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## Bioavailability of Ca, P and Zn and Bone Mineralization in Rats Fed Yoghurt and Soyyoghurt Containing Bifidobacteria

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#### Authors' contributions

This work was carried out in collaboration between all authors. Author IAA designed the study and performed the statistical analysis. Author AMM wrote the first draft of the manuscript. Authors IAA and AMM Wrote the protocol and drafted the manuscript and revision. Authors FAS and EAR managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

**Original Research Article** 

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## ABSTRACT

Bioavailability of calcium, phosphorus and Zinc from probiotic dairy foods and other food sources has been an important issue of studies over recent years.

The aim of the present study was designed to asses and to compare therapeutic effect of milk yoghurt and soy yoghurt containing bifidobacteria with regards to their effect on the bioavailability of Ca, P and Zn and bone mineralization in rats.

Eight groups of rats were fed basal diet, cow milk, probiotic-free or probiotic-containing milk yoghurts (*Bifidobacterium lactis* Bb-12 or *Bifidobacterium longum* Bb-46), soymilk and soy-yoghurts containing only the above mentioned probiotics for 45 days.

Upon feeding probiotic-containing milk and soymilk yoghurts, rat's serum Ca and P content were about two fold that of control and surprisingly the rat's serum Zn content was about 19-21 fold that of control. The increment of apparent absorption % as compared with control was ranged between 24.7–26.6, 24–38 and 51-70% for Ca, P and Zn, respectively.

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The above mentioned results clearly demonstrate the enhancement of Ca, P and Zn bioavailability when probiotic diets were fed. A significant increase (n=6;p<0.05) was observed in ash content and breaking force of femur of rats fed probiotic milk-and soy-yoghurts.

Moreover probiotic milk yoghurts were much better than soy-yoghurts fermented by *Bifidobacteria* in enhancing bioavailability of Ca, P and Zn as well as bone mineralization. These results suggest that intake of probiotic milk-and soy- yoghurts may be useful in enhancing mineral bioavailability and bone properties.

Keywords: Yoghurt; soy yoghurt; mineral bioavailability; probiotics; femur bone.

#### **1. INTRODUCTION**

The nutritional and health benefits of milk and other dairy foods are well documented and sound scientific evidence supports their importance in the diet. In fact these foods have a long history of contributing to health and well-being [1]. Milk and milk products are considered functional foods because of their calcium, magnesium, phosphorus, proteins content and presence of viable microorganisms [2] which when administered in adequate amounts confer a health benefits on the host and called probiotics [3]

Although most probiotic foods are derived from milk fermentation, the demand for using other protein-rich substances such as soymilk to make such foods (soy-based yoghurts) is growing due to problems with lactose intolerance and cholesterol content of fermented dairy products and desire for vegetarian alternatives [4] and therefore interest in a soy-based yoghurt type fermentation has developed [5] due to their reported nutritional and health promoting benefits [6]. This is in part because the isoflavones in soymilk can have many benefits such as bone-sparing effects over the long term [7].

Milk and milk products are considered good sources of Ca which has an anti-resorptive effect and can help to prevent bone loss [8] and phosphorus that binds with calcium to form the mineral hydroxyapatite which confers strength and rigidity to bones [9]. Moreover, the role of zinc as an essential trace element required for the growth of all creatures is indispensable in bone calcification and bone resorption and remodeling [10].

Further, several studies in the past 10 years were conducted to investigate the beneficial effects of prebiotics on mineral absorption while there have been few studies published on the effect of probiotics on mineral absorption [11].

On the basis of the above mentioned information and in the light of our previous studies on the anti-tumors and the hypocholestraemic effect of yoghurt and soy-yoghurt containing *Bifidobacterium lactis Bb-12 or Bifidobacterium longum Bb-46* [12] as well as the scarce and variable data regarding the effect of probiotic yoghurt and soy-yoghurt on mineral absorption and bone mineralization, the present study was designed to assess and to compare the therapeutic effect of the two forementioned yoghurts with regards to their effect on the bioavailability of some minerals (Ca, P and Zn) and bone mineralization in rats.

## 2. MATERIALS AND METHODS

#### 2.1 Preparation of Yoghurt from Cow Milk

Yoghurt was made according to the method of the author [13]. Cows' milk was inoculated with 3% (v/v) of *Lactobacillus delbreuckii* subsp *bulgaricus* and *Streptococcus thermophilus* (Chr. Hansen Laboratories, Copenhagen, Denmark) and divided into three portions. One portion (without added bifidobacteria) served as control yoghurt and was denoted as YCM; a second portion was inoculated at a level of 0.07% (w/v) with a freeze-dried *Bifidobacterium lactis Bb-12* and served as an experimented yoghurt and was denoted as YCMBb-12. The third portion was inoculated at a level of 0.07% (w/v) with a freeze-dried *Bifidobacterium longum Bb-46* and served as an experimented yoghurt and was denoted as YCMBb-46. The two strains of bifidobacteria were obtained from Chr. Hansen Laboratories (Copenhagen, Denmark). After the incubation period, the YCM, YCMBb-12 and YCMBb-46 yoghurts were stored at  $4\pm1^{\circ}$ C.

#### 2.2 Preparation of Non-fermented Soymilk and Soy-yoghurts

Fresh and non-beany flavored soymilk was prepared from soybeans according to the method of the author [14] and divided into three portions. One portion served as a non-fermented soymilk and was denoted as NFSM; the other two portions were used for preparation of soy-yoghurts Bb-12 and Bb-46 according to the method described by the author [15], using 0.07% (w/v) *B. lactis Bb-12* or *B. longum Bb-46*. After inoculation at 37°C for 4-5h, both yoghurts were stored at  $4\pm1^{\circ}$ C.

