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Probiotic consumption decreases the number of osteoclasts during orthodontic movement in mice

Camila Alessandra Pazzini[a,*], Luciano José Pereira[b], Tarcília Aparecida da Silva[c], Carina Cristina Montalvany-Antonucci[c], Soraia Macari[c], Leandro Silva Marques[d], Saul Martins de Paiva[a]

[a] Department of Pediatric Dentistry and Orthodontics, Dental School, Universidade Federal de Minas Gerais – UFMG, Belo Horizonte, MG, Brazil
[b] Department of Health Sciences, Universidade Federal de Lavras – UFLA, Lavras, MG, Brazil
[c] Department of Oral Surgery and Oral Pathology, Dental School, Universidade Federal de Minas Gerais – UFMG, Belo Horizonte, MG, Brazil
[d] Department of Pediatric Dentistry and Orthodontics, Dental School, Universidade Federal do Vale do Jequitinhonha e Mucuri – UFVJM, Diamantina, MG, Brazil

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ABSTRACT

Aims: The aim of the present study was to investigate the effect of probiotic (*Bacillus Subtilis*) supplementation on bone remodelling induced by mechanical loading.
Methods: C57BL/6 mice were divided in two groups: (1) Probiotic and (2) Vehicle (water). The probiotic (1.5×10^8 CFU/mL) was administered orally for 14 days, starting two days before the induction of orthodontic tooth movement (OTM). OTM was determined by histomorphometric analysis by comparing the right to the left side of the maxilla. The number of osteoclasts was determined by counting TRAP-positive cells. Osteoblasts were counted on Masson's trichrome-stained slides.
Results: OTM was similar between groups (with and without probiotic supplementation) ($p = 0.46$). The number of TRAP-positive cells increased ($p < 0.01$) on the experimental side (where the spring coil was installed) in comparison to the control side in both groups. However, the number of osteoclasts decreased ($p < 0.01$) in the probiotic group, in comparison to the vehicle group. There was an increase in the number of osteoblasts ($p < 0.05$) in both the Vehicle and Probiotic groups on the side under OTM, independent of probiotic supplementation.
Conclusion: Oral Supplementation with a probiotic influenced the number of osteoclasts adjacent to the tooth root during orthodontic movement in mice.

1. Introduction

Orthodontic tooth movement (OTM) occurs through the application of sustained controlled mechanical forces in the tooth, which leads to pressure and tension zones in the periodontal ligament and alveolar bone, with subsequent remodelling of the alveolar bone (Gameiro et al., 2008). Bone remodelling associated with tooth movement is characterised by a transitory inflammatory reaction involving leukocytes and bone cells (osteoblasts and osteoclasts) (Alhashimi, Frithiof, Brudvik, & Bakhiet, 2000, 2001; Davidovitch, Nicolay, Ngan, & Shanfeld, 1988; Riancho & Delgado-Calle, 2011; Taddei et al., 2012; Tanaka, Nakayamada, & Okada, 2005). The coordinated action of these two cell types is responsible for bone resorption and deposition, in response to stress and mechanical loading (Hadjidakis & Androulakis, 2006).

Probiotics are live microorganisms (e.g., bacteria of the *Lactobacillus, Enterococcus, Bacillus* and *Bifidobacterium* genuses) (Bron, Van Baarlen, & Kleerebezem, 2012) that when administered in adequate amounts confer benefits to the host and are therefore considered as functional dietary supplements (FAO, 2002; Tsubura et al., 2009). Probiotics are mainly involved in the modulation of pathogenic bacterial adhesion to the intestinal epithelium and their popularity has prompted increased interest for their role in promotion of systemic and oral health (Bhardwaj, 2010). Recently, the probiotic market has been estimated at $15 billion, with a growing rate of 5%–30% (Bhadoria & Mahapatra, 2013). Several studies have shown some effects in inflammation (Amit-Romach, Uni, & Reifen, 2010; Lomax & Calder, 2009; Salminen, Gueimonde,

* Corresponding author at: Rua Padre Dehon, 35, centro, Lavras – MG – Zip Code: 37200-000, Brazil.
E-mail addresses: camilapazzini@hotmail.com, camilapazzini@yahoo.com.br (C.A. Pazzini).

