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CLAUDIA CARDOSO NETTO

AVALIAÇÃO DAS CARACTERÍSTICAS MORFOLÓGICAS E DOS MARCADORES BIOQUÍMICOS RELACIONADOS À HOMEOSTASE DO TECIDO ÓSSEO DE RATAS IDOSAS SUPLEMENTADAS COM DIFERENTES TIPOS DE PREBIÓTICOS

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CLAUDIA CARDOSO NETTO

Nutricionista

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Orientador: Prof. Dr. Mário Roberto Maróstica Júnior

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CLAUDIA CARDOSO NETTO

Aprovado em ___/___/___

Banca Examinadora

_____

Prof. Dr. Mário Roberto Maróstica Júnior
Faculdade de Engenharia de Alimentos – UNICAMP
Orientador

_____

Profa. Dra. Gláucia Maria Pastore
Faculdade de Engenharia de Alimentos – UNICAMP
Membro

_____

Prof. Dr. Jaime Amaya Farfán
Faculdade de Engenharia de Alimentos – UNICAMP
Membro

_____

Profa. Dra. Lilia Zago F. Santos
Faculdade de Nutrição – PUC Campinas
Membro

_____

Profa. Dra. Roberta Roesler
Natura Inovação e Tecnologia de Produtos LTDA
Membro

Banca Examinadora (continuação)

_____

Prof. Dr. Valdemiro Carlos Sgarbieri
Faculdade de Engenharia de Alimentos – UNICAMP
Suplente

_____

Prof. Dr. Miguel Arcanjo Areas
Instituto de Biologia – UNICAMP
Suplente

_____

Profa. Dra. Geórgia Álvares de Castro
Kraft Foods Inc.
Suplente

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RESUMO GERAL

Prebióticos são oligossacarídeos não digeríveis (ONDs) seletivamente fermentados pelas bactérias intestinais e agem como agentes bifidogênicos melhorando a saúde e o bem estar do hospedeiro. Os átomos de carbono das moléculas de hexosil fermentados são transformados em ácidos graxos de cadeias curtas (AGCC) e, uma vez absorvidos na mucosa intestinal, estes são metabolizados pelos enterócitos, células hepáticas e musculares. O interesse primário em relação aos AGCC tem sido relacionado à sua função colônica, especialmente o butirato. Porém, esses AGCC também estão relacionados a outros benefícios em relação a saúde do hospedeiro, como por exemplo, podem aumentar a absorção intestinal de minerais e conseqüentemente reduzir a perda de massa óssea. Como a perda de massa óssea é um processo fisiológico associado ao envelhecimento animal, o objetivo deste trabalho foi avaliar se ratas idosas com 10 (dez) meses de idade suplementadas com diferentes tipos de prebióticos (fructooligossacarídeos, inulina e galactooligossacarídeos) apresentavam redução dessa perda de massa óssea. Foram realizadas as seguintes análises: densitometria óssea (DXA) do fêmur e mandíbula; morfometria e propriedades biomecânicas do fêmur; parâmetros estáticos da histomorfometria e microscopia eletrônica de varredura da epífise proximal da tíbia; dosagem sérica de hormônio da paratireóide (PTH), osteocalcina (OC), osteoprotegerina (OPG), fator receptor ativador nuclear κappa β ligante(RANk-L), fragmentos de colágeno tipo I (CTX-I), atividade da fosfatase alcalina (FA) total e cálcio total. Os resultados foram analisados em ANOVA ($p<0,05$) através do software GraphPad versão 6.0 e as diferenças estatísticas determinadas através do teste de Tukey ou t de Student. Todos os protocolos experimentais foram aprovados pelo Comitê de Ética para Experimentação Animal da

Universidade Estadual de Campinas. Os experimentos foram iniciados com ratas Wistar de dez meses de idade, visto que estas apresentaram alterações significativas no metabolismo ósseo associadas à idade quanto comparadas às ratas jovens, como por exemplo, aumento no nível sérico de PTH. O consumo de prebióticos promoveu a redução da perda de massa óssea fisiológica associada ao envelhecimento, porém, outros estudos precisam ser realizados com a finalidade de descrever os mecanismos de ação.

Palavras-chave: Prebióticos; frutanos do tipo inulina; galactooligossacarídeos; metabolismo ósseo; osteoporose, envelhecimento.

ABSTRACT

Prebiotics are non-digestible oligosaccharides (NDO) selectively fermented by intestinal bacteria that are bifidogenic agents with health benefit on the host associated. The carbon atoms of the fermented hexosyl moiety are recovered in the short-chain fatty acids (SCFA) and once absorbed, they are metabolized by cells of cecocolonic epithelium, liver cells and muscle cells. The primary interest in SCFA has been related with colonic function as a result of their uptake and metabolism by colonocytes, specifically butyrate. Although, SCFA are also described with other benefits properties as improving intestinal mineral absorption and consequently reducing bone loss. As bone loss during later life is a physiologic mechanism, the objective of this study was to evaluate if different kinds of prebiotics attenuate age related bone loss in female rats. Old female rats (10 month-old) were treated with different kinds of prebiotics (Fructooligosaccharides, Inulin and Galactooligosaccharides). Femur and mandible bone mass was measured using dual-energy X-ray absorptiometry. Femur quality was examined using morphometry and diaphysis biomechanic properties (proportional limit, rupture limit, resilience, rigidy and tenacity) using three-point bending. Histomorphometry static parameters and scanning electron microscopy images were taken on tibiae methaphysis cancellous bone. The biochemical assays were serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa β ligand (RANk-L), C-terminal peptides of type I collagen (CTX-I), alkaline phosphate activity (ALP) and total calcium. All data were analyzed by two-way ANOVA ($p<0,05$) using GraphPad software version 6.0 and the significant differences were determined with Tukey's test or Student t-test. All the protocol experimental designs were approved by Animal Experimental Ethics Committee of the

University of Campinas. Our experimental were taken with aged female Wistar rats that showed a satisfactory model to evaluate age related changes on bone metabolism as well as to study osteoporosis and their mechanisms. The consumption of prebiotics preserved bone mass and reduced bone turnover. Despite our prebiotics results had been positive, more studies are required to establish mechanisms of action.

Keywords: Prebiotics; fructans type inulin; galactooligosaccharides; bone metabolism; osteoporosis; aging.

SUMÁRIO

INTRODUÇÃO GERAL

Os prebióticos são oligossacarídeos não digeríveis seletivamente fermentados pelas bactérias intestinais e que agem como agentes bifidogênicos, uma vez que promovem mudanças específicas na composição e/ou na atividade dessas bactérias, conferindo efeitos de bem estar e saúde ao hospedeiro (ROBERFROID, 2007).

Porém, segundo ROBERFROID (2007), nem todos os carboidratos são considerados prebióticos e critérios claros precisam ser estabelecidos para classificar um ingrediente alimentar como um prebiótico. Estes critérios são: resistência à acidez gástrica, hidrólise enzimática e absorção gastrointestinal; fermentação pela microflora intestinal; e estímulo seletivo ao crescimento e/ou atividade dessas bactérias intestinais que contribuem para a saúde e bem-estar.

Atualmente, seguindo esses critérios analisados *in vitro* e *in vivo*, ROBERFROID (2007) destaca que somente dois tipos de oligossacarídeos são considerados prebióticos, os fructooligossacarídeos e os galactooligossacarídeos (GOS). Os fructooligossacarídeos são oligômeros de frutose que são compostos de 1-kestose (GF2), nistose (GF3) e frutofuranosil nistose (GF4), em que as unidades de frutosil (F) são ligadas na posição β-2,1 da sacarose, o que os distingue de outros oligômeros (YUN, 1996). São oligossacarídeos de ocorrência natural e, principalmente, de origem vegetal, entretanto, também podem ser obtidos por ação de enzimas microbianas (HARTEMINK et al. 1997). Os GOS são sintetizados enzimaticamente pela ação da β-galactosidase sobre a lactose, esta enzima age como uma enzima hidrolítica e também como uma enzima de condensação, neste caso denominada reação de transgalactosilação, e podem ser compostos de di-, tri-, tetra- ou de pentassacarídeos,

consistindo principalmente em unidades de galactose ligadas à lactose (SANTOS, 2006).

Quanto aos benefícios fisiológicos as reações ocorridas durante a fermentação dos FOS e dos GOS são de tal intensidade que o valor do pH é reduzido (7,0-7,5 para 6,0-6,5) devido à produção de ácidos graxos de cadeias curtas, tais como: acetato, propionato, butirato e lactato (ROBERFROID et al. 1993; MÁRQUEZ, 1999). Esses ácidos graxos são parcialmente utilizados como fonte de energia pelos enterócitos e o restante é absorvido, promovendo efeitos metabólicos na prevenção ou mesmo controle de doenças sistêmicas, conforme descrito por WONG et al. (2006).

Dentre os efeitos metabólicos promovidos pelos produtos da fermentação dos FOS e dos GOS, podemos destacar o aumento da absorção de minerais (ex: cátions como o cálcio e o magnésio) bem como os efeitos benéficos no tecido ósseo (SCHOLZ-AHRENS et al. 2001; ROBERFROID, 2002; ROBERFROID, 2007).

Dessa forma, visto que a população idosa está suscetível a alterações fisiológicas no tecido ósseo, e considerando a importância do mesmo para a saúde desta população, este trabalho teve como objetivo avaliar as características morfológicas e dos marcadores bioquímicos relacionados à homeostase do tecido ósseo de ratas idosas suplementadas com diferentes tipos de prebióticos.

REFERÊNCIAS BIBLIOGRÁFICAS

HARTEMINK, R.; VANLAERE, K.M.J.; ROMBOUTS, F.M. Growth of enterobacteria on fructooligosaccharides. **Journal of Applied Microbiology**. Oxford, v.83, n.3, p.367-74, Aug. 1997.

MÁRQUEZ, L.R. A fibra terapêutica. São Paulo: Americana de Publicações, 1999. 132p.

ROBERFROID, M.; GIBSON, G. R.; DELZENNE, N. The Biochemistry of Oligofructose, a Nondigestible Fiber: An Approach to Calculate Its Caloric Value. **Nutrition Reviews**. Baltimore, v.51, n.5, p.137-146, May 1993.

ROBERFROID, M.B. Functional foods: concepts and application to inulin and oligofructose. **The British Journal of Nutrition**. New York, v.87, Suppl. 2, p.139-143, May 2002.

ROBERFROID, M. Prebiotics: The Concept Revisited. **The Journal of Nutrition**. Philadelphia, v.137, Suppl. 2, p.830-837, Mar. 2007.

SANTOS, R. **Produção de Galactooligossacarídeo por lactase fúngica**. 2006. Dissertação (Mestrado em Ciências dos Alimentos) - Universidade de Campinas, São Paulo, Campinas.

SCHOLZ-AHRENS, K.E.; SCHAAFSMA, G.; VAN DEN HEUVEL, E.G.; SCHREZENMEIER, J. Effects of prebiotics on mineral metabolism. **The American Journal of Clinical Nutrition**, v.73, Suppl. 2, p.459S–64S, Feb. 2001.

WONG, J.M.W.; SOUZA, R.; KENDALL, C.W.C.; EMAM, A.; JENKINS, D.J.A. Colonic Health: Fermentation and short chain fatty acids. **Journal of Clinical Gastroenterology**. New York, v.40, n.3, p.235-43, Mar. 2006.

YUN, J.W. Fructooligosaccharides - Occurrence, preparation and applications. Enzyme **Microbiology and Technology**. New York, v.19, n.2, p.107-17, Aug. 1996.

CAPÍTULO 1

PREBIOTICS REVIEW: CHEMISTRY, PRODUCTION AND BIFIDOGENIC EFFECTS

Claudia Cardoso Netto[1,3], Rosângela Santos[2], Gláucia Carielo[1], Hope Weiler[3], Gláucia Pastore[2], Mario Roberto Maróstica JR[1*]

[1]Food and Nutrition Department / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[1*]Phone +55 19 3521-4078, Fax +55 19 3521-4060

E-mail: mario@fea.unicamp.br

[2]Department of Food Science / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

Prebiotics are non-digestible oligosaccharides (NDO) selectively fermented by intestinal bacteria that are bifidogenic agents with health benefit on the host associated. At present, the main NDOs used for food industry are composites by glucose, fructose, galactose and xylose. The carbon atoms of the fermented hexosyl moiety are recovered in the short-chain fatty acids (SCFA) and once absorbed, SCFA are metabolized at three major sites in the body: cells of cecocolonic epithelium, liver cells and muscle cells. There is no doubt about recognizing of the beneficial effect of prebiotics in health and then of the most promising and dynamically developing segments of food industry. As the production of these SCFA is determined by a number of factors including type of substrate and its quantity, it is necessary that more investigations regarding different kinds of prebiotics are evaluated for equivalent outcomes as soon as the consumption of its mixed.

Keywords: Prebiotics chemistry; prebiotics production; prebiotics bifidogenic effects.

1) Introduction

In the 1980s Japanese people began to realize the importance of maintaining and improving health with a gradual increase in the occurrence of lifestyle related diseases and the Government also started to pay more attention to the aging population. Concurrently, intensive studies were performed on the physiological effects of various foods and their constituents for so called 'the tertiary function of foods'. In brief, the tertiary function of foods is directly involved in the modulation of the human physiological systems such as the immune, endocrine, nerve, circulatory as well as digestive systems, while the primary and secondary functions are related to nutrition and sensory satisfaction, respectively (OHAMA et al. 2006).

In 1984 the term 'functional foods' was first assigned in the project initiated by the Ministry of Education of Japan (presently as The Ministry of Education, Culture, Sports, Science and Technology) and this concept attracted the 'health foods' industry and health-conscious consumers. The Government from Japan, however, prohibited the use of the term 'functional' because of its implication as a drug-like effect. As a result, the term 'health foods' was widely used and recognized by consumers and the concept of the 'functional foods' was ultimately integrated into the 'foods for specified health uses' (FOSHU) system (OHAMA et al. 2006; JONES, 2002).

Another similar functional food term introduced in 1989 was "nutraceutical", defined as an isolated food compound that confers health benefits including prevention and/or illness treatment (MORAES & COLLA, 2006). This compound can be consumed as an isolated nutrient or added to dietetic supplements or processed foods such as cereals, soups, beverages and herbaceous products (NEUMANN et al. 2000;

ROBERFROID, 2002). In the later situation, adding the nutraceutical ingredient can result in a functional food (BAGCHI et al. 2004).

The main nutraceutical ingredients studied until now are the prebiotics. These ingredients showed to promote some health benefits in experimental animals and humans. Some examples of the possible benefits observed thus fare include: improved colonic microflora composition and intestinal function, up regulated calcium intestinal absorption and other minerals, up regulate the gastrointestinal peptides production, improve the immunity system and resistance to infections and reduce the risk for obesity and osteoporosis (WONG & JENKINS, 2007).

2) Prebiotics

A prebiotic was first defined in 1995 as ‘‘a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health’’ (GIBSON & ROBERFROID, 1995).

After this, GIBSON et al. (2004) stated that “a prebiotic is a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota”. These authors also described that component should not be an organism or drug, but should be a substance characterized chemically.

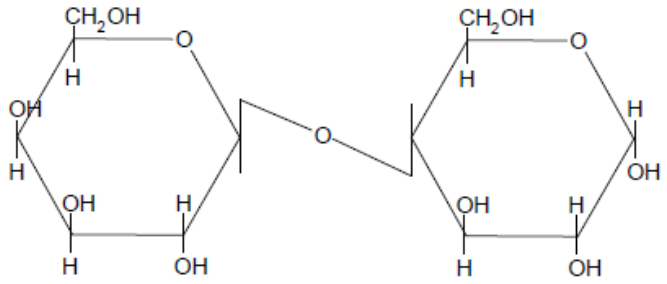
More recently, ROBERFROID (2007) described prebiotics specifically as “non-digestible oligosaccharides (NDO) selectively fermented by intestinal bacterial that are bifidogenic agents that specifically promote modulation of the microbiota through changes on its composition and/or activity, with health benefit on the host associated.

The saccharides, also called carbohydrates, are compounds naturally found in many foods, such as fruits, vegetables, milk and honey and show physiologic activity beyond taste and energy (VORAGEN, 1998).

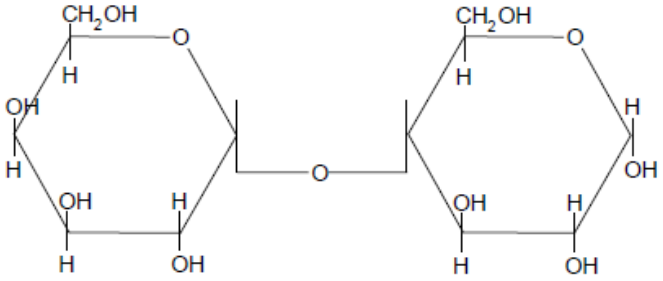
The carbohydrates are usually classified according to their molecular size or degree of polymerization (DP) into sugars, oligosaccharides and polysaccharides, with sub-groups identified by the nature of the constituent monosaccharides (MUSSATTO & MANCILHA, 2007).

The International Union of Pure and Applied Chemistry (IUPAC) classified oligosaccharide as a molecule containing a small number (2 to about 10) of monosaccharides residues, connected by glycosidic linkages. A carbohydrate containing two such residues is a disaccharide; a carbohydrate containing three such residues is a trisaccharide, and *so* on (INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY & JOINT COMMISSION ON BIOCHEMICAL NOMENCLATURE, 1982).

At the same time, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible and soluble or non-soluble (MUSSATTO & MANCILHA, 2007). The concept of non-digestible oligosaccharide (NDO) originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a β configuration that makes their osidic bonds non-digestible to the hydrolytic activity of the human digestive α enzymes (ROBERFROID & SLAVIN, 2000). In the Figure 1 are shown examples of α and β osidic bonds in disaccharides lactose molecules.



β - Lactose



α - Lactose

Figure 1. Esquematic representation of α and β osidic bonds in disaccharides lactose molecules.

The non-digestible carbohydrates can also be called dietary fiber or functional fiber. Dietary fiber consists of non-digestible carbohydrates and lignin, which are found intact in plants. Functional fiber consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in human subjects; and total fiber is the sum of dietary fiber and functional fiber (DONNELLY, 2003).

Regarding fiber solubility, the non-soluble fibers are not viscous and undergo little or no fermentation in the colon. In contrast, the soluble fibers are generally viscous and highly fermented in the colon (LOBO & SILVA, 2001). The classification of dietary fibers and functional fibers can be conferred in Figure 2.

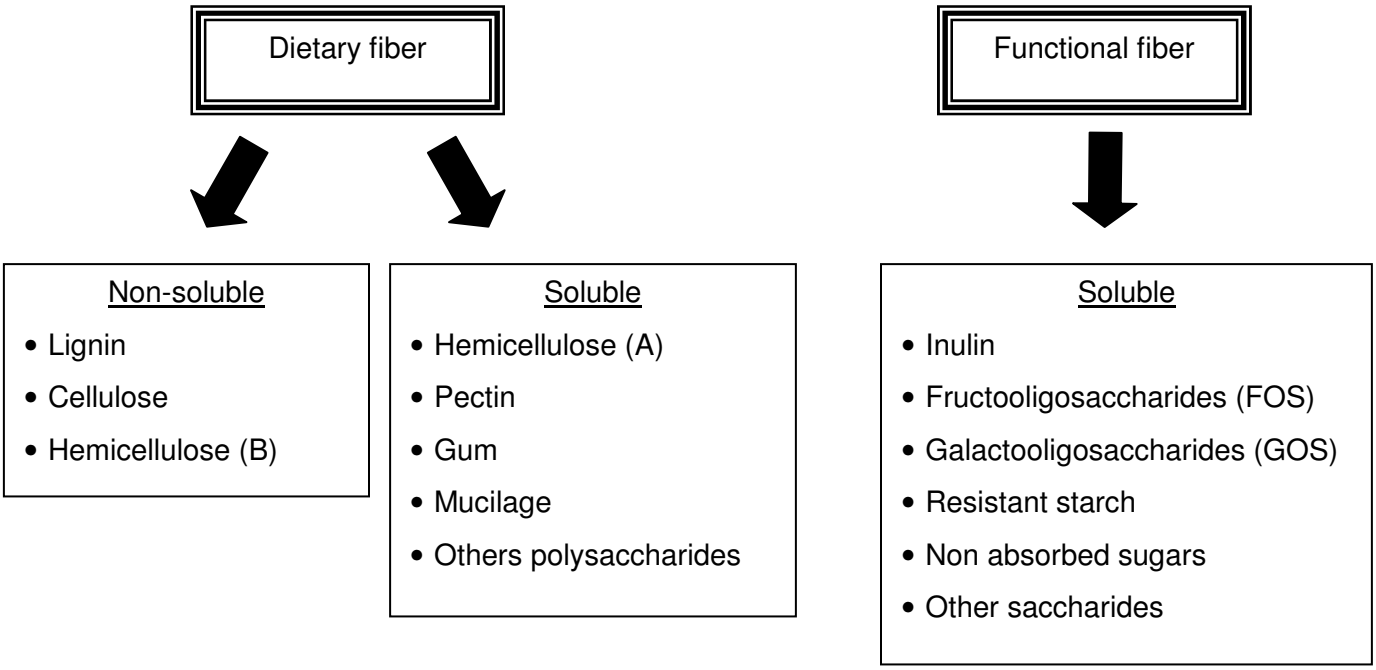


Figure 2. Dietary and functional fibers classification (Adapted from MARQUEZ, 1999).

Actually, the main NDOs used for food industry are made from one, two or even three different types of monosaccharides including glucose, fructose, galactose and xylose (MUSSATTO & MANCILHA, 2007), as shown in Figure 3.

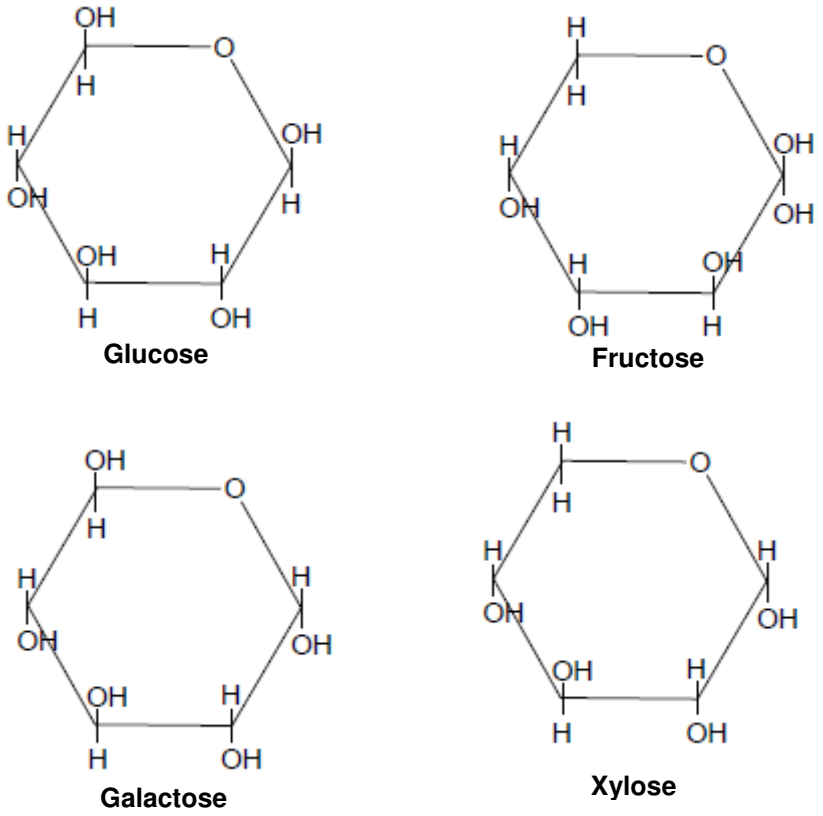


Figure 3. Chemical structure of the main monosaccharides used by food industry to make different types of non-digestible oligosaccharides (NDOs).

Many food components, especially NDOs, have been claimed to have prebiotic activity. However not all NDOs are prebiotics, and clear criteria need to be established for classifying a food ingredient as a prebiotic. These criteria observed *in vitro* and *in vivo* are: 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (ROBERFROID, 2007).

ROBERFROID (2007) still suggested that only two food ingredients fulfill these criteria, inulin-type fructans and galactooligosaccharides (GOS). The inulin-type fructans and GOS have low cost of production and are very important functional ingredients in food industry with the potential to improve the sensory properties of foods and beverages (MACFARLANE et al. 2006).

2.1) Inulin-type fructans

Chemically, inulin-type fructans are linear polydisperse carbohydrate material consisting mainly, if not exclusively, of β-(2→1) fructosyl-fructose linkages. A starting α-D-glucose moiety can be present but is not necessary. $G_{py}F_n$ (glucopyranosyl-(fructofuranosyl)$_{n-1}$-fructose) and $F_{py}F_n$ (fructopyranosyl-(fructofuranosyl)$_{n-1}$-fructose) compounds are included under that same nomenclature and they are both a mixture of oligomers and polymers that are best characterized by the degree of polymerization (DP) and expressed as either average (DP_{av}) and the maximum (DP_{max}) (ROBERFROID, 2005).

The DP of inulin-type fructans varies from 2 to more or less 60 units with DP_{av} = 12. About 10% of the fructan chains have a DP ranging between 2 (F2) and 5 (GF4). The partial enzymatic hydrolysis of inulin using an endo-inulinase (EC 3.2.1.7) produces oligofructose that is a mixture of both $G_{py}F_n$ and $F_{py}F_n$ molecules, in which the DP varies from 2 to 7 with DP_{av} = 4. When the fructans have DP_{max} of 10, they can be also called fructooligoaccharides (FOS) (ROBERFROID, 2005). The inulin-type fructans chemical structures are shown in Figure 4.

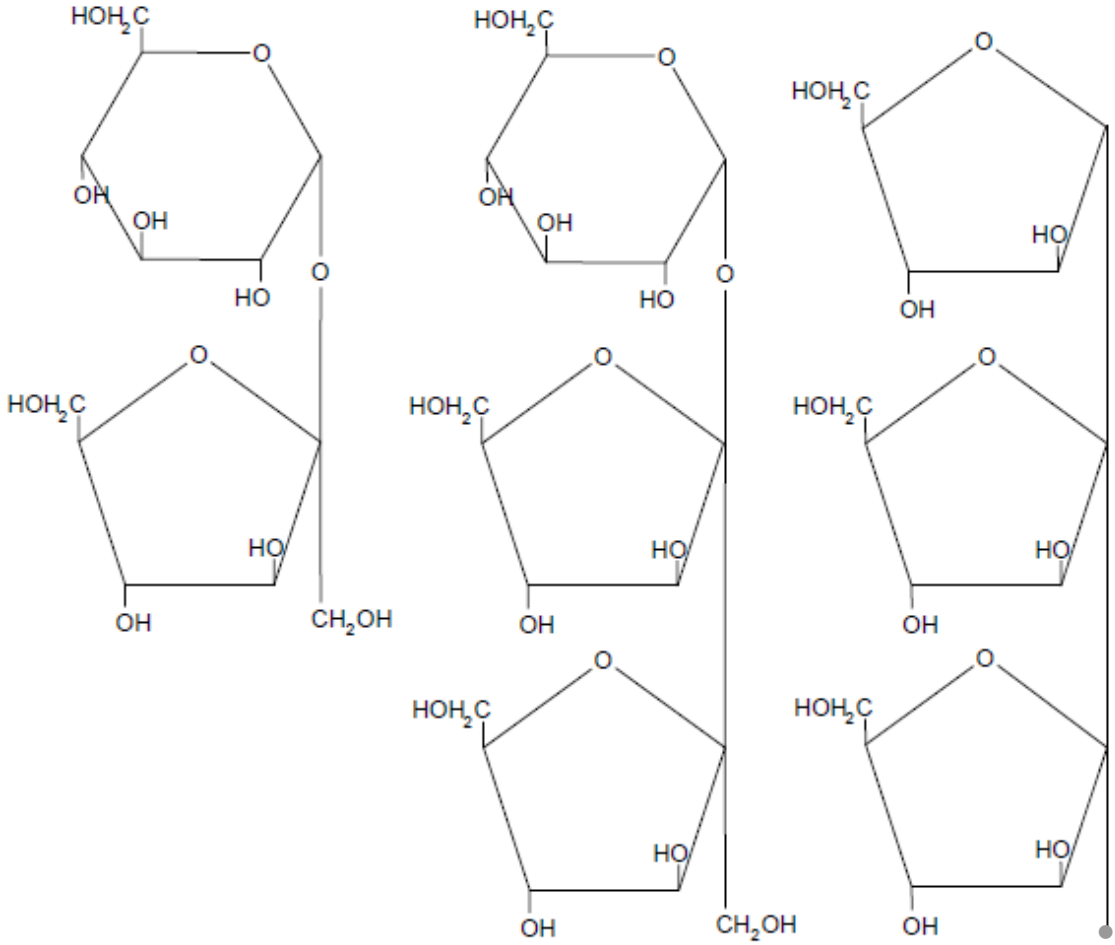


Figure 4. Chemical structures of inulin-type fructans.

Commercially, the FOS can be produced by two ways: the first one is obtained from sucrose by transfructosylation trough fructosyl transferases (FTases), which are enzymes responsible for the microbial production of FOS (SANGEETHA et al. 2005 a,b). Several microorganisms have been reported to show transfructosylation activity, especially *Aspergillus* sp. that are known to produce extracellular or intracellular FTase by aerobic submerged fermentation (YUN, 1996). Fungal strains, such as *Aspergillus*

oryzae CFR 202, *Aspergillus flavus* CFR 203 and *Aureobasidium pullullans* CFR 77 has been recognized as potential sources of FTases and their use is more feasible at industrial level (SANGEETHA et al. 2005b; SANGEETHA et al. 2004).

Parameters of aeration, pH, and temperature should be established for each microorganism to optimize production. However, conditions for the growth of FTase producing microbial cultures have been well demonstrated. Thus, sucrose is the best carbon source for both cell growth and for the enzyme activity. The pH should be above 5.5 and optimum temperature for growth of microorganisms is approximately 30°C. Some studies show that intracellular activity was enhanced with increasing concentration of Mg^{2+} (YUN, 1996; SANGEETHA et al. 2005b).

The second way to produce FOS is from inulin by controlled enzymatic hydrolysis and it consists of linear units fructosyl, with or without a final unit of glucose (SANGEETHA et al. 2005a). The only plant that has been used industrially for the extraction of inulin-type fructans belongs to the *Compositae* family, i.e. chicory. Native chicory inulin is a non-fractionated inulin extracted from fresh roots that always contain glucose, fructose, sucrose and small oligosaccharides (DELZENNE & ROBERFROID, 1994; ROBERFROID & SLAVIN, 2000; ROBERFROID, 2005).

The FOS mixture produced by this process closely resembles the mixture produced by the transfructosylation process. However, not all of the β-(2→1) linked fructosyl chains end with a terminal glucose. Additionally, the oligosaccharide mixture produced from inulin hydrolysis contains longer fructooligomer chains than the produced by the sucrose transfructosylation process (YUN, 1996; SANGEETHA et al. 2005b; MUSSATTO & MANCILHA, 2007).

The natural occurrence of inulin-type fructans and FOS are described in Table 1.

Table 1. Natural occurrence in foods of inulin-type fructans and fructooligosaccharides (FOS) (ROBERFROID et al. 1993).

Source	Inulin-type fructans (%)	FOS (%)	Scientific name
Banana	0,3-0,7	0,3-0,7	*Musa sp.*
Rye	0,5-1,0	0,5-1,0	*Secale cereale*
Leek	3-10	2,5-8,0	*Allium ampeloprasmus*
Wheat	1-4	1-4	*Triticum asetivum*
Garlic	9-16	3,5-6,5	*Allium sativum*
Chicory roots	15-20	8-11	*Cichorium intybus*
Asparagus shoot	2-3	2-3	*Asparagus officinalis*
Jerusalem artichoke	16-20	12-15	*Heliantus tuberosus*
Globe artichoke	3-10	0,3-1,0	*Cynara scolymus*
Onions	1,1-7,5	1,1-7,5	*Allium cepa*
Dandelion	12-15	9,5-12	*Taraxacum officinale*

2.2) Galactooligosaccharides (GOS)

GOS can be synthesized from lactose when this sugar acts as the acceptor and transgalactosylation is catalyzed by the enzyme β-galactosidase (EC 3.2.1.23). However, if this acceptor is a water molecule, galactose is released through a hydrolysis reaction (MARTÍNEZ-VILLALUENGA et al. 2008). The schematic diagram showing production of galactooligosaccharides can be seen in Figure 5.

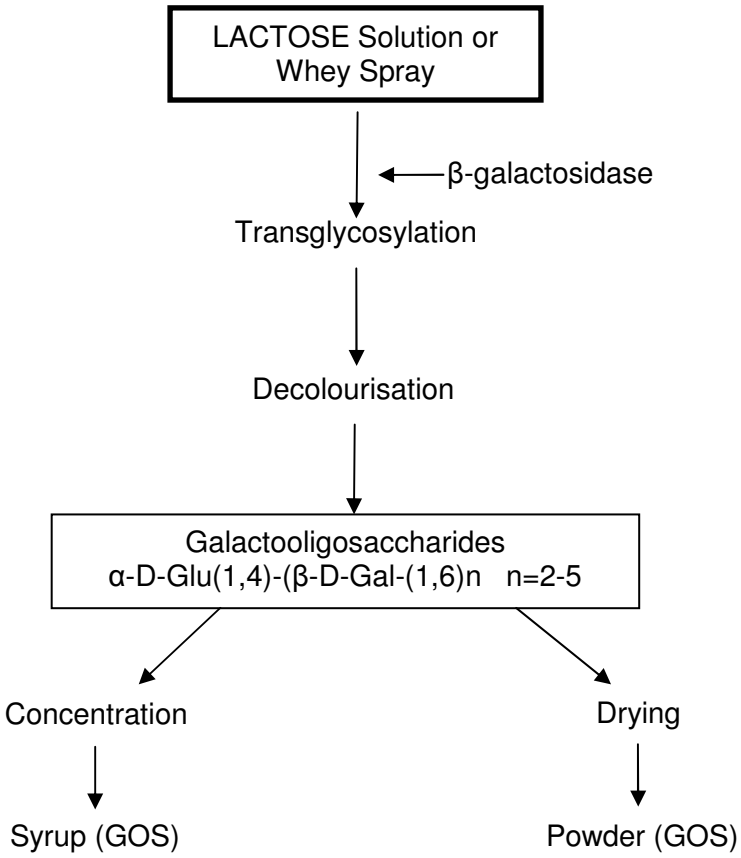
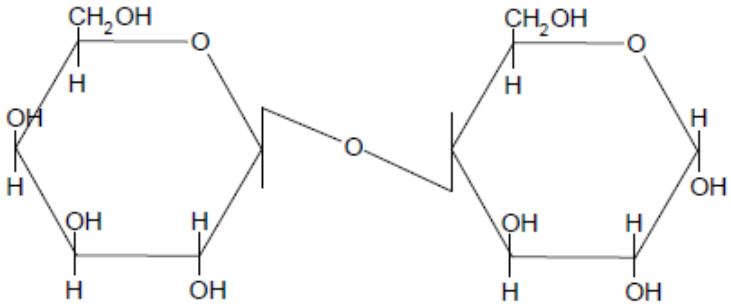
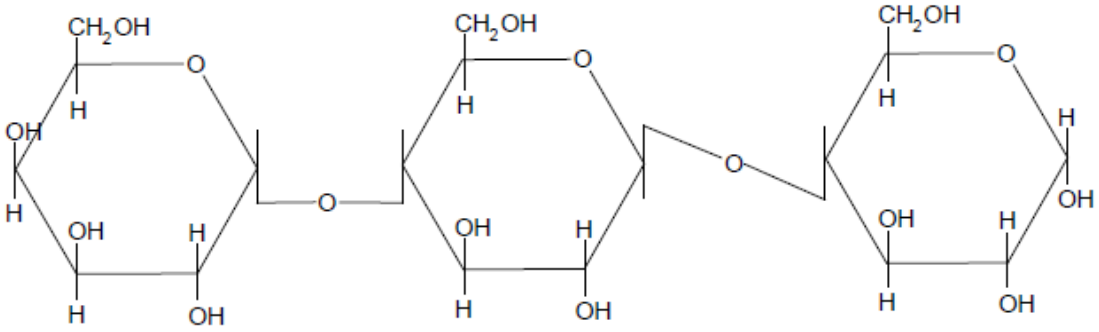


Figure 5. Schematic diagram showing production of galactooligosaccharides (Adapted from MACFARLANE et al. 2008).

