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Instituto de Ciência e Tecnologia

JAQUELINE LEMES RIBEIRO

EFEITOS DE PROBIÓTICOS E POSBIÓTICOS NA SAÚDE ÓSSEA

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Orientadora: Profa. Assoc. Ana Lia Anbinder

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IMPACTO POTENCIAL DESTA PESQUISA

A possibilidade de prevenir a perda óssea pós-menopausa com um método natural e não invasivo tem apelo importante. Bons resultados foram obtidos, e o uso de *L. reuteri* inativado é interessante porque apresentaria menos efeitos adversos, maior facilidade na produção, padronização, estabilidade e tempo de prateleira.

POTENTIAL IMPACT OF THIS RESEARCH

The possibility of preventing postmenopausal bone loss with a natural and non-invasive method has significant appeal. Good results have been achieved, and the use of inactivated *L. reuteri* is interesting because it would have fewer adverse effects, easier production, standardization, increased stability, and shelf life.

BANCA EXAMINADORA

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RESUMO

Ribeiro JL. Efeitos de probióticos e posbióticos na saúde óssea [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2023.

O envelhecimento da população tem sido acompanhado por um aumento significativo nas doenças crônicas, com destaque para a osteoporose. A busca por tratamentos alternativos tem levado ao estudo dos probióticos para prevenção e tratamento da perda óssea pós-menopáusica, com resultados encorajadores. Embora os benefícios dos probióticos sejam numerosos, eles são bactérias vivas e a administração de organismos vivos não é isenta de riscos. Os posbióticos são probióticos inativados ou seus produtos, e poucos são os estudos que avaliam seus efeitos ósseos. Nós revisamos a literatura sobre probióticos e posbióticos na saúde óssea e avaliamos os efeitos de *Limosilactobacillus reuteri* 6475 viável e inativado por calor em camundongos ovariectomizados. Mecanismos de ação semelhantes, como modulação da expressão de citocinas e ativação de sistemas de sinalização celular, são observados em probióticos e posbióticos, e os últimos têm vantagens como maior estabilidade e facilidade de incorporação em alimentos, embora seus efeitos a longo prazo, estabilidade e modo de ação ainda precisem ser estudados. Para que estas terapias sejam validadas clinicamente, é fundamental padronizar metodologias, aumentar o tamanho das amostras, realizar estudos randomizados e cegos, e definir detalhes como cepa, dose e duração do tratamento. Em um estudo in vivo, avaliamos os efeitos de *L. reuteri* (ATCC PTA 6475), e sua forma inativada (*heat-killed*) na perda óssea induzida por ovariectomia (ovx). Camundongos adultos, fêmeas, foram divididos aleatoriamente em quatro grupos: controle (*sham*); Ovx; Ovx + posbiótico; Ovx + probiótico. *L. reuteri* foi administrado aos grupos (10^9 UFC/dia) por gavagem. O tratamento se iniciou uma semana após a ovx e teve duração de 28 dias. Na análise por microscopia eletrônica de varredura, o probiótico manteve sua estrutura intacta, e no posbiótico foram observados alguns rompimentos na superfície celular. Foi avaliada a microarquitetura de fêmur, utilizando microtomografia computadorizada. Análise de expressão gênica em íleo foi feita para avaliar junções intercelulares intestinais e citocinas pró-inflamatórias, por meio dos marcadores *Ocludina*, *Tnf-α* e *Il-6*. Os dados foram submetidos ao teste estatístico mais conveniente ($\alpha = 0,05$). Os grupos Ovx apresentaram menor porcentagem de volume ósseo (BV/TV), menor número de trabéculas ósseas e maior porosidade total, em comparação ao grupo controle, porém os grupos que receberam *L. reuteri* apresentaram maior BV/TV quando comparados ao grupo Ovx. O tratamento com *L. reuteri* em suas duas formas levou à maior espessura trabecular, quando comparados ao controle e ao grupo Ovx. Todos apresentaram semelhança na expressão gênica da *Ocludina*, *Tnf-α* e *Il-6* em intestino. Ambas as formas, viável e inativada por calor, preveniram a perda óssea induzida por depleção de estrógeno.

Palavras-chave: probiótico; posbiótico; *heat-killed*; osteopenia.

ABSTRACT

Ribeiro JL. Effects of probiotics and postbiotics in bone health [doctorate thesis]. São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2023.

The aging of the population has been accompanied by a significant increase in chronic diseases, with osteoporosis standing out. The search for alternative treatments has led to the study of probiotics for the prevention and treatment of postmenopausal bone loss, with encouraging results. Although the benefits of probiotics are numerous, they are live bacteria, and the administration of live organisms is not risk-free. Postbiotics are inactivated probiotics or their products, and few studies have evaluated their bone effects. We reviewed the literature on probiotics and postbiotics in bone health and assessed the effects of both viable and heat-killed Limosilactobacillus reuteri 6475 in ovariectomized mice. Similar mechanisms of action, such as the modulation of cytokine expression and activation of cellular signaling pathways, are observed in probiotics and postbiotics, and the latter have advantages such as greater stability and ease of incorporation into food, although their long-term effects, stability, and mode of action still need to be studied. To clinically validate these therapies, it is crucial to standardize methodologies, increase sample sizes, conduct randomized and blinded studies, and define details such as strain, dosage, and treatment duration. In an in vivo study, we evaluated the effects of L. reuteri (ATCC PTA 6475) and its heat-killed form on ovariectomy (ovx)-induced bone loss. Adult female mice were randomly divided into four groups: control (sham); OVX; OVX + postbiotic; OVX + probiotic. L. reuteri was administered to the groups (10^9 CFU/day) by gavage. Treatment began one week after ovx and lasted for 28 days. Scanning electron microscopy analysis showed that the probiotic maintained its intact structure, while some cell surface disruptions were observed in the postbiotic. Femur microarchitecture was evaluated using computerized microtomography. Gene expression analysis in the ileum was performed to assess intestinal intercellular junctions and pro-inflammatory cytokines, using markers Ocludin, Tnf-α, and Il-6. The data were subjected to the most appropriate statistical test ($\alpha = 0.05$). The Ovx groups showed a lower percentage of bone volume (BV/TV), a lower number of trabecular bones, and greater total porosity compared to the control group. However, the groups that received L. reuteri showed higher BV/TV compared to the Ovx group. Treatment with both forms of L. reuteri led to greater trabecular thickness compared to the control and Ovx groups. All groups exhibited similarity in the gene expression of Ocludin, Tnf-α, and Il-6 in the intestine. Both viable and heat-killed forms prevented estrogen depletion-induced bone loss.

Keywords: probiotic; postbiotic; heat-killed; osteopenia.

SUMÁRIO

1 INTRODUÇÃO

A osteoporose é uma doença sistêmica caracterizada pela redução da massa óssea e degradação da microarquitetura do tecido ósseo, resultando em fragilidade óssea e aumento do risco de fratura (National Institutes of Health, 2001). É uma doença altamente prevalente, e estima-se que no Brasil, aproximadamente 33% das mulheres pós-menopáusicas (International Osteoporosis Foundation, 2020) e mais da metade dos indivíduos acima de 50 anos, nos Estados Unidos, apresentem osteoporose ou osteopenia (Wright et al., 2014). Estima-se que uma a cada duas mulheres e um em cada quatro homens acima de 50 anos sofram alguma fratura devido à osteoporose (McCabe et al., 2013). Calcula-se que a prevalência da osteoporose em mulheres, mundialmente, seja de 18,3% (95% IC 16,2 – 20,7), enquanto nos homens seja de 11,7% (95% IC 9,6 – 14,1) (Salari et al., 2021). Além da alta prevalência, esta doença progride de forma silenciosa, podendo chegar a um estágio grave (geralmente fraturas) sem que o hospedeiro tenha alguma sintomatologia. A osteoporose se tornou um problema de saúde pública em âmbito mundial, causando aproximadamente 8,9 milhões de fraturas, e ainda assim sua magnitude não é totalmente compreendida, devido à variabilidade nos métodos de avaliação (OMS, 2004). Além disso, as despesas com fraturas relacionadas à osteoporose são significantes, custando aproximadamente £4 bilhões por ano no Reino Unido, e US$17,9 bilhões nos Estados Unidos (Clynes et al., 2020). Muitos fatores podem afetar a quantidade de massa óssea, podendo ser fixos ou modificáveis, como idade avançada, disfunções na tireoide e paratireoide, baixo peso (má nutrição), uso prolongado de medicamentos, artrite reumatoide, diabetes, transplantes, deficiência hormonal, deficiência de vitamina D, consumo de álcool e cigarro (Gallagher, Tella, 2014).

Quanto à sua classificação, a osteoporose pode ser primária ou secundária. A osteoporose primária pode ocorrer em ambos os sexos, em qualquer idade, mas frequentemente ocorre após a menopausa em mulheres, e em homens de idade avançada. Já a osteoporose secundária ocorre como consequência de uso contínuo de medicamentos (como glicocorticoides), outras condições ou doenças (National Institutes of Health, 2001). A osteoporose pós-

menopausa (OPM) é o tipo mais comum, e ocorre em consequência da redução de produção hormonal e estimula a rápida reabsorção de osso trabecular (Dar et al., 2018).

Inúmeras terapias têm sido desenvolvidas para o tratamento da osteoporose, com intuito de diminuir a perda óssea, prevenir fraturas e balancear a formação/reabsorção óssea. O tratamento pode ser farmacológico ou não farmacológico. Para indivíduos com baixo risco de fratura, é indicado somente o tratamento não farmacológico, que é baseado na ingestão de cálcio, vitamina D, proteínas, cessação do uso de tabaco e álcool, prática de exercícios físicos, e alterações na dieta. Para indivíduos com médio/alto risco de fratura, além do tratamento não farmacológico, medicamentos antirreabsortivos (como bisfosfonatos) e a terapia de reposição hormonal, nas mulheres, também são indicados (Gallagher, Tella, 2014).

A terapia de reposição hormonal é um dos tratamentos mais utilizados para o alívio dos sintomas associados à menopausa e para prevenção de consequências clínicas da deficiência de estrógeno. Entretanto, o uso hormonal prolongado pode causar efeitos adversos (principalmente aumento no risco de acidentes tromboembólicos, infartos e câncer de mama), o que tem levado a diminuição no seu uso, e a um aumento no desenvolvimento de novas possibilidades terapêuticas como o uso de probióticos e vitaminas (Borrelli, Ernst, 2010).

Pesquisas realizadas em seres humanos e animais têm demonstrado de maneira consistente que a microbiota presente no intestino desempenha um papel crucial na manutenção da saúde do hospedeiro. Essa função abrange diversas áreas, como o metabolismo (incluindo a produção de hormônios como a dopamina e a serotonina), a absorção de nutrientes essenciais, a proteção contra patógenos por meio da integridade da barreira intestinal, e o fortalecimento do sistema imunológico (Bhardwaj et al., 2022). As junções intercelulares (TJs), estruturas proteicas que selam os espaços entre as células epiteliais intestinais, são responsáveis por essa integridade. Adaptações dinâmicas dessas TJs permitem que respondam a fatores externos, como a presença de resíduos alimentares, bactérias benéficas e patógenos (Ulluwishewa et al., 2011). O estrógeno desempenha um papel vital na preservação dessas junções, ativando vias que aumentam a resistência elétrica

através da barreira transepitelial, efetivamente impedindo a entrada de patógenos. Como resultado, uma deficiência neste hormônio enfraquece a barreira intestinal, aumentando o contato com antígenos e bactérias, o que desencadeia uma resposta inflamatória (Xu et al., 2017).

