Available online: http://journal.ipb.ac.id/index.php/jgizipangan ***J. Gizi Pangan, November 2021, 16(3):149-158***
Accredited based on DGHE, Republic of Indonesia No. 28/E/KPT/2019 DOI: https://doi.org/10.25182/jgp.2021.16.3.149-158
ISSN 1978-1059 EISSN 2407-0920

Calcium Bioavailability and Serum Calcium Level in Pregnant Rats After Administration of Milk-Based Drinks Containing Lactic Acid Bacteria

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ABSTRACT

This study aims to evaluate calcium bioavailability through serum calcium level in pregnant rats treated with two Milk-Based Drinks (MBD) containing Lactic Acid Bacteria (LAB), i.e *Lactobacillus Casei Shirota Strain* (LcS) drink, and Four Strains Bacteria (FS) (*Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus delbrueckii* subp. *bulgaricus, Streptococcus thermophiles*). It was a completely randomized experimental study using 24 Sprague Dawley rats. Rats were divided into one negative control group with a normal nutritional status (A0) and three undernourished groups. The undernourished groups were divided into one positive control group (A1), one group receiving MBD containing LcS group (A2), and another group receiving MBD containing FS (A3). All rats received high protein diets during pregnancy. The intervention was started in early pregnancy (D0) until 19 days of pregnancy (D19). Blood samples were collected at the D0 and D19. No significant differences in food intake were found among the rats in all groups. Administration of MBD containing LAB in A2 and A3 showed significant ($p<0.05$) increment of calcium bioavailability (30.79±6.88%; 20.44±9.04%). Both MBDs treatment containing LAB showed no significant difference in serum calcium bioavailability ($p>0.05$). The results suggest that MBDs containing LAB are useful in enhancing calcium bioavailability.

Keywords: bioavailability, calcium, rats, serum, undernourished

INTRODUCTION

The first one thousand days of life starting from conception until two years old is a "golden period" where rapid growth of the brain and internal organs happens. Nutritient deficiency within this periode will cause irreversible growth and development disorders (MoH RI 2014). One of many nutritional problems often encountered within one thousand days of life the is Chronic Energy Deficiency (CED) in pregnant women. In 2018, the prevalence of CED in pregnant women was 17.3 % (MoH RI 2018). CED during pregnancy can cause failure in fetal growth or Intrauterine Growth Restriction (IUGR), Low Birth Weight (LBW), birth defects, stunting, even infant death (Wu *et al.* 2012).

Pathophysiology of stunting starts during the fetal life in the womb and it will manifest once the child is reaching the age of two (Millenium Challange Account Indonesia 2014). One of the risk factors for stunting is the low body mass index of mothers in their early pregnancy, which affects linear growth of their babies (Pusparini *et al.* 2016). The prevalence of stunting in children under five years old in Indonesia in 2018 was 30.8% (MoH RI 2018). Childhood stunting can lead to obesity, which increases the risks for degenerative diseases in the future (Black *et al.* 2013). In addition, stunting can cause loss in productivity. Renyoet (2016) stated that the productivity decreases between USD 214,850 to 966,930 of Indonesia's total (GDP) because of stunting.

Calcium is an important aspect during pregnancy. Adequate calcium intake during this period supports maternal and fetal bone health and reduce the incidence of hypertension in pregnancy (Camargo *et al.* 2013). Total serum calcium usually falls during pregnancy; thus, mothers need to increase their calcium intake during this period. The high levels of total calcium concentration in cord blood compared to maternal serum, showed that 80% of calcium found in the fetal skeleton at birth crossed the placenta during the third trimester and it is mostly derived from

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(Received 25-08-2021; Accepted 11-10-2021; Published 29-11-2021)

dietary absorption of calcium during pregnancy (Kalkwarf & Specker 2002). In Cameroon and Thailand, calcium deficiencies in pregnant women were 94.6% and 55% respectively, meanwhile, in Indonesia such data was not available. However, there was one study conducted by Purnasari *et al.* (2016) showing that 81.2% pregnant women could not meet calcium requirements based on analysis from their daily food intake.

