), who was blinded to the treatments, using the distal root (Dal-Fabbro *et al.* 2019). Three histologic sections were used for each animal, and positive immunoreactivity (IR) was defined as a brownish color in the cytoplasm of the cells and extracellular matrix. Because immunolabeling of both the cells and the extracellular matrix is of great importance for the study, a semiquantitative analysis was performed, providing information about the numbers of immunoreactive cells and immunolabeling intensity of the extracellular matrix. The scores were assigned as follows: 1 = complete absence of immunoreactive cells; 2 = low IR (a few immunoreactive cells and weak labeling of the extracellular matrix; approximately one quarter of the immunoreactive cells); 3 = moderate IR (a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix; approximately one half of the immunoreactive cells); and 4 = high IR (a large number of immunoreactive cells and strong labeling of the extracellular matrix; approximately three-quarters of the immunoreactive cells) (Azuma *et al.* 2017).

Statistical Analysis

The data were analyzed using GraphPad Prism 7 software (La Jolla, CA, USA). After the Shapiro-Wilk test of normality, the Kruskal-Wallis followed by Dunn's test was performed for nonparametric data, and analysis of variance followed by the Tukey test was performed for parametric data. The level of significance was 5%.

Results

General health condition of the animals

The general health condition of the animals remained constant throughout the experimental period. At the end of the experimental period, no statistically significant difference was observed in mean body weight or food and water consumed by the animals (Table 1) ($p>0.05$).

Table 1: Average weight gain, solid and liquid intake the rats throughout the experimental period. The data is expressed in form of mean and standard deviation

Groups	Average Weight Gain (%)	Solid Intake (g/animal/day) Mean±SD	Liquid Intake (ml/animal/day) Mean±SD
Control	66 ^a	17.29±4.64 ^a	60.27±16.37 ^a
<i>Lactobacillus rhamnosus</i>	64.5 ^a	15.84±3.5 ^a	60.1±16.81 ^a
<i>Lactobacillus acidophilus</i>	68.5 ^a	16.02±3.35 ^a	60.0±17.01 ^a

*Different letters indicate significant statistical differences in colunes (p<0.05).

SD: standard deviation

Blood Profile

The effect of supplementation with probiotics on the hematologic tissue was investigated. The results of the complete blood count and differential white blood cell count are shown in Table 2. No statistical difference was found in the total counts of hemoglobin, hematocrit, MCV, mean corpuscular hemoglobin concentration (MCHC), and fibrinogen between the Control, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus* groups (p>0.05) (Table 2). The total leukocyte, neutrophil, lymphocyte, eosinophil, and monocyte counts were greater in the Control group, but no significant difference was seen when compared with the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* groups (p>0.05).

Table 2: Mean and standard deviation of blood cell parameters.

Hematologic parameters	Control	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus acidophilus</i>
	Mean±SD*	Mean±SD*	Mean±SD*
Hemoglobin	151.1±6.85 ^a	152.0±6.63 ^a	150.3±7.30 ^a
Hematocrit	48.3±1.40 ^a	47.6±2.50 ^a	47.0±1.60 ^a
Mean Corpuscular Volume (MCV)	66.1±2.62 ^a	69.2±4.16 ^a	68.4±1.97 ^a
Mean Corpuscular Hemoglobin Concentration (MCHC)	31.2±0.95 ^a	31.9±0.58 ^a	31.9±0.67 ^a
Fibrinogen	0.2±0 ^a	0.3±0.21 ^a	0.2±0.10 ^a
Total leukocytes	19.5±8.8 ^a	16.1±9.0 ^a	15.8±6.1 ^a
Neutrophils	88.2±1.7 ^a	68.0±3.1 ^a	75.7±1.4 ^a
Lymphocytes	62.8±1.4 ^a	46.4±2.3 ^a	47.7± 8.9 ^a
Eosinophils	23.5±1.7 ^a	17.3±2.9 ^a	19.9±1.9 ^a
Monocytes	91.0±1.0 ^a	87.5±1.0 ^a	58.0±7.4 ^a

*Different letters indicate significant statistical differences in lines (p<0.05).
SD: standard deviation

Microbiological analysis

Microbiological analyses of root canals and saliva are shown in Table 3. The CFU counts of the root canals and saliva in the BHI culture medium (total microorganism growth) were lower in the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* groups when compared to the Control group (p<0.05) (Table 3). Between the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* groups, no differences were observed in the CFU counts presented in root canals and the saliva (p>0.05) (Table 3). In contrast, no CFUs were seen in the Rogosa Agar (specific for lactobacillus growth) in the root canal and in saliva of all experimental groups (<50 CFU/ml) (p>0.05).

Table 3: Total microorganisms count (CMT) (log (UFC+1)) in the root canal and saliva according to the experimental groups.

Groups	CMT	CMT
	Root canal	Saliva
	Mean±SD*	Mean±SD*
Control	8,7±0,14 ^a	9,04±0,01 ^a
<i>Lactobacillus rhamnosus</i>	7,6±0,39 ^b	8,0±0,34 ^b
<i>Lactobacillus acidophilus</i>	8,5±0,52 ^b	8,4±0,51 ^b

*Different letters indicate significant statistical differences in columns (p<0.05).
SD: standard deviation

Histopathologic analysis

The groups that had their diets supplemented with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* had lower magnitude of inflammatory response through the evaluation of inflammatory infiltrate when compared to the control group (p<0.05) (Table 4, Figure 1). Between the groups receiving probiotic supplementation, no difference was observed in the inflammatory infiltrate (p>0.05) (Table 4, Figure 1). Histometrically, the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* groups showed a lower periapical lesion area as compared to the Control group (p<0.05) (Table 4, Figure 1). Between the groups receiving probiotic supplementation, no difference was observed in the periapical lesion area (p>0.05).

Table 4: Scores and median of intensity of inflammatory cells, necrosis, AP area (μm^2) and immunohistochemical analysis according to the groups.

Histologic parameters	Control	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus acidophilus</i>
Scores			
1	0/8	0/8	0/8
2	0/8	3/8	3/8
3	2/8	3/8	5/8
4	6/8	2/8	0/8
Median*	4[¶]	3[‡]	3[‡]
Necrosis	100%	100%	100%
Area (x 10⁴ μm^2 - SD*)	47.72±4.4 [¶]	28.62±8.0 [‡]	34.93±10.9 [‡]
Immunohistochemical			
Scores	Cytokine		
1	0/8	0/8	0/8
2	<i>IL 10</i>	6/8	0/8
3		2/8	3/8
4		0/8	5/8
Median**	2^a	4^b	4^b
1	0/8	0/8	0/8
2	<i>IL-1β</i>	0/8	5/8
3		1/8	3/8
4		7/8	0/8
Median**	4^a	2^b	3^b
1	0/8	0/8	0/8
2	<i>IL 6</i>	0/8	2/8
3		0/8	6/8
4		8/8	0/8
Median**	4^a	3^b	2^c

*Histologic parameters: different symbols indicate significant statistical differences in lines (p<0.05).

