Mineral composition, histomorphometry, and bone biomechanical properties are improved with probiotic, prebiotic, and symbiotic supplementation in rats chronically exposed to passive smoking: a randomized pre-clinical study

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ABSTRACT: *Cigarette smoke in large centers is one of the most important causes of chronic inflammatory diseases in public health and is associated with a decrease in bone mass, consolidation process, and bone remodeling. Due to their ability to improve intestinal absorption and compete with pathogenic microorganisms, dietary supplementation with functional foods may contribute to improvement in bone quality. Therefore, the objective of this study was to evaluate the effects of functional, probiotic, prebiotic, or symbiotic food supplementation on mineral composition, histomorphometry, and bone biomechanical properties of rats in the growth phase, chronically exposed to cigarette smoke (T).Sixty-four young male rats were randomly assigned to eight groups (n=8): control (C) [standard diet (SD)]; probiotic (Pro) [SD + probiotic (Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium thermophilum and Bifidobacterium longum) (2–5 109 UFC each)]; prebiotic (Pre) [SD+ prebiotic (mannan oligosaccharide)]; symbiotic (Sym) (SD + probiotic + prebiotic); control smoking (SC) [(SD + exposure protocol to passive smoking (PS)]; probiotic smoking (ProS) (SD + probiotic + PS); prebiotic smoking (PreS) (SD + prebiotic + PS), and symbiotic smoking (SymS)(SD + prebiotic + probiotic + PS). The animals were euthanized after 189 days of the experimental protocol. Results showed that supplementation with probiotics, prebiotics, and symbiotics significantly improved (P<0.05) the parameters: P, Ca, Mg, BMD, BMC, strength, resilience, and size of area of the femoral diaphysis of the animals chronically exposed or not cigarette smoke. We concluded that functional food supplementation improved the bone health of rats chronically exposed or not to cigarette smoke.*
Key words: *functional foods, femur, bone mass, nutrition, cigarette smoke.*

Composição mineral, histomorfometria e propriedades biomecânicas óssea são melhoradas com a suplementação de probióticos, prebióticos e simbióticos em ratos expostos cronicamente ao tabagismo passivo: estudo pré-clínico randomizado

RESUMO: *A fumaça de cigarro em grandes centros é uma das causas mais importantes de doenças inflamatórias crônicas em saúde pública e esta associada à diminuição de massa óssea, processo de consolidação e remodelação óssea. Os alimentos funcionais suplementados na dieta, devido sua capacidade de melhorar a absorção intestinal e competir com microrganismos patógenos, podem contribuir para a melhora da qualidade óssea. Portanto, o objetivo desse estudo foi avaliar os efeitos da suplementação de alimentos funcionais, probiótico, prebiótico ou simbiótico, na composição mineral, histomorfometria e nas propriedades biomecânicas ósseas de ratos em fase de crescimento expostos cronicamente a fumaça do cigarro (T). Sessenta e quatro ratos machos jovens foram randomicamente distribuídos em oito grupos (n=8): controle (C) [dieta basal (DB)]; probiótico (Pro) [DB + probiótico (Lactobacillus acidophilus, Enterecoccusfaecium, Bifidobacterium thermophilumandBifidobacterium longum (2–5 109 UFC cada)]; prebiótico (Pre) [DB + prebiótico (mananoligossacarídeo)]; simbiótico (Sym) (DB + probiótico + prebiótico); controle fumante (CS) [(DB + protocolo de exposição ao tabagismo passivo(PT)]; probiótico fumante (ProS) (DB + probiótico + PT); prebiótico fumante (PreS) (DB + prebiótico + PT); e simbiótico fumante (SymS) (DB + prebiótico + probiótico + PT). Os animais foram mortos após 189 dias de período experimental.Os resultados revelaram que a suplementação com probióticos, prebióticos e simbióticos melhoraram significativamente (P<0,05) os parâmetros: P, Ca, Mg, DMO, CMO, resistência, resiliência e tamanho da área das diáfises dos fêmures dos animais expostos, cronicamente ou não, a fumaça do cigarro. Os resultados permitem concluir que a suplementação dos alimentos funcionais melhorou a saúde óssea de ratos expostos cronicamente ou não a fumaça do cigarro.*
Palavras-chaves: *alimentos funcionais, fêmur, massa óssea, nutrição, fumaça do cigarro.*

INTRODUCTION

Tobacco smoke (T) is a complex, dynamic, and reactive mixture containing approximately 5,000 chemicals (NAGAIE et al., 2014) and is associated with a decrease in bone mass; therefore, being considered a risk factor for the development of osteoporosis in humans (HERMIZI et al., 2009), in

addition, nicotine smoke has a direct action on bone metabolism, influencing the remodeling process (ELSHAWARBI et al., 2014).

