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Research Article
Calcium of *Spirulina platensis* has Higher Bioavailability than those of Calcium Carbonate and High-calcium Milk in Sprague Dawley Rats Fed with Vitamin D-deficient Diet

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Abstract
Background: Calcium is one of the important mineral as a constituent of bone in human, hence critically needed to prevent osteoporosis. Milk is considered as the main source of calcium, however, can not be consumed by those with lactose intolerant. On the other hand, *Spirulina plat ensis*, a species of microalgae is known for its high calcium level, particularly those cultivated with sea water, thus can be used as calcium source alternative. **Objective:** The objective of this study was to determine the bioavailability of calcium from *Spirulina platensis* and compared it with high calcium milk and calcium carbonate by using hypocalcemic Sprague Dawley rats fed with vitamin D-deficient diet. **Methodology:** Thirty male rats were divided into 5 groups of six rats each. The rats were fed different diets for 8 weeks. The diets included standard diet (control), vitamin D-deficient diet (DVD), DVD+calcium carbonate (DVDCa), DVD+high-calcium milk (DVDMCa) and DVD+*Spirulina* diet (DVDSp). **Results:** The results showed that DVDSp group was able to retain calcium, magnesium, phosphorus and alkaline phosphates level in serum compared to control group ($p>0.005$). Femur bone integrity and bone mass density of hypocalcemic rats fed with *S. platensis* contained diet showed better results. **Conclusion:** It was concluded that bioavailability of calcium from *Spirulina platensis* was higher than those of milk and calcium carbonate.

Key words: Bioavailability, calcium, *Sprirulina platensis*, vitamin D deficiency

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Calcium is an important macro-mineral for human. Lack of calcium intake leads to health problems, such as osteoporosis. In Indonesia, the prevalence of osteopenia (early osteoporosis) and osteoporosis were 41.7 and 10.3%, respectively. It is predicted that 2/5 people in Indonesia have a risk of undergone osteoporosis. The numbers were higher than world's prevalence where by one third of population has osteoporosis risk. Furthermore, International Osteoporosis Foundation (IOF) predicts that in 2050, 50% of pelvic bone fractures case will occur in Asia as a result of low calcium intake.

Adequate calcium daily intake is fulfilled by consumption of calcium-rich food. Milk and its derivative products are major sources of calcium from food, besides, supplements like calcium carbonate or calcium lactate are also good source of calcium. Now a days, a large number of milk products with the high-calcium claim are available in the market. High calcium content of these products is achieved through fortification, e.g., using calcium carbonate. However, for various reasons such as health, religious and cultural, some people are restricted from consuming milk and other dairy products and therefore consumption of calcium from other sources is required to meet daily calcium intake.

Spirulina platensis was reported for its potential as a calcium source. *Spirulina* is a blue-green microalgae (Cyanophyceae) and very easily cultivated. Calcium content in *S. platensis* is 700-1000 mg/100 g, much higher compare with vegetables and milk or yogurt[1]. Consumption of 4 g day^{-1} *Spirulina* is adequate to meet daily requirement for calcium recommended by Habib *et al.*[2]. Gupta *et al.*[3] revealed that the administration of 500 mg kg^{-1} b.wt., *Spirulina fusiformis* daily for 28 days improves bone integrity in diabetic rats. In Indonesia, *Spirulina* is mainly consumed as a supplement.

Bioavailability of calcium is determined by measuring digestibility, solubility, absorption, organ's uptake and release, enzyme transformation and post-ingestion excretion[4], both by *in vitro* and *in vivo* method. *In vitro* analysis is carried out based on calcium solubility and dialysis rate, while the later considers absorption rate, excretion or calcium retention in bone. *In vivo* method is usually applied on a healthy animal in infancy by calcium source supplementation into the diet[5], post-ovariectomy rats[6] or hypocalcemic rats with vitamin D-deficient diet[7]. This study aimed to determine the bioavailability of calcium from *Spirulina platensis* and compare it with high-calcium milk and calcium carbonate on animals fed with vitamin D-deficient diet.

