

The application and effectiveness of *Difructose Anhydride III* to increase absorption of calcium in calcium-deficient rats

Ainia Herminiati¹, Rimbawan Rimbawan², Budi Setiawan², Dewi Apri Astuti³,
Linar Zalinar Udin⁴ and Sri Pudjiastuti⁴

¹Research Center for Appropriate Technology, Indonesian Institute of Sciences, Indonesia;

²Department of Community Nutrition, Faculty of Human Ecology, IPB University, Indonesia;

³Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Indonesia; ⁴Research Center for Chemistry, Indonesian Institute of Sciences, Indonesia.

Corresponding Author: Ainia Herminiati, Research Center for Appropriate Technology, Indonesian Institute of Sciences, Indonesia

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ABSTRACT

Background: Dried yogurt enriched by *Difructose Anhydride III* when used as a functional food has been observed to increase calcium absorption, making it useful in osteoporosis prevention. The objective of this study was to analyze the effectiveness of *Difructose Anhydride III* in increasing the absorption of calcium in female rat models, strain Sprague Dawley, in the pre-menopausal age during which they're calcium deficient.

Methods: The effectiveness test of *Difructose Anhydride III* to increase absorption of calcium in pre-menopausal Sprague Dawley rats was performed in calcium-deficient conditions. A completely randomized experimental design was used with 4 treatments for 6 weeks and 4 replications: normal rats fed with purified diet (C), calcium-deficient rats fed with calcium-deficient diet (CD), calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia tubers) fortified in dry yogurt (CD+DFA III dahlia), and calcium-deficient rats fed with a calcium-deficient diet and DFA III (chicory roots) fortified in dry yogurt (CD+DFA III chicory). The parameters measured were serum calcium concentration, femur bone calcium concentration, femur bone matrix condition, and femur bone strength.

Results: DFA III (dahlia tubers and chicory roots) fortified in dry yogurt contained 0.334% and 0.322% of calcium concentration. The provision of a calcium-deficient diet for 12 weeks was shown to reduce the serum calcium concentration of the deficient calcium rat to $7.72 \pm 1.08 \text{ mg dL}^{-1}$ and the control rat to $11.60 \pm 0.85 \text{ mg dL}^{-1}$. CD+DFA III chicory treatments also showed a high calcium concentration in the femur bone ($34.94 \pm 3.21\%$), a relatively higher bone strength ($9.34 \pm 3.61 \text{ kg cm}^{-2}$), and a denser femur bone matrix condition than the control. The femur bone calcium level of rats treated with CD+DFA III dahlia and chicory tubers was $28.95 \pm 1.95\%$ and $34.94 \pm 3.21\%$, respectively. These results were significantly different than the CD treatment ($17.49 \pm 4.38\%$).

Conclusion: The evidence from this study suggests that sufficient calcium intake could provide high calcium deposits in the bones. Diets containing 3.60% w/w DFA III fortified in dry yogurt have been shown to enhance calcium absorption in calcium-deficient rats. Additionally, the effectiveness of dried yogurt enriched by DFA III from chicory tubers was higher than that of the dried yogurt enriched by DFA III from dahlia tubers.

Preclinical Trial Registration: Animal Ethics Committee at IPB University No. 12-2013

Keywords: Bone femur; calcium deficiency; effectivity of *Difructose Anhydride III*

BACKGROUND

The increase in bone density observed during the growth period is a factor that determines the osteoporosis occurrence in later life [1]. In order to maintain bone health, pre-menopausal women need to regularly consume high calcium foods [2]. Higher bone density in the pre-menopausal period helps maintain bone calcium deposits, thus reducing calcium loss during menopause. As a result, women with higher bone densities from growth to the pre-menopausal period could be spared from osteoporosis in their post-menopausal period [3]. High calcium yogurt can be consumed daily by pre and post-menopausal women to maintain bone health and prevent osteoporosis [2]. Due to interferences in calcium absorption, premenopausal women may benefit from a functional food that can enhance calcium absorption [4]. Additionally, many other demographics can benefit from the identification of functional food components that positively affect calcium absorption [5]. One functional food candidate found to have these positive effects is *Difructose Anhydride III* [6].

