EDUARDA PIRES COSTA

EFEITO DO LEITE DE VACA E BEBIDA DE SOJA (TRANSGÊNICA E NÃO TRANSGÊNICA) NO FÊMUR DE CAMUNDONGOS BALB/C

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Magister Scientiae*.

Orientadora: Reggiani Vilela Gonçalves

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Reggiani Vilela Gonçalves
Orientadora

Aos meus pais e irmãos.

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RESUMO

COSTA, Eduarda, M.Sc., Universidade Federal de Viçosa, agosto de 2023. **Efeito do leite de vaca e bebida de soja (transgênica e não transgênica) no fêmur de camundongos Balb/C**. Orientadora: Reggiani Vilela Gonçalves. Coorientadora: Mariáurea Matias Sarandy.

Introdução: A saúde dos ossos é influenciada pela nutrição, e uma das fontes dietéticas mais importantes de cálcio é o leite. No entanto, cada vez mais pessoas estão limitando seu consumo de leite e optando por consumir bebidas à base de plantas, como a soja transgênica ou não transgênica. **Objetivo**: Comparar os efeitos da suplementação de leite de vaca e bebida de soja (transgênica e não transgênica) no fêmur de camundogos BALB/c. **Materiais e métodos:** Vinte e oito animais foram randomizados em quatro grupos experimentais com 7 animais em cada grupo. G1: água destilada, G2: bebida de soja não transgênica, G3: bebida de soja transgênica, e G4: leite de vaca. Todos os grupos receberam os tratamentos durante 42 dias, por gavagem. Os animais foram anestesiados e eutanasiados. Os fêmures foram removidos para análises biomecânicas, biométricas, histomorfológicas e moleculares. **Resultados:** O grupo G3 obteve menor ganho de peso. A resistência óssea foi menor em G3. O diâmetro da diáfise do fêmur foi menor em G2, e a espessura do osso cortical foi reduzida em G2 e G3. O diâmetro do canal medular em G3 foi reduzido. O grupo G4 obteve os maiores parâmetros de densidade volumétrica trabecular, área óssea e largura trabecular na epífise distal, e os menores parâmetros na densidade volumétrica medular e separação trabecular na mesma região. Níveis de osteocalcina foram maiores em G4. Houve uma redução na quantidade de colágeno I em G2 e G3. Os níveis de magnésio aumentaram em G4, e os níveis de cálcio estavam aumentados em todos os grupos em relação ao controle. G4 apresentou menor número de poros na diáfise óssea e G3 apresentou maior número de poros. **Discussão**: A bebida de soja transgênica foi prejudicial ao osso cortical, gerando potencial desequilíbrio entre a reabsorção e deposição óssea devido a uma demanda maior de nutrientes para o osso cortical em G3, além de aumentar a distribuição de poros na diáfise óssea e comprometer a resistência óssea, possivelmente porque alimentos geneticamente modificados podem conter toxinas e fatores antinutricionais prejudiciais à osteogênese. O leite de vaca manteve a integridade morfofuncional do osso cortical, além de apresentar benefícios para

microarquitetura trabecular e regular aumentar os níveis circulantes de osteocalcina e a deposição óssea de colágeno tipo III. **Conclusão**: O consumo de leite de vaca pode ser uma alternativa mais segura do que a bebida de soja para promover e/ou manter a saúde óssea.

Palavras-chave: Microarquitetura óssea. Leite. Nutrição. Transgênico. Soja. Osteocalcina.

ABSTRACT

COSTA, Eduarda, M.Sc., Universidade Federal de Viçosa, August, 2023, **Effect of cow's milk and soy drink (transgenic and non-transgenic) on the femur of Balb/C mice.** Adviser: Reggiani Vilela Gonçalves. Co-adviser: Mariáurea Matias Sarandy.

Introduction: Bone health is influenced by nutrition, and one of the most important dietary sources of calcium is milk. However, more and more people are limiting their milk consumption and opting to consume plant-based beverages, such as transgenic or non-transgenic soy. **Objective:** Compare the effects of cow's milk supplementation and soy beverage (transgenic and non-transgenic) on the femurs of BALB/c mice. **Materials and Methods**: Twenty-eight animals were randomized into four experimental groups, with seven animals in each group. G1: distilled water, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk. All treatments were administered by gavage for 42 days. Then, the femurs were collected for biomechanical, biometric, histomorphological, and molecular analyses. **Results:** Group G3 showed lower weight gain. Bone strength was lower in G3. The diaphysis diameter of the femur was smaller in G2, and the cortical bone thickness was reduced in G2 and G3. The diameter of the medullary canal in G3 was reduced. Group G4 had the highest parameters of trabecular volumetric density, bone area, and trabecular width in the distal epiphysis, and the lowest parameters in medullary volumetric density and trabecular separation in the same region. Osteocalcin levels were higher in G4. There was a reduction in the amount of collagen I in G2 and G3. Magnesium levels increased in G4, and calcium levels were elevated in all groups compared to the control. G4 had a lower number of pores in the bone diaphysis, and G3 had a higher number of pores. **Discussion**: Transgenic soy drink was detrimental to cortical bone, causing an imbalance between bone resorption and deposition due to a greater demand for nutrients for cortical bone in G3, as well as increased distribution of pores in the bone diaphysis and compromised bone strength, possibly because transgenic foods can act as toxins and antinutritional factors impairing bone deposition. Cow's milk maintained the balance between bone resorption and deposition in cortical bone, as well as presenting benefits for trabecular microarchitecture, regulating cellular markers such as osteocalcin and increasing collagen type III deposition. **Conclusion**: Consumption of cow's milk may be a safer alternative than soy drink to promote bone health.

Keywords: Bone microarchitecture. Milk. Nutrition. Genetically modified. Soy. Osteocalcin.

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1. REVISÃO DE LITERATURA:

O tecido ósseo é um tecido conjuntivo especializado, rígido e inflexível, altamente dinâmico, vascularizado, metabolicamente ativo, responsável por oferecer proteção, mobilidade e sustentação a órgãos vitais, além de ser um importante reservatório cálcio e fosfato, fundamentais para manutenção da homeostase corporal (Lopes *et al*., 2018). É constituido de uma matriz extracelular (MEC) composta por uma parte inorgânica e orgânica, sendo que a parte orgânica é sintetizada pelas células osseas (Ansari, 2019).

A parte orgânica contém proteínas colagenosas, principalmente colágeno do tipo I, que representam cerca de 98% do total das proteínas ósseas (Saito; Marumo, 2015). As fibras de colágeno do tipo III estão presentes nas regiões de transição entre o osso e os tecidos circundantes, como os tendões e ligamentos, facilitando a ligação musculoesquelética, ajudando a transmitir as cargas geradas pelas contrações musculares para o esqueleto (Saino *et al*., 2003). Além disso, também fazem parte da parte orgânica a substância fundamental amorfa (proteoglicanos e glicoproteinas) e proteínas não colagenosas (PNC), como a osteocalcina (OC), que é a mais abundante PNC do osso e tem um papel importante na regulação do metabolismo ósseo (Bonjour, 2016). Em conjunto, esses componentes orggânicos da MEC possuem um papel essencial na formação, reabsorção, mineralização e remodelação óssea (Nakamura; Imaoka; Takeda, 2021), e também podem atuar como fatores de crescimento e sinalizadores celulares, afetando a proliferação e diferenciação de células osteogênicas e a atividade de osteclastos; podendo afetar também as características biomecânicas do osso, como os mecanismos de tração e compressão óssea (Licini; Vitale-Brovarone; Matiolli-Belmonte, 2019).

O componente inorgânico do tecido ósseo é composto principalmente por fosfato e cálcio, minerais organizados em cristais de hidroxiapatita ($Ca_{10}(PO_4)_6(OH)_2$), mas também estão presentes em menor quantidade íons como magnésio, potássio, manganês, zinco, sódio e enxofre (Sailaja *et al*., 2016), os quais conferem as propriedades de rigidez aos ossos e contribuem para manutenção funcional e metabólica das células deste tecido (Abou Neel *et al*., 2016).

Os elementos celulares do osso incluem as células osteogênicas como osteoblastos, osteoclastos e osteócitos; as quais estão interligadas como unidades multicelulares básicas (UMB). Essas células participam da remodelação óssea,

permitindo o crescimento e adaptação as tensões aplicadas (Holick; Nieves, 2015). Os osteoblastos são responsáveis pelo crescimento ou remodelação do osso, além de síntese de colágeno e da matriz óssea (Kim *et al*., 2020). Os osteócitos as celulas mais abundantes no tecido ósseo, se diferenciam a partir dos osteoblastos e são responsáveis pela manutenção do metabolismo ósseo (Franz-Odendaal; Hall; Witten 2006). São reguladores centrais da resistência óssea, e seu mau funcionamento pode levar a perda de resistência dos ossos (Ansari; Sims, 2020). Os osteoclastos são grandes células derivadas de monócitos que participam da destruição da matriz óssea, processo denominado de reabsorção (Martin; Sims, 2015).

Os ossos podem ser divididos macroscopicamente em osso cortical (ou compacto) ou trabecular (ou esponjoso)(Steiner, 2009). O osso cortical envolve o espaço medular e é caracterizado pela presença de fibras colágenas organizadas em lamelas circulares e compactas, chamadas canais de Havers, altamente inervadas e vascularizadas (Ramchand; Seeman, 2018). O osso esponjoso é constituído de trabéculas ósseas entrelaçadas, revestidas por osteoblastos e osteoclastos, e delimitam uma cavidade preenchida pela medula óssea (De Boer; Van Der Merwe, 2016). Dentre os ossos longos, podemos destacar o fêmur, cuja diáfise (porção central do fêmur) apresenta formato alongado e cilíndrico. A diáfise é predominantemente composta por tecido ósseo cortical, o qual confere resistência e rigidez. As extremidades do fêmur são compostas por epífises, que são envolvidas por uma camada fina de osso compacto e contêm principalmente tecido ósseo esponjoso em seu interior (Buck; Dumanian, 2012). A região intermediária entre a diáfise e epífise é chamada de metáfise, que apresenta uma expansão em formato de cone e contém principalmente tecido ósseo esponjoso em seu interior (Clarke, 2008a; Rehfeld; Nylander; Karnov, 2017). Considerando a importância do fêmur como um osso longo de composição complexa, todas estas características o tornam um osso altamente qualificado quando se deseja estudar resistência, porosidade, conteúdo de minerais, formação e reabsorção óssea, por isto foi o osso investigado neste estudo.