The β-galactosidases present transgalactosylation activies, which results in the production of 4'- or 6'-galactosyl lactose, longer oligosaccharides. They usually contain 24-55% oligosaccharides, as di-, tri-, tetra- or pentasaccharides of galactose units linked with lactose and smaller amounts of lactose, glucose and galactose (SANTOS et al. 2009). The chemical structures of galactooligosaccharides are shown in Figure 6.



β–D-Gal*p*-(1-4)-β–D-Gl*cp*



β–D-Gal*p*-(1-4)-β–D-Gl*p*-(1-4)-β–D-Gl*cp*

Figure 6. Chemical structures of galactooligosaccharides (GOS).

The enzymes and conditions used in the production of GOS determine the various glycosidic linkages in the final products. β(1,2), β(1,3) and β(1,4) linkages and branched glucose residues occur, while (1,4) and (1,6) linkages are present in the galactan fragment (TOMOMATSU, 1994). The ratio of transferase and hydrolase activities of the enzyme affects the amount and nature of the formed oligosaccharides, since the enzyme source, the concentration and nature of the substrate and the reaction conditions (pH, temperature and time) are the main affecting factors (ONISHI et al. 1996; ALMEIDA, 2003; GAUR et al. 2006; SANTOS et al. 2009). Although hydrolysis of synthesized oligosaccharides competes with transgalactosylation, the latter can be favored at high lactose concentration, elevated temperature and lower water activity (GAUR et al. 2006).

The amount of GOS produced from lactose has also been shown to depend on the initial concentration of β-galactosidase. The resulting products can then be purified by activated carbon, filtered and concentrated before being added to foodstuffs (MACFARLANE et al. 2008).

CHO et al. (2003), for example, used a technique for purification of β-galactosidase, the enzyme was extracted from the yeast, *Bullera singularis* KCTC 7534 for the production of GOS. They used pure lactose and cheese whey permeate as substrates resulting in the oligosaccharide conversion of over 34%. The enzyme was purified by two chromatographic steps giving 96-fold purification with a yield of 16%. The optimum conditions was pH 5 and 50°C in conversion of GOS, including tri- and tetrasaccharides with a yield of 50%, corresponding to 90 g/L of GOS from 180 g/L lactose by the purified enzyme.

SANTOS (2006) extracted the β-galactosidase from *Scopulariopsis* sp, and evaluated the role of temperature, reaction time, lactose and enzyme concentrations, on GOS yields and compared with GOS obtained by a commercial enzyme produced by *Aspergillus oryzae*. The enzyme from *Scopulariopsis* sp converted 20% of lactose into oligosacharides (80.8mg/mL of 4'galactosyl-lactose); a higher GOS yield compared to β-galactosidase from *Aspergillus oryzae*, which converted 6% of lactose into oligosacharides (25.6 mg/mL of 4'galactosyl-lactose), using lactose 40% (w/v), at 45ºC, pH 5.0 and 10 U/mL, and the best reaction time was 12h of reaction.

Human milk is also a GOS source. The GOS molecule contains a large amount of galactose in the backbone structure, which is based on lactose (galactose-glucose) plus a further external galactose residue. This leads to the formation of three galactosyl-lactoses, 3', 4' and 6'-galactosyl-lactose (MACFARLANE et al. 2008).

GOS is a very attractive product to food industry as it is stable in conditions of high acidity and high temperatures, making its use possible in a wide variety of foods. It is already used as sweeteners in milky products, bread products, and drinks (SAKO, 1999).

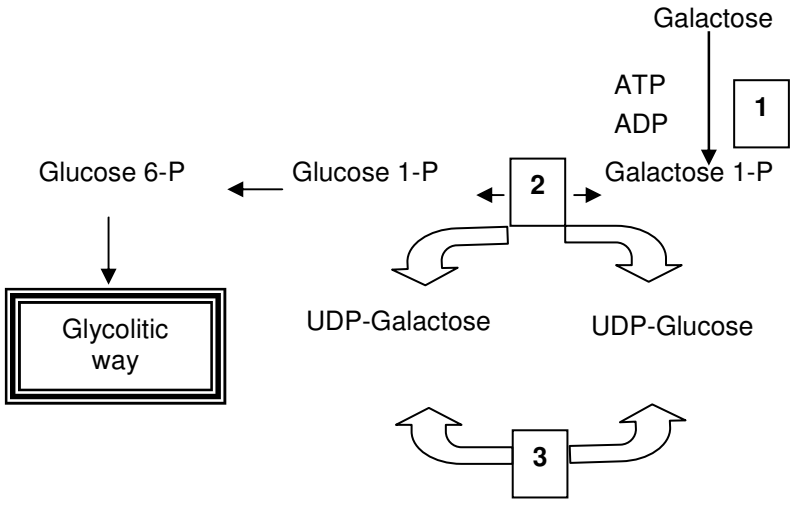
3) Prebiotics bifidogenic effects

The intestinal flora is the metabolic potential beyond the organism and produce a lot of enzymes, for example: glicosidases, ureases, decarboxilases, azorreductases, nitrorreductases and deaminases. The enzyme that presents greater activity is β-galactosidase that is present in *Lactobacillus* and *Bifidobacterium*. Moreover, *Peptostreptococcus* and *Clostridia* frequently show activity of β-glicuronidases and *Bacteroides* are characterized by activity of β-glicosidases (CAMBRODÓN & MARTÍN-CARRÓN, 2001).

The predominant saccharolytic bacteria include: *Bacteroides, Bifidobacterium, Eubacterium, Lactobacillus* and *Clostridia*. These bacteria use carbohydrates as C-source, since they have capacity to produce a variety of polyhydrolases and glycosidase (CUMMINGS & MACFARLANE, 1997). The main saccharolytic bacteria are *Bifidobacterium* that is strictly anaerobic and constitutes 25% of the total bacterial population in adult and 95% in the newborn (MODLER et al. 1990).

Once released by inulin-type fructans and GOS hydrolysis, β-D-fructose and β-D-galactose respectively, serve as substrates for the distinct metabolic pathway by which bifidobacteria oxidize carbohydrates, the so co-called *Bifidus pathways* (ROBERFROID et al. 1993).

Firstly, galactose is transformed through the activity of the enzymes galactokinase and transferase uridil-1-phosphogalactose in 1-phosphoglucose, as represented in Figure 7, and than this follows the glycolytic way normally (KOOLMAN & ROEHM, 2005).



1- Galactokinase

2- Hexose-1-phosphate uridyltransferase

3- UDP glucose 4-epimerase

Figure 7. Transformation of the galactose into glucose-6-phosphate by metabolic way (adapted of KOOLMAN & ROEHM, 2005).

Bifidobacteria do not carry out the usual glycolysis pathway or the hexose monophosphate shunt pathway, since they lack both aldolase (E.C.4.1.2.13.) and glucose-6-phosphate $NADP^+$ oxidoreductase (E.C.1.1.1.). Phosphoketolases phosphoryle of the fructose-6-phosphate (E.C.4.1.2.22) and xylulose-5-phosphate (E.C.4.1.2.9.), which produce acetylphosphate plus erythrose 4-phosphate and glyceraldehydes 3-phosphate, respectively. The pathways of anaerobic metabolism of fructose by bifidobacteria are shown in Figure 8 and 9 (ROBERFROID et al. 1993).

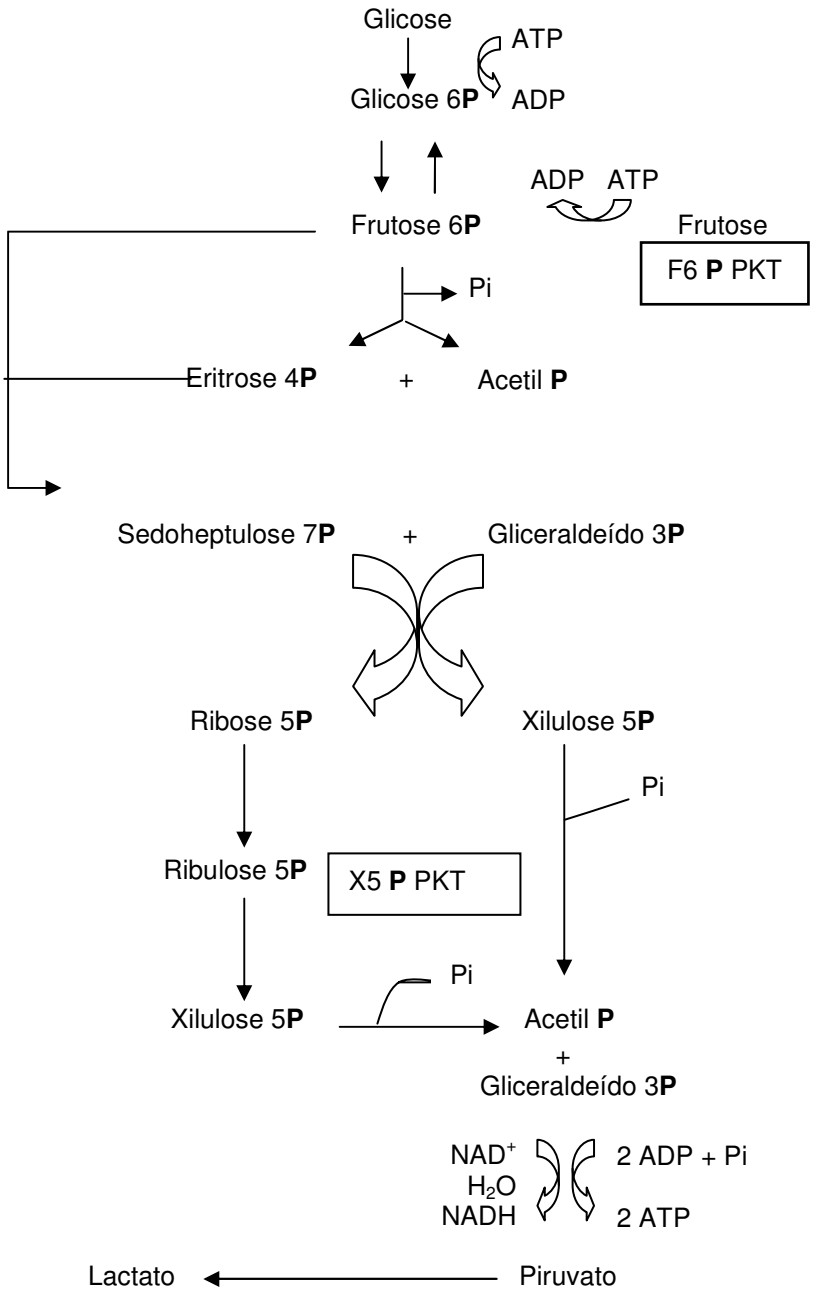


Figure 8. The *bifidus* pathway by hexose fermentation. The specific enzymes are as follow: F6 PKT = fructose 6 phosphoketolase; X5 PKT = xylose 5 phosphoketolase (ROBERFROID et al. 1993).

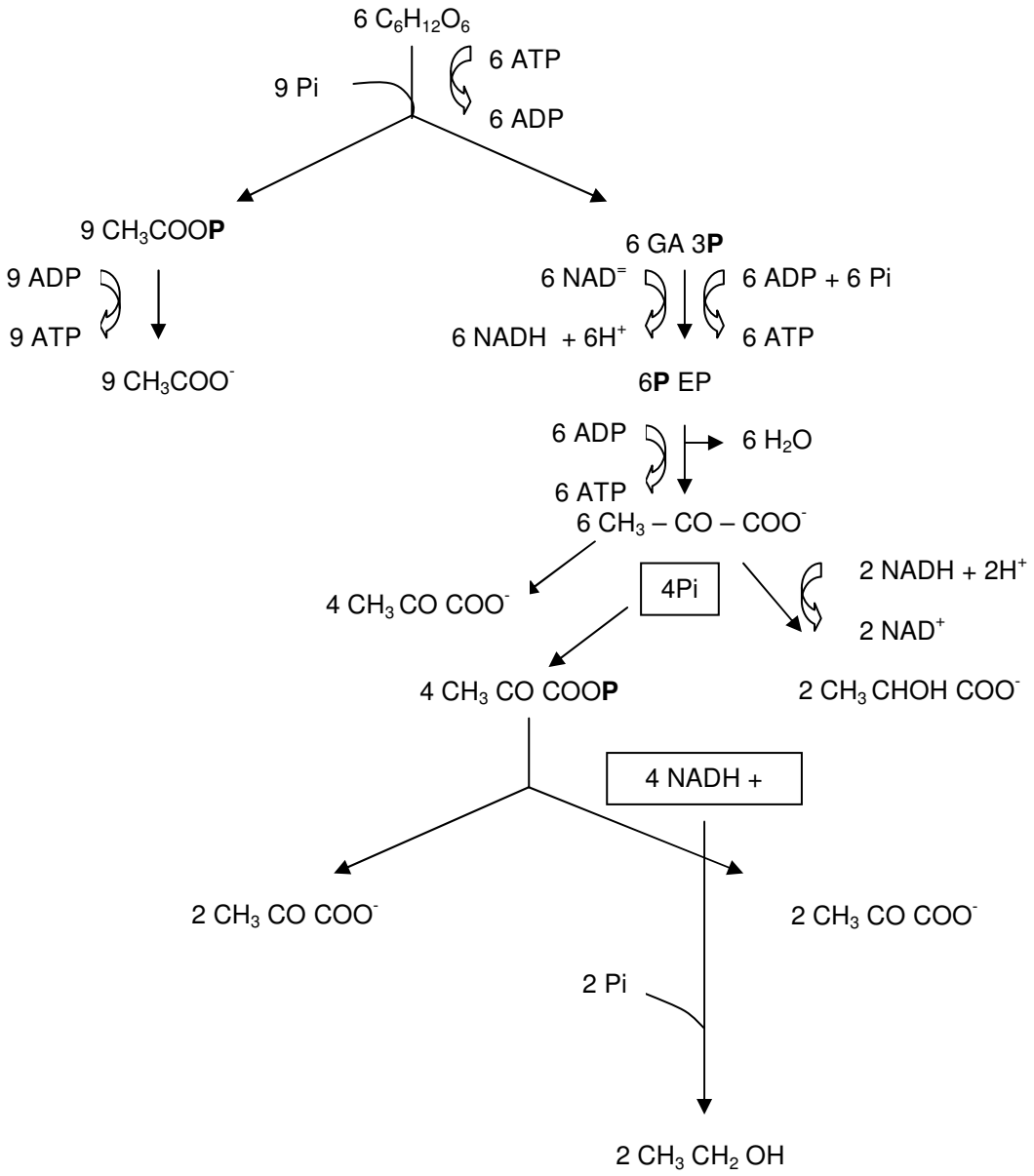


Figure 9. Bifidus pathway of anaerobic oxidative metabolism of hexose (GA 3 P = glyceraldehyde 3 P; P EP = phosphoenolpyruvate) (ROBERFROID et al. 1993).

In agreement with DE VRIES & STOUTHAMER (1968) for the stoichiometry of the bifidus pathway, the overall reaction for hexose fermentation by bifidbacteria is the following:

$$6C_6H_{12}O_6 + 17ADP + Pi \rightarrow 17\ ATP + 4H\ COO^- + 2CH_3CHOH\ COO^- + 2CH_3CH_2OH$$

However, this reaction has to be taken as an average balance for hexose metabolism by bifidobacteria. Both the absolute yield and the ratio of fermentation product formation (in particular, acetate:lactate) vary between strains. These parameters are also influenced by the nature and concentration of the substrate. Moreover, in the colon, some of the products of bifidobacteria metabolism (e.g., pyruvate and lactate) may be fermented further (ROBERFROID et al. 1993), as shown in Figure 10.

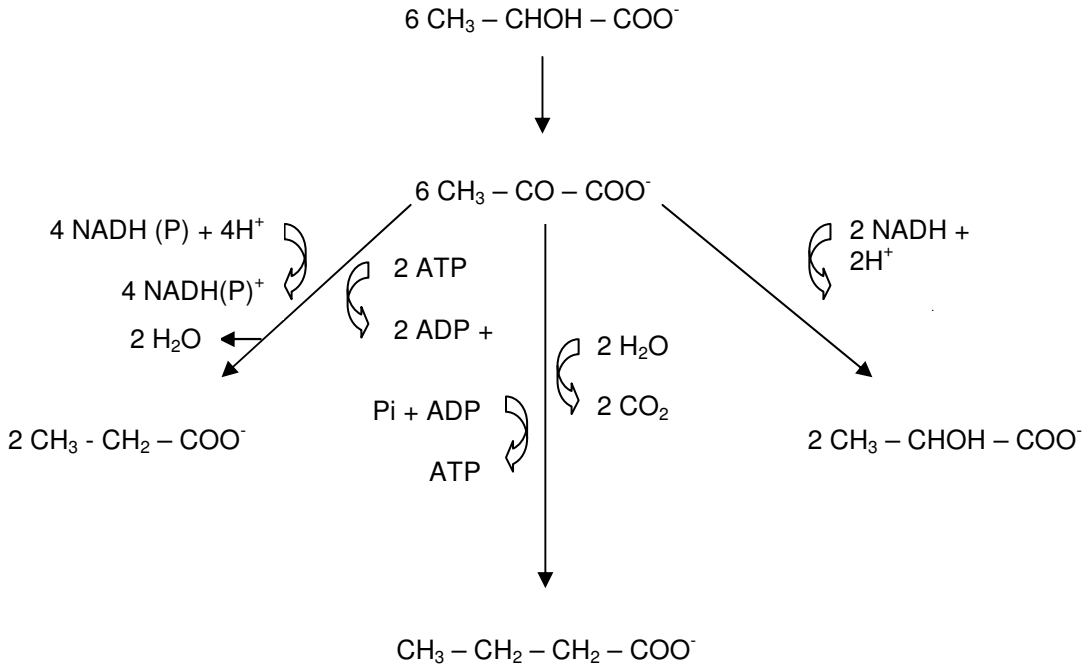


Figure 10. Microbial pathway of anaerobic metabolism of lactate/pyruvate to form short-chain fatty acids (ROBERFROID et al. 1993).

Then the equation for the fermentation of a fructosyl moiety by colonic bacteria could be (ROBERFROID et al. 1993):

$$6C_6H_{12}O_6 + 14\ ADP + 14\ Pi \rightarrow 9CH_3\,COO^- + 2\ CH_3\ CHOH\ COO^-$$
$$+ 2\ CH_3\ CH_2\ COO^- + CH_3(CH_2)_2\ COO^- + 2CO_2 + 14\ ATP + 2H_2O + 14H^+$$

Based on this equation, 78% of the carbon atoms of the fermented hexosyl moiety are recovered in the short-chain fatty acids (SCFA) acetate, propionate and butyrate, with a ratio of 64:21:15%. In addition, 17% is produced as lactate and 5% as ethanol, formate, and CO_2 (ROBERFROID et al. 1993).

In general, fecal SCFA production is in the order acetate > propionate > butyrate in a molar ratio of ~ 60:20:20, respectively. Absorption of SCFA in the cecum and the colon is a very efficient process with only 5–10% being excreted in the feces. Two proposed mechanisms of absorption are diffusion of protonated SCFA and anion exchange. Once absorbed, SCFA are metabolized at 3 major sites in the body: 1) cells of cecocolonic epithelium that use butyrate as the major substrate for maintenance-energy-producing pathways; 2) liver cells that metabolize residual butyrate with propionate used for gluconeogenesis and 50–70% of acetate is also taken up by the liver; 3) muscle cells generate energy from the oxidation of residual acetate (WONG et al. 2006; WONG & JENKINS, 2007).

The primary interest in SCFA has been in relation to colonic function as a result of their uptake and metabolism by colonocytes, specifically butyrate. Although SCFA are also metabolic substrates for other tissues of the host as soon as composition and

activities of the gut microflora, stool production, absorption of calcium and other minerals, production of gastrointestinal endocrine peptides, immunity and resistance to infections lipid homeostasis, reduction of disease risks, intestinal infections, irritable bowel diseases, colon cancer, obesity and osteoporosis (WONG & JENKINS, 2007; ROBERFROID, 2007).

4) Conclusion

There is no doubt about recognizing of the beneficial effect of prebiotics in health and then of the most promising and dynamically developing segments of food industry. However, the physiological traits attributed to prebiotics may be caused by metabolites produced in the caeco-colon by intestinal bacteria fermentation, as SCFA. As the production of these SCFA is determined by a number of factors including type of substrate and its quantity, it is necessary that more investigations regarding different kinds of prebiotics are evaluated for equivalent outcomes resulting from the consumption of mixture of them.

5) References

ALMEIDA, M. M. **Síntese de galactooligossacarídeo por β-galactosidase de *Scopulariopsis sp* a partir da lactose**. 2003. Tese (Doutorado em Ciências de Alimentos) – Universidade Estadual de Campinas, São Paulo, Campinas.

BAGCHI, D.; PREUSS, H. G.; KEHRER, J. A. Nutraceutical and functional food industries: aspects on safety and regulatory requeriments. **Toxicology Letters**. Amsterdam, v.150, n.1, p.1-2, Apr. 2004.

CAMBRODÓN, I. G.; MARTÍN-CARRÓN, N. **Fibra dietética en Iberoamérica: Tecnología y salud**. São Paulo: Varela, 2001.

CHO, Y. J.; SHIN, H. J.; BUCKE, C. Purification and biochemical properties of a galactooligosaccharide producing beta-galactosidase from *Bullera singularis*. **Biotechnology Letters**. Dordrecht, v.25, n.24, p.2107-2111, Dec. 2003.

CUMMINGS, J. H.; MACFARLANE, G. T. Role of intestinal bacteria in nutrient metabolism. **Journal of Parenteral and Enteral Nutrition**. California, v.21, n.6, p.357-365, Nov-Dec. 1997.

DE VRIES, W.; STOUTHAMER, A. H. Fermentation of glucose, lactose, mannitol and xylose by bifidobacteria. **Journal of Bacteriology**. Washington, v.96, n.2, p.472-478, Aug. 1968.

DELZENNE, N.; ROBERFROID, M. B. Physiological effects of nondigestible oligosaccharides. **Lebensm-Wiss Technology**. Amsterdam, v.27, n.1, p.1–6, Sep. 1994.

DONNELLY, B. National Academies of Science definitions relating to food fibre only add confusion. **The British Journal of Nutrition**. New York, v.90, n.2, p.481-483, Aug. 2003.

GAUR, R.; PANT, H.; JAIN, R.; KHARE, S.K. Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. **Food Chemistry**. Barking, v.97, n.3, p.426-430, Aug. 2006.

GIBSON, G. R.; ROBERFROID, M. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. **The Journal of Nutrition**. Bethesda, v.125, n.6, p.1401–1412, Jun. 1995.

GIBSON, G. R.; PROBERT, H. M.; VAN LOO, J.; RASTALL, R. A.; ROBERFROID, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. **Nutrition Research Review**. New York, v.17, n.2, p.259–275, Dec. 2004.

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY & JOINT COMMISSION ON BIOCHEMICAL NOMENCLATURE. Abbreviated terminology of oligosaccharide chains. **Journal of Biology and Chemistry**. Baltimore, v.257, n.7, p.3347–3351, Apr. 1982.

JONES, P. J. Functional foods — more than just nutrition. **Canadian Medical Association Journal**. Toronto, v.166, n.12, p.1555-1563, Jun. 2002.

KOOLMAN, J.; ROEHM, K. H. **Color Atlas of Biochemistry.** 2nd ed. New York: Thieme Stuttgart, 2005.

LOBO, A. R.; SILVA, G. M. L. Implicações nutricionais no consumo de fibras e amido resistente. **Nutrição em Pauta**. São Paulo, v.1, p.28-30, Jan-Fev. 2001.

MACFARLANE, G. T.; STEED, H.; MACFARLANE, S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. **Journal of Applied Microbiology**. Oxford, v.104, n.2, p.305–344, Feb. 2008.

MACFARLANE, S.; MACFARLANE, G. T.; CUMMINGS, J. H. Review article: prebiotics in the gastrointestinal tract. **Alimentary pharmacology & therapeutics**. Oxford, v.24, n.5, p.701-714, Sep. 2006.

MÁRQUEZ, L. R. **A fibra terapêutica**. São Paulo: Americana de Publicações, 1999.

MARTÍNEZ-VILLALUENGA, C.; CARDELLE-COBAS, A.; CORZO, N.; OLANO, A. VILLAMIEL, M. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 LHPG). **Food Chemistry**. Barking, v.107, n.1, p.258-264, Mar. 2008.

MODLER, H. W.; MCKELLAR, R. C.; YAGUCHI, M. Bifidobacteria and bifidogenic factors. **Canadian Institute Food Science and Technology Journal**. Ontario, v.23, n.1, p.29-41, Sep. 1990.

MORAES, F. P.; COLLA, L. M. Functional foods and nutraceuticals: definition, legislation and health benefits. **Electronic Pharmacy Journal**. Goiás, v.3, n.2, p.99-112, Jan. 2006.

MUSSATTO, S. I.; MANCILHA, I. M. Non-digestible oligosaccharides: A review. **Carbohydrate Polymers**. Barking, v.68, n.3, p.587–597, Apr. 2007.

NEUMANN, A. I. C. P.; ABREU, E. S.; TORRES, E. A. F. S. Alimentos saudáveis, Alimentos funcionais, Fármaco-alimentos, Nutracêuticos...: Você já ouviu falar neles? **Higiene Alimentar**, v.14, n.71, p.19-23, Fev. 2000.

OHAMA, H.; IKEDA, H.; MORIYAMA, H. Health foods and foods with health claims in Japan. **Toxicology**. Limerick, v.221, n.1, p.95–111, Apr. 2006.

ONISHI, N.; KIRA, I.; YOKOZEKI, K. Galacto-oligosaccharide production from lactose by *Sirobasidium magnum* CBS6803. **Letters in Applied Microbiology**. Oxford, v.23, n.4, p.253-256, Oct. 1996.

ROBERFROID, M.; SLAVIN, J. Nondigestible oligosaccharides. **Critical Review in Food Science and Nutrition**. Philadelphia, v.40, n.6, p.461–480, Nov. 2000.

ROBERFROID, M.; GIBSON, G. R.; DELZENNE, N. The Biochemistry of Oligofructose, a Nondigestible Fiber: An Approach to Calculate Its Caloric Value. **Nutriton Reviews**. Washington, v.51, n.5, p.137-146, May. 1993.

ROBERFROID, M. B. Functional food concept and its application to prebiotics. **Digestive and Liver Disease**. Amsterdam, v.34, Suppl.2, p.105-110, Sep. 2002.

ROBERFROID, M. B. Introducing inulin-type fructans. **The British Journal of Nutrition**. New York, v.93, Suppl.1, p.13–25, Apr. 2005.

ROBERFROID, M. B. Prebiotics: The Concept Revisited. **The Journal of Nutrition**. Bethesda, v.137, Suppl. 2, p.830-837, Mar. 2007.

SAKO, T. Recent progress on research and applications of non-digestible galactooligosaccharides. **International Dairy Journal**. Barking, v.9, n.1, p.69-80, Jul. 1999.

SANGEETHA, P. T.; RAMESH, M. N.; PRAPULLA, S. G. Production of frutooligosaccharides by fructosyl transferase from Aspergillus oryzae CFR 77. **Process Biochemistry**. Barking, v.39, p.753-758, Jul. 2004.

SANGEETHA, P. T.; RAMESH, M. N.; PRAPULLA, S. G. Maximization of fructooligosaccharide production by two stage continuous process and its scale up. **Journal of Food Engineering**. London, v.68, n.1, p.57–64, May. 2005a.

SANGEETHA, P. T.; RAMESH, M. N.; PRAPULLA, S. G. Recent trends in microbial production, analysis and application of Fructooligosaccharides. **Trends in Food Science & Technology**. Cambridge, v.16, n.10, p.442–457, Oct. 2005b.

SANTOS, R. **Produção de Galactooligossacarídeo por lactase fungica**. 2006. Dissertação (Mestrado em Ciências dos Alimentos) - Universidade de Campinas, São Paulo, Campinas.

SANTOS, R.; SIMIQUELI, A. P. R.; PASTORE, G. M. Produção de galactooligossacarídeos por *Scopulariopis* sp. **Revista de Ciência e Tecnologia de Alimentos** (*in press*), 2009.

TOMOMATSU, H. Health effects of oligosaccharides. **Food Technology**. Chicago, v.48, n.10, p.61–65, Oct. 1994.

VORAGEN, A. G. J. Technological aspects of functional food-related carbohydrates. **Trends in Food Science & Technology**. Cambridge, v.9, n.8, p.328–335, Aug. 1998.

WONG, J. M. W.; JENKINS, D. J. A. Carbohydrate digestibility and metabolic effects. **The Journal of Nutrition**. Bethesda, v.137, Suppl.11, p.2539-2546, Nov. 2007.

WONG, J. M. W.; SOUZA, R.; KENDALL, C. W. C.; EMAM, A.; JENKINS, D. J. A. Colonic Health: Fermentation and short chain fatty acids. **Journal of Clinical Gastroenterology**. New York, v.40, n.3, p.235-243, Mar. 2006.

YUN, J. W. Fructooligosaccharides: occurrence, preparation and application. **Enzyme and Microbial Technology**. New York, v.19, n.2, p.107-117, Aug. 1996.

CAPÍTULO 2

CALCIUM METABOLISM AND ITS AGE-RELATED ALTERATIONS

Claudia Cardoso Netto[1,3], Gláucia Maria Pastore[2], Hope Weiler[3] and Mário Roberto Maróstica JR[1*]

[1]Food and Nutrition Department / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[1*]Phone +55 19 3521-4078, Fax +55 19 3521-4060

E-mail: mario@fea.unicamp.br

[2]Department of Food Science / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

Calcium is an essential element in all organisms and participates in a variety of structural and functional roles ranging from the formation and maintenance of the skeleton to the temporal and spatial regulation of neuronal function and inhibition of proliferation of cancer cells. The Ca^{2+} metabolism in humans involves different physiologic systems including: ingestion, digestion, intestinal transit (trans and paracellular absorption and fecal excretion), renal turnover, and bone remodeling. During bone remodeling for growth, bone formation exceeds bone resorption. From the age of 30 to about 50 years, the amount of bone formed approximately equals the amount resorbed. From the time of the menopause in women and perhaps later in men, bone resorption exceeds bone formation. The mass of bony tissue present at any time during adult life is the difference between the amount accumulated, i.e. the so-called peak bone mass, and that lost with aging. Pathogenetic factors favoring the osteoporotic process include those impairing bone mass accumulations during growth and the accelerated bone loss during later life. As many factors can be related bone loss during later life, the objective is to describe the Ca^{2+} metabolism and its age-related alterations.

Keywords: Aging; calcium metabolism; intestinal calcium absorption; bone metabolism; calcium renal metabolism.

1) Introduction

Calcium (Ca) in its elemental form has two electrons in the valence shell. When in solution, Ca looses these electrons giving yield to the ionized form (Ca^{+2}) and this eletrostatic property is responsible for the behavior of Ca in aqueous solutions and biological processes (WOOD, 2000). Calcium is thus an essential ion in all organisms and participates in a variety of structural and functional roles ranging from the formation and maintenance of the skeleton to the temporal and spatial regulation of neuronal function and inhibition of proliferation of cancer cells (KHANAL & NEMERE, 2008a).

The typical calcium content of the adult human is 1 kg and located primarily in the skeleton with the remaining tissues accounting for <1% of the total. It is thus obvious that almost all calcium that is ingested and retained resides in the skeleton (BRONNER & PANSU, 1999).

Intracellular Ca^{2+}, particularly cytosolic free Ca^{2+}, is an important second messenger and cofactor for proteins and enzymes, regulating key cellular processes such as neurotransmission, motility, hormonal secretion and cellular proliferation. Extracellular Ca^{2+} is an integral part of the mineral phase of the bone, act as a cofactor for adhesion molecules, clotting factors and other proteins and regulates neuronal excitability (KHANAL & NEMERE, 2008a).

The dietary reference intakes for calcium across the life span were described by Food and Nutrition Board (YATES et al. 1998) and are shown below in Table 1.

Table 1. The calcium dietary reference intake for life stage group (YATES et al. 1998).

Life stage group	RDA[1]/AI[2] (mg/d)	UL[3] (mg/d)
Infants		
0-6 months	210	ND[4]
7-12 months	270	ND[4]
Children		
1-3 years	500	2500
4-8 years	800	2500
Males		
9-18 years	1300	2500
19-50 years	1000	2500
>51 years	1200	2500
Females		
9-18 years	1300	2500
19-50 years	1000	2500
>51 years	1200	2500
Pregnancy		
≤18 years	1300	2500
>19 years	1000	2500
Lactation		
≤18 years	1300	2500
>19 years	1000	2500

[1]RDA=Recommended Dietary Allowances
[2]AI=Adequate Intakes
[3]UL=Upper Intake Level
[4]ND=No Detected

While these values are under revision, as much as seventy percent of dietary calcium intake is obtained from milk and milk products, while 16% of green vegetables and dry fruits and 6% of the water (including mineral water) (GUEGUEN & POINTILLART, 2000).

2) Calcium (Ca^{2+}) metabolism

The Ca^{2+} metabolism in mammals involves different physiologic systems as: ingestion, digestion, intestinal transit (transcellular and paracellular absorption and fecal excretion), renal turnover and bone remodeling (BRONNER & PANSU, 1999; GUEGUEN & POINTILLART, 2000; CASHMAN, 2002), as showed in Figure 1.

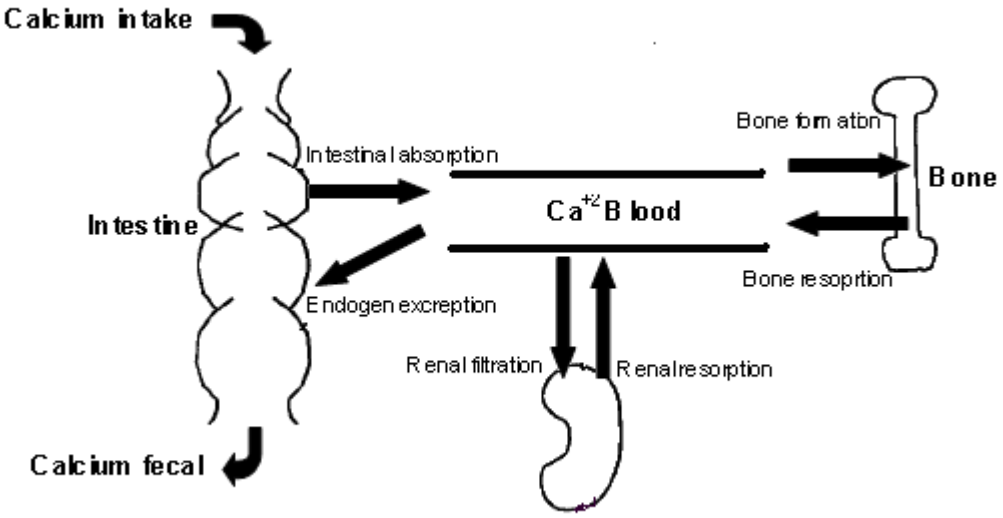

Figure 1. Schematic diagram of calcium metabolism.