Probiotics or good bacteria provide many health benefits and has become a trend in health and nutrition for gut health in 2021 (KHNI 2021). This benefit is also applied for calcium bioavailability, where probiotics could improve calcium metabolism. Abd El-Gawad *et al.* (2014) showed that yogurt containing *Lactobacillus delbrueckiis* subsp. *bulgaricus, Streptococcus thermophilus, Bifidobacterium longum Bb-46, and Bifidobacterium lactis Bb-12* improve bioavailability of calcium, phosphorus, and zinc in rats. Moreover, Raveschot *et al.* (2020) showed that *Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus kefiranofaciens, Lactobacillus plantarum, Lactobacillus fermentum*, and *Lactobacillus helveticus* improved absorption of calcium in vitro. There are many researches on probiotics and absorption of calcium which had been done, but so far there are limited reports on the effects of Lactic Acid Bacteria (LAB) consumption on calcium bioavailability and serum calcium both in animal and human with undernourished condition.

In Indonesian market currently there are two popular Milk-Based Drinks (MBDs), the first contain a single species of LAB and the other contain a mixture of four species containing different species of LAB. Considering the positive effect of LAB administration on calcium bioavailability in previous studies and the paucity of data regarding its effect in undernourished pregnant subjects, this study aimed to better understand the effect of MBD with single LAB versus mixtures of LAB in improving calcium bioavalability and serum calcium in undernourished pregnant rats.

METHODS

Design, location, and time

The study was a completely randomized experimental study. The rats were randomly assigned into four gropus. The first group was the negative control group with normal nutritional status (-A0) and the three groups were undernourished groups. The undernourished groups were assigned as the positive control group (-A1) a Milk-Based Drinks (MBD) containing *Lactobacillus Casei Shirota Strain* (LcS) (-A2), and an MBD containing Four Strains Bacteria (FS) (-A3). In the MBD containing LcS and MBD containing FS contain $1x10^9$ CFU/ml and $7.2x10^7$ CFU/ml of total Lactic Acid Bacteria (LAB), respectively. The experiment was conducted in December 2020 to May 2021 at the *Unit Pengelola Hewan Laboratorium* (UPHL), Faculty of Veterinary Medicine, IPB University. The study was approved by Animal Ethics Commission, Faculty of Veterinary Medicine, IPB University No. 025 / KEH / SKE/ 2020. Analysis of serum calcium, feces, and urine ware carried out at the Physiology Laboratory, Faculty of Animal Science, IPB University. Total LAB in the drink products were analyzed at the SEAFAST Center Laboratory, IPB University.

Materials and tools

The main materials used in the study was two MBD brands containing LAB which was obtained from Greenfields Indonesia and Yakult. Six to eight weeks old female (150–250 g) rats were purchased from the Indonesian Rat Company Laboratory (iRATco), Bogor. Male Sprague Dawley (200–250 g) rats were also purchased from iRATco for mating.

Materials and equipment for animal maintenance and treatment were MBD containing LcS (4.3% milk and 76.5% water), MBD containing FS (62.4% milk and 20.8% water), standar rat diet with 18% casein (AIN-93M formulation), low-protein rat diet with 2% casein (Smith *et al.* 2013), high-protein rat diet with 21% casein (AIN93G and Nutrient Requirements of Laboratory Animals 1995), portable digital scales (Sigma), plastic zip, OneMed PCR tube plastic 1.5 ml, individual cage with cage area 33.5x27x12 cm, husk, and drinking bottle sized 250 ml.

Materials used for estrous cycle observation were 0.9% NaCl, 10% giemsa, methanol, distilled water, sensi gloves, cotton buds, object glass, (GEA) and microscope. Materials used for intervention were 1 ml syringe (Terumo) and gavage tube. Materials and equipment used for necropsy and serum collection were 90% alcohol xylazine and ketamine (3:7), surgical

board, surgical blade no.20, operating scissors sharp blunt straight 14 cm, cotton, 1 ml syringe (Terumo), 18 g needle (Terumo), 16 m x 150 mm centrifuge test tube (Kokusan, Japan), gloves (Sensi). Bioavailability of calcium was measured by calculation of the calcium absorption and calcium retention. Calcium absorption, calcium retention, and serum calcium were obtained from analysis of calcium in the urin, feces, and blood serum using spectrophotometry method at wavelength of 422.7 nm.