MATERIALS AND METHODS

Materials: Materials used in this study were *S. platensis* obtained from PT Transpangan Spirulindo, Jepara, Indonesia, high-calcium milk obtained from a local market, calcium carbonate from E-merck, DiSys kit for analysis Ca, Mg, P, alkaline phosphatase in serum and other materials for AIN-93 maintenance standard feed. All chemicals used were pro-analysis grade.

Animal and diet preparation: Thirty male Albino Sprague Dawley rats aged 2 months weighed 100-150 g were placed in an individual cage (45×35.5×14.5 cm) at room temperature (27±1°C) with a 12 h light-12 h dark cycle. After adaptation, rats were divided into 5 groups of six rats. They were fed different diets of a standard diet (control), vitamin D-deficient diet (DVD), DVD+calcium carbonate (DVDCa), DVD+high-calcium milk (DVDMCa) and DVD+*Spirulina* (DVDSp) for 8 weeks. The DVDCa and DVDMCa and DVDSp were adjusted to be iso-calcium, iso-calorie and iso-protein diet. Feed composition is presented in Table 1.

***In vivo* experiment:** During adaptation period, rats were fed AIN-93M[8] for 7 days. After adaptation, diet was given for 8 weeks. Biweekly blood sampling (0, 2, 4, 6 and 8 weeks) was carried out through sinus orbitalis using micro hematocrit or capillary pipette. Serum was obtained by 1 h centrifugation at 10000 rpm. Calcium, magnesium, phosphorus and alkaline phosphatase level in blood were analyzed using DiSys kit. Body weight was measured once a week. In the final stage of intervention, rats were put in overnight fasting, anesthetized and were dissected, femur bone was taken, washed and measured for its length and diameter. Bone density was measured using x-ray digital radiography, while its structure was observed using scanning electron microscopy (JFC-1100E, JEOL Co, Japan). All protocols were approved by the Pre-clinical Ethical Clearance Commission of Integrated Laboratory for Research and Experiment, Universitas Gadjah Mada (number 223/KEC-LPPT/III/15).

Data analysis: Statistical analysis was performed using SPSS 20 software (Chicago, IL, USA). Normal distribution was measured using Kolmogorov-Smirnovtest. Analysis of variance was performed to determine significant difference among

Table 1: Feed composition

	Iso-calcium diet (10.8 mg/200 g b.wt., of rats)				
Composition (g)	Control	DVD	DVDCa	DVDMCa	DVDSp
Maize starch	620.69	620.69	620.69	599.09	578.75
Casein	140.00	140.00	140.00	131.53	63.59
Sucrose	100.00	100.00	100.00	100.00	100.00
Soybean oil	40.00	40.00	40.00	38.80	39.92
α-cell non nutritive bulk	50.00	50.00	50.00	50.00	50.00
L-cystine	1.80	1.80	1.80	1.80	1.80
Choline bitartrate	2.50	2.50	2.50	2.50	2.50
Spirulina platensis					126.81
Calcium carbonate			1.80		
High-calcium milk				48.00	
Mineral mix	35.000	35.000	35.000	35.000	35.000
Vitamin mix (except vitamin D_3)	9.9975	9.9975	9.9975	9.9975	9.9975
Vitamin D_3, cholecalciferol (400.000 IU g^{-1})	0.0025	-	-	-	-
Total	954.990	954.99	956.79	971.72	963.37
Ca (mg kg^{-1} diet)			720.00	720.00	720.00

treatment, while mean separation was measured using least-significant-difference test[9].

RESULTS AND DISCUSSION

Body weight gain rate: All groups showed significant weight gain compared to baseline (p<0.05). Figure 1 shows that the average weight gain of DVDSp diet group was similar to the control group, whereas DVDCa and DVDMCa diet group showed similar trend in gaining weight, while the lowest weight gain was seen in the DVD group. This result was similar to that reported by Patwardhan *et al.*[7]. Researchers found that rats given a hypocalcemic vitamin D deficient diet have a very low weight gain during the 12 weeks of dietary intervention.