O tecido ósseo é dinâmico e está em constante remodelação, sendo mantido o equilíbrio entre a deposição de matriz óssea, desempenhado pelos osteoblastos e o processo de reabsorção óssea, típico dos osteoclastos (Hankenson; Gagne; Shaudhnessy, 2015). A remodelação óssea é ativada pela resposta dos osteócitos a algum dano ósseo por apoptose, ou naturalmente, quando as demandas biomecânicas e metabólicas direcionadas ao osso se modificam, recrutando os

osteoclastos, que vão degradar a matriz óssea (Schaffler *et al*., 2014). Subsequentemente, os osteoblastos sintetizam uma nova matriz óssea nas cavidades deixadas pelos osteoclastos de reabsorção (Langdahl; Ferrari; Dempster, 2016). Uma interrupção desse processo pode resultar em distúrbios ósseos, como a diminuição da densidade mineral e deterioração da microarquitetura óssea (Bu *et a*l., 2021).

Estudos epidemiológicos e clínicos têm demonstrado que o consumo de produtos lácteos pode ser benéfico para a saúde óssea devido à alta biodisponibilidade de nutrientes como cálcio, vitamina D e proteínas do leite, como a caseína, que regulam positivamente a remodelação atenuando a perda óssea (Rizzoli *et al*., 2018). Estudos em humanos tem confirmado essa relação positiva entre o consumo de produtos lácteos e a saúde óssea, sendo associados a um aumento da densidade/massa óssea e crescimento esquelético (Rozenberg *et al*., 2016, Bielman *et al*., 2018);

Evidências emergentes mostraram que não só as proteínas do leite, mas também seus peptídeos atuam de forma positiva na remodelação óssea e atenua a perda óssea, especialmente por meio da regulação de marcadores celulares (Bu *et al*., 2021) como a osteocalcina, que promove um aumento do número de osteoblastos e degradação de osteoclastos, contribuindo para a formação óssea quando aumentada (Filip *et al*., 2015). Além disso, esses peptídeos possuem propriedades bioativas, como ação antioxidante, imunomoduladoras e antimicrobianas (Nongonierma; Fitzgerald, 2015). Consequentemente, eliminar o leite da dieta pode resultar em carências nutricionais, como por exemplo a predisposição para o desenvolvimento de doenças como a osteoporose e osteopenia (Santos; Rocha; Santana, 2019).

Nas últimas décadas, o consumo de leite de vaca tem diminuído drasticamente, sendo substituído por bebidas não lácteas a base de plantas, como a soja (Cakebread *et al.*, 2019). A bebida de soja é frequentemente considerada uma opção mais saudável e sustentável em comparação ao leite de vaca (Constantine *et al*., 2017), devido ao fato de ser livre de colesterol, lactose e glúten, tornando-a uma escolha popular para pessoas com intolerância à lactose, doenças cardíacas, doenças inflamatórias ou para aqueles que desejam evitar produtos lácteos, como veganos (Sethi; Tyagi; Anurag, 2016). Além disso, a bebida de soja contém uma mistura de nutrientes semelhantes ao leite de vaca e é conhecida por seus potenciais benéficos na prevenção e tratamento de doenças crônicas (Dirkes *et al*., 2018).

Estudos epidemiologicos têm demonstrado que as isoflavonas presentes na soja possuem propriedades antioxidantes e antiinflamatorias (Yamagata, 2019), efeitos antitumorais (Zheng; Lee; Chu, 2016) e podem favorecer o metabolismo ósseo (George *et al*., 2020). Além disso, a bebida de soja tem sido associada a benefícios para a densidade mineral óssea (DMO) e a resistência mecânica dos ossos, além de aumentar a absorção intestinal de cálcio (Jagga *et al*., 2021). Estudos em camundongos machos C57BL/6 sugerem que a proteína da soja tem beneficios para o osso equivalentes ou superiores a caseína, uma das proteínas do leite, aumentando a DMO do fêmur, e as propriedades do osso esponjoso, como o volume ósseo e número de trabéculas (Yan *et al*., 2015). No entanto, um estudo controverso, substituindo o leite de vaca por leite vegetal a base de soja, para bebês de 4 a 14 meses exclusivamente por 1 a 3 meses, devido à alergia ao leite bovino levou à interrupção do crescimento de altura e peso dos bebês (Le Louer *et al*., 2014). Além disso, outro estudo com camundongos mostrou parâmetros ósseos prejudicados com a dieta de soja em comparação à dieta de caseína, demonstrado pela redução da superficie osteóide, taxa de formação óssea e aposição periosteal cortical do osso (Rouy *et al*., 2014). Portanto, para uma melhor elucidação destes achados, faz-se necessário uma análise investigando os principais efeitos e mecanismos histomoleculares envolvidos nas mudanças na estrutura do fêmur em modelos pré-clínicos que ingeriram leite de vaca e bebida de soja (transgênica e não transgênica). Então, com o objetivo de preencher as lacunas do conhecimento nesta área, foi realizado um estudo experimental usando camundongos BALB/c machos para comparar o efeito do leite de vaca e da soja transgênica e não transgênica na saúde óssea.

2. OBJETIVOS

2.1 OBJETIVO GERAL:

O objetivo deste estudo foi comparar os efeitos da suplementação com leite de vaca e bebida de soja (transgênica e não transgênica) no fêmur de camundogos adultos.

2.2 OBJETIVOS ESPECÍFICOS:

- ✓ Determinar a ação do leite de vaca e bebida de soja (transgênica e não transgênica) sobre parâmetros biométricos e biomecânicos do fêmur de camundongos BALB/c ;
- ✓ Avaliar a ação do leite de vaca e bebida de soja (transgênica e não transgênica) sobre estrutura histomorfológica do fêmur de camundongos BALB/c;
- ✓ Determinar a ação do leite de vaca e da bebida de soja (transgênica e não transgênica) sobre a distribuição óssea da proteína osteocalcina no fêmur de camundongos BALB/c;
- ✓ Comparar o nível de porosidade no osso compacto da diáfise do fêmur de camundongos BALB/c que receberam leite de vaca e bebida de soja (transgênica e não transgênica) através de análise de microscopia eletrônica de varredura;
- ✓ Avaliar a densidade volumétrica, distribuição de trabéculas, bem como o percentual de osso esponjoso e compacto na epífise e diáfise de cada amostra através de análises histológicas;
- ✓ Determinar a ação do leite de vaca e bebida de soja (transgênica e não transgênica) sobre o conteúdo de colágeno e minerais na matriz óssea do fêmur de camundongos BALB/c.

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4. Artigo

EFFECT OF COW'S MILK AND SOY DRINK (TRANSGENIC AND NON-TRANSGENIC) IN BALB/C MICE'S FEMUR.

Abstract

Introduction: Bone health is influenced by nutrition, and one of the most important dietary sources of calcium is milk. However, more and more people are limiting their milk consumption and opting to consume plant-based beverages, such as transgenic or non-transgenic soy. **Objective**: Compare the effects of supplementation with cow's milk and soy drink (transgenic and non-transgenic) on femur structure and mechanical function in BALB/c mice. **Materials and Methods**: The animals (n= 28, adults, ± 30 g) were randomized into four experimental groups of seven animals each: G1: distilled water, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk. All treatments were administered by gavage for 42 days. Then, the femurs were collected for biomechanical, biometric, histomorphological, and molecular analyses. **Results:** The G3 group had lower dody mass gain. Maximum compressive resistance was lower in G3. The diaphysis diameter of the femur was smaller in G2, and cortical bone thickness was reduced in G2 and G3. Medullary canal diameter in G3 was reduced. G4 had the highest trabecular volumetric density, bone area, and trabecular width, the lowest medullary canal volumetric density and trabecular separation. Osteocalcin levels were higher in G4. There was a reduction in type I collagen content in bone samples from G2 and G3. Magnesium levels increased in G4, and calcium levels increased in all groups compared to the control. G4 had a lower number of pores in the bone diaphysis, and G3 had a higher number of pores. **Discussion**: Transgenic soy drink was harmful to cortical bone, causing an imbalance between bone resorption and deposition due to a greater demand for nutrients for cortical bone in G3. In this group, there was a reduction in pores, compromising bone strenght, indicating a detrimental effect on tissue quality possibly because genetically modified foods can act as toxins and antinutritional factors. **Conclusion**: The consumption of cow's milk may be a safer alternative than soy-based drinks to promote bone health.

Keywords: bone microarchitecture. Milk. Nutrition. Genetically modified. Soy. Osteocalcin.

1. Introduction

The change in dietary behavior among the population has raised concerns regarding bone health since a balanced diet is fundamental for the developing and maintaining of bone mass and adequate biomechanical function (Lee et al., 2021). Alterations in bone metabolism, microstructure, matrix mineralization, and bone mineral density (BMD) are associated with nutritional factors and dietary components, in which we can highlight different types of milk (Rizzoli, 2014).

Milk, a traditional food product, is recognized as a nutrient-rich food with important minerals for bone health (Bu et al., 2021). It has a high bioavailability of calcium, presence of vitamin D, and proteins like casein that positively regulate bone remodeling, attenuating bone loss (Rizzoli 2022). Studies have shown that the consumption of dairy products can be beneficial for bone health, mainly due to the antioxidant, antimicrobial, and anticarcinogenic properties reported in milk proteins and peptides (Nongonierma & Fitzgerald, 2015; Nakamura et al., 2013).

Reduced or absent consumption of dairy products may increase the risk of developing osteoporosis and osteopenia, which can significantly impact the quality of life for individuals of all age groups (Santos; Rocha; Santana, 2019). According to the International Osteoporosis Foundation (IOF), osteoporosis is responsible for more than 8.9 million yearly fractures in Europe. In terms of costs, the IOF estimates that the total cost spent on osteoporotic fractures worldwide in 2019 was US$ 52 billion. This amount includes expenses on medical care, hospitalization, medication, and rehabilitation (Kanis et al., 2021). In this context, the milk diet represents today a simple and cheaper Ca and P source relevant to keep healthy bone, especially by the absorption and deposition of essential minerals in bone that may play an important role in supporting bone health. However, consumption of milk and its derivatives has decreased drastically in the last few years. This can be attributed to the increased diagnosis of lactose intolerance, the replacement of traditional dietary patterns (e.g., veganism) where animal-derived products are not consumed (Matia-Martín et al., 2019), concerns about milk allergies, and the high-calorie content of dairy products (Sethi; Tyagi; Anurag, 2016).

The consumption of plant-based drinks, such as soy, has significantly increased as a substitute for cow’s milk due to the absence of lactose and no cholesterol content (Jagga et al., 2021). In addition to having an aminoacid composition with a nutritional

value equivalent to animal proteins (Dirkes et al.,2018), soy is rich in flavonoids, widely known for their beneficial effects in the treatment of diseases with strong inflammatory and pro-oxidant bases (Garcia et al.,2018). Studies have shown that soy proteins can improve several trabecular bone characteristics, such as the trabecular number, bone volume, and increasing bone mineral density (BMD) of the femur (Yan et al., 2015). However, the nutritional content of these alternative beverages can vary significantly based on several factors, including the raw materials used, processing and storage methods, and whether they are fortified or not (Cakebread et al., 2021).