Bone tissue is a multifunctional, metabolically very active tissue, composed of a heterogeneous population of cells at different stages of differentiation. This tissue can be altered by a series of conditions: age, osteometabolic diseases, decreased mobility, and drug action, which can lead to an imbalance between bone formation and resorption, resulting in the development of skeletal diseases, among them osteoporosis. This skeletal disease is characterized as systemic, progressive, with low bone mass, deterioration of the micro-architecture of the bone tissue, and a decrease in bone mineral density (BMD)(AMUGONGO et al., 2014; CHANG et al., 2017).

The most important minerals in bone composition are phosphorus (P), calcium (Ca), and magnesium (Mg), established in an organized manner on an organic matrix, whose main constituent is collagen (ELKOMY; ELSAID, 2015; HERNÁNDEZ-BECERRA et al., 2017). Some functional foods and bioactive ingredients have been shown to act beneficially on mineral composition and bone biomechanical properties, improving bone health (COSMAN et al., 2014; HERNÁNDEZ-BECERRA et al., 2017).

Among functional foods, probiotics are living microorganisms that promote benefit to the host by regulating the homeostasis of the microbiota (UMBRELLO; ESPOSITO, 2016). Prebiotics are natural or synthesized food components with carbohydrates in their structure and are selectively metabolized by probiotic microorganisms, conferring benefits to the host (CORRIE; CASTILLO, 2017). Symbiotics are composed of association of probiotics and prebiotics and have demonstrated the ability to beneficially modify the composition of the intestinal microbiota and mineral metabolism, thus contributing to better utilization of dietary nutrients and providing a bone structure with higher mineral content (SCHOLZ-AHRENS et al., 2016).

We did not find any pre-clinical studies using male rats in the growth phase investigating changes in mineral composition, histomorphometric parameters, and bone biomechanical properties in rats supplemented with functional foods: probiotic (Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium thermophilum, and Bifidobacterium longum), prebiotic [mannan oligosaccharide (MOS), oligosaccharide from the cell wall of Saccharomyces cerevisiae], and symbiotics, chronically exposed to cigarette smoke.

Rats has become a standardized physiological and toxicological model and has been used in many experimental protocols, including dietary manipulations. Although, there are several limitations to its resemblance to human condition, these can be overcome through detailed knowledge of the specific characteristics or with certain techniques (ABBOTT, 2004; ABUBAKAR et al., 2016; IANNACCONE; JACOB, 2009; LELOVAS et al., 2008). Therefore, the objective of the present study was to evaluate the effects on mineral composition, biomechanical properties, and histomorphometric parameters of rats in the growth phase supplemented with functional foods in the diet: probiotics, prebiotics, and symbiotics, and chronically exposed to cigarette smoke.

MATERIALS AND METHODS

Cigarette and smoke generation

In the adaptation and experimental period, commercially branded cigarettes were used (Tabacalera del Este S.A., Hernandarias, Paraguay), presenting mean concentrations of tar, nicotine, and CO of 10.2 ± 0.1 mg/cigarette, 0.8 ± 0.0 mg/cigarette of nicotine, and 10.1 ± 0.1 mg/cigarette of carbon monoxide, determined according to RENNE et al. (2006). The methods of conditioning to exposure and smoke generation were performed as described by TSUJI et al. (2013).

Characterization of the atmosphere of exposure to smoke

The concentrations of total wet particulate material (WTPM) and carbon monoxide (CO) were monitored via a real-time aerosol monitor (RAM, Microdust, Pro, Casella, Amherst, NH, USA) and CO monitor (TxiPro® - BioSystems Diagnostics Pvt. Ltd., USA), respectively. The coefficient of variation in percentage (% CV) of the exposure concentration (WTPM) was within $\pm$ 10% through gravimetric analysis using a Cambridge47 mm glass fiber filter (Performance Systematix Inc., Grand Rapids, MI, USA). The mean real concentrations of exposure were calculated from the mass collected in the filters and the total volume of air extracted by the filters (TSUJI et al., 2013).

The temperature and humidity of the exposure atmosphere were measured daily using a humidity / temperature detector (Hygrotherm, Qualitäts-Erzeugnis, TFA, Germany).

Animals and care

All the rats used in this study were from the Central Bioterium of the Universidade do Oeste

Paulista (Presidente Prudente, São Paulo, Brazil). The basal diet was formulated to meet the nutritional needs of the rats according to the NRC (Table 1) (HAN et al., 2016) and tap drinking water (City of Presidente Prudente, São Paulo, Brazil) except during periods of exposure.

Sixty-four male Wistar rats in the growth phase (*Rattus norvegicus albinus*), with a mean initial body mass of 46.3 ± 2.6 g, 21 days of age, were housed in collective cages with four animals each during the experimental period of 189 days. Animals were housed in animal boxes with 12 h light/12 h dark cycles at 22 ± 2 °C, humidity $55 \pm 10\%$, and mean air exchanges of 15 exchanges/hour.