Differences in the rate of weight gain might be due to the differences in feed intake associated with energy availability. The average of daily feed intake in control, DVD, DVDSp, DVDCa and DVDMCa group were 11.64, 10.82, 11.49, 10.78 and 10.32 g, respectively. Despite all diets were set to have similar calorie, the actual feed intake among groups was different. It was possibly due to a distinctive smell/taste of *S. platensis* added feed compared to another diet. Addition of *Spirulina platensis* made the feed green in color with fishy/seaweed aroma. This characteristic was beneficial as it improved palatability, indicated by a higher feed intake in DVDSp group.

Feed Efficiency Ratio (FER) ranged from 5.62-7.61% (Table 2) and the value of FER was in parallel with the weight gain (p<0.05). The FER obtained in this study was lower than those reported by Park *et al.*[6] on ovariectomy rats fed with various sources of calcium, which ranged from 7.6-9.3%.

In DVDSp rats, the average weight gain were similar to the control rats, whereas the DVD, DVDCa and DVDMCa showed

Table 2: Feed Efficiency Ratio (FER) during 8 weeks of dietary intervention

Dietary group	Total feed (g)	Body weight gain (g)	FER (%)
Control	639.50[a]	48.67[a]	7.61[a]
DVD	591.33[b]	26.67[c]	4.51[d]
DVDCa	578.50[bc]	32.50[b]	5.62[c]
DVDMCa	566.50[c]	35.00[b]	6.19[b]
DVDSp	632.33[a]	47.67[a]	7.54[a]

[a-d]Different superscripts in the same column showing significant difference at p<0.05

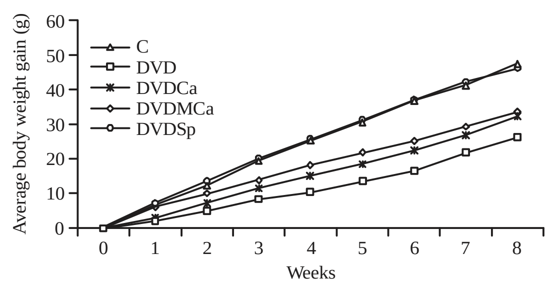

Fig. 1: Weight gain of rats

a lower weight gain, in line with the FER and total feed intake. This phenomenon may be due to the palatability of the diets, lead to the higher nutrients availability. Similar finding was reported by Toba *et al.*[5], who compared the feed intake and average weight gain between normocalcemic rats fed with milk component+$CaCO_3$ and diet+$CaCO_3$. However, average daily weight gain in the previous study was higher than this study, which was possibly caused by the calcium content of the diet. Diet with calcium addition was able to promote body weight gain when given to normal rats compare to hypocalcemic group[7].

Serum calcium, magnesium, phosphorus and alkaline phosphatase level as affected by *S. platensis*, $CaCO_3$ and high-calcium milk: At the initial stage, the level of Ca, Mg, P

Table 3: Profile of Ca, Mg, P and alkaline phosphates in serum as affected by *S. platensis*, $CaCO_3$ and high-calcium milk intake in hypocalcemic rats

	Analysis period							
	Initial stage				Final stage			
	Ca	Mg	P	Alkaline phosphatase	Ca	Mg	P	Alkaline phosphatase
Groups	(mg dL^{-1})			(U L^{-1})	(mg dL^{-1})			(U L^{-1})
Control	12.73^a	4.28^a	9.07^a	40.44^a	12.36^a	4.38^a	8.91^a	45.95^a
DVD	12.69^a	4.25^a	9.03^a	40.90^a	6.50^d	1.83^d	3.85^d	86.16^d
DVDCa	12.73^a	4.35^a	9.04^a	41.36^a	9.24^b	2.46^b	5.21^b	59.96^b
DVDMCa	12.74^a	4.33^a	9.11^a	40.67^a	8.17^c	2.27^c	4.57^c	65.48^c
DVDSp	12.74^a	4.37^a	9.06^a	40.67^a	12.39^a	4.38^a	8.84^a	47.10^a