The increase in soy dietary intake has led to the development of genetically modified soy, a modified organism (GMO) designed to increase production and reduce costs. In this modification, the plant becomes resistant to herbicides used to eradicate harmful weeds that could affect the crop. There is no certainty about the maleficent effects of transgenic compounds on human health, but some studies have reported that this modification can cause risks, including hepatic, pancreatic, and gonadal toxicity in mice (Azevedo et al., 2010; Alvarez-Moya; Reynoso-Silva, 2023). However, organic soy is grown using ecologically sustainable methods, without the use of chemicals that could alter its composition, but this method of production often leads to a reduction in productivity and an increase in costs (Soares; Lucas; Boaventura, 2005).

Although studies have been conducted comparing the effects of cow's milk of soy drink on human health, the results are still controversial and need to provide a conclusive answer. Furthermore, there is no evidence from studies investigating the effects of consuming different types of soy drinks, such as transgenic and non-transgenic, on bone health. In this context, the present study aims to compare the effects of cow's milk, transgenic and non-transgenic soy drinks on bone development, analyzing the biometric, biomechanical, histomorphometric, immunofluorescence, organic and inorganic composition of the bone matrix, as well as the ultrastructural aspects in the femurs of BALB/C mice.

2. Materials and Methods

2.1 Animals

BALB/c mice, with an average body mass of ±30g, were obtained from the Central Animal House of the Biological and Health Sciences Center of the Federal University of Viçosa. The animals were kept in individual cages and experimental facilities with lighting cycles (12/12-h light/dark) and temperature (21º ±1 C) controlled. Water and food were provided *ad libitum*. Body mass was recorded weekly. The use of animals in this study was approved by the Animal Ethics Committee of the Federal University of Viçosa (registration number 23/2022).

2.2 Experimental Model and Experimental Diets

A total of twenty-eight BALB/c mice were randomized into four groups with seven animals per group. The groups were administered water (G1), non-transgenic soy drink (G2), transgenic soy drink (G3), or cow milk (G4), once a day. All treatments were given via gavage over a 42-day period, with a dosage of 0.7 ml. The nutritional composition of the various commercially available drink types is detailed in Table 1.Twenty-four hours after the last meal, the animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and euthanized by exsanguination via cardiac puncture. Femurs were removed for biomechanical, biometric, histomorphological, and molecular analyses.

	Milk type			% (DV)*		
Parameters	Cow's milk (1)	Non-transgenic soy drink (2)	Transgenic soy drink (3)	1	2	3
Energy Value	529kJ	399 kJ	290 kJ	6	5	3
Carbohydrates	10 g	12g	2.3g	3	4	1
Proteins	7.0 g	5.4g	6.4g	9	7	9
Total Fats	6.4 g	2.9g	3.6g	12	5	7
Saturated Fats	4.0	1.7g	0.7g	18	8	3
Sodium	120 mg	95 mg	178 mg	5	4	7
Calcium	240 mg	240 mg	264 mg	24	24	26
Vitamin A	-	-	162 mcg	-	-	27
Vitamin D	-	-	2.8 mcg	-	-	56
Vitamin E	-	-	2.7 mg	-	-	27
Vitamin B6	-	-	0.22 mg	-	-	17
Folic Acid	-	-	1.2 mcg	-	-	50
Zinc	-	-	1.1 mcg	-	-	16
Vitamin B12	-	-	1.2 mcg	-	-	50

Table 1. Nutritional information (Whole Cow's Milk (1), Transgenic Soy Drink (2), and Non-Transgenic Soy Drink (3). *(DV)% Daily values based on a 2000 kcal or 8400 kJ diet. Your daily values may be higher or lower depending on your energy needs.

2.3 Biometric Analysis

After the complete removal of the soft tissue of the left femur, the bone anatomical dimensions were measured using a universal analog caliper (Mitutoyo Sul Americana Ltda®, São Paulo, Brasil) and the support of a magnifying glass. The following measurements were obtained: (i) femur length (Measured from the femoral head's most proximal point to the femur's most distant end), (ii) width of the proximal femur (measured from the femoral head's anterior point to the greater trochanter's tip), (iii) width of the distal femur (referring to the width of the condyles in the anterior-

posterior direction that is perpendicular to the femur's length), and (iv) width of femur diaphysis (measured at the mid-femur's narrowest point).

2.4 Biomechanical Test

The biomechanical analysis method was used to evaluate the mechanical properties of the mice's femur. The left femur was wrapped in gauze moistened with saline solution and stored at -20 °C. After 3-hours period at room temperature, the femurs were subjected to mechanical testing performed on the Universal Testing Machine (EMIC® DL 10.000, Laboratório de Bioengenharia, Universidade Federal de São Paulo, Ribeirão Preto, São Paulo, Brasil) belonging to the Bioengineering laboratory of FMRP/USP. The femurs were subjected to a three-point bending mechanical test, in which the with force was applied to the center of the diaphysis. The bones were positioned on two supports with a free span of 10 mm. The mechanical load was applied to the femur through the blunt-tipped accessory fixed to the moving axis of the machine. A 500 N load cell was used, with load application in the vertical direction at a speed of 1 mm/min, until mechanical failure occurred. The accommodation time was 3 seconds. The Tesc® program (version 13.0, EMIC®, Brazil) was used to generate a load versus displacement graph for each test and obtain the mechanical properties of maximum force (N), relative stiffness (N/mm), and displacement. A representative image of the biomechanical assay can be observed in Figure 1.

Figure 1. Photograph of the three-point bending biomechanical test on the diaphysis of a mouse femur.

2.5 Bone Histomorphometric Analysis

The distal epiphyses of the right femurs were decalcified by immersion in a decalcifying solution containing formic acid (20% diluted in distilled water) for 30 days until complete decalcification of the material was achieved (Torres et al., 2019). Subsequently, the epiphyses were washed in distilled water, dehydrated in an increasing series of ethanol, and clarified in xylene. The anteroposterior thickness of the epiphysis was measured, and the medial portion was sectioned. Both fragments, positioned with the cutting base down, were embedded in paraffin in a vertical orientation relative to the longitudinal axis. Some four-micrometer-thick histological sections were stained with hematoxylin and eosin (H&E) for histomorphometric analysis, and others were stained with picrosirius for I and III collagen fibers analyses. To avoid repeated analyses of the same histological area, the sections were evaluated in semi-series, using one every 15 sections. The slides were viewed using a x20 and x40 objective lenses, and histological images were captured using a light photomicroscope (Olympus BX-60®, Tokyo, Japan) integrated with a digital camera (Olympus QColor-3®, Tokyo, Japan). Five measurements were taken in various directions for the variables bone diameter (DB), diameter of the medullary canal (DM), and cortical thickness (CT), using Image-Pro Plus 4.5 image analysis software (Media Cybernetics, Silver Spring, MD, USA) to ensure greater accuracy of the results, with the simple mean used as the final outcome.

To evaluate the cortical bone thickness, histological sections were stained with H&E. Images were captured using a x4 objective lens and the parameters BD, DM, and CT were measured in micrometers (µm) (PARFITT, 1988). Five measurements were taken in various directions for these variables using the Image-Pro Plus 4.5 image analysis software (Media Cybernetics, Silver Spring, MD, USA) to ensure greater results accuracy, with the simple mean used as the final outcome. In order to estimate the volume densities occupied by trabecular bone (Vv[bone], %) and bone marrow (Vv[marrow],%), point counting was used with the formula: Vv[trabeculae/marrow]= ΣPP[trabeculae/marrow] / ΣPT; where ΣPP[trabecula/marrow] represents the number of points that intersect with bone trabeculae or marrow, and ΣPT represents the total

number of test points (Sequetto et al., 2017). In this study, 81 test points were used. To estimate the bone area (B.Ar), trabecular width (Tb.Wi), and trabecular separation (Tb.Sp), we randomly selected and digitized ten regions of interest (ROI) for each animal, using a x20 objective lens on the photomicroscope. The marrow was manually deleted, and only trabecular bone was considered. Each ROI was segmented into a black-and-white image, and its trabecular-covered area was automatically calculated (Egan; Brennan; Pignolo, 2012). The same images were used to calculate trabecular separation and width using the sphere-fitting method, which computes the mean diameter of all spheres that can be fitted within trabeculae (Tb.Wi) and between trabeculae (Tb.Sp). The analyses were performed using the ImageJ software with the BoneJ plugin (Doube et al., 2010).

2.6 Immunofluorescence Analysis

The proximal bone epiphysis fragments were embedded in resin and submitted to immunohistochemistry using the streptavidin-biotin-peroxidase method, using the polymer method to identify the bone formation marker osteocalcin (OC). Immunofluorescence was analyzed in bone fragments fixed in 4% paraformaldehyde solution for 1 hour and, after fixation, washed three times for 1 hour each in phosphate-buffered saline (PBS 0.1 M, pH 7.2) in 1% Tween 20, followed by incubation for 24 h at 4°C in the anti-osteocalcin primary antibody solution (1:100 dilution, Abcam: ab1824222). After incubation, samples were washed three times (10 min each) with PBS and incubated for 24 h at 4°C with FITC-conjugated secondary antibodies (Sigma-Aldrich Corp., St Louis, MO, USA) (1:1000) dilution). The samples were dehydrated in ethanol and embedded in Leica historesin for sections (3 µm thickness) on a Leica RM 2255 rotary microtome (Leica et al.) using glass knives. The nuclei of bone cells were stained with 4',6-diamidino-2'-phenylindole (DAPI) (dilution 1:1000). The slides were visualized and the images captured using an EVOS M5000 microscope (Thermo Fisher Scientific, Pudong, Shanghai, China), with a 20x objective lens. Cells were counted using Image pro-plus software version 7.0.1 (National Institutes of Health, Rockville, MD, USA).

2.6 Collagen Analyses

To analyze collagen fibers, slides stained with picrosirius red F3Ba (Direct Red 80; Fluka, Buchs, Switzerland) were observed under a polarizing microscope (Olympus AX-70, Tokyo, Japan). Ten histological fields were randomly sampled using a x20 objective lens. The images were analyzed using the Image Pro-plus software version 7.0.1 (National Institutes of Health, Rockville, MD, USA). A grid with 300 test points was overlaid onto each image. The volume density occupied by collagen fibers (Vv%) was estimated by point counting, using the formula Vv[Collagen fibers] = ΣPP/ΣPT; where ΣPP is the number of points that occur on type I or type III collagen fibers, and ΣPT is the total number of test points (Santos et al., 2018). The collagen fibers were analyzed according to their birefringence properties, given that type I collagen fibers appear in bright colors that can range from red to yellow, while type III collagen fibers appear in a bright green color under polarization (Sarandy et al., 2017).