Allocation, management strategy, and animal treatment concealment were performed to reduce bias in the study(MA, N. et al., 2017). The experiment lasted 189 days, with 5 days of adaptation and 184 days of experimental management strategy, exposure to cigarette smoke, and diet with standard or experimental diets (Table 1 and Figure 1).

The study was conducted in accordance with the ethical principles of the Universal Declaration of Animal Rights of the United Nations Educational, Scientific and Cultural Organization (UNESCO). The protocol of the study was approved by the Ethics Committee in Animal Studies of the test facility before the beginning of the experiment, under protocol 2686, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, Brazil.
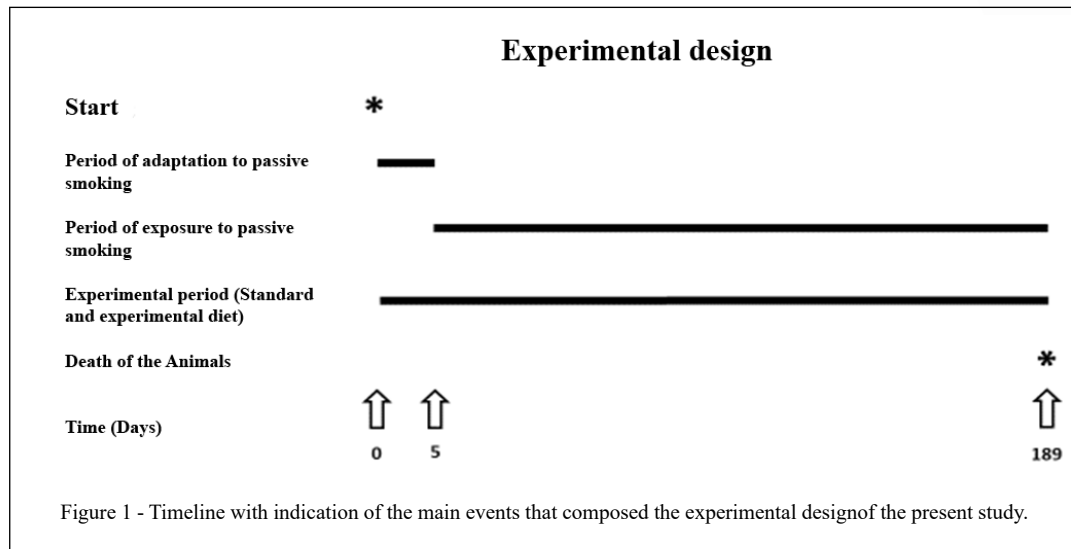
Study design

The animals were randomly distributed to the eight experimental groups (n=8) by means of a sequence table generated by the program R (R DEVELOPMENT CORE TEAM, 2016), and fed with the following diets: Control (C), standard diet (SD); Probiotic (Pro), SD supplemented with 20 g Kg-1 of an association of probiotic microorganisms [Lactobacillus acidophilus, *Enterococcus faecium*, *Bifidobacterium thermophilum*, and *Bifidobacterium longum* (2–5 109 UFC each) (Brazilian Enterprise to Increase Livestock Productivity – Embraupec, Paranavaí – PR, Brazil)]; Prebiotic (Pre), SD supplemented with 10 g Kg-1 of prebiotic [mannan oligosaccharide (MOS)], composed of the active fraction α-1,3 and α-1,6, presenting 30% α-mannan and derivative of the yeast strain *Saccharomyces cerevisiae*]; Symbiotic (Sym), SD supplemented with 20 g Kg-1 of an association of probiotic microorganisms and 10 g Kg-1 of prebiotic. The groups Control Smoking (CS), Probiotic Smoking (ProS), Prebiotic Smoking (PreS), and Symbiotic Smoking (SymS) were fed the diets C, Pro, Pre, and Sym and submitted to the protocol of exposure to cigarette smoke (Table 1).

Table 1 - Composition of dietary ingredients and nutrients.

Ingredients (g)	---Diets---			
	C	Prob	Pre	Sym
Corn meal	82.95	80.95	81.95	79.95
Soybean oil	7.00	7.00	7.00	7.00
L-Cysteine	0.30	0.30	0.30	0.30
Cellulose	5.00	5.00	5.00	5.00
Sodium Chloride	0.25	0.25	0.25	0.25
Vitamin mix[*]	1.00	1.00	1.00	1.00
Mineral mix[**]	3.50	3.50	3.50	3.50
Probiotic[***]	0.00	2.00	0.00	2.00
Prebiotic[****]	0.00	0.000	1.00	1.00
Total	100.00	100.00	100.00	100.00

