RENATA DE CARVALHO FOUREAUX

EFEITOS DA TERAPIA PROBIÓTICA (*Bacillus subtilis*) SOBRE PARÂMETROS METABÓLICOS E INFLAMATÓRIOS EM RATOS COM PERIODONTITE INDUZIDA POR LIGADURA ASSOCIADA OU NÃO AO ESTRESSE CRÔNICO

LAVRAS – MG

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APROVADA em 27 de outubro de 2014.

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“Sonhe com aquilo que você quiser. Seja o que você quer ser, porque você possui apenas uma vida e nela só se tem uma chance de fazer aquilo que se quer. Tenha felicidade bastante para fazê-la forte. Tristeza para fazê-la humana. E esperança para fazê-la feliz. A felicidade aparece para aqueles que choram. Para aqueles que se machucam. Para aqueles que buscam e tentam sempre. E para aqueles que reconhecem a importância das pessoas que passam por suas vidas.”

Clarice Lispector

RESUMO

A doença periodontal é caracterizada por um processo inflamatório crônico dos tecidos de suporte dos dentes, dependente da resposta do hospedeiro, em reação à presença de periodontopatógenos no biofilme subgengival. Medidas locais e sistêmicas de modulação da resposta imunológica e do ambiente microbiano têm sido investigadas. Nesse contexto surgem os probióticos que são organismos vivos que quando administrados em quantidades adequadas conferem benefícios à saúde, despertando o interesse de sua aplicação na terapia periodontal. O presente estudo foi dividido em dois capítulos. No primeiro, foi feita uma revisão sistemática avaliando-se a eficácia dos probióticos na resposta imune em ratos submetidos a desafios experimentais. Os trabalhos foram pesquisados em três bases Pubmed, ISI Web of Science e Scielo. As espécies bacterianas mais empregadas foram os *Lactobacillus* e *Bifidobacterium.* Oitenta e seis porcento dos artigos selecionados mostraram efeito benéfico na resposta imune associado ao uso de probióticos. No segundo capítulo foi avaliado o efeito da terapia probiótica em ratos com indução de doença periodontal associada ao estresse de restrição. O delineamento experimental foi inteiramente casualizado em esquema fatorial 2x2x2 (com e sem doença periodontal (DP), com e sem estresse crônico (EC), com e sem o uso de probióticos) com seis repetições em cada grupo. O probiótico *Bacillus subtilis* foi administrado oralmente na proporção de $1,5x10^8$ unidades formadoras de colônia (UFC)/mL na água de bebida dos animais durante 45 dias. O EC foi realizado por meio de imobilização diária por 2,5 horas a partir do 16º dia durante 30 dias, enquanto que a DP foi induzida com o protocolo de ligadura nos primeiros molares mandibulares direito e esquerdo a partir do 31º dia por 14 dias. Foram medidas a altura de vilosidades e profundidade de criptas do duodeno, jejuno e íleo. A eutanásia foi feita no 45º dia do experimento. O nível de significância foi fixado em $p < 0,05$. A DP aumentou a perda óssea alveolar, os níveis de ciclooxigenase-2 (COX-2), telopeptídeo carboxiterminal do colágeno tipo-1 (CTX), p38 quinases ativadas por mitógeno (MAPK), ligante do receptor ativador da NF-κB (RANKL) e diminuiu os níveis de osteoprotegerina (OPG) ($p < 0,05$). Os ratos estressados apresentaram níveis mais altos de peptídeo-C, corticosterona e glicose ($p < 0.05$). A presença do estresse reduziu a expressão de CTX e p38 ($p < 0.05$). O probiótico reduziu a perda óssea alveolar nos ratos não estressados, bem como diminuiu a expressão do CTX e induziu ao aumento da expressão da OPG em ratos não estressados com DP ($p < 0,05$). Embora o probiótico não tenha sido efetivo na prevenção da perda óssea ou alteração da expressão dos marcadores inflamatórios em ratos estressados, o número de células inflamatórias diminuiu ($p < 0.05$). Os grupos com estresse e

DP apresentaram diminuição na altura das vilosidades e profundidade de criptas intestinais ($p < 0,05$). Em conclusão geral, os probióticos foram benéficos na modulação da resposta imune e se mostraram promissores no controle da doença periodontal.

Palavras-chave: Modelos animais. Estresse. Periodontite. Ligadura. Perda de inserção periodontal.

ABSTRACT

Periodontal disease is characterized by chronic inflammation of the tissues supporting the teeth, depending on the host response, in reaction to the presence of periodontal pathogens in sub-gingival biofilm. Local and systemic measures modulation of the immune response and of microbial environment has been investigated. In this context emerge probiotics which are live organisms that when administered in adequate amounts give beneficial health effects, arousing the interest of its application in periodontal therapy. The present study was divided in two chapters. In the first, it was made a systematic review evaluating the probiotics effect on immune response in rats submitted to different experimental challenges. The survey was using PubMed, ISI Web of Science and Scielo databases. The bacteria species most used were *Lactobacillus* and *Bifidobacterium*. Eighty-six percent of the selected articles reported a beneficial effect on the immune response associated to the probiotics use. In the second chapter it was evaluated the probiotic therapy effect in rats with periodontal disease induction associated with restriction stress. The experimental design was completely randomized factorial 2x2x2 (with and without periodontal disease (PD), with and without chronic stress (CS), with and without probiotics use) with six replicates in each group. The probiotic *Bacillus subtilis* was administered orally at 1.5 x 10^8 proportion of colony-forming units (CFU)/mL to the drinking water of the animals for 45 days. The CS was performed by immobilization daily for 2.5 hours from the 16^{th} day during 30 days, while the PD was induced by the ligation protocol in the first right and left mandibular molars from 31^{st} day for 14 days. Villus height and crypt depth of the duodenum, jejunum and ileum were measured. Euthanasia was performed on the 45^{th} day of the experiment. The significance level was set at $p<0.05$. PD increased alveolar bone loss, levels of cyclooxygenase-2 (COX-2), carboxyterminal telopeptide of collagen type-1 (CTX), p38 mitogen-activated kinases (MAPK), ligand receptor activator of NF-κB (RANKL) and decreased levels of osteoprotegerin (OPG) ($p<0.05$). The stressed rats had higher levels of C-peptide, corticosterone and glucose ($p<0.05$). The presence of stress decreased expression of p38 and CTX ($p<0.05$). The probiotic reduced alveolar bone loss in the non-stressed rats, as well as decreased expression of CTX and induced the increased expression of OPG in unstressed rats with PD ($p<0.05$). Although the probiotic has not been effective in preventing bone loss or alteration of the expression of inflammatory markers in stressed rats, the number of inflammatory cells decreased ($p<0.05$). Groups with stress and PD showed decrease in villous height and depth of intestinal crypts ($p<0.05$). In general conclusion, probiotics were beneficial in the modulation of immune response and have a promising in the control of periodontal disease.

Keywords: Animal models. Stress. Periodontitis. Ligature. Periodontal attachment loss.

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SEGUNDA PARTE - ARTIGOS

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LISTA DE ABREVIATURAS E SIGLAS

ACTH	Hormônio Adrenocorticotrófico
DP	Doença periodontal
CH201	Bioplus 2B - CH Hansen (Hørsholm, Denmark)
cm	Centímetro
CO	Crista Óssea
COX-1	Ciclooxigenase-1
COX-2	Ciclooxigenase-2
CRF	Hormônio de Liberação de Corticotropina
CTX	Telopeptídeo Carboxiterminal do Colágeno Tipo 1
DCR	Doença Crônica Renal
EC	Estresse crônico
EDTA	Ácido etilenodiamino tetra-acético
FAO	Organização das Nações Unidas para Alimentação e Agricultura
HPA	Eixo hipotálamo-hipófise-adrenal
IBD	Doença Inflamatória do Intestino
IFN-γ	Interferon-gama

Ig	Imunoglobulina
IL	Interleucina
JEC	Junção Cemento-Esmalte
LPS	Lipopolisacarídeos
kg	Quilograma
kV	Quilovolts
mA	Miliampere
MAPK	Quinases ativadas por mitógeno
mg	Miligrama
MMP	Matriz Metaloproteinase
NFκβ	Fator Nuclear kappa Beta
NK	Células Natural *Killer*
OPG	Osteoprotegerina
PGE_2	Prostaglandina E_2
pH	Potencial Hidrogeniônico
PIE	Perda de Inserção Epitelial
PMN	Polimorfonucleares
POA	Perda Óssea Alveolar

RAR	Raspagem e Alisamento Radicular
RNA	Ácido Ribonucleico
RANK	Receptor ativador do fator kappa B (NF-κβ)
RANKL	Ligante do Receptor ativador do fator kappa B (NF-κβ)
SOP	Suporte Ósseo Periodontal
SP	Substância P
TIMPs	Inibidores de Metaloproteinases Teciduais
TNF-α	Fator de Necrose Tumoral alfa
UFC	Unidades Formadoras de Colônias
VIP	Peptídeo Vasoativo Intestinal
WHO	Organização Mundial de Saúde

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A doença periodontal é caracterizada por um processo inflamatório crônico dos tecidos de suporte dos dentes, dependente da resposta do hospedeiro, em reação à presença de periodontopatógenos no biofilme subgengival (HAYTAC et al., 2014). A etiologia da forma mais comum de DP – a gengivite - está relacionada à presença de biofilme microbiano (estrutura bem organizada de bactérias) aderido a superfície dentária (TERHEYDEN et al., 2014). As principais espécies patógenas que compõem o biofilme dentário e que determinam as várias formas de periodontite são: *Porphyromonas gingivalis, Prevotella intermedia, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum*, entre outros (SOCRANSKY et al., 1998).

Adicionalmente aos microrganismos existentes na cavidade bucal, o estresse pode ter efeitos negativos sobre o sistema imunológico e/ou mediar efeitos comportamentais nas defesas do organismo, contribuindo sobremaneira na etiologia e perpetuação da periodontite crônica (BOYAPATI; WANG, 2007). O estresse pode alterar a resistência tecidual do hospedeiro por mecanismos autonômicos e endócrinos, resultando principalmente na elevação dos níveis de corticosteroides e catecolaminas, reduzindo a microcirculação da gengiva e do fluxo salivar, com relatos de redução das funções de neutrófilos e linfócitos, o que facilita a invasão bacteriana e o dano tecidual (BOYAPATI; WANG, 2007; HORNING; COHEN, 1995; JOHNSON; ENGEL, 1986).

A remoção do biofilme e diminuição do número de agentes bacterianos por meios físicos, como: escovação, raspagem e alisamento coronoradicular, irradiação laser, fototerapia (ALMEIDA et al., 2007; CARVALHO et al., 2011; LOPES et al., 2010) e químicos locais - clorexidina, óleos essenciais

(HAFFAJEE et al., 2009; KRAYER et al., 2010) são utilizados no tratamento convencional da periodontite crônica. Porém, outras medidas, tais como antibioticoterapia local ou sistêmica também têm sido empregadas em casos graves especialmente (FERNANDES et al., 2010). Mas, o desenvolvimento da resistência aos antibióticos por certos patógenos tem aumentado a possibilidade do retorno a uma época pré-antibióticos (HAYTAC et al., 2014).

Portanto, uma vez que o processo inflamatório é dependente da reação imunológica individual frente à presença do biofilme, uma resposta inadequada ou excessiva a agressão bacteriana é também fator preponderante na progressão da doença periodontal (DOMON et al., 2014; TAKAHASHI et al., 2014; TERHEYDEN et al., 2014). O controle da resposta imunológica e do ambiente microbiano, como por exemplo, o emprego de probióticos, tem surgido como terapia adjuvante no controle da doença periodontal (DEVINE; MARSH, 2009). Esses podem promover uma defesa natural contra bactérias patógenas, o que pode ser favorável para manutenção da saúde bucal. Porém, a identificação de probióticos úteis, o estabelecimento da dose mais apropriada e o veículo de seu uso necessitam de mais investigações (HAYTAC et al., 2014).

Nos sistemas imune e inflamatório a comunicação celular é organizada por proteínas, peptídeos ou moléculas mensageiras chamadas citocinas. Essas moléculas interagem seletivamente com receptores transmembranas, ativando outros mensageiros intracelulares que amplificam, transportam o sinal ao núcleo celular, onde modulam a atividade gênica e síntese proteica (TERHEYDEN et al., 2014). O entendimento dessas vias de sinalização na imunologia da periodontite é fundamental para o entendimento das citocinas e quimiocinas envolvidas na comunicação celular e na defesa do organismo (EBERSOLE et al., 2013) de forma a propociar e desenvolver modalidades terapêuticas alternativas.

Diante do exposto, no presente estudo objetivou-se avaliar os efeitos da ingestão de probióticos em uma revisão sistemática e seu uso em ratos *Wistar* com ou sem indução da doença periodontal por ligadura associado ou não ao estresse crônico de imobilização.

2 REFERENCIAL TEÓRICO

2.1 Doença periodontal

A doença periodontal afeta extensivamente as populações e é o principal fator de risco para a perda dentária (DYE 2012; GHIZONI et al., 2012; HUMPHREY et al., 2008; KURITA-OCHIAI et al., 2014; MACRI et al., 2014; OLIVER et al., 1998).

Segundo a *American Academy of Periodontology* (2000), a gengivite induzida pelo biofilme é definida como uma inflamação da gengiva sem a perda da inserção epitelial. Pode ser caracterizada pela presença de alguns dos seguintes sinais: vermelhidão e edema dos tecidos gengivais, sangramento provocado, mudanças do contorno e consistência, presença de cálculo e/ou biofilme e sem evidência radiográfica de perda da crista óssea. É caracterizada pela presença de bactérias Gram-negativa, bastonetes móveis e filamentos.