2.7 Inorganic Matrix Analyses

The bone mineral content of the femur's diaphysis and epiphysis was investigated by energy-dispersive X-ray spectroscopy (EDS) using a scanning electron microscope (LEO 1430VP, Carl Zeiss, Gernamy) equipped with an X-ray detection system (Tracor TN5502, Tracor Northern Inc., Middleton, WI, USA) (De Souza et al., 2021). The bone fragments were immersed in fresh histological fixative (2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose, and 5 mM $CaCl_2$ prepared in 0.1 M sodium cacodylate buffer, pH 7.2), dehydrated in ethanol, and subjected to critical point drying (CPD030; Bal-tec, Witten, North Rhine-Westphalia, Germany), followed by carbon coating (Quorum Q150 T, Laughton, East Sussex, UK). The analysis was conducted with at ×500 magnification. The proportion of the chemical elements calcium (Ca), phosphorus (P), manganese (Mn), copper (Cu), zinc (Zn), selenium (Se), magnesium (Mg), and sulfur (S) was measured by EDS, and was expressed as the mean for all analyzed areas of the diaphysis and epiphysis.

2.8 Scanning Electron Microscopy of Diaphysis

The bone fragments were immersed in fresh histological fixative (in 2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose, and 5 mM $CaCl_2$ prepared in 0.1 M

sodium cacodylate buffer, pH 7.2), dehydrated in ethanol, and subjected to critical point drying (CPD030; Bal-tec, Witten, North Rhine-Westphalia, Germany), followed by gold coating (Quorum Q150RS, Laughton, East Sussex, UK). The analysis was performed on bone fragments obtained from the proximal, middle, and distal femoral diaphysis. For each animal and diaphysis segment, five random sampling fields were selected at ×3000 magnification. To evaluate cortical bone porosity, number of pores per histological area (N/mm^2) and the proportion of tissue occupied by pores (Vv [porosity],%) were determined from the internal surface of the shaft (Sequetto et al., 2017). The number of pores was determined according to the formula N[pores]= Q^-[pores]/A_T; where Q^-[pores] is the number of pores inside an unbiased two-dimensional test area (AT) of 1.01 × 10^4 µm^2 at tissue level. The proportion of pores was determined according to the formula Vv[pores]= ΣP_P [pores] / ΣP_T, where ΣPP [pores] represents the number of points that reach the pores and ΣPT is the total number of test points, 360 in this study.

2.9 Statistical Analysis

The statistical analyses were performed using the GraphPad Prism 8.0.1 program (GraphPad Software Inc., Boston, MA, USA). The data were expressed as mean and standard deviation (mean ± S.D.). Data distribution was analyzed from the Kolmogorov-Smirnov test. Parametric data were compared using the One-Way ANOVA method, followed by the Turkey test. Non-parametric data were compared using the Kruscal-Wallis method. All results with $P \leq 0.05$ were considered statistically significant.

3. Results

3.1 Animal's Weight

The results obtained regarding the initial and final weight of the experimental groups did not show a statistically significant difference. All animals gained weight, but no significant differences were observed between groups G1, G2, and G4. On the other hand, group G3 obtained the lowest values regarding weight gain, with no statistical difference only in relation to group G2.

Table 1. Variation of weight in mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink.

Groups	Initial weight (g)	Final weight (g)	Weight gain (g)
G1	34.51 ± 2.98	36.17 ± 2.64	1.66 ± 0.34[a]
G2	31.61 ± 2.72	32.70 ± 2.59	1.09 ± 0.13[a.b]
G3	30.92 ± 3.08	30.95 ± 2.70	0.03 ± 0.38[b]
G4	33.70 ± 2.22	35.74 ± 1.97	2.04 ± 0.25[a]

G1: Distilled water; G2: Non-transgenic soy drink; G3: Transgenic soy drink; G4: Whole cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters in the rows indicate statistical difference ($P<0.05$) between the groups. Groups with the same letter in the columns do not show statistical difference ($P>0.05$).

3.2. Biometric Analyses

The anatomical parameters such as width of femur diaphysis, femur length and bone weight were similar in all experimental groups (Figure 2C, 2D and 2E). However, the width of the proximal femur in groups G2, G3, and G4 was reduced compared to the control group (Figure 2A). When analyzing the width of the distal femur, it was observed that the group that received only non-transgenic soy drink (G2) presented significantly higher values than animals receiving only transgenic soy drink (G3), but there was no difference in G2 and G3 compared to G1 and G4 groups (Figure 2B).

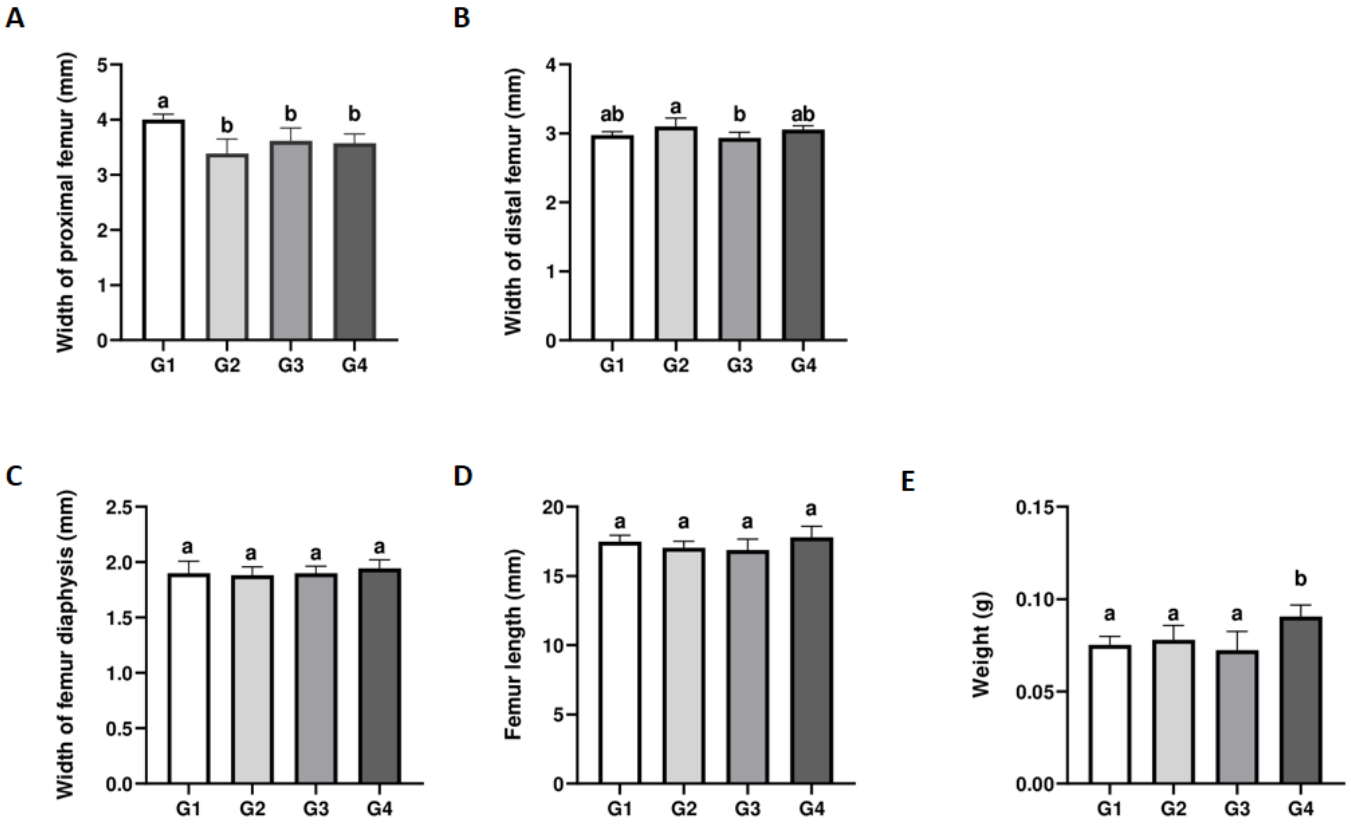

Figure 2. Anatomical dimensions of the femur from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. (A) Width of the proximal femur (mm); (B) Width of the distal femur (mm); (C) Width of the femur diaphysis (mm); (D) Femur length (mm); (E). G1: distilled water; G2: non-transgenic soy drink; G3: transgenic soy drink; G4: whole cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters in the columns indicate statistical difference ($P<0.05$) between the groups. Groups with the same letter do not present statistical differences ($P>0.05$).

3.3 Biomechanical Test

The maximum force required to cause bone fracture was lower in animals receiving the transgenic soy drink (G3) compared to the other groups (Figure 3A). Regarding the maximum displacement at fracture (Figure 3B) and bone stiffness (Figure 3C), there was no statistical difference between the groups.

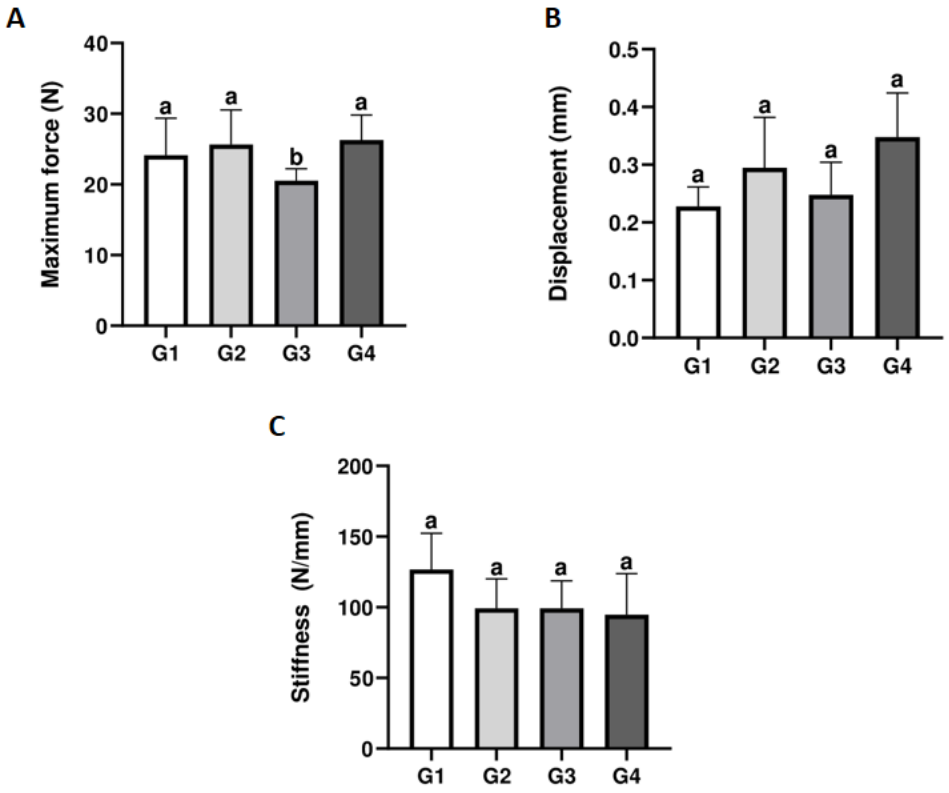

Figure 3. Mechanical resistance (A), displacement (B), and stiffness (C) of the femur from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. G1: distilled water; G2: non-transgenic soy drink; G3: transgenic soy drink; G4: whole cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters in the columns indicate statistical difference ($P<0.05$) between the groups. Groups with the same letter do not present statistical differences ($P>0.05$).