Procedures

Determination of total LAB in MBD, total LAB, and total coliform bacteria in feses. Total LAB in MBD (from Greenfields Indonesia and Yakult), total LAB, and total coliform bacteria in feses were analyzed using methods according to Bacteriological Analytical Manual (2001). de Man Ragosa and Sharp Agar (MRSA) was used in the total LAB analysis. Meanwhile, Violet Red Bile Lactose Agar (VRBA) was used in the total coliform bacteria analysis. Samples that have been diluted (1 ml) were put into a petri dish, then added with 15–20 ml MRSA or VRBA and shake. Samples were incubated at 37^{o}C in a reverse position for 48 hours. Countable colonies were in the range of 25–250 ml.

Induction of undernutrition. Experimental rats were set and put into separated cages (each cage contained one rat) and exposed to 12-hours light-dark cycle. Induction of undernutrition was done by combination of 50% restrictive diet (Nurliyani *et al.* 2014) and low protein diet with 2% casein (Smith *et al.* 2013). Undernutrition was set at mild condition with 8.3–22.6% body weight loss (Merino-Sanjuan 2014).

Measurement of estrous cycle. The estrous cycle was done by vaginal smears. Sterile cotton-tipped swabs wetted in 0.9% NaCl were gently and quickly introduced into the vaginal orifice, Small cotton swab was wetted with 0.9% NaCl and then swab into the rat's vagina. The swab cotton was carefully rotated (one twist) against the vaginal wall. Rats were not anesthetized during smear collection. The collected sample of vaginal epithelial celss was placed on glass slides, soaked in methanol for five minutes, soaked in 0.1% giemsa for 15 minutes, dried at 37^{o}C and fixed in distilled water for one minute. The cotton swab was then observed under photomicroscope (olympus).

Intervention of MBD containing LAB and measurement of body weight and food intake. The mating process was carried out under a ratio of 1:1 starting from 04.00 p.m to 05.30 a.m. Day 0 of pregnancy was declared if there was sperm from the vaginal smears in the morning after mating. All rats were fed with high-protein diet during pregnancy. Administration of MBD containing LAB was done for A2 and A3 as much as 1.5 ml/day by oral gavage based on stomach capacity of rats of 10 ml/kgBW (McConnell *et al.* 2008). Meanwhile, A0 and A1 groups were given mineral water with the same amount. The MBD containing LcS had 76.9 kcal energy, 1.5 g protein, and 47.7 mg calcium per 100 ml. Meanwhile, 100 ml MBD containing FS had 112 kcal energy, 3.2 g protein, and 80 mg calcium. The female rats delivered their off springs by caesarean surgery within 19 days in accordance with ethical guidelines. Measurement of body weight was done every three days. Meanwhile, the total food intake was measured by food given minus food waste. The food waste was weighted every morning.

Analysis of serum calcium and calcium bioavailability. Blood sampels, urine, and feces were collected at D0 and D19 (before the rats was sacrificed). At the D19, rats were sacrificed for other data collection regarding the fetus sample. The blood, urine, and feces samples were stored in a deep freezer -70^{0}C temporarily until all samples were collected. Analysis of serum calcium, feces, and urine were performed at the end of intervention using spectrophotometer.

Two cc of blood from the tail vein was obtained for the serum collection. The rats were sacrificed at the end of the intervention with a heart pucture under ketamine/xylazine anesthesia and blood was drawn directly from the heart for serum collection (after intervention) and other data collection. The blood was collected in a centrifuge tube and centrifuged at 1,500 rpm for 20 minutes to get the serum. Serum calcium analysis was done using spectrophotometer. As much as 1 ml of serum sample was pipetted into a test tube then added with 4 ml of 5% TCA. The solution wax vortexed and centrifuged for 30 minutes at 3,000 rpm. One ml of the supernatant formed was pipetted into another test tube, then added with 1 ml of 5% Strontium (Sr) solution and 8 ml distilled water. Next, the samples were analyzed using spectrophotometer at a wavelength 422.4

nm. The standard calcium solution used was Calcium Carbonate ($CaCO_3$) (Suhartini 2013).