Difructose Anhydride III (DFA III) is a cyclic disaccharide compound categorized as a functional food. This compound can be produced through the enzymatic processing of chicory roots by using *Arthrobacter* sp. H65-7 *fructosyltransferase* enzyme [7]. Pudjiraharti and Asano (2012) also used inulinfructo-transferase enzyme isolated from the actinomycete *Nonomuraea* sp. ID06-A0189 for the manufacturing process of DFA III from dahlia tubers [8]. Previously, DFA III has been shown to increase calcium absorption in the intestine of rats, cows, and humans [6, 9, 10, 11, 12, 13].

Probiotic yogurt can be enhanced by DFA III, facilitating the increased calcium absorption and reduced risk of osteoporosis. Based on this reasoning, it is necessary to examine the effects of consuming dry yogurt fortified with DFA III from dahlia tubers and chicory roots on bone strength. The ultimate objective of this study was to analyze the effects of *Difructose Anhydride III* (DFA

III) on the calcium absorption of femur bones in pre-menopausal, calcium-deficient (Sprague Dawley) rats in an effort to prevent osteoporosis.

METHODS

Materials and Equipment

The used materials include DFA III from dahlia tubers (Research Center for Chemistry, Indonesian Institute of Sciences), DFA III from chicory roots (Nippon Beet Sugar Mfg. Co. Ltd. Obihiro, Hokkaido, Japan), and fresh yogurt (Laboratory of Animal Product Technology of Faculty of Animal Science, IPB University) as well as female Sprague Dawley rats aged 12 months (“Satwa Harapan” Ranch of Faculty of Animal Science, IPB University). Rats were fed a purified, calcium diet [14] modified according to the calcium kit (FAST, PZ Cormay SA, Polandia) [15], and a solution of hydrazinium hydroxide. Raw materials used for the production of the purified diet consisted of rice flour, casein, corn oil, glucose, vitamin mix, DL-methionine, mineral mix, Carboxy Methyl Cellulose (CMC), salt, and distilled water obtained from a chemical store in Bogor, Indonesia. The calcium-deficient feed was made with the same materials as the purified diet but without the calcium addition of the mineral mix [16].

The equipment used included a spray dryer (Buchi 190, Swedia), homogenizer (Armfield L4R, United Kingdom), glass apparatus for chemical analysis, Atomic Absorption Spectrometer (Genesys 10S UV-VIS, Thermo Fisher Scientific, America), Scanning Electron Microscopy (SEM) instrument and Energy Dispersive X-ray (EDX) analyzer (JEOL JSM 6510 LA, Japan), rats surgical instruments, equipment for rat maintenance and treatment, cabinet dryer, and a Universal Testing Machine (Instron, America).

Stages of Research

1. Fortification of dry yogurt with Diffructose Anhydride III Diffructose Anhydride III fortification in functional foods was done using dried yogurt. Dried yogurt has several advantages, namely shelf-life stability [17]. In order to do this, a modified spray drying method [17, 18] was used. The fresh yogurt was mixed with corn starch, DFA III, skim milk, sucrose, citric acid, and gum arabic prior to the drying process. Subsequently, the mixture was homogenized using a homogenizer at 1000 rpm for 10 minutes. The homogenized mixture was then spray dried with the 120°C inlet and 80°C outlet temperatures. Table 1 shows the food formulation of the two treatments using dried yogurt.

Table 1. Food formulation of the two dried yogurt treatments

Composition	Dried yogurt with DFA III (dahlia tubers) fortification	Dried yogurt with DFA III (chicory roots) fortification
Fresh yogurt	500 g	500 g
Corn starch 10.0%	50 g	50 g
DFA III 3.0%	15 g	15 g
Skim milk 1.9%	9.5 g	9.5 g
Sucrose 2.0%	10 g	10 g
Citric acid 0.1%	0.5 g	0.5 g
Gum arabic 0.1%	0.5 g	0.5 g

2. Calcium-deficient rat preparation

The preparation of calcium-deficient rat models and the subsequent testing using DFA III fortified dry yogurt received ethical approval from the Animal Ethics Committee at IPB No. 12-2013 [19].