Probióticos são microrganismos não patogênicos, que quando administrados em quantidades adequadas, podem ser benéficos à saúde do hospedeiro (Guidelines for the Evaluation of Probiotics in Food, 2002). Muitos gêneros de bactérias são utilizados devido a seus efeitos probióticos, como *Lactobacillus*, *Enterococcus*, *Bacilllus*, *Escherichia* e *Bifidobacterium*. Bactérias probióticas são naturalmente encontradas em membranas mucosas como boca, pele, órgãos genitais, urinários, trato gastrointestinal, e comumente adicionadas a produtos fermentados como laticínios e cerveja (Collins et al., 2017b). O consumo de probióticos tem demonstrado diversas vantagens, como melhora na saúde óssea, manutenção na função da barreira intestinal e na função do sistema imune, resultando em uma melhora sistêmica na saúde do hospedeiro, incluindo saúde óssea (Mccabe, Parameswaran, 2018). O uso de *Limosilactobacillus reuteri*, em especial, da cepa ATCC PTA 6475, tem sido associado à redução da perda óssea devido à deficiência de hormônios ovarianos em estudo em animais e humanos (Britton 2014, Nilsson 2018).

Apesar dos bons resultados obtidos pelo uso dos probióticos, as células vivas contidas nos produtos eventualmente perdem sua viabilidade, e uma quantidade de células inativas permanece (Nighswonger et al., 1996). Devido ao uso de bactérias vivas, a preocupação com a segurança de alguns grupos, como de pacientes pediátricos e populações vulneráveis, tem crescido, levando a um aumento no interesse no uso de bactérias mortas, ou não-viáveis (Piqué et al., 2019).

Na literatura têm sido propostos termos como “probióticos não-viáveis”, “paraprobióticos”, “probiótico fantasma”, “probiótico inativado”, e mais recentemente, “posbiótico”, para se referirem ao uso de microrganismos inativados e/ou seus componentes, em quantidade adequada para promover benefícios à saúde (Vinderola et al., 2022). Os posbióticos possuem vantagens como maior facilidade na padronização, armazenagem e transporte (Piqué et al., 2019) em relação ao uso de células vivas. Os possíveis mecanismos de ação

pelos quais os posbióticos melhoram a saúde do hospedeiro ainda vêm sendo estudados.

A inativação das bactérias pode ser feita de diversas formas, como calor, métodos químicos (formalina), raios ultravioleta ou gama, e sonicação, sendo o calor o método mais utilizado (Deshpande et al., 2018). Os métodos de inativação podem afetar de diferentes formas os componentes estruturais celulares, e influenciar a atividade imunomodulatória (Taverniti, Guglielmetti, 2011).

Frente à necessidade de novas alternativas terapêuticas ou adjuntas ao tratamento da osteoporose, doença crônica que pode ser considerada um problema de saúde pública na população com a maior expectativa de vida, e ao crescente uso de probióticos e posbióticos, levantamos a hipótese de que *L. reuteri* na sua forma viável e inativada, podem ser efetivos na melhora da saúde e prevenção da perda óssea que ocorre pós-menopausa. O estudo dos efeitos de *L. reuteri* viável e inativado pode esclarecer os mecanismos de ação do probiótico e possibilitar o uso comercial de outras preparações, dado que os posbióticos teriam produção facilitada, maior estabilidade e tempo de prateleira, além de ser um tratamento de fácil acesso à população.

2 ARTIGOS

2.1 Artigo 1 – Ribeiro JL, Santos TA, Anbinder AL. Benefícios que até a morte não separa: a promessa dos probióticos e posbióticos para a saúde óssea / *Benefits that death does not part: the promise of probiotics and postbiotics for bone health**

RESUMO

A osteoporose pós-menopáusica (OPM) é um problema de saúde global generalizado caracterizado pela deficiência hormonal. As terapias existentes para OPM muitas vezes têm efeitos colaterais indesejáveis, necessitando o desenvolvimento de tratamentos alternativos seguros e eficazes. Os probióticos, que são microrganismos vivos que promovem a saúde do hospedeiro, têm sido explorados como uma solução potencial. No entanto, surgiram preocupações quanto ao seu uso, principalmente quanto à segurança quando administrado a populações vulneráveis (por exemplo, idosos e indivíduos imunodeficientes). Além disso, o processamento industrial e o armazenamento de produtos probióticos apresentam desafios tecnológicos que podem comprometer sua viabilidade. Para lidar com essas desvantagens, o foco mudou para probióticos não viáveis, conhecidos como posbióticos. Posbióticos são definidos como células microbianas inativadas (mortas) ou seus subprodutos que fornecem benefícios à saúde dos consumidores. Eles exibem propriedades imunomoduladoras e demonstram efeitos antagônicos contra patógenos. Além disso, os posbióticos oferecem maior segurança, vantagens tecnológicas e benefícios práticos. Ao alavancar esses atributos únicos, os posbióticos apresentam uma oportunidade promissora de introduzir novas terapias mais seguras e estáveis. Esta revisão fornece uma visão geral das principais considerações em torno dos posbióticos, resume a literatura recente sobre sua aplicação na OPM e ressalta a necessidade urgente de mais ensaios clínicos randomizados. Esses ensaios devem avaliar a segurança, eficácia e mecanismos de ação dos produtos posbióticos para apoiar os processos de tomada de decisão.

Palavras-chave: Posbiótico. Osteoporose. Microbiota intestinal.

ABSTRACT

Postmenopausal osteoporosis (PMO) is a widespread global health concern characterized by hormone deficiency. The existing therapies for PMO often have undesirable side effects, necessitating the development of safe and effective alternative treatments. Probiotics, which are live microorganisms that promote the host health, have been explored as a potential solution. However, concerns

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have been raised regarding their use, particularly the safety when administered to vulnerable populations (e.g., elderly and immunodeficient individuals). Additionally, the industrial processing and storage of probiotic products present technological challenges that can compromise their viability. To address these drawbacks, the focus has shifted towards non-viable probiotics known as postbiotics. Postbiotics are defined as inactivated (dead) microbial cells or their byproducts that provide health benefits to consumers. They exhibit immunomodulatory properties and demonstrate antagonistic effects against pathogens. Furthermore, postbiotics offer enhanced safety, technological advantages, and practical benefits. By leveraging these unique attributes, postbiotics present a promising opportunity to introduce novel therapies that are safer and more stable. This review provides an overview of key considerations surrounding postbiotics, summarizes recent literature on their application in PMO, and underscores the urgent need for further randomized clinical trials. These trials should evaluate the safety, efficacy, and mechanisms of action of postbiotic products to support regulatory decision-making processes.

Keywords: Postbiotics. Osteoporosis. Gut microbiota.

1. Introduction

Osteoporosis is one of the diseases most associated with the aging population. It is a systemic inflammatory condition characterized by an imbalance between bone formation and resorption, resulting in the degradation of bone microarchitecture and loss of bone mass. Osteoporosis can be classified as either primary or secondary. Primary osteoporosis often occurs in postmenopausal women and elderly men. Secondary osteoporosis develops as a result of other underlying diseases or conditions, as well as the prolonged use of certain medications (such as glucocorticoids)[1]. The recommended treatment depends on the individual's fracture risk, which is related to the bone mineral density (BMD)[2]. For those at low risk, non-pharmacological treatment is recommended, such as adequate intake of proteins, calcium, and vitamin D, as well as smoking cessation and alcohol moderation. However, for individuals at moderate/high risk, in addition to non-pharmacological treatment, the use of antiresorptive or anabolic medications may be necessary. Bisphosphonates (such as alendronate, zoledronic acid, for example) and denosumab (monoclonal antibody against receptor activator of nuclear factor kappa-B ligand [RANKL]) are the most prescribed medications, as they inhibit bone resorption through anti-osteoclastogenic effects (American Association of Clinical Endocrinologists and American College of Endocrinology)[3]. Despite their effectiveness, systemic side effects related to the use of bisphosphonates and denosumab have been reported, including gastrointestinal, renal, immunological effects, and osteonecrosis of the jawbones[3]. Hormonal replacement therapy is often used for prevention rather than treatment and it also has adverse effects as increased risk of thromboembolic events, heart attacks and breast cancer[4]. In light of the adverse effects associated with current treatment options, there has been a pursuit for new therapeutic alternatives, among which the use of probiotics is being considered[5].

Probiotics are defined as “non-pathogenic microorganisms which, when administered in adequate amounts, confer health benefits to the host”[6], and their use in clinical trials has shown promising results in numerous diseases and situations, such as high blood pressure and cholesterol levels, inflammation, immune system, mental health[7], periodontal disease[8], but specially in

gastrointestinal tract[9] and bone health[10–12]. It has been demonstrated that probiotics exert their beneficial effects through various mechanisms, including immunomodulation, antimicrobial effects, and improvement of gut epithelial barrier function[13].

The use of live microorganisms carries potential risks for certain groups, such as immunosuppressed patients, leading to an increasing interest in the use of non-viable bacteria or their byproducts, known as postbiotics[14]. Studies evaluating the use of postbiotics in osteoporosis are still limited, and in the following sections, we will address the potential mechanisms of action of pro- and postbiotics in bone health. We will discuss the existing literature, as well as the limitations and potential methodologies to translate these findings into clinical practice.

2. Osteoporosis: estrogen levels and the role of intestinal microbiota in shaping host health

Estrogen regulates bone health through modulation of the immune system and its direct action on bone cells[15]. Estrogen increases the maturation and differentiation of osteoblasts from mesenchymal stem cells (MSCs), thereby enhancing bone formation[16]. Hormonal deficiency also causes an increase in the production of RANKL and pro-inflammatory cytokines (such as tumor necrosis factor [TNF]-α, interleukin [IL]-1, IL-6, and IL-17), and suppression of the differentiation of Th1, Th2, and Treg cells, leading to a decrease in the production of anti-inflammatory cytokines (IL-4, IL-10, interferon [IFN]-γ, and CTLA4) and osteoprotegerin (OPG) by B cells. This entire process results in increased osteoclastogenesis and consequent bone resorption[17].

The intestinal epithelium serves as a vital barrier that selectively absorbs water, nutrients, while also physically restricting the entry of harmful microorganisms. The integrity of this barrier is maintained by dynamic structures, the tight junctions, which consist of proteins complexes including occludin, claudin, and junctional adhesion molecules. Under pathological or physiological stimuli, these intercellular junctions can undergo alterations[18]. Estrogen activates the Ras protein and a cascade of kinases (Raf, MEK1/2, ERK1/2) that play a crucial role in maintaining intercellular junctions. By binding to receptors in the

intestinal epithelium, estrogen increases the transepithelial electrical resistance, effectively preventing the entry of pathogens. However, in the absence of sufficient estrogen, this protective barrier weakens, allowing bacteria and other antigens to breach the intestinal lining, come into contact with antigen-presenting cells, and trigger a cascade of events, leading to inflammation[19]. The antigen-presenting cells activate CD4 T cells, which then differentiate into two distinct subtypes: Th1 and Th17 cells. Once differentiated, Th1 and Th17 cells start producing cytokines that are both pro-inflammatory and osteoclastogenic, leading to bone resorption[20] (Figure 1).