A periodontite crônica com leve a moderada perda de suporte periodontal é definida como a inflamação da gengiva extendendo aos tecidos de suporte adjacentes. A doença é caracterizada pela perda de inserção associada à destruição do ligamento periodontal e perda do osso de suporte adjacente. A destruição leve à moderada é geralmente caracterizada pela sondagem das bolsas periodontais até 6 mm com perda de inserção clínica até 4mm. As evidências radiográficas mostram perda óssea e aumento da mobilidade dentária que pode estar presente. Apresenta uma população predominante de bactérias Gram-negativa, com uma grande proporção de espiroquetas (AMERICAN ACADEMY OF PERIODONTOLOGY, 2000).

A periodontite crônica com perda avançada de suporte periodontal é definida como inflamação da gengiva e tecidos adjacentes. A doença é caracterizada pela perda de inserção clínica acompanhada pela destruição do

ligamento periodontal e perda do tecido ósseo adjacente de suporte. A destruição é geralmente caracterizada pela sondagem das bolsas periodontais maiores que 6mm com perda de inserção maior que 4mm. A evidência radiográfica de perda óssea é aparente e o aumento da mobilidade pode estar presente. Apresenta uma população predominante de bactérias Gram-negativa, com uma grande proporção de espiroquetas (AMERICAN ACADEMY OF PERIODONTOLOGY, 2000). Na periodontite crônica *A. actinomycetemcomitans* e *P. gingivalis* mostraram ser capazes de invadir o epitélio gengival *in vitro* (FERES et al., 2004).

A periodontite agressiva envolve diferentes tipos de periodontia que afetam as pessoas que, na maioria dos casos, podem parecer saudáveis. Pode ser familiar e há uma rápida taxa de progressão da doença. A periodontite agressiva ocorre de forma localizada e generalizada (AMERICAN ACADEMY OF PERIODONTOLOGY, 2000).

A periodontite agressiva localizada, usualmente, tem um começo circumpubertal com o início da destruição periodontal localizado nos primeiros molares permanentes e incisivos, embora padrões atípicos de dentes afetados serem possíveis. A doença é frequentemente associada com o patógeno periodontal *Actinobacillus actinomycetemcomitans* e anomalias das funções dos neutrófilos, além de coccos e bastonetes facultativos. Uma forte resposta dos anticorpos séricos aos agentes infectantes é detectada frequentemente (AMERICAN ACADEMY OF PERIODONTOLOGY, 2000).

A periodontite agressiva generalizada, usualmente, afeta pessoas com 30 anos ou menos, mas podem ser em mais velhos. Há pelo menos três dentes permanentes, sem ser os primeiros molares e incisivos, afetados pela perda de inserção interproximal. A perda de inserção ocorre em períodos pronunciados de destruição. A doença é frequentemente associada aos patógenos periodontais *Actinobacillus actinomycetemcomitans* e *Porphyromonas gingivalis* e anomalias

das funções dos neutrófilos, além de coccos e bastonetes facultativos. Uma pobre resposta dos anticorpos séricos aos agentes infectantes é detectada frequentemente (AMERICAN ACADEMY OF PERIODONTOLOGY, 2000).

A doença periodontal é uma infecção crônica, assintomática, com incidência mundial e especialmente prevalente em populações mais idosas. Relatos recentes mostram as associações e o potencial dos mecanismos biológicos entre a doença periodontal e outras doenças sistêmicas, associando a doença periodontal como um fator de risco para enfermidades cardíacas, aterosclerose, doença crônica pulmonar obstrutiva, diabetes *mellitus* e câncer, além de causar um estado crônico de inflamação sistêmica (BOYLAN et al., 2014; GHIZONI et al., 2012; KURITA-OCHIAI et al., 2014; MACRI et al., 2014; SCARABELOT et al., 2014; TSAI et al., 2014).

As bactérias *Porphyromonas gingivalis e Aggregatibacter actinomycetemcomitans,* agentes periodontopatógenos muito prevalentes podem acelerar a deposição de ateromas em modelos animais (GHIZONI et al., 2012; KURITA-OCHIAI et al., 2014). Além dessas bactérias ainda existem outras espécies que estão fortemente ligadas à progressão da doença periodontal, tais como *Bacteroides forsythus*, *Prevotella intermedia*, *Peptostreptococcus micros* e *Fusobacterium nucleatum* (ALJEHANI, 2014).

A doença periodontal é caracterizada pela destruição irreversível das estruturas de suporte dos dentes, incluindo ligamento periodontal, osso alveolar e tecidos gengivais, levando a um colapso do cemento pela condição inflamatória dos tecidos moles ao redor dos dentes (KURITA-OCHIAI et al., 2014; MACRI et al., 2014). A reabsorção óssea alveolar patológica na periodontite é um resultado da infecção crônica com remissões e progressões, apresentando patogênese semelhante a outras doenças que afetam os ossos (GURSOY et al., 2013). Assim, o plano de tratamento deve incluir tanto considerações locais como sistêmicas (TSAI et al., 2014).

Existem microrganismos colonizadores da pele, boca, tratos digestivo e reprodutivo, que constituem estruturas tridimensionais de agregados de bactérias que se unem umas as outras e também à superfície formando biofilmes. Principalmente àqueles associados a bactérias anaeróbias gram-negativas, têm uma participação bem estabelecida nas doenças orais, tais como nas cáries dentária, gengivite e periodontite, existindo mais de quinhentas espécies bacterianas distintas (KURITA-OCHIAI et al., 2014; MANCL; KIRSNER; AJDIC, 2013). Porém, somente um pequeno número está associado à doença e considerado etiologicamente importante (ALJEHANI, 2014). Estudos sugerem que os agentes etiológicos específicos da doença periodontal incluem espiroquetas, fusiformes e estreptococos (FERES et al., 2004).

Os patógenos periodontais presentes nos biofilmes microbianos iniciam a doença difundindo produtos deletérios e enzimas, tais como hialuronidases, colagenases e proteases que quebram as matrizes extracelulares, tais como colágeno e membranas celulares, com o intuito de produzir nutrientes para seu crescimento e subsequente invasão tecidual (GULATI et al., 2014), levando os tecidos periodontais a atuar como reservatórios de endotoxinas, citocinas, lipídeos e mediadores inflamatórios que podem afetar outras partes do organismo (SCARABELOT et al., 2014).

A invasão bacteriana resulta em uma resposta inflamatória nos tecidos periodontais caracterizada por uma produção de citocinas, tais como interleucinas (IL), fator de necrose tumoral (TNF-α), prostanoides, tais como prostaglandina E2 e enzimas incluindo as matrizes metaloproteinases (MMPs) (ALJEHANI 2014; GULATI et al., 2014; SCARABELOT et al., 2014) e diminuição dos níveis de osteoprotegerina (OPG) não só nos tecidos gengivais e saliva, mas também no plasma em indivíduos afetados pela doença periodontal (JIANG et al., 2013). Em indivíduos saudáveis, os níveis desses mediadores nos tecidos periodontais são equilibrados pelas citocinas e enzimas anti-

inflamatórias do sistema imune que tem a função de eliminar os patógenos microbianos e proteger o hospedeiro (GULATI et al., 2014).

2.2 Modelos experimentais de doença periodontal

Modelos animais podem ser utilizados para se avaliar a progressão e o efeito de diferentes tratamentos em doença periodontal induzida (MOLON et al., 2014; WEINBERG; BRAL, 1999), bem como os efeitos do estresse sobre a progressão da perda óssea alveolar (TAKADA et al., 2004; ZHAO et al., 2012).

O modelo experimental de indução da doença periodontal em ratos por ligadura com fio de algodão mostra ser adequado na obtenção das alterações na estrutura óssea periodontal, atuando como um local de colonização bacteriana (MOLON et al., 2014; OZ; PULEO, 2011; YAGAN et al., 2014). Os ratos também têm várias similaridades com os humanos com relação à anatomia periodontal, formação e composição do biofilme dental, histopatologia das lesões periodontais e imunologia básica (CHANG et al., 2014; EBERSOLE et al., 2013; MACRI et al., 2014), facilitando dessa forma a extrapolação dos resultados (GENCO et al., 1999; KLAUSEN; EVANS; SFINTESCU, 1989; MOLON et al., 2014).

O modelo experimental de indução da doença periodontal por ligadura é caracterizado pelo acúmulo de biofilme, aplainamento e deslocamento da crista gengival, aumento da proliferação do epitélio abaixo da linha do tecido conjuntivo e infiltração de células mononucleares inflamatórias (ALMEIDA et al., 2007). A inflamação associada à indução da doença periodontal por ligadura iniciada na margem gengival é causada tanto pelo trauma mecânico bem como o aumento do acúmulo de microrganismos no biofilme (HOLZHAUSEN et al., 2002). O modelo experimental de indução da doença periodontal por injeção de lipopolissacarídeo (LPS) é um método relativamente simples em que uma única

bactéria infecta o animal levando a destruição periodontal (NAKAJIMA et al., 2006). O lipopolissacarídeo compreende a superfície exterior de todas as bactérias gram-negativas subgengivais, capaz de penetrar nos tecidos conjuntivos (CHANG et al., 2014) e pode induzir a inflamação no tecido periodontal, podendo afetar os leucócitos circulantes diretamente e ativar os osteoclastos (DO et al., 2013). Existem diferenças nos métodos de indução de doença periodontal em modelo de rato na ativação da cinética das vias de sinalização inflamatórias, dependendo do tipo de resposta do hospedeiro. A indução por lipopolissacarídeo (LPS) foi associada com uma ativação mais lenta das vias de sinalização comparada com o modelo de ligadura, exceto para ERK MAPK (AQUINO et al., 2009).

A partir da indução, vários métodos podem ser empregados para avaliação da evolução da doença periodontal, tais como os métodos radiográfico (KESAVALU et al., 2006; REED; POLSON, 1984), morfométrico (BAKER et al., 1983; YU et al., 2007) e histométrico (ALMEIDA et al., 2007; WOLFSON; SELTZER, 1975).

Os sinalizadores inflamatórios podem destruir a integridade tecidual levando ao acesso bacteriano mais profundo no tecido, podendo estar relacionado à gravidade da doença periodontal (TSAI et al., 2014). Além disso, a sinalização e a regulação da expressão desses marcadores inflamatórios representam um papel crítico na remodelação óssea do periodonto (KANZAKI et al., 2002).

No processo inflamatório ocorre a liberação de citocinas que interagem com os tecidos (células endoteliais, macrófagos, plaquetas, entre outras), promovendo a ativação de vias inflamatórias diversas, como por exemplo, a do ácido araquidônico. A ativação da fosfolipase A2 pelas citocinas promove a conversão dos fosfolipídeos da membrana em ácido araquidônico, que por sua vez é utilizado como substrato das cicloxigenases (COX) para formação de

prostaglandinas e prostaciclinas que são importantes mediadores da inflamação (MESA et al., 2012; TSAI et al., 2014). Existem duas isoformas de cicloxigenase: COX-1 e COX-2. A COX-1 é uma enzima geralmente constitutiva responsável pela formação de prostaglandinas nas funções fisiológicas (proteção gastrintestinal, por exemplo), enquanto COX-2 é uma enzima induzida principalmente por citocinas proinflamatórias envolvidas nos processos patofisiológicos, tais como vasodilatação, aumento da permeabilidade vascular, diapedese e migração leucocitária. As prostaglandinas são potentes estimuladores da formação e reabsorção óssea e são produzidas pelos osteoblastos e células do ligamento periodontal (KAYAL et al., 2013). A prostaglandina E_2 (PGE_2) é associada como potente estimuladora na reabsorção óssea e perda de inserção epitelial na doença periodontal (TSAI et al., 2014). A expressão de COX-2 nos tecidos gengivais tem sido relacionada à severidade da doença periodontal e inflamação gengival, indicando um papel importante dessa proteína na patogenia da periodontite crônica (MESA et al., 2012).

O receptor ativador do fator kappa B (RANK), o ligante do receptor ativador do fator kappa B (RANKL) e osteoprotegerina (OPG) são citocinas pertencentes à super família de fator de necrose tumoral alfa (TNFα) (KAYAL et al., 2013). O RANK está presente nos precursores de osteoclastos e é capaz de iniciar a sinalização da transdução osteoclastogênica, depois da ligação com RANKL ou com o agonista anti-RANK. O RANK é um receptor capaz de mediar a função do RANKL durante a homeostasia do osso normal e na doença e nenhum outro receptor para RANKL foi identificado.

O RANKL é produzido pelas células T ativadas e sua expressão é regulada para cima por muitos fatores solúveis que afetam a reabsorção óssea, incluindo citocinas pró-inflamatórias. O RANKL é um fator crítico no sistema imune como um importante coestimulador na ativação das células T, aparecendo também como um elo entre a inflamação e a perda óssea (EBERSOLE et al.,

2013; KANZAKI et al., 2002). Esse sistema é regulado pela osteoprotegerina (OPG) que neutraliza a habilidade do RANKL se ligar com RANK e induzir o sinal para diferenciação dos osteoclastos (DOMON et al., 2014). A via OPG/RANKL é a chave reguladora do metabolismo ósseo e seu efeito produz o desenvolvimento e ativação ou não dos osteoclastos.

Ainda no processo inflamatório periodontal, o telopeptídeo do colágeno tipo I de ligação cruzada carboxiterminal (CTX) é um marcador bioquímico de reabsorção e formação óssea (FLEISHER et al., 2010; KWON et al., 2009). Trata-se de um produto da degradação do colágeno usado como medidor de reabsorção óssea (FLEISHER et al., 2010).

Outros fatores de transcrição, tais como fator nuclear κB (NF- κB), proteína ativadora-1 (AP-1) e p38 são expressos em quantidade aumentada no processo inflamatório, estimulando a produção de várias citocinas, muitas delas direta ou indiretamente estimuladoras da formação dos osteoclastos (KAYAL, 2013).