The preparation of calcium-deficient rat models using the ovariectomy method can cause pain and stress to the animal. According to Sajuthi *et al.* (2012), the use of animals in biomedical research needs to fulfill internationally standardized scientific principles, one of them being the fulfillment of the animal welfare principle [20]. In order to minimize negative impacts, the treatment of a calcium-deficient diet was conducted to prepare the calcium-deficient rat models.

Twenty-four rats aged 12 months were divided into 2 groups; 6 rats were fed with the purified diet in the control group (C) and 18 rats were fed with the calcium-deficient diet in the calcium-deficient group (CD). The food and drinking water were available *ad libitu* and the feeding trial was conducted over 12 weeks.

Blood sampling was conducted at the initial treatment (t_0), week 4 (t_1), week 8 (t_2), and at the end of the study, week 12 (t_3), using cardiac puncture, or blood sampling in the heart. Blood samples were centrifuged to obtain serum and the serum calcium concentration was determined by using O-C FAST® calcium reagent kits (FAST, PZ Cormay S.A., Poland) followed by measurement using a UV-Vis spectrophotometer with a wavelength of 570 to 580 nm.

3. Product effectivity test on calcium-deficient rat models

The product effectivity test was conducted over 6 weeks (after treatment for the calcium-deficient rat models) using DFA III (dahlia tubers and chicory roots) fortified dry yogurt given to Sprague Dawley pre-menopausal rats aged 15 months with a calcium-deficient condition.

The experimental rats were assigned into a completely randomized design consisting of 4 treatment groups and 4 replications. The treatment groups were normal rats fed with a purified diet (C), calcium-deficient rats fed with a calcium-deficient diet (CD), calcium-deficient rats fed with a calcium-deficient diet and DFA III (dahlia tubers) fortified dry yogurt (CD+DFA III dahlia), and calcium-deficient rats fed with a calcium-deficient diet and DFA III (chicory roots) fortified dry yogurt (CD+DFA III chicory). Table 2 shows the feed composition for the 4 treatments (modified AIN 93 M) [15].

Table 2. Feed composition for the 4 treatment variants

Composition		C	CD	CD+DFA III dahlia	CD+DFA III chicory
Rice flour	(%)	25.0	25.0	24.6	24.6
Casein	(%)	18.0	18.0	16.0	16.0
Corn oil	(%)	3.5	3.5	3.5	3.5
Glucose	(%)	49.0	49.0	48.0	48.0
Vitamin mix	(%)	0.5	0.5	0.5	0.5
DL-methionine	(%)	0.3	0.3	0.3	0.3
Mineral mix	(%)	0.5	0	0	0
Mineral mix without calcium	(%)	0	0.5	0.3	0.3
Carboxy methyl cellulose	(%)	3.0	3.0	3.0	3.0
Salt	(%)	0.2	0.2	0.2	0.2
Dried yogurt & DFA III dahlia	(%)	0	0	3.6	0
Dried yogurt & DFA III chicory	(%)	0	0	0	3.6

Source: Modification of National Research Council, 1995

Annotation: Normal rats fed with purified diet (C); calcium-deficient rats fed with calcium-deficient diet (CD); calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia) fortified dry yogurt (CD+DFA III dahlia); and calcium-deficient rats fed with calcium-deficient diet and DFA III (chicory) fortified dry yogurt (CD+DFA III chicory).

After 6 weeks, the experimental rats were sacrificed by anesthesia and surgery was conducted to isolate the femur bone. The parameters measured using the femur were the serum calcium concentration, calcium concentration of the femur, matrix condition of the femur, and the strength of the femur.