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& Isolauri, 2005), including alveolar bone resorption (Foureaux et al., 2014; Messora et al., 2013). To the authors' knowledge, there is no previous study evaluating the effect of probiotic therapy in OTM. The scientific interest in *Bacillus* species as probiotics has occurred only in the last few years. Among the species that have been most extensively examined are *Bacillus subtilis*. Spore probiotics are being used widely in humans as dietary supplements (Cutting, 2011). A significant increase in patients seeking for better quality of life, health and esthetics (Dos Santos, Meneghim, Ambrosano, Filho, & Vedovello, 2017) may show in the future a clear interaction between probiotic consumption and orthodontic treatment. However, the number of consumers is drastically increasing (Saini, Saini, & Sharma, 2010) but the consequences to orthodontc treatment are sitll unknown. The beneficial effects of probiotics has been studied in various bone diseases such as in postmenopausal women (Parvaneh, Jamaluddin, Karimi, & Erfani. 2014), osteoporosis (Britton et al., 2014; McCabe, Britton & Parameswaran, 2015; McCabe, Irwin, Schaefer, & Britton, 2013) and bone loss in type 1 diabetic mice (Zhang et al., 2015). However, more studies are needed to help advance the science and recognition of the value of probiotics in bone (Sanders et al., 2014).

Because OTM involves inflammatory reactions and because in theory probiotics may modulate the time needed for orthodontic treatment (Budzyński, Wiśniewska, Ciecierski, & Kędzia, 2016; Ettinger, MacDonald, Reid, & Burton, 2014; Terai et al., 2015), the aim of this study was to evaluate the influence of supplementation with probiotic *Bacillus Subtilis* in OTM.

2. Materials and methods

2.1. Experimental mice

A total of 36 eight-week-old male C57BL6/J mice weighing approximately 25 g were used in this experiment. All animals were treated under the ethical regulations for animal experiments, defined by the Institutional Ethics Committee of the Federal University of Lavras, Brazil (protocol number 012/2015). Mice were randomly divided in two groups: (1) Probiotic and (2) Vehicle (water). A probiotic product based on *Bacillus subtilis* (Bioplus® PS – CHR HANSEN, Hørsholm, Denmark) was orally administered for 14 days, starting two days before the installation of a coil spring (on the right side), for up to 12 days of OTM. We dissolved the probiotic in drinking water at dose of 1.5×10^8 CFU/mL daily (Selvam et al., 2009).

All mice received regular diet *ad libitum* throughout the entire experiment. Mice weight was recorded during the entire experiment and mice whose weight loss was higher than 15% were excluded from the analysis.

2.2. Experimental protocol

The experimental protocol was based on previous studies (Taddei et al., 2012, 2013). Briefly, mice were anesthetised with 0.2 mL of a xylazine (0.02 mg/mL) and ketamine (50 mg/mL) solution. An orthodontic appliance consisting of a nickel-titanium 0.25×0.76-mm coil spring (Lancer Orthodontics, San Marcos, CA) was bonded by light-cured resin (Transbond, Unitek/3M, Monrovia, CA) between the maxillary right first molar and the upper incisors. The force magnitude was calibrated by a tension gauge (Shimpo Instruments, Ithaca, IL) in order to exert a force of 0.35 N in the mesial direction. No re-activation was performed during the experimental period. The left side of the maxilla (without an orthodontic appliance) was used as control. Mice were euthanized after 12 days of mechanical loading. For each set of experiments, 18 animals were used.