As quinases ativadas por mitógeno (MAPKs) são uma família evolutiva e mediam processos fundamentais biológicos e respostas celulares a diferentes estímulos extracelulares por receptores múltiplos. As três principais subfamílias de MAPKs são quinases reguladoras extracelulares (ERK-1/-2), quinases ativadoras do c-Jun N-terminal (JNK) e p38. Os lipopolissacarídeos (LPS) facilitam a ativação dessas três principais subfamílias (FUJITA et al., 2014). A ativação da p38 leva ao aumento da expressão de vários genes de citocinas pela modulação de ambos os mecanismos transcricional e pós- transcricional. A contribuição de cada mecanismo na mudança global da expressão gênica varia

de acordo com o tipo de células e natureza do estímulo externo, mas os genes que são modulados pelos mecanismos pós-transcricionais envolvendo a modificação das proteínas que se ligam ao RNA como substratos para p38 são TNF-α, IL-8, IL-6, IL-2 e ciclooxigenase-2 (FUJITA et al., 2014; LIANG et al., 2014; SOUZA et al., 2012; TRAVAN et al., 2013).

A sinalização da MAPK p38 é requerida na inflamação e associada à perda óssea decorrente de doença periodontal nos modelos animais, com uma significativa correlação positiva na severidade da doença. Os sinalizadores MAPK são vitais para a síntese e amplificação de mediadores pró-inflamatórios e matriz metaloproteinases pelas células sinoviais, quimioatração de células mononucleares e angiogênese de células endoteliais, bem como a apoptose de células sinoviais. Assim, a p38 parece estar predominantemente envolvida nos processos inflamatórios (TRAVAN et al., 2013).

2.3 Estresse e doença periodontal

O estresse é um estado de tensão fisiológico ou psicológico causado por estímulos adversos, tais como físico, mental ou emocional, interno ou externo, que tende a perturbar o funcionamento do organismo (GOYAL et al., 2013). O fator socioeconômico, tipo de ocupação, rotina, competitividade no trabalho, distúrbios emocionais podem levar ao aumento dos níveis de estresse. Em modelos animais existe a influência de alguns fatores como imobilização, temperaturas adversas, restrição, conflitos na ordem social, carência de água ou comida, manipulação, acomodações na gaiola (umidade e inclinação), entre outros e têm um efeito direto no eixo hipotálamo-hipófise-adrenal (SUTANTO; KLOET, 1994).

Há a hipótese que a ativação prolongada desse eixo pode deteriorar a saúde e pode promover um elo entre o estresse mental e a doença física (doença

periodontal, por exemplo). A secreção de hormônios do estresse prejudica a defesa do hospedeiro e ajuda no crescimento de organismos oportunistas no sulco gengival (GOYAL et al., 2013). Doenças sistêmicas, tais como gastrite e colite ulcerativa também estão ligadas ao estresse. Assim, o impacto do estresse sobre a saúde periodontal não é apenas pela sua presença ou ausência, mas o tipo, duração e como um indivíduo lida com ele. Os indivíduos sob estresse tendem a adotar mudanças comportamentais, como da má higiene bucal, tabagismo, apertamento ou ranger de dentes (GOYAL et al., 2011).

O estresse pode mediar efeitos na imunidade e/ou no comportamento das defesas do organismo, contribuindo com a etiologia e perpetuação da doença periodontal. O estresse pode mudar a resistência dos tecidos do hospedeiro por mecanismos autonômicos e endócrinos, resultando primariamente em aumento dos níveis de catecolaminas e corticoides (FOUREAUX et al., 2014).

O estresse psicológico resulta em secreção de adrenalina e noradrenalina das células da medula adrenal. Através da interação com receptores adrenérgicos, noradrenalina e adrenalina mediam efeitos cardiovasculares e metabólicos. Em amostras de sangue coletadas imediatamente antes e depois de uma situação de estresse emocional, a concentração circulante de linfócitos T-helper, células T citotóxicas (CD8 +) e as células natural killer (células NK), é aumentada, mas uma hora depois é reduzida para os valores iniciais. Além disso, os níveis plasmáticos de imunoglobulinas IgM, IgG e componente C3 do sistema complemento são elevados depois de uma situação de estresse agudo. Além disso, a liberação de neuropeptidos, tais como a substância P (SP) que causa dilatação e aumento da permeabilidade vascular, tanto diretamente como através do estímulo à liberação e à produção de eucosanoides (prostaglandinas e leucotrienos) pelos mastócitos, também modulam a atividade do sistema imunológico e a liberação de citocinas (GOYAL et al., 2013).

Durante uma resposta ao estresse, o hipotálamo secreta o fator liberador de corticotropina (CRF), o que estimula ainda mais o córtex adrenal e induz a produção e liberação de hormônios glicocorticoides. Esses glicocorticoides exercem os seus principais efeitos supressores, reduzindo o número e atividade (quimiotaxia, secreção e degranulação) de células inflamatórias, incluindo os linfócitos, monócitos, macrófagos, neutrófilos, eosinófilos e mastócitos e também inibem a produção de mediadores pró-inflamatórios, citocinas IL-1, IL-2, IL-3, IL-6, fator de necrose tumoral (TNF), interferon-γ, de granulócitos e monócitos e da cascata da resposta imunitária através da inibição da apresentação de antígeno de macrófagos, proliferação de linfócitos, e a diferenciação de células efetoras de linfócitos, tais como linfócitos auxiliares, linfócitos citotóxicos, as células NK e as células formadoras de anticorpos B (GOYAL et al., 2013).

Os dois hormônios do eixo HPA, hormônio corticotrófico (CRF) e hormônio adrenocorticotrófico (ACTH), além de aumentarem e manterem a resposta inflamatória (MOLON et al., 2014), também modulam separadamente a atividade do sistema imunológico, regulando a produção de substâncias sinalizadoras a partir de células do sistema imune (citocinas), tais como IL-1 pelos monócitos e bloqueando a ativação dos macrófagos. Eles também promovem a proliferação de células B, mas inibem a produção de anticorpo (GOYAL et al., 2013).

A periodontite é uma doença multifatorial causada primariamente por microrganismos, mas é significativamente dependente da resposta do hospedeiro à invasão bacteriana (MUWAZI et al., 2014; TSAI et al., 2014). Indivíduos com comportamento inadequado frente ao estresse têm maior risco de desenvolver a doença periodontal severa. O estresse está associado à higiene oral deficiente, aumento da secreção de glicocorticoides que podem deprimir a função

imunológica, aumento da resistência à insulina e aumenta o risco da periodontite (ALJEHANI et al., 2014).

Mousavi Jazi et al. (2013) mostraram que o estresse pode aumentar os níveis de citocinas pró-inflamatórias IL-1β, IL-6 e IL-10 e diminuir a produção de IFN-γ que pode induzir a resposta Th1, sugerindo que existe uma interação entre os sistemas endócrino e a resposta imune a um estresse fisiológico. Além disso, os indivíduos com transtornos de humor também tiveram uma resposta inflamatória exagerada ao estresse psicológico em comparação com indivíduos saudáveis. Nesses estudos o valor médio de IL-1β no grupo periodontite agressiva foi observado como sendo cerca de duas vezes maior do que no grupo de pacientes com periodontite crônica e cerca de quatro vezes mais elevada do que o grupo saudável.

Os níveis totais de IL-1β, IL-6 e IL-8 são, significativamente, elevados em pacientes com doença periodontal, quando comparados com indivíduos saudáveis. Adicionalmente, a gravidade do estresse tem uma forte relação com a quantidade de IL-1β, tanto em pacientes com periodonte agressiva como crônica (GIANNOPOULOU et al., 2003).

2.4 Probióticos e doença periodontal

A *Food and Agriculture Organization* (FAO) e *World Health Organization* (WHO) definiram como probióticos os organismos vivos que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro (FOOD AND AGRICULTURE ORGANIZATION – FAO / WORLD HEALTH ORGANIZATION - WHO, 2002).

O objetivo do tratamento periodontal é reduzir a infecção dos tecidos periodontais através de rigorosa educação para higiene oral e pelo tratamento mecânico (raspagem e alisamento corono-radicular e/ou cirurgia periodontal),

associados à terapia antimicrobiana química incluindo a administração sistêmica de antibióticos nas formas crônicas graves e formas agressivas de periodontite (HUCK et al., 2011).

Porém, o biofilme apresenta um problema terapêutico à medida que oferece resistência à terapêutica microbiana convencional (MANCL; KIRSNER; AJDIC, 2013). Teughels et al. (2011) investigaram o uso dos probióticos influenciando a microbiota e a saúde periodontal e concluíram que os probióticos podem melhorar a saúde periodontal pela interação microbiológica ou pela interação imunomodulatória.

Assim, a identificação de probióticos úteis, o estabelecimento da dose mais apropriada e o veículo para seu uso são áreas de investigação ativa. Embora os probióticos estejam nos estágios iniciais da pesquisa científica e de sua aplicação, eles aparentam ser uma ferramenta promissora (HAYTAC et al., 2014). O impacto dos probióticos na saúde oral é relativamente novo com várias pesquisas surgindo (KARUPPAIAH et al., 2013).

Os mecanismos de ação dos probióticos variam de acordo com a linhagem ou combinações de linhagens específicas utilizadas, a presença de prebióticos e da condição de tratamento, bem como a fase do processo da doença em que o probiótico é administrado. Existem discussões comuns emergentes nos estudos sobre os mecanismos de ação dos probióticos e numerosas hipóteses têm sido propostas, incluindo:

a) a inibição da adesão de patógenos, colonização e a formação do biofilme;
b) a indução de expressão de proteínas citoprotetoras na superfície das células hospedeiras;
c) inibição de colagenases e redução de moléculas de inflamação associadas;

d) a estimulação e modulação da resposta imune do hospedeiro, por exemplo, por redução da produção de citocinas pró-inflamatórias através de ações sobre NFkB aumentando a produção de citocinas anti-inflamatórias tais como IL-10;
e) modulação da proliferação celular e da apoptose;
f) destruição ou inibição do crescimento de patógenos através da produção de bacteriocinas ou outros produtos, tais como o ácido ou peróxido, que são antagônicos em relação a bactérias patogênicas;
g) os probióticos também podem modificar o ambiente, através da modulação do pH e/ou o potencial de oxidação-redução, que pode comprometer a capacidade de agentes patogênicos de se tornarem estabelecidos (GUPTA, 2011).

Nos estudos de Teughels et al. (2011) foram avaliados os efeitos de pastilhas de *Lactobacillus reuteri* contendo probióticos como um adjunto para raspagem e alisamento radicular (RAR). Após 12 semanas de tratamento, todos os parâmetros clínicos foram significativamente diminuídos, com destaque para a redução da profundidade da bolsa, ganho de inserção nas bolsas moderadas e profunda e redução da contagem de *Porphyromonas gingivalis*.

Um estudo prévio do nosso grupo avaliou a influência do uso do probiótico *Bacillus subtilis* (CH201) administrado oralmente e os níveis de perda óssea alveolar foram significativamente reduzidos em animais com doença periodontal que receberam probiótico (MESSORA et al., 2013).

No trabalho de Maekawa e Hajishengallis (2014), animais com doença periodontal e tratados com *L. brevis* CD2 mostraram significativa diminuição da perda óssea e menor expressão do fator de necrose tumoral e interleucina-1β, IL-6 e IL-17A quando comparados com o tratamento controle.

2.5 Probióticos e imunidade

O trato gastrintestinal é um local onde a microbiota e antígenos são mais expostos ao sistema imune (VITETTA et al., 2013). Tem sido sugerido que a composição da microbiota intestinal esteja associada a condições alérgicas, doenças inflamatórias intestinais, câncer, diabetes, doenças cardiovasculares e dislipdemia (GOMES et al., 2014).

A microbiota do trato gastrintestinal é altamente estruturada e exerce ampla influência protetora, estrutural, metabólica e imune dentro do intestino e de forma sistêmica. A microbiota comunica-se com o sistema imune do hospedeiro e, além disso, sinaliza vias de interação com órgãos tais como fígado, músculos e cérebro, compreendendo uma série de ligações metabólicas hospedeiro-microrganismos (VITETTA et al., 2013).

Os probióticos têm sido mostrados como moderadores em uma variedade de funções fisiológicas do trato gastrintestinal que incluem controle regulador sobre as respostas imunes, a função de barreira epitelial e proliferação celular (GOMES et al., 2014; VITETTA et al., 2013).

A resposta do sistema imune, fatores genéticos e fatores ambientais afetam o risco de desenvolvimento de doenças periodontais (MESA et al., 2014), dependendo de como o hospedeiro responde à microbiota, tanto comensal como patógena (EBERSOLE et al., 2013).

3 CONSIDERAÇÕES GERAIS

O uso de probióticos e suas aplicações vêm ganhando espaço. Existe um aumento de evidências de que a espécies de probióticos podem ser benéficas à saúde oral e sistêmica. Mais estudos serão necessários para otimizar o uso, dose, administração e quantificar a extensão dos benefícios, bem como entender a habilidade de sobrevivência da bactéria, o crescimento e o mecanismo de ação sobre as diferentes doenças em que os organismos são usados. Foi observado que o estresse crônico diminuiu os efeitos dos probióticos diminuindo os efeitos imuno-modulatórios nos tecidos periodontais e ainda o uso de probiótico protegeu o tecido intestinal.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Effect of Probiotic Administration on the Immune Response: A Systematic Review of Experimental Models in Rats

Silva, V. O.; Foureaux, R. C.; Araujo, T. S.; Peconick, P.; Zangeronimo, M. G.; Pereira, L. J. Effect of Probiotic Administration on the Immune Response. Brazilian Archives of Biology and Technology, Curitiba, v.55 n.5: pp. 685-694, Sept/Oct 2012.