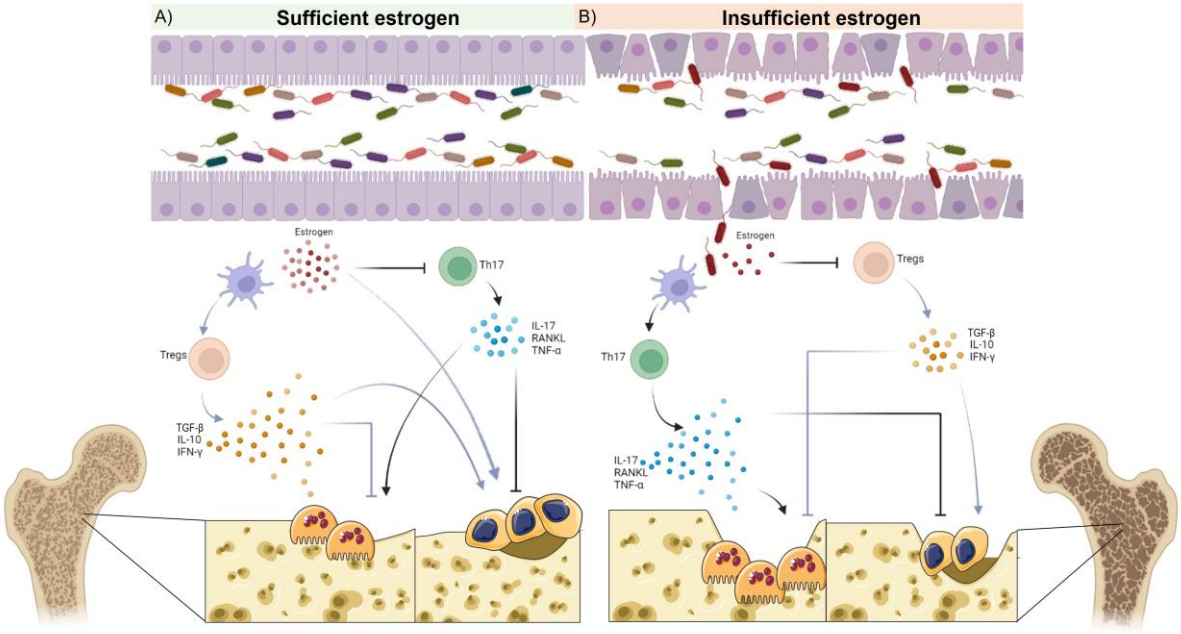

Figure 1: Microbiota-estrogen-bone interaction. A) The beneficial gut bacteria and appropriate levels of estrogen activate Tregs, which produce TGF-β and anti-inflammatory cytokines, to prevent osteoclastogenesis and induce osteoclastic apoptosis. Estrogen also acts directly on the bone by binding to receptors on the bone surface, leading to an increase in osteoblast proliferation and differentiation, favoring bone formation. Additionally, it reduces the formation of Th17 cells, which are associated with bone resorption. B) Estrogen deficiency occurring in osteoporosis reduces the function of the epithelial barrier, leading to pathogen invasion. This activates CD4+ T cells, which differentiate into Th17 cells, increase the production of TNF-α and pro-inflammatory cytokines, and stimulate RANKL, promoting osteoclastogenesis. Consequently, there is a reduction in osteoblast formation, resulting in bone loss and microstructural destruction. Created with BioRender.com.

Estrogen deficiency also can lead to alterations in the composition and structure of the microbial community residing in the gut[21]. This disruption in the

balance of the microbiota can further contribute to inflammatory responses and immune dysregulation, exacerbating the consequences of estrogen deficiency.

The intestinal microbiota plays a crucial role in maintaining host health, since it exerts a significant influence on metabolism, pathogen resistance, and the immune system[15]. The intestinal microbiota has been recognized as a "virtual endocrine organ" due to its ability to influence the host's hormonal levels and the production and secretion of hormones by specific microorganisms[22]. This occurs, in part, due to the production of β-glucuronidase, an enzyme responsible for converting estrogens into their active forms, by some microorganisms[23]. Numerous microorganisms inhabiting the human gastrointestinal tract have a symbiotic relationship with their host[21], but there are multiple factors that contribute to host dysbiosis, such as diet and metabolite production[24]. A growing number of studies have demonstrated a clear link between dysbiosis and the development of various clinical conditions, including obesity[25], metabolic disease[26], inflammatory bowel disease[27], and cardiovascular disease[28].

There is a strong association between bone homeostasis and a healthy microbiota, while intestinal dysbiosis can worsen osteoclastic activity and contribute to the development of osteoporosis[29]. Bone health is influenced by the intestinal microbiota due to its role in regulating the absorption of minerals[30], and vitamin D[31], in the secretion of hormones like serotonin and insulin-like growth factor (IGF)-I and in the production of short-chain fatty acids (SCFAs), which have anti-inflammatory effects by inhibiting the activation of the NF-κB pathway and reducing inflammatory immune responses[32]. In addition, SCFAs have the remarkable ability to reprogram osteoclasts, effectively inhibiting osteoclastogenesis and bone resorption[33]. Furthermore, the gut-brain axis is known to have an impact on bone health, with the intestinal microbiota playing a significant role in this complex interplay[34]. A bidirectional communication takes place between the intestinal microbiota and components of the gut-brain axis, which occurs primarily through three pathways: the vagus nerve, immune response, and microbial metabolites[35].

Recognizing the importance of the intestinal microbiota in the development of postmenopausal osteoporosis, it is essential to be considered as a potential target for future osteoporosis treatments.

3. Probiotics and posbiotics: Benefits that death does not part

Despite Metchnikoff's initial observation over 100 years ago, it was only in recent decades that the use of probiotics gained some popularity[36].

There has been a marketing frenzy surrounding probiotics to the point where people are led to believe that consuming them is always beneficial and free of risks[37]. While probiotics are considered non-pathogenic living microorganisms, there is concern about potential adverse effects in certain vulnerable patients such as neonates and immunosuppressed[36]. In these groups, there are reports that certain probiotic strains can cause more severe clinical conditions such as pneumonia, endocarditis, and sepsis[37].

For over a decade, there has been an increasing body of evidence suggesting that probiotics' health benefits are not solely associated with viability. Probiotic preparations often contain a small portion of non-viable cells and bacterial by-products, leading to a rise in interest in utilizing non-viable cell preparations[36]. In 2021, the International Scientific Association of Probiotics and Prebiotics (ISAPP) proposed the definition of postbiotics as "preparations of inanimate microorganisms and/or their components that confer a health benefit on the host"[38]. Derived from Greek, 'post' means after, and 'bios' means life, therefore 'after-life'[14]. Unlike probiotics, postbiotics seems to be risk-free in vulnerable individuals[39] and there is no issue in administering it concomitantly with an antibiotic or antifungal, for example[36].

Postbiotics have been investigated in various studies, demonstrating positive outcomes in conditions such as *H. pylori* infection[40,41], improvement of pain from colorectal distension[42], viral infections[43] and ulcerative colitis[44–46], among others (Figure 2).

Evidence demonstrated the successful eradication of the *H. pylori*, through competition for adhesion sites, after the combined administration of antibiotics and heat-inactivated *L. acidophilus*[40]. Singh et al. (2017) also demonstrated that non-viable *L. reuteri* retains its capacity for adherence and acts as an antagonist against pathogens[47].

Furthermore, postbiotics have been found to exhibit immunomodulatory effects. In a review conducted by Adams (2010), it was shown the ability of heat-inactivated bacteria belonging to the genus *Enterococcus faecalis* to stimulate

the gastrointestinal immune system in chickens[48]. In an in vitro study, when exposed to the heat-killed *L. brevis*, macrophages demonstrated increased production of TNF-α, lL-6, and nitric oxide and enhancement of phagocytosis. Further investigation revealed that the postbiotic positively regulated the MAPK signaling pathway, specifically targeting the activation of ERK, JNK and p38. These signaling pathways play vital roles in macrophage activation and immune response[49]. All these effects are similar to those produced by probiotics.

Probiotic products require stricter control during formulation and manufacturing to ensure an adequate survival rate of bacteria in the product. This is crucial for maintaining both the physical and genetic stability of the microorganism throughout the product manufacturing process and during storage[50]. Several factors, such as oxygen, temperature, and humidity can influence the viability of microorganisms, thereby limiting their shelf life[50,51], representing a challenge for probiotic products. Even the product packaging, including the choice of materials, must be carefully considered to mitigate these challenges[51]. In the case of postbiotics, which are inanimate microorganisms, standardization, storage and transportation may be easier[52].

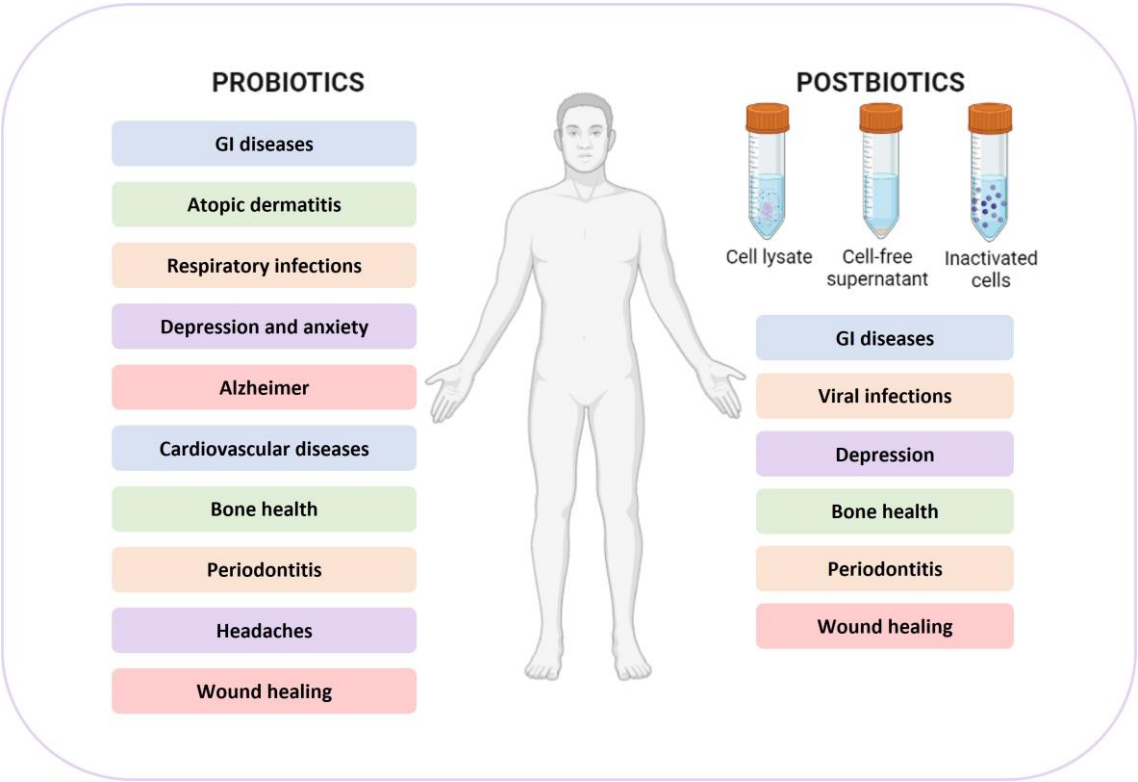

Figure 2: Diseases and conditions in which the effects of pro and postbiotics are studied[53–62]. Created with BioRender.com.

4. The role of probiotics and postbiotics in preserving bone health

Considering the significant role of the host's intestinal microbiota in overall health, probiotics have emerged as a potential therapeutic approach to mitigate estrogen deficiency-induced bone loss, with good results in rodent models[63–69] and clinical studies[10–12].

Due to the promising outcomes, the mechanisms of action by which probiotics act on the host's bone health are being investigated[70]. In the intestinal tract, certain strains of probiotics have been shown to decrease the production of pro-inflammatory and pro-osteoclastogenic cytokines[71–74], which may directly enhance the transport of calcium across the intestinal barrier[30].

Although the number of studies linking the use of postbiotics to bone health is still limited (Table 1 and 2), there is a growing interest in this area of research. In our research group, we investigated the effects of *L. reuteri*, both in its viable and heat-inactivated form, in a rat model of induced periodontitis. The treatments with probiotic and postbiotic showed similar preservation of bone volume, reducing alveolar bone loss, and improving parameters of bone microarchitecture[8].

All the studies, both in vitro and in vivo, demonstrated similar and promising results. In these studies, the administration of postbiotics led to reduced bone loss, decreased levels of bone resorption markers, increased bone density, and improved bone microarchitecture, apart from one study, where no changes in bone density were observed[75].