#### **2.3 Animal Feeding Experiments**

Forty eight male albino rats of average weight between 110-140 g were separately housed in a temperature  $(23\pm1^{\circ}C)$ , light-(12 h day/night cycle), relative humidity  $(60\pm5\%)$ -controlled room and fed a basal diet for one week. After this adaptation period, the rats were divided randomly by weight into 8 experimental groups, each of 6 animals. The experimental diets given to the eight groups as described in Table 1.

| Diet treatment                               | Diet code | Diet formula                     |
|--|-----------|----------------------------------|
| Basal diet                                   | Control   | 100g basal diet + 50 ml water    |
| Basal diet + Cow milk                        | CM        | 100 g basal diet + 50 g CM       |
| Basal diet + yoghurt from Cow milk (YCM)     | YCM       | 100 g basal diet + 50 g YCM      |
| Basal diet + YCM with added B. lactis Bb-12  | YCMBb-12  | 100g basal diet + 50 g YCMBb-12  |
| Basal diet +YCM with added B.longum Bb-46    | YCMBb-46  | 100 g basal diet + 50 g YCMBb-46 |
| Basal diet + non-fermented soy milk          | NFSM      | 100 g basal diet + 50 g NFSM     |
| Basal diet + yoghurt from soy milk fermented | YSMBb-12  | 100 g basal diet + 50 g YSMBb-12 |
| with B. lactis Bb-12                         |           |                                  |
| Basal diet + yoghurt from soy milk fermented | YSMBb-46  | 100 g basal diet + 50 g YSMBb-46 |
| with B.longum Bb-46                          |           | _                                |

#### Table 1. Experimental groups of rats and diets used in 45 days the trial x, y

<sup>x</sup> The basal diet consisted of: 20% (w / w) casein, 53.2% (w / w) corn starch, 7% (w / w) corn oil, 3.5% (w / w) mineral mixture, 1% (w / w) vitamin mixture, 5% (w / w) cellulose, 0.3 % (w / w) L. cysteine and 10% (w / w) sucrose. [16]. <sup>y</sup> The mineral mixture consisted of [mg (except as noted) / kg diet]:Ca HPO<sub>4</sub>, 15 g; K<sub>2</sub>HPO<sub>4</sub>, 2.5 g; KCl, 5 g; NaCl, 5 g; MgCl<sub>2</sub>, 2.5 g; Fe<sub>2</sub>O<sub>3</sub>, 2.5 g; MnSO<sub>4</sub>, 125; CuSO<sub>4</sub>. 5 H<sub>2</sub>O, 25; CoSO4. 7H<sub>2</sub>O, 0.2; ZnSO<sub>4</sub>. H<sub>2</sub>O, 100 and KI, 0.4

The rats were allowed free access to experimental diets and water. The daily food consumption of each rat and weekly body weights were monitored throughout the test period. Feces were collected every week and were kept frozen (- $23\pm1^{\circ}$ C) until analysis.

Blood samples were collected at zero time and 45 days of study period from the rat's plexus Venus by using fine capillary glass tubes, samples were taken into tubes and then centrifuged at 3000rpm for 10min to obtain the serum, which stored at -23±1°C until analysis.

On the final day of the experiment, after rats were killed, the whole cecum was taken, weighed and kept frozen until analysis and the right femur from each rat were removed, cleaned and weighed, then were kept until analysis.

## 2.4 Analytical Methods

#### 2.4.1 Chemical and microbial analysis of the used products

Protein, fat and sugar content in the used products were determined by micro kjeldahl procedure, rose-gottlieb and HPLC methods according to [17-19], respectively. Whereas Ca and Zn content were determined by wet ashing by atomic absorption according to the method of the author [20] While P was determined by spectrophotometer according to the method of the author [21]. Table 2 shows the chemical composition of the products used in the investigation.

| Product  | Protein (%)     | Fat (%)                    | Sugar (%)           | Mineral (mg/kg) |            |           |
|----------|-----------------|----------------------------|---------------------|-----------------|------------|-----------|
|          |                 |                            |                     | Ca              | Р          | Zn        |
| СМ       | 3.1±0.04        | 3.2±0.10                   | 4.5±0.08            | 1142.9±4.14     | 107.1±1.65 | 0.4±0.02  |
| YCM      | 3.1±0.08        | 3.4±0.05                   | 2.6±0.09            | 1191.2±4.75     | 110.0±0.97 | 0. 5±0.03 |
| YCMBb-12 | 3.4±0.06        | 3.4±0.05                   | 2.3±0.13            | 1189.4±2.91     | 109.8±2.93 | 0.5±0.03  |
| YCMBb-46 | 3.3±0.21        | 3.3±0.05                   | 2.2±0.06            | 1190.9±1.37     | 110.3±1.76 | 0.5±0.02  |
| NFSM     | 4.5±0.06        | 2.3±0.06                   | 0.9±0.05            | 246.9±2.55      | 45.0±2.60  | 0.1±0.01  |
| YSMBb-12 | 4.7±0.06        | 2.4±0.03                   | 0.5±0.08            | 262.5±2.24      | 46.8±1.26  | 0.1±0.06  |
| YSMBb-46 | 4.8±0.04        | 2.4±0.02                   | 0.5±0.11            | 257.30±3.57     | 47.1±1.36  | 0.1±0.03  |
|          | Xaaa Tabla A as | and the set for an all the | He of distance atom | t- V t          |            |           |

#### Table 2. Chemical composition of the products used in the study <sup>x,y</sup>

<sup>x</sup>see Table 1 and text for details of diet treatments. <sup>y</sup>values are mean  $\pm$  SD (n=6)

The count of bifidobacteria in both soy-yoghurts (YSMBb-12 and YSMBb-46), which did not contain yoghurt culture and in cow milk yoghurts (YCMBb-12 and YCMBb-46), which contain yoghurt culture as well as bifidobacteria, were determined according to the methods described by authors [22-23], respectively. Whereas yoghurt culture was enumerated according to the method of [24]. Table 3 shows the viable count of bifidobacteria and yoghurt culture in the experimental products.

#### 2.4.2 Serum assay

Ca, P and Zn in rat's serum were determined according to the method of authors [25-27], respectively.