[*] Vitaminmix/Kg: Nicotinicacid, 30 mg; Pantothenate, 15 mg; Pyridoxine, 6 mg; Thiamine, 5 mg; Riboflavin, 6 mg; Folicacid, 2 mg; Biotin, 0.2 mg; Vitamin B12, 25 mg; Vitamin E, 75 IU; Vitamin A, 4000 IU; Vitamin D3, 1000 IU; Vitamin K, 900 mg; Choline, 1000 mg. [**] Mineral mix mg/Kg: Calcium, 5000; Phosphorus, 1.561; Potassium, 3600; Sulfur, 300; Sodium, 1019; Chlorine, 1.574; Magnesium, 507; Ferro, 35; Zinc, 30; Manganese, 10; Copper, 6; Iodine, 0.2; Molybdenum, 0.15; Selenium, 0.15. [***] *Lactobacillus acidophilus*, *Enterococcus faecium*, *Bifidobacterium thermophilum,* and *Bifidobacterium longum* (2–5 10^9 UFC each). [****] Mannan oligosaccharides (MOS), composed of the active fraction α-1,3 and α-1,6, presenting 30% of α-mannans and derivative of yeast strain *Saccharomyces cerevisiae*.

Experimental design

Start   *

Period of adaptation to passive
smoking

Period of exposure to passive
smoking

Experimental period (Standard
and experimental diet)

Death of the Animals   *

Time (Days)

0   5   189

Figure 1 - Timeline with indication of the main events that composed the experimental designof the present study.

Protocol and period of exposure to passive smoking

During the five-day adaptation period, animals from the CS, ProS, PreS, and SymS groups were exposed daily to cigarette smoke in a chamber, at a controlled temperature of 22 °C, for a period of 10 minutes, and the C, Pro, Pre, and Sym groups were exposed to filtered air for the same period. After this adaptation, the experimental period of 184 days began, in which the CS, ProS, PreS, and SymS groups were exposed to cigarette smoke for 60 minutes daily: 30 minutes in the morning (7:00 a.m.) and 30 minutes in the afternoon (7:00 p.m.), five days a week. The C, Pro, Pre, and Sym groups were exposed to filtered air for the same period of time and periods of the day. The mean concentration of cigarette smoke was adjusted daily to contain 350 parts per million (ppm) of carbon monoxide (CO) during the exposure period (TxiPro® - BioSystems Diagnostics Pvt. Ltda., USA) (KOZMA et al., 2014).

Euthanasia and histomorphometry

At 210 days of age, all the rats were anesthetized (Thiopentax, Cristália - Produtos Químicos Farmacêuticos Ltda., Itapira, São Paulo, Brazil), euthanized by exsanguination, and theright and left femurs of all animals were disarticulated from the hip and the surrounding soft tissues removed.

The right femur was cleaved transversally in the medial region and the lower half was decalcified in 5.5% ethylenediaminetetraacetic acid (EDTA) solution in 10% formalin solution, for a period of two to three weeks. The decalcification was tested every two to three days when the solution was changed. After decalcification, the samples were processed for inclusion in paraffin and subsequent microscopic analysis in histological sections of 5 μm and stained with hematoxylin and eosin (HE). Cross sections of the diaphysis were analyzed and the cortical bone thickness was analyzed through images acquired from the medial parts of the diaphysis, with a final increase of 40x (10x eyepiece and 4x objective). Three measurements were made of each region: proximal, medial, and distal, of each histological section (BERGMANN DE CARVALHO et al., 2010).

Determination of bone mineral density (BMD), area, maximum strength (Fmax), resilience, stiffness, and bone mineral content (BMD)

In the dissected left femur, bone mineral density (BMD) and bone area (g/cm2 and cm2, respectively) were measured by means of dual X-ray densitometry (DXA) (DPX-ALFA model GE Medical Systems Lunar - USA), using a specific program for rats. Subsequently, femurs were submitted to mechanical tests (three-point flexion and axial compression) in a universal machine EMIC®, model DL 3000 (Instron Brazil, Brazil) and the maximum strength, resilience, and stiffness were determined. The remaining left femur samples used in the BMD and biomechanical tests were rehydrated, weighed, and placed in an oven at 800 °C for 6 hours to obtain the ashes after calcination. After cooling in a desiccator, samples were weighed to determine the amount of mineral matter (ashes) (BARBOSA et al., 2011, 2012; PAJAMÄKI et al., 2008).

Ashes were used for the quantification of phosphorus (P), calcium (Ca), and magnesium (Mg), using inductively coupled plasma mass spectrometry

(ICP-MS) and ICP-EOS Variant 730-ES equipment (Kyoto, Japan). All tests were performed according to official methods (SIMSEK et al., 2017).

Statistical analysis

The data were analyzed by the Shapiro-Wilk test for normality of the data. As normal distribution was demonstrated, one-way ANOVA was used, followed by the Tukey's test (P<0.05). Statistical analyzes were performed in the BioEstat 5.0 program.