The serum calcium concentration was measured at week 2 and week 6 by using calcium reagent kits. The mineral composition of the femur bone (i.e. calcium, phosphorus, magnesium, carbon, oxygen, aluminum, and sodium) and the femur bone matrix conditions were examined using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Analysis (EDX) after week 6. EDX is a method of material characterization that detects the X-rays emitted when the material collides with electrons. These X-rays are emitted from the transition of electrons between atomic shells. Therefore, the energy levels emitted depend on the energy levels of the atomic shell [21]. Radiographic tests of the experimental rat bones were conducted to analyze the bone mass density. The dense bone will show a radiopaque image while the less compact bone will show a radiolucent image [22].

The bone strength test used was a modification of the glulam compression strength test [23] and timber compression strength tests [24]. By using compression strength tests in conjunction with the analysis of the perpendicular compression load, we were able to test rat tibia bone strength [25].

The test of femur bone strength for the experimental rats was conducted after 6 weeks of treatment by first measuring the surface area of the bone manually with a digital caliper. Furthermore, the bone was laid horizontally on the Universal Testing Machine. The bone strength test analyzed the type of perpendicular compression load ($F_c = P/A$) by measuring the weight-bearing bone strength (kg cm^{-2}) and the load (kg) against the surface area (cm^2).

Statistical Analysis

The data parameter measurements were analyzed by ANOVA at a 95% confidence interval and followed by Duncan's multiple range test [26].

RESULTS and DISCUSSION

Diffructose Anhydride III Fortification in Yogurt

The fresh yogurt used in the production of dried yogurt for the study complied with the Indonesian National Standards No. 01.2981-2009 for fresh yogurt, making it eligible for use during the study [27]. The fresh yogurt used contained 92.24% moisture, 0.78% ash, 2.80% protein, 0.97% fat, 3.21% carbohydrate, 0.5% acidity as lactic acid, 3.85 pH or acidity, and 6.1×10^7 colonies g^{-1} viability of lactic acid bacteria.

Yogurt was selected for DFA III fortification because it contains higher calcium concentrations (0.179%) compared to fresh milk. According to Tamime and Robinson (2007), calcium concentrations of yogurt and fresh milk are 0.145% and 0.119%, respectively [28]. The concentrations of calcium in the dried yogurt fortified with DFA III (dahlia tubers) and dried yogurt fortified with DFA III (chicory roots) were 0.334% and 0.322%, respectively.

The viability of lactic acid bacteria was still high after drying (6.61×10^6 and 5.43×10^6 CFU g^{-1}) and the standard was $>10^6$ CFU g^{-1} . According to the FAO/WHO (2002), probiotics are live microorganisms that provide health benefits to the host when present in sufficient quantities (10^6 -

10^8 CFU ml^{-1}) [29]. Donkor *et al.* (2007) explained that yogurt containing probiotics could enhance the concentration of acetic and lactic acid and that the presence of lactic acid and short-chain fatty acid (SCFA) in the large intestine can enhance calcium absorption [30].

Calcium-Deficient Rat Model

The deficiency period was conducted until the serum calcium concentration was less than 9.2 mg dL^{-1} . Calvo and Park (1996) stated that in animals, calcium deficiency is a major causative factor for osteoporosis with other factors being malnutrition and phosphorus deficiency [31]. At the end of the 12 week treatment, the serum calcium content was $7.72 \pm 1.08 \text{ mg dL}^{-1}$ in the calcium-deficient rats and $11.60 \pm 0.85 \text{ mg dL}^{-1}$ in the control group. According to Murray *et al.* (2003), a normal level of serum calcium ranges from 9.2 to 10.4 mg dL^{-1} [32]. Table 3 shows calcium content measurement for fed with a purified diet and rats fed with a calcium-deficient diet after 12 weeks of treatment. The serum calcium content of rats fed with calcium-deficient diets was significantly lower than that of the rats fed with a purified diet as a control ($P < 0.05$).