**Immunohistochemical analysis: different letters indicate significant statistical differences in lines (p<0.05).

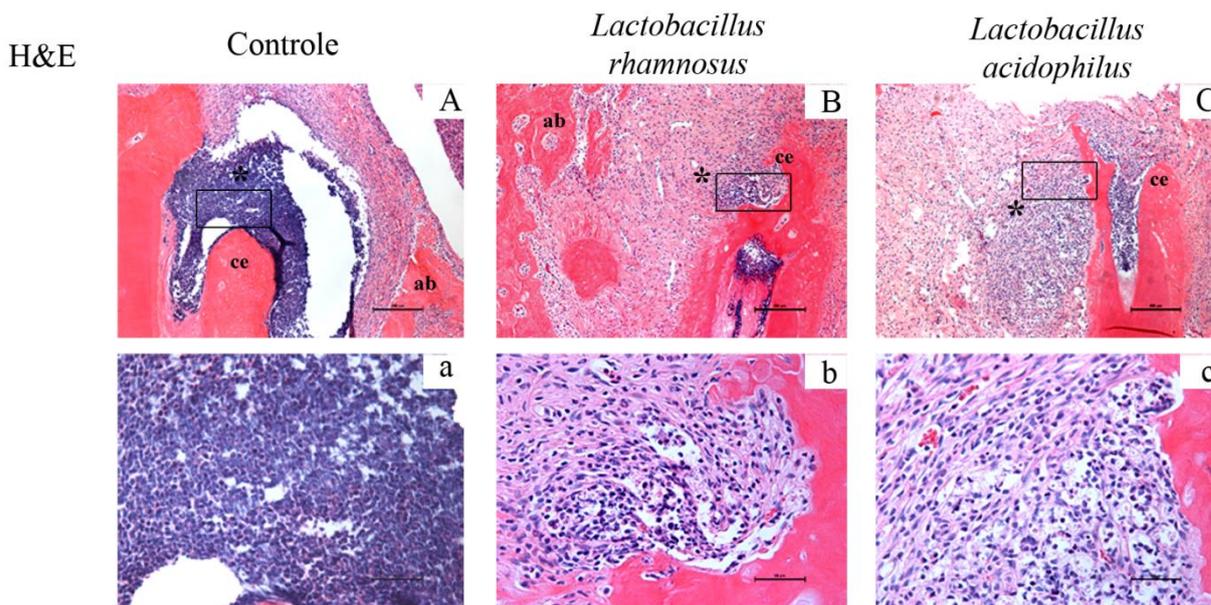


Figure 1: Photomicrographs showing histologic aspects of periapical regions (A-C, a-c). Apical periodontitis of greater severity were observed in Control group (A,a) than groups that consumed probiotics: *Lactobacillus rhamnosus* (B,b) and *Lactobacillus acidophilus* (C,c). ab, alveolar bone; ce, cementum. *Inflammatory infiltrate. Haematoxylin–eosin staining. Rectangle shows area elected for 400X magnification. Original magnification: A, B, C 100X; a, b, c 400X.

Immunohistochemistry

The immunoreactivity patterns for IL-10, IL-1 β , and IL 6 are described below and in the Table 4.

IL-10: The groups receiving *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* had a median score of 4 for IL-10. No statistically significant difference was seen between them ($p>0.05$) (Table 4, Figure 2). All specimens of the Control group had a median score of 2, which was significantly lower when compared to groups receiving probiotic supplementation ($p<0.05$) (Table 4, Figure 2).

IL-1 β : The groups that received *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* had a median of score 3 for *Lactobacillus acidophilus* and a median score of 2 for *Lactobacillus rhamnosus*, but no statistically significant difference was found between them ($p>0.05$) (Table 4, Figure 2). The specimens of the Control group had a median of score 4, which was significantly higher when compared to the groups receiving probiotic supplementation ($p<0.05$) (Table 4, Figure 2).

IL-6: The groups that received *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* had a median of score 2 for *Lactobacillus acidophilus* and a median score of 3 for

Lactobacillus rhamnosus with significant difference between them ($p < 0.05$) (Table 4, Figure 2). All specimens of the Control group had a median score of 4, which was significantly higher when compared to the groups receiving probiotic supplementation ($p < 0.05$) (Table 4, Figure 2).

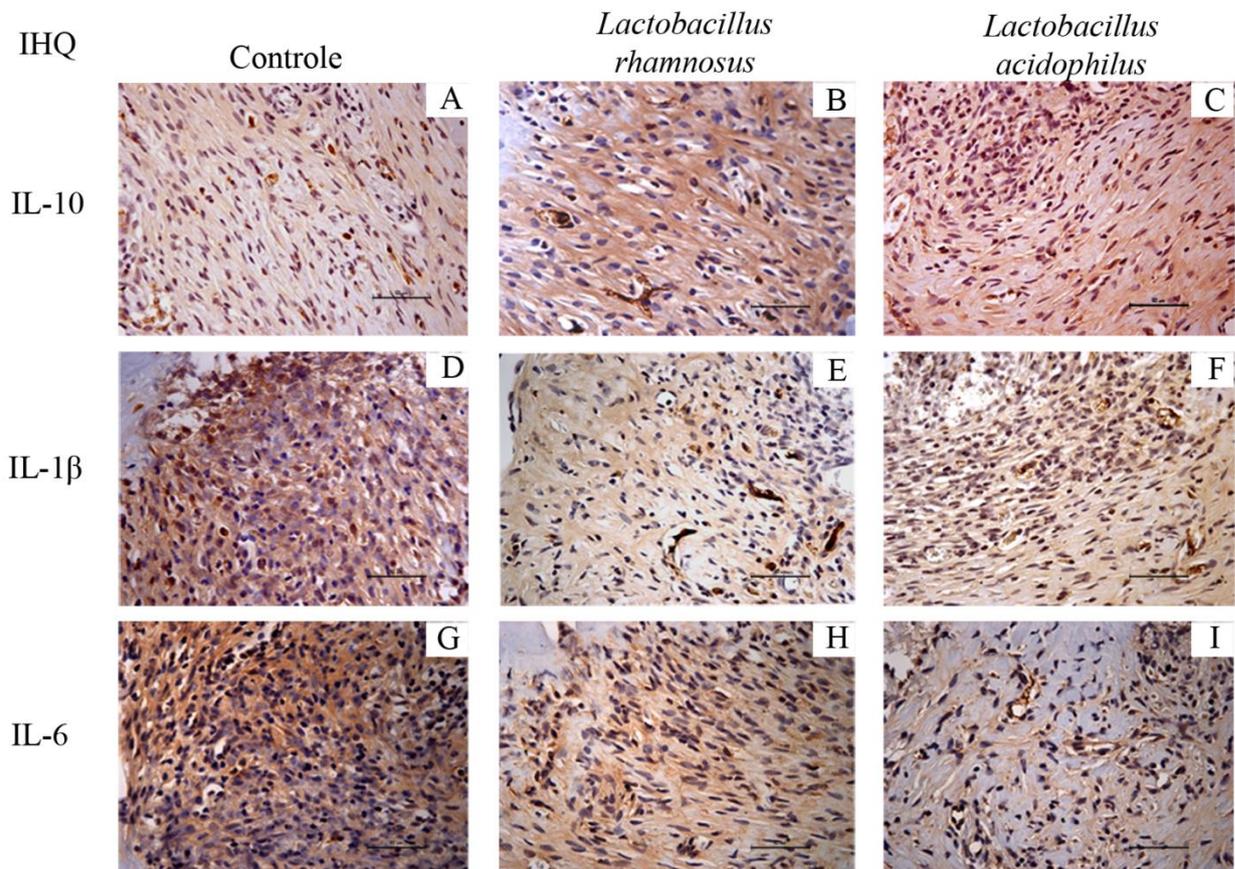


Figure 2: Photomicrographs showing the histological appearance of immunolabeling in periapical regions (A-I). More intense immunolabeling pattern for IL-10 in groups that consume probiotics: *Lactobacillus rhamnosus* (B) and *Lactobacillus acidophilus* (C); More intense immunolabeling pattern for IL-1 β in the control (D) groups when compared to *Lactobacillus rhamnosus* (E) and *Lactobacillus acidophilus* (F) groups; More intense immunolabeling pattern for IL 6 in the control (G) and smaller groups in *Lactobacillus rhamnosus* (H) and *Lactobacillus acidophilus* (I) groups. Original magnification: 400X.