RESULTS

Exposure and clinical observations

The concentrations of tar, nicotine, and CO during the period of exposure to cigarette smoke, both adaptation and experimental, were well controlled during the study. There were no unscheduled removals of animals due to premature death or a moribund condition after chronic exposure to cigarette smoke or air ventilation. Immediately after exposure, animals in the CS group exhibited decreased locomotor activity, ataxic gait, irregular breathing, salivation, and nasal noise. Groups supplemented with functional foods and exposed to cigarette smoke, ProS, PreS and SymS, presented a slight salivation and nasal noise.

Bone densitometry, mechanical assay, and histomorphometry

The mean BMD and BMC of the groups supplemented with functional foods and exposed to cigarette smoke were higher than the CS group; although, this difference was not significant (P>0.05). The groups exposed to cigarette smoke did not differ from group C (P>0.05).Groups supplemented with functional foods and not exposed to cigarette smoke did not differ significantly regarding BMD only from groups C and SymS (P> 0.05). The groups supplemented with functional foods and not exposed to cigarette smoke differed significantly (P<0.05) from the groups exposed to cigarette smoke in relation to the BMC parameter (Table 2).

The mean values of the femur areas of the CS group were lower in relation to the SymS group, but not significantly (P>0.05); however, this value was significant in relation to the other treatments (P<0.05). Groups supplemented and not exposed to cigarette smoke were significantly higher than the groups exposed to cigarette smoke (P<0.05) (Table 2).

For the Maximum Strength parameter, the groups supplemented with the functional foods and the C group presented significantly larger means of the results (P<0.05) than the CS group. The maximum strength means were higher in the groups supplemented with functional foods than in group C, but this difference was not significant (P>0.05) (Table 2).

The mean values of the Resilience parameter of the CS group were lower (P<0.05) than the groups not exposed to cigarette smoke. The mean values of the groups supplemented with functional foods and not exposed to cigarette smoke were higher than group C, but this difference was not significant (P>0.05) (Table 2).

The mean of the Stiffness (R) parameter of the CS group was lower (P<0.05) than the means of the groups not exposed to cigarette smoke. Supplementation with functional foods increased the means of this parameter in groups exposed or not to cigarette smoke (Table 2).

Concentration of minerals in the femur

The mean concentrations of P, Ca, and Mg in the CS group were significantly (P<0.05) lower in relation to group C and the other groups. Supplementation with functional foods significantly increased (P<0.05) the mean P, Ca, and Mg concentrations in the groups exposed or not to cigarette smoke. The mean Mg concentration of groups supplemented with functional foods exposed to passive smoking did not differ (P>0.05) from group C (Table 2).

Thickness of the diaphysis

The mean femoral diaphysis measurements of the treatment groups (proximal, medial, and distal regions) are shown in Table 3 and Figures 2 and 3. Results revealed a significant increase (P<0.05) in the mean bone thickness in the analyzed regions of the animals of the Pro, Pre, and Sym groups, when compared to group C. It was also possible to observe a significant decrease in the mean femoral diaphysis thickness of the CS group when compared to group C. Results showed a significant increase (P<0.05) in the femoral diaphysis mean values of the ProS, PreS, and SymS groups compared to the CS group.

DISCUSSION

The experimental design of the present study sought to demonstrate the benefits to bone tissue of the use of functional foods (probiotics, prebiotics, and symbiotics) in a murine model (*Rattus norvegicus albinus*) exposed or not to cigarette smoke. Similar studies using nicotine and probiotics alone demonstrated the usefulness and safety of

Table 2 - Mean and standard deviation values of mineral concentration and bone mineral density (BMD), bone mineral content (BMC), area, maximum strength (Fmax), resilience, and stiffness (R) obtained for groups of rats supplemented or not with probiotics , prebiotics, and symbiotics, and exposed for 189 days to passive smoking.