Effect of Probiotic Administration on the Immune Response: A Systematic Review of Experimental Models in Rats

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ABSTRACT

The probiotic influence on the immune system, especially under pathogenic challenge conditions, still remains controversial. To address this, a systematic review of current studies concerning the efficacy of probiotics on the immune response of rats subjected to experimental challenges was conducted. The survey was conducted using PubMed, ISI Web of Science and Scielo databases. Only studies which tested probiotics in vivo *in rats were included. The experimental design, methodological quality, and results of the articles were analyzed. In total 21 articles were selected for this study. The most commonly used microorganisms in the experiments were those of the genus* Lactobacillus*, which was reported in 12 articles. The second most often used genus was* Bifidobacterium *(*B. animalis *and* B.longum*). In general, the probiotics use against experimental pathogenic challenges was successful: 86% of the selected articles reported a beneficial effect on the immune response associated with the use of probiotics.*

Keywords: immunity, probiotics, rats, dietary supplements

1 INTRODUCTION

It is well known that the nutrition, through a series of complex interactions, is able to improve the health status of the animals. In animal production, several substances have been used as growth promoters, including probiotics, which are live microorganisms that improve the microbial balance in the gastrointestinal tract, thereby increasing the efficiency with which the nutrients are used. In other areas, probiotics have been used for preventive purposes, to inhibit the proliferation of microorganisms that cause gastrointestinal disturbances (Chaucheyras-Durand et al. 2008; Vanderpool et al. 2008; Mountzouris et al. 2009; Chaucheyras-Durand and Durant 2010; Maragkoudakis et al. 2010).

By definition, probiotics are microorganisms that are regulated as dietary supplements when ingested in sufficient quantities, have beneficial effects on the health of the host (FAO 2002; Budiño et al. 2005; Siró et al. 2008; Tsubura et al., 2009). Most probiotics contain bacteria of the genus *Lactobacillus* and *Bifidobacterium* (Brizuela et al. 2001; Peran et al. 2006; Zeng et al. 2009; Bloise et al. 2010; de Roock et al. 2010).

However, certain bacteria of the genus *Enterococcus* (Maragkoudakis et al. 2010), *Leuconostoc* and *Streptococcus* (Zanini et al. 2007) and yeasts, such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Baptista et al. 2005; Generoso et al. 2010) can be considered to be probiotic microorganisms.

Numerous studies have demonstrated the effectiveness of these microorganisms at improving the intestinal health of the animals and, thereby, their metabolic and physiological status Brizuela et al. 2001). Besides the direct effect of probiotics on the adherence of pathogenic bacteria in the intestinal epithelium, several studies have also correlated probiotic

administration with the positive effects on the immune response in animals (Borchers et al. 2009; Amit-Romach et al. 2010; Generoso et al. 2010; Fink 2010) and humans (Nomoto 2005; Salminen et al. 2005; Lomax and Calder 2009). Other benefits identified in *in vitro* studies include significant inhibition of infection by *L. monocytogenes* (Corr et al., 2007); strong induction of IL-12 and TNF-α in monocytes and cultured human peripheral blood mononuclear cells (PBMC) (Fink 2010); and inhibition of the growth of *C. albicans* (Verdenelli et al., 2009), among others. However, no consensus exists in the literature on the preventive or therapeutic use of probiotics to improve the immune system's ability to defend against different infectious agents.

Animal models, such as rats, are often used to simulate the physiological and pathological mechanisms *in vivo*. Results are then extrapolated to other species, which cannot be directly investigated, due to ethical, financial and/or facilities management issues, or simply because of a lack of physical space (Fagundes and Taha 2004; Da Matta 2010). Thus, detailed studies on a single species are necessary for comparative analysis. Therefore, the objective of the present study was to conduct a systematic review of the efficacy of probiotics on the immune response in rats.

2 MATERIALS AND METHODS

Research strategy

An electronic search of the PubMed database (http://www.ncbi.nlm.nih.gov) was conducted in October 2010, using the following keywords: immunity, probiotics, rats. To confirm the findings and obtain supplementary studies, a similar strategy was employed for the ISI Web of Science database (http://apps.isiknowledge.com) and Scielo database (http://www.scielo.org/php/index.php), using the same keywords (also in Portuguese and Spanish, when applicable).

Study Selection

For the present review, only in vivo studies using probiotics and rats were selected. Studies conducted on mice, rabbits, guinea pigs, or other types of animal models were excluded.

No restrictions were made for the type of probiotic used in the study, administration form, or administration period against an experimental challenge (for prevention and treatment). Additionally, no date, language or number of animals were restricted as selection criteria.

Data extraction and Quality criteria

Two researchers conducted article searches separately, and independently verified the compliance of the selected papers with the inclusion criteria. In the cases of divergence between the papers, all the criteria were reviewed and discussed. Table 1 displays the data related to the experimental design of the retrieved articles. After study selection, quality analysis was conducted and scores were assigned to specific scientific criteria as described in

Table 2. Selection criteria were defined to evaluate both the protective effects of probiotics in relation to the immune system and the methodological quality of the selected articles.

However, not all the parameters used were scored on the quality scale (such as animal strain, type of microorganism used and evaluated technique, among others), but were taken into consideration as they were relevant to the subsequent discussion. The scientific criteria used were adapted from other systematic reviews (Noli and Auxilia 2005; Negre et al. 2009; Pereira et al. 2010). The parameters were classified as either adequate (score: 2) or unclear/partially adequate (score: 1). The following parameters were scored:

- Sample number: Studies with sample groups containing $\geq$ 6 animals received a score of 2 and studies with less than 6 animals per group received a score of 1.
- Randomization: Studies reporting nonrandomized experiments or studies for which the degree of randomization was not clearly described in the text received a score of 1, while studies using randomized experimental designs received a score of 2.
- Control group: Studies that included a control group received a score of 2, while studies that did not include a control group or did not clearly mention a control group in the text received a score of 1.
- Blind evaluation: Studies which included blind assessments in their experimental design received a score of 2, while studies whose experimental designs did not include blind assessments, or for which blind assessments were not clearly reported in the text received a score of 1.
- Interference factors: Studies that did not evaluate interference factors received a score of 1, while studies which considered

additional factors, such as stress, hormonal evaluation, and variations between the males and females received a score of 2.

- Pathogenic challenge: Studies which did not include an experimental challenge received a score of 1, while studies which subjected the animals to an experimental challenge received a score of 2.

The maximum total score was 12 points.

3 RESULTS

An initial search of the PubMed database retrieved 24 articles. Of these, three were excluded because were conducted in humans or mice; one evaluated the isolated action of prebiotics;three others were also excluded because they were literature reviews. Thus, of the initial 24 articles retrieved, 18 were selected for this study.

A search of the ISI Web of Science database also retrieved 24 articles, eight of which were duplicates of articles retrieved from PubMed. Of the 16 remaining articles, five were excluded because they were studies on humans, sows and piglets, prebiotics, or were performed *in vitro*; two others were excluded because they did not evaluate probiotics, and six more were excluded because they were literature reviews. Therefore, three additional papers were selected from this search. A search of the database Scielo did not identify additional articles. Thus, in total 21 articles met the inclusion and exclusion criteria, and were selected for this review. Table 1 presents a summary of the selected studies.

The following rat lineages were used in the studies included in this review: Wistar, Lewis, Sprague Dawley and Fischer. The combinations of two distinct lineages of rats were also used, as well as the combinations of rats with Balb-C mice. In three studies, the authors did not report the rat lineages used.

Bacteria from the genus *Lactobacillus* were the most commonly used microorganisms in the selected studies, and were reported in 57% of the papers. The second most common genus was *Bifidobacterium* (*B. animalis* and *B. longum*). The combinations of microorganisms were used in 38% of the papers, such as *Lactobacillus helveticus* + *Streptococcus thermophilus* or *Streptococcus thermophilus* + *Lactobacillus acidophilus* + *Bifidobacterium lactis*, among others.

In 48% of the articles, probiotics were administered in the feeding, while in 38% of the studies, probiotics were administered by gavage. Other forms of administration, such as water or castor oil were also mentioned. There was a large variability in the amount of colony forming units (CFU) used among the surveys, and no consensus technique emerged, even among the studies dealing with the same species of bacilli. The duration of probiotic administration (e.g. before, during or after experimental challenge) also varied considerably: probiotics were administered both before and after the challenge in 33% of the studies; only during the challenge in 14%; and only after the challenge in 19%. In 19% of the studies, probiotics were administered throughout the study period, independent of the timing of the experimental challenge. Other studies administered probiotics only a few days before the animals were killed, in the pre- and post-operatory period.

Of the 21 selected articles, only one reported having conducted a blind evaluation (So et al. 2008), while 16 articles reported randomization of the sample. The number of animals per group ranged from 1 to 32, although three papers did not report the number of animals used per experimental group.

A total of 90% of the articles induced a pathogenic challenge: 38% introduced an intestinal challenge (e.g. colitis or tumors, among others); 9.5% introduced encephalomyelitis; 9.5% induced liver injury; 4.8% induced respiratory allergies; 4.8 % induced arthritis; 4.8% challenged the animals with *Escherichia coli*; 4.8% induced ischemia and infusion; 4.8% induced intracerebroventricular cannulation; and 4.8% were challenged by the introduction of air pockets into the back of the animals. In 4.8% of the papers, both encephalomyelitis and respiratory allergy were induced simultaneously (Ezendam et al. 2008).

With respect to the interference factors 24% of the articles separated male and female groups, while 5% used the models of stress. However, the

vast majority (71% of the articles) did not report any interference factor. In the work of Laudanno et al. (2008), both the sexes (male/female) and stress were evaluated. All the studies used control groups.

4 DISCUSSION

Literature reviews are useful to the scientific community in general, and can provide significant insight into a particular research field, since they enable a more complete view of current results. In addition, they can suggest the best protocols to be employed and/or future directions for research (Snodgrass 2006). The present literature review on the efficacy of probiotics at improving the immune response in rats focused on targeting which therapeutic protocols were associated with the best (or more promising) results in this species, and could be used as a guide to future studies attempting to reproduce these experiments in other species.

Research using animal models are important, especially given the limitations of investigating certain diseases directly in humans, which often involves the ethical issues and/or risks related to the disease under study. Diseases which can be induced in animal models have the potential to reveal the pathological mechanisms that can be extrapolated to humans, increasing the understanding of human disease. Thus, the use of animal models can help overcoming numerous research limitations and often provides causal relationships more quickly. For these reasons, experimentation on animal systems often represents the first step in many research projects (Taha and Fagundes 2004; DaMatta 2010).

According to Nomoto (2005), excessive use of the antibiotics can induce an imbalance in the intestinal microbiota, encouraging the emergence of antibiotic-resistant bacterial infections, and, at the same time, reducing the possible activation of the immune system prior to infection. Because of this problem, interest in the use of probiotics as a complement to antibiotics has been growing.

Although it is known that probiotics have different properties and functions, the mechanisms by which individual probiotics act in a host are not fully understood. As described in the literature, probiotics are assumed to act via several mechanisms, including: a) competitive exclusion, where probiotics compete with the pathogens for fixation sites and nutrients, thereby temporarily preventing the pathogenic action; b) production of antimicrobial substances, such as bacteriocins, hydrogen peroxide and volatile organic acids; c) induction of direct changes in the immune response, through immune stimulation of residing cells in the enteric tract, which then initiate activation of macrophages, increasing phagocytosis; and d) modulation of enzyme activity by changing the microbial metabolism (Audisio et al. 2000; de Vrese et al. 2001; Ogawa et al. 2001; Cross 2002; Puupponen-Pimia et al. 2002; Hamilton-Miller 2004; Boirivant and Strober 2007; Gillor et al. 2008; Borchers et al. 2009; Ng et al. 2009; Rijkers et al. 2010; Yan and Polk, 2010).

Of the 21 articles selected, 86% reported the beneficial effects from the administration of probiotics on the immune response in rats. Two studies, one conducted by Baken et al. (2006) and one by Guitard et al. (2006) reported unsatisfactory results from the use of probiotics, suggesting that further studies were necessary. Baken et al. (2006) induced autoimmune encephalomyelitis, the same challenge experiment used by Maassen et al. (2008), who concluded that probiotics could suppress this disease. Ezendam et al. (2008) observed a significant reduction in the duration of clinical symptoms, and an improvement in weight gain versus the control group. Guitard et al. (2006) investigated the effect of probiotic administration on the development and progression of an experimental parasite infection (cryptosporidiosis) in lactating rats. Although the rats administered probiotics tended to display faster parasite clearance than the controls, no significant effect was observed in terms of weight gain, parasite burden, mucosal damage or cytokine kinetics in the mucosa during the

course of the infection. Overall, these authors found that daily administration of probiotic mixtures containing *Lactobacillus casei* was not able to eradicate the parasite in their experimental model. However, differences in probiotic strains and dosages could justify the discrepancies between these studies.

The animals underwent intestinal challenge in 48% of the assessed studies, and all responded positively to the use of probiotics, with the exception of Guitard et al. (2006). The immunostimulant effect associated with probiotic administration could be related to the ability of these microorganisms to interact with Payer's patches and intestinal epithelial cells, thereby activating the mucosal immunity by stimulating the plasma cells, IgA secretion and migration of intestinal T cells (Park et al. 2002; de Vrese et al. 2005).

Of the articles investigating induced respiratory allergies (n=4 articles), only two reported a positive response. However, probiotics were found to increase the phagocytic activity of alveolar macrophages, suggesting that they could act systemically by inducing the secretion of mediators which could then stimulate the adaptive immune system (Cross 2002).

Significant differences were observed with respect to the doses of probiotics used. However, no differences in results were noted between the highest (Beaulieu et al. 2007) and lowest administered doses (Aguilar-Nascimento *et al*. 2006): both showed positive immune responses. The immune response to the use of probiotics was also not dependent on the time of administration: positive responses were noted when probiotics were administered before pathogen challenge, during the challenge, or both before and after the challenge.