2.3. Histopathological analysis

The maxillary halves were dissected and fixed in 10% buffered formalin (pH 7.4). First, the anterior maxillary fragment containing the incisors was removed with a scalpel; second, the scalpel was positioned in palatal suture separating the maxillae in right and left helves. Then, right and left maxillae halves, including first, second and third molars were dissected, fixed in 10% buffered formalin for 2 days, decalcified in 14% EDTA (pH 7.4) for 21 days and embedded in paraffin. The entire blocks containing the samples were cut into sagittal sections, after roughing the microtome approximately 120 times of 10 μm thickness. After that, slides of 4 μm thickness were obtained. The slides selected for H&E, and histochemistry presented the first and second molars mesial and distal-buccal root, and the third molar, and adjacent structures as periodontal ligament and alveolar bone. At least five serial vertical sections containing above mentioned structures were evaluated for each animal per analysis. Samples were cut into sagittal sections of 5-μm thickness, stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, Saint Louis, MO) and counterstained with haematoxylin. The mesial side of the first molar distal-buccal root was used for osteoclast counts, which were determined in five consecutive microscopic fields (400×), on five sections per animal. Osteoclasts were identified as TRAP-positive, multinucleated cells on the bone surface. The slides were counted by two examiners, who were blinded to group status. Osteoblasts were counted on Masson's trichrome-stained slides on the distal side of the first molar distal-buccal root.

2.4. Measurement of OTM

Images of the first and second molars were obtained using an optical microscope (Axioskop 40, Carl Zeiss, Gottingen, Germany) and an adapted digital camera (PowerShot A620, Canon, Tokyo, Honshu, Japan). Image J software (National Institutes of Health) was used to quantify the OTM by measuring the difference between the distance at the cement-enamel junction (CEJ) from the right first and second molars in relation to the opposite (control) side of the same animal. Five vertical sections per animal were evaluated, and three measurements were conducted for each evaluation; the variability was below 5%.

2.5. Statistical analysis

The data were expressed as mean ± SEM. Comparisons between groups and between sides were performed using an unpaired and paired "*t*"-test, respectively. For the trap was performed two-way Anova. Data were processed with GraphPadPrism (version 5.01, GraphPad Software, San Diego, USA). The level of significance was adjusted to $p < 0.05$. To validate the consistency of the evaluations, the intraclass correlation coefficient was determined, and there was a significant positive correlation ($p < 0.001$).

3. Results

Histomorphometric analysis showed similar results ($p > 0.05$) in the amount of OTM between groups (Fig. 1A). The number of TRAP-positive cells increased ($p < 0.05$) on the right side (where the spring coil was installed) in comparison to the left side (without movement) in both groups. However, the number of osteoclasts decreased ($p < 0.05$) in the probiotic group in the tooth to which loading was applied (on the right side), in comparison to the control group (which also received loading) (Fig. 1B). After 12 days, we found greater amounts of TRAP-positive cells in the vehicle group (Fig. 1C and D) as compared to the probiotic group (Fig. 1E and F) for the same condition (tooth receiving loading).