[a-d]Different superscripts in the same column showing significant difference at $p<0.05$, Ca: Calcium, Mg: Magnesium, P: Phosphorus

and alkaline phosphates were not significantly different in all groups. However, after 8 weeks of dietary intervention, a lower Ca, Mg and P concentration in the DVD, DVDCa and DVDMCa group were observed but not in the DVDSp groups (Table 3). Similar trend was also found between control groups and DVDSp groups, indicating that *Spirulina* has a potential to cover mineral (Ca, Mg and P) deficiency condition. Serum Ca concentration reflected the availability and absorption of the minerals. Since in this experiment the contents of Ca in the diets were adjusted at the same level, higher level of Ca in the serum of rats fed with *Spirulina* indicated that a higher absorption of Ca occurs in DVDSp group compared to the DVDCa and DVDMCa group. It is an interesting finding and this is probably due to the effect of dietary fibre of the *Spirulina* diets. Fermentation of dietary fiber increase the Short Chain Fatty Acids (SCFA) that increase the acidity of the digested material in lumen. The lower pH increase the solubility and absorption of the Ca in the intestine.

Increase in alkaline phosphatase (ALP) level represents the role of parathyroid gland in maintaining calcium in the blood under normal conditions, which then lead to the reduction of calcium deposits. The DVDSp group showed similar ALP level to the control group, whereas DVDCa and DVDMCa group showed 20% higher level of ALP activity than DVDSp group. Under hypocalcemic condition, the absorption of calcium in DVDMCa and DVDCa groups was inadequate. In DVDMCa group, rats were fed with calcium from milk and fortified with calcium carbonate, while DVDCa were only fed with calcium carbonate. It is reported that calcium carbonate is not a good source of calcium in the diet. Patwardhan *et al.*[7] demonstrated the lower *in vivo* bioavailability of calcium carbonate than calcium lactate and calcium phosphate when added to the diet of both hypocalcemic and normocalcemic rats.

Increase in ALP was observed in hypocalcemic group except DVDSp group. Under hypocalcemic condition, the secretion of parathyroid hormone occurs which increases the secretion of the alkaline phosphatase enzyme that triggers the process of calcium mobilization from the bones. This mobilization process may also lead to a reduction of magnesium and phosphorus concentration in the blood[10]. This phenomenon was observed in the DVD group and it is suspected that these conditions were in accordance to the reduction of calcium content in bone. Meanwhile, the final level of calcium, magnesium and phosphorus concentration in blood serum of DVDSp group was similar to the initial stage. This suggests that the calcium contained in *S. platensis* was satisfactory absorbed in hypocalcemic conditions, resulting to the stable activity of the ALP.

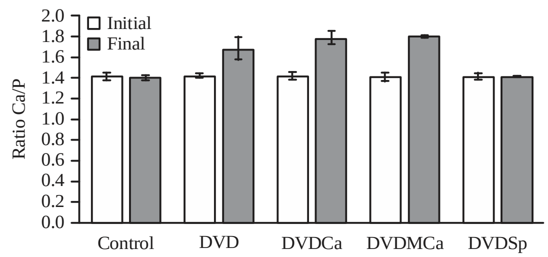

Fig. 2: Ca/P ratio in blood serum pre and post intervention

The adequate calcium absorption is characterized by a balance ratio of Ca and P in blood serum. The Ca and P ratio in serum during pre and post-intervention is presented in Fig. 2. The Ca/P ratio was ranged from 1: 1.4 to 1:1.8. The control and DVDSp groups showed a balance ratio of Ca/P through the period of experiment, while uprising of Ca/P ratio at the final stage was observed in those of DVDCa and DVDMCa groups. Raupp *et al.*[11] and Pointillart *et al.*[12] in separate studies proved that Ca and P supplementation in balanced amount is very important for human and animals. Wolters *et al.*[13] explained that deficiency and excess uptake of both result to negative effects for health. An appropriate ratio of dietary Ca/P in intestinal tract should be ranged from 1:1-1:3[14].

The change of Ca, Mg, P and alkaline phosphatase level in serum during 8 weeks experiment is presented in Fig. 3.