Table 3. Calcium levels in the serum of control (C) and calcium-deficient (CD) rats, their consumed diets, and feces calcium measurements

Parameters	Normal rats (C)	Calcium-deficient rats (CD)
Consumption of dry matter ($\text{g rat}^{-1} \text{day}^{-1}$)	7.9966 ± 0.8770^a	7.8856 ± 0.6470^a
Consumption of calcium ($\text{g rat}^{-1} \text{day}^{-1}$)	0.0480 ± 0.0050^a	0.0315 ± 0.0030^a
Calcium in feces ($\text{g rat}^{-1} \text{day}^{-1}$)	0.0044 ± 0.0010^a	0.0031 ± 0.0010^a
Calcium absorption ($\text{g rat}^{-1} \text{day}^{-1}$)	0.0435 ± 0.0060^a	0.0284 ± 0.0030^a
Serum calcium (mg dL^{-1})	11.6000 ± 0.8500^a	7.7200 ± 1.0800^b
Daily gain ($\text{g rat}^{-1} \text{day}^{-1}$)	0.1000 ± 0.1200^a	0.0600 ± 0.0400^a

Different superscripts in the same row indicate significant differences in the test ($P < 0.05$) by ANOVA

Product Effectivity on Calcium-Deficient Rat Model

Product effectivity of DFA III fortified dry yogurt was tested by using 15 months pre-menopausal calcium-deficient Sprague Dawley rats after 12 weeks of treatment for the calcium-deficient rat model. Safrida (2013) found that the pre-menopausal period of female Sprague Dawley rats occurs at the age of 12 to 18 months, as indicated by the decrease of bone density and the calcium to phosphorus ratio of the tibia bone [25]. DFA III fortified dry yogurt was added to a calcium-deficient diet (3.60%) calculated based on the energy needs of feed formulation for female rats about 3800 to 4100 kcal MEg^{-1} [14].

For all four treatments, serum calcium concentrations were decreased at weeks 2 and 6, as shown in Table 4. The result of product effectivity measurement in the groups fed with fortified dried yogurt from dahlia tubers and chicory root showed that there was no significant difference in the decrease of serum calcium levels. However, control rats that consumed calcium as much as 0.60% of the needs of rats with corresponding age and conditions (0.50%), had a lower decrease in serum calcium concentration as compared to the calcium-deficient rats.

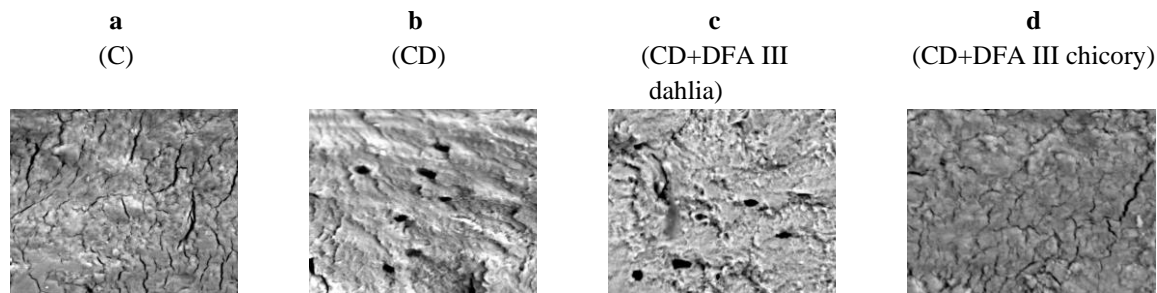
Table 4. Serum calcium levels of different rat groups at weeks 2 and 6

Treatments	Week 2 (mg dL ⁻¹)	Week 6 (mg dL ⁻¹)	Δ	Serum calcium (mg dL ⁻¹)
C	11.85 ± 0.08 ^c	10.88 ± 0.10 ^b		0.97 ± 0.18 ^a
CD	9.53 ± 0.31 ^b	7.99 ± 0.53 ^a		1.54 ± 0.22 ^b
CD + DFA III dahlia	9.29 ± 0.11 ^b	8.06 ± 0.02 ^a		1.23 ± 0.13 ^{ab}
CD + DFA III chicory	8.76 ± 0.07 ^a	7.66 ± 0.00 ^a		1.10 ± 0.07 ^{ab}