However, no trend was found among the studies analyzed regarding the type of microorganism used for the treatment, preventing the establishment of a general protocol. *Lactobacillus* were used against several different types of pathogenic challenges, including encephalomyelitis (Baken *et al*. 2006;

Maassen and Claassen 2008); colitis (Amit-Romach et al. 2010); laparotomy with colon anastomosis (Aguilar-Nascimento et al. 2006); and E. coli infection (Ishida-Fujii et al. 2007), among others. With the exception of Baken et al. (2006), all of these articles reported satisfactory results associated with the probiotic administration. However, numerous other microorganisms were also used in the analyzed studies, both alone and in combination. This variation probably stemmed from the fact that the objective of the research was to generally stimulate the immune response of the animals, not to evaluate the specific infections. No relationship was identified between the type of probiotic, pathogenic challenge and the effectiveness of probiotic administration.

Only one of the papers analyzed was conducted by blind assessment. However, 71% of the articles included a randomized experimental design (the remaining 29% did not clearly state if the study was randomized or not). Use of blind assessments and randomized evaluations improved the reliability of scientific works, by preventing study investigators from knowing which treatment was administered and in the case of randomized trials, distribution was done randomly (Snodgrass 2006; Taylor and Yildirim 2011).

5 CONCLUSION

In the studies assessed in this review, the administration of probiotics has been shown to be associated with a positive induction of the immune response in the presence of a wide range of experimental pathogenic challenges. Therefore, further studies should be encouraged in this field in order to develop new protocols with respect to the microorganism type, dosage and the timing of probiotic administration for specific illnesses.

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Table 1 - Summary of the Selected Studies.

1	2	3	4	5	6	7	8	9	10	11	12	13
A	Wistar	*Lactobacillus helveticus and Streptococcus thermophilus* 10^6 CFU	Y	Pre- and post-operative period	30	Y	U	Y	U	Laparotomy with colon anastomosis	Intestine (colon)	Evaluation of the IGA, total protein, albumin and globulin; analysis of DNA content by the method Gyles and Meyers.
B	Wistar	*Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium lactis* 10^9 CFU	Y	Before the challenge and throughout the experiment	6	Y	U	Y	N	Induction of colitis	Intestine (colon and lower end of the ileum)	Evaluation of colonized tissues by real-time PCR. Morphology of the colon and damaged tissue were histologically evaluated.
C	Lewis, Wistar and Balb/c	*Lactobacillus casei* 10^9 CFU	N	Before the challenge and throughout the experiment	16	Y	U	Y	U	EAE induction	Ears (epidermis) or central nervous system	Isolation and proliferation of lymph nodes, IL-4 and IFN-γ by ELISA, cytokine through standard curves of recombinant IL-4 or IFN-γ, analysis of gene expression in liver and thymus tissue, as well as frozen MLN; analysis of the amount of RNA by spectrophotometry and RNA integrity by gel electrophoresis; microarray analysis
D	Wistar and Lewis	*Lactobacillus kefiranofaciens* 6 x 10^{11} CFU	N	Throughout the experiment	5	N	U	Y	Y	N	-	ELISA and blood cell count
E	Sprague Dawley	*Lactobacillus sp.* 10^9 CFU	U	Before the challenge and 9, 3 and 10 days after challenge	3	Y	U	Y	N	Cecum perforation for polymicrobial infection	Cecum	Intestine histology; counting bacterial colonies; Backlight analysis; serum TNF analysis by ELISA.
F	Wistar	*Lactobacillus casei* $2x10^9$ CFU.	U	Before and after the challenge	6	Y	U	Y	N	*Listeria monocytogenes* (sensitization caused by oral infection)	Gastro-intestinal tract and visceral organs	Bacteriological analysis; liver and spleen histological analysis; ALT levels and concentration of total serum bile acids by a Beckman Synchron CX7, cell-mediated immunity measured using the DTH assay
G	Wistar	Lactobacillus $2x10^9$ CFU	N	After the challenge	4	Y	U	Y	U	*Listeria monocytogenes infection.*	Spleen and liver	Liver and spleen bacteriological analysis and measurement of *L. monocytogenes* specific DTH.
H	Sprague Dawley	*Bifidobacterium longum* 1×10^{10} CFU	N	From birth until the end of the experiment	10	U	U	Y	U	N	-	RNA concentration by spectrophotometry, reverse transcription, RT-PCR, cytokine and immunoglobulin (by ELISA)

(Cont. …)

(Cont. Table 1)

1	2	3	4	5	6	7	8	9	10	11	12	13
I	Lewis and Balb/c	*Lactobacillus casei* 2 - 4x10^8 CFU or 1 - 2x10^9 CFU	N	Before the challenge and throughout the experiment	8	Y	U	Y	Y	Allergy Induction	Lymphocytes in the lungs and ovalbumin-specific cytokines in the spleen	Specific ovalbumin IgE and IgG1 titres in sera were determined by ELISA. Th1 and Th2 cytokines were measured in the supernatants of spleen cells that were cultured with ovalbumin; IL-4, IL-5, IL-10, IL-13 and IFN-γ analysis.
J	Lewis and Balb/c	*Bifidobacterium animalis* 1x10^9 CFU	N	Before the challenge and throughout the experiment	16	Y	U	Y	Y	OVA (respiratory allergy) or EAE	Lung or central nervous system	OVA-specific antibodies; Cytokine; IgE ova-specific (by ELISA)
K	Albino rats	*Lactobacillus sp.* 10^8 CFU	N	Throughout the experiment	6	Y	U	Y	Y	Induction of diarrhea (castor oil used as a laxative)	Gastro-intestinal tract	Protein levels were determined by the method of Buiret; blood cell count
L	Sprague Dawley	*Lactobacillus casei, Lactobacillus bulgaricus, Streptococus thermophilus, Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium infantis* 2x10^7 CFU or 4x10^8 CFU	Y	From the second day of the experiment until sacrifice	1	U	U	Y	U	Cryptosporidiosis	Small intestine (cecum)	Estimated amount of parasites in the mucosa of the cecum by Ziehl-Neelsen staining and *C. parvum* by real-time PCR, histological analysis of the cecum; IFN-γ, IL-10 and TNF-α
M	U	*Lactobacillus casei* 5x10^{10} CFU or 1x10^{11} CFU	N	Before the challenge and throughout the experiment	10	Y	U	Y	N	Infection with *E. coli*	U	IgA (ELISA), cytotoxicity of NK cells, macrophages, TNF-α, IL-6 and IL-12
O	Fisher and Balb/c	*Lactobacillus paracasei* 5 x 10^8 CFU	N	After the challenge	5	U	U	Y	N	Air bags (injection of sterile air)	Back of the animal	PMN accumulation and phagocytic activity of these cells, IFN-γ, TNF-α and IL-10 (ELISA), histopathology; immunohistochemistry
P	Wistar	*Lactobacillus brevis, Lactobacillus plantarum, Streptococcus faecalism and Bifidobacte-rium brevis* 6 x 10^8 CFU	Y	For 3 days before sacrifice	10	Y	U	Y	Y	Indomethacin	Gastro-intestinal tract	Percentage of damaged area (macroscopically); Histology of the gastric mucosa, ileum and colon, immunohistochemistry of lymphocytes B (CD 20) and T (CD 4 +)
Q	U	*Lactobacillus plantarum and Lactobacillus murines* CFU (UC)	N	During the challenge	U	U	U	Y	N	EAE	Central nervous system	Cytokines and DNA
R	Sprague Dawley	*Lactobacillus acidophilus, L.. helveticus and Bifidobacterium* CFU (UC)	Y	Throughout the experiment	U	Y	U	Y	U	Azinomethane (colon carcinoma)	Colon	Analysis of the proliferation rate of the mucosa, mesenteric lymph nodes were removed from rats for analysis of intestinal immune system markers, ACF determination; tumor detection
S	Wistar	*Lactobacillus paracasei* 3 x 10^7 CFU	N	During the challenge	7	Y	U	Y	U	Ischemia and reperfusion	Liver	Hepatic microcirculation, liver histology, Western blotting analysis, plasma assessment; bacteriological evaluation in the small intestine

(Cont. …)

(Cont. Table 1)

1	2	3	4	5	6	7	8	9	10	11	12	13
T	Fischer	*Lactobacillus rhamnosus and Bifidobacterium lactis* $5x10^8$ CFU or $5.5x10^8$ CFU	Y	U	32	Y	U	Y	U	Azinomethane (colon carcinoma)	Colon	Immunofluorescence of lymphocyte subpopulations through the spleen and MLN, flow cytometry analysis; IL-10 and IFN-γ by ELISA
V	Lewis	*Lactobacillus casei* $2x10^{10}$ CFU	N	After induction and during the whole experiment	U	Y	Y	Y	Y	Induction of rheumatoid arthritis (collagen type II)	Ankle (foot)	Histopathological analysis of the hind paws; cytokines by RT-PCR, IgG (ELISA), TNF-α, IL-10 and Foxp3 by FACS Calibur Flow Cytometer
X	Sprague Dawley	*Lactobacillus acidophilus* 2.5 x 10^8 CFU	N	After the challenge	7	U	U	Y	N	ICV cannulations	Brain tissue	Histopathology; immunohistochemistry; mRNA and cDNA (RT-PCR), positive colonies were confirmed by DNA sequencing, Western-blotting of the intestines and retroperitoneal adipose tissue.

A: Aguilar-Nascimento et al. 2006; B: Amit-Romach et al. 2010; C: Baken et al. 2006; D: Beaulieu et al. 2007; E: Bu et al. 2006; F: de Waard et al. 2002 a; G: de Waard et al. 2002 b; H: Dong et al. 2010; I: Ezendam and van Loveren 2008; J: Ezendam et al. 2008; K: Flore et al. 2010; L: Guitard et al. 2006; M: Ishida-Fujii et al. 2007; O: Kourelis 2010; P: Laudanno et al. 2008; Q: Maassen and Claassen 2008; R: Marotta et al. 2003; S: Nardone et al. 2010; T: Roller et al. 2004; V: So et al. 2008; X: Sousa et al. 2008; CFU: colony forming unit; U: Unclear; Y: YES; N: NO; IgA: immunoglobulin A; IgE: immunoglobulin E; IgG1: immunoglobulin G; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; mRNA: Messenger ribonucleic acid; ELISA: Enzyme Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction; RT-PCR: reverse transcription polymerase chain reaction; EAE: experimental autoimmune encephalomyelitis; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-10: interleukin-10; IL-12: interleukin-12; IL-13: interleukin-13; ICV: intracerebroventricular; MLN: mesenteric lymph nodes; PMN: polymorphonuclear leukocyte; ACF: aberrant crypt foci ALT: alanine aminotransferase; DTH: delayed-type hypersensitivity ; Th1: T helper cell type 1; Th2: T helper cell type 2; IFN-γ: interferon-gamma; TNF-α: tumor necrosis factor-α; OVA: ovalbumin; NK: natural killer; 1: author and year of publication; 2: lineage; 3: microorganisms used; 4: association of microorganisms; 5: period of probiotic administration; 6: number of animals per experimental group°; 7: randomization; 8: blind assessments; 9: control group: 10: interference factors°°, 11: pathogenic challenge; 12: tissue where the challenge was induced; 13: technical evaluated. °Studies in which the "n" experimental varied, was considered the smallest n; °°Stress, hormone assessment, gender, etc.

Table 2 - Evaluation criteria and scores for the selected articles.

Author	Mean number of animals per group*	Type of assay**	Control group***	Blind assessment+	Interference fators++	Pathogenic challenge+++	Total
Ezendam and van Loveren 2008	2	2	2	1	2	2	11
Ezendam et al. 2008	2	2	2	1	2	2	11
Laudanno et al. 2008	2	2	2	1	2	2	11
So et al. 2008	1	2	2	2	2	2	11
Aguilar-Nascimento et al. 2006	2	2	2	1	1	2	10
Amit-Romach et al. 2010	2	2	2	1	1	2	10
Baken et al. 2006	2	2	2	1	1	2	10
Flore et al. 2010	2	2	2	1	1	2	10
Bu et al. 2006	2	2	2	1	1	2	10
Ishida-Fujii et al. 2007	2	2	2	1	1	2	10
Roller et al. 2004	2	2	2	1	1	2	10
de Waard et al. 2002a	2	2	2	1	1	2	10
Beaulieu et al. 2007	2	1	2	1	2	1	9
Marotta et al. 2003	1	2	2	1	1	2	9
Nardone et al. 2010	2	1	2	1	1	2	9
Sousa et al. 2008	2	1	2	1	1	2	9
de Waard et al. 2002b	1	2	2	1	1	2	9
Kourelis 2010	1	1	2	1	1	2	8
Guitard et al. 2006	1	1	2	1	1	2	8
Maassen and Claassen 2008	1	1	2	1	1	2	8
Dong et al. 2010	2	1	2	1	1	1	8

*Scores for the sample number were 1 (less than 6 animals/group) and 2 (6 or more animals/group)

** Nonrandomized experiments or when randomization was not described clearly in the text (score 1) and randomized experiments (score 2)

*** Studies without control groups or those which did not clearly mention a control group in the text (score 1) and studies with a control group (score 2)

+ Experiments without blind assessments or those in which blind assessments were not clearly reported in the text (score 1) and experiments with blind assessments (score 2)

++ Studies that did not evaluate interference factors (score 1) and studies which evaluated additional factors such as: stress, hormonal evaluation, variations between males and females (score 2)

+++ Studies in which animals were not subjected to experimental challenge (score 1), and studies in which animals were subjected to an experimental challenge (score 2).

ARTIGO 2

Effects of probiotic therapy on metabolic and inflammatory parameters of rats with ligature-induced periodontitis associated with restraint stress

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Effects of probiotic therapy on metabolic and inflammatory parameters of rats with ligature-induced periodontitis associated with restraint stress

Running title: Probiotics, periodontitis and stress

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Summary Sentence: Stress seems to affect the action of probiotic agents, modulating their effects in intestinal and periodontal tissues.