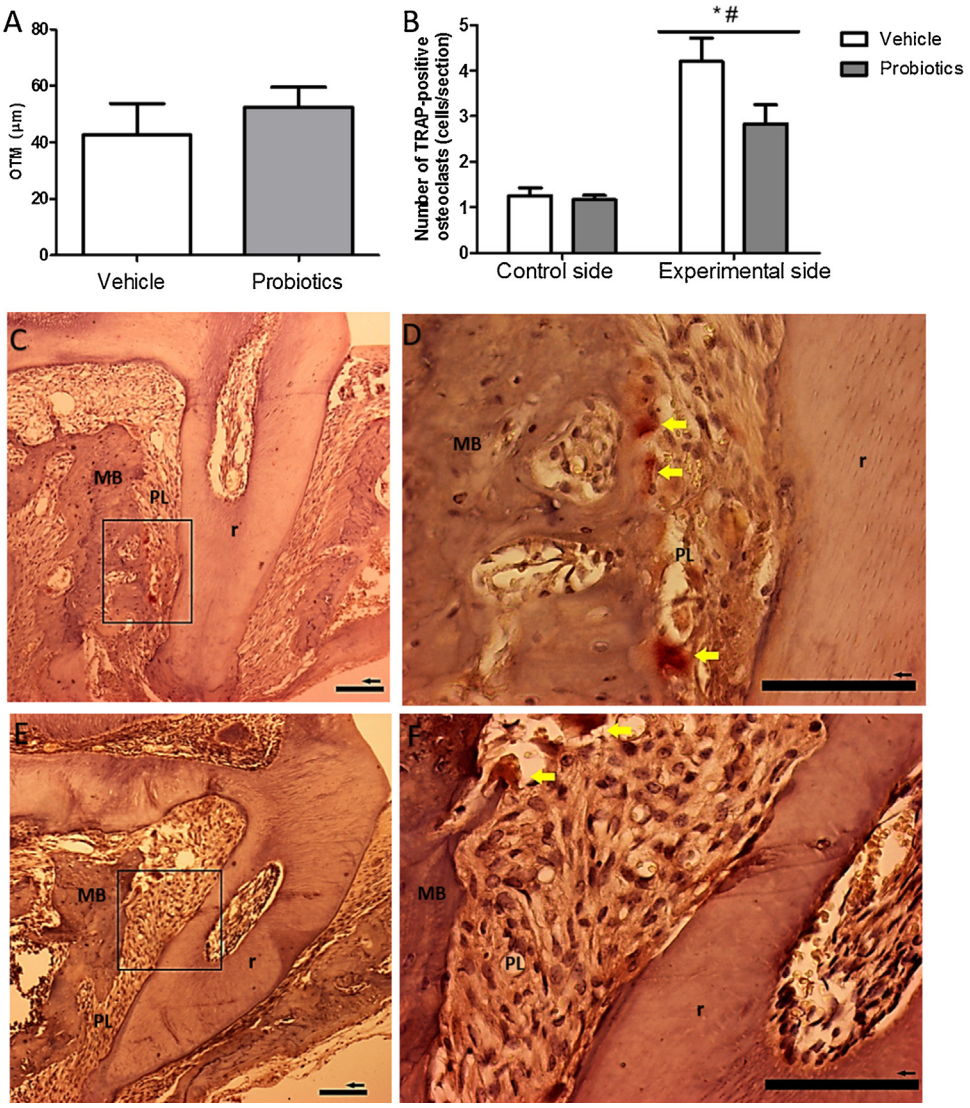

Fig. 1. A OTM in vehicle and probiotic groups; B Number of TRAP-positive osteoclasts; C and E Histologic changes related to OTM in Vehicle (C) and Probiotic (E) groups; D and F higher views of the areas identified in C and E. *Yellow arrows*, TRAP-positive osteoclasts; *MB*, mesial alveolar bone; *PL*, periodontal ligament; *r*, root; *black arrows* indicate the direction of tooth movement. Data are expressed as means and standard deviations. *$p \leq 0.05$ comparing the results between control and experimental sides of the same group. #$p \leq 0.05$ comparing the results between vehicle and probiotic groups in experimental side. Bar = 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

There was no significant difference ($p. > 05$) for the number of osteoblasts between groups (Vehicle × Probiotics) for right (under OTM) and left sides (without OTM) (Fig. 2A). There was an increase in the number of osteoblasts ($p < 0.01$) when the right and left sides of each group were compared (Fig. 2B–E).

4. Discussion

In this study, 12 days of probiotic therapy promote a decrease in the number of osteoclasts in the tissues around the tooth submitted to mechanical loading. The number of osteoblasts was influenced by OTM but not by probiotic supplementation. Similarly, previous studies showed that probiotic supplementation increases osteoblasts and decreases osteoclasts in a model of ovariectomised rats (Parvaneh et al., 2015). Furthermore, bone resorption markers such as Trap5 and RANKL, as well as osteoclastogenesis, were significantly decreased in mice treated with probiotics (Britton et al., 2014).