Different superscripts in the same column indicate significant differences in the test ($P < 0.05$) by ANOVA

Annotation: Normal rats fed with purified diet (C); calcium-deficient rats fed with calcium-deficient diet (CD); calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia) fortified dry yogurt (CD+DFA III dahlia); and calcium-deficient rats fed with calcium-deficient diet and DFA III (chicory) fortified dry yogurt (CD+DFA III chicory).

Using SEM-EDX to analyze femur bones, it was shown that calcium absorption is increased in the calcium-deficient rats fed with DFA III fortified dry yogurt for 6 weeks. Figure 1 shows the cross-section of the bone matrix for all four groups of experimental rats. Table 5 shows the composition of the bone constituents by SEM-EDX analysis.

**Figure 1.** Scanning electron microscopy sections of femur bone matrix for different rat groups (1000 X)

Annotation: Normal rats fed with purified diet (C); calcium-deficient rats fed with calcium-deficient diet (CD); calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia) fortified dry yogurt (CD+DFA III dahlia); and calcium-deficient rats fed with calcium-deficient diet and DFA III (chicory) fortified dry yogurt (CD+DFA III chicory).

Table 5. The mineral composition of the femur bone constituents by SEM-EDX analysis of rats after 6 weeks of feeding

Treatments	Ca (%)	P (%)	Mg (%)	C (%)	Na (%)	Al (%)	O (%)
C	36.52±0.75 ^c	16.35±0.38 ^c	0.49±0.01 ^a	8.47±1.04 ^a	0.52±0.11 ^a	0.22±0.06 ^b	37.43±1.35 ^a
CD	17.49±4.38	8.66±0.25 ^a	0.52±0.14 ^a	29.09±2.18 ^c	0.53±0.09 ^a	0.21±0.14 ^b	43.51±2.22 ^b
CD + DFA III dahlia	28.95±1.95 ^b	12.70±0.56 ^b	0.37±0.10 ^a	17.30±1.67 ^b	0.48±0.01 ^a	0.00±0.00 ^a	40.20±0.90 ^{ab}
CD + DFA III chicory	34.94±3.21 ^c	15.26±1.44 ^c	0.54±0.04 ^a	10.73±2.29 ^a	0.48±0.08 ^a	0.00±0.00 ^a	38.05±2.63 ^a

Different superscripts in the same column indicate significant differences in the test ($P < 0.05$) by ANOVA

Annotation: Normal rats fed with purified diet (C); calcium-deficient rats fed with calcium-deficient diet (CD); calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia) fortified dry yogurt (CD+DFA III dahlia); and calcium-deficient rats fed with calcium-deficient diet and DFA III (chicory) fortified dry yogurt (CD+DFA III chicory).

Figures 1a and 1d show SEM images of denser and more radiopaque femur bones, which indicate normal femur conditions. Meanwhile, Figures 1b and 1c show femur bone conditions with osteoporosis. Figure 1b of the femur bones shows the bone thinning with a large empty space in the bone structure, making it prone to fracture. According to Compston *et al.* (1993), the bone

density is its mass density related to the bone formation process (particularly bone mineral or calcium metabolism), in which a decrease in bone density leads to bone fragility or osteoporosis [3]. The mass of bone tissue is a risk determinant of bone fragility. Increased calcium absorption depends on the formation of 1,25-dihydroxycholecalciferol [33]; the small intestine responds to increased 1,25- dihydroxycholecalciferol by increasing the absorptions of calcium, phosphorus, and magnesium.