Table 1 – In vitro studies using postbiotics

Author/Year	Cell type	Postbiotic preparation	Strain	Methodology	Results
Jang, 2021[76]	Macrophages	Cell-free supernatant	*L. curvatus* Wikim 38 (LC38)	Cultured with RANKL for 4 days, and then treated with LC38 for 24 hours.	↓ Formation of TRAP-positive multinucleated cells; formation of F-actin rings in osteoclasts; expression of TRAF6, MAPKS, and NF-kB; genes *NFATc1*, *TRAP*, *DC-STAMP*, and *cathepsin* K.
Yeom, 2021[77]	Human fetal osteoblasts (hFOB 1.19)	Heat-killed	*Propionibacterium freudenreichii* MJ2 (hkMJ2)	Treated with hkMJ2 ($1x10^6$, $1x10^7$ or $1x10^8$ CFU/ml) for 14 days	↑ ALP activity and osteoblast mineralization; *Opg*/*Rankl*, *Fibronectin* and *Osteocalcin* mRNA levels; activated BMP2/RUNX2 signaling pathway.
Jhong, 2022[78]	RAW 264.7	Heat-killed	*L. paracasei* GMNL-653	GMNL-653 for 2h, then *E. coli* LPS for 20h	↓ IL-6 and nitric oxide levels
	MG63			GMNL-653 $1x10^8$ CFU/ml for 28 days	↑ calcium deposits
Myeong, 2023[79]	Macrophages	Heat-killed	*L. plantarum* MD35	*L. plantarum* (5, 10 and 50 µg/ml) and MCSF (30 ng/ml) for 3 days; *L. plantarum*, MCSF and/or RANKL (100 ng/ml) for 7 days	↓ TRAP activity, osteoclastic formation, and RANKL-induced resorption activity; inhibited activation of the Nfatc1 pathway

Legend: ALP – Alkaline phosphatase; LPS – lipopolysaccharide; MCSF – Macrophage colony-stimulating factor.

Table 2 – In vivo studies using postbiotics

Author/Year	Species	Postbiotic preparation	Strain	Informed dose/concentration	Duration	Results
Kimoto-Nira, 2007[80]	Senescent male SAMP6 mice	Heat-killed	*Lactococcus lactis* subsp. *cremoris* H61	0,5 g/kg (w/w) in the diet	5 months	↑IL-12, IFN-γ, and bone density
Kimoto-Nira, 2009[75]	Senescent male SAMP6 mice	Heat-killed	*L. lactis* G50	0,5 g/kg (w/w) in the diet	11 months	No difference in bone density was observed
Jang, 2021[76]	C57BL/6 – OVX	Cell-free supernatant	*L. curvatus* Wikim 38	Diluted 0,5 and undiluted	12 weeks	↑BMD and BV/TV
Montazeri-Najafabady, 2021[81]	Sprague-Dawley female rats – OVX	Lysate and supernatant	*L. acidophilus, L. reuteri, L. casei, B. longum, B. coagulans*	$1x10^9$ CFU/ml/day	4 weeks	↑BMD: *L. casei* and *B. coagulans* lysate and supernatant; *B. longum* and *L. acidophilus* lysate; and *L. reuteri* supernatant.
Yeom, 2021[77]	Sprague-Dawley female rats – OVX	Heat-killed	*Propionibacterium freudenreichii* MJ2	$1x10^7$ or $1x10^8$ CFU/ml	4 months	↑BMD; cortical bone thickness, bone trabeculae; gene and protein expression levels of OPG/RANKL
Jhong, 2022[78]	ICR mice – OVX	Heat-killed	*L. paracasei* GMNL 653	$1x10^{10}$ CFU/ml	4 weeks	↑BV/TV; *Bmp-2*, *Tgf-β* and *Il-10* mRNA levels; ↓*Rankl*, *Trap-5* and *Il-17a* mRNA levels
Myeong, 2023[79]	C57BL/6 – OVX	Heat-killed	*L. plantarum* MD35	10 and 50 mg/kg	8 weeks	Suppressed weight gain and uterine atrophy; ↑BMD, BV/TV, Tb.Th e Tb.N; ↓growth plate destruction.

Legend: BV/TV – bone volume fraction; Tb.Th – trabecular thickness; Tb.N – trabecular number.

4.1 How the magic happens: probiotics and postbiotics mechanisms of action

The effects on host health exerted by postbiotics often closely resemble those exerted by probiotics, including the stimulation/supression of cytokine expression, activation of cellular signaling and antimicrobial activity. These findings suggest that the observed effects are not always reliant on cell viability[36]. The mechanisms by which probiotics and postbiotics exert their benefits and help maintain specifically bone health are summarized in Figure 3.

The presence and proliferation of probiotics in the gastrointestinal tract can directly influence the microbial balance. Additionally, probiotics can compete with other bacterial strains for resources and colonization sites, by attaching to epithelial cells and blocking physically the pathogen's adherence, further influencing the overall microbiota composition[82]. Furthermore, several lactic acid bacteria can produce bacteriocins (such as reuterin), which are peptides with antimicrobial properties[83].

One of the mechanisms of action of probiotics is the decrease of the permeability of the intestinal epithelial barrier. Research has focused on how microorganisms influence intercellular junction proteins, and it is believed that this regulation is mediated by SCFAs, particularly butyrate, which is a fermentation by-product. Butyrate plays a crucial role in stimulating the reorganization of intercellular junction proteins and increasing the production of protein kinases. As a result, the electrical resistance across the intestinal epithelium is strengthened, leading to an enhanced barrier function[70]. This improved barrier function has a direct impact on calcium absorption in the intestine[19]. In addition, fatty acids produced by probiotics can exert actions at a distance, since once released into the bloodstream, they can inhibit the differentiation of osteoclasts by binding to receptors on osteoclast precursor cells[84,85]. Another suggested mechanism of probiotic action at a distance from the gut involves the secretion of extracellular vesicles (EVs) which traverse the intestinal epithelial barrier, enter the systemic circulation, and reach bone tissue, where they directly regulate bone formation[86].

The beneficial effects of probiotics on animal and human health are largely attributed to their capacity to modulate the host's immune system. T cells are recognized as key mediators of inflammation as they can differentiate into different subsets (such as Th17 or Tregs) that promote or suppress the inflammatory response.

Probiotics influence this differentiation process through membrane receptors, particularly Toll-like receptor (TLR)-6 and TLR-2, expressed in macrophages and dendritic cells, which can decrease Th17 polarization and direct T cells towards Tregs[87]. Treg differentiation promotes the production of high levels of IL-10 and lower levels of TNF-α, reducing the inflammatory state. In the gut, probiotics can also activate B cells in the lamina propria, leading to the production of OPG[17] that binds to RANK, preventing RANK-RANKL interaction and inhibiting osteoclastogenesis. Probiotics can alter intracellular pathways of immune cells through kinases (such as MAP kinase cascade), which ultimately activate or suppress transcription factors (such as NF-kB)[87].

The gut microbiota is currently considered an “endocrine organ” because of its influence in the host’s hormonal levels (e.g., serotonin and incretins)[88]. Hormones and growth factors play a crucial role in regulating bone metabolism. Insulin-like growth factor-I (IGF-1), primarily secreted by the liver, is a key factor in longitudinal bone growth, skeletal maturation, and bone mass acquisition[89]. IGF-1 promotes the differentiation and growth of osteoblasts and osteoclasts[89], and the impact of the microbiota on IGF-1 has been investigated in germ-free models[90,91]. However, there is limited research available on the effects of probiotics on IGF-1 and its underlying mechanisms in the existing literature.

Probiotics are also involved in the improvement of absorption of minerals, such as calcium[92] and vitamin D[93]. Indigestible carbohydrates consumed in the diet are fermented by the gut microbiota, resulting in the production of SCFAs within the lumen of the cecum and colon[94]. SCFAs have been found to benefit bone metabolism by increasing the availability of various vitamins and minerals, such as vitamin K, vitamin B12, calcium, and magnesium[95] and decreasing the gut pH[96]. With a lower pH, there is an increase in calcium absorption[96].

Similar to probiotics, the postbiotics have mainly immunomodulatory properties (Tables 1 and 2), and these effects are not dependent on the viability of the probiotics themselves.

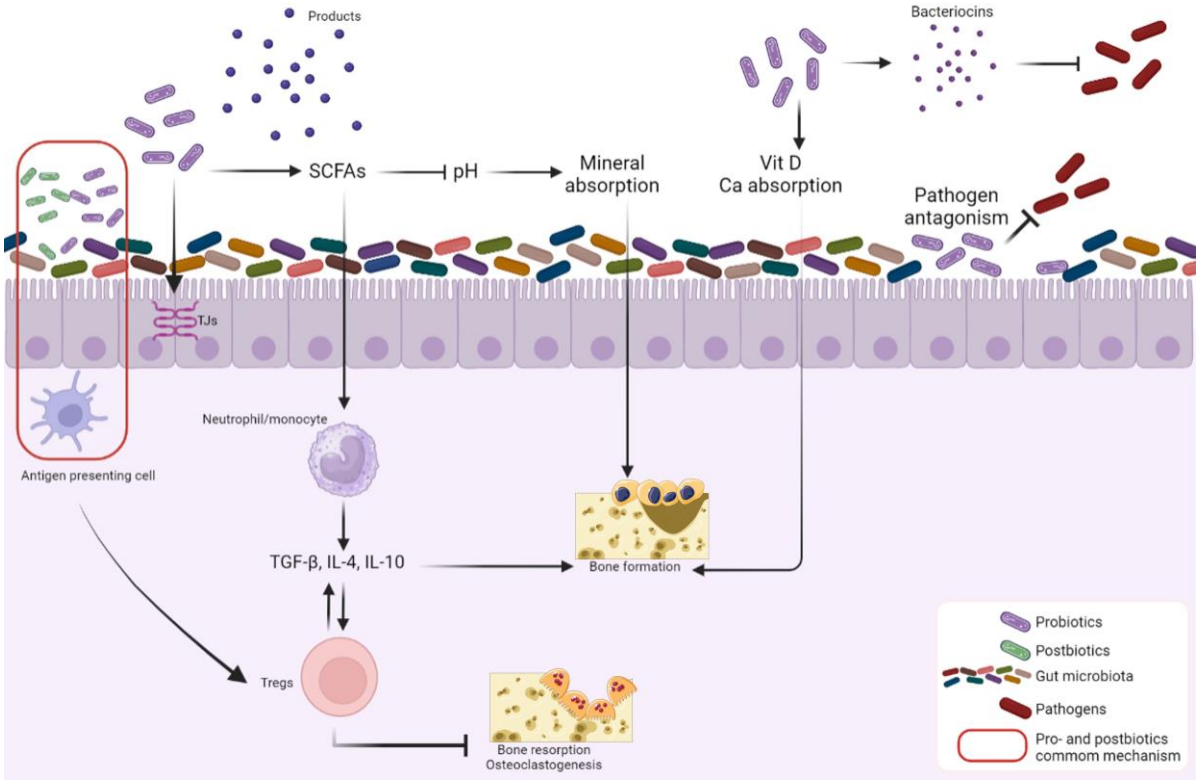

Figure 3: Mechanisms of action of pro- and postbiotics. Probiotics have antimicrobial properties, such as pathogen antagonism (through competition for sites and nutrition) and production of bacteriocins (e.g., reuterin). Probiotics also reduce intestinal permeability by positively regulating tight junctions, preventing the passage of microbial products that act as antigens. They increase the production of short-chain fatty acids (SCFAs), which cross the epithelial barrier and reach the systemic circulation. SCFAs activate neutrophils, which produce anti-inflammatory cytokines, leading to the differentiation of T cells into Tregs that also produce anti-inflammatory cytokines. This inhibits bone resorption and osteoclast differentiation. The anti-inflammatory cytokines also induce osteoblast differentiation, promoting bone formation. Probiotics activate antigen-presenting cells, which promote the generation of Tregs, preventing bone resorption and favoring bone formation. Additionally, SCFAs reduce intestinal pH, facilitating the absorption of minerals, vitamin D, and calcium. On the other hand, postbiotics (red square) also possess the ability to adhere to and antagonize pathogens. They are recognized by antigen-presenting cells, which induce the production of anti-inflammatory cytokines by Tregs, favoring bone formation and inhibiting bone resorption and osteoclastogenesis. This immunomodulatory effect does not depend on viability. Created with BioRender.com.

5. If it has good results, why is it still not used?

For some conditions, such as antibiotic-associated diarrhea, infectious diarrhea, and prevention of *Clostridioides difficile* infection, the use of probiotics is already recommended[97,98]. However, there is no consensus indicating the use of probiotics as an adjunct treatment for osteoporosis or several other diseases.