Measurement of calcium bioavailability was done by calculating calcium absorption and calcium retention. The data on calcium absorption and calcium retention was obtained by calculating calcium intake from the diet (including calcium content of MBD) and analyzing calcium in urine and feces. Urine and feces of the rats were collected and put into a 100 ml Erlenmeyer two days before the intervention and two days before the rats were sacrificed. As much as 5 ml HNO_3 was added into the sample and then the samples were put in the acid chamber. After one hour, the samples were heated on a hot plate at low temperature for 4–6 hours in the acid chamber. After keeping it for overnight, 0.4 ml H_2SO_4 was added into the samples and they were heated on a hot plate again to concentrate the solution. Then, 2–3 drops of a mixed solution of $HClO_4$:HNO_3 (2:1) was added into the samples. The heating was continued until the color changed from brown to dark yellow, and then back to light yellow (approx. one hour). After the color change had been seen, the heating process was continued for 10–15 minutes. The samples were cooled and added with 2 ml distilled water and 0.6 ml HCI and then heated again so that the samples dissolved. The samples were next put into a 100 ml volumetric flask. If there was a precipitate, it was filtered with a glass wool. The results of wet ashing were analyzed using spectrophotometry to determine the calcium concentration at of 422.4 nm. The bioavailability of calcium was measured by calculating the calcium absorption and calcium retention.

Data analysis

Data on body weight, food intake, serum calcium, and calcium bioavailability were processed using Ms.Excel 2013. The results were presented as mean±Standard Deviation. Statistical significance was evaluated using one-way ANOVA and continued with Duncan Multiple Range Test (DMRT) at 5% (0.05). The statistical analysis was done using SPSS software version 16.0.

RESULTS AND DISCUSSION

Effects of milk-based drink containing lactic acid bacteria on food intake and body weight of rats

Data of food intake of rats was collected by weighing the leftover of feed every day. Table 1 shows the food intake of rats during pregnancy. Table 1 show that there was no significant difference in food, energy, and protein intake between all groups. Data of rats' body weight is shown in Table 2.

Table 1 shows that there was no significant difference in body weight loss because of induction undernutrition among A1, A2, and A3. This due to the same decrease in food intake. Weight loss in a short period of time can be caused by treatment of decreasing food intake (Estrela *et al.* 2014). Food restriction by 50% and low protein diet (2% casein) can lead to loss of skeletal muscle, which is very sensitive to protein deficiency because it is a protein reservoir to the organism. Protein deficiency makes depletion of the tissue, leading to significant muscle loss and consequently decreased body mass. Thus, mothers who are undernutrition have higer risk to deliver Low Birth Weight (LBW) babies (Yongki *et al.* 2014).

MBD containing *Lactobacillus casei shirota strain* (LcS) drink was given to A2, and MBD containing four strains bacteria (FS) (*Lactobacillus rhamnosus, Lactobacillus*

Table 1. Food and nutrient intake of rats during pregnancy

Group	Food intake (g)	Energy (kcal)	Protein (g)
A0	18.05±2.53	63±9.10	4.12±0.58
A1	20.88±1.05	73±4.00	4.76±0.24
A2	21.08±2.05	75±7.20	4.83±0.47
A3	21.42±1.68	76±5.90	4.92±0.00
p[1]	0.090	0.069	0.078

[1] One way-ANOVA

Table 2. Effect induction of undernutrition on rats weight loss and effects of MBD containing LAB on rats body weight during pregnancy (day 0, day 19, and increment body weight)

Group	Weight loss (%)	Day 0 (D0)	Day 19 (D19)	Increment (D19–D0)
A0	-	153.67±19.37[a]	224.33±23.29	70.67±5.43[a,b]
A1	10.15±1.69	201.67±17.50[b]	276.33±15.57	74.67±2.31[b]
A2	12.37±1.82	182.50± 21.21[a,b]	245.00±30.01	62.75±9.74[a]
A3	12.69±2.76	172.00±19.80[a,b]	253.00±27.62	81.00±8.54[b]
p[1]	0.322*	0.026*	0.069	0.031*