2.1) Ca^{2+} epithelial absorption

The two distinct processes involved in the Ca^{2+} absorption of across epithelia are transcellular and paracellular transport. The transcellular transport occurs predominantly in the duodenum and kidney by movement across the plasma membrane of the cell, requires energy and involves three steps: entry across the cell wall, diffusion through the cytoplasm and exit at the basolateral cell pole. Entry of Ca^{2+} into the epithelial cells at the brush border membrane is the first of three steps associated with this route. Ca^{2+} is postulated to enter the epithelial cells via selective Ca^{2+} channels at the luminal membrane under the influence of steep, inwardly directed electrochemical gradient. Entry of Ca^{2+} into the cell is considered as the rate-limiting step, with epithelial Ca^{2+} channels the gatekeepers. It is possible that Ca^{2+} may also enter the cells using transporter protein pumps, such as H^+/Ca^{2+} and Na^+/H^+, or by co-transport with appropriate anions (KHANAL & NEMERE, 2008a).

These Ca^{2+} channels are represented by a protein family called “transient receptor potential” (TRP). The mammalian TRP protein family can be divided into seven families; TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, and TRPN. The TRPC (‘Canonical’) and TRPM (‘Melastatin’) subfamilies consist of seven and eight different channels, respectively (i.e. TRPC1–TRPC7 and TRPM1–TRPM8). The TRPV (‘Vanilloid’) subfamily presently comprises six members (TRPV1–TRPV6). The TRPML (‘Mucolipin’) family comprises three members, and the TRPP (‘Polycystin’) family three channel-like and five non-channel members, respectively. These families are not sufficiently characterized, but it is gain increasing interest because of their involvement in several human diseases. The most recently proposed subfamily, TRPA (‘Ankyrin’), has only one mammalian member, TRPA1, and finally, the TRPN (no mechanoreceptor

potential C, or NOMPC) has so far only been detected in *Caenorhabditis elegans*, *Drosophila*, and zebra fish (PEDERSEN et al. 2005).

The Ca^{2+} influx channels of the TRP family compromise all the TRPCs, all TRPVs, TRPM1, 2, 3, 6, 7, and 8, TRPA1, TRPP2, 3, and 5 and TRPML1, 2, and 3. The permeability ratios *P*Ca/*P*Na for these channels vary considerably, ranging from 0.3 for TRPM2 to >100 for TRPV5 and TRPV6 (PEDERSEN et al. 2005).

Three models have been proposed to explain subcellular routes for transcellular Ca^{2+} movement across the cell. The first to be conceived was diffusion through the cytoplasm, also known as facilitated diffusion, the second is the vesicular transport model and the third could be tunneling through intracellular stores. Facilitated diffusion proceeds with the involvement of the 'cytoplasmic' proteins calbindin D9K and calbindin D28K, to ferry the cation from one end of the cell to the other (BELLATON et al. 1992; CHOI & JEUNG, 2008; KHANAL & NEMERE, 2008a).

The efflux of Ca^{2+} at the serosal side of the cell occurs against a considerable electrochemical gradient, but is not considered a rate-limiting step. Two Ca^{2+} transporters have been identified at the basolateral membrane (BLM) of absorptive epithelia to extrude Ca^{2+}: the plasma membrane Ca^{2+}-ATPase (PMCA) and Ca^{2+} transport ATP-ase (SERCA) within sarco/endoplasmic reticulum. Both types possess a similar kind of activity towards Ca^{+2} and their function is based on a similar mechanism. As a result, Ca^{+2} transport is connected with hydrolysis of ATP (POLAK-JONKISZ et al. 2007).

The paracellular transport allows direct exchange of Ca^{+2} between two compartments; epithelia consist of a continuous layer of bipolar cells. Between the two adjacent epithelial cells lies a tight junction characterized by a specialized membrane

domain at the apical region. Movement of Ca^{2+} through these tight junctions is called paracellular transport. It is primarily passive, non-saturable mechanism that is driven by Ca^{2+} concentration, mineral solubility, electrical gradient across the epithelium, permeability and integrity of tight junctions (KHANAL & NEMERE, 2008a).

The paracellular route is a concentration-dependent diffusion of the ion that takes place throughout the length of the intestine and does not depend on energy. When Ca^{2+} intake is low (<20 mM), the epithelial transcellular transport in the duodenum accounts for approximately 80% of the Ca^{2+} absorbed, but its contribution to overall Ca^{2+} epithelial transport is minor when Ca^{2+} intake is high (50 mM) (PANSU et al. 1981; BELLATON et al. 1992; BRONNER & PANSU, 1999; KHANAL & NEMERE, 2008a; KHANAL & NEMERE, 2008 b).

The physiologic aging process in mammalian animals' can lead to a downregulation on Ca^{2+} epithelial absorption trans and/or paracellular (AMBRECHT, 1990; KINYAMU et al. 1997; COUDRAY et al. 2006).

VAN ABEL et al. (2007), for example, investigated the Ca^{+2} transcellular epithelial transport through of the expression of genes encoding the Ca^{+2} transport proteins in the aged mice kidney and intestine using quantitative real-time PCR. They showed a downregulation of the renal and duodenal TRPV5, TRPV6 and calbindin-D28K in aging. Moreover, TRPV5$^{-/-}$ mice develop age-related hyperparathyroidism and osteoporotic characteristics earlier compared with control mice, demonstrating the importance of these epithelial Ca^{+2} channels in Ca^{+2} homeostasis. BRONNER & PANSU (1999) described that alkaline solutions lower Ca^{2+} solubilization and associate with a higher Ca^{2+} precipitation and consequently a bigger epithelial excretion when compared with acid solutions. Thus, as the Ca^{2+} epithelial paracellular transport occurs mainly on small

intestine and it is pH dependent, age related achlorhydria impair the absorption of this micronutrient (GARIBALLA & SINCLAIR, 1998; GARIBALLA, 2004).

2.2) Bone metabolism

Bone plays several important functions including: protection against trauma, locomotion, represent hematopoietic and endocrine functions and regulate the calcium phosphate reservoir. It is a specialized form of connective tissue composed by cells (osteoblasts, osteoclasts and osteocytes) and an organic matrix mineralized (proteins and inorganic elements) by the deposition of calcium phosphate. This gives rigidity and strength to the skeleton together with some elasticity. Morphologically, there are two forms of bone: cortical or compact, and cancellous or spongy (WORLD HEALTH ORGANIZATION, 2003; LEE et al. 2007).

Osteoblasts are bone-forming cells that originate from local mesenchymal stem cells (bone marrow stroma or connective tissue mesenchyme), which undergo proliferation and differentiate to pre-osteoblasts and then to mature osteoblasts (TRIFFITT, 1996). The osteoblast transport systems located in the plasma membrane are responsible for the transfer of bone mineral ions, mainly calcium and phosphate, from the extracellular space of the bone marrow to the osteoid layer (CAVERZASIO & BONJOUR, 1996). Towards the end of the production of the bone matrix and the deposition of mineral ions, the osteoblasts become either flat lining cells or osteocytes (NIJWEIDE et al. 1996).

Osteocytes are embedded in a mineralized matrix, residing individually in caves called lacunae. These cells are stellateshaped or dendritic cells that communicate with each other and with osteoblasts and lining cells on the bone surface via their prominent

cell processes within tunnels called canaliculi, thereby forming a neuron-like network throughout the skeleton (TATSUMI et al, 2007).

Osteoclasts are giant cells that originating from haematopoietic stem cells, probably of the mononuclear/phagocytic lineage (SUDA et al. 1996), and they are found in contact with the calcified bone surface (also known as resorptive lacunae) that result from their resorptive activity. Osteoclastic resorption takes place at the cell/bone interface in a sealed-off microenvironment. The mechanism of bone resorption involves the secretion of hydrogen ions and proteolytic enzymes into the sub-osteoclastic resorbing compartment. The hydrogen ions dissolve the bone minerals, thereby exposing the organic matrix to the proteolytic enzymes (BARON, 1996; TEITELBAUM et al. 1996). These enzymes, which include collagenases and cathepsins, are responsible for the breakdown of the organic matrix. The process releases the minerals that contribute to calcium and phosphate homeostasis (EYRE, 1996; GARNERO & DELMAS, 1998).

The organic matrix (proteins) accounts for approximately 35% of the total dry weight of bone. Approximately 90% of this matrix consists of bone-specific collagen; the remainder consists of non-collagenous proteins, such as osteonectin, osteocalcin (formerly referred to as bone Gla protein), osteopontin and bone sialoprotein. The matrix proteins are synthesized and laid down by osteoblasts (YOUNG, 2003).

The mineral component of bone accounts for about 65% of its total dry weight. Chemically, it is predominantly hydroxyapatite, ($Ca_{10}(PO_4)_6(OH)_2$). Other constituents, such as carbonates, citrate, magnesium, sodium, fluoride and strontium, are either incorporated into the hydroxyapatite crystal lattice or adsorbed on to the surface. Some

substances, e.g. bisphosphonates, have a special affinity for bone mineral (FLEISCH, 1997).

Bone remodelling maintains the mechanical integrity of the skeleton by replacing old bone with new. Bone resorption and bone formation occur at the same place, so that there is no change in the shape of the bone. This constant process of turnover enables the skeleton to release calcium phosphate whenever the net intestinal absorption of this mineral is less than the amount excreted in urine (BROADUS, 1996).

The morphological dynamic structure of turnover is the “basic multicellular unit” (BMU), also called the “bone remodelling unit” (BRU). The morphological entity formed when the process is terminated is called the “bone structural unit” (BSU) (PARFITT, 1992) and it corresponds to a “packet” in cancellous bone, and to an osteon in cortical bone.

The integrity of the skeletal system (cortical and cancellous bone) is maintained by a continuous remodeling process, where bone resorption by osteoclasts is followed by bone formation by osteoblasts. Further, the recruitment and activity of osteoclasts and osteoblasts are under hormonal, neuronal, immunological and mechanical control (MANOLAGAS, 1995; TATSUMI et al. 2007).

Osteoclasts differentiate from the monocyte/macrophage lineage under the stimulation of two essential cytokines, macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa β ligand (RANkL), and through the coordinated actions of nuclear transcription factors, such as c-Fos, nuclear factor of activated T cells c1 (NFATc1), and nuclear factor of activated kappa B cells (RANk) (TEITELBAUM & ROSS, 2003). The generation and death of osteoclasts are also kept

in check by local and systemic negative regulators, most notably osteoprotegerin (OPG), a decoy receptor of RANKL, and estrogen, respectively (BOYLE et al. 2003).

However, several other cytokines and colony-stimulating factors have been implicated in osteoclast development. This list includes the interleukins (IL-1, IL-3, IL-6, IL-11), tumor necrosis factor (TNF), granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), leukemia inhibitory factor (LIF), and stem cell factor (SCF) (MANOLAGAS, 1995; TEITELBAUM, 2004; KOBAYASHI et al. 2005). Moreover, transforming growth factor-β (TGF- β) has been shown an inhibitor of osteoclast activity and, incidentally, is a powerful enhancer of osteoclast apoptosis (MUNDY et al. 1993).

Osteoclastic bone resorption is followed by the recruitment of osteoblasts. The osteoblasts actively synthesize extracellular matrix, represented by type I collagen, followed by mineralization. Bone formation and resorption are not independent processes, but are in fact mutually and intimately linked in that osteoblastic/stromal cells provide an osteoclastogenic microenvironment through presentation of RANKL and in that the bone formation by osteoblasts depends on the preceding resorption by osteoclasts through the action of a putative ‘‘coupling factor’’ (MARTIN & SIMS, 2005; TATSUMI et al. 2007).

The main cytokines and colony-stimulating factors that have been implicated in osteoblast development are interleukins (IL-1ra, IL-4, IL-10, IL-18), interferon-γ, transforming growth factor β (TGF-β), platelet-derived growth factors (PDGF), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), the heparin-binding fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs). Presumably,

they undergo apoptosis through negative *feedback* or by tumor necrosis factor-α (TNF-α) produced by their neighborhood cells (MUNDY et al. 1993; MAUGERI et al. 2005).

During bone remodeling for growth, bone formation exceeds bone resorption. From the age of 30 to about 50 years, the amount of bone formed approximately equals the amount resorbed. From the time of the menopause in women and perhaps later in men, bone resorption exceeds bone formation. The mass of bony tissue present at any time during adult life is the difference between the amount accumulated, i.e. the so-called peak bone mass, and that lost with ageing. Pathogenetic factors favoring the osteoporotic process are those impairing bone mass accumulations during growth and that accelerating bone loss during later life (WORLD HEALTH ORGANIZATION, 2003).

Despite that bone turnover occurs via a similar mechanism for cancellous and cortical bone, the cost of remodeling is approximately 25% and only 3%, respectively (RIGGS & MELTON, 1995), thus, in woman bone loss during later life is higher in cancellous than in cortical bone.

RIGGS et al. (1981) described that bone mineral density of the vertebrae in women declines linearly at a rate of approximately 0.0092 g/cm^2 per year during the age span of 30 to 96 years. Moreover, MELTON et al. (1989) indicated that this decline is increased in the periods after menopause leading to an average bone loss of approximately 30% at age 70. Such loss in turn is associated with a 30% life-time risk for vertebral fractures. ENSRUD et al. (1995) measured the rate of bone loss in 5698 women of 65 years or older. He reported an average rate of bone loss of 2.5 mg/cm^2/year at an age of 67±69 years and an increase to 10.4 mg/cm^2/year at an age of

over 85. Additionally CUMMINGS & NEVITT (1989) suggested that at an age of 80 years most women will have had at least one partial fracture of a vertebra.

Owing to the severe consequences of bone loss culminating in fractures, animal models of bone loss have been established to facilitate development of interventions. LI et al. (1991), for example, observed in Sprague-Dawley female rats that the peak bone mass is maintained without significant changes until 8-months of age. Thereafter, bone mass begins to decrease with aging and reaches a low value of 14% of the metaphyseal tissue area at 12 months of age indicating that loss is from primarily trabecular bone. YAMAGUCHI et al. (1989) affirmed this observation when they described that the femur diaphysis calcium content did not decrease appreciably in female older rats (age up 28 week-old) when compared with female young rats (3 week-old). Likewise, AKKUS et al. (2004) did not observe a statistical (p<0.05) difference on rupture limit of Sprague-Dawley female rats with 3-month old (young group), 8-month old (middle-age) and 24-month old (older), but did observe an age related reduction on proportional limit between older and younger groups. Furthermore the middle-age group proportional limit was 52% and 63% greater than the younger and older groups, respectively. In addition KIEBZAK et al. (1988) did not observe a statistical (p<0.05) change in the maximum breaking force required to fracture femurs with senescence.

Beyond bone densitometry, morphometry and biomechanics measurements, some biochemical assays may be useful in risk assessment the skeletal turnover age related too. KIEBZAK et al. (1988) showed that serum osteocalcin (OC) concentration decreased progressively from 6 to 12-months old (21%) and from 12 to 24-months old (23%) in female rats. These results suggested that the bone remodeling shows age related down regulation. Further, IIDA & FUKUDA (1993) also showed a total ALP

activity decreased up to 9-months old in Wistar female and male rats while VIEIRA (2003) and YOSHIKUBO et al. (2005) described an increase of total ALP activity in conditions where the bone remodeling is increased. KHOSLA et al. (2002) showed serum OPG levels increased with age in both men and women, moreover they still described that these elevation in OPG in older population can be explained occurring as a compensatory phenomenon to slow down the enhanced bone resorption. In addition, IIDA & FUKUDA (1993) observed that the value of serum calcium decreased up to 12 months of age, but then increased after 18 months of age in Wistar rats of the both sexes. However, KIEBZAK et al. (1988) did not find age related changes in the serum calcium concentration in female rats at 6, 12, and 24 months of age.

3) Endocrine regulation role of Ca^{+2} metabolism

3.1) Parathyroid hormone (PTH)

PTH plays a central role in Ca^{+2} homeostasis. When the extra cellular flux (ECF) of Ca^{+2} decreases, a transient fall is detected by the Ca^{+2} sensing receptor (CaR) in the parathyroid gland, leading to induction of PTH synthesis and secretion. PTH can restore ECF of Ca^{+2} to normal by stimulating renal tubular reabsorption of Ca^{+2} and renal production of 1,25-dihydroxyvitamin D_3 spell it out, and by promoting osteoclastogenesis (ANDERSON et al. 2005).

Blood PTH concentration can be regulated by Ca^{+2} doses as well as Ca^{+2} intake. KARKKAINEN et al. (2001) showed that Ca^{+2} dosages from 250 mg increased serum Ca^{+2} and downregulated circulating PTH levels. REGINSTER et al. (2002) described that the timing of Ca^{+2} intake had a negative correlation on serum PTH levels.

In the aging process, many investigators showed that blood PTH is higher in aged animals when compared with young animals (KIEBZAK et al. 1988; LIPS, 2001; MOSEKILDE, 2005).

3.2) Vitamin D: 1,25-dihydroxyvitamin D_3

Certainly the most important role for 1,25-dihydroxyvitamin D_3 is to stimulate dietary Ca^{+2} and phosphate absorption in the small intestine and renal conservation of calcium. The 1,25-dihydroxyvitamin D_3 increases intestinal Ca^{+2} absorption through a vitamin D receptor (VDR) mediating an increase in the transcription of specific genes involved in transcellular Ca^{+2} absorption (ANDERSON et al. 2005).

Moreover, vitamin D deficiency in normal animals and humans produces defects in bone mineralization, characterized by an increase in osteoid (unmineralised bone matrix protein) and impaired calcium phosphate deposition (PARFITT, 1997). Many *in vitro* studies have shown that 1,25-dihydroxyvitamin D_3 is capable of regulating osteoblast gene transcription, differentiation and mineralization. Further, 1,25-dihydroxyvitamin D_3 in association with other factors including PTH, also induces osteoclastogenesis by stimulating the differentiation of bone marrow-derived pro-myelocytes and monocytes to active osteoclasts (ABE et al. 1981; TANAKA et al. 1982). This process occurs indirectly through increased expression of the RANkL, which promotes osteoclastic differentiation (SUDA et al. 1995). EASTELL et al. (1991) showed that VDR receptors, their sensitivity and renal vitamin D hydroxylation decrease with age.

3.3) Estrogen

Estrogen deficiency in women produces a number of effects on bone and Ca^{+2} homeostasis, including increased bone resorption and formation, increased urine excretion of Ca^{+2}, and intestinal malabsorption of Ca^{+2}, resulting in significant bone loss (SCHULZ & MORRIS, 1999; KHANAL & NEMERE, 2008b).

Estrogen receptors have been detected on bone cells which are necessary for a direct effect of estrogen on the modulation of bone cell activity. This model would be expected to produce increased Ca^{+2} flux from the bone with subsequent hypercalcemic suppression of calciotropic hormones, resulting in intestinal Ca^{+2} malabsorption and increased urine Ca^{+2} excretion. Alternatively, increased bone loss in the estrogen-deficient state may occur secondary to loss of Ca^{+2} at the kidney, and/or impairment of intestinal Ca^{+2} absorption. This model is supported by the detection of estrogen receptors in both intestinal and kidney cells (SCHULZ & MORRIS, 1999).

3.4) Calcitonin

Calcitonin (CT) is a polypeptide of 32 amino acids produced by thyroidal C cells that when administered at high pharmacological doses, it triggers a hypocalcemic response that is partially mediated through an inhibition of bone resorption (HUEBNER et al. 2006).

The effects of CT are mediated through binding to a G-protein-coupled receptor (CTR). Besides its expression in epithelial cells of the kidney, the CTR has subsequently been found to be present in several other cell types, including neurons of the central nervous system, placental cells or lymphocytes. In terms of CT's effects on bone remodeling, it has been established through many experiments that the major action lies

in the inhibition of bone resorption, since osteoclasts, unlike bone-forming osteoblasts, express high levels of the CTR. However, the demonstration that this also reflects a physiological function of CT was delayed for a long time, since a human CT deficiency model has never been described, and since thyroidectomy did not lead to major changes in bone mineral density (HUEBNER et al. 2008).

GÜRKAN et al. (1986) showed the plasma levels of CT decreased at 2 weeks after castration in female rats when compared to both controls and precastration levels. High CT levels were however, observed in castrated female and male groups compared to their controls 12 weeks after the operation.

3.5) Glucocorticoids

Glucocorticoids reduce bone mineral density as a result of their impact on intestine, kidney, parthryoid and bone. Malabsorption of Ca^{2+} in the intestine or reabsorption in the kidney is believed to play an important role in the etiology of low bone mineral density. Glucocorticoids have been shown to have mixed effects on the intestinal absorption of Ca^{2+}. Whereas glucocorticoids enhanced both para- and transcellular transport in the jejunum and increased transcellular transport in colon, their effect in the duodenum (where net absorption of Ca2+ actually occurs) was largely inhibitory, suggesting, overall, that they are hypocalcaemic hormones (KHANAL & NEMERE, 2008b).

Blood glucocorticoid levels are upregulated in elderly (PERLMAN et al. 2007). Further, glucocorticoids and their chemical analogs, as prednisolone, are often used in the clinical context because of their anti-inflammatory and immunosuppressive properties (KHANAL & NEMERE, 2008b). As GARIBALLA (2004) described, since the

elderly population frequently experiences age-related inflammatory problems as immune dysregulation, high glucocorticoid use is common.

3.6) Thyroid hormone

There is some evidence available linking thyroid dysfunction to disturbances in Ca^{2+} and phosphorus homeostasis and some other disorders. The action of thyroid hormone in the intestine was to further enhance the effects of 1,25D (KHANAL & NEMERE, 2008b). It was demonstrated that both Ca^{2+} influx and efflux from enterocytes were significantly increased in hyperthyroid rats and decreased in hypothyroid rats (KUMAR & PRASAD, 2003). Similarly, thyroid hormones increased Ca^{2+} reabsorption in rat kidney (KUMAR & PRASAD, 2002). Moreover, aging is associated with alterations in the hypothalamic-pituitary-thyroidal axis which can lead to hypothyreosis (PAPIEZ et al. 2008).

4) Conclusion

From the time of the menopause in women and perhaps later in men, bone resorption exceeds bone formation and many factors can be related with this bone loss during later life, including calcium intake as well as its bioavailability. As age related alterations in calcium metabolism are physiologic mechanisms, will be interesting to establish interventions that could change these alterations and reduce bone loss.

5) References

ABE, E.; MIYAURA, C.; SAKAGAMI, H.; TAKEDA, M.; KONNO, K.; YAMAZAKI, T.; YOSHIKI, S.; SUDA, T. Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. **Proceedings of the National Academy of Sciences of the United States of America**. Washington, v.78, p.4990-4994, Aug. 1981.

AKKUS, O.; ADAR, F.; SCHAFFLER M. B. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. **Bone**. New York, v.34, p.443– 453, Mar. 2004.

AMBRECHT, H. J. Effect of age on calcium and phosphate absorption. Role of 1,25-dihydroxyvitamin D. **Mineral Electrolyte Metabolism**. New York, v.16, n.(2-3), p.159–166, Dec. 1990.

ANDERSON, P. H.; MAY, B. K.; MORRIS, H. A. Vitamin D Metabolism: New Concepts and Clinical Implications. **The Clinical Biochemistry Review**. Chippendale, v.24, n.1, p.13-26, Feb. 2003.

BARON R. Molecular mechanisms of bone resorption: therapeutic implications. **Revue du Rhumatisme (English ed.)**. v.63, n.10, p.633–638, Nov. 1996.

BELLATON, C.; ROCHE, C.; REMY, C.; PANSU, D. Calcium absorption. Recent physiological data. Dietary consequences. **Gastroentérologie Clinique et Biologique**. Paris, v.16, n.3, p.239-247, Mar. 1992.

BOYLE, W. J.; SIMONET, W. S.; LACEY, D. L. Osteoclast differentiation and activation. **Nature**. Basingstoke, v.423, n.6937, p.337-42, May. 2003.

BROADUS, A. E. Mineral balance and homeostasis. In: Favus MJ, editor. **Primer on the metabolic bone diseases and disorders of mineral metabolism**. 3rd ed. Philadelphia (PA): Lippincott-Raven,1996. p.57–63.

BRONNER, F.; PANSU, D. Nutritional aspects of calcium absorption. **The Journal of Nutrition**. Bethesda, v.129, n.1, p.9-12, Jan. 1999.

CASHMAN, K. D. Calcium intake, calcium bioavailability and bone health. **The British Journal of Nutrition**. New York, v.87, Suppl. 2, p.169-77, May. 2002.

CAVERZASIO, J.; BONJOUR, J. P. Characteristics and regulation of Pi transport in osteogenic cells for bone metabolism. **Kidney International**. New York, v.49, n.4, p.975–80, Apr. 1996.

CHAKRABORTY, T. R.; GORE, A. C. Aging-Related Changes in Ovarian Hormones, Their Receptors, and Neuroendocrine Function. **Experimental biology and medicine**. Maywood, v.229, n.10, p.977-987, Nov. 2004.

CHOI, K. C.; JEUNG, E. B. Molecular mechanism of regulation of the calcium-binding protein calbindin-D9k, and its physiological role(s) in mammals: a review of current research. **Journal of cellular and molecular medicine**. London, v.12, n.2, p.409-420, Apr. 2008.

COUDRAY, C.; FEILLET-COUDRAY, C.; RAMBEAU, M.; TRESSOL, J. C.; GUEUX, E.; MAZUR, A.; RAYSSIGUIER, Y. The effect of aging on intestinal absorption and status of calcium, magnesium, zinc, and copper in rats: A stable isotope study**. Journal of trace elements in medicine and biology**. Stuttgart, v.20, n.2, p.73–81, Dec. 2006.

CUMMINGS, S. R.; NEVITT, M. C. A hypothesis: the causes of hip fractures. **Journal of Gerontology**. Washington, v.44, n.4, p.107-11, Jul. 1989.

EASTELL, R.; YERGEY, A. L.; VIEIRA, N. E.; CEDEL, S. L.; KUMAR, R.; RIGGS, B. L. Interrelationship among vitamin D metabolism, true calcium absorption, parathyroid function, and age in women: evidence of an age-related intestinal resistance to 1,25-

dihydroxyvitamin D action. **Journal of Bone and Mineral Research**. Washington, v.6, n.2, p.125–32, Feb. 1991.

ENSRUD, K. E.; PALERMO, L.; BLACK, D. M.; CAULEY, J.; JERGAS, M.; ORWOLL, E. S.; NEVITT, M. C.; FOX, K. M.; CUMMINGS, S. R. Hip and calcaneal bone loss increase with advancing age: longitudinal results from the study of osteoporotic fractures. **Journal of Bone and Mineral Research**. Washington, v.10, n.11, p.1778-1787, Nov. 1995.

EYRE, D. R. Biochemical basis of collagen metabolites as bone turnover markers. In: Bilezikian JP, Raisz LG, Rodan GA, editors. **Principles of bone biology**. San Diego (CA): Academic Press, 1996. p.143–153.

FLEISCH, H. Bone and mineral metabolism. In: **Bisphosphonates in bone disease: From the laboratory to the patient**. 3rd ed. New York (NY): The Parthenon Publishing Group, 1997. p.11–31.

GARIBALLA, S. E.; SINCLAIR, A. J. Nutrition, ageing and ill health. **The British Journal of Nutrition**. New York, v.80, n.1, p.7-23, Jul. 1998.

GARIBALLA, S. E. Nutrition and older people: special considerations relating to nutrition and ageing. **Clinical Medicine**. Northfield, v.4, n.5, p.411–414, Sep.-Oct. 2004.

GARNERO, P.; DELMAS, P. D. Biochemical markers of bone turnover. Applications for osteoporosis. **Endocrinology and metabolism clinics of North America**. Philadelphia, v.27, n.2, p.303–323, Jun. 1998.

GUEGUEN, L.; POINTILLART, A. The bioavailability of dietary calcium. **Journal of the American College of Nutrition**. New York, v.19, Suppl. 2, p.119-36, Apr. 2000.

GÜRKAN, L.; EKELAND, A.; GAUTVIK, K. M.; LANGELAND, N.; RONNINGEN, H.; SOLHEIM, L. F. Bone changes after castration in rats. A model for osteoporosis. **Acta Orthopaedica Scandinavica**. Basingstoke, v.57, n.1, p.67-70, Feb. 1986.

HUEBNER, A. K.; KELLER, J.; CATALA-LEHNEN, P.; PERKOVIC, S.; STREICHERT, T.; EMESON, R. B.; AMLING, M.; SCHINKE, T. The role of calcitonin and a-calcitonin gene-related peptide in bone formation. **Archives of biochemistry and biophysics**. New York, v.473, n.2, p.210–17, May. 2008.

HUEBNER, A. K.; SCHINKE, T.; PRIEMEL, M.; SCHILLING, S.; SCHILLING, A. F.; EMESON, R. B.; RUEGER, J. M.; AMLING, M. Calcitonin deficiency in mice progressively results in high bone turnover. **Journal of Bone and Mineral Research**. Washington, v.21, n.12, p.1924-1934, Dec. 2006.

IIDA, H.; FUKUDA, S. Age-related changes in bone weights and their components in rats. **Jikken Dobutsu**. Tokyo, v.42, n.3, p.349-56, Jul. 1993.

KARKKAINEN, M. U.; LAMBERG-ALLARDT, C. J.; AHONEN, S.; VALIMAKI, M. Does it make a difference how and when you take your calcium? The acute effects of calcium on calcium and bone metabolism. **The American Journal of Clinical Nutrition**. Bethesda, v.74, n.3, n.335–342, Sep. 2001.

KHANAL, R. C.; NEMERE, I. Regulation of intestinal calcium transport. **Annual review of nutrition**. Palo Alto, v.28, p.179-196, Aug. 2008a.

KHANAL, R. C.; NEMERE, I. Endocrine regulation of calcium transport in epithelia. **Clinical and experimental pharmacology & physiology**. Oxford, v.35, n.11, p.1277-1287, Nov. 2008b.

KHOSLA, S.; ARRIGHI, H. M.; MELTON, L. J; ATKINSON, E. J.; O'FALLON, W. M.; DUNSTAN, C.; RIGGS, B. L. Correlates of osteoprotegerin levels in women and men. **Osteoporosis International**. London, v.13, n.5, p.394-399, May. 2002.

KIEBZAK, G. M.; SMITH, R.; HOWE, J. C.; GUNDBERG, C. M.; SACKTOR, B. Bone status of senescent female rats: chemical, morphometric, and biomechanical analyses. **Journal of Bone and Mineral Research**. Washington, v.3, n.4, p.439-46, Aug. 1988.

KINYAMU, H. K.; GALLAGHER, J. C.; BALHORN, K. E.; PETRANICK, K. M.; RAFFERTY, K. A. Serum vitamin D metabolites and calcium absorption in normal young and elderly freeliving women and in women living in nursing homes. **The American Journal of Clinical Nutrition**. Bethesda, v.65, p.790–797, Oct. 1997.

KOBAYASHI, Y,; KIM, K. J.; ITOH, T. Gene expression of bone-resorbing cytokines in rat osteolysis model. **Journal of Orthopaedic Science**. Tokyo, v.10, n.1, p.62-69, Feb. 2005.

KUMAR, V.; PRASAD, R. Molecular basis of renal handling of calcium in response to thyroid hormone status of rat. **Biochimica et Biophysica Acta**. Amsterdam, v.1586, n.3, p.331–343, Apr. 2002.

KUMAR, V.; PRASAD, R. Thyroid hormones stimulate calcium transport systems in rat intestine. **Biochimica et Biophysica Acta**. Amsterdam, v.1639, n.3, p.185–194, Nov. 2003.

LEE, N. K.; SOWA, H.; HINOI, E.; FERRON, M.; AHN, J. D.; CONFAVREUX, C.; DACQUIN, R.; MEE, P. J.; MCKEE, M. D.; JUNG, D. Y.; ZHANG, Z.; KIM, J. K.; MAUVAIS-JARVIS, F.; DUCY, P.; KARSENTY, G. Endocrine regulation of energy metabolism by the skeleton. **Cell**. Cambridge, v.130, n.3, p.456-469, Aug. 2007.

LI, X. J.; JEE, W. S. S.; KE, H. Z.; MORI, S.; AKAMINE, T. Age-related changes of cancellous and cortical bone histomorphometry in female Sprague-Dawley rats. **Cells and Materials Supplement**. Chicago, v.1, p.25-35, Jan. 1991.

LIPS, P. VitaminD deficiency and secondary hyperparathyroidism in the elderly: Consequences for bone loss and fractures and therapeutic implications. **Endocrine Reviews**. Baltimore, v.22, n.4, p.477–501, Aug. 2001.

MANOLAGAS, S. C. Role of cytokines in bone resorption. **Bone**. New York, v.17, Suppl.2, p.63-67, Aug. 1995.

MARTIN, T. J.; SIMS, N. A. Osteoclast-derived activity in the coupling of bone formation to resorption. **Trends in Molecular Medicine**. Oxford, v.11, n.2, p.76-81, Feb. 2005.

MAUGERI, D.; MAMAZZA, C.; GIUDICE, F. L.; PUGLISI, N.; MUSCOSO, E. G.; RIZZOTTO, M.; TESTAÌ, M.; BENNATI, E.; LENTINI, A.; PANEBIANCO, P. Interleukin-18 (IL-18) and matrix metalloproteinase-9 (MMP-9) in post-menopausal osteoporosis. **Archives of gerontology and geriatrics**. Amsterdam, v.40, n.3, p.299-305, May-Jun. 2005.

MELTON, L.J.; KAN, S. H.; FRYE, M. A.; WAHNER, H. W.; O'FALLON, W. M.; RIGGS, B. L. Epidemiology of vertebral fractures in women. **American journal of epidemiology**. Baltimore, v.129, n.5, p.1000-1011, May. 1989.

MOSEKILDE, L. Vitamin D and the elderly. **Clinical Endocrinology**. Oxford, v.62, n.3, p.265–281, Mar. 2005.

MUNDY, G. R.; HARRIS, S. E.; SABATINI, M.; GUTIERREZ, G.; GARRETT, I. R.; LZBICKA, E. The use of osteosarcoma cells to characterize factors which regulate bone cell function. In: Novak JF, McMaster JH, editors. **Frontiers of Osteosarcoma Research**. Seattle (WA): Hogrefe & Huber, 1993. p.449-456.

NIJWEIDE, P. J.; BURGER, E. H.; NULEND, J. K.; PLAS, A. V. The osteocyte. In: Bilezikian JP, Raisz LG, Rodan GA, editors. **Principles of Bone Biology**. San Diego (CA): Academic Press, 1996. p.115–26.

PANSU, D.; BELLATON, C.; BRONNER, F. Effect of Ca intake on saturable and nonsaturable components of duodenal Ca transport. **The American Journal of Physiology**. Bethesda, v.240, n.1, p.32-37,Jan. 1981.

PAPIEZ, M. A.; KAJA, M.; GEBAROWSKA, A. Age-dependent different action of curcumin in thyroid of rat. **Folia Histochemica et Cytobiologica**. Warszawa, v.46, n.2, p.205-211, Jun. 2008.