The effective colonization of the gastrointestinal tract plays a vital role in enabling probiotics to establish meaningful interaction with the host and confer permanent health advantages. The limited long-term effects of probiotics might be

attributed, in part, to colonization resistance[99]. Vestman et al.[100] found that 12-week consumption of two probiotic strains changed the microbial composition in tooth biofilms, but the overall diversity of species remained unchanged. Notably, the shifts in composition returned to normality within one month after discontinuation of probiotic exposure. Intriguingly, even though participants received daily booster doses, approximately 30% of them did not exhibit detectable levels of the test strains[100], demonstrating that the effectiveness of probiotic colonization can differ across individuals. Therefore, the continuous use of probiotics is necessary to exert their beneficial effects, which can be considered a potential drawback for their official recommendation.

Although preclinical and some clinical studies have shown promising results, the evidence is still limited, and further research is needed to establish their effectiveness in treating osteoporosis.

As far as we know, studies linking postbiotics and osteoporosis are still in the early stages and there are no randomized clinical trials. The studies on postbiotics and osteoporosis have certain limitations: they demonstrate benefits; however, they present significant heterogeneity in their methodology, varying greatly in the analysis methods, doses, strains, and species, making difficult to compare these studies to one another. The studies must be reproducible, therefore, the doses given to the animals (volume, concentration, for example) must always be correctly and precisely described. To the date (June, 2023), there are a few clinical trials registered associating probiotics and postmenopausal osteoporosis (ClinicalTrials.gov), with different methodologies and probiotics strains.

Currently, there are more studies in the literature involving animals than in humans[101]. The small number of human studies, along with their significant heterogeneity[101–103], poses a major limitation for meta-analyses. There is considerable variation in the number and age of participants, gender, bone health status, chronic conditions, strain used, dose, and duration of treatment[101–103]. Similarly, in animal studies, there is the use of different species, varying sample sizes, different forms of probiotic administration (such as through feed or gavage), as well as diverse strains, doses, and treatment durations[101]. Therefore, the results should be interpreted with caution. Due to the small number of studies available, meta-analyses often combine

studies involving different strains to draw conclusions, which introduces a significant risk of bias[101–103], as the effects of probiotics are strain-dependent.

As far as we known, there are no meta-analyses in the literature that have analyzed the use of postbiotics in bone health.

5.1 Maybe we can change the course of the river…

Based on studies on the use of probiotics in osteoporosis, how can we improve the methodology to avoid making the same mistakes in future studies with postbiotics? This is not an easy question to be answered, and the greatest challenge is to walk the talk, once guidelines for in vitro, animal and clinical trial studies are well known by the scientific community[104–107]. However, some recommendations could be highlighted.

Following promising preclinical studies, it is important to conduct randomized controlled clinical trials in humans to evaluate the efficacy and safety of postbiotics in osteoporosis. These studies should follow ethical guidelines, have appropriate control groups, and be adequately sized. Since osteoporosis is a chronic condition that develops over time, it is important to conduct long-term studies[108] and control the variables responsible to the heterogeneity of an elderly cohort, such as background inflammation levels, age, nutritional status, and frailty, which have the potential to influence the observed effect size[109]. In addition to bone mineral density, the evaluation of other relevant bone markers such as bone formation and bone resorption may allow a comprehensive understanding of the possible mechanisms of action of postbiotics on bone metabolism.

Postbiotics are a broad category of compounds, and like probiotics, their effects are strain- and disease-specific[9]. It is critical to select the postbiotics to be studied based on previous evidence of specific activities related to osteoporosis. According to a systematic review and meta-analysis, probiotic supplementation with *L. reuteri*, *L. casei*, *L. paracasei*, *L. bulgaricus*, *L. acidophilus*, and *B. subtilis*, demonstrated to improve bone health parameters in in vivo studies, both animal and human[101]. Including appropriate probiotic control groups is also important to compare the effects of postbiotics[62].

Furthermore, the cell inactivation method should be standardized as it can alter the beneficial effects. The inactivation of probiotics can be performed through different

methods, such as heat, chemicals, and sonication, for example, and they can affect bacterial structural components differently, influencing the exerted effects[52,110]. Different methods should be tested to choose the one that best preserves the benefits of the microorganisms.

To promote a global collaborative effort and ensure the reproducibility of positive results, another crucial point is to make data readily accessible and encourage data sharing. By sharing data, researchers can facilitate the verification and replication of findings, which is essential before drafting or modifying guidelines. Open access to data promotes transparency, scientific rigor, and the advancement of knowledge in a collaborative manner. The adverse events and side effects also should be more reported[13].

6. Conclusions

The recognition that non-viable cellular components and metabolic substances produced by probiotic bacteria hold significant health benefits has opened up new avenues for research and potential applications. Although limited, current data supports the idea that postbiotics have the potential to improve bone health and represent an important opportunity for the development of safe treatments for at-risk groups. Well-planned preliminary studies and clinical trials are needed to further evaluate the efficacy and safety of postbiotics and translate the promising results from bench to bed.

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2.2 Artigo 1 – Ribeiro JL, Santos TA, Garcia MT, Carvalho BFC, Esteves JS, Moraes RM, Anbinder AL. *Limosilactobacillus reuteri* ATCC PTA 6475 inativado por calor previne a perda óssea em camundongos ovariectomizados / *Heat-killed Limosilactobacillus reuteri ATCC PTA 6475 prevents bone loss in ovariectomized mice**

RESUMO

A osteoporose é um problema de saúde importante, que ocorre devido a um desequilíbrio entre a formação e a reabsorção óssea. A deficiência hormonal pós-menopausa é um fator de risco significativo. Foi relatado que o probiótico *Limosilactobacillus reuteri* previne a perda óssea induzida por ovariectomia (Ovx) em camundongos e reduz a perda óssea em mulheres na pós-menopausa. Apesar dos inúmeros benefícios à saúde, por serem bactérias vivas, a administração de probióticos não é isenta de riscos para determinados grupos (por exemplo, neonatos e pacientes imunossuprimidos). Nós avaliamos os efeitos de *L. reuteri* (ATCC PTA 6475) e sua forma morta pelo calor (posbiótico) na perda óssea induzida por Ovx. Camundongos fêmeas adultas foram aleatoriamente divididas em quatro grupos: grupo C - controle (sham); grupo OVX-C - Ovx; grupo OVX-POS - Ovx + probiótico morto pelo calor; grupo OVX-PRO - Ovx + probiótico. *L. reuteri* ou o posbiótico foi administrado aos grupos ($1,3x10^9$ UFC/dia) por gavagem. A morfologia bacteriana após o tratamento térmico foi avaliada por microscopia eletrônica de varredura (MEV). O tratamento começou uma semana após a Ovx e durou 28 dias (4 semanas). Os animais foram sacrificados ao final do período de tratamento. A microarquitetura óssea do fêmur e a expressão gênica de *Ocludina* e citocinas pró-inflamatórias do íleo foram avaliadas por técnicas de microtomografia computadorizada e qPCR, respectivamente. Os grupos Ovx apresentaram menor percentual de volume (BV/TV) e número de trabéculas ósseas, bem como maior porosidade total em relação ao grupo controle. O tratamento com *L. reuteri* vivo e inativado resultou em maior BV/TV e espessura trabecular do que o grupo OVX. O posbiótico mostrou algumas rupturas na superfície da célula, mas sua estrutura permaneceu semelhante à do probiótico na análise por MEV. Não houve diferenças estatísticas na expressão dos genes *Occludina*, *Il-6* e *Tnf-α*. Tanto o *L. reuteri* viável quanto o inativado preveniram a perda óssea em camundongos ovariectomizados, independentemente da *Ocludina* intestinal e da expressão gênica intestinal de *Il-6* e *Tnf-α*.

Palavras-chave: probiótico; posbiótico; osteopenia; osteoporose.

ABSTRACT

Osteoporosis is an important health problem, occurring due to an imbalance between bone formation and resorption. Hormonal deficiency post-menopause is a significant risk factor. The probiotic Limosilactobacillus reuteri has been reported to prevent ovariectomy (Ovx)-induced bone loss in mice and reduce bone loss in postmenopausal women. Despite the numerous health benefits of probiotics, as they are live bacteria,

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the administration is not risk-free for certain groups (e.g., neonates and immunosuppressed patients). We evaluated the effects of L. reuteri (ATCC PTA 6475) and its heat-killed (postbiotic) form on Ovx-induced bone loss. Adult female mice were randomly divided into four groups: group C - control (sham); group OVX-C - Ovx; group OVX-POS - Ovx + heat-killed probiotic; group OVX-PRO - Ovx + probiotic. L. reuteri or the postbiotic was administered to the groups ($1.3x10^9$ CFU/day) by gavage. Bacterial morphology after heat treatment was accessed by scanning electron microscopy (SEM). The treatment started one week after Ovx and lasted 28 days (4 weeks). The animals were euthanized at the end of the treatment period. Bone microarchitecture and ileum Occludin and pro-inflammatory cytokines gene expression were evaluated by computed microtomography and qPCR techniques, respectively. The Ovx groups had lower percentage of bone volume (BV/TV) and number of bone trabeculae as well as greater total porosity compared to the control group. Treatment with live and heat-killed L. reuteri resulted in higher BV/TV and trabecular thickness than the Ovx group. The postbiotic showed some disruptions on the cell surface, but its structure resembled that of the live probiotic in SEM analysis. There were no statistical differences in Occludin, Il-6 and Tnf-α gene expression. Both viable and heat-killed L. reuteri prevented bone loss on ovariectomized mice, independently of gut Occludin and intestinal Il-6 and Tnf-α gene expression.

Keywords: Probiotic; Postbiotic; Osteopenia; Osteoporosis.

1. Introduction

Osteoporosis is a systemic disease characterized by reduced bone mass and microarchitectural degradation, leading to bone fragility and an increased risk of fracture[1]. It represents a significant public health problem as it is estimated that over half of the individuals over 50 in the United States have osteoporosis or osteopenia[2]. One in three women and one out of five men over 50 will experience an osteoporosis-related fracture[3]. Postmenopausal osteoporosis (PMO) is the most common due to reduced hormone production[4]. During estrogen deficiency, there is an increase in the production of pro-inflammatory and pro-osteoclastogenic cytokines such as RANKL, Tumor necrosis factor (TNF)-α[5] and interleukin (IL)-17[6]. Additionally, there is a decrease in the production of anti-inflammatory cytokines such as IL-10, IL-4, Interferon (IFN)-γ, as well as osteoprotegerin (OPG), resulting in increased osteoclastogenesis and subsequent bone resorption[6].

"Osteomicrobiology" is a research field that focuses on the role of the microbiota in host health and bone diseases[7]. Both human and animal studies have made it clear that the gut microbiota is essential for the overall health of the host playing a critical role in metabolism (e.g., production of hormones, such as dopamine and serotonin), nutrition (e.g., minerals absorption), pathogen resistance (e.g., intestinal barrier), and the immune system[8,9]. The intercellular space of the intestinal epithelium is sealed by tight junctions (TJs) composed of complex protein structures, such as junctional adhesion molecules (JAM), zonula occludens (ZO) proteins, claudin, and occludin. TJs are dynamic structures that adapt to external stimuli, such as food residues, commensal and pathogenic bacteria[10]. Estrogen activates a series of pathways essential for maintaining TJs, thus increasing the transepithelial electrical resistance in the barrier, preventing the entry of pathogens. Therefore, estrogen deficiency weakens the intestinal epithelial barrier, allowing the invasion of antigens and bacteria. This invasion initiates an inflammatory immune response associated with postmenopausal bone loss[11].

Numerous therapies have been developed for the treatment of osteoporosis ranging from non-pharmacological measures (e.g., healthy lifestyle habits) to pharmacological treatments. These are recommended for patients at an increased risk of fracture and include medications such as bisphosphonates, selective estrogen

receptor modulators, estrogen replacement therapy, and anabolic and antiresorptive drugs[12]. However, pharmacological therapy is associated with various adverse effects, including gastrointestinal reactions, osteonecrosis of the jaw[12], an increased risk of thromboembolic events, myocardial infarction, and breast cancer. These side effects have led to exploring new preventive and therapeutic possibilities, such as probiotics[13].