3.4 Bone Histomorphometric Analysis

A significant increase in trabecular volumetric density was observed in all groups evaluated compared to the control group. Groups G2 and G3 did not show significant differences between them, while group G4 obtained the highest values compared to the other groups (Figure 4A). Regarding medullary volumetric density and trabecular separation, all groups presented significantly reduced values compared to the control, with G2 and G3 not differ statistically. In contrast, group G4 presented reduced values compared to the other groups (Figures 4B and 4C). Bone area and trabecular width was similar in the groups G1 and G3, while the other groups showed

increased values. G2 and G3 obtained similar values between them, and group G4 showed a significant increase compared to all other groups (Figures 4D and 4E).

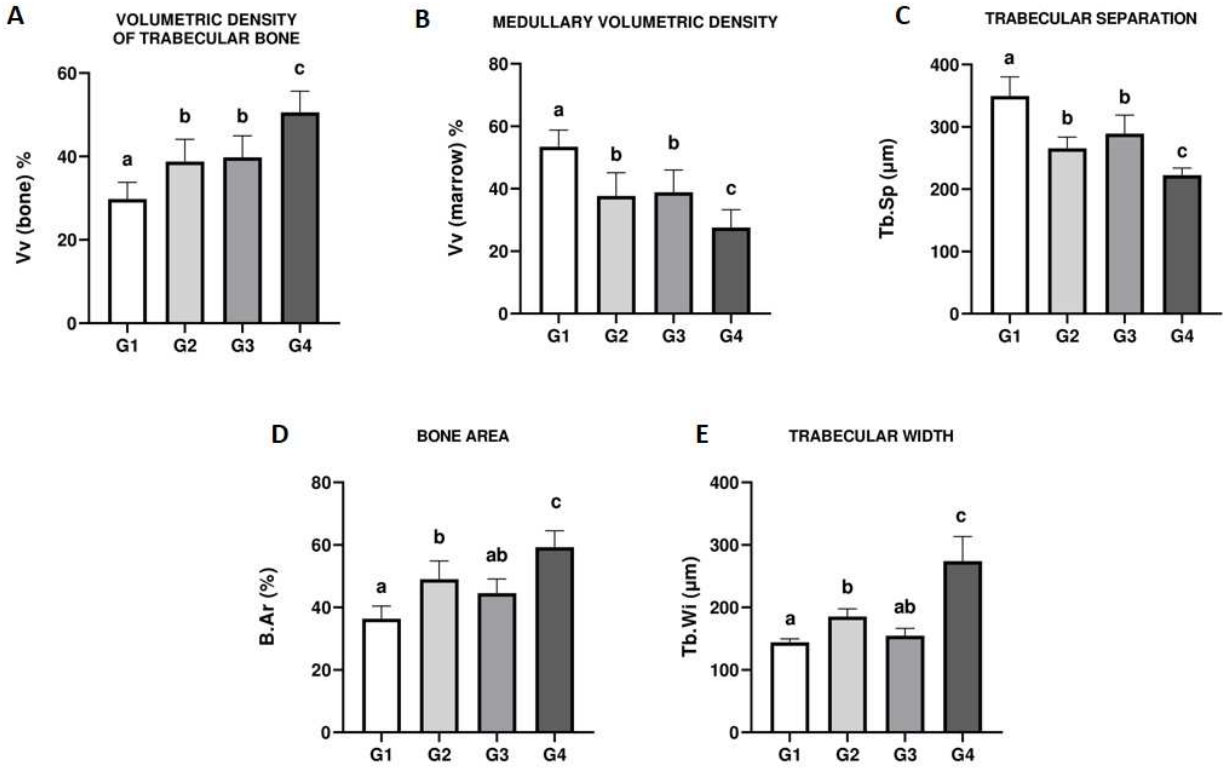

Figure 4. Trabecular bone microarchitecture in mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. (A) Volumetric density of trabecular bone (Vv bone); (B) Medullary volumetric density (Vv medulla); (C) Trabecular separation (Tb.Sp) (D) Bone area (B.ar); (E) Trabecular width (Tb.Wi). G1: treated with distilled water, G2: non-transgenic soy drink (G2). G3: transgenic soy drink, G4: cow's milk. Data are expressed as mean ± standard deviation. ^{a,b,c} Different letters on the columns indicate statistical differences ($P<0.05$) between the groups. Groups with the same letter do not show statistical differences ($P>0.05$).

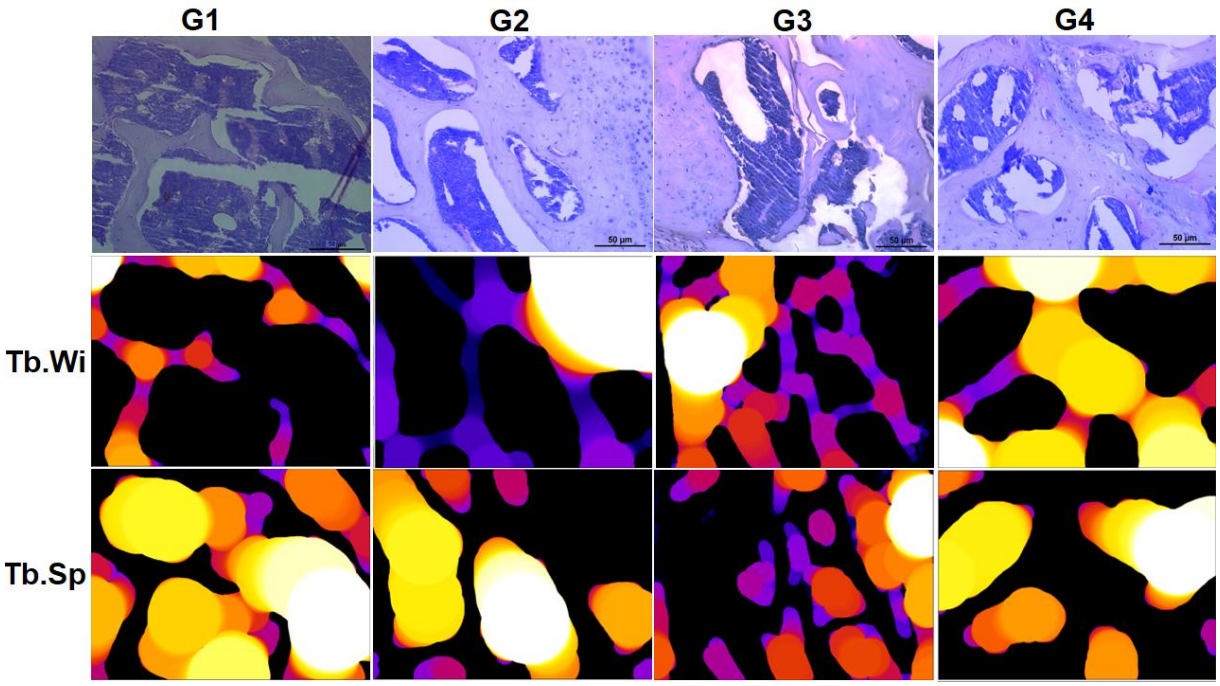

Figure 5. 3D computational method for calculating trabecular bone width (Tb.Wi) and trabecular bone separation (Tb.Sp) from the Image J software. Different colors represent the overlap of spheres positioned within and between trabeculae. G1: distilled water treated, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk.

The results of the microstructural analysis of the femur diaphysis are presented in figure 6. The femur diaphysis diameter (Figure 6A) was significantly smaller in G2 compared to the other groups, whereas G1, G3, and G4 had similar values. As the medullary diameter (6B), all groups were similar to the control, and only groups G2 and G3 showed a statistical difference between them. Cortical bone thickness was reduced in groups G2 and G3 compared to the other groups (Figure 6C). In contrast, group G4, which received cow's milk, did not negatively impact this parameter, exhibiting a response profile similar to the control group (G1).