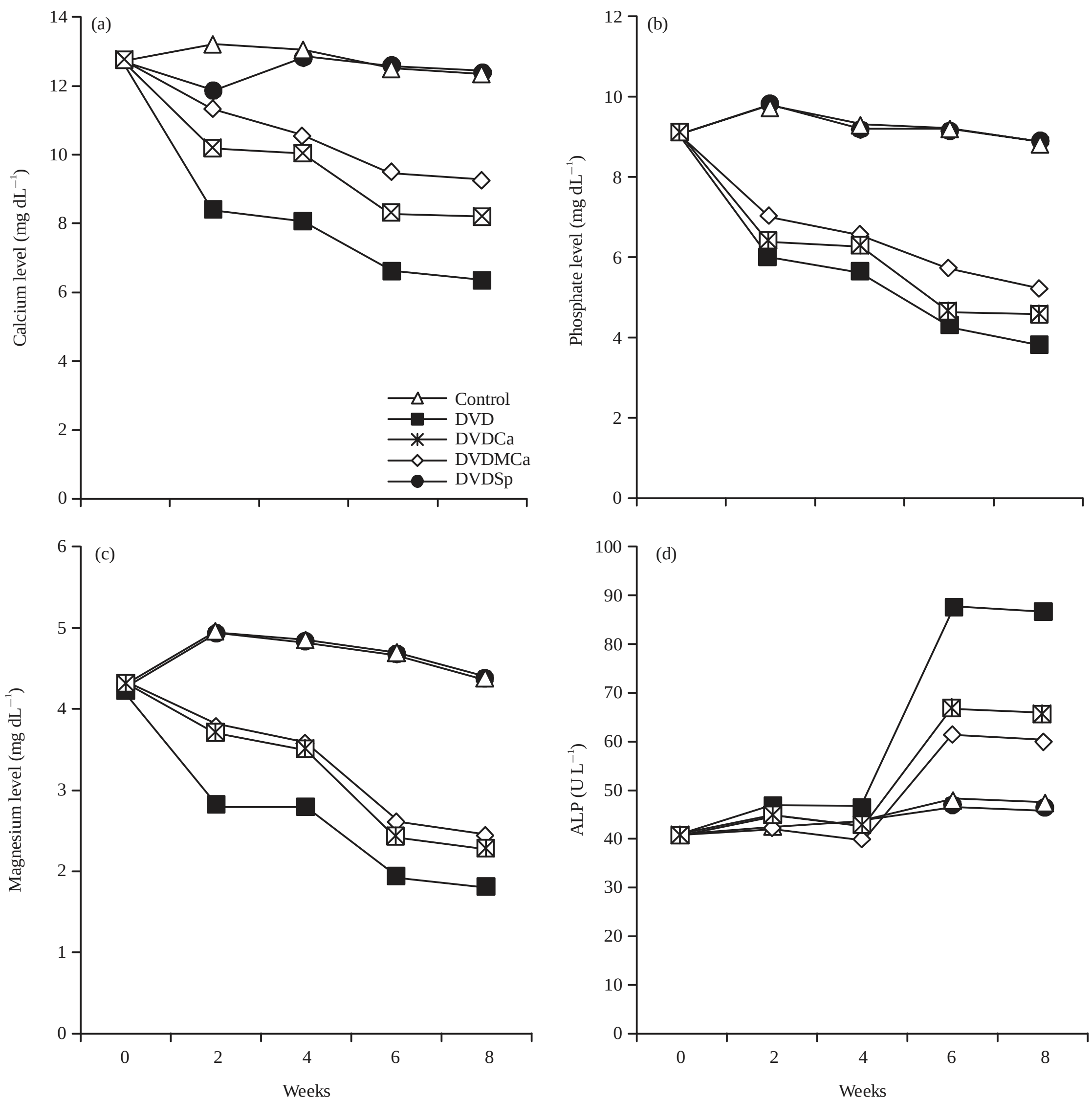

Fig. 3(a-d): Changes of (a) Calcium, (b) Phosphate, (c) Megnesium and (d) alkaline phosphatase (ALP) levels in serum

Hypocalcemic rats showed significant difference ($p<0.05$) in alkaline phosphatase activity during intervention compared to control. Increase in alkaline phosphatase activity of hypocalcemic rats started after 4th week of diet intervention. Figure 3 revealed that the calcium from *S. platensis* was able to maintain normal level of alkaline phosphatase activity in serum as observed in DVDSp group.