#### 2.4.3 Feces assay

Ca, P and Zn in feces were determined according to the method of the author [20].

| Product  | Bifidobacterial count <sup>w</sup> | Yoghurt culture count <sup>z</sup> |
|----------|------------------------------------|------------------------------------|
|          | (cfu ×1                            | 0 <sup>8</sup> / ml) <sup>a</sup>  |
| YCM      | ND <sup>b</sup>                    | 75.8                               |
| YCMBb-12 | 3.1                                | 68.9                               |
| YCMBb-46 | 3.8                                | 47.7                               |
| YSMBb-12 | 4.6                                | ND                                 |
| YSMBb-46 | 5.3                                | ND                                 |

## Table 3. The viable count of yoghurt culture and Bifidobacteria in experimental products <sup>x, y</sup>

<sup>x</sup>values are means (n = 6). <sup>y</sup>see Tables 1,2 and text for details of diet treatments. <sup>z</sup>yoghurt culture: Lactobacillus delbreuckii subsp bulgaricus and Streptococcus thermophilus. <sup>w</sup>Bifidobacteria: B. lactis Bb-12 or B.longum Bb-46. <sup>a</sup>cfu: colony forming unit. <sup>b</sup>ND: Not determined

#### 2.4.4 Cecum assay

Cecum pH was measured by pH meter according to the method of the author [28]. While the count of bifidobacteria in rat's cecum was determined according to the method described by the author [23]. Whereas carboxylic acids concentration (acetic, lactic, propionic and pyruvic acids) were determined by HPLC according to the method of the author [29]

#### 2.4.5 Femur length, thickness, bone density and breaking force

The length and thickness of each right femur were measured with a vernier caliper. Femur bone density was measured by Archimedes' principle [30], while breaking force was determined according to the method of the author [31].

## 2.4.6 Mineral and non-mineral content of femur

The right femurs were scraped clean from remaining flesh and weighed (wet weight). They were then dried at 80°C for 18h and weighed again (dry weight). The dried bones were ashed at 550°C for 24h and reweighed again (ash weight). The amount of Ca, P and Zn in the ashed samples of femur was determined according to [32]. The mineral bone weight was calculated using the following formula:

Non-mineral weight = Dry weight – Ash weight.

## 2.4.7 Statistical analysis

Data are presented as means and standard deviation. The significance differences between groups / treatments were analyzed by analysis of variance (ANOVA) using the general linear model procedure of Statistical Analysis System [33] Multiple comparison between treatment means was made by the LSD (Least Significant Difference ) at P<0.05.

## 3. RESULTS AND DISCUSSION

## 3.1 Body Weight Gain, Food Intake and Food Efficiency

Results in Table 4 showed that there were no significant differences (P<0.05) in body weight, body weight gain, food intake and food efficiency among the eight experimental rat

groups throughout the study period. This is in agreement with the results obtained by the author [11] who found similar results when growing male and ovariectomised female rats were fed high calcium milk powder plus or minus probiotics (*L. rhamnosus* HN001).

| Table 4. Food intake, | body weight gain and | I food efficiency         | of rats fed o | n experimental |
|-----------------------|----------------------|---------------------------|---------------|----------------|
|                       | diets during         | g 45 days <sup>x, y</sup> |               |                |

| Treatment | Body weight (g)        |                      | Body                              | Food intake          | Food efficiency      |
|-----------|------------------------|----------------------|-----------------------------------|----------------------|----------------------|
|           | Initial body<br>weight | Final body<br>weight | weight gain<br>/ day <sup>z</sup> | (g/day)              | (%) <sup>w</sup>     |
| Control   | 123 <sup>a</sup> ±11   | 236 <sup>ª</sup> ±22 | 2.5 <sup>ª</sup> ±0.3             | 16 <sup>a</sup> ±0.6 | 16 <sup>a</sup> ±2.4 |
| CM        | 124 <sup>a</sup> ±10   | 235 <sup>ª</sup> ±19 | 2.4 <sup>a</sup> ±0.6             | 16 <sup>a</sup> ±1.1 | 15 <sup>ª</sup> ±3.3 |
| YCM       | 124 <sup>a</sup> ±10   | 231 <sup>ª</sup> ±15 | 2.3 <sup>a</sup> ±0.0             | 16 <sup>a</sup> ±1.1 | 15 <sup>ª</sup> ±1.9 |
| YCMBb-12  | 125 <sup>ª</sup> ±10   | 229 <sup>a</sup> ±15 | 2.3 <sup>a</sup> ±0.2             | 17 <sup>a</sup> ±1.5 | 14 <sup>a</sup> ±2   |
| YCMBb-46  | 127 <sup>a</sup> ±16   | 235 <sup>°</sup> ±27 | 2.4 <sup>a</sup> ±0.9             | 16 <sup>a</sup> ±0.2 | 15 <sup>ª</sup> ±5.5 |
| NFSM      | 127 <sup>a</sup> ±18   | 235 <sup>ª</sup> ±21 | 2.4 <sup>a</sup> ±0.1             | 16 <sup>a</sup> ±0.7 | 15 <sup>ª</sup> ±0.2 |
| YSMBb-12  | 129 <sup>a</sup> ±12   | 236 <sup>a</sup> ±20 | 2.4 <sup>a</sup> ±0.2             | 17 <sup>a</sup> ±1.3 | 14 <sup>a</sup> ±0.2 |
| YSMBb-46  | 126 <sup>ª</sup> ±12   | 234 <sup>ª</sup> ±6  | 2.4 <sup>a</sup> ±0.4             | 16 <sup>ª</sup> ±0.9 | 15 <sup>ª</sup> ±2.9 |

<sup>x</sup>values are means ± Standard deviation (n = 6). Means not sharing common letters within column are significantly different (P<0.05). <sup>y</sup>See Tables 1,2 and text for details of diet treatments. <sup>z</sup>Body weight gain / day = (final body weight – initial body weight) / study period. <sup>w</sup>Food efficiency (%) =body weight gain / food intake ×100

## 3.2 Cecum pH and Bifidobacterial Count

Results in Fig. 1 indicated that feeding on each of the supplemented diets led to significant decrease in the rat's cecum pH as compared with that of control group. Moreover, the incorporation of probiotics in rat's diet resulted in significant (P<0.05) reduction in pH of rat's cecum than probiotic-free diets. It is worthy to note that B. lactis Bb-12 was more effective on pH reduction than B. longum Bb-46 either when present in yoghurt from cow milk or soy milk when compared with that of control group. These results are in general agreement with those of the authors [34-35] who illustrated that the addition of bifidobacteria and / or prebiotic to the diets reduced the pH of the cecal content.