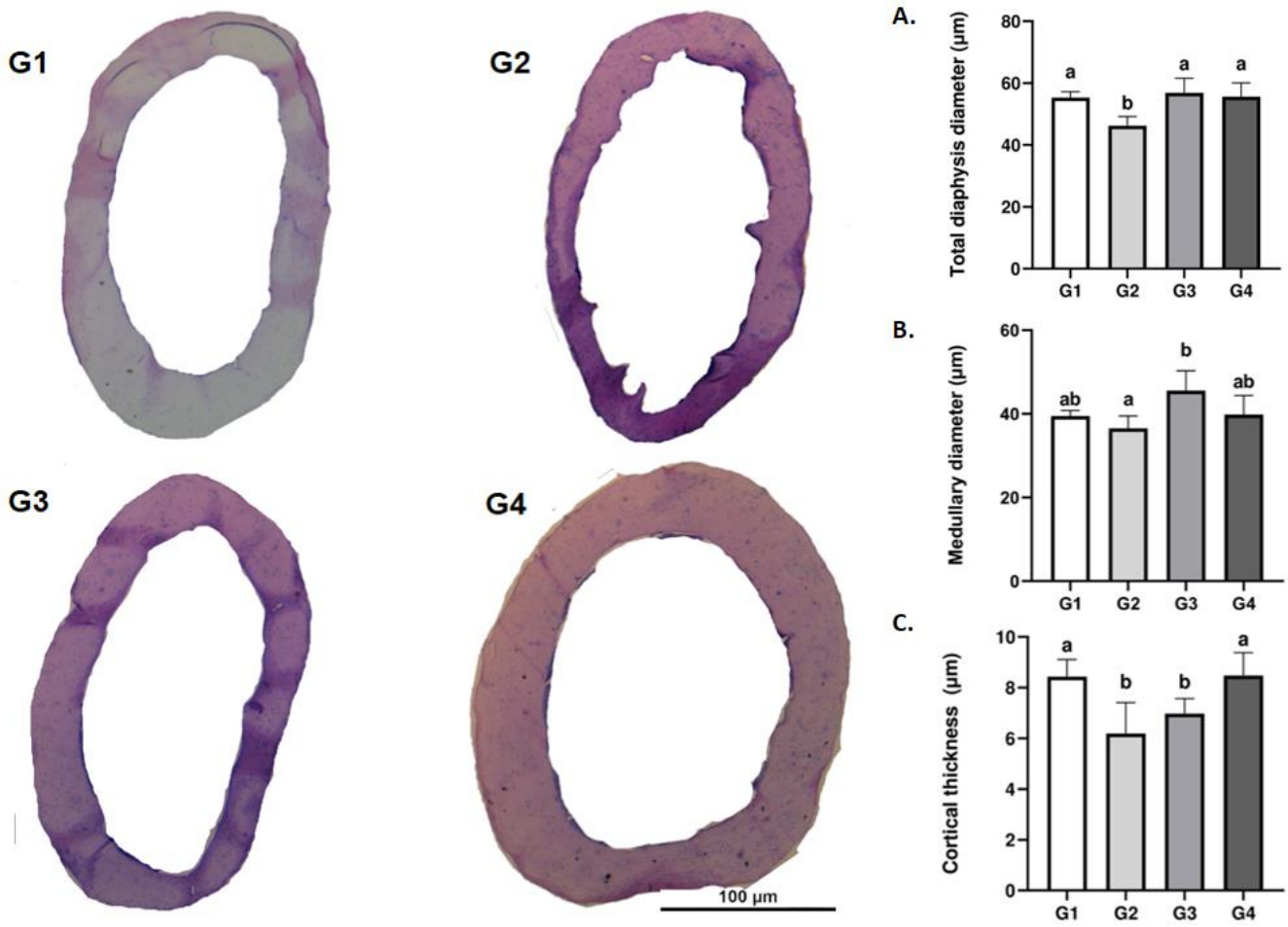

Figure 6. Representative microscopic images of the femur diaphysis (H&E staining) and morphometric parameters of the femur diaphysis from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. (A) Diaphysis diameter; (B) Medullary diameter; (C) Cortical thickness. G1: treated with distilled water, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters in the columns indicate statistical difference ($P<0.05$) between the groups. Groups with the same letter do not present statistical difference ($P>0.05$).

3.5 Immunofluorescence Analysis

The immunostaining of osteocalcin protein is shown in figure 7. Immunohistochemical analysis showed greater positivity for osteocalcin in the group treated with cow's milk (G4). In groups G1, G2, and G3, immunostaining was similar, with no statistical difference between them.

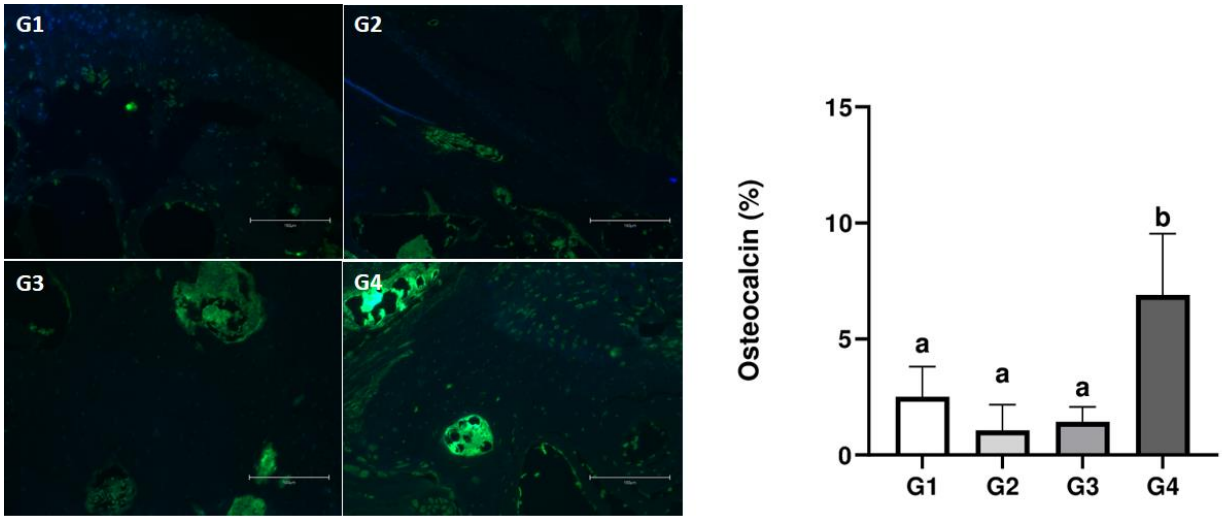

Figure 7. Microscopic images showing osteocalcin immunostaining (left images) and osteocalcin relative levels (right graph) in the femoral epiphysis from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. G1: treated with distilled water, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters on the columns indicate statistical differences between the groups in the graph, ($P<0.05$). Groups with the same letter do not present statistical differences ($P>0.05$).

3.6 Collagen Analysis

A significant reduction was observed in type I collagen content in G2 and G3 compared to the groups G1 and G4. Conversely, the G4 group did not present a significant difference in relation to the control group. In addition, increased type III collagen distribution was observed in the group G4 compared to the other groups. There were no statistically significant differences in type III collagen amount between the groups G1, G2, and G3 (Figure 8).

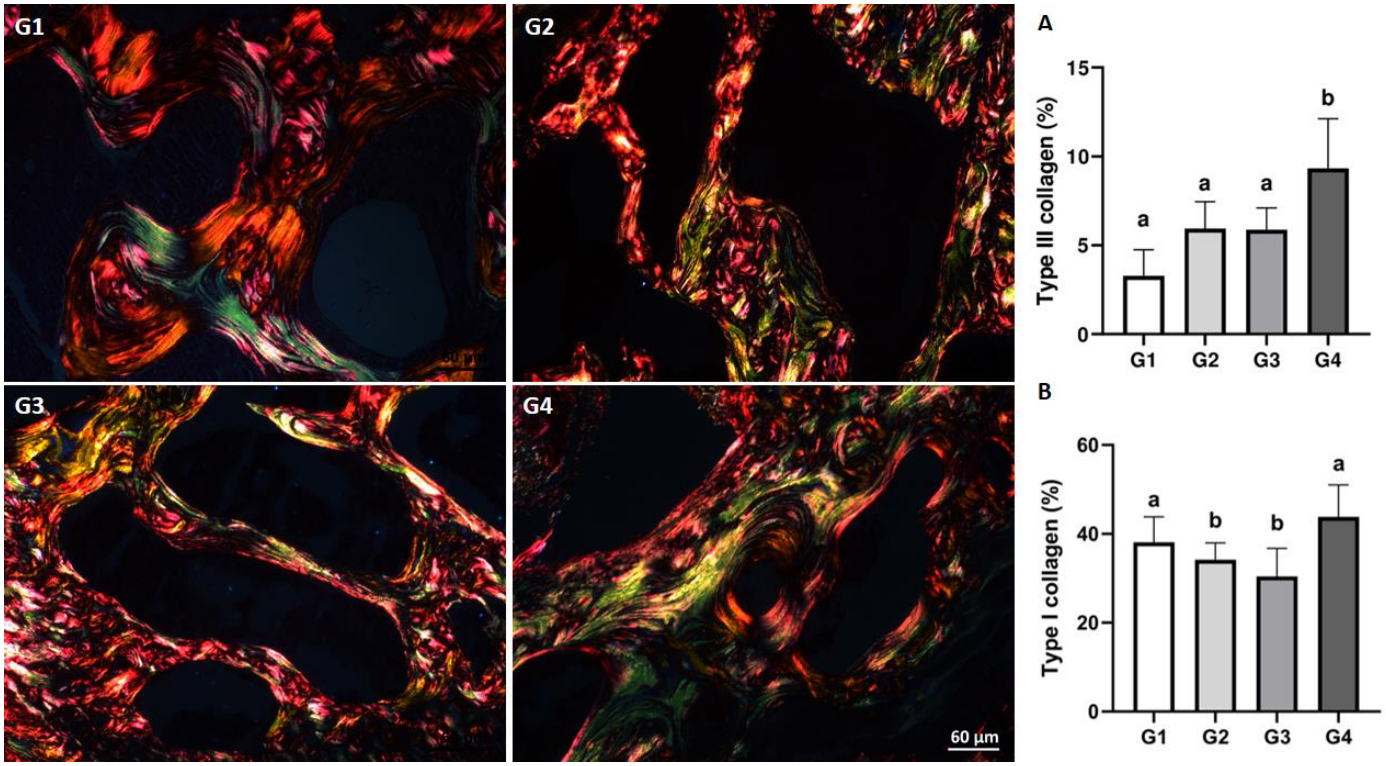

Figure 8. Representative microscopic images and proportion of type-I and type-III collagen distribution in the femoral epiphysis from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink (Sirius Red staining, polarized light microscopy). Type I collagen fibers appear in bright yellow, orange and red. Type III collagen fibers appear in bright green. The medullary space is shown in black. G1: treated with distilled water, G2: non-transgenic soy drink, G3: transgenic soy drink, G4: cow's milk. Data are expressed as mean ± standard deviation. [a,b] Different letters on the columns indicate statistical differences ($P<0.05$) between the groups. Groups with the same letter do not present statistical differences ($P>0.05$).

3.7 Mineral Analysis (inorganic matrix):

The quantitative analysis of minerals in the femurs of the different experimental groups revealed that, regarding magnesium, the group that received cow's milk (G4) showed a significant increase only compared to the control group (G1), while the other groups had similar values. Compared to the control group, calcium levels were significantly increased in all other groups; however, there was no statistical difference between the G2, G3, and G4 groups. Regarding phosphorus levels, there was no statistical difference among the experimental groups compared to the control group,

but there was an increase in the G4 group compared to the G3 group. No statistical difference was found for the mineral's manganese, copper, zinc, selenium, and sulfur.