Group[*]	Parameters								
	Bone Densitometry and Mechanical Assay						Concentration of minerals in the femur (mg/g)		
	BMD (g/cm^2)	BMC (g)	Area (cm^2)	Fmax (N)	Resilience (mJ)	R (10^3N/m)	P[*]	Ca[**]	Mg[***]
C	0.15 ± 0.01^{AB}	0.28 ± 0.02^{AB}	1.85 ± 0.02^{C}	138.18 ± 11.27^{C}	59.65 ± 6.24^{BC}	275.92 ± 15.01^{BCD}	172.59 ± 2.08^{B}	350.59 ± 3.69^{B}	5.99 ± 0.11^{B}
Pro	0.17 ± 0.02^{B}	0.30 ± 0.02^{BC}	1.90 ± 0.02^{D}	147.36 ± 10.50^{C}	61.62 ± 5.89^{BC}	296.18 ± 10.62^{D}	179.47 ± 2.61^{C}	364.81 ± 3.41^{C}	6.73 ± 0.17^{D}
Pre	0.17 ± 0.01^{B}	0.32 ± 0.01^{BC}	1.90 ± 0.04^{D}	145.37 ± 13.19^{C}	63.58 ± 6.66^{BC}	288.64 ± 21.49^{CD}	177.79 ± 1.84^{C}	362.28 ± 2.00^{C}	6.44 ± 0.20^{C}
Sym	0.17 ± 0.01^{B}	0.33 ± 0.03^{C}	1.88 ± 0.03^{CD}	139.92 ± 8.61^{BC}	67.24 ± 1.93^{C}	276.31 ± 15.66^{BCD}	179.43 ± 3.01^{C}	363.75 ± 3.30^{C}	6.45 ± 0.22^{CD}
CS	0.13 ± 0.01^{A}	0.25 ± 0.01^{A}	1.72 ± 0.02^{A}	117.88 ± 8.94^{A}	50.26 ± 4.64^{A}	225.01 ± 12.39^{A}	162.98 ± 3.33^{A}	340.32 ± 2.65^{A}	5.23 ± 0.09^{A}
ProS	0.14 ± 0.01^{A}	0.26 ± 0.01^{A}	1.80 ± 0.03^{B}	137.24 ± 9.71^{B}	57.39 ± 2.91^{AB}	273.82 ± 15.53^{BCD}	169.92 ± 2.67^{B}	348.76 ± 5.61^{B}	5.87 ± 0.19^{B}
PreS	0.14 ± 0.01^{A}	0.26 ± 0.01^{A}	1.80 ± 0.04^{B}	125.68 ± 7.54^{AB}	57.22 ± 3.80^{AB}	268.48 ± 11.78^{BC}	168.83 ± 3.41^{B}	347.50 ± 5.89^{B}	5.75 ± 0.22^{B}
SymS	0.14 ± 0.01^{AB}	0.26 ± 0.03^{A}	1.76 ± 0.02^{AB}	121.40 ± 8.35^{A}	58.11 ± 3.35^{AB}	255.76 ± 13.13^{B}	170.17 ± 2.97^{B}	348.62 ± 4.55^{B}	5.72 ± 0.22^{B}

[*]Phosphorus. [**]Calcium. [***]Magnesium.The values correspond to the mean ± SD. Means followed by the same letter in the column do not differ from each other, using the Tukey test (P> 0.05). [*] C: control group (standard diet); Pro: standard diet + 2 g probiotic [*Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium thermophilum,* and *Bifidobacterium longum (*2–5 10^9 UFC each)]; Pre: standard diet + 1 g prebiotic (active fraction, α-1,3 and α-1,6 derived from a mannan oligosaccharide, presenting 30% α-mannans, a derivative of the yeast strain *Saccharomyces cerevisiae*); Sym: symbiotic group (standard diet + 2 g probiotic + 1 g prebiotic); CS: control group + + smoking protocol (SP); ProS: probiotic group + SP; PreS: prebiotic group + SP; SymS: symbiotic group + SP.

this experimental model to evaluate parameters of bone composition, tissue organization, and resistance (CAHILL et al., 2012; FORD et al., 2014; ŚWIATKIEWICZ et al., 2010).

The passive chronic exposure of rats to cigarette smoke, for 189 days, led to a decrease in BMD and BMC parameters, which in humans is indicative of a predisposition to osteoporosis, as well as an increased risk of fractures (ELSHAWARBI et al., 2014; HERMIZI et al., 2009), reinforcing the already known deleterious effects of smoking. In both humans and animals, these deleterious effects have been related to the ability of cigarette smoke to modify the composition of the intestinal microbiota, inducing, in four weeks, in rats, a decrease in the population of Bifidobacterium spp. in the cecum. In mice, an increase in Clostridium spp. and decrease in the phylum Firmicutes and segmented filamentous bacteria in the cecum were observed, increasing the risk of onset of various diseases, including inflammatory (BIEDERMANN et al., 2013; TOMODA et al., 2011; WANG, H. et al., 2012).

The beneficial results, in BMD and BMC, observed with dietary supplementation of the prebiotic, mannan oligosaccharide, may result from its contribution to maintaining pathogens at bay, as this food supplement contains mannose residues and is known to inhibit the adhesion of many enteric pathogens, including Salmonella and E. coli (GANNER; SCHATZMAYR, 2012), which enables the colonization of beneficial lactic bacteria, mainly *Bifidobacterium* and *Lactobacillus* (GIBSON, G. R.; ROBERFROID, 1995) and; consequently, leads to lowering of the pH of the intestinal bolus (FERKET, 2002), as a result of stimulated production of lactic acid and short chain fatty acids, particularly propionate and acetate, and at a lower level, but at a higher rate, butyrate (LEVRAT et al.; 1991), increasing the solubility of the minerals, improving the integrity of the intestinal mucosa, and increasing the crypt depth of the distal colon (WEAVER et al., 2011), as well as increasing the expression of binding proteins to Ca2+. In addition, this functional food may promote the release of bone modulating factors,

Table 3 - Thickness (μm) of the diaphysis of the three femur regions of rats fed with diets supplemented or not with probiotics, prebiotics, or symbiotics and exposed or not for 189 days to passive smoking.