Regarding the bifidobacterial count in cecum (Fig. 2), it was found that the addition of B. longum Bb-46 significantly increased the count of bifidobacteria as compared with that of other all experimented diets and control groups, respectively. This was explained by the author [36] who reported that bifidobacteria have been shown to decrease the number of ammonia producing bacteria such as clostridia, resulting in a decreased production of ammonia and possibly explaining the low pH.



Fig. 1. Cecum pH in rats fed experimental diets at the end of study period



Fig. 2. Bifidobacterial count (cfu x 10<sup>7</sup>/g) in cecum of rats fed experimental diets at the end of study period

## 3.3. Carboxylic Acids in Rats' Cecum

It is obvious from Table 5 that presence of probiotic B. lactis Bb-12 and B. longum Bb-46 (YSMBb-12 followed by YSMBb-46,YCMBb-12 followed by YCMBb-46) and yoghurt culture (YCM) in the rat's diets increased significantly (P<0.05) the amount of acetic acid as compared with that of cow milk, soymilk diets and basal diet. Also, the data showed that the incorporation of probiotic products in rat's diets increased significantly the amount of lactic acid and propionic acid as compared with that of control group. The data showed that there were insignificant changes in the amount of pyruvic acid in the cecal content of rats fed various diets except YSMBb-46 containing diet which led to significant increase in the amount of pyruvic acids content was in the following descending order 11.4, 11.1, 10.9, 10.4, 9.1,

8.5, 7.3, 6.2 for the YSMBb-12, YSMBb-46, YCMBb-12, YCMBb-46, YCM, NFSM, CM, control groups, respectively. These findings are in accordance with the previously reported data by the author [37] who concluded that probiotic bacteria produce fermentation by-products including short chain fatty acids (SCFAs)

| Table 5. Carboxylic acids in cecum of rats fed experimental diets at the end of stu | ıdy |
|---|-----|
| period <sup>x, y</sup>  | -   |

| Treatment | Car                   | Total <sup>z</sup>     |                        |                         |                         |
|-----------|-----------------------|------------------------|------------------------|-------------------------|-------------------------|
|           | Acetic                | Lactic                 | Propionic              | Pyruvic                 | _                       |
| Control   | 5.7 <sup>b</sup> ±1.0 | 0.3 <sup>c</sup> ±0.0  | 0.11 <sup>c</sup> ±0.0 | $0.10^{b} \pm 0.0$      | 6.2 <sup>e</sup> ±1.1   |
| CM        | 6.8 <sup>b</sup> ±1.0 | $0.4^{c} \pm 0.0$      | 0.12 <sup>b</sup> ±0.0 | 0.11 <sup>b</sup> ± 0.0 | 7.3 <sup>de</sup> ±.0   |
| YCM       | 8.5 <sup>ª</sup> ±1.3 | 0.4 <sup>c</sup> ±0.0  | 0.14 <sup>b</sup> ±0.0 | 0.11 <sup>b</sup> ± 0.0 | 9.1 <sup>bc</sup> ±1.3  |
| YCMBb-12  | 9.6 <sup>a</sup> ±0.5 | 1 <sup>b</sup> ±0.0    | 0.16 <sup>a</sup> ±0.0 | 0.11 <sup>b</sup> ± 0.0 | 10.9 <sup>a</sup> ±0.4  |
| YCMBb-46  | 9.4 <sup>b</sup> ±0.5 | 1. <sup>b</sup> ±0.0   | 0.17 <sup>a</sup> ±0.0 | 0.16 <sup>a</sup> ± 0.0 | 10.4 <sup>ab</sup> ±0.8 |
| NFSM      | 6.9 <sup>a</sup> ±0.5 | 1.4 <sup>a</sup> ±0.2  | 0.11 <sup>c</sup> ±0.0 | 0.10 <sup>b</sup> ± 0.0 | 8.5 <sup>cd</sup> ±0.4  |
| YSMBb-12  | 9.9 <sup>a</sup> ±0.3 | 1.2 <sup>ab</sup> ±0.3 | 0.16 <sup>a</sup> ±0.0 | 0.11 <sup>b</sup> ± 0.0 | 11.4 <sup>a</sup> ±0.2  |
| YSMBb-46  | 9.8 <sup>ª</sup> ±0.3 | 1. <sup>b</sup> ± 0.1  | 0.16 <sup>ª</sup> ±0.0 | $0.13^{b} \pm 0.0$      | 11.1 <sup>ª</sup> ±0.4  |

<sup>x</sup>values are means ± Standard deviation (n = 6). Means not sharing common letters within column are significantly different (P<0.05). <sup>y</sup>See Tables 1,2 and text for details of diet treatments. <sup>z</sup>Total = (acetic, lactic, propionic and pyruvic)

## 3.4 Concentration of Ca, P and Zn in rat's serum

Serum Ca, P and Zn concentration at the end of study period was presented in Fig. (3a+3b) Significant (P<0.05) increase was observed between values of serum Ca concentration (mg/dl) in rats of experimental groups YCMBb-12, YSMBb-12, YCMBb-46, YSMBb-46, YCM, NFSM and CM (14.3±2.4, 13.7±2.3, 13.5±3.3, 13.2±1.5, 12.4±2.0, 12.2±1.4 and 12±1.8, respectively) as compared with that of control group (7.2±2.0). On the other hand, no significant differences were existed between the values of serum calcium concentrations in rats test groups other than control group.

The serum Ca concentration of rats fed probiotic milk - or soy-yoghurt was about two fold that of rats fed the control diet at the end of study period. It is worthy to note that feeding diets containing probiotic milk yoghurt YCMBb-12 had greater effect than that of YCMBb-46, soy-yoghurt YSMBb-12 and YSMBb-46 on serum Ca concentration (Fig. 3a). This might be due to the partial digestion of existed calcium form occurred during manufacturing of probiotic yoghurt or soy yoghurt which increase the soluble calcium and consequently enhance its absorption and bioavailability by the author [38]. This observation was explained by the author [39] who reported that milk fermentation by *L. helveticus* bacteria had a positive effect on calcium metabolism by suppressing serum parathyroid hormone (PTH) and increasing serum calcium concentrations. However the dietary lactose stimulates Ca utilization and also increases Ca absorption and tissue uptake of Ca and Zn in the adolescent rats. Compared to control and other sugars [41].