Element (%)	G1	G2	G3	G4
Mg	1.48 ± 0.41^{a}	1.60 ± 0.18^{ab}	2.14 ± 0.14^{ab}	3.32 ± 0.70^{b}
S	2.14 ± 1.42	1.98 ± 1.41	2.80 ± 1.17	2.02 ± 0.63
P	33.59 ± 7.3^{ab}	35.69 ± 4.19^{ab}	31.78 ± 0.90^{a}	37.94 ± 3.55^{b}
Ca	33.15 ± 18.9^{a}	57.13 ± 3.55^{b}	60.21 ± 2.32^{b}	61.31 ± 2.35^{b}
Mn	0.40 ± 0.19	0.28 ± 0.17	0.43 ± 0.24	0.34 ± 0.22
Cu	0.42 ± 0.21	0.34 ± 0.18	0.51 ± 0.22	0.22 ± 0.04
Zn	0.53 ± 0.26	0.54 ± 0.20	0.49 ± 0.27	0.47 ± 0.17
Se	1.18 ± 0.74	1.17 ± 0.74	1.73 ± 1.23	1.02 ± 0.977

Table 2. Mineral content in the femur of mice treated with distilled water (G1); non-transgenic soy drink (G2), transgenic soy drink (G3), and cow's milk (G4). Data are expressed as mean ± standard deviation. ^{a,b} Different letters in the rows indicate statistical difference ($P<0.05$) between the groups. Groups with the same letter do not present statistical difference ($P>0.05$).

3.8 Scanning Electron Microscopy of Diaphysis

Scanning electron microscopy images revealed bone porosity of the femoral diaphysis in the different experimental groups (Figure 9). The number of pores per histological area and the proportion of bone tissue occupied by pores in group G4 were lower compared to the other groups (G1, G2, and G3). On the other hand, group G3 showed a higher pores distribution compared to the other groups (G1, G2, and G4). In turn, group G2 presented similar results to the control group (G1).

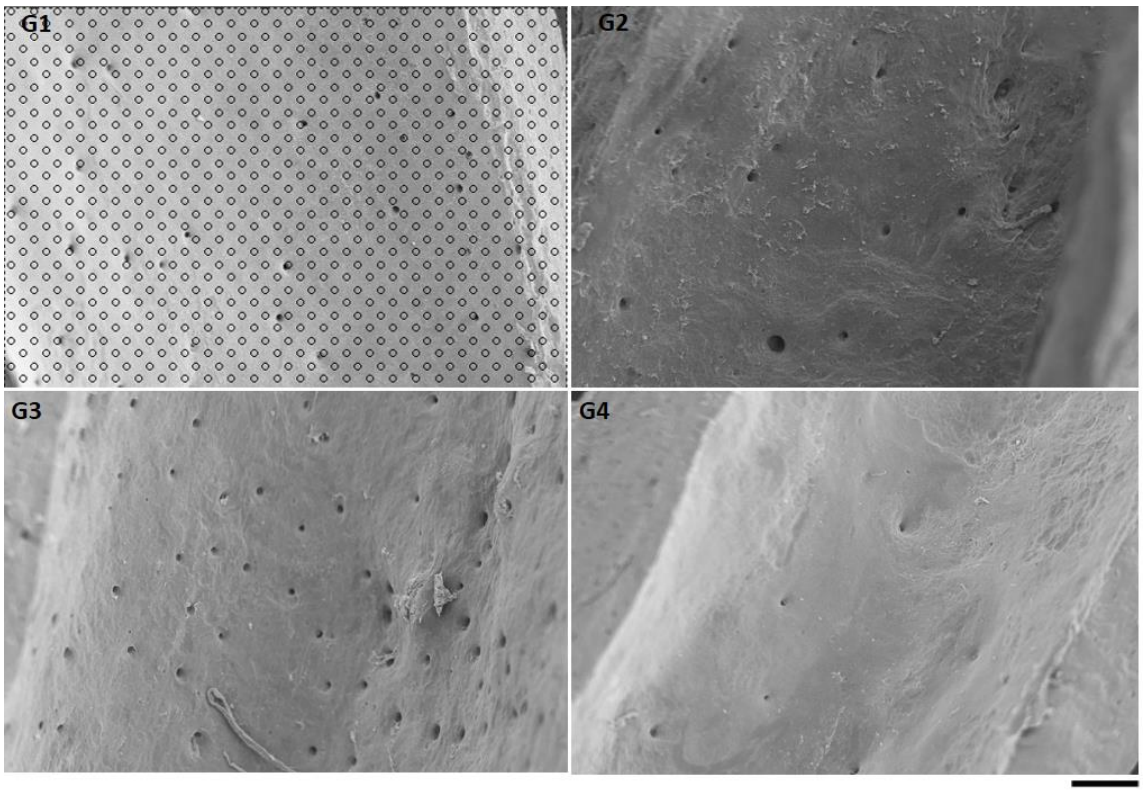

Figure 9. Representation of the method applied to determine the number and proportion of pores in the bone diaphysis using scanning electron micrographs (Scale bar= 200 µm). G1: treated with distilled water, G2: non-transgenic soy drink (G2). G3: transgenic soy drink, G4: cow's milk.

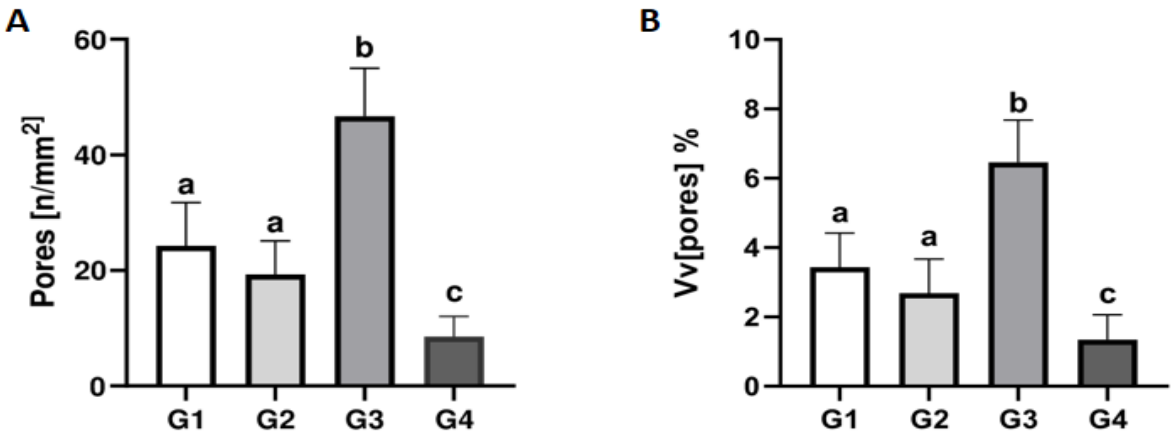

Figure 10. Number of pores per histological area (A) and volume density of pores – Vv (B) in the diaphysis from mice untreated and treated with cow's milk, non-transgenic and transgenic soy drink. G1: treated with distilled water, G2: non-transgenic soy drink

(G2). G3: transgenic soy drink, G4: cow's milk. Data are expressed as mean ± standard deviation. [a,b,c] Different letters on the columns indicate statistically significant differences ($P<0.05$) between the groups. Groups with the same letter do not present statistical differences ($P>0.05$).

4. Discussion

The nutritional composition of plant-based and animal-based foods has been the subject of many studies, and it is widely accepted that they differ in their composition (Scholz Ahrens; Ahrens; Barth, 2020; Khor et al., 2021). Plant-based beverages such as soy are often considered viable alternatives for many people (Thorning et al., 2016). However, there is no consensus about these products effect on human health. There is a research group that defends their daily use as safe, and another group radically against it (Soares; Lucas; Boaventura, 2005; Walther et al., 2022). In this sense, our study is the first to understand the comparative effects of exposure to cow milk and soy drink (transgenic and non-transgenic) and the structural and ultrastructural consequences of this exposure in the femurs of BALB/c mice. The results showed that consuming these different soy-based beverages caused changes in the biomechanics and microstructure of the mice's femurs, affecting the organic and inorganic composition of the bone matrix and cortical porosity when compared to control (water) and cow milk.

Studies have suggested that soy may have a modulatory effect on body weight, with isoflavones involved in adipogenesis and lipid metabolism without specifying if this effect is associated with the transgenic or non-trangenic soy (Yae Rim Choi et al., 2020; Kim et al., 2020b). Our results indicated that transgenic soy drink reduced weight gain compared to the control group and animals that received cow's milk, but animals that received non-transgenic soy drink did not differ from the control group. Although there is no evidence explaining the reduction in weight gain observed in animals that received transgenic soy drink, it is possible that some component present in this food may have influenced this result, especially thermally stable antinutrients (e.g., phytoestrogens, glucinins, phytic acid) (Dona; Arvanitoyannis, 2009) and toxins produced by genetically modified plants during the growth period (Kramkowska et al., 2013). However, further studies are needed to identify and evaluate these components and their possible effects on body metabolism.

The cortical bone is a critical determinant of bone strength, and alterations in bone diameter due to decreased cortical thickness and changes in porosity, can reduce bone resistance and increase susceptibility to fractures. Its growth occurs through a series of events characterized by independent actions of osteoblasts and osteoclasts on the outer (periosteal) and inner (endosteal) surface of the cortex (Maggiano et al., 2015). Periosteal apposition, where osteoblasts add a new layer of bone along the outer surface, and endosteal resorption, a process by which osteoclasts degrade the bone matrix on the inner surface, regulate the expansion and thickness of the cortical bone throughout life (Chen; Baron; Cori, 2022). Under normal conditions, periosteal apposition and endosteal resorption are balanced, maintaining skeleton integrity and strength (Isojima; Sims, 2021). Continuous or greater resorption associated with less apposition is responsible for the reduction in cortical bone thickness (Seeman, 2008). In our study, we observed that there was a reduction in cortical thickness and an increase in medullary cavity diameter in G3 suggesting a possible imbalance between endosteal resorption and periosteal apposition. On the other hand, the results indicate that bone resorption and apposition were balanced in G2, maintaining the medullary cavity diameter equal to the control group. Knowing that the demand for greater amounts of nutrients for bone can increase medullary cavity size (Veldhuis-Vlug et al., 2016), we suggest that resorption and apposition imbalance may have occurred due to a higher demand for nutrients for the cortical bone of the femur of animals that received transgenic soy drinks. Although genetically modified foods can play important beneficial roles, they can also contain toxins and antinutritional factors, having harmful effects on human and animal health (Kramkowska et al., 2013).

The groups that received transgenic and non-transgenic soy drink showed beneficial effects on bone health in all trabecular microarchitecture parameters. An explanation for these effects is that isoflavones, compounds present in soy, are structurally similar to estrogen and can bind to beta and alpha estrogen receptors (Boutas et al., 2022), having a higher affinity for the estrogen receptor beta (ER-β) than for the estrogen receptor alpha (ER-α). Trabecular bone has higher ER-β expression compared to cortical bone, which may explain why soy has positive effects on trabecular bone density, but not on cortical bone (Dirkes et al., 2018). In addition, bone remodeling and turnover occur in both cortical and trabecular bone, but with greater intensity in trabecular bone due to its functions and demands (Clarke, 2008b).