The femur calcium concentration of calcium-deficient rats fed with a calcium-deficient diet was $(17.49 \pm 4.38\%)$. This concentration indicated significantly lower levels of calcium than the normal rats fed with a purified diet $(36.52 \pm 0.75\%)$. Calcium-deficient rats fed with calcium-deficient diet and dry yogurt fortified by DFA III (chicory roots) showed femur bone calcium concentrations of $34.94 \pm 3.21\%$ and calcium-deficient rats fed with calcium-deficient diet and dry yogurt fortified by DFA III (dahlia tubers) showed femur bone calcium concentrations of $28.95 \pm 1.95\%$; these numbers substantially higher than the calcium-deficient rats and comparable to the control value. Low concentrations of calcium in the femur bone indicate a less dense matrix. The results of Mitamura and Hara (2005) also showed that the supplementation of DFA III in rat models deficient in estrogen may improve bone strength and bone mineral concentrations of the femur by increasing the absorption of calcium [34]. Furthermore, Eastwood (2003) stated that generally calcium will be stored in skeleton [35].

Figure 1c shows the decreasing diameter of empty space, suggesting an increase of bone density in the calcium-deficient rats fed with calcium-deficient diets and dried yogurt fortified with 3.60% DFA III (dahlia tubers). Administration of yogurt fortified with DFA III from dahlia tubers and DFA III from chicory roots increased the calcium content of bones in calcium deficiency rats to the levels of $28.95 \pm 1.95\%$ and $34.94 \pm 3.21\%$, respectively. This shows that the physiological activity of DFA III increases calcium absorption and, ultimately, improves bone condition.

According to Suzuki *et al.* (1998, 2004), DFA III stimulates calcium absorption in the small intestine as well as fermentation in the cecum and large intestine [9, 10]. Mitamura and Hara (2005) further stated that DFA III may increase the absorption of calcium by mucosal calbindin-D9k in the large intestine. Calbindin-D9k levels of cholecalciferol induce the main calcium-binding protein (CaBP) of the intestinal mucosa which has a high affinity for calcium in the intestine, where CaBP has an active role in transcellular calcium transport during calcium deficiency [34]. The results of the study by Mineo *et al.* (2006) further showed that feeding a 3% DFA III supplemented ration to rats for 14 days resulted in DFA III degradation by microbial fermentation, which produced short-chain fatty acids (SCFA) that increased calcium absorption in the large intestine [36].

In the body, the process of bone formation involves osteoblasts as the main cells for matrix production. The osteoblasts regulate calcium ion concentrations in the matrix through the release of intracellular calcium [37]. Higher calcium levels and denser bone matrices in pre-menopausal, calcium-deficient rats fed with DFA III fortified dry yogurt could prevent the osteoporosis in the post-menopausal period. This is in agreement with work by Compston *et al.* (1993), which stated that higher bone mass density in the pre-menopausal period maintained calcium deposits in the bones that will protect the body from osteoporosis and fractures that may result from calcium declines during aging [3].

Bones are minerally composed of inorganic compounds, most commonly calcium phosphate or calcium hydroxyapatite $[3\text{Ca}_3(\text{PO}_4)_2\text{Ca}(\text{OH})_2]$ crystals. SEM and EDX can be used to detect

constituents of the femur including calcium, phosphorus, magnesium, carbon, sodium, aluminum, and oxygen. Goldstein *et al.* (1992) showed that every element in the periodic table emits different x-rays and energy, which can be detected [21]. Using this detection of elements, the types of atoms and their relative percent compositions can be determined for any material.

There was no aluminum detected in the treatment of calcium-deficient rats fed with calcium-deficient diets and dried yogurt, with or without DFA III fortification. According to Urbiola *et al.* (2012), bone mineral composition is influenced by several factors including age, nutritional conditions, diseases, and habits [38].