It was also found that at the end of the study period (Fig. 3a), there was significant (P<0.05) increase between values of serum P concentration in rats of groups fed any of the diets containing YCMBb-12, YCMBb-46, YSMBb-12 or YSMBb-46 (11.7±0.2, 12.4±1.7, 12.1±2.1, and 12.8±0.5, respectively) and that of control group rats (6.8±1.6). These data also showed that values of serum P concentrations were not significantly different between CM, YCM and

NFSM (9.4±2.1, 8.7±0.5 and 8.9±1.0, respectively) when compared with that of control group. The serum P concentration of rats fed probiotic milk or soy-yoghurt as indicated in Fig. 3a was ranged between 1.7 to 1.9 fold that of rats fed the control diet at the end of study period. It was observed that feeding probiotic soy-yoghurt (YSMBb-46 and YSMBb-12) had slightly greater effects than that of its correspondence of probiotic milk yoghurt. These results are in a general agreement with those of the author [35] who studied the effect of seven infant formulas on bioavailability of calcium, magnesium and phosphorus. They reported that addition of probiotic and prebiotic to the infant formulas either singly or jointly stimulated the bioavailability of minerals in rats during different periods of time.

Surprisingly, it was shown from the data in Fig. 3b that serum zinc concentration of rats fed YCMBb-12, YCMBb-46, YSMBb-12 and YSMBb-46 was about 18.5-21 fold that of rats fed the control diet at the end of study period (their values were  $2.6\pm0.9$ ,  $2.3\pm0.4$ ,  $2.2\pm1.8$ ,  $2.5\pm0.5$ , respectively). On the other hand, a marginal and insignificant increase was seen in serum Zn concentration in rats fed CM, YCM and NFSM ( $0.16\pm0.04$ ,  $0.17\pm0.02$  and  $0.15\pm0.01$ , respectively) as compared with that of the control group ( $0.12\pm0.02$ ).



Fig. 3a. Ca and P concentration in rats' serum



Fig. 3b. Zn concentration in rats' serum

## 3.5 Apparent absorption of Ca, P and Zn

Our results also showed there were no significant differences in food intake (Table 4) or intake of Ca, P and Zn (data not shown) between rats fed supplemented diets (71 $\pm$ 5.1 to 74 $\pm$ 6.7, 62 $\pm$ 4.3 to 64 $\pm$ 5.9 and 0.62 $\pm$ 0.01 to 0.62 $\pm$ 0.01 to 0.64 $\pm$ 0.02, respectively) when compared with control group (70 $\pm$ 2.5, 61 $\pm$ 1.3 and 0.64 $\pm$  0.05, respectively).The data reported in Fig. 4 Showed that the apparent absorption of Ca in all test groups was significantly (p<0.05) higher than in the control group.



Fig. 4. Ca, P and Zn apparent absorption % in rats fed diets for 45 days.

The highest value of Ca apparent absorption % was for rats fed diet containing YSMBb-12 (84±1.2) followed by rats fed diets containing YSMBb-46, YCMBb-12, YCMBb-46, YCM, CM, NFSM (83.40±3.9, 83±2.3, 82±1.4, 78±1.7, 76±1.9 and 73±3.3, respectively) as compared with that of control group (66±4.3). The increment percentage of Ca apparent absorption as a result of feeding probiotic-containing yoghurts ranged between 24.7 to 26.6%. These results are in accordance with the results obtained by the author [37] who found that probiotic yoghurts containing strains of *L. casei, L. reuteri* and *L. gasseri* increased apparent calcium absorption and bone mineral content in growing rats. Also this was confirmed by data of the author [42] who indicated that fermenting of calcium fortified soymilk with selected probiotics could potentially enhanced the calcium bioavailability due to increased calcium solubility and bioactive isoflavone aglycone enrichment. In addition, degradation of the caseins by digestive enzymes increases the likelihood of peptide liberation [43]. Which may promote calcium uptake by intestinal cells by preventing precipitation of insoluble salts [44]? It was also showed that *Bifidobacterium bifidum* and *Bifidobacterium longum* improve apparent absorption and apparent retention of Ca in weanling rats [45].

The obtained results (Fig. 4.) also showed that apparent absorption of P was significantly higher in rats fed all treatments when compared with that of control .The incorporation of YCMBb-12, YCMBb-46, YSMBb-12 and YSMBb-46 into the diets led to increase the apparent absorption of P with high degree ( $69\pm2.6$ ,  $69\pm2.8$ ,  $64\pm1.7$  and  $62\pm1.3\%$ , respectively) than that of CM, YCM, NFSM and control as well ( $56\pm2.4$ ,  $59\pm3.5$ ,  $55\pm1.4$  and  $50\pm2.8\%$ , respectively). The apparent absorption increment % of P ranged from 24 - 38% when probiotic-containing yoghurts were fed. These data are in line with the author [46], who reported that urinary P excretion was significantly lowered when prebiotics or symbiotics were given to rats as compared with ovariectomised control rats.

Regarding the apparent absorption % of Zn, it was significantly higher (P<0.05) in rats fed YCMBb-12, YCMBb-46, YSMBb-12 and YSMBb-46 (68±2.3, 64±4.3, 60±6.0, 64±3.7%, respectively) followed by that of rats fed CM and YCM (56±5.9 and 53±6.9%, respectively). On the other hand, it was insignificantly increased in case of rats fed NFSM (44±7.6%) in comparison with that of control group (40±2.3%). This means that milk- and soy-yoghurts containing Bb-12 or Bb-46 were effective in the enhancement of apparent absorption % of Zn in rats.

The promising effect of probiotic-containing yoghurts on serum Zn content (Fig. 3b) is confirmed by the increase percentage in Zn apparent absorption which ranged between 51–76%. The obtained results are in accordance with that reported by the author [47] who studied bioavailability of zinc from nonfat dry milk, low fat plain yoghurt and soy flour in pigs. They found that apparent absorption was greater when nonfat dry milk and yoghurt containing diets were fed.