PARFITT, A. M. The physiologic and pathogenetic significance of bone histomorphometric data. In: Coe FL, Favus MJ, editors. **Disorders of Bone and Mineral Metabolism**. New York (NY): Raven Press, 1992. p.475–489.

PARFITT, A. M. Vitamin D and the pathogenesis of rickets and osteomalacia. In Feldman D, Glorieux FH, Pike JW, editors**. Vitamin D.** San Diego (CA): Academic Press, 1997. p.645-662.

PEDERSEN, S. F.; OWSIANIK, G.; NILIUS, B. TRP channels: an overview. **Cell Calcium**. Amsterdam, v.38, n.3-4, p.233-252, Sep.-Oct. 2005.

PERLMAN, W. R.; WEBSTER, M. J.; HERMAN, M. M.; KLEINMAN, J. E.; WEICKERT, C. S. Age-related differences in glucocorticoid receptor mRNA levels in the human brain. **Neurobiology of Aging**. New York, v.28, n.3, p.447-458, Mar. 2007.

POLAK-JONKISZ, D.; ZWOLINSKA, D.; PURZYC, L.; MUSIAL, K. Ca^{2+}-Mg^{2+}-dependent ATP-ase activity and calcium homeostasis in children with chronic kidney disease. **Pediatric Nephrology**. Berlin, v.22, n.3, p.414-419, Mar. 2007.

REGINSTER, J. Y.; ZEGELS, B.; LEJEUNE, E.; MICHELETTI, M. C.; KVSAZ, A.; SEIDEL, L.; SARLET, N. Influence of daily regimen calcium and vitamin D supplementation on parathyroid hormone secretion. **Calcified tissue international**. New York, v.70, n.2, p.78–82, Feb. 2002.

RIGGS, B. L.; MELTON, L. J. The worldwide problem of osteoporosis: insights afforded by epidemiology. **Bone**. New York, v.17, Suppl. 5, p.505-511, Nov. 1995.

RIGGS, B. L.; WAHNER, H. W.; DUNN, W. L.; MAZESS, R. B.; OFFORD, K. P.; MELTON, L. J. Differential changes in bone mineral density of the appendicular and axial skeleton with aging: relationship to spinal osteoporosis. **The Journal of Clinical Investigation**. Ann Arbor, v.67, n.2, p.328-35, Feb. 1981.

SCHULZ, S. R.; MORRIS, H. A. Ionized Calcium and Bone Turnover in the Estrogen-Deficient Rat. **Calcified tissue international**. New York, v.65, n.1, p.78–82, Jul. 1999.

SUDA, T.; UDAGAWA, N.; NAKAMURA, I.; MIYAURA, C.; TAKAHASHI, N. Modulation of osteoclast differentiation by local factors. **Bone**. New York, v.17, Suppl.2, p.87-91, Aug. 1995.

SUDA, T.; UDAGAWA, N.; TAKAHASHI, N. Cells of bone: osteoclast generation. In: Bilezikian JP, Raisz LG, Rodan GA, editors. **Principles of bone biology**. San Diego (CA): Academic Press, 1996. p.87–102.

TANAKA, H.; ABE, E.; MIYAURA, C.; KURIBAYASHI, T.; KONNO, K.; NISHII, Y.; SUDA, T. 1 alpha, 25-Dihydroxycholecalciferol and a human myeloid leukaemia cell line (HL-60). **The Biochemical Journal**. London, v.204, n.3, p.713-19, Jun. 1982.

TATSUMI, S.; ISHII, K.; AMIZUKA, N.; LI, M.; KOBAYASHI, T.; KOHNO, K.; ITO, M.; TAKESHITA, S.; IKEDA K. Targeted ablation of osteocytes induces osteoporosis with

defective mechanotransduction. **Cell Metabolism**. Cambridge, v.5, n.6, p.464-475, Jun. 2007.

TEITELBAUM, S. L.; ROSS, F. P. Genetic regulation of osteoclast development and function. **Nature Reviews. Genetics**. London, v.4, n.8, p.638-649, Aug. 2003.

TEITELBAUM, S. L.; TONDRAVI, M. M.; ROSS, F. P. Osteoclast biology. In: Marcus R, Feldman D, Kelsey J, editors. **Osteoporosis**. San Diego (CA): Academic Press, 1996. p.61–94.

TEITELBAUM, S. L. Postmenopausal osteoporosis, T cells, and immune dysfunction. **Proceedings of the National Academy of Sciences of the United States of America**. Washington, v.101, n.48, p.16711-16712, Nov. 2004.

TRIFFITT, J. T. The stem cell of the osteoblast. In: Bilezikian JP, Raisz LG, Rodan GA, editors. **Principles of bone biology**. San Diego (CA): Academic Press, 1996. p.39–50.

VAN ABEL, M.; HUYBERS, S.; HOENDEROP, J. G. J.; VAN DER KEMP, A. W. C. M.; VAN LEEUWEN, J. P. T. M.; BINDELS, R. J. M. Age-dependent alterations in Ca^{+2} homeostasis: role of TRPV5 and TRPV6. **American Journal of Physiology. Renal Physiology**. Bethesda, v.291, p.1177-1183, May. 2006.

VIEIRA, J. G. H. **Bone metabolics illnesses**. São Paulo (SP): Sonopress-Rimo, 2003. 1 CD-ROM.

WOOD, R. J. Searching for the determinants of intestinal calcium absorption. **The American Journal of Clinical Nutrition**. Bethesda, v.72, n.3, p.675-676, Sep. 2000.

WORLD HEALTH ORGANIZATION. **Prevention and management of osteoporosis prevention**. Technical Report Series n° 921. Geneva, 2003.

YAMAGUCHI, M.; OZAKI, K.; SUKETA, Y. Alteration in bone metabolism with increasing age: Effects of Zinc and Vitamin D3 in aged rats. **Journal of pharmacobio-dynamics**. Tokyo, v.12, n.2, p.67-73, Feb. 1989.

YATES, A. A.; SCHLICKER, S. A.; SUITOR, C. W. Dietary Reference Intakes: the new basis for recommendations for calcium and related nutrients, B vitamins, and choline. **Journal of the American Dietetic Association**. Chicago, v.98, n.6, p.699-706, Jun. 1998.

YOSHIKUBO, H.; SUZUKI, N.; TAKEMURA, K.; HOSO, M.; YASHIMA, S.; IWAMURO, S.; TAKAGI, Y.; TABATA, M. J.; HATTORI, A. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. **Life Science**. Amsterdam, v.76, n.23, p.2699-2709, Apr. 2005.

YOUNG, M. Bone matrix proteins: their function, regulation, and relationship to osteoporosis. **Osteoporosis International**. London, v.14, Suppl. 3, p.35-42, Mar. 2003.

CAPÍTULO 3

AGED FEMALE RATS ARE A SUITABLE MODEL TO STUDY SENILE OSTEOPOROSIS

Claudia Cardoso Netto[1,3], Rosângela Santos[2], Gláucia Maria Pastore[2], Sherry Agellon[3], Hope Weiler[3] and Mário Roberto Marostica JR[1*]

[1]Food and Nutrition Department / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[*]Corresponding author: Phone +55 19 3521-4078, Fax +55 19 3521-4060

e-mail: mario@fea.unicamp.br

[2]Department of Food Science / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

Many pre-clinical trials for osteoporosis prevention and management have relied on young ovariectomized rats to mimic natural menopause. However, it is postulated that the physiology of a young rat is not representative of that observed in aging. The objective of this study was to evaluate the effects of aging on bone metabolism of female rats. Female Wistar rats at 6 weeks (younger group n=8) and 36 weeks (older group n=8) of age were fed a nutritionally complete diet *ad libitum*, isocaloric and equal in Ca (5 mg/kg) for 14 weeks. After the aging period, femur and tibia were excised for measurements of dual-energy X-ray absorptiometry scans (DXA), morphometry, biomechanics properties, scanning electron microscopy (SEM) and histomorphometry. Biochemical indicators of bone metabolism in serum included: parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa β ligand (RANk-L), C-terminal peptides of type I collagen (CTX-I), alkaline phosphate activity (ALP) and total calcium. Data were analyzed by Student t-test ($p<0.05$) and all data are reported as means (SEM). The BMD of whole femur and diaphysis BMD, wet weight, length, head, diaphysis and distal epiphysis widths, osteoclastic-covered bone surface (Oc.S/BS), plus serum PTH and OPG were highest ($p<0,05$) in the older rats. Furthermore they presented several and deep resorption lacunae. Femur diaphysis proportional limit, relative bone volume (BV/TV), serum OC, CTX-I and ALP were highest ($p<0,05$) in the younger group while neck and hipaxis width, serum RANk-L and calcium were not statistically ($p<0,05$) different between the age groups. Based on the results of this study the older female Wistar rat is a satisfactory model to evaluate age related changes on bone metabolism as well as to study osteoporosis and their mechanisms.

Keywords: Aging, bone metabolism, senile osteoporosis, bone markers**.**

1) Introduction

As life expectancy in industrialized nations continues to increase, concerns over various health issues related to aging have also grown. It is estimated that in the next 30 years the proportion of people over the age of 65 years will double from 12% to 22–25% of the population in the USA and Canada (DANILOVICH et al. 2004). Similarly, in Europe it is estimated that this age group exceeds one in every thousand people and will grow from 21.4 million in year 2000 to 35.7 million in year 2025 (WORLD HEALTH ORGANIZATION, 2003).

The most dramatic population growth is in the oldest age group, over 80 years and is mainly represented by women. In Europe, for example, there are 15.2 million women aged 80 and above compared to only 6.2 million men; virtually doubling from 7.2 million women and 3.3 million men 80 years of age and over since 1970 (WORLD HEALTH ORGANIZATION, 2002).

Estrogen metabolism is intricately intertwined with bone metabolism (BROWN et al. 1974; WHITE et al. 1992). With aging of the female population around the world and consequently deficiency of estrogen with menopause, the prevalence of osteoporosis will increase markedly (WORLD HEALTH ORGANIZATION, 2002). Owing to the rapid decline in estrogen with menopause, rates of bone loss accelerate during the menopausal years resulting in accelerated rates of osteoporosis. After the menopausal years, the rate of bone loss in women slows slightly and becomes more comparable to that seen in older men (GAFFNEY-STOMBERG et al. 2009).

According World Health Organization (WHO) Study Group, by age 75 years, approximately 30% of Caucasian women would be classified as having osteoporosis. This is based on declining BMD at the femoral neck which is predictive of fractures with severe clinical consequences (WORLD HEALTH ORGANIZATION, 2002; DANILOVICH et al. 2004). Despite the highest risk with advanced aging, studies to prevent, manage and/or study osteoporosis have focused heavily on the ovariectomized (OVX) rat model (PATLAS et al. 2000; NETTO & MIYASAKA, 2009). However, the physiology of bone metabolism and bone mass are not static across aging in any species. Li et al. (1991), for example, observed static and dynamic bone histomorphometric changes in female Sprague-Dawley rats at the proximal tibial metaphysis that is primarily cancellous bone from 3 to 12 mo of age. The rate of longitudinal bone growth decreased rapidly between 3 and 7 months of age and the rates of bone growth continued to decline slowly between 8 and 11 months, stopping altogether in some at 12 months of age. Further, maximum or peak cancellous bone mass (percent of trabecular area) accumulates during the first 3 months of life and it is maintained until they are 8 months old. At 9 months, age-related cancellous bone loss begins and continues until 12 months. Thus it is clear that advanced aging in a rat would be accompanied by loss of cancellous bone and strategies to enhance bone during this physiological state require investigation. However, a comprehensive assessment of key biomarkers of bone metabolism along with measures of bone mass, structure and strength in both young and aged rats have not been forthcoming. Therefore, the objective of this study was to evaluate the effects of advanced ageing on bone mass, structure and metabolism of female rats to demonstrate that this animal model is superior over the younger model in the study of osteoporosis.

2) Materials and methods

2.1) Animals and Diets

Eight (6 week-old) female Wistar rats weighing approximately 250 g (younger group) and eight (36 week-old) female Wistar rats weighing approximately 320 g (older group) were obtained from the Center of Multidisciplinary Investigation in Biology (CEMIB) of the University of Campinas (São Paulo, Brazil). Animals were housed individually in stainless steel hanging cages with temperatures (21–24˚C), humidity (55 %) and a light:dark cycle of 12:12 h throughout the study. Animals were aged over a 20 week period during which they were fed *ad libitum* a nutritionally complete diet based on the AIN-93M formulation (REEVES et al. 1993) (Table 1), adequate in Ca (5 mg/kg). The protocol was approved by Animal Experimental Ethics Committee of the University of Campinas (Protocol nº 1625-1).

Table 1. Diets formulated composition based in AIN-93M (REEVES et al. 1993).

Ingredients	**(g/Kg of diet)**
Cornstarch	465.692
Casein (≥ 85% protein)	140.000
Dextrinized cornstarch	155.000
(90-94% tetrasaccharides)	
Sucrose	100.000
Soybean oil	40.000
Cellulose	50.000
Mineral mix (AIN-93M-MX)	35.000
Vitamin mix (AIN-93-VX)	10.000
L-Cystine	1.800
Choline bitartrate (41.1% choline)	2.500
Tert-butylhydroquinone	0.008

2.2) Tissue collection

All animals were killed by guillotine and trunk blood was collected and stored on ice until centrifuged to obtain serum (3.000 rpm / 10 min) (FANEM Excelsa Baby I – model 206 / São Paulo - Brazil) and stored at -80˚C until analysis. Femur and tibia were excised and stored at -20˚C until analysis.

2.3) Dual-energy X-ray absorptiometry scans (DXA)

The femur (whole, hipaxis and diaphysis) and whole mandible were scanned for bone area (BA), bone mineral content (BMC), bone mineral density (BMD) in a water bath by dual-energy X-ray absorptiometry (DXA, 4500A; Hologic Inc., Bedford, MA, USA; small animal software high resolution option QDR 12.3) (JAMIESON et al. 2008). Sub region analysis was used to assess hip axis defined as the distance from the lateral border of the femur along the central axis of the femoral neck to the medial pelvic wall (Figure 1); and diaphysis defined as mid section of the long bone compound for cortical bone.

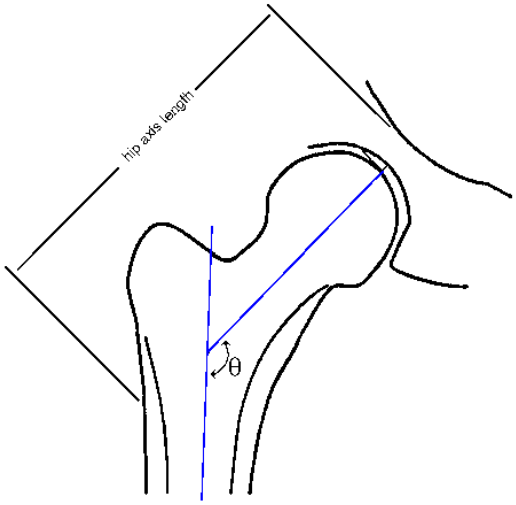


Figure 1. Hip axis demonstrative image (the distance from the lateral border of the femur along the central axis of the femoral neck to the medial pelvic wall).

2.4) Bone morphometry

Following DXA analysis, right and left femurs were thoroughly cleaned of soft tissue. Morphometric measures were taken with a digital caliper as previously described (JAMIESON et al. 2008) and recorded to the nearest 0.01 mm. All measures were reproduced in triplicate by the same trained examiner and included length, head, neck, hipaxis, diaphysis and distal epiphysis width.

2.5) Biomechanic properties

The femur diaphysis was selected to represent cortical bone known as a site of bone loss with aging (RHO et al. 1998). Biomechanical properties included proportional limit, rupture limit, resilience, rigid and tenacity conducted using three-point bending rheolometer (Hydraulic Equipment Service, INC.; 810 TestStar II MTS; Houston, Texas / USA) according as previously described (NETTO & MIYASAKA, 2009). The

measurement conditions were the following: sample space, 18 mm; plunger speed, 2 mm/min.; and load range, 100kN.

2.6) Bone histomorphometry

The left tibiae were cleaned of surrounding soft tissue and fixed for 48 hours in formalin solution (10%) followed by demineralization and routine histology procedures. Tibiae were then embedded in paraffin (Merck Chemicals / Darmstadt – Germany) and blocks sectioned (5 µm), stained using hematoxilin and eosin. Images of proximal epiphysis cancellous bone were taken with 10 X 10 magnification using a digital camera COHU 2700 (Cohu Inc. Electronics Division, California, Unites States) coupled a light microscope Nikon Eclipse H550S (Nikon Corporation, Tokyo, Japan). Standard histology parameters were quantified in accordance with nomenclature described previously (PARFITT et al. 1987; PARFITT, 1988) using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

2.7) Scanning electron microscopy (SEM)

The right tibiae proximal epiphysis cancellous bone was prepared for scanning microscopy as described elsewhere by NETTO & MIYASAKA (2009) and using a Scanning Microscope (JSM-5800LV/Jeol Serving Advanced Technology) with 500X magnification.

2.8) Biochemical assays

Serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa B ligand (RANk-L), C-terminal peptides of type I collagen (CTX-I) were measured by enzyme linked immunosorbent assays specific for rat (Rat Bone Panel 3 – PTH and Osteocalcin; Rat Bone Panel 1 – osteoprotegerin; Rat Bone Panel 2 – RANK-L of the Millipore, Billerica, Massachusetts, United States; CTX-I of the Immunodiagnostic Systems Ltd., Arizona, Unites States). Serum total calcium and alkaline phosphatase (ALP) activity were measured by colorimetric method (Calcium CAT n° 00800 of the Laborlab – São Paulo / Brazil; alkaline phosphatase activity LB 170123-800 of the Biodiagnostic – Paraná / Brazil) in Beckman Coulter DU-70 spectrophotometer (Beckman Coulter Inc., California, Unites States). All the biochemical assays were performed according to the manufacturer's instructions in duplicate. The methods sensitivity, intra-assay and inter-assay were respectively: PTH = 0.3 pg/mL, 3.5 CV% and 8.1 %CV; OC = 1.6 pg/mL, 2.9 %CV and 4.6 %CV; OPG = 2.3 pg/mL, 3.1 %CV and 3.4 %CV; RANk-L = 1.1 pg/mL, 3.1 %CV and 9.0 %CV; CTX-I = 2 ng/mL, 5.6 %CV and 10.5 %CV, ALP = 24 U/l, 1.2 %CV and 0.99 %CV.

2.9) Statistical methods

Differences between age groups (n=8) were tested using a Student t-test as the criteria of normal and equal variances between groups was met with a level of significance of 0.05 using GraphPad Prism 5.0 version (GraphPad Software, Inc.; La Jolla, CA, USA). All data are reported as means with their standard errors of the mean (SEM).

3) Results

3.1) Feed intake and growth

Body weight was 43% higher ($p<0.05$) in the older group than the younger group (233.5 ± 2.5 g vs 333.9 ± 0.9 g) upon arrival to the facility. The daily feed intake during the experimental period was not different between age groups (younger group = 15.2 ± 0.6 g; older group = 15.6 ± 0.6 g).

3.2) Bone mass (DXA) and morphometry

Femur diaphysis bone area, BMC and BMD, whole femur BMD and hipaxis BA were statistically ($p<0,05$) higher (45; 54; 8; 7; 12% respectively) in the older group when compared with younger group (Table 2). However, hipaxis BMC and BMD were statistically ($p<0.05$) equal in both groups. Femur wet weight, length, head, diaphysis and distal epiphysis width were significantly ($p<0,05$) higher (41; 11; 8; 16 and 3%, respectively) in the older group than the younger group (Table 3). Neck and hipaxis widths, however, were not significantly ($p>0,05$) different between the groups. The precision error (CV %) for triplicate scans of bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) was 25.6, 24.3 and 6.1, respectively for the whole femur; 6.1, 6.2 and 6.0, respectively for the hipaxis; 3.9, 4.7 and 5.6, respectively for the femur diaphysis; and 16.3, 17.1 and 5.3, respectively for the mandible.

Table 2. Bone area (BA), mineral content (BMC) and density (BMD) of femur and mandible from female rats 20 and 50 months of age.

	Younger group (n=8)	**Older group** (n=8)
Whole femur		
BA (cm^2)	1.22 ± 0.08	1.20 ± 0.11
BMC (g)	0.271 ± 0.02	0.280 ± 0.03
BMD (g/cm^2)*	0.221 ± 0.004[a]	0.237 ± 0.004[b]
Hipaxis		
BA (cm^2)**	0.28 ± 0.008[a]	0.31 ± 0.003[b]
BMC (g)	0.066 ± 0.003	0.073 ± 0.002
BMD (g/cm^2)	0.233 ± 0.005	0.236 ± 0.004
Femur diaphysis		
BA (cm^2)***	0.31 ± 0.003[a]	0.44 ± 0.007[b]
BMC (g)***	0.070 ± 0.002[a]	0.108 ± 0.002[b]
BMD (g/cm^2)*	0.225 ± 0.005[a]	0.243 ± 0.004[b]
Mandible		
BA (cm^2)	0.51 ± 0.03	0.54 ± 0.02
BMC (g)	0.063 ± 0.004	0.066 ± 0.004
BMD (g/cm^2)	0.125 ± 0.002	0.122 ± 0.002

Data are mean (SEM). Abbreviations: BA, bone area; BMC, bone mineral content, BMD, bone mineral density.
Within rows, values with different letters indicates significant differences ($p<0.05$) between the age groups; *, $P = 0.01$; **, $P = 0.001$; ***, $P = 0.0001$.

Table 3. Morphometry measurements of femur and mandible from female rats 20 and 50 months of age.

Measurements	**Younger group** (n=8)	**Older group** (n=8)
Wet weight (mg)**	488.4 ± 16.0[a]	690.3 ± 6.7[b]
Length (mm)**	30.3 ± 0.2[a]	33.8 ± 0.2[b]
Head (mm)*	3.55 ± 0.09[a]	3.82 ± 0.04[b]
Neck (mm)	2.03 ± 0.04	2.06 ± 0.02
Hipaxis (mm)	7.45 ± 0.05	7.65 ± 0.09
Diaphysis (mm)**	3.67 ± 0.09[a]	4.27 ± 0.04[b]
Distal epiphysis (mm)*	5.40 ± 0.05[a]	5.59 ± 0.05[b]

Data are mean (SEM). Within rows, values with different letters indicates significant differences ($p<0.05$) between the age groups; *, $P = 0.01$; **, $P = 0.0001$.

3.3) Bone biomechanic properties

The biomechanical properties of femur diaphysis (Table 4) showed that proportional limit (the end point of the elastic region) was significantly (p<0,05) highest (7%) on younger group while rupture limit, resilience, tenacity and rigidity were not different between groups.

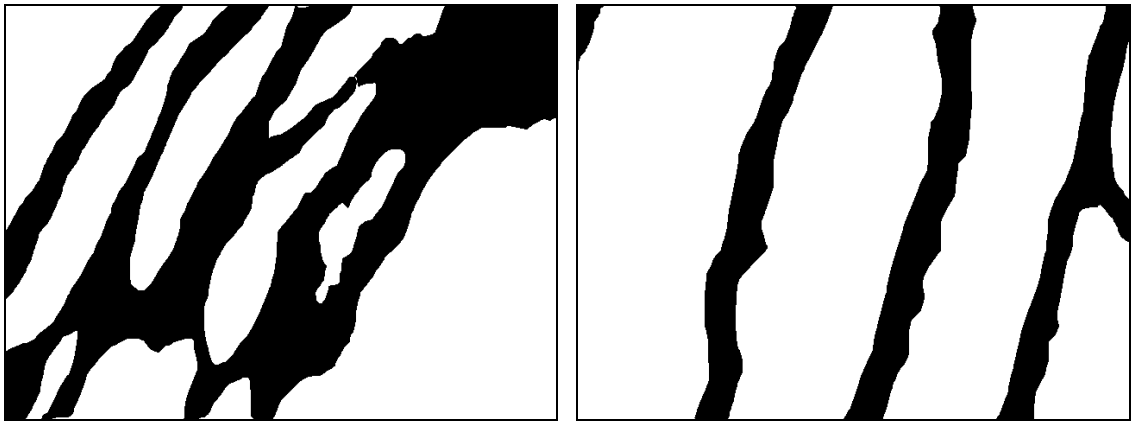
Table 4. Biomechanics properties of femur from female rats 20 and 50 months of age.

	Younger group (n=8)	**Older group** (n=8)
Proportional limit *	87.3 ± 1.7[a]	81.3 ± 0.8[b]
Rupture limit	95.3 ± 1.2	93.8 ± 1.1
Resilience	12.3 ± 0.7	11.6 ± 1.3
Tenacity	306.5 ± 25.4	332.6 ± 32.7
Rigidity	330.4 ± 16.6	319.1 ± 36.3

Data are mean (SEM). Within rows, values with different letters indicates significant differences (p<0.05) between the age groups; *, P = 0.005

3.4) Bone histomorphometry and scanning electron microscopy (SEM)

Relative bone volume (BV/TV) was significantly (p<0.05) higher in younger group (44%) while osteoclastic-covered bone surface (Oc.S/BS) was significantly (p<0.05) higher (71%) in the older group. The osteoblast-covered bone surface (Ob.S/BS) was not different between groups (Figure 2; Table 5). Using SEM the proximal epiphysis of the right tibia from the older group presented with several and deep resorption lacunae when compared with the younger group (Figure 3).



Younger group **Older group**

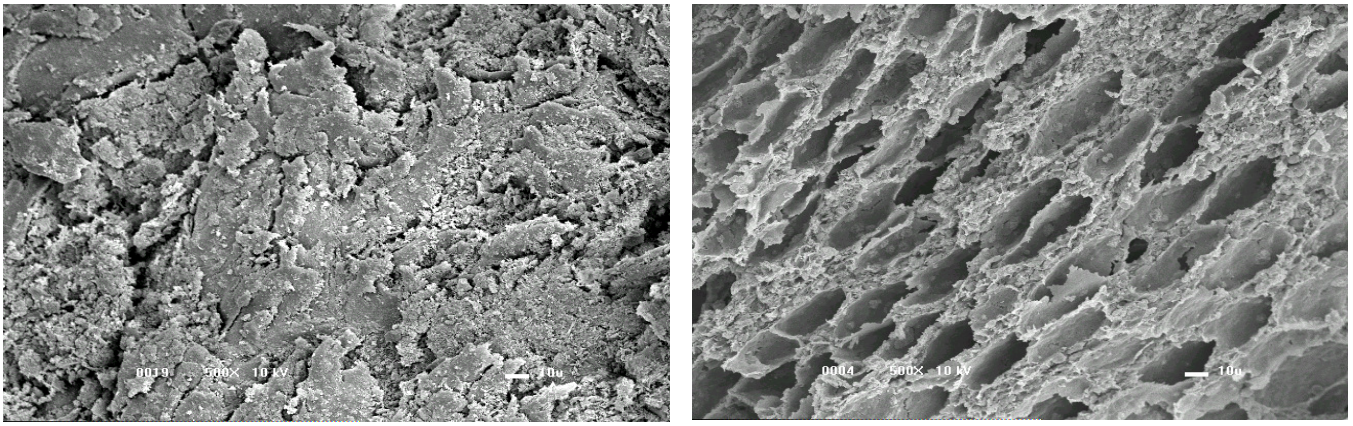
Figure 2. Representative histomorphometry images showing the differences between trabecular volume (BV/TV) in the two age groups (Hematoxilin and eosin stain, 10 X 10 magnification)*.

*These images were transformed to 8 bit archive using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

Table 5. Histomorphometry parameters of tibiae from female rats 20 and 50 months of age.

Parameters analyzed	**Younger group** (n=8)	**Older group** (n=8)
BV/TV (%)**	50.0 ± 1.4[a]	34.6 ± 2.2[b]
Ob.S/BS	22.6 ± 1.4	26.9 ± 2.9
Oc.S/BS*	13.3 ± 1.1[a]	22.7 ± 2.8[b]

Data are mean (SEM). Abbreviations: BV/TV, relative bone volume, Ob.S/BS, osteoblast-covered bone surface, Oc.S/BS, osteoclast-covered bone surface.
Within rows, values with different letters indicates significant differences (p<0.05) between the age groups; *, P = 0.01; **, P = 0.0001.



Younger group **Older group**

Figure 3. Representative images of the proximal epiphysis of the tibia using scanning electron microscopy (SEM) at 500X magnification.

3.5) Serum markers of bone metabolism

Serum PTH (Figure 4) and OPG (Figure 5) concentrations were significantly (p<0.05) higher (166 and 677%, respectively) in the older group. Serum OC (46%) (Figure 6), CTX-I (112,5%) (Figure 7) and ALP activity (younger group = 80.6 ± 6.9 U/L; older group = 46.6 ± 7.6 U/L) were significantly (p<0.05) lower in the older group. Serum RANk-L concentration (Figure 8) and serum total calcium (younger group = 9.7 ± 0.4 mg/dL; older group = 9.0 ± 0.3 mg/dL) were not different between the groups. However, serum OPG and RANk-L showed a positive correlation (r^2 = 0,2865; P = 0.0326) (Figure 9).

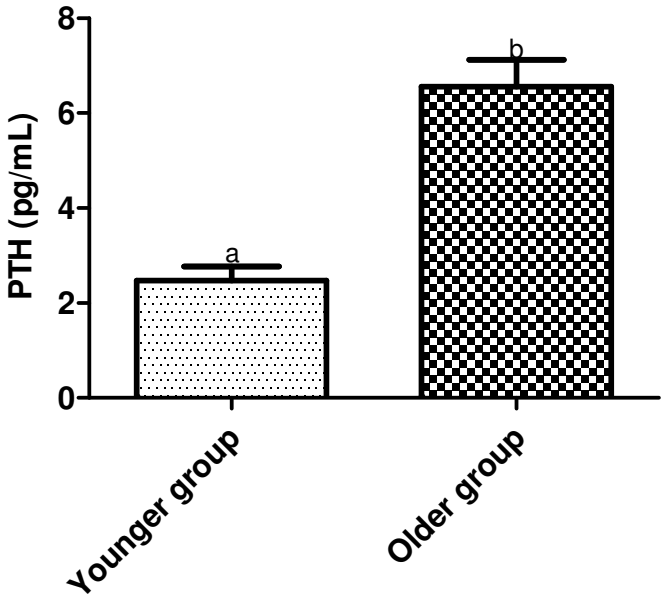


Figure 4. Serum parathyroid hormone (PTH) concentration of female rats at 20 and 50 weeks of age (n=8/group).

Bars with different letters indicate significant difference (p<0.0001) between the groups.

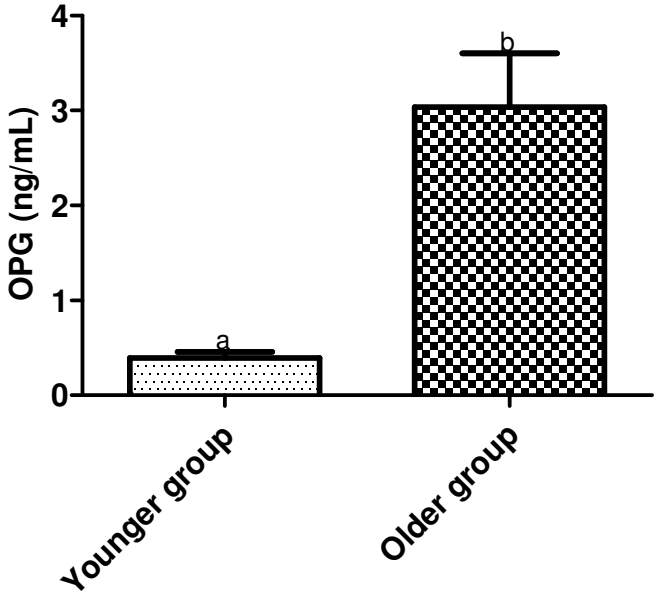


Figure 5. Osteoprotegerin (OPG) concentration of female rats at 20 and 50 weeks of age (n=8/group).

Bars with different letters indicate significant difference (p<0.0004) between the groups.

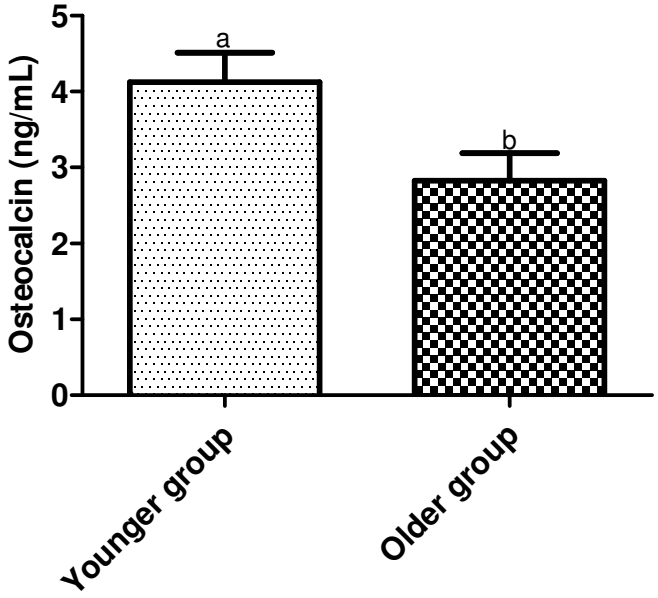


Figure 6. Osteocalcin concentration of female rats at 20 and 50 weeks of age (n=8/group).

Bars with different letters indicate significant difference (p<0.03) between the groups.

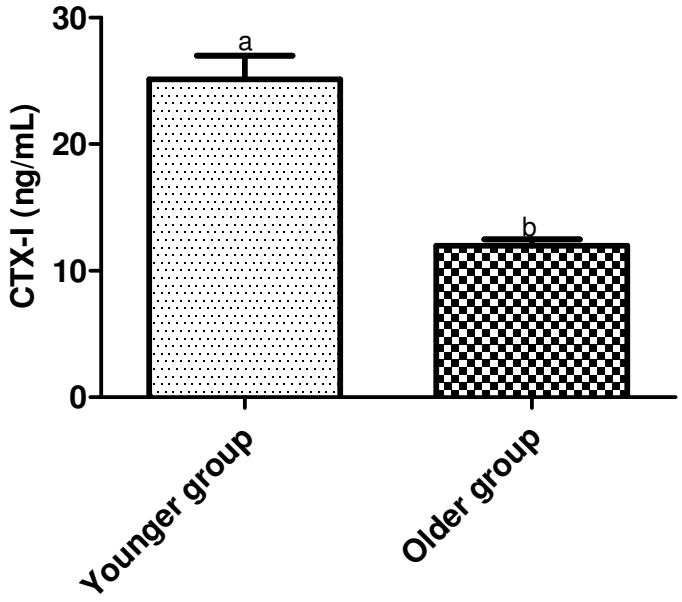


Figure 7. C-terminal peptides of type I collagen (CTX-I) concentration of female rats at 20 and 50 weeks of age (n=8/group).

Bars with different letters indicate significant difference (p<0.0001) between the groups.