The average strength, length, and weight of the experimental rat bones are presented in Table 6. Statistical analysis of the bone weight and strength showed no significant difference. However, calcium-deficient rats fed with dry yogurt fortified by DFA III (chicory) showed longer femurs (3.40 ± 0.10 cm), higher bone weights (0.31 ± 0.07 g), higher bone strength (9.34 ± 3.61 kg cm⁻²), and higher calcium concentrations in the femur bone ($34.94 \pm 3.21\%$). These rats had bone strengths similar to the control rats fed purified diets (9.34 ± 4.81 kg cm⁻²) and high calcium concentrations ($36.52 \pm 0.75\%$), allowed by a bone matrix condition that is still dense and radiopaque.

Table 6. Length, weight, and strength of the femurs from pre-menopausal rats

Treatments	Length of femur bone(cm)	Weight of femur bone (g)	Bone strength (kg cm ⁻²)
C	3.07 ± 0.25^a	0.28 ± 0.04^a	9.34 ± 4.81^a
CD	3.33 ± 0.15^{ab}	0.32 ± 0.06^a	6.46 ± 2.68^a
CD+DFA III dahlia	3.33 ± 0.58^{ab}	0.31 ± 0.02^a	7.87 ± 0.64^a
CD+DFA III chicory	3.40 ± 0.10^b	0.31 ± 0.07^a	9.34 ± 3.61^a

Different superscripts in the same column indicate significant differences in the test ($P < 0.05$) by ANOVA

Annotation: Normal rats fed with purified diet (C); calcium-deficient rats fed with calcium-deficient diet (CD); calcium-deficient rats fed with calcium-deficient diet and DFA III (dahlia) fortified dry yogurt (CD+DFA III dahlia); and calcium-deficient rats fed with calcium-deficient diet and DFA III (chicory) fortified dry yogurt (CD+DFA III chicory).

Based on the study by Mitamura and Hara (2005), it is known that DFA III supplementation in estrogen-deficient rat models improves bone strength and plays a role in bone metabolism [34] because estrogen receptors induced by DFA III increased the absorption of calcium, affecting bone mineral density. Calcium absorption is also influenced by age and according to Shiga *et al.* (2003), supplementation of 3% DFA III in the diet of 4 week old Sprague Dawley male rats can improve the strength of the femur bone, as compared to rats aged 15 months [39]. Bone strength is also influenced by body condition as reported by Hawkins *et al.* (2010) in studying the risk of decline in bone mineral density for lean and obese rats [40].

Previous findings by Boivin and Meunier (2003) also stated that bone strength depends on the bone matrix volume, bone microarchitecture distribution, and the mineralization degree of bone tissue [41]. The mineralization degree of bone tissue affects not only the bone mechanical resistance but also bone mineral density. Safrida (2013) stated similar conclusions that the improvement of bone quality was characterized by increased bone calcium levels, bone density, and bone strength [25].

Consumption of dry yogurt fortified with DFA III did not affect the weight of the femur bone in the pre-menopausal rats. This is because the rats used were 15 months old, meaning that they

had already reached the bone mass peak, and bone growth had stopped. This result confirms the previous research findings by Wronski and Yen (1991) which showed that bone growth is minimal in rats aged 9 to 12 months [42].

CONCLUSIONS:

The femur bones of rats fed with CD+DFA III chicory were stronger ($9.34 \pm 3.61 \text{ kg cm}^{-2}$) and contained higher bone calcium concentrations ($34.94 \pm 3.21\%$), as indicated by higher concentrations of dense bone matrix. These results were compared to rats fed with CD+DFA III dahlia that showed calcium concentrations of $28.95 \pm 1.95\%$ and a bone strength of $7.87 \pm 0.64 \text{ kg cm}^{-2}$ as well as a less dense bone matrix.

Normal rats fed with a purified diet (C) containing adequate calcium (0.60%) showed relatively higher calcium concentrations in the femur bone ($36.52 \pm 0.75\%$), higher bone strength ($9.34 \pm 4.81 \text{ kg cm}^{-2}$), and a more dense bone matrix compared to other treatments. The evidence from this study suggests that sufficient calcium intake could provide high calcium deposits in the bones. Ultimately, diets containing 3.60% w/w DFA III fortified dry yogurt have been shown to enhance calcium absorption in calcium-deficient rats.

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