Probiotics are live, non-pathogenic microorganisms that, when administered in adequate amounts, offer benefits to the health of the host[14]. The consumption of probiotics has been associated with several advantages, including the maintenance of intestinal barrier function and strengthened immune system, which contribute to an overall improvement in host health, including bone health[15]. Studies conducted with *Limosilactobacillus reuteri* ATCC PTA 6475 have demonstrated that this specific strain suppresses the expression of pro-inflammatory and pro-osteoclastogenic cytokines in both the intestine and bone marrow[16–18]. Furthermore, apart from the encouraging outcomes observed in animal studies, the regular intake of this strain for 12 months among older women with low bone mineral density (BMD) decreased overall tibial bone mineral density (BMD) loss[19] compared to the placebo group.

It is essential to consider the challenges associated with the product composition and storage conditions when dealing with nutrition supplements or drugs. To be classified as probiotics, microorganisms must be alive, and the viability of products containing probiotics can be affected by variables such as temperature, moisture and oxygen[20]. Although probiotics are generally considered safe for healthy adults, using live bacteria has been linked to an increased risk of infection or complications in specific populations. This includes young infants and neonates, critically ill adult and infant patients in intensive care units, and postoperative, hospitalized, or immunocompromised patients. The risks are partially attributed to the occurrence of bacteremia and fungemia[21,22]. We must emphasize that, as far as we know, there have been no reports of any adverse effects in healthy individuals. This concern has led to a growing interest in the use of dead or non-viable bacteria[23].

The term “postbiotic” refers to using inactivated microorganisms or their components in adequate amounts to promote health benefits[24]. Postbiotics offer advantages such as easier standardization, storage, and transport[23] compared to live cells. Despite the growing interest in postbiotics, there is a limited number of studies investigating their effects on bone health[25–29], and none of them assessed the effects

of heat-killed *L. reuteri* 6475. However, these studies have shown promising results, as the administration of postbiotics has been associated with reduced bone loss, lower bone resorption markers, increased bone density and improved bone microarchitecture.

The potential to prevent postmenopausal bone loss through a natural, non-invasive, and non-stressful method holds significant appeal. Using heat-killed *L. reuteri* is interesting because it would offer fewer potential adverse effects, easier production, and a product with improved stability and longer shelf-life. Considering the demand for new therapeutic alternatives or adjuncts in osteoporosis treatment and the growing use of probiotics and postbiotics, we hypothesize that heat-killed form of *L. reuteri* could be so effective as the viable form in preventing postmenopausal bone loss.

2. Materials and methods

This is a randomized, controlled, and blinded experimental study. This study was approved by the Institutional Ethics Committee (nº 18/2019). Thirty-two 18-week-old specific pathogen-free female mice (*Mus musculus*, BALB/c) were housed in a temperature- and light-controlled environment (22 °C; light/dark cycle of 12/12h) and received water ad libitum. They were randomly divided into four groups: 1) Sham-Control group (C): *Sham*-operated animals that received MRS broth (vehicle of probiotic suspension); 2) Ovariectomy (Ovx)-Control group (OVX-C): ovariectomized animals that received MRS broth; 3) OVX-Postbiotic group (OVX-POS): ovariectomized animals that received heat-killed *L. reuteri*; and 4) OVX-Probiotic (OVX-PRO): ovariectomized mice that received viable *L. reuteri*. Mice were treated by gavage with 0.3 ml ($1.3x10^9$ CFU/day)[16] with *L. reuteri* or MRS broth.

Power analysis showed that a sample of 8 mice per group has 80% power to detect an effect size of 5.13 or a 22% change, assuming a significance level of 5% and a two-sided test[30].

The animals received standardized AIN-93-M chow. We determined the average food intake by weighing the remaining food in each cage three times a week for two weeks before the Ovx. Following the surgery, the animals were provided with only the average amount of food intake to prevent weight gain from the Ovx. Body weight

measurements were taken on the day of surgery and weekly thereafter until the day of euthanasia.

2.1 Ovx and sham surgery

All animals, previously weighed, were anesthetized with isoflurane (2% for induction and 1.8% for maintenance; oxygen 2 L/min) and submitted to Ovx or sham surgery on day 1 (Fig. 1). Bilateral Ovx was performed through a bilateral longitudinal skin incision in the lateral abdominal region, below the last rib, and total excision of the ovaries, after ligation of the uterus with absorbable suture (4–0, Shalon Ltda). Sham surgery was done by exposing the ovaries without excision. The peritoneal muscle was sutured with an absorbable suture thread, and skin with a silk thread (4–0, Ethicon, Johnson & Johnson). The animals received analgesic and anti-inflammatory medication subcutaneously in the three days following surgery (meloxicam: 2 mg/kg; tramadol: 5 mg/Kg). The efficacy of Ovx was confirmed during euthanasia by uterine atrophy.

2.2 Bacterial strains and culture

One week after the Ovx/sham surgery, the administration of probiotic, postbiotic or MRS broth was started (Fig. 1). *L. reuteri* ATCC PTA 6475 (BioGaia AB) was cultured under anaerobic conditions in de Man Rogosa Sharpe (MRS, Difco) broth at 37 °C. Single colonies were confirmed by Gram stain from the agar. After 24 hours of growth, cells were harvested by centrifugation at 5000 rpm for 10 minutes, the supernatant was discarded, the bacterial pellet was resuspended in MRS broth, and the standardization of the microorganism was performed in a spectrophotometer with 600 nm wavelength[16]**.** For postbiotic preparation, the suspension with live microorganisms was autoclaved at 121 °C for 20 minutes and then plated on MRS agar to ensure no viable cells remained. The postbiotic was prepared in sufficient quantity once per week and stored in a refrigerator. Probiotics and postbiotics were administered to the mice in MRS broth.

2.3 Euthanasia

Euthanasia was performed after four weeks of treatment (on day 36 – 35 days after the day of Ovx or sham surgery) (Fig. 1). Animals were anesthetized using intraperitoneal overdose injection of ketamine and xylazine followed by decapitation. The uterus was weighed immediately to assess atrophy after Ovx. The right femur was fixed in paraformaldehyde 4% (phosphate buffer pH 7.4; 0.1 M). The ileum was immediately frozen in liquid nitrogen and stored in a -80 °C freezer. An external examiner coded the samples, and the researchers were blinded before data evaluation.

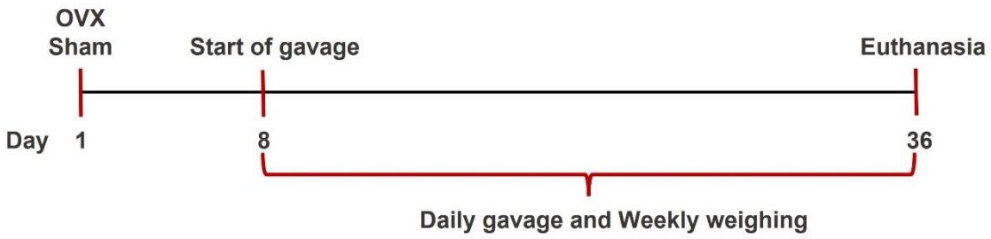

Fig 1. Experimental design.

2.4 Scanning electron microscopy (SEM)

Probiotic and postbiotic samples were analyzed by scanning electron microscopy (SEM) to visualize bacterial structural alterations after heat inactivation. The SEM (JEOL JSM-7900 F Scanning Electron Microscope, JEOL USA, Inc.) procedures were performed according to Garcia et al[31]. Collected from the culture in MRS broth (1 µl), the bacteria were placed in a small pre-delimited area of the Petri dish and fixed in 2% glutaraldehyde for 1 h. The samples were dehydrated in increasing ethanol concentrations (10, 25, 50, 75 and 90%) for 20 min each, followed by 1 h immersion in 100% alcohol. The samples were then incubated at 37 °C for 24 h for total drying and transferred to aluminum stubs and sputter coated with gold for 160 s at 40 mA (Denton Vacuum Desk II).

2.5 Micro-computed tomography (µ-CT)

Three-dimensional µ-CT analyses of the distal femoral metaphysis were performed using SkyScan (SkyScan 1176 Bruker MicroCT). Tomographic images were acquired

at 50 kV and 500 µA with an isotropic voxel size of 17.48 µm; aluminum filter of 0.5 mm, and an integration time of 380 ms. The images were reconstructed three-dimensionally, with the aid of the NRecon (SkyScan, Version 1.6.6.0) and Data Viewer (SkyScan, Version 1.4.4 64-bit) software, and a volume of interest (VOI) was standardized from a transaxial section, using the CT Analyzer software (CTAn-2003-11, SkyScan, Version 1.12.4.0). The analyzed VOI corresponded to 100 CT scans of the trabecular bone at the tibial metaphysis located at 25 cuts after the most caudal point of the growth plate. Bone volume fraction (BV/TV), trabecular number (Tb.N), thickness (Tb.Th), separation (Tb.Sp) and total porosity (Po.tot) were calculated using standard methods.

2.6 RT-qPCR

The tissues were pulverized using a liquid N_2 cooled mortar and pestle and then transferred to TRIzol (Invitrogen). RNA was extracted, and the quality was confirmed by the presence of rRNA bands on agarose gel electrophoresis. After treatment with DNase (RQ1 RNase-Free DNase, Promega), 1 µg of RNA was transcribed into cDNA using the GoScript™ Reverse Transcriptase Kit (Promega), and all protocols were performed as recommended by the manufacturers. After analyzing the *Gapdh*, *Tbp* and *Hprt* genes, *Gapdh* was the endogenous control of choice. Primer sequences are summarized in Table 1. The specificity of the reaction was checked by melting curve analysis and amplification efficiency. Evaluation of gene expression was performed using the $2^{-\Delta\Delta CT}$ method.

Gene	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tbp	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Ocl	CCTTCTGCTTCATCGCTTCC	AGCGCTGACTATGATCACGA
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG

Table 1. Primer sequences for RT-qPCR reaction.

2.7 Statistical analysis

The statistical analysis was performed in two stages – in the first, the Ovx groups (OVX-Control, OVX-Postbiotic and OVX-Probiotic) were compared with the Sham-Control group; and in the second, the OVX groups were compared to each other. Data were evaluated using the analysis of variance (ANOVA) or non-parametric Kruskal-Wallis' test (α = 0.05). The Dunnett, Dunn and Tukey posthoc tests were used in case of statistical difference. Tests were performed using GraphPad Prism version 6.01 (GraphPad Software, Inc).

3. Results

The body weight (g) on day 1 and day 36 was compared. At both time points, no statistically significant difference was observed among the groups, confirming the effectiveness of the feeding control (p = 0.2527, Kruskal-Wallis and p = 0.4188, ANOVA; day 1 and day 29, respectively). Bilateral Ovx leads to uterine atrophy, confirmed by weighing the uterus at the time of euthanasia[32]. When compared to the control, the Ovx groups had lower uterine weight (g), with a statistically significant difference (p = 0.0004, Kruskal-Wallis, Dunn).

3.1 Heat inactivation does not alter the overall integrity of L. reuteri

The images acquired through SEM confirmed the structural integrity of the probiotic (Fig. 2A and 2B), as well as the preservation of the heat-killed *L. reuteri*'s structure, which remained largely intact (Fig. 2C). Only a few disruptions were observed on the cell surface (Fig. 2D), resembling the appearance of the live probiotic.