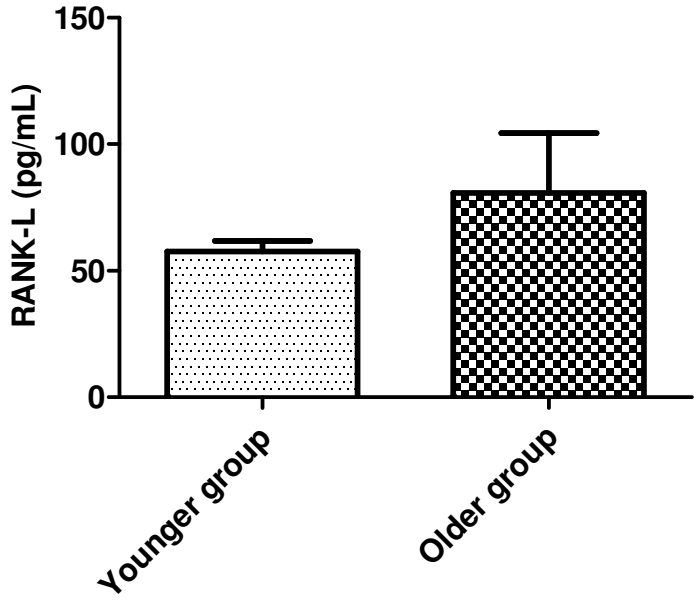


Figure 8. Receptor activator for nuclear factor κ B ligand (RANk-L) concentration of female rats at 20 and 50 weeks of age (n=8/group).

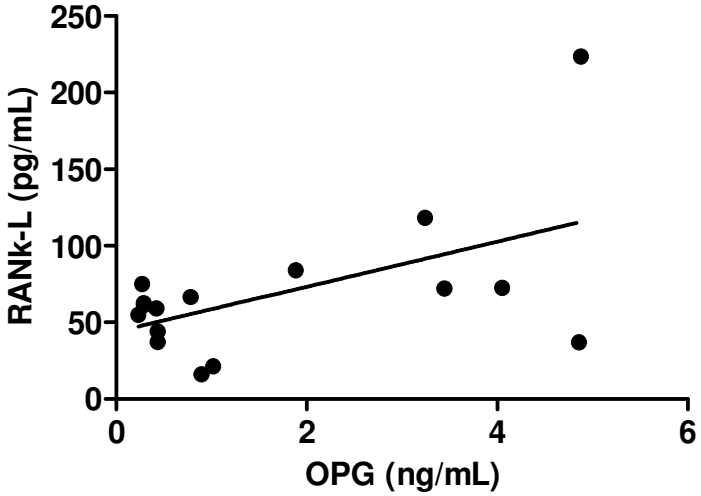


Figure 9. OPG and RANk-L correlation of female rats at 20 and 50 weeks of age (n=8/group)*.

* r^2 = 0,2865; *P* = 0.0326

4) Discussion

In the aging female, bone loss occurs in both trabecular and cortical bone that may in some circumstances result in osteoporosis. Using the Wistar rat, a common strain for the study of bone, we have demonstrated that aging results in markedly compromised bone structure and strength. Both the DXA obtained BMD and morphometry of the femur in the older female rat model were consistent with a natural postmenopausal bone loss. Using the diaphysis of the femur no differences were observed in BA, BMC or BMD and width between younger and older groups rats, suggesting that in the female rat, cortical bone is not lost readily with aging. This is in accordance with what was described previously by others in humans (RIGGS & MELTON, 1995; BROUNS & VERMEER, 2000). RIGGS & MELTON (1995) reported that the cortical bone remodeling represents 3% and cancellous bone remodeling represent approximately 25% of the bone loss in human adults. Similarly, BROUNS & VERMEER (2000) suggest that bone loss in osteoporosis occurs in all skeletal sites, however, the postmenopausal bone loss affects primarily cancellous bone tissue. By examining the hip axis in our study, the older group indirectly showed cancellous bone depletion based on the higher BA, but lack of higher BMC or BMD compared to the younger rats. This region was measured using DXA technology that is not capable of examining trabecular matrix of the hip without inclusion of the dense cortical shell. Therefore, we also commissioned histology and SEM technology to more precisely examine tracebular bone. Indeed, age related depletion on cancellous bone was confirmed by the lower BV/TV and higher Oc.S/BS in the older rats. Similar loss of cancellous bone was also shown in Sprague-Dawley female rats by LI et al. (1991). These authors observed that the peak bone mass is maintained without significant

changes until 8-months of age. Thereafter, bone mass begins to decrease with aging and reaches a low value of 14% of the metaphyseal tissue area at 12 months of age. Further, our scanning electron microscopy images show several and deep resorption lacunae in tibiae epiphysis cancellous bone of the older group when compared with younger group, confirming age related loss of cancellous bone.

Consistent with no age related changes in diaphyseal BMD, bone strength was also unaltered with aging based on rupture limit. Such testing is sufficient to indicated changes in bone strength however, since BMD is positively related to diaphysis BMD and proportional limit was statistically (p<0.05) lowest in older group, indicating an age related reduction in the cortical bone architecture. AKKUS et al. (2004) did not observe statistical (p<0.05) difference on rupture limit of Sprague-Dawley female rats at 3, 8 and 24 months of age. KIEBZAK et al. (1988) also did not observe a statistical (p<0.05) difference in the maximum breaking force required to fracture femurs with senescence. However, still in accordance with our findings, AKKUS et al. (2004) observed an age related reduction on proportional limit between the 3 and 24 month old rats. Moreover, the middle-age 8 month group proportional limit was 52% and 63% greater than younger and older groups, respectively. As our animals were approximately one year old at the end of the aging period and the proportional limit is related with the end point of the elastic region, our findings suggest that our older group was in the early stages of age related changes in the cortical bone architecture, despite no apparent difference in diaphysis BMD. Further, despite that NORIAN et al. (2009) had described previously that the BMD is a major determinant of bone strength, some changes as perforation and/or disappearance of cancellous bone without major repercussions on BMD could account for the modifications in bone strength (AMMANN & RIZZOLI, 2003).

Aligned with the structural observations, the biochemical observations are consistent with aging in humans as well. For example, the serum PTH was elevated with aging and similar to that described by KIEBZAK et al. (1988). LIPS (2001) and MOSEKILDE (2005) previously reviewed that elevated PTH is common in aged animals. Typically osteoblasts directly and osteoclasts indirectly respond to elevated PTH with the net result being increased bone resorption and the rapid release of Ca^{2+} from the bone matrix (SCHLUTER, 1999; KHANAL & NEMERE, 2008). However, both biomarkers of bone turnover, serum OC and CTX-I, were reduced in the older group. However, KIEBZAK et al. (1988) also showed a serum OC decreased progressively from 6 to 12-months of age (21%) and from 12 to 24-months of age (23%) in female rats. These results suggest that bone remodeling slows with age and, moreover, PEREZ-CASTRILLON et al. (2009) described that maintenance of bone mass depends on the proper balance between bone formation and bone resorption. Further, our OC and CTX-I results were confirmed by lower ALP activity observed with aging, again that bone remodeling is reduced in this group. IIDA & FUKUDA (1993) also showed that total ALP activity decreased up to 9 months of age in Wistar female and male rats and others (VIEIRA, 2003; YOSHIKUBO et al. 2005) report that ALP activity is proportional to bone remodelling. Lastly, it is important to distinguish our model from humans in that the rats were fed a nutritionally complete diet throughout aging that would provide ample calcium to sustain calcium balance (REEVES et al. 1993).

A diet adequate in calcium would explain why the elevated PTH did not result in higher bone resorption. While serum OPG concentration was elevated in the older rats, serum RANk-L concentration was not different between the groups. RANk-L can remain on cell surfaces or can be proteolytically cleaved into soluble forms that possess

osteoclast-stimulating activities within their TNF-homology domains. It is produced by numerous cell types including cells of the osteoblast lineage and activated T cells (KEARNS et al. 2008). Differentiation of cultured osteoblasts was associated with reduced RANk-L expression and decreased ability to support osteoclastogenesis, suggesting that the mature bone-forming osteoblast might not be capable of directing osteoclast activity via RANk-L (GORI et al. 2000). NORIAN et al. (2009) suggested that RANk-L may not be a reliable biomarker reflecting the state of bone metabolism in young women, further elucidation of this relationship in the premenopausal period is warranted.

FAHRLEITNER et al. (2003) showed a direct correlation between low serum OPG levels and vertebral fractures (lower BMD) and MIZUNO et al. (1998) showed beneficial influences of exogenous OPG on skeletal phenotype (i.e., higher BMD) in a rodent model. However, KHOSLA et al. (2002) measured serum OPG levels in an age-stratified, random sample of men (n = 346 age range, 23-90 years) and women (n = 304; age range 21-93 years) and related them to sex steroid levels, bone turnover markers and BMD. Serum OPG levels increased with age in both men and women. Premenopausal women had higher OPG levels than men under age 50 years (171 ± 6 pg/mL vs. 134 ± 6 pg/ml, respectively, $p < 0.001$), whereas serum OPG levels were not different in postmenopausal women compared with men = 50 years (195 ± 7 pg/ml vs. 188 ± 7 pg/ml, respectively, $p = 0.179$). OPG levels correlated inversely with serum bioavailable testosterone levels in men = 50 years ($R = -0.27$, $p < 0.001$), but no associations were present with either estrogen or testosterone levels in the women. Moreover, our Ob.S/BV and Oc.S/BV results are consistent with described for KHOSLA et al. (2002) who hypothesized that elevations in OPG in older population can be

explained as a compensatory phenomenon to slow down the enhanced bone resorption. In our results, despite Oc.S/BV had been higher in older group, Ob.S/BV averages were equal in both groups. The serum calcium concentration did not show age-related reductions as supported by IIDA & FUKUDA (1993) and KIEBZAK et al. (1988). The value of serum calcium decreases up to 12 months of age, but then increases after 18 months of age in Wistar rats of the both sexes IIDA & FUKUDA (1993), while in female rats 6, 12, and 24-months of age it is constant (KIEBZAK et al. 1988). Further, TAKADA et al. (1997) described that only in critical situations such as undernutrition or hyperparathyroidism serum calcium concentration change.

5) Conclusion

Based on the results of this study the older female Wistar rat is a satisfactory model to evaluate age related changes on bone metabolism as well as to study osteoporosis and their mechanisms. Moreover, as this older female rat model showed important differences when compared with the younger group, the physiological response to any intervention is likely to be different as well. For example, the elevated PTH even with adequate dietary Ca might attenuate response to interventions to attenuate age-related bone loss.

6) Acknowledgments

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7) References

AKKUS, O.; ADAR, F.; SCHAFFLER M. B. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. **Bone**. New York, v.34, p.443– 453, Mar. 2004.

AMMANN, P.; RIZZOLI, R. Bone strength and its determinants. **Osteoporosis International**. London, v.14, Suppl. 3, p.13-18, Mar. 2003.

BROUNS, F.; VERMEER, V. C. Functional food ingredients for reducing the risks of osteoporosis. **Trends in Food Science & Technology**. Oxford, v.11, n.1, p.22-33, Jan. 2000.

BROWN, D. M.; JOWSEY, J.; BRADFORD, D. S. Osteoporosis in ovarian dysgenesis. **The Journal of Pediatrics**. St. Louis, v.84, p.816-820, Jun. 1974.

DANILOVICH, N.; MAYSINGER, D.; SAIRAM, M. R. Perspectives on reproductive senescence and biological aging: studies in genetically altered follitropin receptor knockout [FORKO] mice. **Experimental Gerontology**. Amsterdam,v.39, n.11-12, p.1669–1678, Nov.-Dec. 2004.

FAHRLEITNER, A.; PRENNER, G.; LEB, G.; TSCHELIESSNIGG, K. H.; PISWANGER-SOLKNER, C.; OBERMAYER-PIETSCH, B.; PORTUGALLER, H. R.; BERGHOLD, A.; DOBNIG, H. Serum osteoprotegerin is a major determinant of bone density development and prevalent vertebral fracture status following cardiac transplantation. **Bone**. New York, v.32, p.96–106, Jan. 2003.

GAFFNEY-STOMBERG, E.; INSOGNA, K. L.; RODRIGUEZ, N. R.; KERSTETTER, J. E. Increasing Dietary Protein Requirements in Elderly People for Optimal Muscle and Bone Health. **Journal of the American Geriatrics Society**. Malden, v.57, n.6, p.1073-1079, Jun. 2009.

GORI, F.; HOFBAUER, L. C.; DUNSTAN, C. R.; SPELSBERG, T. C.; KHOSLA, S.; RIGGS, B. L. The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. **Endocrinology**. Chevy Chase, v.141, n.12, p.4768–4776, Dec. 2000.

IIDA, H.; FUKUDA, S. Age-related changes in bone weights and their components in rats. **Jikken Dobutsu**. Tokyo, v.42, n.3, p.349-356, Jul. 1993.

JAMIESON, J. A.; RYZ, N. R.; TAYLOR, C. G.; WEILER, H. A. Dietary long-chain inulin reduces abdominal fat but has no effect on bone density in growing female rats. **The British Journal of Nutrition**. New York, v.100, n.2, p.451-459, Aug. 2008.

KEARNS, A. E.; KHOSLA, S.; KOSTENUIK, P. J. Receptor activator of nuclear Factor κ B ligand and osteoprotegerin regulation of bone remodeling in health and disease. **Endocrine Reviews**. Chevy Chase, v.29, n.2, p.155-192, Apr. 2008.

KHANAL, R. C.; NEMERE, I. Endocrine regulation of calcium transport in epithelia. **Clinical and experimental pharmacology & physiology**. Oxford, v.35, n.11, p.1277-1287, Nov. 2008.

KHOSLA, S.; ARRIGHI, H. M.; MELTON, L. J; ATKINSON, E. J.; O'FALLON, W. M.; DUNSTAN, C.; RIGGS, B. L. Correlates of osteoprotegerin levels in women and men. **Osteoporosis International**. London, v.13, n.5, p.394-399, May. 2002.

KIEBZAK, G. M.; SMITH, R.; HOWE, J. C.; GUNDBERG, C. M.; SACKTOR, B. Bone status of senescent female rats: chemical, morphometric, and biomechanical analyses. **Journal of Bone and Mineral Research**. Washington, v.3, n.4, p.439-46, Aug. 1988.

LI, X. J.; JEE, W. S. S.; KE, H. Z.; MORI, S.; AKAMINE, T. Age-related changes of cancellous and cortical bone histomorphometry in female Sprague-Dawley rats. **Cells and Materials Supplement**. Chicago, v.1, p.25-35, Jan. 1991.

LIPS, P. VitaminD deficiency and secondary hyperparathyroidism in the elderly: Consequences for bone loss and fractures and therapeutic implications. **Endocrine Reviews**. Baltimore, v.22, n.4, p.477–501, Aug. 2001.

MIZUNO, A.; AMIZUKA, N.; IRIE, K.; MURAKAMI, A.; FUJISE, N.; KANNO, T.; SATO, Y.; NAKAGAWA, N.; YASUDA, H.; MOCHIZUKI, S.; GOMIBUCHI, T.; YANO, K.; SHIMA, N.; WASHIDA, N.; TSUDA, E.; MORINAGA, T.; HIGASHIO, K.; OZAWA, H. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. **Biochemical and biophysical research communications**. New York, v.247, n.3, p.610–615, Jun. 1998.

MOSEKILDE, L. Vitamin D and the elderly. **Clinical Endocrinology**. Oxford, v.62, n.3, p.265–281, Mar. 2005.

NETTO, C. C.; MIYASAKA, C. K. Fructooligosaccharides (FOS) and Hormonal Replacement Therapy (HRT) by estrogen suppressed bone resorption in the ovariectomized rat. **Journal of Medical and Biological Science**. Salvador (*in press*), 2009.

NORIAN, J. M.; ZEITLIAN, G.; ADEL, G.; FREEMAN, R. G.; SANTORO, N. F.; PAL, L. Low serum osteoprotegerin levels in premenopausal infertile women with low bone density: an association of relevance? **Fertility and Sterility**. New York, v.91, n.2, p.542-548, Feb. 2009.

PARFITT, A. M; DREZNER, M. K.; GLORIEUX, F. H; KANIS, J. A.; MALLUCHE, H.; MEUNIER, P. J.; OTT, S. M.; RECKER. R. R. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. **Journal of Bone and Mineral Research**. New York, v.2, n.6, p.595-610, Dec. 1987.

PARFITT, A. M. Bone histomorphometry: proposed system for standardization of nomenclature, symbols, and units. **Calcified Tissue International**. New York, v.42, n.5, p.284-6, May. 1988.

PATLAS, N.; ZADIK, Y.; YAFFE, P.; SCHWARTZ, Z.; ORNOY, A. Oophorectomy-induced osteopenia in rats in relation to age and time postoophorectomy. **Cells, Tissues, Organs**. New York, v.166, n.3, p.267-274, May. 2000.

PEREZ-CASTRILLON, J. L.; OLMOS, J. M.; NAN, D. N.; CASTILLO, J.; AROZAMENA, J.; MONTERO, A.; PEREZ-NUNEZ, M. I.; RIANCHO, J. A. Polymorphisms of the WNT10B Gene, Bone Mineral Density, and Fractures in Postmenopausal Women. **Calcified Tissue International**. New York, May 21st. 2009 [Epub ahead of print].

REEVES, P. G.; NIELSEN, F. H.; FAHEY, G. C. JR. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **The Journal of Nutrition**. Bethesda, v.123, n.11, p.1939-1951, Nov. 1993.

RHO, J. Y.; SPEARING, L. K.; ZIOUPOS, P. Mechanical properties and the hierarchical structure of bone. **Medical engineering & physics**. Oxford, v.20, n.2, p.92-102, Mar. 1998.

RIGGS, B. L.; MELTON, L. J. The worldwide problem of osteoporosis: insights afforded by epidemiology. **Bone**. New York, v.17, Suppl. 5, p.505-511, Nov. 1995.

SCHLUTER, K. D. PTH and PTHrP: Similar structures but different functions. **News in physiological sciences**. Bethesda, v.14, p.243-249, Dec. 1999.

TAKADA, Y.; MATSUYAMA, H.; KATO, K.; KOBAYASHI, N.; YAMAMURA, J. I.; YAHIRO, M.; ACE, S. Milk whey protein enhances the bone breaking force in

ovariectomized rats. **Nutrition Research**. New York, v.17, n.11-12, p.1709-1720, Nov.-Dec. 1997.

VIEIRA, J. G. H. **Bone metabolics illnesses**. São Paulo (SP): Sonopress-Rimo, 2003. 1 CD-ROM.

WHITE, C. M.; HERGENROEDER, A. C.; KLISH, W. J. Bone mineral density in 15- to 21-year-old eumenorrheic and amenorrheic subjects. **American Journal of Diseases of Children.** Chicago, v.146, n.1, p.31-35, Jan. 1992.

WORLD HEALTH ORGANIZATION. **Prevention and management of osteoporosis prevention**. Technical Report Series n° 921. Geneva, 2002.

YAMAGUCHI, M.; OZAKI, K.; SUKETA, Y. Alteration in bone metabolism with increasing age: Effects of Zinc and Vitamin D3 in aged rats. **Journal of pharmacobio-dynamics**. Tokyo, v.12, n.2, p.67-73, Feb. 1989.

YOSHIKUBO, H.; SUZUKI, N.; TAKEMURA, K.; HOSO, M.; YASHIMA, S.; IWAMURO, S.; TAKAGI, Y.; TABATA, M. J.; HATTORI, A. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. **Life Science**. Amsterdam, v.76, n.23, p.2699-2709, Apr. 2005.

CAPÍTULO 4

EFFECTS OF THE PREBIOTICS FRUCTOOLIGOSACCHARIDES (FOS) AND INULIN ON BONE METABOLISM OF AGED FEMALE RATS

Claudia Cardoso Netto[1,3], Vivian Cristine Correia Vieira[1], Nathália Ono[1], Celio Kenji Miyasaka[1], Rosângela Santos[2], Gláucia Maria Pastore[2], Sharry Agellon[3], Hope Weiler[3] and Mário Roberto Maróstica JR[1*]

[1] Department of Food and Nutrition / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[1*]Phone +55 19 3521-4078, Fax +55 19 3521-4060

E-mail: mario@fea.unicamp.br

[2]Department of Food Science / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

The objective of this study was to establish if the prebiotics fructooligosaccharides (FOS) and inulin alone or together attenuate age related bone loss in female rats. Forty 10-month old female rats were randomly assigned to four diet groups for 2 months (Control group = 10% sucrose; FOS group = 5% FOS and 5% sucrose; Inulin group = 5% inulin and 5% sucrose; FOS + Inulin group = 2.5% FOS + 2.5% inulin and 5% sucrose). Femur and mandible bone mass was measured using dual-energy X-ray absorptiometry. Femur quality was examined using morphometry and diaphysis biomechanic properties (proportional limit, rupture limit, resilience, rigidy and tenacity) using three-point bending. Histomorphometry static parameters and scanning electron microscopy images were taken on tibiae methaphysis cancellous bone. The biochemical assays were serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa β ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I). Data were analyzed by two-way ANOVA ($p<0,05$) using GraphPad software version 6.0 and the significant differences were determined with Tukey's test. The FOS increased hipaxis bone mineral density (BMD) and femur neck width while FOS + Inulin increased the proportional limit. FOS and FOS + Inulin reduced serum PTH and total calcium, however, only FOS + Inulin reduced ALP activity. All the prebiotics showed a positive effect on bone volume (BV/TV), reduced serum OPG and RANk-L and did not show differences on serum OC and CTX-I. Despite the fact that consumption of FOS alone or with inulin preserved bone mass and reduced bone turnover, more studies are required to establish mechanisms of action.

Keywords: Aged related bone loss; prebiotics; osteoporosis; menopause.

1) Introduction

Prebiotics are described as “non-digestible oligosaccharides (NDOs) selectively fermented by intestinal bacterial and are bifidogenic agents that specifically promote modulation of the host microbiota through changes on its composition and/or activity, with health benefit on the host associated” (ROBERFROID, 2007).

Actually, many NDOs have been claimed to have prebiotic activity. However not all NDOs are prebiotics and clear criteria need to be established for classifying a food ingredient as a prebiotic. These criteria observed *in vitro* and *in vivo* are: 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being (ROBERFROID, 2007).

Inulin-type fructans are food components well established as prebiotic since they show all the criteria described above by ROBERFROID (2007). They are a linear polydisperse carbohydrate material consisting mainly, if not exclusively, of β-(2→1) fructosyl-fructose linkages. $G_{py}F_n$ (glucopyranosyl-(fructofuranosyl)$_{n-1}$-fructose) and $F_{py}F_n$ (fructopyranosyl-(fructofuranosyl)$_{n-1}$-fructose) compounds which are included under that same nomenclature and they are both a mixture of oligomers and polymers that are best characterized by the degree of polymerization (DP) and expressed as either average (DP_{av}) and the maximum (DP_{max}). The DP of inulin-type fructans varies from 2 to more or less 60 units with DP_{av} = 12. About 10% of the fructan chains have a DP ranging between 2 (F2) and 5 (GF4). The partial enzymatic hydrolysis of inulin using an endo-inulinase (EC 3.2.1.7) produces oligofructose that is a mixture of both $G_{py}F_n$ and $F_{py}F_n$ molecules, in which the DP varies from 2 to 7 with DP_{av} = 4. When the fructans have

DP_{max} of 10, they can be also called fructooligoaccharides (FOS) (ROBERFROID, 2005).

The inulin-type fructans are used as substrate for the distinct metabolic pathways by which bifidobacteria oxidize carbohydrates, the so co-called *Bifidus pathways* and 78% of the carbon atoms of the fermented hexosyl moiety are recovered as the short-chain fatty acids (SCFA) acetate, propionate and butyrate, with a ratio of 64:21:15%. In addition, 17% is produced as lactate and 5% as ethanol, formate, and CO_2 (WONG et al. 2006; WONG & JENKINS, 2007).

The primary interest in SCFA has been related to colonic function as a result of their uptake and metabolism by colonocytes, specifically butyrate. Although, SCFA can be used as metabolic substrates for other tissues of the host such as bone metabolism (WONG & JENKINS, 2007). However, the inulin-type fructans have not been clearly tested for benefits to bone mass in elderly people. So, the objective of this study was to establish if the prebiotics fructooligosaccharides (FOS) and inulin, alone or together, attenuate age related bone loss in female rats.

2) Experimental methods

2.1) Animals and diets

Forty (10-month old) female Wistar rats weighing approximately 300-320g were obtained from the Center of Multidisciplinary Investigation in Biology (CEMIB) of the University of Campinas (São Paulo, Brazil). The animals were randomly assigned to four diet treatments groups (n=10 animals per group) and they were fed *ad libitum* for 2 months. All diets were based on the AIN-93M formulation (REEVES et al. 1993) (Table 1), isocaloric and equal in Ca (5mg/kg). The prebiotics fructooligossaccharides (FOS –

RAFTILOSE®P95) and Inulin (Orafti®LGI) utilized in this study were supplied by Beneo Orafti Ingredients (Tienen, Belgium). The animals were housed individually in stainless steel hanging cages in a temperature (21–24˚C) and humidity (55%) controlled room with a light:dark cycle of 12:12 h. The protocol was approved by the Animal Experimental Ethics Committee of the University of Campinas (Protocol nº 1625-2).

Table 1. Diets formulated composition based in AIN-93M (REEVES et al. 1993).

Ingredients	**Control diet (g/Kg of diet)**	**FOS diet (g/Kg of diet)**	**Inulin diet (g/Kg of diet)**	**FOS + Inulin diet (g/Kg of diet)**
Cornstarch	465.692	465.692	465.692	465.692
Casein (≥85% protein)	140.000	140.000	140.000	140.000
Dextrinized cornstarch (90-94% tetrasaccharides)	155.000	155.000	155.000	155.000
Sucrose	100.000	50.000	50.000	50.000
FOS	-	50.000	-	25.000
Inulin	-	-	50.000	25.000
Soybean oil	40.000	40.000	40.000	40.000
Cellulose	50.000	50.000	50.000	50.000
Mineral mix (AIN-93M-MX)	35.000	35.000	35.000	35.000
Vitamin mix (AIN-93-VX)	10.000	10.000	10.000	10.000
L-Cystine	1.800	1.800	1.800	1.800
Choline bitartrate (41.1% choline)	2.500	2.500	2.500	2.500
Tert-butylhydroquinone	0.008	0.008	0.008	0.008

2.2) Tissue collection

All animals were killed by guillotine and trunk blood was collected and stored on ice until centrifuged to obtain serum (3.000 rpm / 10 min) (FANEM Excelsa Baby I – model 206 / São Paulo - Brazil). Serum samples were stored at -80˚C until analyzed. The bones were collected and stored at -20˚C until analyzed.

2.3) Dual-energy X-ray absorptiometry scans (DXA)

The femur (hip axis and diaphysis) and mandible were scanned for bone area (BA), bone mineral content (BMC), bone mineral density (BMD) *ex vivo* in a water bath by dual-energy X-ray absorptiometry (DXA, 4500A; Hologic Inc., Bedford, MA, USA; small animal software high resolution option) (JAMIESON et al. 2008). Sub region analysis was used to assess hip axis defined as the distance from the lateral border of the femur along the central axis of the femoral neck to the medial pelvic wall; and diaphysis defined as mid section of the long bone compound for cortical bone.

2.4) Bone morphometry

Following *ex vivo* in a water bath DXA analysis, right and left femurs were excised and thoroughly cleaned of soft tissue. Morphometric measures were taken with digital calipers as previously described (JAMIESON et al. 2008) and recorded to the nearest 0·01 mm. All measures were reproduced in triplicate by the same trained examiner and included head, neck and hip axis width.

2.5) Biomechanic properties

The femur diaphysis biomechanical properties (proportional limit, rupture limit, resilience, rigidy and tenacity) were measured using three-point bending rheolometer (hydraulic serv equipment; 810 TestStar II MTS model – USA) according to previously described procedures (NETTO & MIYASAKA, 2009). The measurement conditions were the following: sample space, 18mm; plunger speed, 2 mm/min.; and load range, 100kN.

2.6) Bone histomorphometry

The left tibiae from each animal were excised at death, and the surrounding soft tissue was cleaned off. After cleaned, tibiae were maintained 48 hours in formol solution (10%), demineralized and submitted to routine hystologic procedures. Then, the tibiae were included in histological paraffin (Merck Chemicals / Darmstadt – Germany), the paraffin blocks were sliced on 5 µm sections, put in laminas and submitted to routine histological procedures using hematoxilin and eosin to stain. The images were in the left tibiae metaphysis cancellous bone with 10 X 10 magnification with a digital camera COHU 2700 (Cohu Inc. Electronics Division, California, Unites States) coupled a light microscope Nikon Eclipse H550S (Nikon Corporation, Tokyo, Japan) following, quantified in accordance with nomenclature described previously (PARFITT et al. 1987; PARFITT, 1988) using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

2.7) Scanning electron microscopy (SEM)

The right tibiae metaphysis cancellous bone was prepared by a procedure similar to that described elsewhere by NETTO & MIYASAKA (2009). Then it was examined on a Scanning Microscope JSM-5800LV/Jeol Serving Advanced Technology with 500X of increase.

2.8) Biochemical assays

Serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa β ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) were measured by enzyme linked immunosorbent assays specific for rat (Rat Bone Panel 3 – PTH and Osteocalcin; Rat Bone Panel 1 – osteoprotegerin; Rat Bone Panel 2 – RANK-L of the Millipore, Billerica, Massachusetts, USA; CTX-I of the Immunodiagnostic Systems Ltd., Arizona, USA). Serum total calcium and alkaline phosphatase (ALP) activity were measured by colorimetric method (Calcium CAT n° 00800 of the Laborlab – São Paulo / Brazil; alkaline phosphatase (ALP) activity LB 170123-800 of the Biodiagnostic – Paraná / Brazil) in Beckman Coulter DU-70 spectrophotometer (Beckman Coulter Inc., California, USA). All the biochemical assays were performed according to the manufacturer's instructions in duplicate. The methods sensitivity, intra-assay and inter-assay were respectively: PTH = 0.3 pg/mL, 3.5 CV% and 8.1 %CV; OC = 1.6 pg/mL, 2.9 %CV and 4.6 %CV; OPG = 2.3 pg/mL, 3.1 %CV and 3.4 %CV; RANk-L = 1.1 pg/mL, 3.1 %CV and 9.0 %CV; CTX-I = 2 ng/mL, 5.6 %CV and 10.5 %CV, ALP = 24 U/l, 1.2 %CV and 0.99 %CV.

2.9) Statistical methods

Data were analyzed by two-way ANOVA with a level of significance of 0.05 using GraphPad Prism 5 version (GraphPad Software, Inc.; La Jolla, CA, USA). Significant differences were determined among treatment groups with Tukey's multiple range test. All data are reported as means with their standard errors of the mean (SEM).

3) Results

3.1) Growth and feed intake

Initial and end-point body weight (Control group = 332.4 ± 1.8g; FOS group = 334.1 ± 1.8g; Inulin group = 321.1 ± 0.8g; FOS + Inulin group = 325.8 ± 2.1g) and daily feed intake (Control group = 15.6 ± 0.6g; FOS group = 15.5 ± 1.0g; Inulin group = 14.8 ± 0.7g; FOS + Inulin group = 14.8 ± 1.0g) during the entire experiment there were not statistical ($p<0.05$) difference between the groups.

3.2) Bone mass (DXA)

The FOS + Inulin group hipaxis BA was statistically ($p<0.05$) lower (8%) when compared with the control group and it was statistically ($p<0.05$) equal when compared with FOS and Inulin groups. However, the FOS and Inulin groups were statistically ($p<0.05$) equal with control group. The FOS group hip axis BMD was statistically ($p<0.05$) higher (8%) when compared with control group and it was statistically ($p<0.05$) equal than Inulin and FOS + Inulin groups. The prebiotics groups mandibles BA, BMC and BMD did not show statistical ($p<0.05$) differences when compared with control group. However, FOS + Inulin group was statistically ($p<0.05$) higher (39; 51; 8% respectively) than Inulin group. All DXA results are described in Table 2.

The coefficient of variation (CV%) for triplicate scans of bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) were 31.0, 26.4 and 7.4, respectively for the whole femur; 6.2, 10.6 and 6.0, respectively for the hipaxis; 6.0, 9.5 and 6.6, respectively for the femur diaphysis; and 17.0, 20.0 and 5.0, respectively for the mandible.

Table 2. Effect of the prebiotics FOS and/or Inulin on bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) in the *ex vivo* femur and mandible of aged female rats.

	Control group	**FOS group**	**Inulin group**	**FOS + Inulin group**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Hipaxis				
BA (cm^2)	0.31 ± 0.003^{a}	0.30 ± 0.004^{ab}	0.29 ± 0.010^{ab}	0.29 ± 0.005^{b}
BMC (g)	0.073 ± 0.002	0.076 ± 0.003	0.071 ± 0.003	0.071 ± 0.002
BMD (g/cm^2)	0.236 ± 0.005^{a}	0.255 ± 0.005^{b}	0.246 ± 0.005^{ab}	0.241 ± 0.005^{ab}
Diaphysis				
BA (cm^2)	0.44 ± 0.01	0.45 ± 0.01	0.42 ± 0.01	0.43 ± 0.01
BMC (g)	0.107 ± 0.002	0.116 ± 0.003	0.105 ± 0.004	0.110 ± 0.004
BMD (g/cm^2)	0.241 ± 0.003	0.259 ± 0.006	0.248 ± 0.005	0.255 ± 0.006
Mandible				
BA (cm^2)	0.54 ± 0.02^{ab}	0.53 ± 0.04^{ab}	0.44 ± 0.03^{a}	0.61 ± 0.02^{b}
BMC (g)	0.066 ± 0.004^{ab}	0.065 ± 0.005^{ab}	0.051 ± 0.003^{a}	0.077 ± 0.004^{b}
BMD (g/cm^2)	0.122 ± 0.002^{ab}	0.123 ± 0.002^{ab}	0.118 ± 0.002^{a}	0.127 ± 0.002^{b}

- Abbreviations: BA, bone area; BMC, bone mineral content, BMD, bone mineral density.
- Different letters mean statistical difference ($p<0.05$) between the groups.

3.3) Bone morphometry

FOS group femur neck width was statistically ($p<0.05$) higher (7%) when compared with control and Inulin group and it was statistically ($p<0.05$) equal FOS + Inulin group; however, FOS + Inulin group was also statistically ($p<0.05$) equal to control and Inulin group. These results are shown in Table 3.

Table 3. Effect of the prebiotics FOS and/or Inulin on femur morphometry of aged female rats.

Femur width	**Control group**	**FOS group**	**Inulin group**	**FOS + Inulin group**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Head (mm)	3.84 ± 0.04	3.79 ± 0.05	3.74 ± 0.08	3.72 ± 0.04
Neck (mm)	2.04 ± 0.02^{a}	2.19 ± 0.01^{b}	2.04 ± 0.03^{a}	2.12 ± 0.03^{ab}
Hip axis (mm)	7.63 ± 0.09	7.46 ± 0.06	7.42 ± 0.09	7.44 ± 0.07

- Different letters mean statistical difference ($p<0.05$) between the groups.

3.4) Bone biomechanic properties

FOS + Inulin group proportional limit was statistically ($p<0.05$) higher (7%) than control group and it was statistically ($p<0.05$) equal than FOS and Inulin groups; however, FOS and Inulin groups were also statistically ($p<0.05$) equal control group. All the femur biomechanic properties results are described in Table 4.

Table 4. Effect of the prebiotics FOS and/or Inulin on femur biomechanic properties of aged female rats.

	Control group	**FOS group**	**Inulin group**	**FOS + Inulin group**
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Proportional limit	81.3 ± 0.8[a]	84.7 ± 1.2[ab]	84.3 ± 1.7[ab]	87.2 ± 1.0[b]
Rupture limit	93.8 ± 1.1	93.8 ± 0.8	91.5 ± 2.6	95.7 ± 0.6
Tenacity	332.6 ± 32.7	401.2 ± 40.1	356.6 ± 22.8	327.4 ± 21.2
Rigidity	319.1 ± 36.3	395.1 ± 38.9	341.0 ± 27.8	324.7 ± 21.6
Resilience	11.6 ± 1.3	10.1 ± 1.6	10.4 ± 1.1	12.3 ± 1.0

- Different letters mean statistical difference ($p<0.05$) between the groups.