Discussion

To the authors' knowledge, this is the first study evaluating the effect of probiotics on the severity of AP. The influence of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* lowered the amount of bacteria present in the root canals of necrotic teeth and also in saliva, the severity of inflammatory infiltrate, and proinflammatory cytokine expression (IL-1 β and

IL6) concomitantly with higher anti-inflammatory cytokine expression (IL-10). Therefore, the null hypothesis was rejected.

In this study, rats were used as the experimental model because the oral bacterial microflora and the apical response to pulp exposure in these animals are similar to those seen in humans (Tani-Ishii *et al.* 1994). AP was induced by exposing the pulp of rat molars to the oral environment, allowing pulp tissue infection and culminating in pulp necrosis and subsequent AP development. The 30-day experimental period was chosen because it is considered long enough for chronic AP development (Gomes-Filho *et al.* 2015, Cintra *et al.* 2016; Dal-Fabbro *et al.* 2019). In addition, the chronic AP model can guarantee the absence of systemic signs of acute infection, such as abscesses, which may influence the expression of proinflammatory mediators in periapical tissues (Azuma *et al.* 2018).

The administration of probiotics by gavage (10^9 CFU) was the method of choice in this study, besides the treatment occurring during the development of AP. This approach was chosen because the aim of this investigation was to evaluate the systemic effect of the probiotics during the development of AP. If the administration of probiotics was via drinking water of the animals or by oral inoculation or for a period prior to the induction of AP, then the aim of this research would not have been achieved. To validate the systemic supplementation method, we performed a microbiological analysis of lactobacillus in the saliva and root canal. No CFUs of lactobacillus were found in saliva/root canal, showing that the results of this study are related to the systemic effects of probiotics. The mode of administration of probiotic therapy is a factor that may influence treatment outcomes. However, Gatej *et al.* (2018) demonstrated that regardless of the administration mode (oral inoculation or oral gavage), the beneficial effect of probiotics on periodontal disease remained the same. Furthermore, the dose/frequency can also have an influence. Ricoldi *et al.* (2017) used a dose of 10^9 CFUs and were successful in the adjuvant treatment of periodontitis. Thus, this dosage was chosen for the present study.

This study opted for *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* probiotics. The use of noninvasive and natural approaches in the prevention and treatment of oral diseases has been investigated (Ricoldi *et al.* 2017). Kitazawa *et al.* (2002) demonstrated that probiotics of the genus lactobacillus have antimicrobial activity and antioxidant and immunostimulatory properties that are factors related to the decrease of pathogens. The use of probiotics, especially in periodontics, has shown a beneficial effect on periodontal disease due to the reduction of plaque index and less destruction of periodontal tissues (Toiviainen *et al.*

2015, Ricoldi *et al.* 2017). In addition, better clinical parameters such as depth of probing, inflammation, and gingival bleeding were observed in periodontitis (Toiviainen *et al.* 2015).

No changes in blood cell counts were observed in the current study. It was expected that the presence of infection could be reduced with probiotics, including using hematologic analyses. The expectation was postulated due to a possible correlation of periodontal disease and anemia (Agarwal *et al.* 2009, Anand *et al.* 2013). Leukocytes are involved in defense against pathogens and can be altered when there is local infection, since a relationship between bacterial infection and blood cell numbers is commonly observed (Gkrania-Klotsas *et al.* 2011, López *et al.* 2012).

In the microbiological analysis of this study, we observed lower bacterial counts in root canals and saliva in rats supplemented with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*. The microbiota of the infected root canals is highly diversified and for the development of the AP it is essential that these microorganisms and their byproducts are in constant action (Gomes *et al.* 2004). These results are in agreement with the literature, in which Kobayashi *et al.* (2017) demonstrated that administration of lactobacillus by gavage controlled oral inflammation, decreased bone resorption, and significantly decreased the number of periodontal pathogens in the subgingival microbiota in rats with periodontal disease. In addition, the authors showed that the expression of β -defensin (antimicrobial peptides that exert immunomodulatory and chemotactic functions) was increased in the gut, but also in the gingival tissue, tongue, and saliva. It was shown that defensins could suppress early events in inflammation and enhance systemic antibody responses (Kohlgraf *et al.* 2010).

It was also observed that *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were able to decrease the severity of the inflammatory infiltrate in AP when compared to the control. In addition, lower area of periapical lesion was observed in the groups that consumed probiotics. During AP development, inflammatory cells (T cells, B cells, and macrophages) are recruited to the periapical tissues to eliminate infection, resulting in a “cascade” of inflammatory cytokine release (Liapatas *et al.* 2013). *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were chosen because previous studies have shown a considerable anti-inflammatory property associated with a decrease in the synthesis of proinflammatory cytokines (e.g., IL-6 and IL-1 β), but they also positively promote the synthesis of anti-inflammatory cytokines, such as IL-10 (Amdekar *et al.* 2013, Amdekar *et al.* 2016).

We observed that *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* influenced the reduced release of proinflammatory cytokines (IL-6 and IL-1 β) and the increased release

of the anti-inflammatory (IL-10) cytokine in periapical tissues. Although inflammation is an important biological process to protect the host and contain pathogens, excessive inflammation aggressively affects healthy tissues and may compromise tissue functionality (Bian *et al.* 2012). It is known that IL-1 β positively regulates the synthesis of collagenases and prostaglandins and is associated with degradation through proteases, acting directly in the process of inflammation (Bickel *et al.* 2001). In contrast, IL-10 is a cytokine that exhibits anti-inflammatory properties and plays a central role in reducing infection by limiting the immune response to pathogens and thus preventing damage to the host (Bambirra *et al.* 2015). Similar results to this study were found when probiotics were used as adjuvants in the treatment of periodontitis (Messora *et al.* 2016, Ricoldi *et al.* 2017, Kobayashi *et al.* 2017).

It is noteworthy that in the present study the group that consumed *Lactobacillus acidophilus* showed lower expression of IL-6 in AP when compared to the *Lactobacillus rhamnosus* group. Interleukin-6 stimulates inflammatory response by recruiting inflammatory mediators to restrict a pathogenic agent but may cause exaggerated responses that damage healthy tissue (D'Aiuto *et al.* 2004). It has been demonstrated that IL-6 acts as a mediator of the immunopathogenesis of dental granulomas contributing to the differentiation of B lymphocytes and also to the proliferation of T lymphocytes (Radics *et al.* 2003). In addition, larger periapical lesions have greater IL-6 expression, evidencing the role of IL-6 in osteoclast formation and bone resorption of AP (Huang *et al.* 2001). Clearly, probiotic therapy, mainly *Lactobacillus acidophilus*, influenced the severity of inflammation in the periapical region, revealing a possible protective effect on the development of AP.

The present study provided substantial findings related to the probiotic therapeutic potential in AP. A systemic oral administration of *Lactobacillus rhamnosus* or *Lactobacillus acidophilus* can decrease bacteria in root canals and saliva, attenuate the inflammation in AP, and decrease IL-6 and IL-1 β and increase IL-10 expression. Although promising results have been reported in the scientific literature, it is important to remember that probiotic findings cannot be generalized since they are dependent on the strain, dosage, frequency, and experimental model. In this way, new studies are essential for exploring the mechanism of action and to define a solid protocol for probiotic use in the endodontics.