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Abstract

Background: This study evaluated the effects of Probiotic Therapy (PT) in rats with ligature-induced periodontitis associated with restraint stress. **Methods:** Sixty-four rats were divided into: control (C), stress (STR), probiotic (PROB), periodontal disease (PD), STR-PROB, STR-PD, STR-PROB-PD and PROB-PD goups. The probiotic was added to the drinking water for 44 days. PD was induced by a ligature. In STR groups, the animals were subjected to restraint stress for 2.5 hours during 30 days. **Results:** PD increased alveolar bone loss ($p<0.05$). PD also increased levels of Cyclooxygenase-2 (COX-2), serum C-terminal telopeptide (CTX), p38 mitogen-activated protein kinase (p38), receptor activator of NF- κ B ligand (RANKL) and decreased levels of osteoprotegerin (OPG). Stressed rats presented high levels of C-peptide, corticosterone and glucose ($p<0.05$). In general, the presence of stress reduced the expression of CTX and p38 ($p<0.05$). PT reduced alveolar bone loss in unstressed animals. It also decreased expression of CTX and induced increased expression of OPG in unstressed animals with PD. However, PT was not effective in preventing bone loss or altering the expression of inflammatory markers in stressed animals. PT decreased the number of inflammatory cells in the periodontal tissue ($p<0.05$). Groups with stress and PD presented decreased villous height and crypt depth. Stress seemed to prevent part of probiotic beneficial effects on small intestine. **Conclusion:** Based on the methodology used, PT may reduce tissue destruction resulting from PD in unstressed rats. The protocol used for restraint stress decreased the immuno-modulatory effects in intestinal and periodontal tissues of probiotics.

Keywords: Inflammation, periodontal diseases, physical restraints, animal models.

1 Introduction

Periodontal disease (PD) is characterized by a chronic inflammatory process of tooth supporting tissues.[1-4] The most common form, chronic periodontitis, affects 35% to 60% of the adult population in developed countries,[5] and severe forms are estimated to affect 5 to 15% of the world's population.[6] It was reported that one in two Americans aged 30 or older has periodontits.[7] Besides being the most common cause of tooth loss, there is some evidence that periodontal infection can also be involved in systemic disease processes, such as cardiac abnormalities and preterm low birth weight.[8-10]

The presence of biofilm is essential for initiating PD. However, the clinical course of the condition depends on host-pathogen interactions.[6] Bacterial products start a local response in the gingival tissue [11] leading to resorption of alveolar support bone, mobility and consequent tooth loss.[1] Nevertheless, the presence of biofilm alone accounts for only a small proportion (20%) of the variations in expression of PD. [12] The main contributor to the destruction of hard and soft tissues is the result of activation of an immune-inflammatory host response to bacterial aggression.[13] Acquired risk factors and environmental factors (e.g. *diabetes mellitus*, smoking and stress) as well as genetically transmitted characteristics (e.g. polymorphisms for interleukin-1) may exacerbate the inflammatory response.[13]

Thus, in addition to microorganisms, stress can mediate immune and/or behavioural effects on the body's defences, contributing to the aetiology and perpetuation of chronic periodontitis.[14] Stress can change the resistance of the host tissue by autonomic as well as endocrine mechanisms, resulting primarily in increased levels of catecholamines and corticosteroids, reduced microcirculation in the gums and salivary flow, interference with the normal functioning of neutrophils and lymphocytes and facilitation of bacterial invasion and tissue damage.[15,16]

In this context, probiotics (live microorganisms that confer health benefits[17] – e.g. bacteria of the genus *Lactobacillus*, *Enterococcus*, *Bacillus* and *Bifidobacterium)*[18] appear as new adjuvants for controlling PD.[19]

As far as we are concerned, there is no previous study evaluating the effect of Probiotic Therapy (PT) in association to PD and stress. In a previous study from our group, it was shown that PT reduced epithelial attachment and alveolar bone loss in rats with ligature-induced periodontitis.[20] Since probiotics can interfere both with microorganism load and with the immune response, their systemic application is of interest.

The impact of stress on exacerbating inflammatory processes has a particular role in the management of PD.[4,14,21] Due to the diversity and complexity of the interactions of probiotic species with the immune system, a careful selection process should be conducted before using them in clinical trials.[22] Since similar immune-inflammatory mechanisms occur in periodontal tissue and intestinal mucosa, it is believed that the action of probiotics in the oral cavity is analogous to that described in intestinal mucosa. *Bacillus subtilis* along with other probiotics is known to be valuable for prevention of enteric infections. We hypothesized that its use in drinking water should adhere to and colonize part of the oral and intestinal bacteria with beneficial effects on intestinal and periodontal tissues even under stress situations. Therefore, this study aimed to evaluate the effects of Probiotic Therapy (PT) in rats with ligature-induced periodontitis associated with restraint stress

2 Materials And Methods

All procedures were approved by the Ethics Committee on Animal Experimentation (CEUA – UFLA protocol number 036/10). A total of 64 healthy adult male rats (*Rattus norvegicus Albinus, Wistar*) weighing approximately 200 g were used in this study. A completely randomized design in a 2x2x2 factorial scheme (stressed or not, with periodontal disease or not, treated or not with probiotics) was employed. After acclimatizing for 7 days, the animals were divided into eight groups with eight animals each: control, stress (STR), probiotic (PROB), periodontal disease (PD), STR-PROB, STR-PD, PROB-PD and STR-PROB-PD. The animals were housed in individual metabolic cages. The room was kept at a temperature of 22 ± 2°C with light-dark cycles of 12/12 h. Food and water were supplied *ad libitum* throughout the experimental period.

The rats were immobilized in plastic containers adapted to their size/weight. [21] The restriction of movements was maintained for 2.5 hours per day over 30 days (Figure 1).[18] The immobilization was performed in a quiet room and always at the same time to avoid additional stress or interference with the circadian cycle.

Probiotic product based on *Bacillus subtilis* (CH201)[§] was orally administered for 44 days (1.5 x 10^8 colony-forming units CFU/mL daily).[23] PD was induced by a ligature protocol in both mandibular first molars. The animals received general anaesthesia with intraperitoneal injection of 10 mg/kg xylazine[‖] and 80 mg/kg[¶] ketamine. Once anesthetized, the animals received a ligature with cotton[#] according to the methodology described by Holzausen et al.[24]

All animals were killed after 44 days and their tissues used for subsequent analyses. The rats were decapitated with a guillotine. Blood samples were collected for assessment of glucose levels (by colorimetric assay),

corticosterone[**] and C-peptide[††] (by enzyme immunoassay - ELISA) using commercial kits.

After collecting blood, the jaws were removed and fixed in buffered formalin in a volume of liquid at least 30 times greater than the removed tissue. The right hemi-mandibles were destined for histological slide preparation for counting inflammatory cells, while the left side of each mandible was used for morphometric analysis and also to collect tissues for semi-quantitative assays of proteins by Western Blotting. These analyses are described below.

Inflammatory cell counts

The right hemi-mandibles were demineralized in 10% EDTA solution for 60 days,[25] paraffin embedded, then cut into a half series of slices with thickness 6.0 μm in the mesio-distal direction. The sections were fixed on slides, dewaxed and stained with hematoxylin and eosin. Their images were captured under a light microscope. Before the analysis, criteria were established in order to conduct a more objective evaluation of inflammatory infiltrates in the interproximal sub-epithelial connective tissue between the distal root of the first mandibular molar and the mesial root of the second mandibular molar. The counts of inflammatory cells such as eosinophils, lymphocytes, plasma cells and macrophages[26] in the interproximal papilla between the first and second molars were performed using a computer program [‡‡].

The analyses were performed by two calibrated examiners who were blinded to the experimental groups. A second sample was measured again 48 hours after the first. A paired t test was used to calculate the intra-examiner error. A Pearson correlation analysis between the data obtained by the two examiners was also performed. P values > 0.05 in the paired t test and $r > 0.90$ in the Pearson correlation test were considered in estimating the feasibility of the proposed method.

Western blotting

Quantification of proteins: p38 mitogen-activated protein kinase (p38), Cyclooxygenase-2 (COX-2), receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) were visualised from total protein extracts of gingival tissues. Samples of 40 μg of total protein were added in a buffer containing glycerol and SDS DTT, heated and denatured at 95°C for 5 min, and loaded onto SDS polyacrylamide gels. Proteins on the gel were separated by electrophoresis in 10 mM Tris-HCl buffer subjected to a voltage of 100 V for 90 min. Subsequently they were electro-transferred to 0.2 mM nitrocellulose membranes (300 mA for 60 min). The membranes were then incubated with antibodies against a housekeeping protein (α-tubulin) used as a positive control. Images obtained on radiograph films were scanned by a chemiluminescent documentation system and further analysis was performed by densitometry of the bands using specific software.

ELISA for serum C-terminal telopeptide (CTX) protein

CTX levels were determined in capture ELISA assays using microtiter plates[§§] coated for 24 h at 4^{o}C with 2 μg/ml of goat IgG anti-rat CTX in carbonate-bicarbonate buffer, pH 9.6. Unless stated elsewhere, all antibodies were affinity purified and obtained from Rheabiotech (Campinas/SP, Brazil). ELISA reactions were performed in 100 μl volumes. After coating, the plates were washed 3 times with a solution of 0.9% NaCl, 0.05% Tween 20 and 0.02% NaN3, then blocked for 1 hour at room temperature with phosphate buffer (PBS), pH 7.5, and 0.01% BSA. After a new series of washes, gingival proteins were applied in triplicate and the plates were incubated for 2 hours at room temperature. After additional washes, the plates were incubated for 2 hours at room temperature with a 1:5000 dilution of biotin-conjugated goat IgG anti-rat CTX. The plates were then washed and incubated for 1 h with a 1:500 solution of streptavidine. The plates were washed again and the reactions were revealed

by incubation with the TMB substrate. Colour development was measured at 450 nm in an ELISA plate reader[|||].

Morphometric analysis of periodontal tissue

The left hemi-mandibles were fixed in 10% formalin solution. Subsequently, they were immersed in 30% hydrogen peroxide for 2 hours and the soft tissues were removed with gauze, followed by staining with 1% methylene blue for 30 minutes in order to differentiate bone from teeth and enhance the visibility of the cement-enamel junction (CEJ). Using a stereomicroscope[##] with a colour video camera mounted on it and coupled to a computer, the lingual surfaces of the defleshed jaws were recorded in a standardized manner (20 x magnification). The distance between the alveolar bone crest (ABC) and the CEJ (ABC–CEJ, mm) was measured on the three distal root surface of the first molar, using image analysis software [##].

Histomorphometric analysis of small intestine

Tissues samples from the small intestine (duodenum, jejunum and ileum) were collected and fixed in neutral formalin for 48 hours. The samples were routinely processed and embedded in paraffin. Serial sections, 6 μm thick, were obtained. The sections were stained with H&E for analysis by light microscopy. Villous height was estimated by measuring the vertical distance from the villous tip to the villous-crypt junction level for 10 villi per section; crypt depth was estimated by measuring the vertical distance from the villous-crypt junction to the lower limit of the crypt for 10 corresponding crypts per section, as described previously. [20]

Statistical analysis

Analysis of variance (Three-way ANOVA) was performed to compare the means between groups in a 2x2x2 factorial design (with and without probiotic, with and without stress, with and without periodontal disease). When F values indicated significant differences in the interactions between these factors, they were deployed. The number of inflammatory cells were analysed by Kruskal-wallis test among all groups. Analyses were performed using the SigmaStat 3.1 program (Sigma StatStatistical Package Software Inc., USA) and Statistical Analysis System (Statistical Analysis System Institute - SAS Institute (1996). The significance level was set at $p < 0.05$.

3 Results

Animals subjected to chronic restraint stress showed higher levels of plasma corticosterone, C-peptide (referencing insulin production) and glucose ($P < 0.05$) compared to unstressed animals, indicating that the model was adequate for inducing stress. The use of probiotic and induction of PD did not interfere with these parameters ($p > 0.05$) (Figure 2).

The animals subjected to stress showed decreased levels of CTX, and placement of the ligature induced an increase in this marker. However, there were significant interactions between stress and PD, leading to higher levels of CTX. The use of probiotic decreased the levels of CTX in unstressed animals with PD ($p<0.05$). In stressed animals with PD, PT had no effect (Figure 3).

The expression of COX-2 was higher in the unstressed groups with PD. The stress protocol did not alter the expression of this enzyme in relation to the respective controls ($p <0.05$), nor did PT. Similarly, in the groups with PD there was increased expression of p38 ($p <0.05$), but this expression was reduced in the presence of stress ($p<0.05$) (Figure 3). In addition, there was a significant interaction effect between probiotic and PD ($p<0.05$), where the use of probiotic in animals with ligature leaded to smaller amounts of p38.

In groups with PD there was increased expression of RANKL ($p <0.05$). The use of probiotic did not affect the expression of this protein, nor the stress induction. Nonetheless, significant interaction between Probiotic and PD was found ($p<0.05$), where the use of probiotic mitigated the effects of PD. OPG expression was decreased in the groups with PD ($p <0.05$). There was no significant influence of stress. PT induced an increase in the expression of this protein in unstressed animals with PD ($p <0.05$) (Figure 3).

The number of inflammatory cells was higher in the groups with PD and stress ($p <0.05$). The use of probiotic significantly decreased the numbers of

these cells ($p<0.05$), as shown in Figure 3. Groups PD, STR-PD, PD-PROB and STR-PROB-PD presented foreign bodies on the surface of the gingival epithelium, loss of the interdental papillae, apical migration of the junctional epithelium, interdigitation towards the underlying connective tissue and presence of moderate to severe mononuclear inflammatory infiltrate in subepithelial connective tissue.

There was no difference in bone loss in stressed and unstressed animals that received ligature ($p>0.05$) (Table 1). PD increased alveolar bone loss ($p< 0.05$) and probiotic had a positive effect against this loss in unstressed animals ($p<0.05$). However, it was observed that in stressed groups there was no preventive effect of probiotic on bone loss.