Treatments	Regions		
	Proximal	Medial	Distal
C	589.30 ± 46.50^{D}	580.90 ± 59.44^{BCD}	558.35 ± 80.16^{BCD}
Pro	632.83 ± 59.44^{E}	614.59 ± 120.12^{DE}	582.86 ± 57.67^{CD}
Pre	649.83 ± 65.52^{E}	596.55 ± 96.39^{CDE}	588.36 ± 162.25^{CD}
Sym	623.88 ± 57.26^{E}	626.11 ± 62.84^{E}	608.36 ± 99.49^{D}
CS	440.29 ± 49.50^{A}	504.93 ± 100.21^{A}	510.78 ± 77.85^{A}
ProS	501.30 ± 59.60^{B}	544.04 ± 78.00^{AB}	551.90 ± 80.99^{BC}
PreS	554.09 ± 67.12^{C}	554.30 ± 114.68^{B}	550.31 ± 66.10^{AB}
SymS	516.94 ± 38.34^{B}	561.24 ± 53.11^{BC}	591.87 ± 73.44^{CD}

The values correspond to the mean ± SD. Means followed by the same letter in the column do not differ from each other, using the Tukey test (P> 0.05). [*]C: control group (standard diet); Pro: standard diet + 2 g probiotic [*Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium thermophilum,* and *Bifidobacterium longum (*2–5 10^9 UFC each)]; Pre: standard diet + 1 g prebiotic (active fraction, α-1,3 and α-1,6 derived from a mannanoligosaccharide, presenting 30% α-mannans, a derivative of the yeast strain *Saccharomyces cerevisiae*); Sym: symbiotic group (standard diet + 2 g probiotic + 1 g prebiotic); CS: control group + + smoking protocol (SP); ProS: probiotic group + SP; PreS: prebiotic group + SP; SymS: symbiotic group + SP.

enhancing the degradation of phytates by probiotic bacterial enzymes, resulting in improved bowel health and maximization of nutrient absorption, as well as improving the health of the host in general (SCHOLZ-AHRENS et al., 2007).

Among the compounds of cigarette smoke, studies have observed that nicotine may cause deleterious effects on the chemical composition and organization of bone micro architecture (ELSHAWARBI et al., 2014; RODRÍGUEZ-MARTÍNEZ; GARCÍA-COHEN, 2002), since it has a vasoconstrictor effect and entails the reduction in nutrient supply. In addition, nicotine also causes increased levels of IL-1 and tumor necrosis factor (TNF), which exert an indirect effect on osteoblasts and osteoclasts (NORAZLINA et al., 2007). The stimulation of osteoclast formation, through the proliferation of its precursors in the bone marrow and activation of pro-osteoclastogenic activity in stromal cells, interrupt the normal balance of formation, leading to increased bone resorption (ELSHAWARBI et al., 2014) and the inhibition of osteogenesis (FUNG et al., 1999). In addition, the direct cellular effects of smoking on bone include alterations in calciotropic hormone metabolism (KRALL; DAWSON-HUGHES, 1999) and may decrease the activity
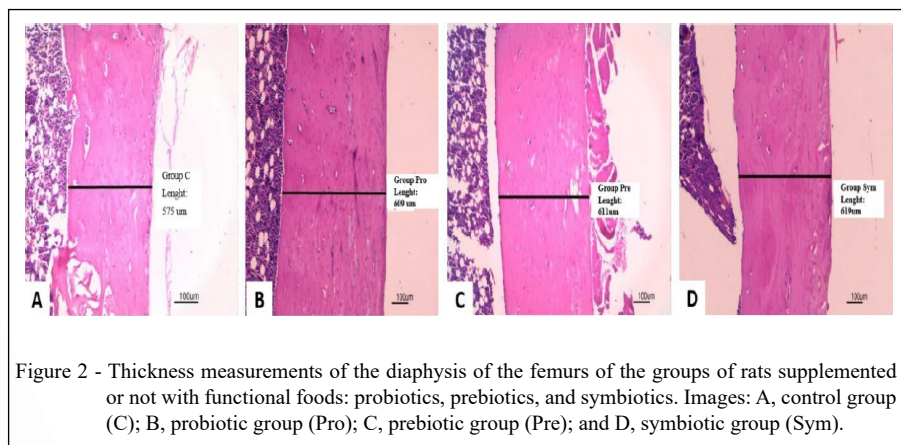