3.5) Bone histomorphometry

In FOS, Inulin and FOS + Inulin groups, the relative bone volume (BV/TV) was statistically ($p<0.05$) higher (54; 26; 36%, respectively) when compared with control group. FOS and Inulin groups Ob.S/BS were statistically ($p<0.05$) equal and lower (78; 118%, respectively) than control and FOS + Inulin groups while these last two groups were statistically ($p<0.05$) equal. Inulin group Oc.S/BS was statistically ($p<0.05$) lower (84%) when compared with all the other groups and these groups were statistically ($p<0.05$) equal. These histomorphometry results are showed in Figure 1 and Table 5.

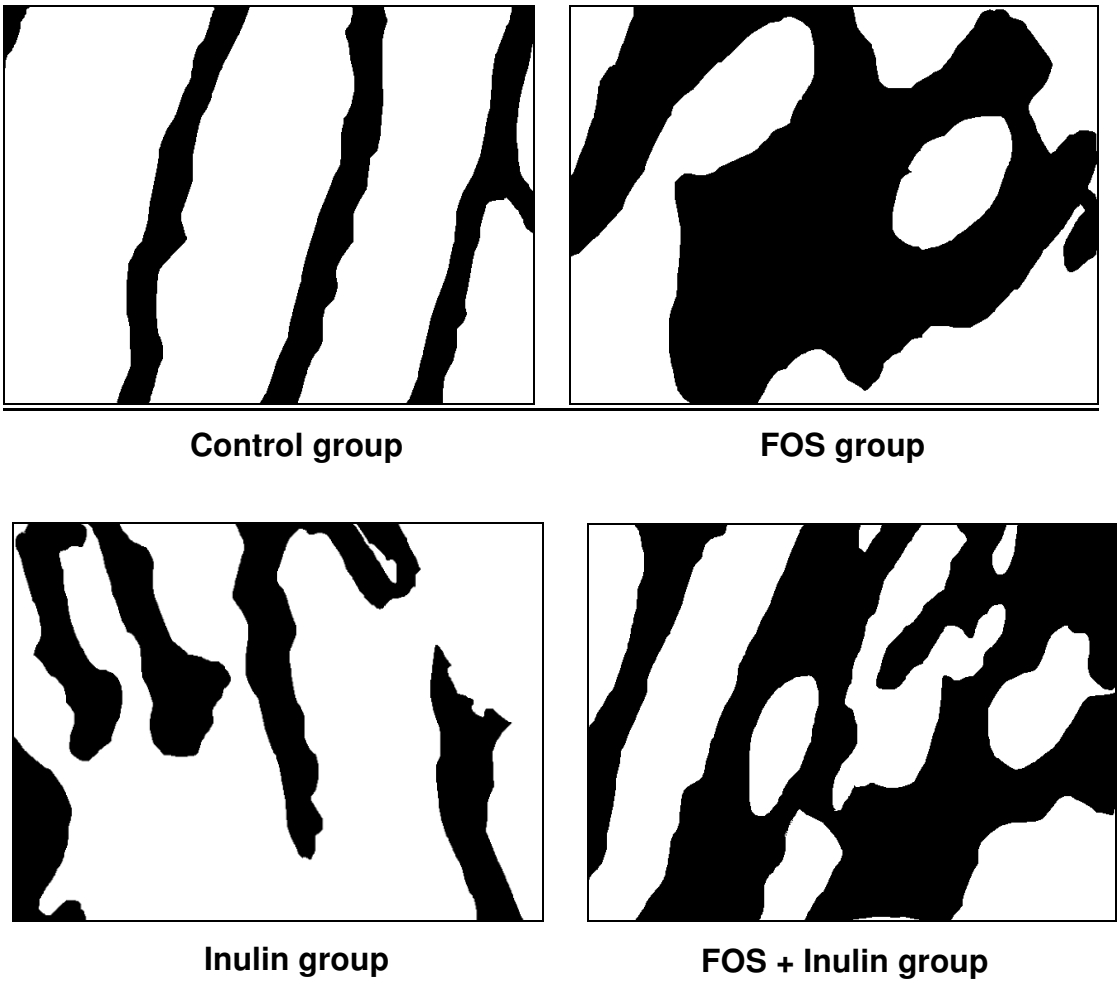

Figure 1. Example of photos used for histomorphometry showing the differences between trabecular volume (BV/TV) in the groups (Hematoxilin and eosin stain, 10 X 10 magnification)*.

* These pictures were transformed to 8 bit archive using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

Table 5. Effect of the prebiotics FOS and/or Inulin on proximal epiphysis tibiae histomorphometry parameters of aged female rats.

Histomorphometry parameters	**Control group** Mean ± SEM	**FOS group** Mean ± SEM	**Inulin group** Mean ± SEM	**FOS + Inulin group** Mean ± SEM
BV/TV (%)	34.6 ± 2.2[a]	53.2 ± 2.4[b]	43.6 ± 2.6[c]	47.2 ± 2.2[bc]
Ob.S/BS	26.9 ± 2.9[a]	13.6 ± 1.4[b]	11.1 ± 1.3[b]	21.6 ± 1.3[a]
Oc.S/BS	22.7 ± 2.8[a]	22.8 ± 3.0[a]	12.8 ± 1.6[b]	25.1± 2.3[a]

- Abbreviations: BV/TV = relative bone volume; Ob.S/BS = osteoblast-covered bone surface; Oc.S/BS = osteoclast-covered bone surface.
- Different letters mean statistical difference ($p<0.05$) between the groups.

3.6) Scanning electron microscopy (SEM)

Scanning electron microscopy of the proximal epiphysis of the right tibiae showed that the control group presented several and deep resorption lacunae inducing to a fragile bone when compared with prebiotics groups. The scanning electron microscopy images can be seen in Figure 2.

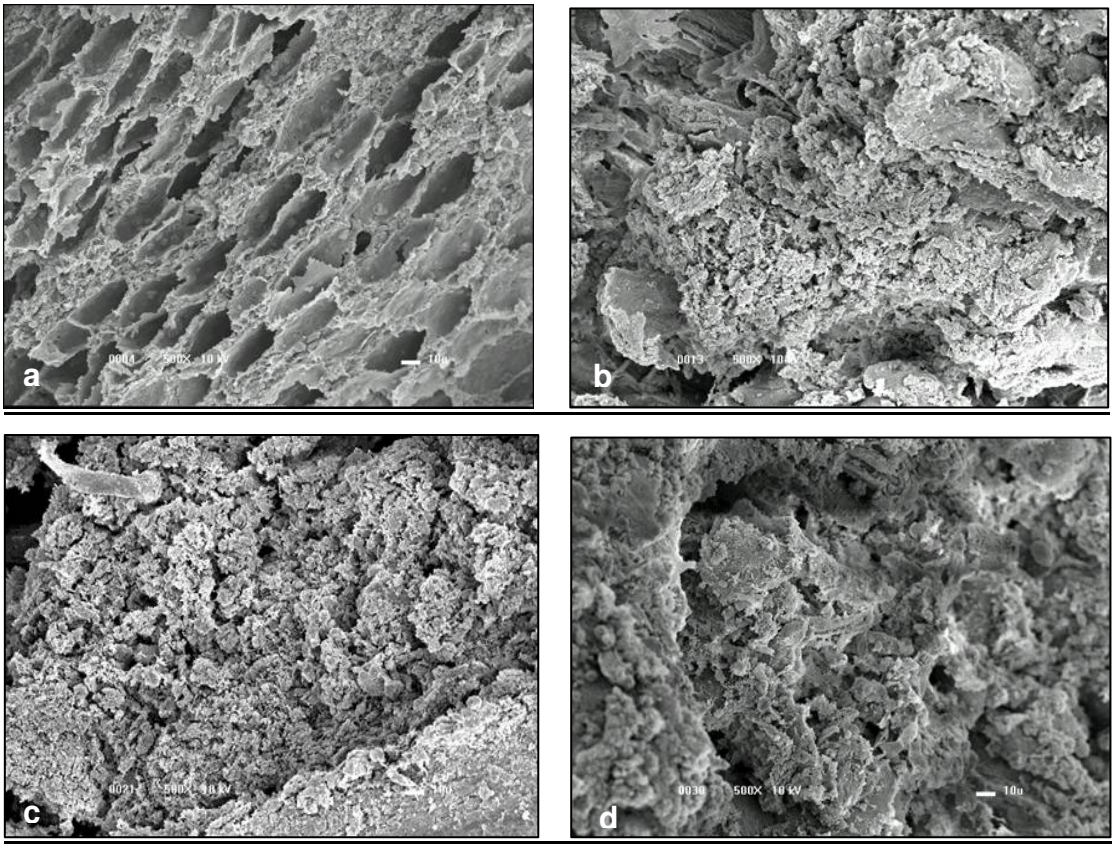

Figure 2. Image of the proximal epiphysis of the tibia scanning electron microscopy (SEM) with 500X of increase in aged female rats supplemented by prebiotics FOS and/or Inulin (a = control group; b = FOS group; c = Inulin group; d = FOS + Inulin group).

3.7) Serum markers of bone metabolism

FOS and FOS + Inulin groups serum PTH concentration and total calcium were statistically ($p<0.05$) equal and lower (approximately 99% and 34%, respectively) when compared with control group while inulin group was statistically ($p<0.05$) equal for all the groups (Figure 3 and Figure 8, respectively). The prebiotics groups serum OPG and RANk-L concentration were statistically ($p<0.05$) equal and lower (approximately 151 and 286%, respectively) when compared with control group (Figure 5 and 6). FOS + Inulin group ALP activity was statistically ($p<0.05$) lower (108%) than control group and it was statistically ($p<0.05$) equal when compared with FOS and Inulin groups (Figure 9). However, the FOS and Inulin groups were statistically ($p<0.05$) equal when compared with control group (Figure 10). All the groups serum OC and CTX-I did not show statistical ($p<0.05$) differences (Figure 4 and 7).

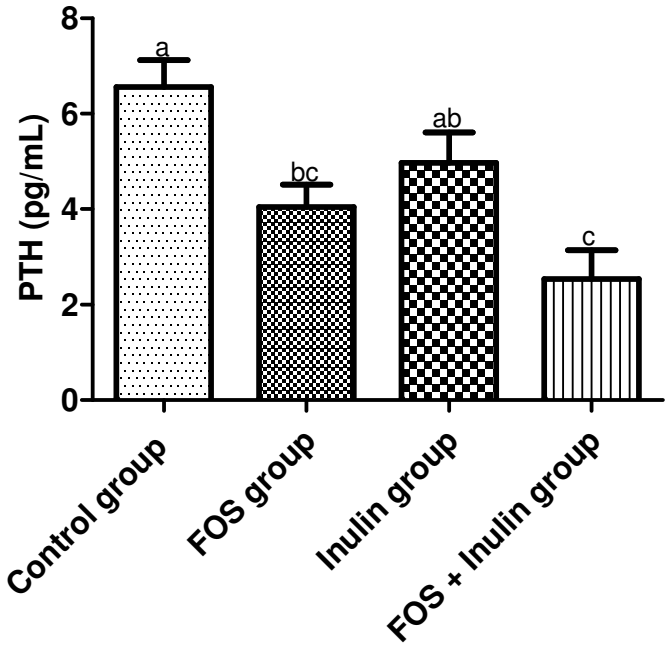

Figure 3. Effect of the prebiotics FOS and/or Inulin on serum parathyroid hormone (PTH) concentration of aged female rats.

- Different letters mean statistical difference ($p<0.05$) between the groups.

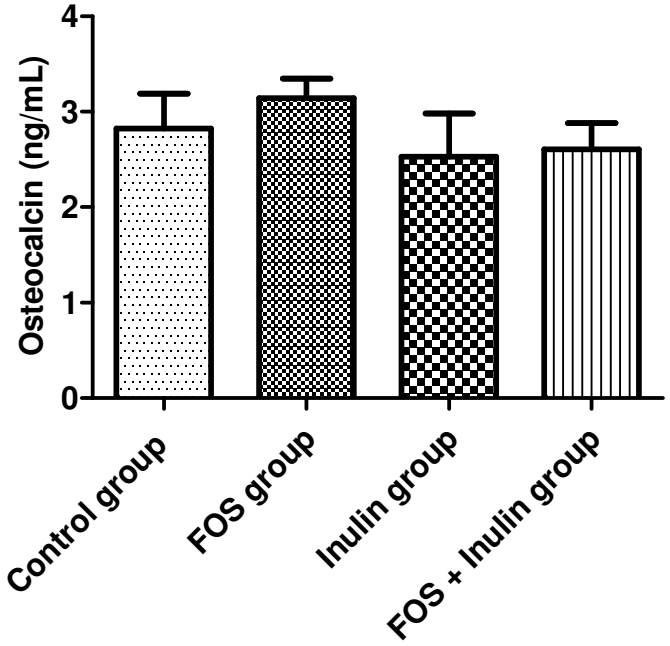

Figure 4. Effect of the prebiotics FOS and/or Inulin on serum osteocalcin (OC) concentration of aged female rats.

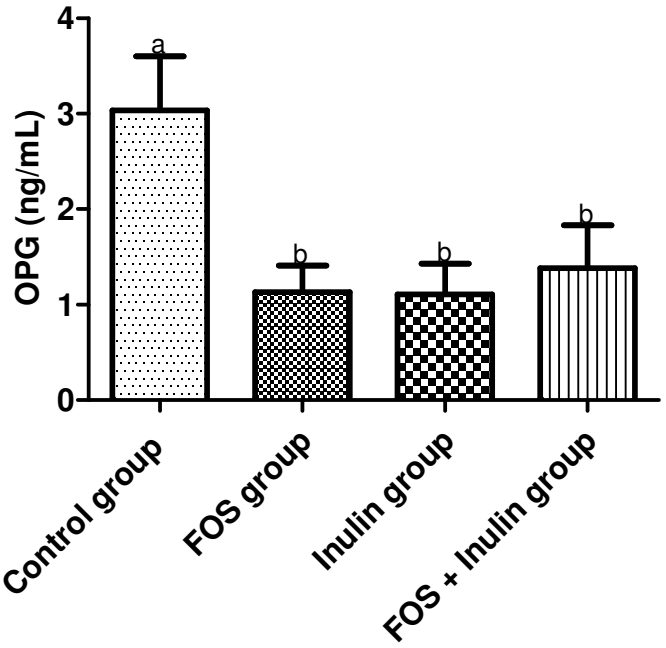

Figure 5. Effect of the prebiotics FOS and/or Inulin on serum osteoprotegerin (OPG) concentration of aged female rats.

- Different letters mean statistical difference ($p<0.05$) between the groups.

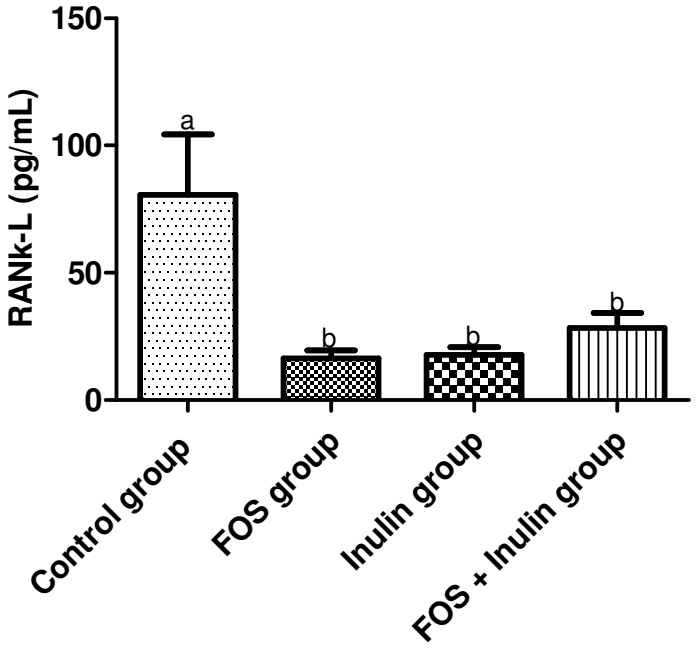

Figure 6. Effect of the prebiotics FOS and/or Inulin on serum receptor activator for nuclear factor κappa B ligand (RANk-L) concentration of aged female rats.

- Different letters mean statistical difference ($p<0.05$) between the groups.

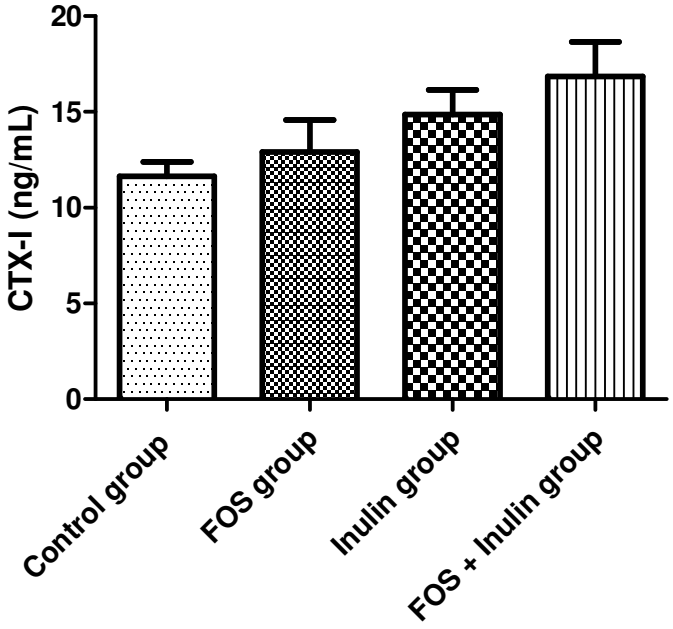

Figure 7. Effect of the prebiotics FOS and/or Inulin on serum bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) concentration of aged female rats.

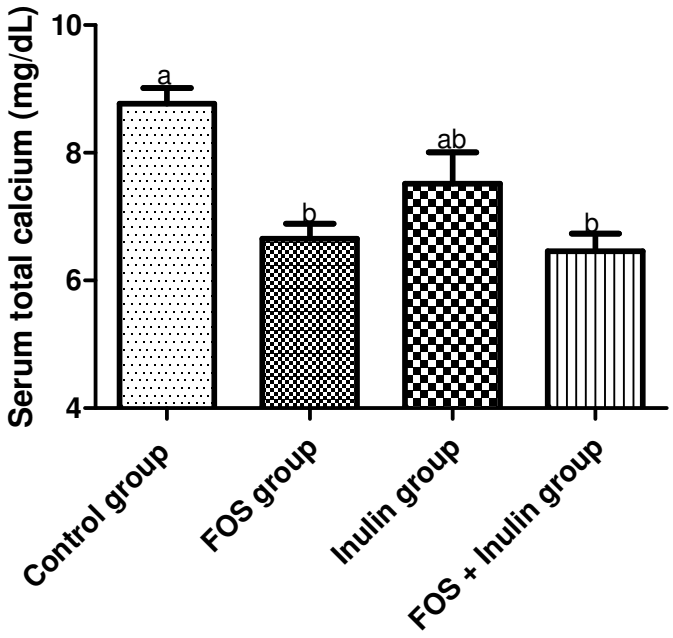

Figure 8. Effect of the prebiotics FOS and/or Inulin on serum total calcium concentration of aged female rats.

- Different letters mean statistical difference ($p<0.05$) between the groups.

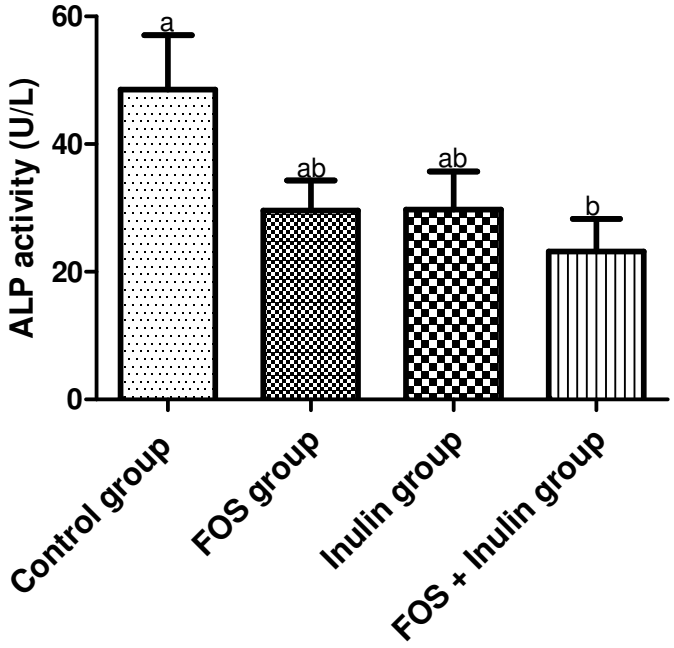

Figure 9. Effect of the prebiotics FOS and/or Inulin on serum alkaline phosphatase activity (ALP) of aged female rats.

- Different letters mean statistical difference ($p<0.05$) between the groups.

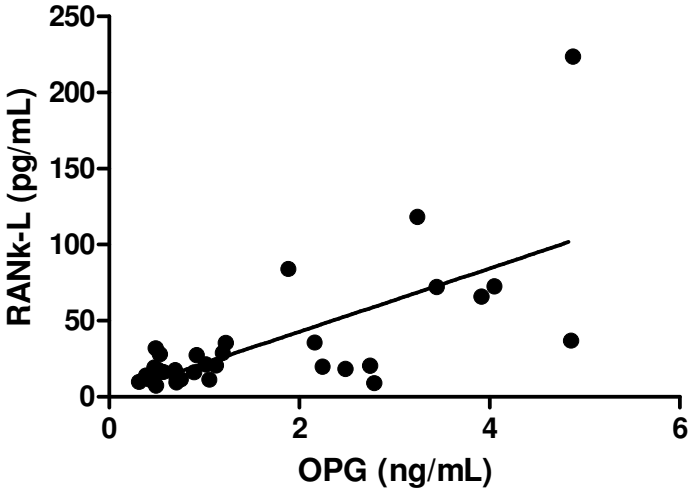

Figure 10. Correlation between serum osteoprotegerin (OPG) and receptor activator for nuclear factor κappa B ligand (RANk-L) in aged female rats supplemented with FOS and/or Inulin.

$r^2 = 0.4554$ ($P = 0.0001$)

4) Discussion

Our hipaxis BMD and femur neck width results showed that only FOS group had shown a positive effect on cancellous bone. DEVAREDDY et al. (2006) also described that ovariectomized rats supplemented by FOS had lumbar BMC and BMD and tibiae BMC increased. This positive effect for group treated with FOS (results of bone BMC, BMD and morphometry) is significant mainly for bone loss age related processes. The losses on bone density markedly increase the rates of fracture, giving rise to significant morbidity and some mortality (WORLD HEALTH ORGANIZATION, 2003). Moreover, the most serious osteoporotic fracture is that of the hip including femur neck. These fractures are typically result from falls or can occur spontaneously; they are usually painful and nearly always necessitate hospitalization. In many countries, hip fracture among women is a serious problem as it is very impactant - the number of hospital bed-days accounted is similar to that for cardiovascular disease, breast cancer and chronic obstructive pulmonary disease (WORLD HEALTH ORGANIZATION, 2003).

In our findings, the rupture limit did not show statistical difference ($p<0.05$) between the groups, however, this result is supported by AKKUS et al. (2004); these investigators did not observe statistical ($p<0.05$) difference on rupture limit of Sprague-Dawley female rats with 3-month old (younger group), 8-month old (middle-age) and 24-month old (older group); and KIEBZAK et al. (1988) did not observe statistical ($p<0.05$) change in the maximum breaking force required to fracture femurs with senescence too.

Considering the proportional limit results, the FOS + Inulin group was statistically ($p<0.05$) higher (7%) than control group. As AKKUS et al. (2004) observed a physiologic age related reduction on femur diaphysys proportional limit, our results suggest that FOS + Inulin together had a positive and synergistic effect on femur diaphysys end point

of the elastic region, differently from the groups that received the same prebiotics separately.

The relative bone volume (BV/TV) results found for FOS, Inulin and FOS + Inulin groups were statistically ($p<0.05$) higher (54; 26; 36%, respectively) when compared with control group. SCHOLZ-AHRENS et al. (1997) described previously similar results with treated ovariectomized rats supplemented by 5% of oligofructose for 8 and 16 weeks. They observed that the oligofructose conserved more trabecules than control group. Moreover, our results for BV/TV could also emphasize the biomechanics properties results described above (SCHOLZ-AHRENS & SCHREZENMEIR, 2002).

Despite our prebiotics groups had been statistically ($p<0.05$) equal and higher than control group for BV/TV analysis, the FOS and Inulin groups Ob.S/BS were statistically ($p<0.05$) equal and lower (78; 118%, respectively) than control and FOS + Inulin groups; and, Inulin group Oc.S/BS was statistically ($p<0.05$) lower (84%) when compared with all the other groups. The Ob.S/BS and Oc.S/BS results suggest that FOS and Inulin groups reduced the bone turnover age related on Wistar female rats. However, despite the fact that EASTELL et al. (1993) had described that in the normal 'coupled' bone system, a decrease in resorption is followed by a decrease in formation, in our study, it seems that the FOS group bone formation became temporarily 'uncoupled' from resorption. Some pharmacological treatments, such as intermittent doses of PTH, suggest their anabolic effect to an initial 'uncoupled' increase in bone formation (HOLLOWAY et al. 2007).

The histomorphometry results can be confirmed by scanning electron microscopy images once that the control group presented several and deep resorption lacunae inducing to a fragilized bone when compared with prebiotics groups and that both

analyzes were taking in the same portion of the bone (tibiae metaphysis cancellous bone).

Our findings showed that FOS and FOS + Inulin groups reduced the serum PTH and total calcium, however, the inulin prebiotic alone did not show the same effect. As some prebiotics are appointed by many investigators (ZAFAR et al. 2004; HOLLOWAY et al. 2007) as enhancer of intestinal calcium absorption and KARKKAINEN et al. (2001) described that calcium doses from 250mg can increase serum ionized calcium and decrease circulating PTH, we can suggest that FOS alone and FOS + Inulin prebiotics in this study reproduced a similar effect in aged female rats metabolism. HOLLOWAY et al. (2007) did not report a significant decrease in serum PTH in post-menopausal women (72.2 ± 6.4 years) treated with 5g per day of 1:1 mixture of chicory oligofructose (chicory fructan with a degree of polymerization average of 4 and 25) or placebo for 6 weeks despite the intestinal calcium absorption increased. However the same authors reported that PTH analisys in blood was taken after a fast of at least 10 h, minimizing the ability to detect the acute effect of Ca intake on circulating PTH level (REGINSTER et al. 2002).

Our prebiotics groups serum OPG and RANk-L concentration were statistically ($p<0.05$) lower when compared with control group and further theses values also showed a positive correlation ($r^2 = 0.4554$; $P = 0.0001$), therefore we suggest that FOS and Inulin separately or together could reduced the bone turnover in aged female rats. This suggestion is supported by KHOSLA et al. (2002) which these authors showed that serum OPG levels increased with age in both men and women and described that the age related OPG higher levels can be a compensatory phenomenon to slow down the enhanced bone resorption.

Further, the OPG and RANk-L results described above can be confirmed by FOS + Inulin group ALP activity found in our study, since only this group showed statistically ($p<0.05$) lower ALP activity average when compared with control group. NETTO AND MIYASAKA (2009) also showed a reduction on ALP activity in ovariectomized rats supplemented with 5% of FOS when compared control group. And, VIEIRA (2003) and YOSHIKUBO et al. (2005) confirmed our statement as they described an increase of total ALP activity when the bone remodeling is also increased.

In our study, all the groups did not show statistical ($p<0.05$) differences on serum OC and CTX-I (Figure 4 and 8). These results suggest that prebiotics supplementation were not sufficient to change both biomarkers in aged female rats. TAHIRI et al. (2003) also reported that 10g FOS daily for five weeks had no effect on plasma osteocalcin and urinary deoxypyridinoline excretion in postmenopausal women (50-70 years of age going through menopause for > 2 years). However, HOLLOWAY et al. (2007) supplemented fifteen postmenopausal women (72.2 ± 6.4 years) with 5g per day of 1:1 mixture of chicory oligofructose (chicory fructan with an average degree of polymerization of 4 and 25) for 6 weeks and showed an initial transient decrease at 3 weeks deoxypyridinoline cross-link levels in the mixture of chicory oligofructose group, but rebounded to levels greater than baseline for 6 weeks of treatment. Further, serum osteocalcin was increased at 6 weeks of treatment with a mixture of chicory oligofructose group too. Although TAHIRI et al. (2003) and HOLLOWAY et al. (2007) had analyzed deoxypyridinoline cross-links and in our study we had analyzed serum CTX-I, both are considered bone resorption biomarkers.

Despite consumption of FOS alone or with inulin had preserved bone mass and reduced bone turnover, more studies are required to establish mechanisms of action.

5) Acknowledgments

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6) References

AKKUS, O.; ADAR, F.; SCHAFFLER M. B. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. **Bone**. New York, v.34, p.443– 453, Mar. 2004.

DEVAREDDY, L.; KHALIL, D. A.; KORLAGUNTA, K.; HOOSHMAND, S.; BELLMER, D. D.; ARJMANDI, B. H. The effects of fructo-oligosaccharides in combination with soy protein on bone in osteopenic ovariectomized rats. **Menopause**. New York, v.13, n.4, 692-699, Jul.-Aug. 2006.

EASTELL, R.; ROBINS, S. P.; COLWELL, T.; ASSIRI, A. M.; RIGGS, B. L.; RUSSELL, R. G. Evaluation of bone turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption. **Osteoporosis International**. London, v.3, n.5, p.255–260, Sep. 1993.

HOLLOWAY, L.; MOYNIHAN, S.; KENT, K.; HSU, A. R.; FRIEDLANDER, A. L. Effects of oligofructose enriched inulin on mineral absorption and markers of bone turnover in postmenopausal women. **The British Journal of Nutrition**. New York, v.97, n.2, p.365–372, Feb. 2007.

JAMIESON, J. A.; RYZ, N. R.; TAYLOR, C. G.; WEILER, H. A. Dietary long-chain inulin reduces abdominal fat but has no effect on bone density in growing female rats. **The British Journal of Nutrition**. New York, v.100, n.2, p.451-459, Aug. 2008.

KARKKAINEN, M. U.; LAMBERG-ALLARDT, C. J.; AHONEN, S.; VALIMAKI, M. Does it make a difference how and when you take your calcium? The acute effects of calcium on calcium and bone metabolism. **The American Journal of Clinical Nutrition**. Bethesda, v.74, n.3, n.335–342, Sep. 2001.

KHOSLA, S.; ARRIGHI, H. M.; MELTON, L. J; ATKINSON, E. J.; O'FALLON, W. M.; DUNSTAN, C.; RIGGS, B. L. Correlates of osteoprotegerin levels in women and men. **Osteoporosis International**. London, v.13, n.5, p.394-399, May. 2002.

KIEBZAK, G. M.; SMITH, R.; HOWE, J. C.; GUNDBERG, C. M.; SACKTOR, B. Bone status of senescent female rats: chemical, morphometric, and biomechanical analyses. **Journal of Bone and Mineral Research**. Washington, v.3, n.4, p.439-46, Aug. 1988.

NETTO, C. C.; MIYASAKA, C. K. Fructooligosaccharides (FOS) and Hormonal Replacement Therapy (HRT) by estrogen suppressed bone resorption in the ovariectomized rat. **Journal of Medical and Biological Science**. Salvador, 2009 [Epub ahead of print].

PARFITT, A. M; DREZNER, M. K.; GLORIEUX, F. H; KANIS, J. A.; MALLUCHE, H.; MEUNIER, P. J.; OTT, S. M.; RECKER. R. R. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. **Journal of Bone and Mineral Research**. New York, v.2, n.6, p.595-610, Dec. 1987.

PARFITT, A. M. Bone histomorphometry: proposed system for standardization of nomenclature, symbols, and units. **Calcified Tissue International**. New York, v.42, n.5, p.284-6, May. 1988.

REEVES, P. G.; NIELSEN, F. H.; FAHEY, G. C. JR. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **The Journal of Nutrition**. Bethesda, v.123, n.11, p.1939-1951, Nov. 1993.

REGINSTER, J. Y.; ZEGELS, B.; LEJEUNE, E.; MICHELETTI, M. C.; KVSAZ, A.; SEIDEL, L.; SARLET, N. Influence of daily regimen calcium and vitamin D

supplementation on parathyroid hormone secretion. **Calcified Tissue International**. New York, v.70, n.2, p.78–82, Feb. 2002.

ROBERFROID, M. B. Introducing inulin-type fructans. **The British Journal of Nutrition**. New York, v.93, Suppl.1, p.13–25, Apr. 2005.

ROBERFROID, M. B. Prebiotics: The Concept Revisited. **The Journal of Nutrition**. Bethesda, v.137, Suppl. 2, p.830-837, Mar. 2007.

SCHOLZ-AHRENS, K. E.; ACIL, Y.; SCHREZENMEIR, J. Effect of oligofructose or dietary calcium on repeated calcium and phosphorus balances, bone mineralization and trabecular structure in ovariectomized rats. **The British Journal of Nutrition**. New York, v.88, p.365–377, Oct. 2002

SCHOLZ-AHRENS, K. E.; HEIN, A.; BOBMANN, K. Quantification of bone structure as parameter of calcium bioavailability. In: University of Wageningen, editor. **Bioavailability, Proceedings of the International Symposium on Bioavailability**. Netherland: Agricultural University of Wageningen, 1997. p.124.

TAHIRI, M.; TRESSOL, J. C.; ARNAUD, J.; BORNET, F. R. J.; BOUTELOUP-DEMANGE, C.; FEILLET-COUDRAY, C.; BRANDOLINI, M.; DUCROS, V.; PÉPIN, D.; BROUNS, F.; ROUSSEL, A. M.; RAYSSIGUIER, Y.; COUDRAY, C. Effect of short chain fructooligosaccharides on intestinal calcium absorption and calcium status in postmenopausal women: a stable-isotope study. **The American Journal of Clincal Nutrition**. Bethesda, v.77, p.449-457, Feb. 2003.

VASHISHTH, D. Small animal bone biomechanics. **Bone**. New York, v.43, n.5, p.794-797, Nov. 2008.

VIEIRA, J. G. H. **Bone metabolics illnesses**. São Paulo (SP): Sonopress-Rimo, 2003. 1 CD-ROM.

WONG, J. M. W.; JENKINS, D. J. A. Carbohydrate digestibility and metabolic effects. **The Journal of Nutrition**. Bethesda, v.137, Suppl.11, p.2539-2546, Nov. 2007.

WONG, J. M. W.; SOUZA, R.; KENDALL, C. W. C.; EMAM, A.; JENKINS, D. J. A. Colonic Health: Fermentation and short chain fatty acids. **Journal of Clinical Gastroenterology**. New York, v.40, n.3, p.235-243, Mar. 2006.

WORLD HEALTH ORGANIZATION. **Prevention and management of osteoporosis prevention**. Technical Report Series n° 921. Geneva, 2002.

YOSHIKUBO, H.; SUZUKI, N.; TAKEMURA, K.; HOSO, M.; YASHIMA, S.; IWAMURO, S.; TAKAGI, Y.; TABATA, M. J.; HATTORI, A. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. **Life Science**. Amsterdam, v.76, n.23, p.2699-2709, Apr. 2005.

ZAFAR, T. A.; WEAVER, C. M.; ZHAO, Y.; MARTIN, B. R.; WASTNEY, M. E. Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. **The Journal of Nutrition**. Bethesda, v.134, n.2, p.399-402, Feb. 2004.