[1]One-way ANOVA analysis ; *Significant at p<0.05
MBD: Milk-Based Drinks ; LAB: Lactic Acid Bacteria

paracasei, Lactobacillus delbrueckii subp. bulgaricus, Streptococcus thermophiles) was given to A3. Statistical analysis showed that A2 (62.75±9.74 g) had significantly lower weight gain compared to A1 (74.67±2.31 g) and A3 (81.00±8.54 g). The gross body weight gain of rats in positive control group (A0) at D19 of pregnancy was ±45.9% of initial body weight. Study conducted by Rahayu (2020) showed that gross body weight gain of rats was up to 72.83% at partus. In this study, the smaller weight gain in A2 at 34% of initial body weight can be associated with the administration of MBD containing LcS. Another animal study by Karimi *et al.* (2015) also showed the same results where the administration of LcS to rats which were given high-fat diet had caused a significantly lower final body weight compared to rats which were fed with a high-fat diet only.

On the other hand, an animal study conducted by Benakriche *et al.* (2014) showed that in malnourished rats treated with *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium bifidum, Streptococcus thermophiles,* and fructooligosaccharides at 0.5 mg/g of body weight/day managed to improve the rats' intestinal atrophy that was caused by malnutrition; thus, the rats' body weight increased from 123 g±9.9 g to 181.08±9.9 g. However, the weight gain of A3 (81.00±8.54 g) which was given FS in this study was not significantly different from the control groups (A0 and A1). This might be due to the dose of MBD containing FS that was not enough to increase the rats' body weight during pregnancy higher than the standard high protein diet given to the control groups. It was noted that beverage product for A3 contained $7.2x10^7$ CFU/ml total LAB. Based on the World Health Organization, the recommended dose of probiotic to provide health benefits is between 10^8–10^9 CFU/ml.

Some probiotics can help increase body weight while other probiotics work adversely. Several mechanisms underlie how bacteria strains elicit their function. Oral administration of probiotics increases the activity of the sympathetic nervous system in white and brown adipose tissues. Meanwhile, intragastric administration of probiotics increases lipolysis in white adipose tissue and thermogenesis in brown adipose tissue (Tanida *et al.* 2008). This process facilitates thermogenic and lipolytic responses via stimulation of the sympathetic nervous system, which leads to weight reduction. Another mechanism underlying how probiotic induce weight reduction is the bacteria's metabolite known as Short Chain Fatty Acids (SCFA), especially butyrate. Butyrate plays as the main regulator of energy production and mitochondrial function. It induces gene expression of peroxixome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) in the muscle and brown adipose tissue where PGC-1α is involved in thermogenesis and glucose metabolism process. Therefore, it affects body weight loss (Gao *et al.* 2009).

Probiotics administration also benefits the host through vitamin synthesis and amino acids release. The MDB yogurt given to A3 contained 62.4 % milk. There are activities from *Lactobacillus delbrueckii subsp.* bulgaricus and *Streptococcus thermophiles* that allow the pre-digestion of milk protein. Those processes help

produce more lactose which can be digested and it is more tolerable than liquid milk (Savaiano 2014). This mechanism helps increase the body weight of experimental rats. Dainese–Plichonet *et al.* (2014) also showed that the consumption of yoghurt and cheese was recommended as part of diet to help people with lactose intolerance to still be able to gain benefits from dairy products.

Effects of milk-based drink containing lactic acid bacteria on calcium bioavailability

Calcium from food is usually unable to be 100% absorbed by the body because it depends on biological availability or bioavailability of it. Moreover, positive calcium balance is required, especially during growth, pregnancy, and lactation period. Average values from absorption, retention, bioavailability, and increment of bioavailability of calcium in pregnant rats before and after the intervention are displayed in the Table 3.

Based on the baseline data shown from Table 3, it showed that calcium absorbtion, calcium retention, and calcium bioavailability of rats with undernutrition induction (A1, A2, and A3) were significantly lower compared to rats with normal weight (A0). Table 3 shows absorption, retention, and calcium bioavailability in A1, A2, and A3 that were significantly lower than A0. This might be due to differences in calcium content for standard and low-protein diet. The low-protein diet for induction of undernutrition, had 0.88 g calcium per 100 g while the standard diet had 0.90 g calcium per 100 g. In addition, the amount of food was also restricted for A1, A2, and A3.