Bone remodeling is a dynamic process necessary for bone homeostasis and orchestrated by bone-producing osteoblasts, and bone-resorbing osteoclasts and their molecular signaling (Xiao, Wang, Pacios, Li, & Graves, 2016). In the present study, the number of TRAP-positive cells has increased on the experimental side in comparison to the control side. While, the number of osteoclasts decreased in the probiotic group. However, the number of osteoblasts has not changed. OTM was similar between groups with and without probiotic intake. The possible explanation for the similarity of OTM between groups is that despite the fact that there is lower osteoclasts account, they still active promoting bone resorption and tooth movement. Previous studies from our group (Andrade, Silva, Silva, Teixeira, & Teixeira, 2007; Macari et al., 2016; Moura et al., 2014) demonstrated that 12 days reproduces better results of orthodontic tooth movement. Once this study was related to probiotic intake, which the systemic effect on bone is directly dependent on the mice consumption – what could take more time to see the results – we have decided to analyze the effects of the mechanical loading on the tooth in only one time point period of 12 days. We consider our study pioneer as the first one linking probiotic consumption and tooth movement. News studies are encouraged varying not only the time but also other

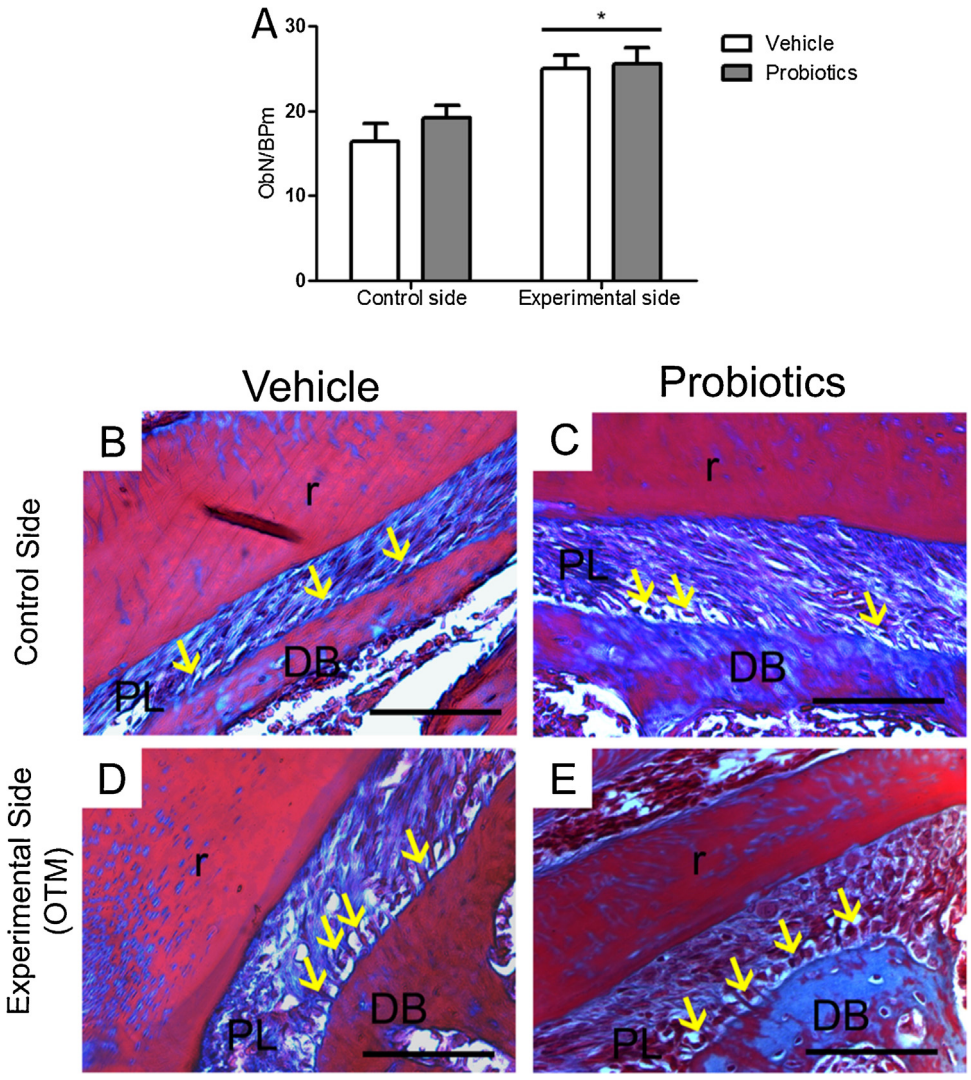

Fig. 2. A Number of osteoblasts per bone perimeter; B and D Histologic changes between control (B) and experimental (D) sides in the vehicle group; C and E Histologic changes between control (C) and experimental (E) sides in the probiotics group *; DB,* distal bone; *PL*, periodontal ligament; *r*, root; *yellow arrows*, osteoblasts. Data are expressed as means and standard deviations. $^{*}p \leq 0.05$ comparing the results between control and experimental sides of the same group. Bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species of probiotic microorganisms and doses in order to elucidate the mechanisms involved in its consumption and tooth movement (Sanders et al., 2014).

Probiotics can modulate the immune response by reducing the production of pro-inflammatory cytokines and increasing the production of anti-inflammatory cytokines (Borchers et al., 2009; Selvam et al., 2009; Yan & Polk, 2010). In this sense, we can suppose that the consumption of probiotic-enriched products can provide many benefits (especially for osteoporotic patients), but it can impair orthodontic treatment. One of the goals of orthodontic movement is to move the teeth in an efficient manner with a minimum of adverse effects for these and supporting tissues (Von Böh & Kuijpers-Jagtman, 2009). To our knowledge, this is the first study evaluating probiotic supplementation with simulated orthodontic treatment. The consumption of probiotics during treatment could interfere with the success of orthodontic movement as the bone remodelling during tooth movement is characterised by an inflammatory reaction (Davidovitch et al., 1988; Taddei et al., 2012), and probiotics alter osteoclastogenesis.