CAPÍTULO 5

EFFECTS OF THE FRUCTOOLIGOSACCHARIDES (FOS) IN THE AGED FEMALE RATS BONE LOSS

Claudia Cardoso Netto[1,3], Rosângela Santos[2], Gláucia Pastore[2], Sherry Agellon[3], Hope Weiler[3], Mário Roberto Maróstica JR[1*]

[1]Food and Nutrition Department / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

*Corresponding author e-mail: mario@fea.unicamp.br

Phone +55 19 3521-4078, Fax +55 19 3521-4060

[2]Department of Food Science / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

This study aimed to evaluate the role of prebiotic fructooligosaccharides (FOS) on the age related bone loss in female rats. Twenty (10 month-old) female Wistar rats weighing approximately 300-320g were randomly assigned to two diet treatment groups fed *ad libitum* for 3 months. Both diets were isocaloric and equal in Ca (5mg/kg); however, FOS group received 5% FOS (Corn Products) and 5% sucrose and control group received 10% sucrose. The analyses performed were: bone dual-energy X-ray absorptiometry scans (DXA), morphometry and histomorphometry; biochemical assays: serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa B ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I). Data were analyzed by Student t-test ($p<0.05$) and all data are reported as mean plus or minus standard error (SEM). L-3 spine BMD, femur length, head, neck and hipaxis width, relative bone volume (BV/TV), osteoclastic-covered bone surface (Oc.S/BS) and CTX-I were statistically ($p<0,05$) highest in FOS group; PTH was statistically ($p<0.05$) lowest in FOS group; and, osteoblast-covered bone surface (Ob.S/BS), OC, OPG and RANk-L were not statistically different ($p<0,05$) between the groups. The FOS improved spine BMD, morphometric parameters and bone turnover, and it reduced serum PTH. Despite the fact that consumption of FOS showed a bone mass preservation, more studies are required to establish mechanisms of action.

Keywords: Prebiotics; fructooligosaccharides; age related bone loss; osteoporosis.

1) Introduction

In the 1980s Japanese people began to realize the importance of maintaining and improving their health with a gradual increase in the occurrence of lifestyle and dietary related diseases and the Government also started to pay more attention to the aging population. Concurrently, intensive studies were performed on the physiological effects of various foods and their constituents the so called 'the tertiary function of foods'. In brief, the tertiary function of foods is directly involved in the modulation of the human physiological systems such as the immune, endocrine, nerve, circulatory as well as digestive systems, while the primary and secondary functions are related to nutrition and sensory satisfaction, respectively (OHAMA et al. 2006).

In 1984 the term 'functional foods' was first assigned in the project initiated by the Ministry of Education (presently as The Ministry of Education, Culture, Sports, Science and Technology) and this concept attracted the 'health foods' industry and health-conscious consumers. The Government, however, prohibited the use of the term 'functional' because of its implication as a drug-like effect. As a result, the term 'health foods' was widely used and recognized by consumers and the concept of the 'functional foods' was ultimately integrated into the 'foods for specified health uses' (FOSHU) system (JONES, 2002; OHAMA et al. 2006).

Many specified health claim ingredients called prebiotics are been designed for clinical management of disease states in nutritional supplements. ROBERFROID (2007) described prebiotics as "non-digestible oligosaccharides (NDO) selectively fermented by intestinal bacterials that are bifidogenic agents that specifically promote modulation of the microbiota by changing its composition and/or activity, with health benefit on the host associated.

Prebiotics ingredients can promote health benefits as: improving colonic microflora composition and intestinal function, stimulating calcium intestinal absorption and other minerals, up regulating the gastrointestinal peptides production, improving the immunity system and resistance to infections and reducing the risk of obesity and osteoporosis (WONG & JENKINS, 2007).

Despite some investigators have shown the benefits prebiotics in some osteoporosis models (MATHEY et al. 2004; NETTO & MIYASAKA, 2009), their effects have not been clearly tested to bone mass and its age related changes. Thus, this study aimed to evaluate the role of prebiotic fructooligosaccharides (FOS) on age related bone loss in female rats.

2) Materials and Methods

2.1) Animals and diets

Twenty (10 month-old) female Wistar rats weighing approximately 300-320g were obtained from the Center of Multidisciplinary Investigation in Biology (CEMIB) of the University of Campinas (São Paulo, Brazil). The animals were randomly assigned to two treatment dietary groups and fed *ad libitum* for 3 months. All diets were based on the AIN-93M formulation (REEVES et al. 1993) (Table 1), isocaloric and equal in Ca (5mg/kg). The fructooligossaccharides (FOS) used were supplied by Corn Products. Five animals were housed per cage in a temperature (21–24˚C) and humidity (55%) controlled room with a light:dark cycle of 12:12 h. At the end of the experimental period the animals were fasted overnight before being euthanized. The experimental protocol was approved by Animal Experimental Ethics Committee of the University of Campinas (Protocol nº 1561-1).

Table 1. Diets formulated composition based in AIN-93M (REEVES et al. 1993).

Ingredients	Control diet (g/Kg of diet)	FOS diet (g/Kg of diet)
Cornstarch	465.692	465.692
Casein (≥85% protein)	140.000	140.000
Dextrinized cornstarch (90-94% tetrasaccharides)	155.000	155.000
Sucrose	100.000	50.000
FOS	-	50.000
Soybean oil	40.000	40.000
Cellulose	50.000	50.000
Mineral mix (AIN-93M-MX)	35.000	35.000
Vitamin mix (AIN-93-VX)	10.000	10.000
L-Cystine	1.800	1.800
Choline bitartrate (41.1% choline)	2.500	2.500
Tert-butylhydroquinone	0.008	0.008

2.2) Tissue collection

All animals were killed by guillotine and trunk blood was collected and stored on ice until centrifuged to obtain serum (3.000 rpm / 10 min) (FANEM Excelsa Baby I – model 206 / São Paulo - Brazil). Serum samples were stored at -80˚C until analysis. The bones were collected and stored at -20˚C until analysis.

2.3) Dual-energy X-ray absorptiometry scans (DXA)

Femur (whole, hipaxis and diaphysis), spine (L1-4 and L-3 alone) and mandible were scanned for bone area (BA), bone mineral content (BMC), bone mineral density (BMD) *ex vivo* in a water bath by dual-energy X-ray absorptiometry (DXA, 4500A; Hologic Inc., Bedford, MA, USA; small animal software high resolution option) (JAMIESON et al. 2008). Subregion analysis was used to assess hipaxis defined as the distance from the lateral border of the femur along the central axis of the femoral neck to the medial pelvic wall; and diaphysis defined as mid section of the long bone compound for cortical bone. The coefficients of variation (CV %) for triplicate scans of BA, BMC and BMD were 6.6, 9.7 and 4.9, respectively for the whole femur; 6.3, 10.8 and 8,0, respectively for the hipaxis; 9.3, 8.4 and 6.6, respectively for the femur diaphysis; and 6.7, 8.2 and 3.7, respectively for the mandible.

2.4) Bone morphometry

Following *ex vivo* in a water bath DXA analysis, right and left femurs were excised and thoroughly cleaned of soft tissue. Morphometric measures were taken with digital calipers as previously described (JAMIESON et al. 2008) and recorded to the nearest 0·01 mm. All measures were reproduced in triplicate by the same trained examiner and

included wet weight, length, femoral head, neck and hip axis, diaphysis and distal epiphysis width.

2.5) Bone histomorfometry

The left tibiae from each animal were excised at death, and the surrounding soft tissue was cleaned off. After cleaned, tibiae were maintained 48 hours in formol solution (10%), demineralizated and submitted to routine hystologic procedures. Then, the tibiae were included in histological paraffin (Merck Chemicals / Darmstadt – Germany), the paraffin blocks were sliced on 5 µm sections, put in laminas and submitted to routine hystologic procedures using hematoxilin and eosin to stain. The images were taken in the left tibiae metaphysis cancellous bone with 10 X 10 magnification with a digital camera COHU 2700 (Cohu Inc. Electronics Division, California, Unites States) coupled a light microscope Nikon Eclipse H550S (Nikon Corporation, Tokyo, Japan) following, quantification in accordance with nomenclature described previously (PARFFIT et al. 1987; PARFFIT, 1988) using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

2.6) Biochemical assays

Serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator of nuclear factor κappa B ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) were measured by enzyme linked immunosorbent assays specific for rat (Rat Bone Panel 3 – PTH and Osteocalcin; Rat Bone Panel 1 – osteoprotegerin; Rat Bone Panel 2 – RANK-L of the Millipore, Billerica, Massachusetts, United States; CTX-I of the Immunodiagnostic

Systems Ltd., Arizona, Unites States). All the biochemical assays were performed according to the manufacturer's instructions in duplicate. The methods sensitivity, intra-assay and inter-assay were respectively: PTH = 0.3 pg/mL, 3.5 CV% and 8.1 %CV; OC = 1.6 pg/mL, 2.9 %CV and 4.6 %CV; OPG = 2.3 pg/mL, 3.1 %CV and 3.4 %CV; RANk-L = 1.1 pg/mL, 3.1 %CV and 9.0 %CV; CTX-I = 2 ng/mL, 5.6 %CV and 10.5 %CV.

2.7) Statistical methods

Data were analysed by Student t-test with a level of significance of 0.05, using GraphPad Prism 5 version (GraphPad Software, Inc.; La Jolla, CA, USA). All data are reported as means with their standard errors of the mean (SEM).

3) Results

3.1) Feed intake and growth

The end-point body weight was statistically ($p<0.05$) higher in FOS group than control group (FOS group = 317.4 ± 2.5g; control group = 302.3 ± 3.4g), however, the daily feed intake during all the experimental period was not different (FOS group = 13.1 ± 0.8g; control group = 11.8 ± 0.7g).

3.2) Bone mass (DXA)

The experimental groups did not show statistical difference ($p<0.05$) in BA, BMC and BMD results for whole femur, hip axis, diaphysis, L1-4 spine and mandible. However, FOS group L-3 spine BMD was statistically higher (10%) than control group. DXA results are shown in Table 2.

Table 2. Effect of FOS on bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) in the femur, spine and mandible *ex vivo* of aged female rats.

	Control group	**FOS group**
	Mean ± SEM	Mean ± SEM
Whole femur		
BA (cm^2)	1.88 ± 0.04	1.87 ± 0.03
BMC (g)	0.46 ± 0.01	0.47 ± 0.01
BMD (g/cm^2)	0.242 ± 0.003	0.250 ± 0.004
Hipaxis		
BA (cm^2)	0.304 ± 0.005	0.306 ± 0.007
BMC (g)	0.078 ± 0.002	0.079 ± 0.003
BMD (g/cm^2)	0.250 ± 0.007	0.249 ± 0.006
Diaphysis		
BA (cm^2)	0.455 ± 0.002	0.454 ± 0.005
BMC (g)	0.112 ± 0.003	0.111 ± 0.003
BMD (g/cm^2)	0.242 ± 0.005	0.245 ± 0.005
Spine (L1-4)		
BA (cm^2)	0.357 ± 0.03	0.368 ± 0.02
BMC (g)	0.043 ± 0.003	0.050 ± 0.004
BMD (g/cm^2)	0.123 ± 0.003	0.132 ± 0.003
Spine (L3)		
BA (cm^2)	0.134 ± 0.007	0.146 ± 0.013
BMC (g)	0.017 ± 0.001	0.020 ± 0.002
BMD (g/cm^2)*	0.117 ± 0.003[a]	0.129 ± 0.003[b]
Mandible		
BA (cm^2)	0.59 ± 0.01	0.61 ± 0.01
BMC (g)	0.071 ± 0.002	0.076 ± 0.001
BMD (g/cm^2)	0.122 ± 0.002	0.124 ± 0.001

- Abbreviations: BA, bone area; BMC, bone mineral content, BMD, bone mineral density.
- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, $P = 0.01$

3.3) Bone morphometry

Femur morphometry as length, head, neck and hip axis width were statistically ($p<0.05$) higher (3; 6; 11 and 2%, respectively) in FOS group than control group. These results can be seen in Table 3.

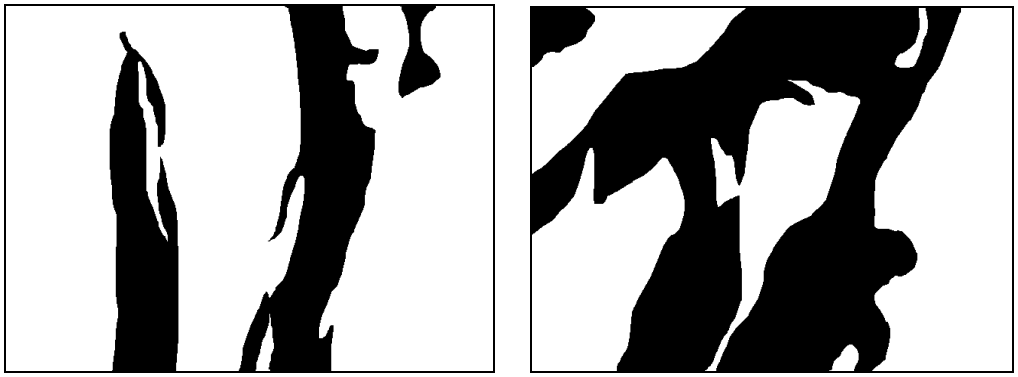
Table 3. Effect of the FOS on femur morphometry measurements of aged female rats.

Femur measurements	**Control group**	**FOS group**
	Mean ± SEM	Mean ± SEM
Wet dry weight (mg)	689.3 ± 8.2	730.4 ± 22.3
Length (mm)*	33.77 ± 0.22[a]	34.64 ± 0.08[b]
Head (mm)**	3.93 ± 0.07[a]	4.15 ± 0.05[b]
Neck (mm)***	2.30 ± 0.05[a]	2.55 ± 0.07[b]
Hipaxis (mm)**	7.49 ± 0.03[a]	7.66 ± 0.06[b]
Diaphysis (mm)	4.26 ± 0.04	4.30 ± 0.06
Distal epiphysis (mm)	5.61 ± 0.06	5.66 ± 0.07

- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* values: *, *P* = 0.002; **P* = 0.02; ***P* = 0.01.

3.4) Bone histomorfometry

Relative bone volume (BV/TV) and osteoclastic-covered bone surface (Oc.S/BS) were statistically ($p<0.05$) highest (33 and 62%, respectively) in the FOS group; however, osteoblast-covered bone surface (Ob.S/BS) was statistically equal in both groups. These results are showed in Figure 1 and Table 4.



Control group **FOS group**

Figure 1. Example of photos used for histomorphometry showing the differences between trabecular volume (BV/TV) in the groups (Hematoxilin and eosin stain, 10 X 10 magnification)*.

*These pictures were transformed to 8 bit archive using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

Table 4. Effect of the FOS on proximal epiphysis tibiae histomorphometry parameters of aged female rats.

Parameters	**Control group**	**FOS group**
	Mean ± SEM	Mean ± SEM
BV/TV (%)*	35.6 ± 2.1[a]	47.4 ± 3.2[b]
Ob.S/BS	12.3 ± 2.0	14.2 ± 2.1
Oc.S/BS*	8.5 ± 0.9[a]	13.8 ± 1.6[b]

- Abbreviations: BV/TV = relative bone volume; Ob.S/BS = osteoblast-covered bone surface; Oc.S/BS = osteoclast-covered bone surface.
- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, *P* = 0.01;

3.5) Biochemical assays

Serum PTH was statistically ($p<0.05$) lowest (29%) in FOS group (Figure 2) while serum CTX-I was statistically ($p<0.05$) highest in the same group (Figure 6). Osteocalcin (OC), OPG and RANk-L did not show statistical difference ($p<0.05$) between the groups and these assays results are shown in Figure 3, 4 and 5, respectively.

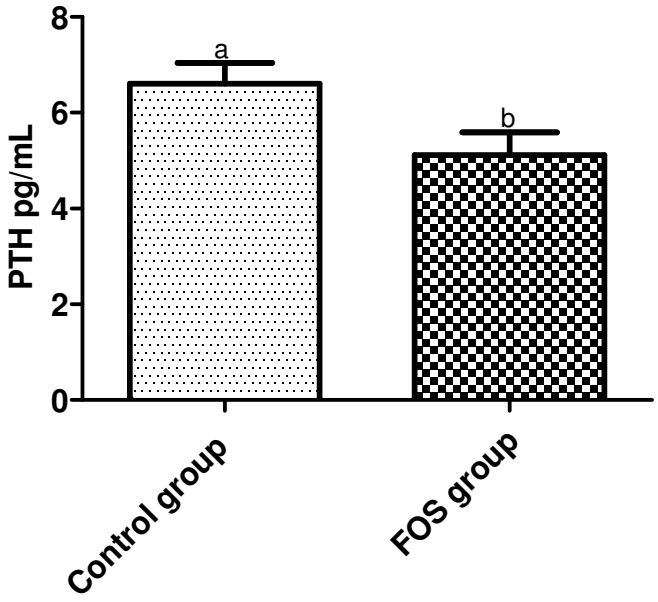


Figure 2. Effect of the FOS on serum parathyroid hormone (PTH) in aged female rats*.

- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, *P* = 0.04

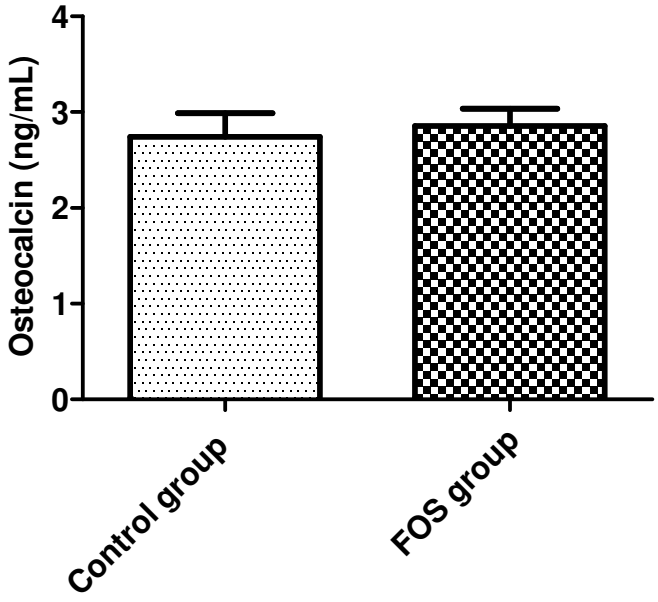


Figure 3. Effect of the FOS on serum osteocalcin (OC) in aged female rats.

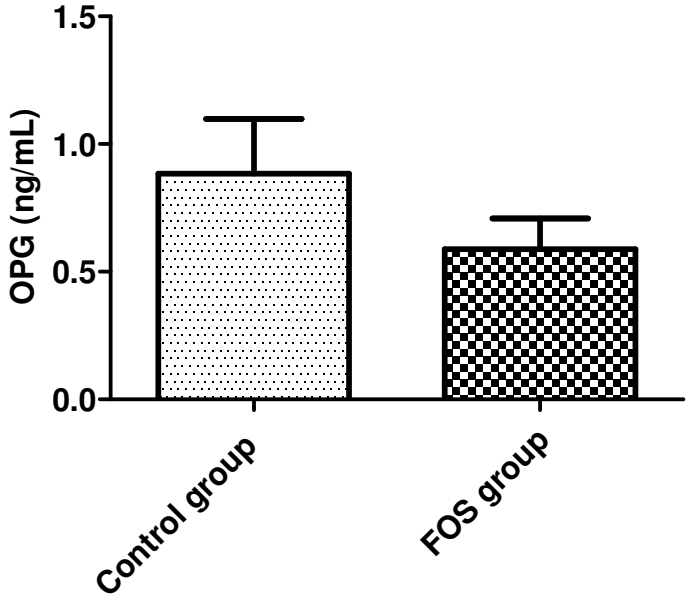


Figure 4. Effect of the FOS on serum osteoprotegerin (OPG) in aged female rats.

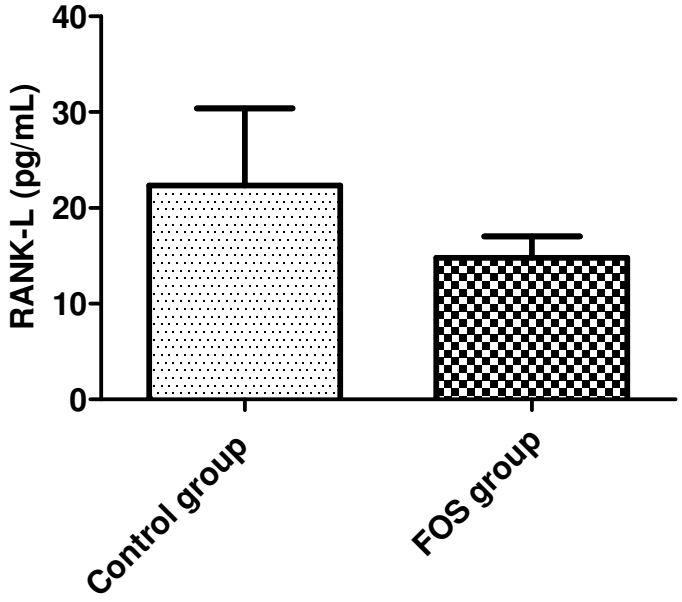


Figure 5. Effect of the FOS on serum nuclear factor kappa B ligant (RANk-L) in aged female rats.

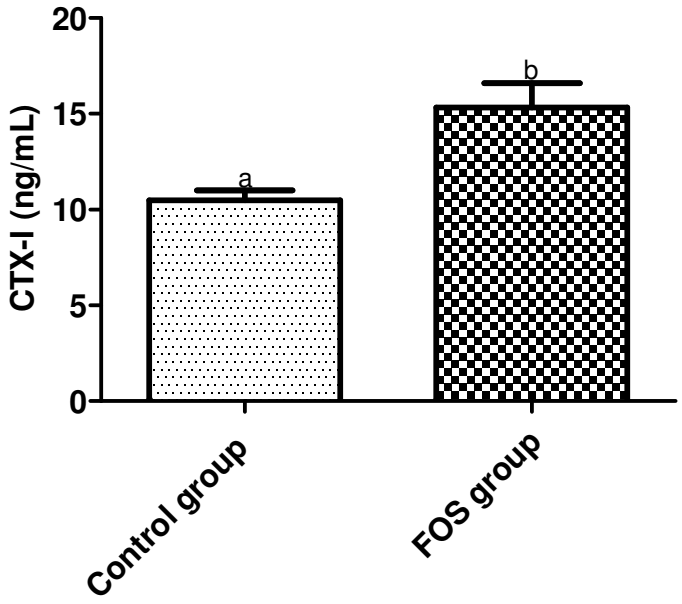


Figure 6. Effect of the FOS on serum bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) in aged female rats*.

- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, $P = 0.002$

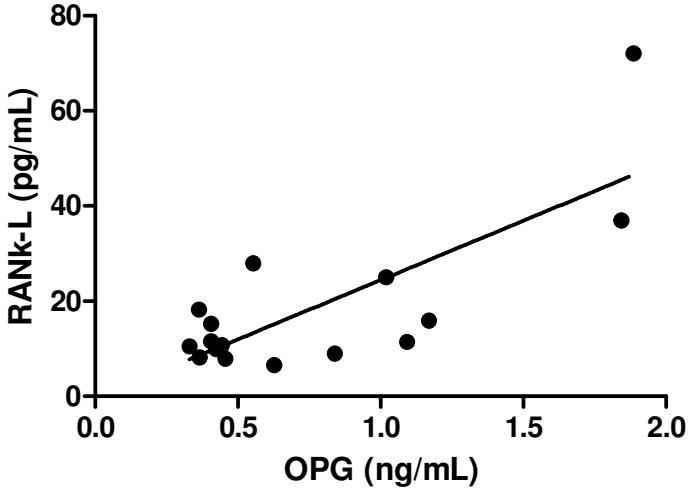


Figure 7. Effects of the FOS on serum OPG and RANk-L correlation in older female rats.

r^2 = 0,5898 (*P* = 0.0005)

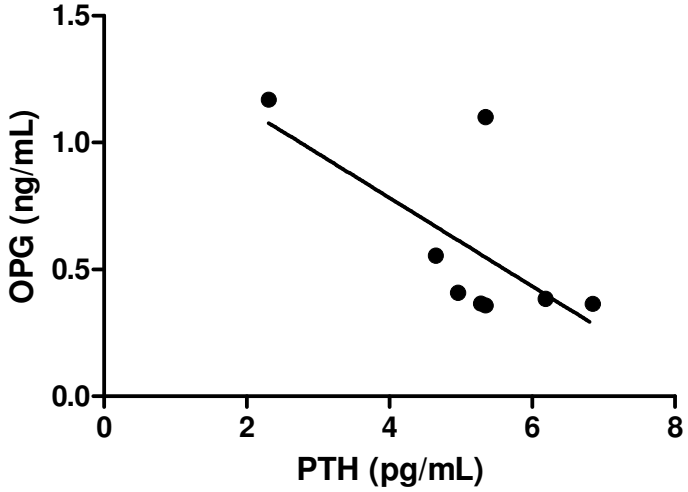


Figure 8. Effects of the FOS on serum PTH and OPG correlation in aged female rats.

$r^2 = 0.4554$ ($P = 0.0664$)

4) Discussion

The daily feed intake during the entire experimental period was not different between the groups. Moreover, MATHEY et al. (2004) did not find differences ($p<0.05$) in fatty mass and lean mass (g/100 g body weight) when compared ovariectomized rats receiving or not supplement of FOS in different concentrations. Thus, the end-point body weight had been highest ($p<0.05$) in FOS group could be explained because of bone alterations by the FOS supplement and will be discussed later.

Our DXA and morphometry results suggest that FOS supplementation could lead to protective effects in the cancellous bone mass (L-3 spine BMD; head, neck and hip axis width results). These results are relevant as that the most common osteoporotic fractures in postmenopausal and older people are those of the hip, vertebrae and forearm (WORLD HEALTH ORGANIZATION, 2003). Our results are supported by SCHOLZ-AHRENS et al. (2002) and DEVAREDDY et al. (2006); furthermore, SCHOLZ-AHRENS et al. (2002) showed that 7- and 9-month aged ovariectomized rats feeding with oligofructose for 8 and/or 16 weeks effectively prevented ovariectomy-induced loss of cancellous structure and DEVAREDDY et al. (2006) described that ovariectomized rats supplemented with by FOS had lumbar BMC and BMD and tibiae BMC increased.

Our results for the cortical bone (femur diaphysis BA, BMC and BMD; diaphysis width results) are not in agreement with the literature. Similar age related cancellous bone loss was also showed in Sprague-Dawley female rats by LI et al. (1991); these authors observed that the peak bone mass is maintained without significant changes until 8-months of age. Thereafter, bone mass begins to decrease with aging and reaches a low value of 14% of the metaphyseal tissue area at 12 months of age. Both results can be explained because in adult's animals, the cortical bone remodeling

represent only 3% while cancellous bone remodeling represent approximately 25% (RIGGS & MELTON, 1995). Further, despite of the bone loss in osteoporosis process to occur in all skeletal sites, in the postmenopausal this bone loss affects primarily cancellous bone tissue (BROUNS & VERMEER, 2000).

The hystomorphometric results also suggest that FOS supplementation showed a beneficial effect on cancellous bone. The BV/TV average of the FOS group was higher ($p<0.05$) than control group, despite of the Oc.S/BS had been higher ($p<0.05$) in this group too.

The results pointed that FOS could down regulate the serum PTH concentration and this finding is in accordance to other authors (LIPS, 2001; MOSEKILDE, 2005) who described that high blood parathyroid hormone (PTH) level is common in aged animals. The prebiotics are appointed by many investigators (ZAFAR et al. 2004; HOLLOWAY et al. 2007) to enhance intestinal calcium absorption; moreover, KARKKAINEN et al. (2001) described that calcium doses of 250mg can increase serum ionized calcium and decrease circulating PTH, confirming our PTH result.

Despite the down regulation of PTH by FOS supplementation, RANk-L (serum osteoclastic activity biomarker), OC and OPG (osteoblastic activity biomarkers) did not show differences ($p<0.05$) between the groups. However, the CTX-I (serum osteoclastic activity biomarker) was higher ($p<0.05$) in the FOS group.

The FOS group serum OPG showed a positive, however, inverse correlation ($p<0.05$) with PTH ($r^2= 0.4554$; $P = 0.0664$) (Figure 8). KHOSLA et al. (2002) described serum OPG levels increased with age in both men and women; moreover, these researchers also described that these observed elevations in OPG in older population

could be explained as a compensatory effect to slow down the enhanced bone resorption.

Thus, our results suggest that the FOS supplementation reduced age related cancellous bone loss. Perhaps a longer supplementation could result in serum bone biomarkers levels beyond bone mass gain.

5) Acknowledgments

This work was supported by FAEPEX – University of Campinas (São Paulo – Campinas - Brazil) and the National Council of Scientific and Technological Development (CNPq).

6) References

BROUNS, F.; VERMEER, V. C. Functional food ingredients for reducing the risks of osteoporosis. **Trends in Food Science & Technology**. Oxford, v.11, n.1, p.22-33, Jan. 2000.

DEVAREDDY, L.; KHALIL, D. A.; KORLAGUNTA, K.; HOOSHMAND, S.; BELLMER, D. D.; ARJMANDI, B. H. The effects of fructo-oligosaccharides in combination with soy protein on bone in osteopenic ovariectomized rats. **Menopause**. New York, v.13, n.4, 692-699, Jul.-Aug. 2006.

HOLLOWAY, L.; MOYNIHAN, S.; KENT, K.; HSU, A. R.; FRIEDLANDER, A. L. Effects of oligofructose enriched inulin on mineral absorption and markers of bone turnover in postmenopausal women. **The British Journal of Nutrition**. New York, v.97, n.2, p.365–372, Feb. 2007.

JAMIESON, J. A.; RYZ, N. R.; TAYLOR, C. G.; WEILER, H. A. Dietary long-chain inulin reduces abdominal fat but has no effect on bone density in growing female rats. **The British Journal of Nutrition**. New York, v.100, n.2, p.451-459, Aug. 2008.

JONES, P. J. Functional foods — more than just nutrition. **Canadian Medical Association Journal**. Toronto, v.166, n.12, p.1555-1563, Jun. 2002.

KARKKAINEN, M. U.; LAMBERG-ALLARDT, C. J.; AHONEN, S.; VALIMAKI, M. Does it make a difference how and when you take your calcium? The acute effects of calcium on calcium and bone metabolism. **The American Journal of Clinical Nutrition**. Bethesda, v.74, n.3, n.335–342, Sep. 2001.

KHOSLA, S.; ARRIGHI, H. M.; MELTON, L. J; ATKINSON, E. J.; O'FALLON, W. M.; DUNSTAN, C.; RIGGS, B. L. Correlates of osteoprotegerin levels in women and men. **Osteoporosis International**. London, v.13, n.5, p.394-399, May. 2002.

LI, X. J.; JEE, W. S. S.; KE, H. Z.; MORI, S.; AKAMINE, T. Age-related changes of cancellous and cortical bone histomorphometry in female Sprague-Dawley rats. **Cells and Materials Supplement**. Chicago, v.1, p.25-35, Jan. 1991.

LIPS, P. VitaminD deficiency and secondary hyperparathyroidism in the elderly: Consequences for bone loss and fractures and therapeutic implications. **Endocrine Reviews**. Baltimore, v.22, n.4, p.477–501, Aug. 2001.

MATHEY, J.; PUEL, C.; KATI-COULIBALY, S.; BENNETAU–PELISSERO, C.; DAVICCO, M. J.; LEBECQUE, P.; HORCAJADA, M. N.; COXAM, V. Fructooligosaccharides maximize bone-sparing effects of soy isoflavone-enriched diet in the ovariectomized rat. **Calcified tissue international**. New York, v.75, n.2, p.169–179, Aug. 2004.

MOSEKILDE, L. Vitamin D and the elderly. **Clinical Endocrinology**. Oxford, v.62, n.3, p.265–281, Mar. 2005.

NETTO, C. C.; MIYASAKA, C. K. Fructooligosaccharides (FOS) and Hormonal Replacement Therapy (HRT) by estrogen suppressed bone resorption in the ovariectomized rat. **Journal of Medical and Biological Science**. Salvador, 2009 [Epub ahead of print].

OHAMA, H.; IKEDA, H.; MORIYAMA, H. Health foods and foods with health claims in Japan. **Toxicology**. Limerick, v.221, n.1, p.95–111, Apr. 2006.

PARFITT, A. M; DREZNER, M. K.; GLORIEUX, F. H; KANIS, J. A.; MALLUCHE, H.; MEUNIER, P. J.; OTT, S. M.; RECKER. R. R. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. **Journal of Bone and Mineral Research**. New York, v.2, n.6, p.595-610, Dec. 1987.

PARFITT, A. M. Bone histomorphometry: proposed system for standardization of nomenclature, symbols, and units. **Calcified Tissue International**. New York, v.42, n.5, p.284-6, May. 1988.

REEVES, P. G.; NIELSEN, F. H.; FAHEY, G. C. JR. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **The Journal of Nutrition**. Bethesda, v.123, n.11, p.1939-1951, Nov. 1993.

RIGGS, B. L.; MELTON, L. J. The worldwide problem of osteoporosis: insights afforded by epidemiology. **Bone**. New York, v.17, Suppl. 5, p.505-511, Nov. 1995.

ROBERFROID, M. B. Prebiotics: The Concept Revisited. **The Journal of Nutrition**. Bethesda, v.137, Suppl. 2, p.830-837, Mar. 2007.

SCHOLZ-AHRENS, K. E.; ACIL, Y.; SCHREZENMEIR, J. Effect of oligofructose or dietary calcium on repeated calcium and phosphorus balances, bone mineralization and trabecular structure in ovariectomized rats. **The British Journal of Nutrition**. New York, v.88, p.365–377, Oct. 2002

WONG, J. M. W.; JENKINS, D. J. A. Carbohydrate digestibility and metabolic effects. **The Journal of Nutrition**. Bethesda, v.137, Suppl.11, p.2539-2546, Nov. 2007.

WORLD HEALTH ORGANIZATION. **Prevention and management of osteoporosis prevention**. Technical Report Series n° 921. Geneva, 2002.

ZAFAR, T. A.; WEAVER, C. M.; ZHAO, Y.; MARTIN, B. R.; WASTNEY, M. E. Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. **The Journal of Nutrition**. Bethesda, v.134, n.2, p.399-402, Feb. 2004.