The group that received cow's milk showed similar results to the control group regarding the diameter of the diaphysis, medullary and cortical areas; suggesting that resorption and apposition balance was maintained, with no impairment in cortical bone formation. In addition, cow's milk had beneficial effects on trabecular microarchitecture in relation to all groups, altering trabecular volumetric density, bone area, width and separation, as well as medullary volumetric density. Literature suggests that specific milk proteins and peptides exert a positive role in bone remodeling through the regulation of cellular markers such as osteocalcin, and signaling pathways associated with osteoclasts and osteoblasts metabolism (Bu et al., 2021). Supporting these findings, a study in growing young mice showed that supplementation with isolated milk basic proteins resulted in a significant increase in femur trabecular volume levels, compared to mice untreated with these proteins (Ono-Ohmachi et al., 2017). Furthermore, Dongen et al. showed that higher milk intake, yogurt-associated milk, and milk associated with yogurt and cheese increased trabecular volumetric density in men (Van Dongen et al., 2018).

Osteocalcin is the main non-collagenous protein (NCP) produced by osteoblasts and is a specific marker of bone formation and resorption (Diaz-Franco; Villafan-Bernal, 2019). This protein modulates bone mass and is involved in the organization of the extracellular matrix and coordination of cell-matrix interactions, regulating overall bone structure and morphology (Bailey et al., 2017). Some studies suggest that an increase in osteocalcin promotes bone formation by osteoblasts, and when combined with calcium ions, it has a positive effect on bone deposition and growth (Li et al., 2019). A more intense immunostaining suggests better bone repair (Vieira et al., 2017), a characteristic observed in the group receiving cow's milk. When osteocalcin levels are increased, there is an acceleration of osteoblastic activity and bone remodeling. From a mechanistic standpoint, the increase of osteocalcin in the femur of animals that consumed cow's milk suggests that milk increases the number of osteoblasts or enhances the activity of existing cells. This, can contribute to osteoclasts depletion (which are responsible for bone resorption) and promote an increase in bone formation (Filip et al., 2015), which explains the increased trabecular volumetric density, Tb.Wi, and B.Ar; as well as lower medullary volumetric density and Tb.Sp in the group treated with cow's milk. Consistent with our results, a study in healthy women showed that supplementation with milk basic protein (MBP) increased bone mineral density and improved bone metabolism. In this study, osteocalcin concentration was higher in the

MBP group than in the control group after 6 months of intervention, indicating an increase in bone formation rate (Uenishi et al., 2007).

Collagen corresponds to 90% of the bone matrix proteins, playing a fundamental role in tissue properties (Kuo; Chen, 2017). Type I collagen is the main organic bone component, contributing significantly to deposition, mineralization (Terajima et al., 2014), and providing greater tensile strength (Kwansa; De Vita; Freemank, 2016). Type III collagen is present in smaller amounts, especially in the regions of tendon and ligament insertion (Miedel et al., 2015). Despite being ignored in some research, bone proteins are fundamental to the modulation of bone mineralization and growth, since they form the morphofunctional basis of these processes (Sequetto et al., 2017). Here, both transgenic and non-transgenic soy drink were shown to be harmful, decreasing the proportion of type I collagen fibers, indicating a negative osteogenic effect, since the main function of type I collagen in the bone extracellular matrix is structural, and the assembly and biochemical characteristics of these fibers interfere with bone mechanical properties (Licini; Vitale-Brovarone; Mattioli-Belmonte, 2019). On the other hand, animals that received cow's milk maintained the pattern of type I collagen fibers similar to the control group, as well as restored type III collagen fibers, indicating a positive role in osteoblastogenesis regulation, trabecular bone formation and maintenance (Volk et al., 2014). In addition, studies show that type III collagen plays a important role in bone repair, accelerating osteoblasts growth and preserving the osteogenic potential of stem cells, increasing angiogenesis (Miedel et al., 2015).

One of the factors that can also modulate bone growth and metabolism are inorganic elements such as zinc (Zn), copper (Cu), phosphorus (P), selenium (Se), and sulfur (S). These minerals are essential for maintaining the metabolic activity of cells, and therefore playing a fundamental role in preserving bone tissue integrity (De Souza et al., 2021), incorporating bone matrix and also regulating cellular formation and resorption processes. The concentration and distribution of these elements in bone tissue are related to biological and environmental factors, such as sex, age, and diet (Ciosek et al., 2021). In this study, the similar levels of these minerals among the experimental and/or control groups suggest that, despite the histomorphological, immunohistochemical, and biomechanical differences between some of the treatments, there was metabolic activity in the bone cells of all of them.

Macrominerals such as calcium and magnesium are considered essential nutrients for bone health (Fontes-Pereira et al., 2018). Calcium is the main mineral in

mammalian bone tissue and influences important extracellular and intracellular processes for bone development and growth (Vannucci et al., 2018). Additionally, it is one of the main components of the hydroxyapatite crystals that make up the inorganic part of bone, and is responsible for tissue mineralization (Reid; Bristow, 2020). Cow's milk is a well-known natural source of calcium, while soy drinks are poor sources of calcium and therefore require fortification with this mineral, primarily using calcium phosphate, which is a supplement (Walther et al., 2022). Previous studies have shown that foods that use calcium substitutes, such as fortified soy drink, although containing similar amounts of calcium to cow's milk, are not nutritionally equivalent as they alter the overall nutritional profile of the diet, potentially causing nutrient deficits (Fulgoni et al., 2011). Therefore, we suggest that although the results of the study show that groups G2, G3, and G4 had an increase in calcium concentration compared to the control, it is important to consider the nutritional composition of each food source to evaluate its nutritional value and potential for long-term health.

Magnesium plays a key role in bone homeostasis and bone cell function, influencing the formation and growth of hydroxyapatite crystals (Rude; Singer; Gruber, 2009). A reduction in magnesium intake in the diet can cause a disturbance in bone and mineral metabolism (Yu et al., 2017). Animals that received cow's milk were the only ones to have increased magnesium levels compared to the control group, and based on the study by Sobczak and West, dairy foods such as milk significantly contribute to the intake of essential nutrients in the diet, such as magnesium, while also demonstrating that fortified soy drink is not a significant source of many dairy nutrients, including this mineral (Fulgoni et al., 2011). Additionally, magnesium is known to have the property of stimulating neovascularization, which may contribute to better bone regeneration and angiogenesis (Zhang et al., 2021). According to the systematic review and meta-analysis titled "Impact of magnesium on bone health in older adults" higher magnesium intake was associated with increased bone mineral density in the femoral neck of adults (Groenendijk et al., 2022). Therefore, we believe that the increase in magnesium levels had a beneficial impact on bone health in the animals that received cow's milk, especially in trabecular microarchitecture.

The structure of cortical bone is crucial for mechanical strength and impact absorption capacity, and porosity in this region can be an indicator of bone loss (Mcnerny et al., 2019). An increase in cortical porosity is commonly attributed to an imbalance of bone remodeling, where resorption exceeds formation. This increase in

cortical porosity is also associated with a decrease in bone stiffness and toughness (Harrison et al., 2020). In our study, we observed an increase in the number of pores in compact bone in animals treated with transgenic soy drink (G3). Based on the results of cortical histological analysis, we can infer that this group presented an imbalance in bone remodeling, leading to this increase in the number of pores. Furthermore, in our study, the number of pores in compact bone was reduced in animals that consumed cow's milk, indicating an acceleration in the restoration of bone microstructure, which is consistent with the results we observed in trabecular bone, where trabecular volumetric density, bone area, and trabecular width were also increased compared to the control group, in addition to an increase in magnesium concentration, a mineral that can contribute to improving bone regeneration.

Mechanical tests allow for the evaluation of the structural and material properties of bone tissue (Bailey; Vashishth, 2018). Bone strength is determined as the maximum capacity to withstand forces before fracture or structural failure occurs (Batty; Bionaz, 2019). There are several factors that contribute to bone strength, which are considered determinants of bone quality, such as overall bone structure, which is determined by the quantity and distribution of tissue (Komori, 2020). Bone compromise can be caused by a decrease in bone mass, changes in microarchitecture or bone geometry, including an increase in cortical porosity, as well as by the morphology and overall composition of the tissue, such as the proportion of type I collagen and other non-collagenous proteins (Fonseca et al., 2014). In addition, factors such as cortical bone thickness and medullary diameter play an important role in determining bone strength (Bailey et al., 2017). Considering the results of the previous analyses, we believed that the transgenic soy drink would compromise bone strength, as animals that received this beverage had higher cortical porosity, lower amount of type I collagen, and also greater diameter of the cortical medullary region, indicating a possible imbalance between resorption and apposition. Similar to our findings, studies on human bones with denatured type I collagen showed that they lost strength compared to bones without denaturation, indicating that these changes can lead to an increased risk of fractures (Wang et al., 2001). After performing mechanical tests, the hypothesis that genetically modified soy beverage would compromise bone strength was confirmed by the results obtained, indicating that this beverage may have a negative effect on tissue quality and strength.

Currently, there are no studies that have specifically investigated the effects of genetically modified foods on bone health, making it difficult to explain the harmful results observed in cortical bone. However, some experimental studies suggest that these foods may have negative effects on human health, including changes in the pancreas and liver (Zareba et al.,2007; Malatesta et al., 2003;Malatesta et al., 2002), organs that are linked to bone tissue homeostasis.

5. Conclusion:

In summary, our findings suggest that soy drink (transgenic and non-transgenic) have comparable effects on mineral composition, type I and III collagen content, osteocalcin levels, weight gain, and trabecular microarchitecture, while transgenic soy drink was harmful to cortical bone, reducing maximum bone strength, cortical thickness, and increasing medullary region, indicating a possible imbalance between apposition and resorption, and an increase in bone porosity. However, it was found that cow's milk has positive effects on cortical and trabecular bone microarchitecture, modifying parameters such as trabecular separation, medullary and trabecular volumetric density, trabecular width and bone area, as well as increasing levels of magnesium, osteocalcin and type III collagen, and reducing cortical porosity. The results suggest that transgenic soy drink, despite the comparable effects to non-transgenic soy beverages on trabecular microarchitecture, brought harmful effects to cortical bone, while cow's milk improved bone parameters, suggesting that cow's milk consumption may be a better and safer alternative than transgenic soy drink to promote bone health. However, further investigations are needed to elucidate the mechanisms by which specific bioactives present in milk and soy drinks may influence bone metabolism. It is also important to obtain a more accurate understanding of the quantitative composition of macro and micronutrients present in these products in order to assess their contribution to bone health.

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