Figure 2 - Thickness measurements of the diaphysis of the femurs of the groups of rats supplemented or not with functional foods: probiotics, prebiotics, and symbiotics. Images: A, control group (C); B, probiotic group (Pro); C, prebiotic group (Pre); and D, symbiotic group (Sym).
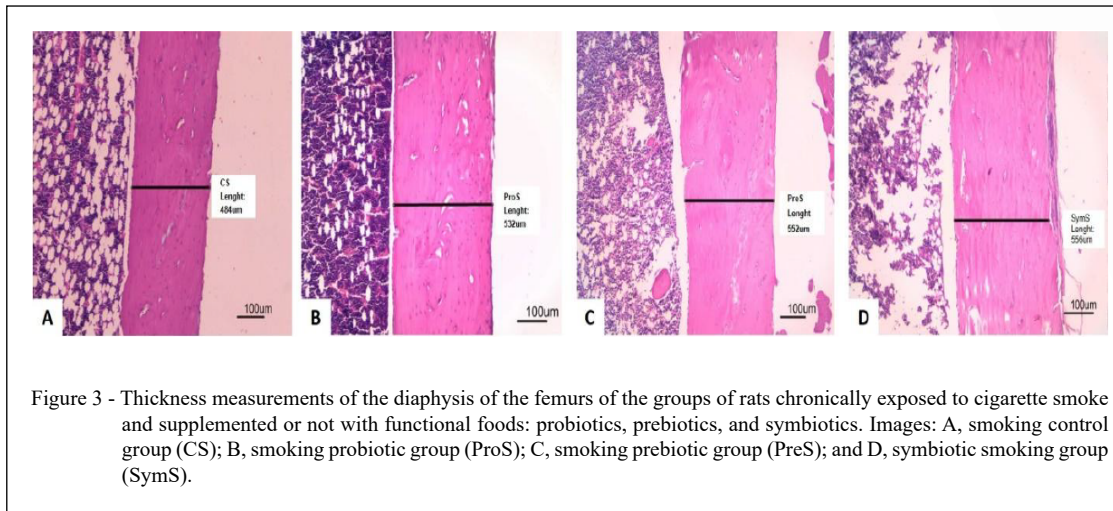
Figure 3 - Thickness measurements of the diaphysis of the femurs of the groups of rats chronically exposed to cigarette smoke and supplemented or not with functional foods: probiotics, prebiotics, and symbiotics. Images: A, smoking control group (CS); B, smoking probiotic group (ProS); C, smoking prebiotic group (PreS); and D, symbiotic smoking group (SymS).

of osteoblasts (RAPURI et al., 2000). FUNG et al. (FUNG et al., 1999)reported that nicotine (active ingredient of cigarettes) reduced vitamin D storage and osteoblast activity in humans.

A significant decrease in the area of the diaphysis and parameters that quantify bone resistance (maximum strength), resilience (energy), and concentration of minerals, phosphorus, calcium, and magnesium have also been reported in rats exposed to cigarette smoke (ELSHAWARBI et al., 2014) and are probably due to the effect of nicotine on the expression of the sialoprotein gene, a protein expressed by osteoblasts and having functions in osteogenic mineralization (NASH; PERSAUD, 1989).

Supplementation with functional foods (probiotics, prebiotics, and symbiotics) in the diet resulted in a significant increase in the area of the diaphysis, stiffness, bone resistance, and concentration of the minerals phosphorus, calcium, and magnesium in the femurs of rats both of the groups exposed or not to cigarette smoke. Other studies reported that some functional foods have acted beneficially in smokers and nonsmokers through modulation of the gut, decreased proinflammatory interleukins IL-1 and TNF-α (DADDAOUA et al., 2007), and the composition and activity of the microbiome (zebra fish, rodents, and chickens) as well as in humans (DAI et al., 2015; DEMIGNÉ et al., 2008; GIBSON et al., 2017; MCCABE et al., 2015; PARVANEH et al., 2015; RODRIGUES et al., 2012; STRÁNSKÝ; RYŠAVÁ, 2009; ŚWIATKIEWICZ et al., 2010); although, using other prebiotics and probiotics. In addition, functional foods can act on several local and systemic responses: reduction in the inflammatory response in the intestine, blood, and

bone; increased levels of SCFA metabolites, which in turn increase calcium uptake and local signaling in the gut and bone; increased secretion of bacterial factors and intestinal hormones such as incretins and serotonin which are known regulators of bone density, promoting decreased osteoclast activity and/ or increased osteoblast activity, leading to structural modulation and increased bone density and strength (MCCABE et al., 2015).

CONCLUSION

Results allowed us to conclude that supplementation with functional foods, probiotics (*Lactobacillus acidophilus*, *Enterococcus faecium*, *Bifidobacterium thermophilum*, and *Bifidobacterium longum*), prebiotics (mannan oligosaccharide), and symbiotics (a combination of probiotic and prebiotic) reduced the detrimental toxic effects of chronic exposure to passive smoking in relation to mineral composition, histomorphometric parameters, and biomechanical properties of the femur of rats in the growth phase, in an experimental model. Dietary supplementation with functional foods, probiotics, prebiotics, and symbiotics in the groups not exposed to cigarette smoke improved the femoral bone health: composition, resistance, resilience, and diaphysis area.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation

of data; in the writing of the manuscript, and in the decision to publish the results.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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