Conclusion

Supplementation with probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) had a significant effect on the severity of AP in rats, demonstrating the effect of probiotics on the development of AP.

Acknowledgements

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Conflict of interest

The authors deny any conflict to interests to this study.

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Legends

Table 1: Average weight gain, solid and liquid intake the rats throughout the experimental period. The data is expressed in form of mean and standard deviation (SD).

Table 2: Mean and standard deviation of blood cell parameters.

Table 3: Total microorganisms count (CMT) (log (UFC+1)) in the root canal and saliva according to the experimental groups.

Table 4: Scores and median of intensity of inflammatory cells, necrosis, AP area (μm^2) and immunohistochemical analysis according to the groups.

Figure 1: Photomicrographs showing histologic aspects of periapical regions (A-C, a-c). Apical periodontitis of greater severity were observed in Control group (A,a) than groups that consumed probiotics: *Lactobacillus rhamnosus* (B,b) and *Lactobacillus acidophilus* (C,c). ab, alveolar bone; ce, cementum. *Inflammatory infiltrate. Haematoxylin–eosin staining. Rectangle shows area elected for 400X magnification. Original magnification: A, B, C 100X; a, b, c 400X.

Figure 2: Photomicrographs showing the histological appearance of immunolabeling in periapical regions (A-I). More intense immunolabeling pattern for IL-10 in groups that

consume probiotics: *Lactobacillus rhamnosus* (B) and *Lactobacillus acidophilus* (C); More intense immunolabeling pattern for IL-1 β in the control (D) groups when compared to *Lactobacillus rhamnosus* (E) and *Lactobacillus acidophilus* (F) groups; More intense immunolabeling pattern for IL 6 in the control (G) and smaller groups in *Lactobacillus rhamnosus* (H) and *Lactobacillus acidophilus* (I) groups. Original magnification: 400X.

Artigo 2

Dietary supplementation with *Lactobacillus Rhamnosus* and *Acidophilus* reduces inflammation and bone resorption in apical periodontitis.

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Running title: Probiotics and apical periodontitis

Keywords: apical periodontitis; endodontics; probiotics.

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Abstract

Aim: The aim of the present investigation was to evaluate the relationship between systemic administration of probiotic and inflammatory process of apical periodontitis (AP).

Methodology: Twenty-four male *Wistar* rats were used. AP was induced in the lower left/right first molars. The animals were arranged into 3 groups: Control, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*. Probiotics were diluted 5 mL of water and daily orally administrated for gavage (10^9 colony-forming units (CFU)) along 30 days, need for AP development. At the 30th, blood collect was performed to analyze the calcium, phosphorus and alkaline phosphatase concentration in plasma. Then the animals were killed and the jaws were removed for micro-computed tomography and immune-histopathological analysis (haematoxylin–eosin, RANKL, OPG and TRAP) of the AP. The data were statistically analyzed and the level of significance was 5%.

Results: Alkaline phosphatase was the highest in the groups that consumed probiotics ($p < 0.05$). The volume of bone reabsorption was lower in probiotic groups when compared to the control ($p < 0.05$). The inflammatory infiltrate and the immunostaining for RANKL and TRAP were lower in probiotic groups when compared to the control ($p < 0.05$). It was observed that OPG was more immunolabeled in the *Lactobacillus acidophilus* group than in the *Lactobacillus rhamnosus* and Control group ($p < 0.05$). There was no statistical difference in the calcium and phosphorus levels among the groups ($p > 0.05$).

Conclusion: Supplementation with probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) had a significant effect reducing inflammation and bone resorption in apical periodontitis.

Keywords: apical periodontitis; endodontics; probiotics

Introduction

Apical periodontitis (AP) occurs as a result of bacterial infection of the root canals that progresses to the apical region causing inflammation (Kawashima *et al.* 1996, Becconsall-Ryan *et al.* 2010). In response to bacterial stimulation, host cells secrete inflammatory cytokines and activation of cells related to bone resorption, such as osteoclasts leading to resorption of the surrounding bone (Kawashima *et al.* 1996, Austah *et al.* 2016, Dal-Fabbro *et al.* 2019b). Osteoclast activators stimulate bone resorption and periapical tissue destruction through an imbalance in the RANK/RANKL/OPG system, which is triggered by the inflammatory process and its products (Boyce & Xing 2008, Eriksen 2010, Britton *et al.* 2014, Dal-Fabbro *et al.* 2019b).

Probiotics are microorganisms that confer benefits to the health of the host and may interfere in the health-disease process when administered in adequate amounts (FAO 2001). Anti-inflammatory activity has been highlighted among the major effects of probiotics on human health (George *et al.* 2018). Recently, probiotics are being used as a novel approach for treating various diseases including inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and diseases related to bone health, such as osteoporosis (Chiang & Pan 2011, Petersen *et al.* 2012, Britton *et al.* 2014). Among the useful probiotics, the lactobacillus strain has been used as adjuvant treatment in several diseases including periodontitis (Tekce *et al.* 2015, Liu *et al.* 2018). Animal and human studies showed that the use of probiotics in periodontitis was able to improve periodontal pocket depth, probing bleeding, insertion loss, and reduction of the periodontal pathogens number (Teughels *et al.* 2013, Tekce *et al.* 2015, Toiviainen *et al.* 2015, Ricoldi *et al.* 2017). However, there are no study that relate consumption of probiotics and apical periodontitis. Thus, the aim of this study was to evaluate effect of the systemic consumption of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in the inflammatory process and bone resorption of rats with AP that consumed or not probiotics. The null hypothesis tested was that there was no difference in the severity of apical periodontitis in rats with receiving or not a systemic probiotic administration.

Material and Methods

Animals

Twenty-four male *Wistar* rats (*Rattus norvegicus*), each weighing 200-250 g, were used in this study. The animals were housed in temperature -controlled environment (22°C±1°C, 70% humidity) with a 12h light -dark cycle and given ad libitum access to water and food. The experimental protocol was performed in compliance with the relevant laws and institutional guidelines in accordance with the ethical standards of the Declaration of Helsinki and approved by the Institutional Ethics Committee on Animal Use (516-2017) of Universidade Estadual Paulista, São Paulo, Brazil.

Sample size calculation

Sample size estimates was based on data from a previous study (Dal-Fabbro *et al.* 2019b). Considering alpha error of 0.05 and 95% power to recognize a significant difference, a minimum number of 7 animals per group was considered necessary. Taking into account possible animal deaths, one more animal was added in each group, resulting in 8 rats/group, totaling 24 animals.

Induction of apical periodontitis

The rats were then administered anesthesia via intramuscular injections of ketamine (87 mg/kg, Francotar; Virbac do Brazil Ind e Com Ltda, Roseira, São Paulo Paulo, Brazil) and xylazine (13 mg/kg, Rompum, Bayer AS, São Paulo, Brazil). The animals had the coronal pulp tissue exposed and disorganized through the access opening of the coronary surface of first right and left lower molars using a spherical carbide bur with 0.5mm diameter (Jet Carbide ¼, Kavo Kerr Group, Orange California, USA). The coronal pulp tissue was exposed and left open to the oral cavity in this way until euthanasia (Cintra *et al.* 2016, Dal-Fabbro *et al.* 2019b).

Probiotic therapy

The animals were randomly assigned into three groups ($n=8$): rats with apical periodontitis and a regular diet (Control); rats with apical periodontitis and, a regular diet, and supplemented with *Lactobacillus rhamnosus* LR 04 (DSM 16605) (Coana Importação e Exportação Ltda, Florianópolis, Brazil) and rats with apical periodontitis, a regular diet and, and supplemented with *Lactobacillus acidophilus* LA 14 (Aché Laboratórios Farmacêuticos S.A. Guarulhos, Brazil).