With induction of undernutrition can interfere calcium absorption. Protein is the largest part in human body after water. Enzymes, hormones, nutritional carriers, blood, and intracellular matrix are made from protein. Protein deficiency causes disturbances in nutrients absorption and transport (Nyaradi *et al.* 2013). In this study, protein deficiency could possibly affect calcium bioavailability.

Administration of MBD containing LAB led to significant increase of calcium bioavailability ($p<0.05$) in all groups (A2 and A3). The Duncan test showed that in calcium bioavailability in A2 (30.79±6.88%) was significantly higher compared to A0 (1.01±4.46%) and A3 (20.44±9.04%). Similar results were also reported by Abd El-Gawad *et al.* (2014) who showed that administration of *Bifidobacterium lactis* and *Bifidobacterium longum probiotics* in rats for 45 days resulted in higher calcium absorption (84% for *Bifidobacterium lactis* and 83.4% for *Bifidobacterium longum*) than the control group

Table 3. Effects MBD containing LAB on the absorbtion, retention, bioavailability of calcium

Parameters	Groups				p[1]
	A0	A1	A2	A3	
Calcium absorption ability (%)					
Before	68.1±11.04b	22.87±5.73[a]	37.35±13.18a	30.13±13.14[a]	0.000*
After	51.08±25.90	71.77±25.36	81.05±2.74	74.40±29.83	0.245
Calcium retention (%)					
Before	62.42±11.54[b]	13.3±4.76[a]	25.12±10.10[a]	23.57±12.19[a]	0.000*
After	49.32±25.28	68.57±27.89	78.45±3.18	71.50±29.10	0.276
Calcium bioavailibility (%)					
Before	91.35±310[c]	57.37±10.02[a]	66.03±6.72[a,b]	75.47±9.39[b]	0.000*
After	92.35±6.02	93.85±7.15	96.79±1.34	95.90±0.99	0.535
Increment bioavailability (%)	1.01±4.46[a]	36.48±14.48[c]	30.79±6.88[b,c]	20.44±9.04[b]	0.000*

[1]ANOVA one-way analysis; MBD: Milk-Based Drinks ; LAB: Lactic Acid Bacteria

(66%). The groups of rats which were given probiotics had an increase in calcium absorption by 24.7%– 26.6%. Administration of probiotics is proven to increase the crypth depth in the large intestine and it has the ability to lower pH due to increasing Short Chain Fatty Acids (SCFA) production in the large intestine. Decreasing pH causes increased calcium absorption (Abd El-Gawad *et al.* 2014).

In vivo study conducted by Raveschot *et al.* (2020) reported that probiotics *Lactobacillus casei, Lactobacillus kefiranofaciens, Lactobacillus plantarum, Lactobacillus helveticus, and Lactobacillus delbrueckii* improved calcium transport and uptake. Moreover, *L.casei9b, L. kefiranofaciens15b,* and *L. helveticus49d* increased total calcium transport by increasing calcium solubility. Meanwhile, *L. delbrueckii50b* strain increase calcium transport through paracellular pathway by upregulating the cld-2 gene where claudin-2 functions as a transport channel for ions and water (Lu *et al.* 2013). The use of LAB *L. delbrueckii* and *L. casei* in this study was assumed to have a role in calcium absorption by increasing calcium solubility and upregulating genes that are involved in paracellular calcium transport through intestinal cells.

Milk also affects calcium bioavailability. The MBD given to A2 contained 4.3% milk while MBD given to A3 had higher milk content (62.4%). Milk and dairy products are the main sources of calcium because it has an anti-resorptive effect and can help to prevent bone loss. Besides, the presence of phosphorus in milk and dairy products can also bind calcium to form the hydroxyapatite mineral that are able to increase bone density (Heaney 2002).