CAPÍTULO 6

EFFECTS OF THE GALACTOOLIGOSACCHARIDES (GOS) IN THE AGED FEMALE RATS BONE LOSS

Claudia Cardoso Netto[1,3], Rosângela Santos[2], Gláucia Maria Pastore[2], Sherry Agellon[3], Hope Weiler[3] and Mário Roberto Maróstica JR[1*]

[1]Food and Nutrition Department / School of Food Engineering

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[1*]Phone +55 19 3521-4078, Fax +55 19 3521-4060

e-mail: mario@fea.unicamp.br

[2]Department of Food Science / Food and Engeneering College

PO Box 6121, University of Campinas - UNICAMP

13083-970, Campinas, SP, Brazil

[3]School of Dietetics and Human Nutrition

McGill University

H9X 3V9, Montreal, QC, Canada

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Abstract

The objective of this study is to evaluate the role of galactooligosaccharides (GOS) on age related bone loss in female rats. Twenty (10 month-old) female Wistar rats weighing approximately 300-320g were randomly assigned to two dietary treatment groups and fed *ad libitum* for 3 months. Both diets were isocaloric and equal in Ca (5mg/kg), however, GOS group received 5% GOS (produced in the Laboratory of Bioaromas – UNICAMP) and 5% sucrose while control group received 10% sucrose. Several analyses were performed: bone dual-energy X-ray absorptiometry scans (DXA), morphometry and histomorphometry; biochemical assays: serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator for nuclear factor κappa B ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I). Data were statistically analyzed by Student t-test ($p<0.05$) and all data are reported as mean plus or minus standard error (SEM). GOS group showed L1-4 spine BMC and BMD, L-3 spine BMD, femur neck width and relative bone volume (BV/TV) statistically ($p<0.05$) higher than control group. Femur wet weight and head width were statistically ($p<0,05$) lowest in GOS group. Oc.S/BS, Ob.S/BS and the biochemical assays as serum PTH, OC, OPG, RANk-L and CTX-I did not show statistical ($p<0.05$) difference between the groups. GOS supplementation reduced cancellous bone losses related to age, however, further researches using GOS supplementation are required to establish mechanisms of this prebiotic action on bone turnover.

Keywords: Prebiotics; galactooligossaccharides; age related bone loss; osteoporosis.

1) Introduction

In the 1980s Japanese people began to realize the importance of maintaining and improving their health and the Government also started to pay more attention to the aging population. Concurrently, intensive studies were performed on the physiological effects of various foods and their constituents for so called 'the tertiary function of foods'. In brief, the tertiary function of foods is directly involved in the modulation of the human physiological systems such as the immune, endocrine, nerve, circulatory as well as digestive systems, while the primary and secondary functions are related to nutrition and sensory satisfaction, respectively (OHAMA et al. 2006).

In 1984 the term 'functional foods' was first assigned in the project initiated by the Ministry of Education (presently as The Ministry of Education, Culture, Sports, Science and Technology) and this concept attracted the 'health foods' industry and health-conscious consumers. The Government, however, prohibited the use of the term 'functional' because of its implication as a drug-like effect. As a result, the term 'health foods' was widely used and recognized by consumers and the concept of the 'functional foods' was ultimately integrated into the 'foods for specified health uses' (FOSHU) system (JONES, 2002; OHAMA et al. 2006).

Nowadays, many specified health ingredients called prebiotics are been designed for clinical management of disease states as nutritional supplements. ROBERFROID (2007) described prebiotics as "non-digestible oligosaccharides (NDO) that are bifidogenic agents selectively fermented by intestinal bacterials that specifically promote modulation of the microbiota composition and/or activity, with health benefit to the host associated.

Prebiotics ingredients can promote a lot of health benefits such as: improved colonic microflora composition and intestinal function, up regulated calcium intestinal absorption and other minerals, up regulate the production of gastrointestinal peptides, improve the immunity system and resistance to infections and reduce the risk for obesity and osteoporosis (WONG & JENKINS, 2007). Despite some investigators have shown the prebiotics benefits in some osteoporosis models (MATHEY et al. 2004; NETTO & MIYASAKA, 2009), their effects have not been clearly tested to bone mass and its age related changes.

Recently, MACFARLANE et al. (2008) reported that almost all the recent studies in the literature are not sufficiently consistent to evaluate the effects of the prebiotics on bone metabolism and they still recommend that specific experimental study should be done in subject groups at risk of osteoporosis, such elderly women and/or postmenopausal women, as well in animals models (rodents) with similar bone metabolism. Thus, this study has for objective to establish if the prebiotic galactooligosaccharides (GOS) attenuate age related bone loss in female rats.

2) Material and methods

2.1) Animals and diets

Twenty (10 month-old) female Wistar rats weighing approximately 300-320g were obtained from the Center of Multidisciplinary Investigation in Biology (CEMIB) of the University of Campinas (São Paulo – Brazil). The animals were randomly assigned to two dietary treatment groups and they were fed *ad libitum* for 3 months. The diets were based on the AIN-93M formulation (REEVES et al. 1993) (Table 1), isocaloric and equal in Ca (5mg/kg). The prebiotics galactooligossaccharides (GOS) were produced in the

Laboratory of Bioflavors, University of Campinas (São Paulo - Brazil) and contained 28% of tetrasaccharides, 50% of lactose, 15% of glucose and 7% of galactose. Five animals were housed per cage in a temperature (21–24˚C) and humidity (55%) controlled room with a light:dark cycle of 12:12 h. At the end of the experimental period the animals were fed overnight before had been euthanized. The experimental protocol was approved by Experimental Animal Ethics Committee of the University of Campinas (Protocol nº 1561-1).

Table 1. Diets formulated composition based in AIN-93M (REEVES et al. 1993).

Ingredients	**Control diet (g/Kg of diet)**	**GOS diet (g/Kg of diet)**
Cornstarch	465.692	465.692
Casein (≥85% protein)	140.000	140.000
Dextrinized cornstarch (90-94% tetrasaccharides)	155.000	155.000
Sucrose	100.000	50.000
GOS	-	50.000
Soybean oil	40.000	40.000
Cellulose	50.000	50.000
Mineral mix (AIN-93M-MX)	35.000	35.000
Vitamin mix (AIN-93-VX)	10.000	10.000
L-Cystine	1.800	1.800
Choline bitartrate (41.1% choline)	2.500	2.500
Tert-butylhydroquinone	0.008	0.008

2.2) Tissue collection

All animals were killed by guillotine and trunk blood was collected and stored on ice until centrifuged to obtain the serum (3.000 rpm / 10 min) (FANEM Excelsa Baby I – model 206 / São Paulo - Brazil). Serum samples were stored at -80˚C until analysis. The bones were collected and stored at -20˚C until analysis.

2.3) Dual-energy X-ray absorptiometry scans (DXA)

Femur (whole, hipaxis and diaphysis), spine (L1-4 and L-3 alone) and mandible were scanned for bone area (BA), bone mineral content (BMC), bone mineral density (BMD) *ex vivo* in a water bath by dual-energy X-ray absorptiometry (DXA, 4500A; Hologic Inc., Bedford, MA, USA; small animal software high resolution option) (JAMIESON et al. 2008).

2.4) Bone morphometry

Following *ex vivo* in a water bath DXA analysis, right and left femurs were excised and thoroughly cleaned of soft tissue. Morphometric measures were taken with digital calipers as previously described (JAMIESON et al. 2008) and recorded to the nearest 0·01 mm. All measures were reproduced in triplicate by the same trained examiner and included wet weight, length, femoral head, neck and hipaxis, diaphysis and distal epiphysis width.

2.5) Bone histomorfometry

The left tibiae from each animal were excised at death, and the surrounding soft tissue was cleaned off. After cleaning, tibiae were maintained 48 hours in formol solution (10%), demineralizated and submitted to routine hystologic procedures. Then, the tibiae were included in histological paraffin (Merck Chemicals / Darmstadt – Germany), the paraffin blocks were sliced on 5 µm sections, put in laminas and submitted to routine hystologic procedures using hematoxilin and eosin to stain. The images were taking with 10 X 10 magnification with a digital camera COHU 2700 (Cohu Inc. Electronics Division, California, Unites States) coupled to a light microscope Nikon Eclipse H550S (Nikon Corporation, Tokyo, Japan) following quantification in accordance with nomenclature described previously (PARFFIT et al. 1987; PARFFIT, 1988) using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

2.6) Biochemical assays

Serum parathyroid hormone (PTH), osteocalcin (OC), osteoprotegerin (OPG), receptor activator of nuclear factor κappa B ligand (RANk-L) and bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) were measured by enzyme linked immunosorbent assays specific for rat (Rat Bone Panel 3 – PTH and Osteocalcin; Rat Bone Panel 1 – osteoprotegerin; Rat Bone Panel 2 – RANK-L of the Millipore, Billerica, Massachusetts, United States; CTX-I of the Immunodiagnostic Systems Ltd., Arizona, Unites States). All the biochemical assays were performed according to the manufacturer's instructions in duplicate. The methods sensitivity, intra-assay and inter-assay were respectively: PTH = 0.3 pg/mL, 3.5 CV% and 8.1 %CV; OC

= 1.6 pg/mL, 2.9 %CV and 4.6 %CV; OPG = 2.3 pg/mL, 3.1 %CV and 3.4 %CV; RANk-L = 1.1 pg/mL, 3.1 %CV and 9.0 %CV; CTX-I = 2 ng/mL, 5.6 %CV and 10.5 %CV

2.7) Statistical methods

Data were analysed by Student t-test with a level of significance of 0.05, using GraphPad Prism 5 version (GraphPad Software, Inc.; La Jolla, CA, USA). All data are reported as means with their standard errors of the mean (SEM).

3) Results

3.1) Feed intake and growth

The end-point experimental body weight was statistically ($p<0.05$) higher in GOS group than control group (GOS group = 316.3 ± 2.0g; control group = 302.3 ± 3.4g); however, the daily feed intake during all the experimental period was not statistically ($p<0.05$) different (GOS group = 12.7 ± 0.5g; control group = 11.8 ± 0.7g).

3.2) Dual-energy X-ray absorptiometry scans (DXA)

The experimental groups did not show statistical difference ($p<0.05$) in BA, BMC and BMD results for whole femur, hipaxis, diaphysis and mandible. However, GOS group L1-4 spine BMC and BMD and L-3 spine BMD were statistically higher (56, 12, 18, 13%, respectively) than control group. DXA results are shown in Table 2.

The coefficients of variation (CV %) for triplicate scans of BA, BMC and BMD were 7.3, 9.0 and 3.7, respectively for the whole femur; 5.3, 7.0 and 7.8, respectively for the hipaxis; 1.9, 8.4 and 6.2, respectively for the femur diaphysis; and 6.9, 8.4 and 4.8, respectively for the mandible.

Table 2. Effect of GOS on bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) in the femur, spine and mandible *ex vivo* in aged female rats.

	Control group	**GOS group**
	Mean ± SEM	Mean ± SEM
Whole Femur		
BA (cm^2)	1.88 ± 0.04	1.81 ± 0.04
BMC (g)	0.46 ± 0.01	0.43 ± 0.01
BMD (g/cm^2)	0.242 ± 0.003	0.241 ± 0.002
Hipaxis		
BA (cm^2)	0.304 ± 0.005	0.309 ± 0.005
BMC (g)	0.078 ± 0.002	0.080 ± 0.002
BMD (g/cm^2)	0.250 ± 0.007	0.253 ± 0.006
Diaphysis		
BA (cm^2)	0.455 ± 0.002	0.457 ± 0.003
BMC (g)	0.112 ± 0.003	0.113 ± 0.003
BMD (g/cm^2)	0.242 ± 0.005	0.243 ± 0.004
Spine (L1-4)		
BA (cm^2)	0.357 ± 0.03	0.459 ± 0.03
BMC (g)*	0.046 ± 0.003^a	0.068 ± 0.007^b
BMD (g/cm^2)**	0.123 ± 0.003^a	0.138 ± 0.004^b
Spine (L3)		
BA (cm^2)	0.134 ± 0.007	0.154 ± 0.011
BMC (g)	0.017 ± 0.001	0.020 ± 0.002
BMD (g/cm^2)***	0.117 ± 0.003	0.132 ± 0.002
Mandible		
BA (cm^2)	0.59 ± 0.01	0.57 ± 0.01
BMC (g)	0.071 ± 0.002	0.073 ± 0.002
BMD (g/cm^2)	0.122 ± 0.002	0.128 ± 0.002

- Abbreviations: BA, bone area; BMC, bone mineral content, BMD, bone mineral density.
- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, $P = 0.02$; **, $P = 0.01$; ***, $P = 0.001$

3.3) Bone morphometry

Femur wet weight and head width results were statistically ($p<0.05$) lower (7%) in GOS group, however, neck width was statistically ($p<0.05$) highest (17%) in the same group. These results can be seen in Table 3.

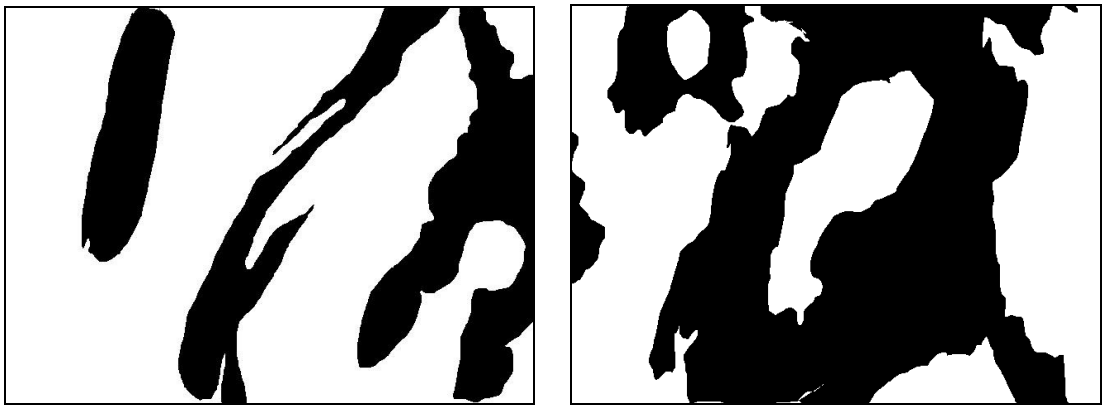
Table 3. Effect of the GOS on femur morphometry measurements in aged female rats.

Femur measurements	**Control group**	**GOS group**
	Mean ± SEM	Mean ± SEM
Wet weight (mg)*	689.3 ± 8.2[a]	640.0 ± 12.5[b]
Length (mm)	33.8 ± 0.2	34.1 ± 0.3
Head (mm)**	3.93 ± 0.07[a]	3.67 ± 0.03[b]
Neck (mm)***	2.30 ± 0.05[a]	2.69 ± 0.07[b]
Hipaxis (mm)	7.51 ± 0.04	7.53 ± 0.05
Diaphysis (mm)	4.26 ± 0.04	4.21 ± 0.02
Distal epiphysis (mm)	5.61 ± 0.06	5.58 ± 0.05

- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* values: *, *P* = 0.002; **P* = 0.006; ***P* = 0.0002.

3.4) Bone histomorphometry

Relative bone volume (BV/TV) was statistically ($p<0.05$) higher (29%) in GOS group than control group; however, osteoclastic-covered bone surface (Oc.S/BS) and osteoblastic-covered bone surface (Ob.S/BS) did not show statistical ($p<0.05$) difference between the groups. These results are showed in Figure 1 and Table 4.



Control group **GOS group**

Figure 1. Example of photos used for histomorphometry showing the differences between trabecular volume (BV/TV) in the groups (Hematoxilin and eosin stain, 10 X 10 magnification)*.

*These pictures were transformed to 8 bit archive using image processing software (ImageJ 1.37v, National Institute of Health, Bethesda, EUA).

Table 4. Effect of the GOS on proximal epiphysis tibiae histomorphometry parameters in aged female rats.

Parameters	**Control group**	**GOS group**
	Mean ± SEM	Mean ± SEM
BV/TV (%)*	35.6 ± 2.1[a]	45.8 ± 2.7[b]
Ob.S/BS	12.3 ± 2.0	10.3 ± 1.6
Oc.S/BS	8.5 ± 0.9	8.5 ± 1.0

- Abbreviations: BV/TV = relative bone volume; Ob.S/BS = osteoblast-covered bone surface; Oc.S/BS = osteoclast-covered bone surface.
- Different letters mean statistical difference ($p<0.05$) between the groups.
- *P* value: *, *P* = 0.01;

3.5) Biochemical assays

The biochemical assays as serum PTH, OC, OPG, RANk-L and CTX-I were not statistically ($p<0.05$) different in both groups. These biochemical results are shown in Figure 2, 3, 4, 5 and 6, respectively.

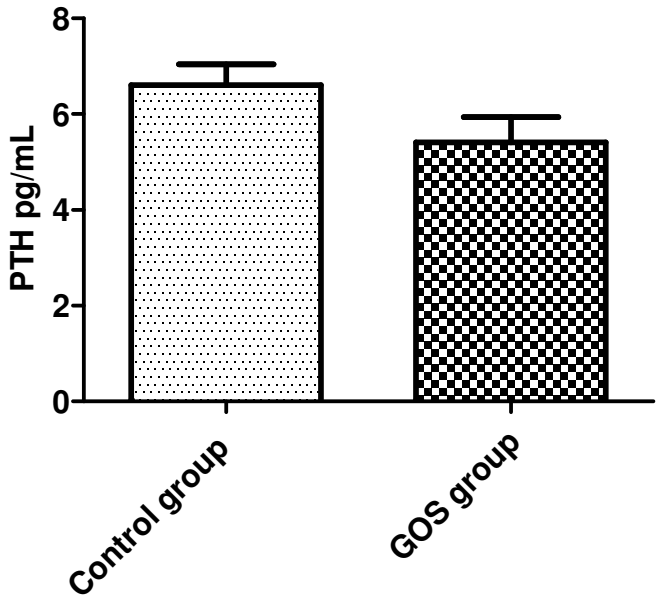


Figure 2. Effect of the GOS on serum parathyroid hormone (PTH) in aged female rats.

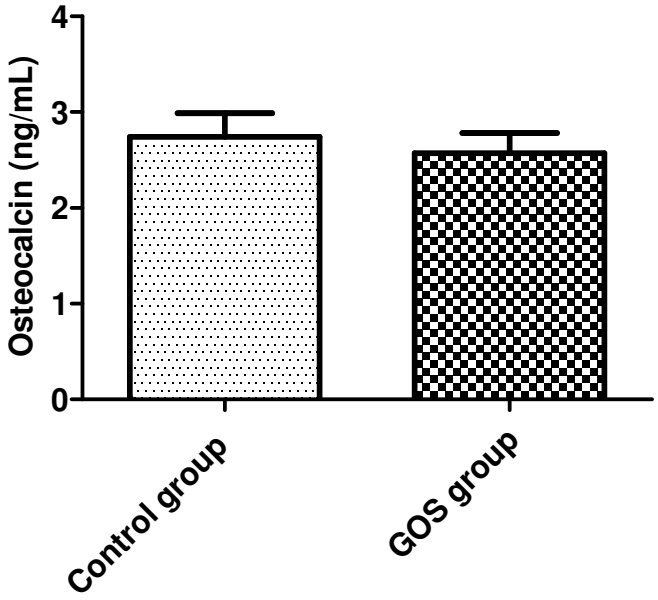


Figure 3. Effect of the GOS on serum osteocalcin (OC) in aged female rats.

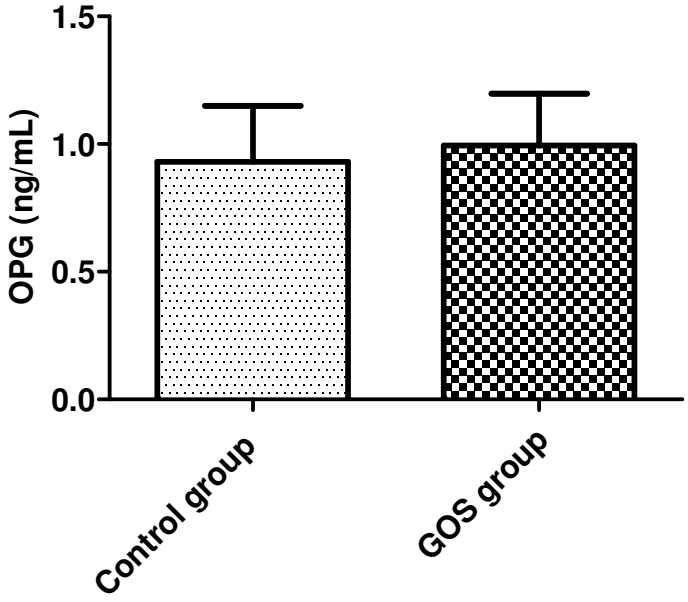


Figure 4. Effect of the GOS on serum osteoprotegerin (OPG) in aged female rats.

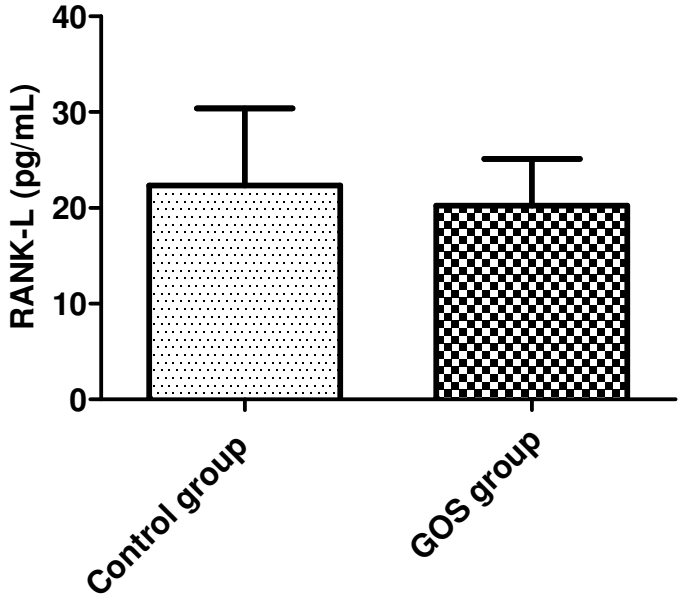


Figure 5. Effect of the GOS on serum nuclear factor kappa B ligant (RANk-L) in aged female rats.

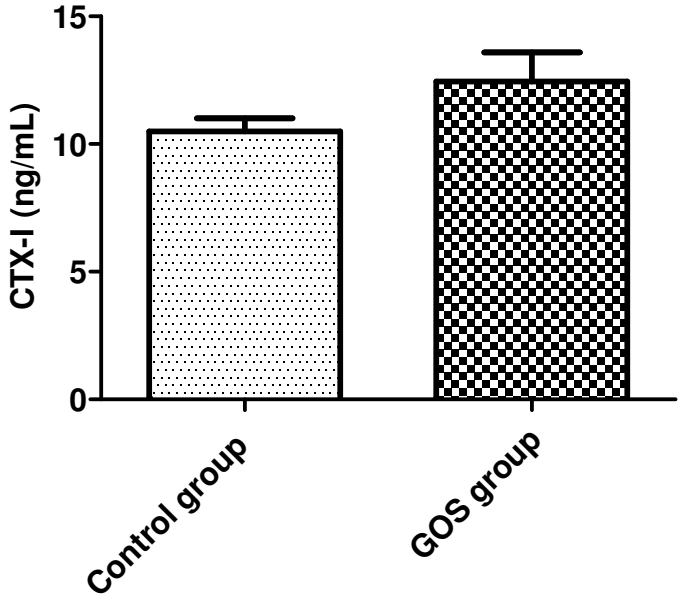


Figure 6. Effect of the GOS on serum bone-related degradation products of C-terminal peptides of type I collagen (CTX-I) in aged female rats.

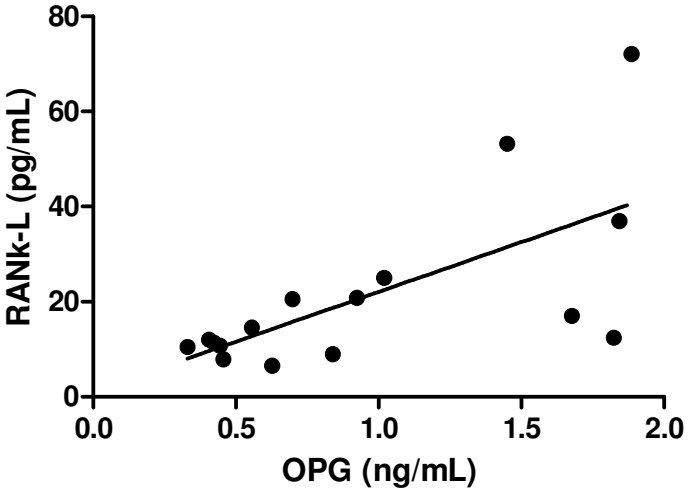


Figure 7. Effects of the GOS on serum OPG and RANk-L correlation in aged female rats*.

r^2 = 0,4417 (*P* = 0.005)

4) Discussion

The daily feed intake during all the experimental period was not different between the groups. The end-point body weight was higher ($p<0.05$) in GOS group and it could be due to bone alterations induced by the prebiotics; however, this will be discussed later.

Our DXA and morphometry results suggest that the GOS prebiotic supplementation had protective effects on the trabecular bone mass as L1-4 spine and L-3 spine BMD and neck width were statistically ($p<0.05$) higher in this group when compared with control group. These are important results once these spines as femur neck (hip axis) are the most common osteoporotic fractures in postmenopausal and older people (femur neck) (WORLD HEALTH ORGANIZATION, 2003).

CHONAN et al. (1995) described that the femur and tibiae ash weight and tibia calcium content of ovariectomized rats fed on 5% of GOS diet were significantly higher than those of the control animals. However, these investigators did not show differences between cancellous and cortical bone. CHONAN & WATANUKI (1996) also showed that the bone (femur and tibiae) Ca content and bone mineralization of male rats fed normal-Ca diet and GOS were significantly higher than those of the control animals.

The results did not show significant ($p<0.05$) results in cortical bone (femur diaphysis BA, BMC and BMD; diaphysis width); however, it was expected that in adult animals, the cortical bone remodeling represent only 3% while cancellous bone remodeling represent approximately 25%, as described by RIGGS & MELTON (1995). LI et al. (1991) observed that, in Sprague-Dawley female rats the peak bone mass is maintained without significant changes until 8-months of age. The bone mass begins to decrease with aging and reaches a low value of 14% of the metaphyseal tissue area

after the age of 12 months. Thus, despite of the bone loss in all skeletal sites osteoporosis process occur, the postmenopausal bone loss affects primarily cancellous bone tissue as described by BROUNS & VERMEER (2000).

The hystomorphometric results also suggest that the GOS prebiotics supplementation had beneficial effect on trabecular bone observing that this group showed BV/TV statistically ($p<0.05$) highest despite of the Ob.S/BS and Oc.S/BS had not been statistically ($p<0.05$) different between the groups.

All the biochemical assays did not show statistical ($p<0.05$) difference between the groups. However, our analysis were performed in serum of fasting overnight animals and the blood PTH level is directly affected such by serum calcium concentration (HERFARTH et al. 1992; KARKKAINEN et al. 2001) as timing of this mineral intake (BLUMSOHN et al. 1994). HOLLOWAY et al. (2007) support our results of PTH analysis; despite of these investigators had observed a true absorption of Ca increased in postmenopausal women after 6 weeks of treatment with mixture of chicory oligofructose and long-chain inulin (Synergy1 - SYN1), they did not observe a significant decrease in the blood PTH when blood analyses were also performed after a fasting period of at least 10 h (overnight).

HOLLOWAY et al. (2007) also measured bone biomarkers as deoxypyridinoline cross-links (an index of bone resorption activity) and OC. The deoxypyridinoline cross-links, for example, showed an initial transient decrease at 3 weeks in the SYN1 group but rebounded to greater levels than baseline by 6 weeks of treatment and there was no significant change in the placebo group and no significant difference between treatments. Levels of serum osteocalcin, a measure of bone formation activity, were increased at 6 weeks of treatment only in the SYN1 group.

PTH stimulates bone resorption by osteoclasts, bone mineralization by osteoblasts can induce the rapid release of Ca^{2+} from the bone matrix (SCHLUTER, 1999; KHANAL & NEMERE, 2008) and in the normal 'coupled' bone system the decrease in resorption is followed by a decrease in formation of bone tissue (EASTELL et al. 1993).

Thus, this PTH result minimized our ability to detect the acute and direct effect of the GOS on intestinal Ca absorption, as described previously (CHONAN & WATANUKI, 1995; 1996; CHONAN et al. 1995; VAN DEN HEUVAL et al. 2000), and its probable indirect effect on bone turnover markers.

Our results suggest that the GOS supplementation reduced some important sites associated with common cancellous bone loss age related like a spine and femur neck. However, further investigations using GOS supplementation without fasting animals prior to blood analyses could be useful in order to compare with fasting animals and to establish mechanisms of this prebiotic action on bone turnover related to age.

5) Acknowledgments

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6) References

BLUMSOHN, A.; HERRINGTON, K.; HANNON, R. A.; SHAO, P.; EYRE, D. R.; EASTELL, R. The effect of calcium supplementation on the circadian rhythm of bone resorption. **The Journal of Clinical Endocrinology and Metabolism**. Chevy Chase, v.79, n.3, p.730–735, Sep. 1994.

BROUNS, F.; VERMEER, V. C. Functional food ingredients for reducing the risks of osteoporosis. **Trends in Food Science & Technology**. Oxford, v.11, n.1, p.22-33, Jan. 2000.

CHONAN, O.; WATANUKI, M. Effect of galactooligosaccharides on calcium absorption in rats. **Journal of Nutritional Science and Vitaminology**. Tokyo, v.41, n.1, p.95–104, Feb. 1995.

CHONAN, O.; WATANUKI, M. The effect of 6-galactooligosaccharides on bone mineralization of rats adapted to different levels of calcium. **International Journal for Vitamin and Nutrition Research**. Bern, v.41, p.95–104, Dec. 1996.

CHONAN, O.; MATSUMOTO, K.; WATANUKI, M. Effect of galactooligosaccarhides on calcium absorption and preventing bone loss in ovariectomized rats**. Bioscience, Biotechnology and Biochemistry**. Tokyo, v.59, n.2, p.236–239, Feb. 1995.

EASTELL, R.; ROBINS, S. P.; COLWELL, T.; ASSIRI, A. M.; RIGGS, B. L.; RUSSELL, R. G. Evaluation of bone turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption. **Osteoporosis International**. London, v.3, n.5, p.255–260, Sep. 1993.

HERFARTH, K.; DRECHSLER, S.; IMHOFF, W.; SCHLANDER, M.; ENGELBACH, M.; MAIER, A.; SCHMIDT-GAYK, H. Calcium regulating hormones after oral and

intravenous calcium administration. **European Journal of Clinical Chemistry and Clinical Biochemistry**. Berlin, v.30, n.12, p.815–822, Dec. 1992.

HOLLOWAY, L.; MOYNIHAN, S.; KENT, K.; HSU, A. R.; FRIEDLANDER, A. L. Effects of oligofructose enriched inulin on mineral absorption and markers of bone turnover in postmenopausal women. **The British Journal of Nutrition**. New York, v.97, n.2, p.365–372, Feb. 2007.

JAMIESON, J. A.; RYZ, N. R.; TAYLOR, C. G.; WEILER, H. A. Dietary long-chain inulin reduces abdominal fat but has no effect on bone density in growing female rats. **The British Journal of Nutrition**. New York, v.100, n.2, p.451-459, Aug. 2008.

JONES, P. J. Functional foods — more than just nutrition. **Canadian Medical Association Journal**. Toronto, v.166, n.12, p.1555-1563, Jun. 2002.

KARKKAINEN, M. U.; LAMBERG-ALLARDT, C. J.; AHONEN, S.; VALIMAKI, M. Does it make a difference how and when you take your calcium? The acute effects of calcium on calcium and bone metabolism. **The American Journal of Clinical Nutrition**. Bethesda, v.74, n.3, n.335–342, Sep. 2001.

KHANAL, R. C.; NEMERE, I. Endocrine regulation of calcium transport in epithelia. **Clinical and experimental pharmacology & physiology**. Oxford, v.35, n.11, p.1277-1287, Nov. 2008.

LI, X. J.; JEE, W. S. S.; KE, H. Z.; MORI, S.; AKAMINE, T. Age-related changes of cancellous and cortical bone histomorphometry in female Sprague-Dawley rats. **Cells and Materials Supplement**. Chicago, v.1, p.25-35, Jan. 1991.

MACFARLANE, G. T.; STEED, H.; MACFARLANE, S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. **Journal of Applied Microbiology**. Oxford, v.104, n.2, p.305–344, Feb. 2008.

MATHEY, J.; PUEL, C.; KATI-COULIBALY, S.; BENNETAU–PELISSERO, C.; DAVICCO, M. J.; LEBECQUE, P.; HORCAJADA, M. N.; COXAM, V. Fructooligosaccharides maximize bone-sparing effects of soy isoflavone-enriched diet in the ovariectomized rat. **Calcified tissue international**. New York, v.75, n.2, p.169–179, Aug. 2004.

NETTO, C. C.; MIYASAKA, C. K. Fructooligosaccharides (FOS) and Hormonal Replacement Therapy (HRT) by estrogen suppressed bone resorption in the ovariectomized rat. **Journal of Medical and Biological Science**. Salvador, 2009 [Epub ahead of print].

OHAMA, H.; IKEDA, H.; MORIYAMA, H. Health foods and foods with health claims in Japan. **Toxicology**. Limerick, v.221, n.1, p.95–111, Apr. 2006.

PARFITT, A. M; DREZNER, M. K.; GLORIEUX, F. H; KANIS, J. A.; MALLUCHE, H.; MEUNIER, P. J.; OTT, S. M.; RECKER. R. R. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. **Journal of Bone and Mineral Research**. New York, v.2, n.6, p.595-610, Dec. 1987.

PARFITT, A. M. Bone histomorphometry: proposed system for standardization of nomenclature, symbols, and units. **Calcified Tissue International**. New York, v.42, n.5, p.284-6, May. 1988.

REEVES, P. G.; NIELSEN, F. H.; FAHEY, G. C. JR. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **The Journal of Nutrition**. Bethesda, v.123, n.11, p.1939-1951, Nov. 1993.

RIGGS, B. L.; MELTON, L. J. The worldwide problem of osteoporosis: insights afforded by epidemiology. **Bone**. New York, v.17, Suppl. 5, p.505-511, Nov. 1995.

ROBERFROID, M. B. Prebiotics: The Concept Revisited. **The Journal of Nutrition**. Bethesda, v.137, Suppl. 2, p.830-837, Mar. 2007.

SCHLUTER, K. D. PTH and PTHrP: Similar structures but different functions. **News in Physiological Sciences**. Bethesda, v.14, p.243-249, Dec. 1999.

VAN DEN HEUVEL, E. G.; SCHOTERMAN, M. H.; MUIJS, T. Transgalactooligosaccharides stimulate calcium absorption in postmenopausal women. **The Journal of Nutrition**. Bethesda, v.130, n.12, p.2938–2942, Dec. 2000.

WONG, J. M. W.; JENKINS, D. J. A. Carbohydrate digestibility and metabolic effects. **The Journal of Nutrition**. Bethesda, v.137, Suppl.11, p.2539-2546, Nov. 2007.

WORLD HEALTH ORGANIZATION. **Prevention and management of osteoporosis prevention**. Technical Report Series n° 921. Geneva, 2002.

CONCLUSÕES GERAIS

- O consumo de FOS isolado ou associado à inulina (Orafti) preservou a perda de massa óssea associada ao envelhecimento. Já o consumo isolado de inulina não apresentou contribuição significativa para preservação da perda de massa óssea associada ao envelhecimento.
- A suplementação de FOS (Corn Products) também preservou a perda de massa óssea associada ao envelhecimento.
- A suplementação com GOS preservou a perda de massa óssea em sítios específicos do esqueleto normalmente relacionados com fraturas, como por exemplo, vértebras e pescoço do fêmur. Porém, outros trabalhos com suplementação de GOS deveriam ser realizados com a finalidade de estabelecer os mecanismos de ação desse prebiótico no metabolismo ósseo.

PERSPECTIVAS DE TRABALHOS CIENTÍFICOS

- Realizar experimentos com diferentes tipos de prebióticos com a finalidade de estabelecer os mecanismos de preservação da perda de massa óssea em animais idosos.
- Realizar experimentos com prebióticos associados em diferentes proporções com a finalidade de estabelecer os efeitos sinérgicos dos mesmos nos mecanismos de preservação da perda de massa óssea em animais idosos.