Starting from the first day of AP induction, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were orally administered to the animals once a day (at 09:00) for 30 days for gavage (10⁹ colony-forming units (CFU)) diluted in 5 mL of water) during the period of development of AP (Ricoldi et al. 2017). The Control group received 5 mL of water (without probiotics)

Blood Sample Collection

On 30 days, the rats were again anesthetized with the same protocol as previously described, and a cardiac puncture was performed to collect 3 mL blood. The blood was packed in tubes without anticoagulant and centrifuged to obtain plasma (3,000 RPM - 10 min - 20°C) for dosage of calcium, phosphorus and alkaline phosphatase. The quantification of calcium, phosphorus and alkaline phosphatase were performed with LabTest™ kits (Lagoa Santa, Minas Gerais, MG, Brazil) according to the manufacturer's instructions. The kits used were: Liquiform Calcium 90; Liquiform Phosphorus 12 and Alkaline Phosphatase 1011)

Micro-Computed Tomography analysis (μCT)

The animals were killed by an overdose of anesthetic solution. The lower right mandible were scanned using μCT system (Bruker SkyScan1272, Aartselaar, Belgium). Each specimen was placed in a vial and positioned with the incisor facing upwards. Samples were scanned with the following settings: 70 kV, 167 μA, 0.5° rotation step, 2100 millisecond exposure, 3 frame averages, 12 a pixel image and 9 a pixel camera size. A 0.5 mm aluminum filter was used during the scans, and a polynomial correction was also used to reduce the hardening effects of the beam during reconstructions. The region of interest (ROI) included the empty space of periradicular destruction and/or space of the periodontal ligament, encompassing the root canal and apical foramen of the distal root of first right molar as a measure of the volume of the apical lesion (mm³). The ROI started at the first transaxial cut, where the molar was completely encapsulated by the crestal bone and continued toward the apex of the root, ending at the last slice where the empty space was seen (Austah *et al.* 2016) The tissue volume (TV), alveolar bone volume (BV) ratio were measured using CTan software (Skyscan, Aartselaar, Belgium). The volume was analyzed and used to determine the effect of treatment.

Histological analysis

The jaw was removed, fixed in a solution of 4% buffered formaldehyde for 24h, and then decalcified in buffered (pH = 8) 17% EDTA (Sigma Chemical Co, St Louis, MO). The first lower left molar was 6µm semi-serial cut in laterolateral direction, allowing sectioning in its longitudinal axis. After, were and stained with hematoxylin and eosin (HE). Slides without staining were reserved for immunohistochemistry. Histopathologic analyzes were performed by a single certified histologist (E.E.) who was blinded to the experimental groups using light microscope (DM 4000 B; Leica) and a color camera (DFC 500; Leica, Wetzlar, Germany). The distal root was standardized for all analyzes (Dal-Fabbro *et al.* 2019b). Histopathological analysis was conducted using the following guidelines: quality of inflammation and the cellularity pattern of dental and periodontal tissues to score the inflammatory infiltrate as follows: absent (0 to few inflammatory cells, score = 1), mild (< 25 cells, score = 2), moderate (25–125 cells, score = 3) and severe (> 125 cells, score = 4) (Azuma *et al.* 2017). Presence or absence of necrosis was also recorded (Gomes-Filho *et al.* 2015, Dal-Fabbro *et al.* 2019b).

Immunohistochemical Analysis

Immunolabeling in the histological sections were performed using an indirect immunoperoxidase technique (Gomes-Filho *et al.* 2015): primary antibodies (1:100) against RANKL (Goat anti-RANKL -SC7627, Santa Cruz Biotechnology), OPG (Rabbit anti-OPG - SC11383, Santa Cruz Biotechnology) and TRAP (Goat anti-TRAP -SC30832, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Universal secondary antibody (Universal LSAB + Kit / HRP, Rb/Mo/Goat, Dako Laboratories, Carpinteria, CA, USA) and was counterstained with Harris's haematoxylin. The sections were incubated with biotinylated secondary antibody for 2 hours and subsequently treated with conjugated streptavidin to the - HRP for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, Carpinteria, CA, USA). The disclosure was performed using 3,3'- diaminobenzidine tetrahydrochloride (DAB chromogen Kit®, Dako Laboratories) as a chromogen. As a negative control, the specimens were subjected to the procedures described above by suppressing the primary antibody use.

RANKL and OPG were analyzed in the vicinity of the periapical region at a 400x magnification (Leica Microsystems, Wetzlar, Germany). A semi-quantitative analysis of the immunostaining was carried out using three histological sections of each block. The adopted immunolabeling pattern (IP) was: 1, complete absence of immunoreactive cells; 2 (low IR), a

few immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immunoreactive cells); 3 (moderate IR), a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 4 (high IR), a large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immunoreactive cells) (Garcia *et al.* 2013, Azuma *et al.* 2017). The number of only the mature osteoclasts as TRAP-positive multinucleated cells was quantified in the perimeter of the lesion. The results were expressed as the number of multinucleated TRAP-positive cells per mm (Wayama *et al.* 2015).

Statistical Analysis

The data were analyzed using GraphPad Prism 7 software (La Jolla, CA, USA). After the Shapiro-Wilk test of normality, the Kruskal-Wallis followed by Dunn's test was performed for nonparametric data, and analysis of variance followed by the Tukey test was performed for parametric data. The level of significance was 5%.

Results

Biochemical analysis

The results of the blood tests are shown in Table 1. The plasma calcium and phosphorus concentration remained constant in all groups analyzed ($p>0.05$). The alkaline phosphatase activity was higher in the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* groups than that in the Control group ($p<0.05$). Between the groups receiving probiotic supplementation, no difference was observed (Table 1) ($p>0.05$).

Table 1: Mean and standard deviation biochemical analysis of blood.

Biochemical analysis	Control	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus acidophilus</i>
	Mean±SD*	Mean±SD*	Mean±SD*
Calcium (mg/dL)	10.9±0.56 ^a	11.2±0.43 ^a	10.9±0.56 ^a
Phosphorus (mg/dL)	8.8±0.49 ^a	7.8±0.79 ^a	7.9±0.66 ^a
Alkaline phosphatase (U/L)	234.8±61.42 ^a	332.3±65.55 ^b	322.6±65.9 ^b

*Different letters indicate significant statistical differences in lines (p<0.05).

SD: standard deviation

mg/dL: milligram per deciliter

U/L: units per liter

Micro-Computed Tomography analysis (μCT)

The groups that had their diets supplemented with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* had lower volume of bone resorption when compared to the control group (p<0.05) (Table 2, Figure 1). Between the groups receiving probiotic supplementation, no difference was observed in the volume of bone resorption (p>0.05). (Table 2, Figure 1).

Table 2: Scores and median of intensity of inflammatory cells, necrosis, AP volume (mm³) and immunohistochemical analysis according to the groups.