It was observed that the animals with stress and PD presented decreased villous height and crypt depth. Significant statistical interactions were found between PD and Probiotics for duodenum and jejunum tissues (in general probiotics promoted greater villi height and crypt depth), and also between stress and PD in jejunum (stress associated to PD promoted shorter villi height)($p<0,05$). (Table 2).

4 Discussion

Previously, beneficial effects of using a probiotic to prevent bone loss caused by PD in unstressed rats were observed in a study from our group.[20] Those results suggested that orally administered probiotics may produce benefits both by passage through the oral cavity and by modulating its oral mucosal immunity systemically.[27]

There is evidence that colonization of the gut by probiotics can cause beneficial systemic effects, providing protection against disease at distant sites.[28] Thus, it is suggested that beneficial effects of the probiotic may include preventing adhesion of pathogens to host tissues while passing through the oral cavity. Additionally, stimulation and modulation of the immune system, reducing the production of pro-inflammatory and increasing the production of anti-inflammatory cytokines,[29] enhancing the integrity of the intestinal barrier and increasing the production of mucins, as well as eliminating or inhibiting pathogen growth by producing bacteriocins or other products such as acids and peroxides[30] and decreasing luminal pH.[31]

However, it was not known if these effects were still present under stressful conditions. In this study, the beneficial effects of probiotics were minimized in animals subjected to restraint stress. The relationship between stress and PD progression is still controversial in the literature.[4,32,33] The feasible mechanism involved in the pathogenesis of stress in PD is related to increased production of glucocorticoids (corticosterone) and catecholamines. Animals subjected to stress showed corticosterone levels significantly above their respective controls without stress, as observed in previous studies, indicating the validity of the proposed model.[4] Stress alone did not induce any significant change in periodontal tissues, as demonstrated in previous studies.,[32,33] However, animals subjected to restraint stress did not show more pronounced bone loss

than unstressed animals in groups with induced PD, despite the numbers of inflammatory cells being significantly higher in those groups. These differences can be justified by variations in restraint periods, manner of restraint [34] and gender susceptibility, since females are more prone to stress models than males.[35] It was observed that bone loss in animals undergoing ligature and the stress protocol showed slightly less bone loss than the unstressed groups (although not significantly less), indicating a possible anti-inflammatory effect of plasma corticosterone levels in stressed animals, reducing bone loss in animals with respect to induction of the disease alone.[4]

Although stress is considered an important risk factor for PD,[36] studies evaluating the influence of probiotics in stressed rats have not yet been performed. It is speculated that stress may produce important effects in the gastrointestinal tract, which may have affected the modulating role of probiotics on the immune system in the performed model. Increased secretion of norepinephrine and corticosterone may influence the interactions of host cells involved in the protection of intestinal mucosa with commensal bacteria that inhabit the mucosal surface[37] by changing the effect of probiotics in the presence of stress.

Studies in rodents have shown that exposure to stressors can lead to diseases of the small and large intestine, including altered ion secretion and increased epithelial permeability. Prolonged exposure to stress can also induce low-grade inflammation, cause ultrastructural epithelial abnormalities and alter interactions between intestinal bacteria, allowing greater microbial translocation.[38] Additionally, stressful events promote changes in the temperature and acidity of the gastrointestinal tract,[39] possibly having a negative impact on the viability of the probiotic. Thus, this set of physiological reactions to stress in the gastrointestinal tract may have contributed to the inefficiency of

the bacilli in stressed animals, as also evidenced by the present intestinal findings.

In this study, the blood glucose levels of animals submitted to the stress were increased. This result was due to activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, which results in increased production of catecholamines and glucocorticoids, respectively, with a consequent increase in glucose production by the liver and decreased use by tissues.[40] With respect to insulin levels, in the present study we chose to quantify them indirectly, through the measurement of plasma C-peptide. Since the principal stimulus for insulin secretion is hyperglycemia,[41] the increases in C-peptide in stressed animals were expected.

With regard to inflammation at periodontal sites, COX-2 expression was higher in the groups with PD. The stress protocol did not alter the expression of this enzyme in relation to these groups' respective controls, corroborating the findings of Rettori et al.[4] for prostaglandin E_2 (PGE_2), the main product of COX-2. The production of PGE_2 from the activity of COX-2 is elevated in subjects with PD compared to healthy subjects, corroborating our findings.[42] The use of probiotics did not influence the expression of this protein, nor did stress.[43]

The p38 MAPK kinase is a subfamily that plays roles in adaptation, homeostasis and the stress response and may also be involved in inflammatory responses. Besides JNK, p38 is responsible for inflammation and the development of stress-induced signalling. The main function of the p38α MAPK in RANKL-induced osteoclastogenesis was determined to be the formation of precursor cells of macrophages/osteoclasts.[44] In the present study it was observed that after PD induction in unstressed groups, increased expression of p38 protein was seen, corroborating Aquino et al.[45] where activation of p38 was significant on the fifth day after induction by PD ligation as well as injection of LPS. It was also observed that the groups subjected to stress induction showed

reduced expression of p38, suggesting that stress may have modulated the expression of this protein.

The proteins RANKL and OPG modulate osteoclastogenesis. RANKL is a key molecule in osteoclast activation and OPG is a decoy receptor for RANKL. Therefore, the relative proportion of OPG on RANKL determines the speed and intensity of bone resorption mediated osteoclastogenesis.[46] In the present study, groups with PD had higher amounts of RANKL, corroborating the study of Jabbar et al.[47] The findings with the OPG also agree with the literature showing a decreased expression of this protein in groups with PD.[48] The use of probiotic induced increased expression of this protein in non-stressed animals with PD.

The measurement of CTX has been considered as a marker of bone resorption. In this study, ligature placement induced an increase in this marker and animals subjected to stress showed decreased levels of CTX, corroborating previous findings. [47,49] The use of probiotic also decreased levels of CTX in unstressed animals with PD. It has been suggested that reducing CTX can determine the degree of suppression of osteoclasts.[50] Thus, it is suggested that the use of probiotics may favour lower levels of CTX, decreasing osteoclast activity and thus preventing periodontal bone loss.

In general, the effects of the probiotic were mostly modified under stress conditions employed in this experiment. However, the number of inflammatory cells was still influenced by the use of probiotics even under restraint stress. Further studies are essential to exploit these mechanisms of action of probiotics in periodontal tissues and other stress protocols should also be investigated.

5 Conclusions

Based on the methodology used, PT may reduce tissue destruction resulting from PD in unstressed rats. Stress seems to affect the action of probiotic agents, modulating their effects in intestinal and periodontal tissues.

Footnotes:

[§] Bioplus 2B (CH Hansen (Hørsholm, Denmark)

[‖] Rompum, Bayer Animal Health, São Paulo, SP, Brazil

[¶] Dopalem, Agribands, Paulínia, SP, Brazil.

[#] Coats-Corrente, São Paulo, SP, Brazil.

[**] DetectX ® Corticosterone Enzyme Immunoassay– Arbor Assays, New Orleans, LA, USA

[††] RayBio ® C-Peptide Immunoassay Protocol, RayBiotech, Inc., Norcross (Georgia), USA

[‡‡] Cell B - Olympus CX31, Olympus Optical Co., Tokyo, Japan

[§§] Costar 3590, Fisher Scientific, PA, USA

[‖‖] Microplate Reader / Model 3550, Bio Rad, Promega Corporation, Fitchburg, Wisconsin, USA.

[¶¶] Leica MZ6, Leica Microsystems GmbH, Wetzlar, Germany

[##] Imagelab - DiraconBio Informática Ltda., Vargem Grande do Sul, SP, Brazil

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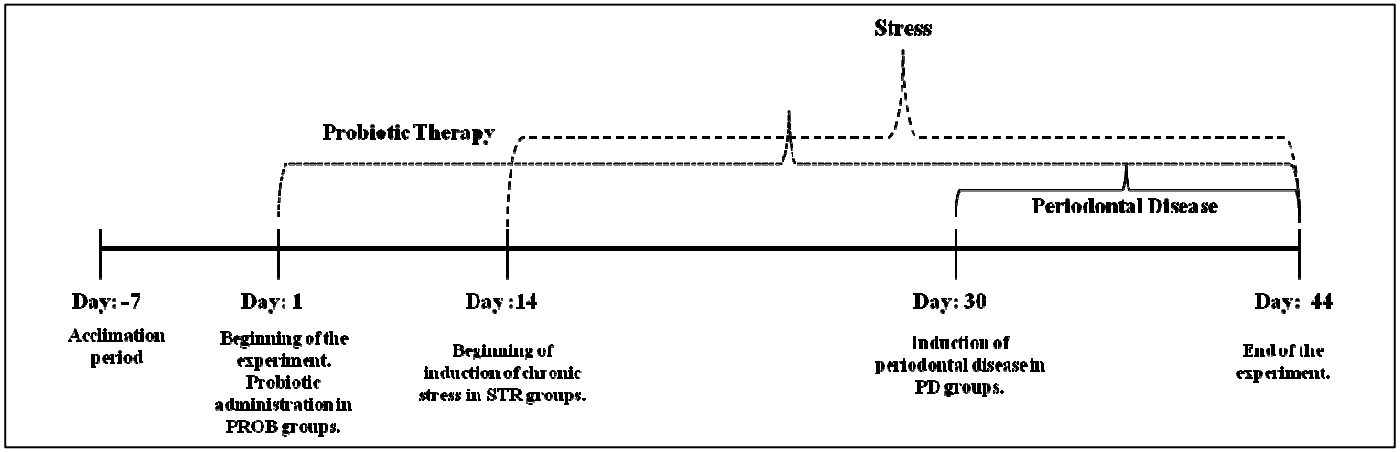
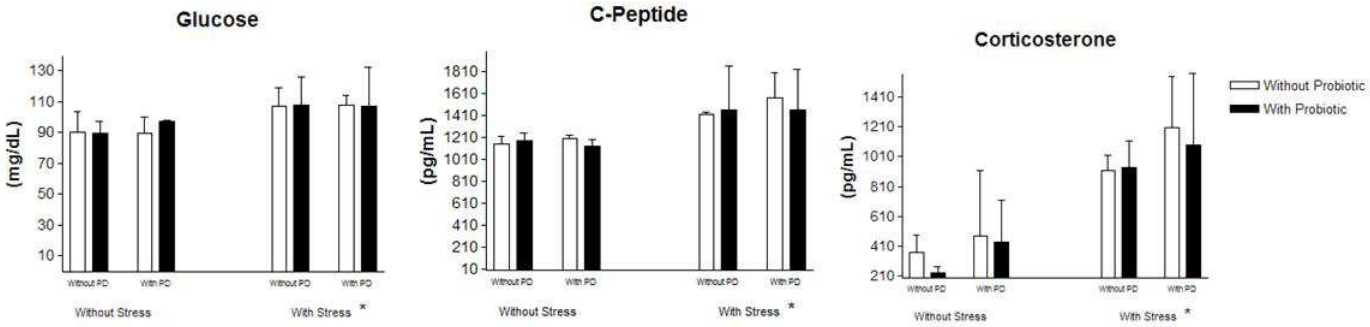
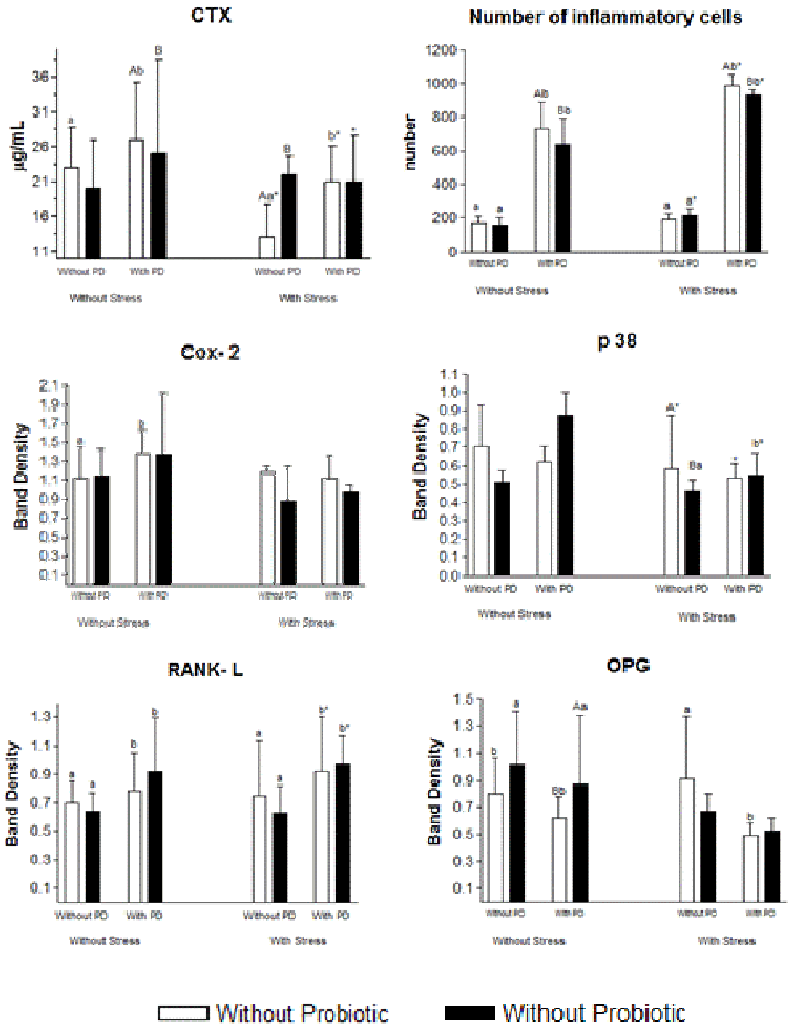


Figure 1 Time schedule of experimental period



* Differs significantly from the respective control (without stress) by F test ($P < 0.05$)

Figure 2 Means and standard deviations of plasmatic corticosterone, C-peptide and glucose levels