## 3.6 Femur ash, bone density and breaking force

The results showed there were no significant changes (P<0.05) in the wet & dry weights of femur in all rats fed the different experimental diets for 45 days (data not shown). While consumption of diets containing YCM, YCMBb-12, YCMBb-46, YSMBb-12 and YSMBb-46 led to significant (P<0.05) changes in the ash weight of femur of the rats as compared with that of other groups (non fermented diets). These data also illustrated that there were no significant changes in the ash weight between rats fed any of the fermented diets, i.e., YCM, YCMBb-12, YCMBb-46, YSMBb-46, YSMBb-12 and YSMBb-46 (data not shown). The incorporation of

YCMBb-12 in the feeding diets led to the highest value of femur ash weight. Similar results were obtained by the authors [48-49].

The bone density values of the femur were significantly (*P*<0.05) affected in rats fed YCMBb-12, YCMBb-46 and YSMBb-12. In addition, there were no significant changes in bone density of femur between CM, YCM, NFSM and YSMBb-46 (Fig. 5).



Fig. 5. Femur bone density (g/cm<sup>3</sup>) in rats fed experimental diets at the end of study period

Bones from rats fed YCMBb-12 had the highest breaking force value (103.78±8.93 N) among all experimental groups. The data in Fig. 6 exhibited that there were a significant increase in femur breaking force of rats fed YCMBb-12 and rats fed YSMBb-12 and those fed other experimental diets. Similar trend was obtained by the author [50-51] who indicated that feeding whey protein increases the level of bone proteins such as collagen in rats, thereby increasing bone breaking force.

## 3.7 Bone minerals composition of rat's femur

The results in Table 6 showed the contents of Ca, P and Zn in femur of rats fed the various experimental diets for 45 days. These results revealed significant (*P*<0.05) increase in calcium content in femur of rats fed CM, YCM, YCMBb-12, YCMBb-46, NFSM, YSMBb-12 and YSMBb-46. However, the increase in calcium content in femur was higher in probiotic yoghurt treatments than in rats fed CM, YCM or NFSM. The increment in femoral calcium content was highest in rats fed YCMBb-46. Our results agreed with results of the author [52], who showed that supplementing probiotic yoghurt, substituting corn starch with a prebiotic or by administering symbiotics in rat's diet could enhance the Ca deposition in their femur the author [45] found that *Bifidobacterium bifidum* and *Bifidobacterium longum* significantly increased femoral and tibial Ca content in weanling rats when they were fed for 30 days. These authors showed that probiotics increase crypt deth in the colon, and lowered colon pH compared to a control diet with no probiotics and Ca absorption was correlated with the pH of the colonic contents.



# Fig. 6. Femur breaking force (N) in rats fed experimental diets at the end of study period

Also, there was significant (*P*<0.05) increase in phosphorus content in femurs of rats fed CM, YCM, YCMBb-12, YCMBb-46, YSMBb-12 and YSMBb-46. Rats fed diet containing YCMBb-12 had the highest value of phosphorus content in femur followed by YCMBb-46, YSMBb-12 and YSMBb-46, respectively. Thus, the incorporation of probiotic in the feeding diets led to increase the accumulation of the minerals such as Ca and P in the femur.

The results in Table 6 revealed a significant increase in femur zinc content in rats fed diet containing YCMBb-12 and YCMBb-46. While, no significant (*P*<0.05) changes in femur zinc content between rats fed CM, YCM, NFSM, YSMBb-12 and YSMBb-46 in comparison with that of rats fed control diet. Thus, the probiotic dairy diets (YCMBb-12 and YCMBb-46) were the most effective in the accumulation of zinc in femur than that of probiotic non-dairy diets (YSMBb-12 and YSMBb-46).

Our results presented above indicate that feeding the probiotic diets (YCMBb-12 and YCMBb-46 and YSMBb-12 and YSMBb-46) may affect positively Ca, P and Zn bioavailability and bone properties. In addition, the suggested mechanisms of the positive effect of probiotics on mineral bioavailability and bone properties may include increased solubility of minerals due to increased bacterial production of short chain fatty acids, and enlargement of the absorption surface due to promotion of proliferation of enterocytes mediated by bacterial fermentation products [35]. It is also possible that short chain fatty acids can directly stimulate calcium and possibly magnesium absorption in the rat colon and that calcium at least could pass through the cell membrane more readily in the form of a less-charged complex (calcium acetate), via a passive pathway [53].

| Treatment | Femoral bone mineral composition (mg/bone dry weight) |                        |                            |  |  |
|-----------|---|------------------------|----------------------------|--|--|
|           | Ca  | Р                      | Zn                         |  |  |
| Control   | 45 <sup>b</sup> ±4.6                                  | 17 <sup>d</sup> ± 0.7  | 0.42 <sup>cd</sup> ±0.04   |  |  |
| CM        | 57 <sup>a</sup> ±2.6                                  | 18 <sup>bc</sup> ±0.5  | 0.4 <sup>7abcd</sup> ±0.01 |  |  |
| YCM       | 57 <sup>a</sup> ±3.6                                  | 19 <sup>abc</sup> ±0.7 | 0.49 <sup>abc</sup> ±0.00  |  |  |
| YCMBb-12  | 58 <sup>a</sup> ±4.9                                  | 20 <sup>a</sup> ±0.8   | 0.50 <sup>ab</sup> ±0.01   |  |  |
| YCMBb-46  | 58 <sup>ª</sup> ±3.1                                  | 19 <sup>ab</sup> ±0.6  | 0.5 <sup>a</sup> ±0.01     |  |  |
| NFSM      | 53 <sup>a</sup> ±2.0                                  | 18 <sup>cd</sup> ± 0.4 | 0.44 <sup>cd</sup> ± 0.01  |  |  |
| YSMBb-12  | 54 <sup>a</sup> ±3.3                                  | $19^{ab} \pm 0.7$      | $0.45^{bcd} \pm 0.01$      |  |  |
| YSMBb-46  | 53 <sup>a</sup> ±2.4                                  | $19^{abc} \pm 0.8$     | 0.46 <sup>abcd</sup> ±0.03 |  |  |

| Table 6. | Bone minerals | composition (  | of femur  | of rats | fed yoghurt and se           | oy-yoghurt |
|----------|---------------|----------------|-----------|---------|------------------------------|------------|
|          | containing    | g bifidobacter | ia at the | end of  | study period <sup>x, y</sup> |            |

<sup>x</sup>values are means ± Standard deviation (n = 6). Means not sharing common letters within column are significantly different (P<0.05). <sup>y</sup>See Tables 1,2 and text for details of diet treatments.