Effects of milk-based drink containing lactic acid bacteria on serum calcium

Table 4 shows mean serum calcium in all groups before and after the intervention. The ANOVA test results showed that the induction of undernutrition had a significant effect on serum calcium level. The groups A1, A2, and A3 had significantly lower level of serum calcium than the control group (A0), whereas A3 had the lowest level of serum calcium when compared to all the other groups

These findings was supported by a previous study conducted by Aminah *et al.* (2017) who concluded that normal level of serum calcium in two months Sprague Dawley rats was 11.36±0.46 mg/dl. In this research, the A1, A2, and A3 had a lower serum calcium as much as 17.08%, 19.45%, and 31.55%, respectively when compared to the control group (A0). This might be due to the induction of undernutrition. Study by Solang (2017) showed that induction of undernutrition in rats by administering an 8.46% protein diet for eight weeks showed a decrease in serum calcium level by 31.4% compared to the control group given 13–15% protein for 8 weeks. Moreover, calcium level in the control group was 11.8±0.20 mg/dl (Solang 2017).

The decrease in serum calcium in undernourished group is presumably caused by low protein diet which led to a decrease in the availability of proteins in the body, such as the availability of albumin. Albumin plays an important role in transporting calcium, which means that there is a relationship between calcium absorption and protein intake. Therefore, low-protein diet causes lower calcium absorption; hence, the level of calcium in the blood decreases (Solang 2017). Moreover, induction

Table 4. The average serum calcium levels in rats before and after intervention

Groups	Before (mg/dl)	After (mg/dl)	Δ (mg/dl)	p[1]
A0	10.97±0.97[c]	9.59±1.85[a]	-1.37±2.51[a]	0.239
A1	9.09±0.53[b]	9.01±0.47[a]	-0.08±0.27[a]	0.642
A2	8.83±0.74[a,b]	8.75±0.66[a]	-0.09±0.96[a]	0.867
A3	7.51±0.89[a]	9.49±1.81[a]	1.99±1.93[a]	0.216
p[2]	p[2]=0.000*	p[2]=0.804	p[2]=0.146	

*[1]Paired t tests analysis before and after intervention; *[2]One-way ANOVA analysis

of undernutrition resulted in lower *Lactobacilli* which caused increased intestinal pH. Eventually, increased intestinal pH caused decreased calcium absorption.

Administration of MBD containing LAB did not significantly affect serum calcium level during pregnancy. This is in line with the results of others studies elaborated before who found that administration of MBD containing LAB decreased intestinal pH and increased calcium absorption. This phenomenol implies that the human body keeps the homeostatic level of serum calcium and it is not affected by calcium absorption. An animal study conducted by Gonen *et al.* (2005) showed that serum calcium level of pregnant rats was 7.86±1.3 mg/dl, which was lower than the results found in this present study. This difference might be caused by the fact that Gonen *et al.* (2005) had no intervention in terms of calcium intake. Descreasing serum calcium in late pregnancy period is associated with decreasing calcium ionization. This might be related to an increase in fetal requirement, and a decrease in serum albumin concentration due to hemodilution, as well as increase in urinary calcium excretion (Prentice 2000). This research showed that although the administration of MBD containing LAB did not provide a significant difference in increasing serum calcium, it was able to maintain the calcium level within the normal range during pregnancy.

CONCLUTION

Intervention of 1.5 ml/day MBD containing LAB during 19 days of pregnancy ($7.2x10^7$ CFU/ml total LAB for A3 and $1.2x10^9$ CFU/ml total LAB for A2) showed significant effect on weight gain and increased calcium bioavailability in undernourished pregnant rats. Administration of MBD containing FS (A3) was associated with higher body weight increase as compared to standard feed. Both MBD containing LcS (A2) and FS (A3) were associated with higher calcium bioavailability. However, MBD containing LAB did not significantly affect the serum calcium in undernourished pregnant rats.

Further research is needed on the development of food product containing a mixture of these LAB to improve calcium bioavailability and serum calcium of undernourished pregnant subjects. The appropriate dosage of the LAB to be administered to undernourished pregnant subjects also needs to be identified.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Research, Technology and Higher Education (KEMENRISTEK DIKTI) for funding this research through 2020/2021 PMDSU scheme. The authors also thank the Greenfields Indonesia for providing the MBD containing FS used in this study.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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