Different capital letters [A,B] indicate difference between groups with and without periodontal disease by F test ($P<0.05$)
Different small letters [a,b] indicate difference between groups with and without probiotic therapy by F test ($P<0.05$)
*Differs significantly from the respective control (without stress) by F test ($P<0.05$)

Figure 3 Means and standard deviations of CTX, Cox-2, p-38, RANK-L, OPG expression and number of inflammatory cells evaluated on periodontal tissues

Table 1 Mean values of morphometric analysis rat mandible

Stress	Periodontal disease	Probiotic	
		Without	With
		Morphometric analysis	
Without	-	0.72 (0.04) a	0.74 (0.01)
	+	1.02 (0.02) bB	0.86 (0.04)A
With	-	0.80 (0.05)a	0.78 (0.13)a
	+	1.00 (0.01)b	0.91 (0.11)b

- : without periodontal disease; + : with periodontal disease

Different capital letters ^{A,B} indicate difference between groups with and without periodontal disease by F test ($P<0.05$)

Different small letters ^{a,b} indicate difference between groups with and without probiotic therapy by F test ($P<0.05$)

Table 2 Means and standard deviations of the villous height and crypt depth in small intestine sections, with comparisons among groups

		Villous height		**Crypt depth**	
Stress	PD	Probiotic		Probiotic	
		without	with	without	with
Duodenum					
without	-	1176 (72,20) A	1306 (66,37) A	559 (5,85) ABb	657 (32,22) Ba
	+	1118 (44,91) Ba	1068 (31,89) Ab	634 (38,87) Ab	775 (66,40) Aa
with	-	1095 (77,72) B	1143 (93,76)BC	610 (46,59) A	629 (23,67) B
	+	984 (81,89)C	1031 (50,85)C	536 (40,51) Bb	614 (49,43) Ba
Jejunum					
without	-	759 (45,64) A	882 (59,90) A	478 (26,43) A	424 (61,34) AB
	+	528 (51,65) B	672 (83,27) BC	269 (48,41) Bb	463 (100,94) Aa
with	-	503 (38,03) B	647 (113,49) BC	302 (41,95) B	365 (79,70) B
	+	614 (51,97) B	522 (45,96) C	279 (53,84) B	352 (24,63) BC
Ileum					
without	-	546 (26,61) A	532 (55,50) A	350 (36,19) A	353 (19,37)
	+	552 (44,98) A	544 (31,62) A	340 (26,28) A	360 (48,88)
with	-	449 (38,05) B	464 (34,04) B	311 (30,48) Bb	351 (18,47) a
	+	488 (39,56) AB	529 (28,77) A	289 (10,96) ABb	369 (19,94) a

PD: Periodontal Disease; - : without periodontal disease; + : with periodontal disease
Different capital letters ^{A,B} indicate difference between groups with and without periodontal disease by F test ($P<0.05$)
Different small letters ^{a,b} indicate difference between groups with and without probiotic therapy by F test ($P<0.05$)

ANEXOS

Certificado fornecido pela Comissão de Ética no Uso de Animais da Universidade Federal de Lavras para a realização da pesquisa

PRÓ-REITORIA DE PESQUISA - PRP
NÚCLEO DE INOVAÇÃO TECNOLÓGICA - Nintec
COMISSÃO DE BIOÉTICA NA UTILIZAÇÃO DE ANIMAIS
Cx.P.3037 - Lavras – MG – 37200-000 – (35) 3829-1591/1127
cba@nintec.ufla.br - nintec@nintec.ufla.br

CERTIFICADO

Certificamos que o **Protocolo nº 036/2010**, relativo ao projeto intitulado **"EFEITO DO USO DE PROBIÓTICO (*Bacillus subtilis*) NA EXPRESSÃO DE MEDIADORES INFLAMATÓRIOS PERIODONTAIS, IMUNIDADE E PARÂMETROS INTESTINAIS EM RATOS SUBMETIDOS A ESTRESSE POR IMOBILIZAÇÃO"**, que tem como responsável **Luciano José Pereira,** está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela **Comissão de Bioética na Utilização de Animais (Nintec/PRP-Ufla),** tendo sido aprovado na reunião de **26/10/2010.**

CERTIFICATE

We hereby certify that the **Protocol nº 036/2010,** related to the project entitled **"EFFECTS OF THE PROBIOTIC (*Bacillus subtilis*) USE IN PERIODONTAL INFLAMMATORY MARKERS EXPRESSION, IMUNITY AND INTESTINAL PARAMETERS IN RATS SUBMITTED TO RESTRAIN STRESS",** under the supervision of **Luciano José Pereira,** is in agreement with the Ethics Principles in Animal Experimentation, adopted by the **Bioethic Committee in Utilization of Animals (NINTEC/PRP-Ufla),** and was approved in October **26, 2010.**

Lavras, 26 de outubro de 2010.

Prof. Luis David Solis Murgas
Presidente da Comissão de Bioética na Utilização de Animais

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Tabela 1A - Variável analisada: CTX

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,0025	0,0025	0,0015	0,9697
Estresse (E)	1	0,8421	0,8421	0,5039	0,4852
Doença (D)	1	2,7504	2,7504	1,6458	0,2129
P*E	1	0,9580	0,9580	0,5733	0,4570
P*D	1	2,7434	2,7434	1,6417	0,2134
E*D	1	8,6288	8,6288	5,1636	0,0332
P*D*D	1	5,7813	5,7813	3,4596	0,0763
erro	22	36,7642	1,6711		
CV %	29,79				

Tabela 2A - Variável analisada: RANK_L

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,1689	0,1689	7,8303	0,0105
Estresse (E)	1	0,0463	0,0463	2,1452	0,1572
Doença (D)	1	0,0746	0,0746	3,4569	0,0764
P*E	1	0,0044	0,0044	0,2053	0,6549
P*D	1	0,1114	0,1114	5,1641	0,0332
E*D	1	0,0354	0,0354	1,6430	0,2133
P*D*D	1	0,0935	0,0935	4,3344	0,0492
erro	22	0,4746	0,0216		
CV %	16,38				

Tabela 3A - Variável analisada: OPG

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,0194	0,0194	0,8155	0,3763
Estresse (E)	1	0,4662	0,4662	19,5995	0,0002
Doença (D)	1	0,0014	0,0014	0,0580	0,8119
P*E	1	0,0199	0,0199	0,8354	0,3706
P*D	1	0,0032	0,0032	0,1347	0,7171
E*D	1	0,0216	0,0216	0,9094	0,3506
P*D*D	1	0,0935	0,0935	3,9306	0,0600
erro	22	0,5233	0,0238		
CV %	17,59				

Tabela 4A - Variável analisada: COX_2

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,0010	0,0010	0,0316	0,8606
Estresse (E)	1	0,4733	0,4733	15,0521	0,0008
Doença (D)	1	0,0090	0,0090	0,2855	0,5985
P*E	1	0,2113	0,2113	6,7208	0,0166
P*D	1	0,1246	0,1246	3,9628	0,0591
E*D	1	0,0060	0,0060	0,1908	0,6665
P*D*D	1	0,0935	0,0935	2,9732	0,0987
erro	22	0,6918	0,0314		
CV %	16,43				

Tabela 5A - Variável analisada: p38

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,0414	0,0414	5,3804	0,0300
Estresse (E)	1	0,2959	0,2959	38,4897	0,0000
Doença (D)	1	0,0075	0,0075	0,9714	0,3350
P*E	1	0,0584	0,0584	7,5945	0,0115
P*D	1	0,0482	0,0482	6,2725	0,0202
E*D	1	0,0294	0,0294	3,8289	0,0632
P*D*D	1	0,0935	0,0935	12,1607	0,0021
erro	22	0,1692	0,0077		
CV %	10,99				

Tabela 6A - Variável analisada: MORFOMÉTRICO

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	0,0049	0,0049	0,7716	0,3902
Estresse (E)	1	0,0065	0,0065	1,0222	0,3241
Doença (D)	1	0,2927	0,2927	46,0869	0,0000
P*E	1	0,0049	0,0049	0,7653	0,3921
P*D	1	0,0221	0,0221	3,4862	0,0766
E*D	1	0,0073	0,0073	1,1417	0,2980
P*D*D	1	0,0334	0,0334	5,2510	0,0329
erro	20	0,1270	0,0064		
CV %	9,20				

Tabela 7A - Variável analisada: GLICEMIA

Opção de transformação: Raiz quadrada - SQRT (Y)

FV	GL	SQ	QM	F	P>F
Probiótico (P)	1	1,1365	1,1365	1,3344	0,2549
Estresse (E)	1	6,6290	6,6290	7,7833	0,0080
Doença (D)	1	0,0088	0,0088	0,0103	0,9197
P*E	1	0,1766	0,1766	0,2073	0,6514
P*D	1	1,3948	1,3948	1,6376	0,2080
E*D	1	2,6645	2,6645	3,1285	0,0846
P*D*D	1	0,0612	0,0612	0,0719	0,7900
erro	40	34,0677	0,8517		
CV %	9,02				

Tabela 8A - Variável analisada: CORTICOSTERONA

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	2523052,9000	2523052,9000	11,313	0,0020
Doença (D)	1	849139,6000	849139,6000	3,807	0,0598
Probiótico (P)	1	90820,9000	90820,9000	0,407	0,5279
E*D	1	136422,4000	136422,4000	0,612	0,4399
E*P	1	344844,9000	344844,9000	1,546	0,2227
D*P	1	751856,4000	751856,4000	3,371	0,0757
E*D*P	1	906010,0000	906010,0000	4,062	0,0523
erro	32	7136990,8000	223030,9625		
CV%	58,45				

Tabela 9A - Variável analisada: PEPTÍDEOC

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	58140,6250	58140,6250	6,960	0,0128
Doença (D)	1	88642,2250	88642,2250	10,612	0,0027
Probiótico (P)	1	256800,6250	256800,6250	30,743	0,0000
E*D	1	186459,0250	186459,0250	22,322	0,0000
E*P	1	185096,0250	185096,0250	22,159	0,0000
D*P	1	316662,0250	316662,0250	37,910	0,0000
E*D*P	1	12075,6250	12075,6250	1,446	0,2380
erro	32	267296,8000	8353,0250		
CV%	7,42				

Tabela 10A - Variável analisada: DUODENO VILOSIDADES

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	103369,9224	103369,9224	18,238	0,0002
Doença (D)	1	142857,4752	142857,4752	25,205	0,0000
Probiótico (P)	1	2184,1884	2184,1884	0,385	0,5391
E*D	1	641,2806	641,2806	0,113	0,7388
E*P	1	10543,0090	10543,0090	1,860	0,1821
D*P	1	38980,0435	38980,0435	6,877	0,0133
E*D*P	1	38243,0928	38243,0928	6,747	0,0141
erro	32	181372,8274	5667,9008		
CV%	6,75				

Tabela 11A - Variável analisada: DUODENO CRIPTAS

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	25398,5760	25398,5760	13,352	0,0009
Doença (D)	1	6124,6350	6124,6350	3,220	0,0822
Probiótico (P)	1	57968,4276	57968,4276	30,473	0,0000
E*D	1	47630,7022	47630,7022	25,039	0,0000
E*P	1	7428,8953	7428,8953	3,905	0,0568
D*P	1	5785,4680	5785,4680	3,041	0,0908
E*D*P	1	263,3742	263,3742	0,138	0,7123
erro	32	60872,5904	1902,2684		
CV%	7,00				

Tabela 12A - Variável analisada: JEJUNO VILOSIDADES

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	212738,2688	212738,2688	32,180	0,0000
Doença (D)	1	71123,0789	71123,0789	10,759	0,0025
Probiótico (P)	1	24802,8900	24802,8900	3,752	0,0616
E*D	1	59818,6230	59818,6230	9,049	0,0051
E*P	1	5902,2273	5902,2273	0,893	0,3518
D*P	1	37267,9725	37267,9725	5,637	0,0237
E*D*P	1	32796,8109	32796,8109	4,961	0,0331
erro	32	211547,7508	6610,8672		
CV%	12,61				

Tabela 13A - Variável analisada: JEJUNO CRIPTAS

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	50944,6200	50944,6200	14,806	0,0005
Doença (D)	1	20485,1286	20485,1286	5,954	00204
Probiótico (P)	1	40168,3426	40168,3426	11,674	0,0017
E*D	1	7608,4947	7608,4947	2,211	0,1468
E*P	1	248,2530	248,2530	0,072	0,7900
D*P	1	59015,4286	59015,4286	17,152	0,0002
E*D*P	1	52146,4515	52146,4515	15,156	0,0005
erro	32	110103,6454	3440,7389		
CV%	16,26				

Tabela 14A - Variável analisada: ÍLEO VILOSIDADES

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	36673,9248	36673,9248	24,840	0,0000
Doença (D)	1	9293,9619	9293,9619	6,295	0,0174
Probiótico (P)	1	744,5964	744,5964	0,504	0,4827
E*D	1	4672,5145	4672,5145	3,165	0,0847
E*P	1	3732,2376	3732,2376	2,528	0,1217
D*P	1	641,9214	641,9214	0,435	0,5144
E*D*P	1	225,2451	225,2451	0,153	0,6987
erro	32	47244,8250	1476,4007		
CV%	7,48				

Tabela 15A - Variável analisada: ÍLEO CRIPTAS

Opção de transformação: Variável sem transformação (Y)

FV	GL	SQ	QM	Fc	Pr>Fc
Estresse (E)	1	4370,8174	4370,8174	5,335	0,0275
Doença (D)	1	889,7205	889,7205	1,086	0,3052
Probiótico (P)	1	13279,8292	13279,8292	16,210	0,0003
E*D	1	1192,7916	1192,7916	1,456	0,2364
E*P	1	5829,5688	5829,5688	7,116	0,0119
D*P	1	116,3833	116,3833	0,142	0,7087
E*D*P	1	259,6412	259,6412	0,317	0,5774
erro	32	26214,9528	819,2172		
CV%	8,40				