## 4. CONCLUSION

The present study indicated that consumption of milk- or soy-based probiotic yoghurts significantly increased the bioavailability of Ca, P and Zn as measured by the apparent absorption (%) as well as the bone mineralization as measured by minerals and ash contents of rat's femur. It is worthy to note that diet containing YCMBb-12 was the most efficient at increasing Ca, P and Zn bioavailability and in general probiotic milk yoghurts were much better than those of soymilk yoghurt in enhancing the bioavailability of such minerals. Thus, the present work directs the attention towards the beneficial role of milk yoghurts and subsequently may be useful lowering loss of body calcium and prevent osteoporosis. Further studies are needed to confirm these findings and to clarify the suggested mechanisms of action of Bifidobacteria and other probiotics on mineral absorption and bone properties.

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## **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

## REFERENCES

- 1. Rayan EA. Bioavailability of Some Minerals in Rats Fed Yoghurt and Soy-yoghurt Containing Bifidobacteria. M.Sc. Thesis. Fac. Agric., Cairo Univ., Egypt. 2010;147.
- 2. Heller KJ. Probiotic bacteria in fermented foods: product characteristics and starter organisms. American Journal of Clinical Nutrition. 2001;73:374-379.
- 3. FAO/WHO. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report; 2001.

- 4. Yoon KY, Woodams EE& Hang YD. Production of probiotic cabbage juice by lactic acid bacteria. Bioresource Technology. 2006;97:1427-1430.
- 5. Abd El-Gawad IA, El-Sayed EM, Hafez SA, El-Zeini HM, Saleh FA. Inhibitory effect of yoghurt and soy-yoghurt containing bifidobacteria on the proliferation of Ehrlich ascites tumour cells *in vitro* and *in vivo* in a mouse tumour model. British Journal of Nutrition. 2004;92:81-86.
- 6. Omoni AO, Aluko RE. Soybean foods and their benefits: potential mechanisms of action. Nutrition Reviews. 2005;63:272-283.
- 7. Setchel KDR, Cassidy A. Dietary isoflavones: biological effects and relevance to human health. Journal of Nutrition. 1999;129:758-767.
- 8. Heaney RP. Calcium, dairy products and osteoporosis. Journal of American College of Nutrition. 2000;19:83-99.
- 9. Institute of Medicine. Dietary reference intakes for calcium, phosphorous, magnesium, vitamin D, and fluoride. Standing committee on the scientific evaluation of dietary reference intakes, Food and Nutrition Board. National Academy Press;1997.
- 10. Swann JC, Reynolds JJ, Galloway WA. Zinc metalloenzyme properties of active and latent collagenase from rabbit bone. Biochemical Journal. 1981;195:41-49.
- 11. Kruger MC, Fear A, Chua WH, Pilmer GG, Scollum LM. The effect of *Lactobacillus rhamnosus* HN001 on mineral absorption and bone health in growing male and ovarictomized female rats. Dairy Science and Technology. 2009;1:1-13.
- 12. Abd El-Gawad IA, El-Sayed EM, Hafez SA, El-Zeini HM, Saleh FA. The hypocholesrolaemic effect of milk yoghurt and soy-yoghurt containing bifidobacteria in rats fed on a cholesterol-enriched diet. International Dairy Journal. 2005;15:37-44.
- 13. Tamime AY, Robinson RK. Yoghurt: Science and Technology. Paramo Press, Oxford: England;1985.
- 14. Kamaly KM. Bifidobacteria fermentation of soybean milk. Food Research International. 1997;30:675-682.
- El-Sayed EM, Hefny AA, Saleh FA, Abd El-Gawad IA. Bifidobacteria as a starter for the manufacture of soy-yoghurt products. 7<sup>th</sup> Egyptian Conference of Dairy Science & Technology. 1998;269-294.
- Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory roudents: final report of American Institute of Nutrition ad hoc writing committee on reformulation of AIN-76A rodent diet. Journal of Nutrition. 1993;123:1939-1951.
- 17. AOAC. Official methods of analysis of AOAC International (13<sup>th</sup> ed). Washington: USA;1984.
- 18. AOAC. Official methods of analysis of AOAC International (15<sup>th</sup> ed). Inc: USA; 1990.
- Black LT, Bagley EB. Determination of oligosaccharides in soybeans by high pressure liquid chromatography using an internal standard. Journal of the American Oil Chemistry Society. 1978;55:228-230.
- 20. Parker MM, Humoller FL, Mahler DJ. Determination of copper, zinc and other trace elements in biological materials. Clinical Chemistry. 1967;13:40-48.
- 21. Astm. Annual book of American society of testing materials standard water. Part 31Dsis-72-Philadelphia: USA; 1975.
- 22. Samona A, Robison RK. Enumeration of bifidobacteria in dairy products. Journal of the Society of Dairy Technology. 1991;44:64-66.
- 23. Dinakar P, Mistry VV. Growth and viability of *Bifidobacterium bifidum* in chedder cheese. Journal of Dairy Science. 1994;77:2854-2864.
- 24. Lee SY, Vedemuthu ER, Washam CJ, Reinbold BW. An agar medium for the differential enumeration of yoghurt starter. Journal of Milk and Food Technology. 1973;37:272.