Hypocalcemia stimulates the release and synthesis of parathyroid hormone from the parathyroid glands. The hormone promotes the activity of 1-ά-hydroxylase in proximal tubular (PT) of kidney's nephron and increases the synthesis of 1, 25 (OH) dihydroxy-vitamin D_3 (1, 25$(OH)_2$-D3). Parathyroid hormone increases Ca^{2+} resorption from the bone by osteoclast and jointly with 1, 25$(OH)_2$-D_3 stimulates Ca^{2+} reabsorption at Distal Convoluted Tubular (DCT). The Ca^{2+} absorption from diet is also promoted by 1, 25 (OH)-D_3 in gastrointestinal tract (GI), which bring back Ca^{2+} level in the blood to normal[15]. In Fig. 3, significant increase was seen after 4th week of dietary intervention but tend to return to the normal level subsequently.

Patwardhan *et al.*[7] noted that the administration of vitamin D-deficient diet in long period (12 weeks) gradually turns back the alkaline phosphatase activity into the normal level. The reduction of ALP activity was observed after acute period of deficiency had been overcame. Prediction of acute period in this study was occurred in 6th week. Increasing rate of Ca, Mg, P level and decreasing level of ALP was observed at 6th and 8th weeks of diet

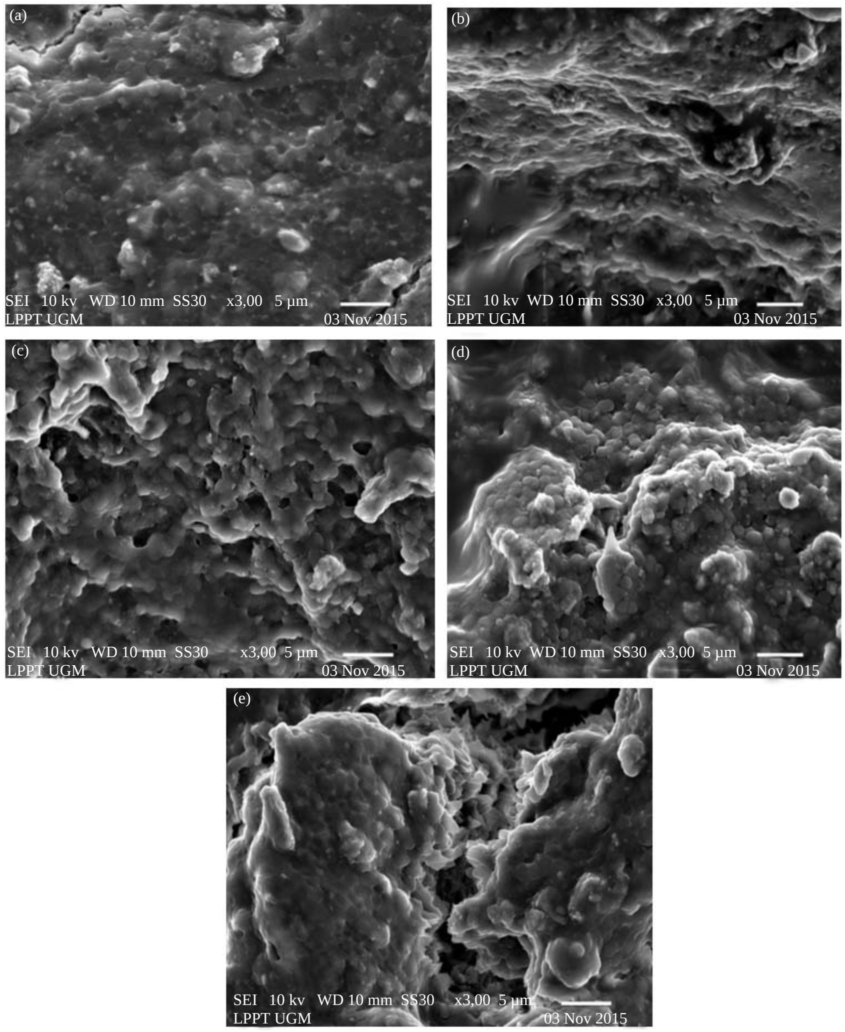

Fig. 4(a-e): Right femur bone profile of rats resulted from scanning electron microscopy (3000x), (a) Group I: Control, (b) Group II: DVD, (c) Gruop III: DVDCa, (d) Group IV: DVDMCa and (e) Group V: DVDSp

intervention in all vitamin D-deficient diet group, except for those fed with *S. platensis*.