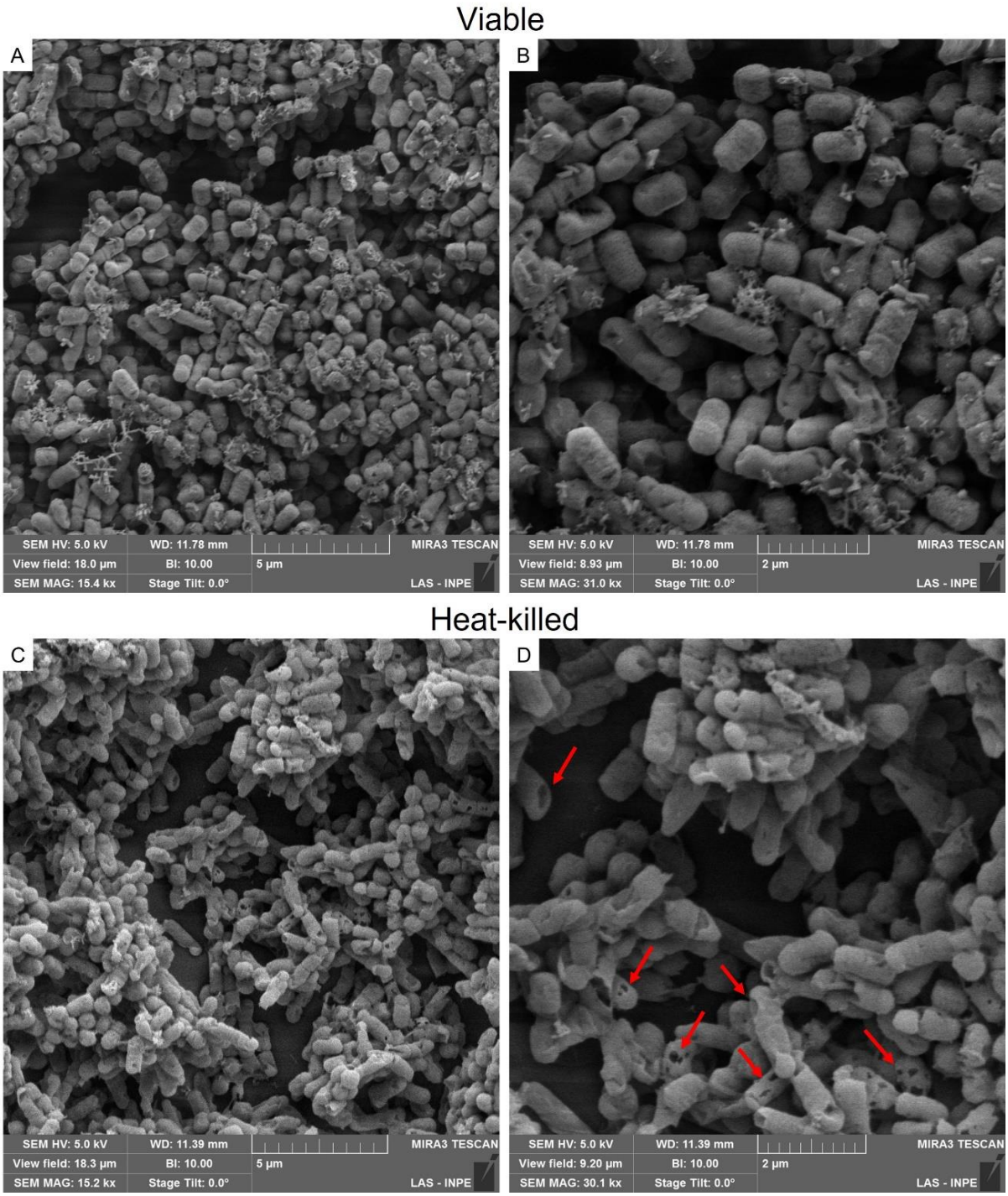

Fig 2. Scanning electron microscopy images of *L. reuteri.* Figures A and C were taken at 15,000x magnification, and B and D at 30,000x magnification. The heat-killed *L.reuteri* showed punctual disruptions on the cell wall (red arrows).

3.2 Heat-killed L. reuteri is as effective as the viable form in preventing estrogen-deficiency-induced bone loss

A statistically lower BV/TV ($p < 0.0001$, ANOVA, Dunnett, Fig. 3A), Tb.N ($p < 0.0001$, ANOVA, Dunnett, Fig. 3B) and greater Po.tot ($p = 0.0004$, ANOVA, Dunnett, Fig. 3E) were found in the OVX groups when compared to the C group.

In addition, the OVX-POS and OVX-PRO groups showed a positive and similar effect of treatment with heat-killed and viable *L. reuteri*, respectively, with a higher BV/TV when compared to OVX-C ($p = 0.0077$, ANOVA, Tukey, Fig. 3A). Treatment with both forms of *L. reuteri* had a positive and similar effect on Tb.Th, where both groups showed greater Tb.Th when compared to C ($p < 0.0001$, ANOVA, Dunnett) and OVX-C ($p = 0.0002$, ANOVA, Tukey, Fig. 3C). In the Tb.Sp analysis, groups were statistically similar ($p = 0.0762$, ANOVA, Fig. 3D).

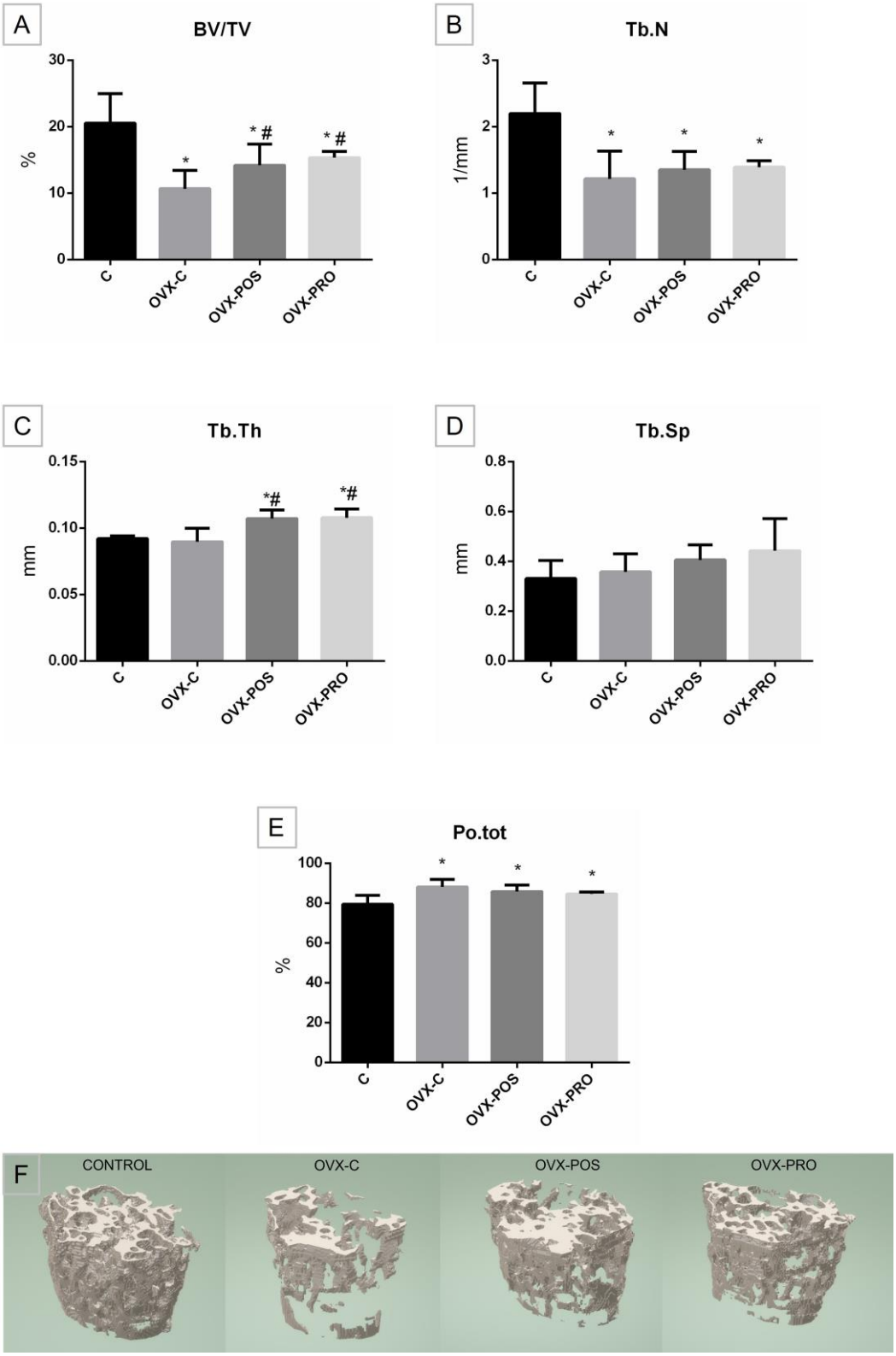

Fig. 3. Graphs of means ±standard deviation of A) bone volume fraction (BV/TV – %), B) trabecular number (Tb.N – 1/mm), C) trabecular thickness (Tb.Th – mm), D) trabecular separation (Tb.Sp – mm), E) total porosity (Po.tot – %), and F) three-dimensional reconstruction of the volume of interest reveals that Ovx led to increased bone loss, while both treatments effectively mitigated it.* $p < 0.05$ when compared to C; # $p < 0.05$ when compared to OVX-C.

3.3 The benefits of L. reuteri on bone microarchitecture of ovariectomized mice occur independently of the gene expression of Occludin, Il-6, and Tnf-α in the intestine

In the analysis of *Occludin*, *Il-6* and *Tnf-α* gene expression, the Ovx groups maintained statistically similar levels to the control (p = 0.1909, ANOVA; p = 0.6508, Kruskal-Wallis; p = 0.0693, Kruskal-Wallis; respectively, Fig. 4). However, a trend to increased expression could be seen due to Ovx (OVX-C), as well a trend to reduction by the treatments.

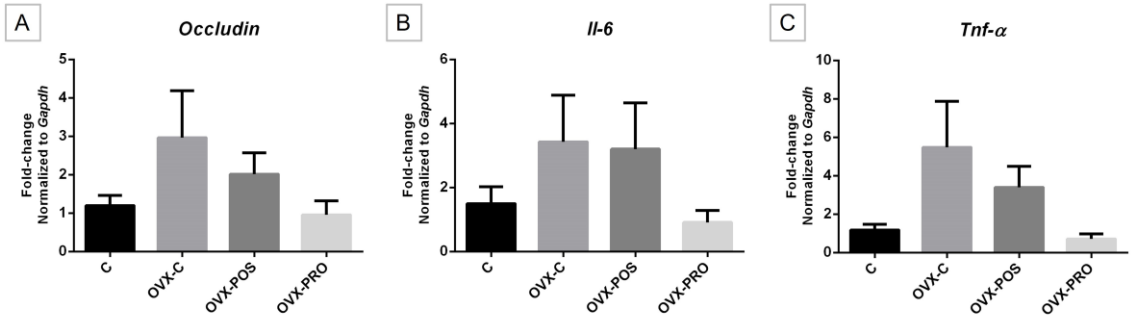

Fig. 4. Graphs of means ± standard error of A) *Occludin* B) *Il-6,* and C) *Tnf-α* mRNA expression in the ileum, showing similar expression levels among groups.

4. Discussion

Given the status of osteoporosis as a significant public health concern, it is crucial to explore novel and effective therapies. Probiotics and postbiotics have gained widespread attention for their potential benefits to the host. Studies have demonstrated the positive impact of probiotics[33–37] and postbiotics[25–29] on bone health. In the present study, we investigated the effects of *L. reuteri* ATCC PTA 6475, both in its viable and heat-killed forms, on the maintenance of bone volume after Ovx in mice.

During menopause, there is a decline in ovarian hormone production, which can result in hyperphagia[38], increasing body weight and adipose tissue mass[39]. Elevated body mass index (BMI) is correlated with increased bone density[40], which can be explained by mechanical and hormonal mechanisms. Bone cells can detect heightened mechanical load, and, in response, increase bone formation[41]. Furthermore, estrogen can be synthesized from adipose tissue, which, in addition to

greater mechanical stress exerted, may also play a role in elevating BMD[42]. We implemented strict animal feeding control to prevent weight gain resulting from Ovx and its potential impact on bone mass. Throughout the experiment, the animals' weight remained relatively stable, indicating the effectiveness of the feeding control measures. Furthermore, immediate weighing of the uterus after euthanasia revealed uterine atrophy in the OVX groups, confirming the success of the Ovx procedure.

Emerging evidence suggests that probiotics and gut microbiota play a crucial role in regulating bone health[7]. In healthy male mice, the administration of *L. reuteri* has been shown to reduce intestinal inflammation, enhance osteoblast activity, and promote increased bone formation[18]. In a previous study, ovariectomized mice treated with *L. reuteri* ATCC PTA 6475 demonstrated a decrease in bone loss[16], which was also observed in this study since the treatment with *L. reuteri* 6475 protected mice from estrogen deficiency-induced bone loss by improving BV/TV and Tb.Th. Furthermore, confirming our hypothesis, and in accordance with other authors, the postbiotic also positively affected bone health[25–29]. We found that the postbiotic had the same effect as the probiotic on the bone microarchitecture.