Histologic parameters		Control	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus acidophilus</i>
Scores				
1		0/8	0/8	0/8
2		0/8	1/8	0/8
3		0/8	5/8	5/8
4		8/8	2/8	3/8
Median*		4^f	3^f	3^f
Necrosis		100%	100%	100%
Volume - μCT (mm³ - SD**)		2,39± 0,40 ^a	1,20± 0,48 ^b	1,21± 0,32 ^b
Immunohistochemical				
Scores				
1		0/8	0/8	0/8
2	<i>RANKL</i>	0/8	1/8	0/8
3		0/8	7/8	6/8
4		8/8	0/8	2/8
Median***		4^a	3^b	3^b
1		0/8	0/8	0/8
2	<i>OPG</i>	8/8	0/8	0/8
3		0/8	8/8	3/8
4		0/8	0/8	4/8
Median***		2^a	3^b	4^c
Number of osteoclasts (mm – SD***)	<i>TRAP</i>	7.6±2.3 ^a	3.9±1.7 ^b	3.9±0.8 ^b

*Histologic parameters: different symbols indicate significant statistical differences in lines (p<0.05).

**Micro-Computed Tomography analysis (μ CT): different letters indicate significant statistical differences in lines ($p < 0.05$).

***Immunohistochemical analysis: different letters indicate significant statistical differences in lines ($p < 0.05$).

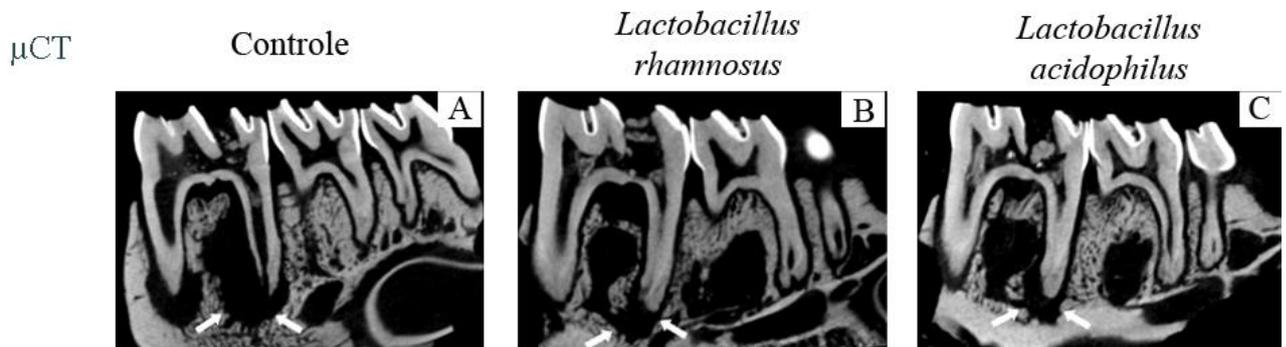


Figure 1: Micro-Computed Tomography analysis (μ CT) aspects of apical periodontitis in sagittal sections in the mandibular first molars control group (A), *Lactobacillus rhamnosus* (B) and *Lactobacillus acidophilus* (C). Arrowheads indicate apical periodontitis. The control group had greater bone loss compared with the other groups.

Histopathologic analysis

The groups that had their diets supplemented with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* had the magnitude of the inflammatory response was lower through the evaluation of inflammatory infiltrate when compared to the control group ($p < 0.05$) (Table 2, Figure 2). Between the groups receiving probiotic supplementation, no difference was observed in the inflammatory infiltrate ($p > 0.05$). In all groups necrosis was observed in the periapical region (Table 2, Figure 2).

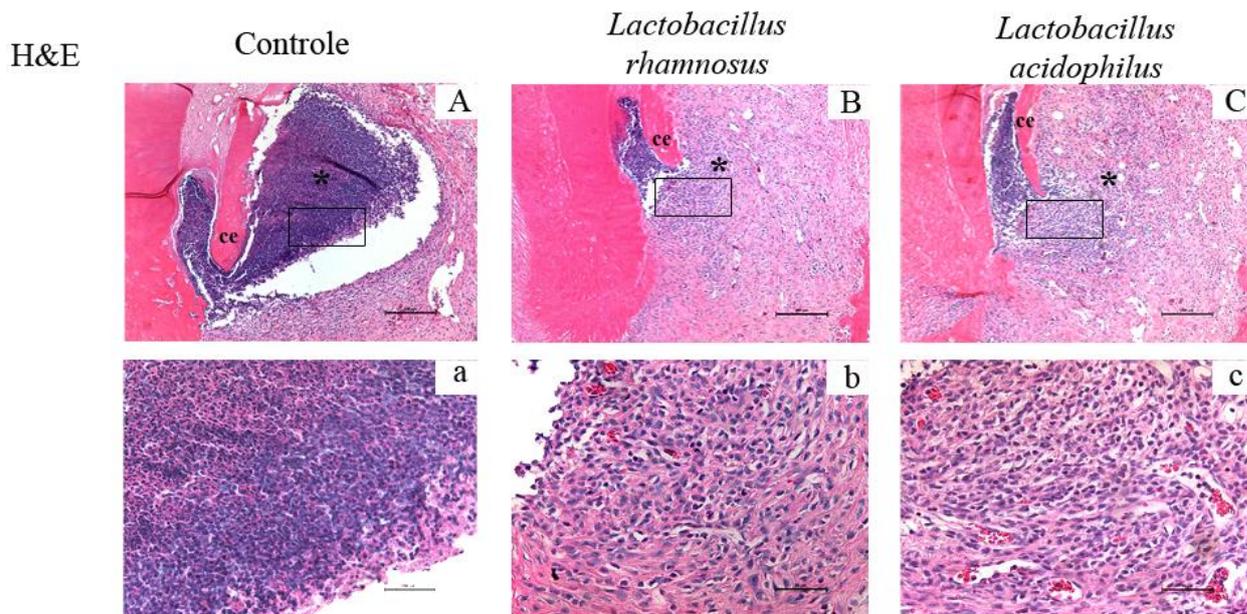


Figure 2: Photomicrographs showing histologic aspects of periapical regions (A-C, a-c). Apical periodontitis of greater severity were observed in Control group (A,a) than groups that consumed probiotics: *Lactobacillus rhamnosus* (B,b) and *Lactobacillus acidophilus* (C,c). ab, alveolar bone; ce, cementum. *Inflammatory infiltrate. Haematoxylin–eosin staining. Rectangle shows area elected for 400X magnification. Original magnification: A, B, C 100X; a, b, c 400X.

Immunohistochemistry

The immunoreactivity pattern for RANKL, OPG and TRAP are described below and in the Table 2.

RANKL: The groups receiving *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* marked a median of score 3. There was no statistically significant difference between them ($p>0.05$) (Table 2, Figure 3). All specimens of the Control group marked score 4 that which was significantly greater when compared to groups receiving probiotic supplementation ($p<0.05$) (Table 2, Figure 3).

OPG: The groups that received *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* marked a median of score 3 for *Lactobacillus rhamnosus* and 4 for *Lactobacillus acidophilus*, with statistically significant difference between them ($p<0.05$) (Table 2, Figure 3). The specimens of the Control group marked a median of score 2 which was significantly lower when compared to groups receiving probiotic supplementation ($p<0.05$) (Table 2, Figure 3).

TRAP: The immunolabeling technique for TRAP was remarkably specific to osteoclasts. The groups that received *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* had a significantly lower load of TRAP -positive multinucleated cells per mm in the periapical region compared to the group Control ($p<0.05$) (Table 2, Figure 3).