The *Bacillus subtilis* used in our study was chosen because it was previously proven to be effective in other oral inflammatory conditions such as periodontal disease and bone loss (Foureaux et al., 2014; Messora et al., 2013; Tsubura et al., 2009). The effects in the oral cavity can induce local and systemic reactions, because it was administered in drinking water, passing through the oral route (Shimauchi et al., 2008). Probiotics act primarily on the gut microbiota. The influence of probiotic consumption on metabolic or inflammatory diseases such as diabetes and inflammatory bowel disease is well established. However, new studies are revealing that interactions with gut microbiota may also be crucial for skin, lungs, arteries, and bone. These interactions are mainly because of its impact on nutrient acquisition (calcium and phosphate), immune regulation, and direct effects mediated by the production of small molecules such as serotonin or oestrogen-like molecules (McCabe et al., 2015).

Previous studies from our group (Foureaux et al., 2014; Messora et al., 2013) have demonstrated that probiotic supplementation reduces alveolar bone loss in rats with periodontitis. Nevertheless, it is worth noting that periodontal disease treatment prevents alveolar bone loss, while orthodontic treatment requires bone remodelling. Published studies pertaining to the use of probiotics in OTM are scarce. More studies are needed to understand the real effects of these supplements. Future studies should include clinical trials, diverse types of animal models and microorganism types, as

well as a range of doses and treatment durations. New studies investigating other treatment durations, doses and other types of probiotic yeast are encouraged. These studies would contribute to a better understanding of the mechanisms involved in OTM accompanied by probiotics consumption.

5. Conclusion

Probiotic therapy for 12 days decreased the number of osteoclasts in the periodontal tissues of teeth submitted to mechanical loading in a specific mouse model.

Conflicts of interest

The authors report no commercial, proprietary, or financial interest in the products or companies described in this article.

Our paper has been professionally proofread (Proof-Reading-Service.com Ltd).

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