Postbiotic preparations encompass intact cells or structural fragments, such as cell walls, bacterial lysates, cell-free supernatants, metabolites, and proteins, all contributing to beneficial health effects[24]. The specific inactivation method can influence the postbiotic effects as bacterial cells can be disrupted, releasing compounds that interact with host cells[43]. Thermal treatment, involving heat application for a specified duration, is the most common method used for inactivation[44]. Our study used sterilization (> 100 °C), which resulted in minimal disruptions on the bacterial surface while maintaining the anticipated bone effects. The mechanisms underlying the health benefits of postbiotics are not fully understood but involve immunomodulatory effects[45–49] and antimicrobial properties[50]. Similar to probiotics, postbiotics can adhere to and counteract pathogens[50]. Inactivated cells are recognized by antigen-presenting cells, triggering the production of anti-inflammatory cytokines while suppressing the production of pro-inflammatory cytokines[48,49] Additionally, probiotics exert their effects through the production of metabolites (such as short-chain fatty acids)[5] and vitamins[51], enhanced mineral absorption[52], and the upregulation of TJs [10]. Based on our findings, the impact of probiotics on bone microarchitecture is not dependent on its viability.

To elucidate a portion of the mechanism underlying the effects of pro- and postbiotic on bone, we investigated the gene expression of a protein associated with intestinal permeability and two pro-inflammatory cytokines. TJs play a crucial role in maintaining the integrity and function of the intestinal barrier50, and sex steroid deficiency increases gut permeability and upregulates osteoclastogenic cytokines through an inflammatory response[54]. Occludin plays an important role in paracellular diffusion[55]. Our study did not find a statistically significant difference in *Occludin* gene expression between the groups. Collins et al. (2017)[56] have reported that estrogen deficiency resulting from Ovx induces region- and time-dependent effects on gut TJs. One limitation of our study is that we only assessed occludin in the ileum at a single time point, while multiple families of proteins are involved in TJs.

Estrogen deficiency has been associated with an increase in the expression of pro-inflammatory cytokines[56], and previous studies have demonstrated the effects of probiotics on cytokine expression[16–18]. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine, has been shown to disrupt TJ proteins and increase intestinal permeability[57] In our study, although not statistically significant, there was a trend towards upregulation of *Tnf-α* and *Il-6* gene expression in the Ovx groups. Collins et al. (2017)[56] found that increased gene expression of *Tnf-α* in the ileum was correlated with upregulated expression of *Occludin*. Also, the compensatory increase in *Occludin* gene expression is believed to play a direct role in enhancing the epithelial barrier function[58]. Moreover, the graphs displayed a coherent pattern, indicating a tendency towards decreased *Ocluddin* and citokines expression in the groups that received *L. reuteri* in its viable and heat-killed form.

In conclusion, our study provides evidence that the beneficial effects of *L. reuteri* ATCC PTA 6475 in the maintenance of bone volume in ovariectomized mice are not contingent upon cell viability. Our findings suggest that viable and heat-killed *L. reuteri* ATCC PTA 6475 had similar beneficial effects on maintaining the bone volume percentage independently of gut *Occludin* and intestinal *Il-6* and *Tnf-α* gene expression. However, the precise mechanisms of action remain unclear, and further investigations are warranted to elucidate them.

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3 CONSIDERAÇÕES GERAIS

Nas últimas décadas, a população brasileira tem envelhecido consideravelmente, com associação ao aumento na prevalência de doenças crônicas (Baccaro et al., 2015). A osteoporose é uma das doenças mais relacionadas ao envelhecimento da população (Zeng et al., 2021). Sendo um problema de saúde pública em âmbito mundial, a busca por novas possibilidades terapêuticas vem sendo explorada, e dentre elas, o uso de probióticos.

O uso de probióticos tem sido extensivamente testado, e demonstrado resultados promissores na prevenção e tratamento da perda óssea pós-menopausa (Jansson et al., 2019; Lambert et al., 2017; Nilsson et al., 2018; Takimoto et al., 2018), assim como o uso de posbióticos (Jang et al., 2021; Jhong et al., 2022; Montazeri-Najafabady et al., 2021; Myeong et al., 2023; Yeom et al., 2021). Os mecanismos e os efeitos na saúde exercidos pelos posbióticos têm se mostrado bastante semelhantes aos observados nos probióticos, como a estimulação/supressão da expressão de citocinas e a ativação do sistema de sinalização celular, além de efeitos antimicrobianos. Essa similaridade sugere que a perda de viabilidade devido à inativação celular não compromete suas funções benéficas (Siciliano et al., 2021).

Em nosso estudo, a administração diária de *L. reuteri* ATCC PTA 6475, em sua forma viável e inativada, levou a uma menor perda óssea em camundongos ovariectomizados. Esses resultados estão alinhados com a compreensão existente sobre a conexão entre o intestino e os ossos e a influência da microbiota intestinal na saúde óssea (Ohlsson, Sjögren, 2018). Entretanto, o mecanismo pelo qual isso acontece, ainda é incerto.

Sabendo que a deficiência hormonal pós-menopausa leva a um aumento da permeabilidade da barreira intestinal (Li et al., 2016), avaliamos um marcador intestinal de junção intercelular, *Ocludina*, no íleo. Entretanto, não obtivemos diferença estatística entre os grupos. Embora não tenhamos encontrado diferença significativa, existem várias famílias de proteínas juncionais que não foram avaliadas (Claudinas, Jam-3, Zônula ocludens, por exemplo). Esta foi uma limitação do nosso estudo, onde apenas avaliamos uma proteína juncional, em um determinado período (28 dias) e em

um segmento intestinal. Segundo Collins et al. (2017), as alterações induzidas pela Ovx são região- e tempo-dependentes (Collins et al., 2017a).

Além da *Ocludina*, avaliamos em íleo as citocinas pró-inflamatórias *Tnf-α* e *Il-6.* Estudos já demonstraram que os probióticos possuem influência na expressão de citocinas inflamatórias (Britton et al., 2014; Collins et al., 2016; McCabe et al., 2013). Não houve diferença estatística entre os grupos, porém os gráficos exibiram um padrão coerente, mostrando uma tendência a diminuição da expressão dessas citocinas nos grupos que receberam *L. reuteri*.

Nosso principal desfecho confirmou nossa hipótese inicial: o posbiótico teve um efeito positivo na manutenção da saúde óssea após ovariectomia. Embora sejam considerados reguladores do metabolismo ósseo, o papel dos probióticos como tratamento adjuvante e preventivo para a osteoporose tem gerado debate devido à falta de consenso sobre sua eficácia (Zeng et al., 2021), e ainda não há estudos clínicos randomizados com o uso de posbióticos. Em nosso estudo, a administração de *L. reuteri* inativado levou a uma menor perda óssea, quando comparado ao grupo Ovx, aumentando BV/TV e Tb.Th, com efeito similar ao do probiótico na microarquitetura óssea. Nossos resultados estão em concordância com o obtido por outros pesquisadores (Jang et al., 2021; Jhong et al., 2022; Kimoto-Nira et al., 2009, 2007; Montazeri-Najafabady et al., 2021; Myeong et al., 2023).

O uso de *L. reuteri* inativado nos trouxe bons resultados como opção terapêutica e ajuda a confirmar que bactérias vivas nem sempre são necessárias para obter efeitos benéficos. Entretanto, o modo de ação dos posbióticos, seu impacto no sistema imunológico do hospedeiro e no metabolismo ósseo ainda foram pouco explorados.

O próprio processo de inativação pode introduzir propriedades únicas ou modulações nos componentes bacterianos (Akter et al., 2020), exigindo uma avaliação e padronização dos métodos utilizados. A inativação pode ser feita por diferentes métodos: aplicação de calor, químicos, raios ultravioleta ou gama, entre outros. O método mais utilizado para inativação dos probióticos é o calor (geralmente entre 70-100 °C) (Piqué et al., 2019). Em nosso estudo, utilizamos temperatura acima de 100 °C, porém, ao avaliarmos a estrutura bacteriana através de microscopia eletrônica de varredura, constatamos que os posbióticos mantiveram sua estrutura, apresentando apenas algumas rupturas na superfície celular. Tratamentos térmicos

normalmente causam danos à membrana celular, perda de alguns nutrientes e íons, além de inativação de algumas enzimas essenciais e coagulação proteica (Akter et al., 2020). Entretanto, mesmo com essas alterações, obtivemos resultados semelhantes entre as formas viável e inativada.

Manter a viabilidade do probiótico em produtos é um fator crítico, tornando o uso de posbióticos de extremo interesse financeiro, uma vez que possuiria maior tempo de prateleira, estabilidade em maior faixa de pH e temperatura, além da incorporação em alimentos antes do processo térmico, sem a perda da funcionalidade (Siciliano et al., 2021). Explorar a estabilidade e o prazo de validade dos posbióticos é crucial para suas aplicações práticas. Compreender a estabilidade e a eficácia desses produtos a longo prazo será essencial para sua implementação bem-sucedida em ambientes clínicos.

Com os numerosos estudos associando o uso de probióticos com saúde óssea, podemos aprender com os erros. Algumas limitações em estudos existentes impedem que a terapia probiótica seja validada e tenha recomendação clínica. Os estudos clínicos são muito heterogêneos, o que impede a comparação dos resultados entre si (Zeng et al., 2021). Em pesquisas futuras, a metodologia deve ser padronizada, com amostras maiores e adequadas, randomização, cegamento, métodos de análise de alta qualidade, estabelecer o desfecho principal, dose, cepa, maior duração do tratamento (superior a 12 meses) (Billington et al., 2021; Zeng et al., 2021), métodos de inativação (no caso de posbióticos), e descrição de eventos adversos ou efeitos colaterais. Outro ponto importante é a padronização da descrição dos métodos – como doses, concentrações, volume administrado por animal, que devem estar descritos de forma precisa, para que os experimentos sejam reprodutíveis.

O uso de posbióticos representa uma grande oportunidade para o desenvolvimento de novas terapias, principalmente para os grupos de risco. Os resultados positivos obtidos com o uso de *L. reuteri*, tanto viável quanto inativado, destacam suas aplicações potenciais na prevenção e no tratamento da perda óssea pós-menopausa. Isso abre caminhos para pesquisas adicionais, com implicações teóricas e práticas. Estudos futuros devem se concentrar em ensaios clínicos randomizados padronizados e de alta qualidade, além de elucidar os mecanismos de ação específicos tanto para as formas viáveis quanto inativadas de *L. reuteri*, e otimizar suas aplicações práticas em ambientes clínicos. Ao fazer isso, os estudos

podem ampliar nosso conhecimento sobre o papel dos probióticos na saúde óssea e desenvolver estratégias inovadoras para prevenir e tratar a perda óssea pós-menopausa.

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ANEXO A – Certificado CEUA

CERTIFICADO
CEUA – Comissão de Ética no Uso de Animais

CERTIFICAMOS, que o protocolo registrado sob o nº 18/2019, intitulado:- "**Influência de Lactobacillus Reuteri Viável, não-Viável e Associações com vitaminas D e K em camundongos ovariectomizados** " sob a **responsabilidade ANA LIA ANBINDER,** tendo como colaboradora **JAQUELINE LEMES RIBEIRO,** e que envolve a utilização de animais pertencentes ao filo Chordata subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009 e com as Normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADO** pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA – ICT – CAMPUS DE SÃO JOSÉ DOS CAMPOS-UNESP), em reunião de 17/12/2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da Autorização	06/01/2020 a 31/12/2022
Espécie/linhagem/raça	Camundongos isogênico Balb/c
Nº de Animais	80
Peso/Idade	10 SEMANAS
Sexo	FÊMEA
Origem	Biotério da Unicamp - CEMIB

São José dos Campos, 17 de dezembro de 2019

Profa.Dra. CRISTIANE YUMI KOGA ITO
Vice-Coordenadora em exercício

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