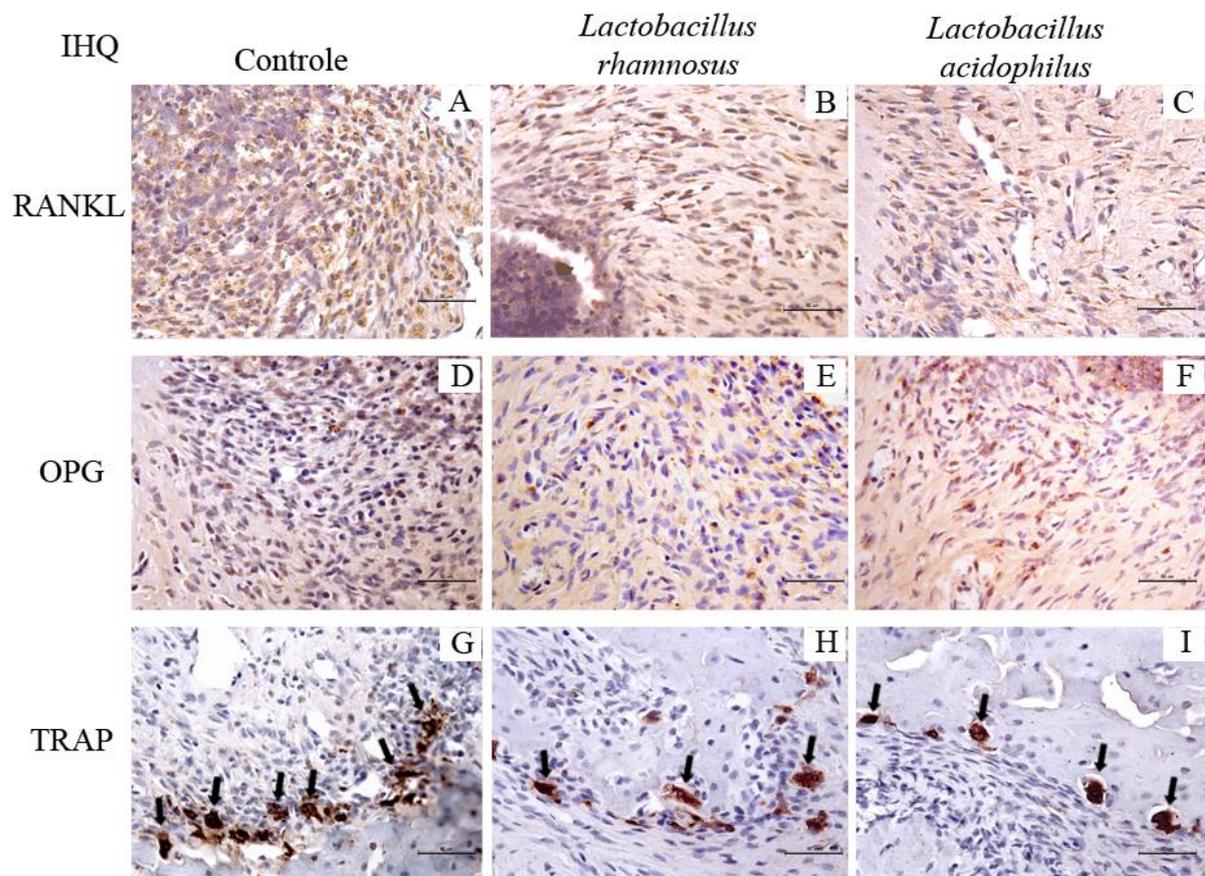


Figure 3: Less intense immunolabeling pattern for RANKL in groups that consume probiotics: *Lactobacillus rhamnosus* (B) and *Lactobacillus acidophilus* (C); More intense immunolabeling pattern for OPG in the *Lactobacillus rhamnosus* (E) and *Lactobacillus acidophilus* (F) when compared to the control group (D); Higher numbers of TRAP-positive multinucleated cells (arrowheads) are in the control group (G) when compared to *Lactobacillus rhamnosus* (H) and *Lactobacillus acidophilus* (I). Original magnification: 400X.

Discussion

Recently, probiotics have been receiving worldwide attention due to their health benefits. For the authors' knowledge, this is the first study that shows the systemic effect of probiotics on apical periodontitis. The null hypothesis of this study was rejected because the pathogenic inflammation/bone resorption induced in the AP by pulp exposure was

significantly suppressed when systemic supplementation with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* was administered demonstrating the beneficial action of probiotics on inflammation and osteoclastogenesis during AP development.

In the present study, the exposure of the pulp tissue to the oral cavity of the animals was used to induce PA for a period of 30 days (Tani-Ishii *et al.* 1994, Gomes-Filho *et al.* 2015, Cintra *et al.* 2016, Dal-Fabbro *et al.* 2019b). It has been shown that the pathogenesis of AP in rats is similar to the pathogenesis of AP in humans (Tani-Ishii *et al.* 1994). In addition, after 30 days of AP induction, chronic disease is observed. In the chronic stage, there are no systemic and local signs of acute infection, which could influence the systemic or local analyses (Tani-Ishii *et al.* 1994, Azuma *et al.* 2017).

Lactobacillus Rhamnosus and *Lactobacillus Acidophilus* were the probiotics chosen for dietary supplementation. Previous studies have shown the role of lactobacillus in inflammation and also in bone health: Lactobacillus has antimicrobial activity, antioxidant and are related to stimulation of the immune system against inflammation (Kitazawa *et al.* 2002). Furthermore, lactobacillus can attenuate bone loss in ovariectomized rats and suggesting lower risk of osteoporosis (Chiang & Pan 2011). In Dentistry, more specifically in Periodontics, the use of lactobacillus improved periodontal health (Toiviainen *et al.* 2015) and significantly reduced alveolar bone loss, detachment and disorganization of the periodontal ligament in rats (Kobayashi *et al.* 2017). In addition, the aim of this study was the systemic evaluation of the action of probiotics in the development of PA, therefore, the gavage method was recommended and daily dose was established according to a previous study that used the dose of 10^9 CFU and obtained satisfactory results as coadjuvant in the treatment of periodontitis (Ricoldi *et al.* 2017).

In the present study, the severity of the inflammatory infiltrate and volume evaluated by μ CT on AP were lower in the group that consumed *Lactobacillus Rhamnosus* and *Lactobacillus Acidophilus*. A correlation between the methods of analysis of apical periodontitis (histological and μ CT analysis) was demonstrated in this study evidenced that probiotic supplementation has influenced on the magnitude of periapical inflammation as well as the relationship between RANKL and OPG and active osteoclast (TRAP) on bone resorption during apical periodontitis development. It was observed through μ CT lower bone damage e through histological analysis lower inflammation when probiotics was used similar to that observed in periodontics (Ricoldi *et al.* 2017). The lower severity of the inflammatory infiltrate in the groups receiving probiotics is showed another study and point out its ability to

enhance immunoregulation via the immune system (Kobayashi *et al.* 2017) and lower count of periodontal pathogens such as *Porphyromonas gingivalis* (Teughels *et al.* 2013).

The present study revealed significantly lower expression of RANKL and higher expression of OPG as well as lower number of osteoclastic cells (TRAP), thereby reducing the areas with periapical bone loss in animals that consumed probiotics corroborating with the probiotic ability to modulate osteoclastogenesis (Britton *et al.* 2014). The OPG/RANKL system is fundamental to the regulation of bone metabolism (Boyce & Xing 2008). The receptor activator of RANKL and OPG are members of the TNF superfamily (Boyce & Xing 2008). In bone, RANKL stimulates osteoclastic differentiation and enhances the activity of mature osteoclasts and inhibits their apoptosis (Boyce & Xing 2008). RANKL exerts its function by binding to the receptor activator of RANK, which is present on the membrane of mononuclear osteoclast precursors (Eriksen 2010). By the other hand, OPG protects the bone tissue from excessive reabsorption by binding to RANKL, thus preventing it from binding to RANK (Boyce & Xing 2008).