- 25. Ray Sarker BC, Chouhan UPS. A new method for determining micro quantities of calcium in biological materials. Analytical Biochemistry. 1967;20:155-166.
- 26. Kaplan LA, Pesce AJ. Clinical Chemistry: Theory, Analysis and Correlation (.3<sup>rd</sup> ed). Mosby Company St. Louis; 1996.
- 27. Butrimovitz GB, Purdy WC. The determination of zinc in blood plasma by atomic absorption spectrometry. Analytica Chimica Acta. 1977;94:63-66.
- 28. Kotula AW, Twigg GG, Young EP. Evaluation of beef batties containing protein during 12-month frozen storage. Journal of Food Science. 1976;41:1142-1147.
- 29. Wodecki ZJ, Torlop B, Slebioda M. Chromatographic separation of citric acid for monitoring the mould process. Journal of Chromatography. 1991;558:302-305.
- Kalu DN, Liu CC, Salerno E, Hollis B, Echon R, Ray M. Skeletal response of ovarectomized rats to low and high doses of 17 B-estradiol. Bone Mineral. 1991;14:175-187.
- Gomez-Aldapa C, Martinez-Bustos F, Figueroa CJ, Ondorica FC. A composition of the quality whole corn tortillas made from instant corn flours by traditional or extrusion. International Journal of Food Science and Technology. 1999;34:391-399.
- Ohta A, Uehara M, Sakai K, Takasaki M, Adlercreutz H, Morohashi T, Ishimi Y. A compination of dietary fructooligosaccharides and isoflavone conjugates increase femoral bone mineral density and equol production in ovariectomized mice. Journal of Nutrition. 2002;132:2048-2054.
- 33. SAS User Guide Statistics, Version 6.0; 1990. SAS Inst., Inc., Cary, NC.
- Campbell JM, Fahey GC, Wolf BW. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. Journal of Nutrition. 1997;127:130-136.
- 35. Perez-Conesa D, Lopez G, Ros G. Effects of probiotic, prebiotic and synbiotic followup infant formulas on large intestine morphology and bone mineralization in rats. Journal of the Science of Food and Agriculture. 2007;87:1059-1068.
- 36. Nilsson U, Nyman M. Carboxylic acids in the hindgut of rats fed highly soluble inulin and *Bifidobacterium lactis (Bb-12), Lactobacillus salivariuos* (UCC500) or *Lactobacillus rhamnosus* (GG). Scandinavian Journal of Food and Nutrition. 2007;51:13-21.
- Ghanem KZ, Badawy IH, Abd EL-Salam AM. Influence of yoghurt and probiotic yoghurt on the absorption of calcium, magnesium, iron and bone mineralization in rats. Milchwissenschaft, 2004;59:472-475.
- 38. Recker RP, Bammi A, Barger-Lux J, Heaney RP. Calcium absorbability from milk products, an imitation milk, and calcium carbaonate. American Journal of Clinical Nutrition. 1988;47:93-95.
- 39. Narva M, Nevala R, Poussa T, Korpela R. The effect of *Lactobacillus helveticus* fermented milk on acute changes in calcium metabolism in postmenopausal women. European Journal of Nutrition. 2004;43:61-68.
- 40. Sato R, Noguchi T, Naito H. Effect of lactose on calcium absorption from the rat small intestine with a non-flushed ligated loop. J Nutr Sci Vitaminol (Tokyo). 1983;29(3):365-73.
- 41. Ghishan FK, Stroop S, Meneely R. The effect of lactose on the intestinal absorption of calcium and zinc in the rat during maturation. Pediatr Res. 1982;16(7):566-8.
- 42. Tang AL, Shah NP, Wilcox G, Walker KZ, Stojanovska L. Fermentation of calciumfortified soymilk with lactobacillus: effects on calcium solubility, isoflavone conversion, and production of organic acids. Journal of Food Science. 2007;72:431-436.
- 43. Mahe S, Marteau P, Huneau J, Thuillier F, Tome D. Intestinal nitrogen and electrolyte movements following fermented milk ingestion in man. British Journal of Nutrition. 1994;71:169-180.

- 44. Meisel H, Bernard H, Fairweather-Tait S, FitzGerald RJ, Hartmann R, Lane CN, McDonagh D, Teucher B, Wal JM. Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. British Journal of Nutrition. 2003;89:351-358.
- 45. Perez-Conesa D, Lopez G, Abell'an P, Ros G. Bioavailability of calcium, magnesium and phosphorus in rats fed probiotic, prebiotic and synbiotic powder follow-up infant formulas and their effect on physiological and nutritional parameters. Journal of the Science of Food and Agriculture. 2006;86:2327-2336.
- 46. Scholz-Ahrens KE, Ade P, Marten B, Weber P, Timm W, Asil Y, Gluer CC, Schrezenmeir J. Prebiotics, probiotics and synbiotics affect mineral absorption, bone mineral content, and bone structure. Journal of Nutrition. 2007;137:838-846.
- 47. Bobilya DJ, Ellersieck MR, Gordon DT, Veum TL. Bioavailabilities of zinc from nonfat dry milk, low fat plain yogurt and soy flour in diets fed to neonatal pigs. Journal of Agriculture and Food Chemistry. 1991;39:1246-1251.
- 48. Kelly O, Cusack S, Cashman KD. The effect of bovine whey protein on ectopic bone formation in young growing rats. British Journal of Nutrition. 2003;90:557-564.
- 49. Yoon GA, Hwang HJ. Effect of soy protein/animal protein ratio on calcium metabolism of the rat. Nutrition. 2005;22:414-418.
- 50. Takada Y, Kobayashi N, Kato K, Matsuyama H, Yahiro M, Ace S. Effects of protein on calcium and bone metabolism in ovariectomized rats. The Journal of Nutritional Science and Vitaminology. 1997;43:199-210.
- 51. Takada Y, Matsuyama H, Kato K, Kobayash, N, Yamamura J, Yahiro M, Ace S. Milk whey protein enhances the bone breaking force in ovariectomized rats. Nutrition Research. 1997;17:1709-1720.
- 52. Schrezenmeir J, de Vrese M. Probiotics, prebiotics and synbiotics-approaching a definition. American Journal of Clinical Nutrition. 2001;73:361-364.
- 53. Lutz T, Scharrer E. Effects of short-chain fatty acids on calcium absorption in the rat colon, Exp. Physiol. 1991;76:615–618.

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