The levels of calcium, phosphorus and alkaline phosphatase were analyzed in order to investigate the effects of biochemical parameters. Calcium and phosphate levels were similar between the groups. Calcium and phosphorus are regulators of bone growth and mineralization (Scholz-Ahrens *et al.* 2007). The results of the present study corroborates with previous one that found lactobacillus had no significant effect on mineral absorption in comparison to control group (Scholz-Ahrens *et al.* 2007).

Alkaline phosphatase is the most commonly used marker for bone metabolism and may reflect the extent of bone formation and osteoblast activity (Anh *et al.* 1998). In the present study, plasma alkaline phosphatase level was higher in groups receiving probiotics and this may be related to increased osteoblastic activity in these groups. It was concurrently with the other changes observed: less extension of apical periodontitis and lower severity of inflammatory infiltrate; lower RANKL expression accompanied by greater OPG expression and lower number of TRAP cells in the groups that received *Lactobacillus Rhamnosus* and *Lactobacillus Acidophilus*. In addition, such results corroborate with another study that also demonstrated a higher alkaline phosphatase level when ovariectomized rats were treated with lactobacilos (Montazeri-Najafabady *et al.* 2018).

The present study is the first one to demonstrate the potential effect of probiotic bacterium of the genus *Lactobacillus* in the development of apical periodontitis. However,

new studies are essential for exploring the mechanism of action and to define a solid protocol for probiotic use in the endodontics.

Conclusion

Dietary supplementation with probiotics (*Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) had a significant effect reducing inflammation and bone resorption in apical periodontitis.

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Conflict of interest

The authors deny any conflict to interests to this study.

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Legends

Table 1: Mean and standard deviation biochemical analysis of blood.

Table 2: Scores and median of intensity of inflammatory cells, necrosis, AP volume (mm³) and immunohistochemical analysis according to the groups.

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Figure 2: Photomicrographs showing histologic aspects of periapical regions (A-C, a-c). Apical periodontitis of greater severity were observed in Control group (A,a) than groups that consumed probiotics: *Lactobacillus rhamnosus* (B,b) and *Lactobacillus acidophilus* (C,c). ab, alveolar bone; ce, cementum. *Inflammatory infiltrate. Haematoxylin–eosin staining. Rectangle shows area elected for 400X magnification. Original magnification: A, B, C 100X; a, b, c 400X.

Figure 3: Less intense immunolabeling pattern for RANKL in groups that consume probiotics: *Lactobacillus rhamnosus* (B) and *Lactobacillus acidophilus* (C); More intense immunolabeling pattern for OPG in the *Lactobacillus rhamnosus* (E) and *Lactobacillus acidophilus* (F) when compared to the control group (D); Higher numbers of TRAP-positive multinucleated cells (arrowheads) are in the control group (G) when compared to *Lactobacillus rhamnosus* (H) and *Lactobacillus acidophilus* (I). Original magnification: 400X.

Anexos

ANEXO A

Referências Introdução Geral

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ANEXO B
Comitê de Ética em Pesquisa
(CEP)



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"



CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado "Efeito de probióticos no desenvolvimento de lesão periapical induzida em ratos", Processo FOA nº 00516-2017, sob responsabilidade de João Eduardo Gomes Filho apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 05 de Julho de 2017.

VALIDADE DESTE CERTIFICADO: 15 de Junho de 2020.

DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 15 de Julho de 2020.

CERTIFICATE

We certify that the study entitled "Effect of probiotics on the development of induced periapical lesion in rats", Protocol FOA nº 00516-2017, under the supervision of João Eduardo Gomes Filho presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on July 05, 2017.

VALIDITY OF THIS CERTIFICATE: June 15, 2020.

DATE OF SUBMISSION OF THE FINAL REPORT: July 15, 2020.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
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ANEXO C

Author Guidelines International Endodontic Journal

Content of Author Guidelines:

1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance

Useful Websites: Submission Site, Articles published in International Endodontic Journal, Author Services, Wiley's Ethical Guidelines, Guidelines for Figures The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.



1. GENERAL International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted. Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in International Endodontic Journal. Authors are encouraged to visit Wiley Author Services for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES International Endodontic Journal adheres to the below ethical guidelines for publication and research. 2.1. Authorship and Acknowledgements Authors submitting a paper do so on the understanding that the manuscript has been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal.

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2.2. Ethical Approvals Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used. When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations. All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors **MUST** upload a copy of the ethical approval letter when submitting their manuscript and a separate English translation. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

2.3 Clinical Trials The International Endodontic Journal asks that authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following public clinical trials registries: www.clinicaltrials.gov, <https://www.clinicaltrialsregister.eu/>, <http://isrctn.org/>. Other primary registries if named in the WHO network will also be considered acceptable. The clinical trial registration number and name of the trial register

should be included in the Acknowledgements at the submission stage. 2.3.1 Randomised control clinical trials Randomised control clinical trials should be reported using the guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material. 2.3.2 Epidemiological observational trials Submitting authors of epidemiological human observations studies are required to review and submit a 'strengthening the reporting of observational studies in Epidemiology' (STROBE) checklist and statement. Compliance with this should be detailed in the materials and methods section. (www.strobe-statement.org) 2.4 Systematic Reviews Systematic reviews should be reported using the PRISMA guidelines available at <http://prisma-statement.org/>. A PRISMA checklist and flow diagram (as a Figure) should also be included in the submission material. Systematic reviews must be registered prospectively in the PROSPERO database. The review registration number should be included in the Acknowledgements at the submission stage. 2.5 DNA Sequences and Crystallographic Structure Determinations Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly. 2.6 Conflict of Interest and Source of Funding International Endodontic Journal requires that all authors (both the corresponding author and co-authors) disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company. If authors are unsure whether a past or present affiliation or relationship should be disclosed in the manuscript, please contact the editorial office at iejeditor@cardiff.ac.uk. The existence of a conflict of interest does not preclude publication in this journal. The above policies are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the International Committee of Medical Journal Editors (<http://www.icmje.org/>). It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms individually (do not combine the forms into one file) together with the manuscript on submission. The disclosure statement

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clearly. Technical jargon should be avoided as much as possible and clearly explained where its use is unavoidable. Abbreviations should also be kept to a minimum, particularly those that are not standard. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist. Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Structure

All manuscripts submitted to International Endodontic Journal should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate

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Abstract for Original Scientific Articles should be no more than 350 words giving details of what was done using the following structure:

- **Aim:** Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
- **Methodology:** Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.
- **Results:** Give the main results of the study, including the outcome of any statistical analysis.
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- **Background:** Provide a brief introduction of the subject and why it is important.
- **Aim:** Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
- **Data sources:** Describe the databases searched.
- **Study eligibility criteria, participants, and interventions:** Briefly describe the methods adopted including exclusion/inclusion criteria.
- **Study appraisal and synthesis methods:** Describe bias, study type and quality
- **Results:** Give the main results of the review, including the outcome of any statistical meta-analysis.
- **Limitations:** Highlight problems with the current review and research area
- **Conclusions and implications of key findings:** State the primary conclusions of the study and their

implications. Suggest areas for further research, if appropriate. Abstract for Review Articles (narrative) The Abstract should be unstructured and no more than 350 words. Abstract for Case Reports should be no more than 350 words using the following structure: • Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed. • Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any. • Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report. Abstract for Clinical Articles should be no more than 350 words using the following structure: • Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed. • Methodology: Describe the methods adopted. • Results: Give the main results of the study. • Conclusions: State the primary conclusions of the study. Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested. Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. (i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material. (ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used. When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for

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