Femur bone characteristic: The characteristic of rats femur bone after intervention is shown in Table 4. The length and diameter of femur as well as femur absorption coefficient and density were affected by diet ($p<0.05$). The DVDSp group had the longest femur bone but not significantly different to control, DVD and DVDCa group, meanwhile, femur bone of DVDMCa groups was shorter than control and DVDSp group ($p<0.05$). However, femur diameter was not different among control, DVD and DVDSp groups. Absorption coefficient demonstrates the ability of bone to absorb x-rays, was influenced by the mineral content of the bone. Low absorption coefficient values impacts the bone mass density. The absorption coefficient ranged from 0.95-1.21 cm^{-1}, DVD group had the lowest value. This finding is similar to the result of Park *et al*.[6] showing that the ovariectomized rats fed with a diet without calcium supplements have the lowest Bone Mass Density (BMD), whereas a diet with calcium-supplemented milk increases the BMD of rat. Moreover, milk with calcium carbonate supplementation resulted to a lower BMD in rat compared with nano calcium. Table 4 shows that DVDMCa group had a higher femur bone density than DVDCa group. The superiority of calcium carbonate and milk as a source of calcium are still controversy among the researchers. Most states that the calcium carbonate is better than milk but others indicate otherwise.

The increasing number of resorptive pit (shaped like a small mound) was observed in DVDSp group as shown in Fig. 4. On the other hand, group of DVD had plateau-like bone surface without resorptive pit. The number of restorative pit in

Table 4: Characteristics of femur bone of rats post-intervention

Diets	Length (cm)	Diameter (mm)	Absorption coefficient (cm^{-1})	Density ($g\ cm^{-3}$)
C	2.96[a]	2.78[a]	1.21[a]	1.402[a]
DVD	2.56[b]	2.58[a]	0.95[c]	1.065[c]
DVDCa	2.55[b]	2.31[c]	1.15[b]	1.264[b]
DVDMCa	2.57[b]	2.38[b,c]	1.13[b]	1.324[a,b]
DVDSp	3.00[a]	2.76[a,b]	1.18[a]	1.340[a,b]

[a-c]Different superscripts in the same column showing significant difference at $p<0.05$

the DVDCa, DVDMCa and DVDSp groups were higher than that of the DVD group. This could be due to resorption of calcium and phosphorous through calcium supplementation from the diet. Demineralization activity under hypocalcemic condition can be substantially treated with *Spirulina* supplementation by stimulating on the bone formulation. In DVDSp group, resorptive pit was observed more frequent compare to DVDMCa and DVDCa group.

The SEM pictures are consistent with the bone mass density measurement. The bone mass density of DVDSp group was greater than DVDSCa and DVDCa group. This result suggests that the absorption of calcium from *S. platensis* in the intestinal tract is superior thus improving bone metabolism. This phenomenon explained that *S. platensis* contain insoluble and soluble dietary fiber. Dietary fiber plays an important role in creating the conditions of acidity in the lumen's stool as fermentation of dietary fiber produce Short Chain Fatty Acids (SCFA) which increase acidity and subsequently increase calcium absorption.

Gupta *et al.*[3] reported that *Spirulina fusiformis* was effective in the treatment of osteoporosis and prevent negative effect of rosiglitazone diabetic drug. *Spirulina fusiformis* given to diabetic mice with a dose of 500 mg kg^{-1} b.wt., stimulated the formation of restorative pits and prevented osteoporosis. In this study feed intake of DVDSp group was 1457 mg day^{-1} or approximately 7.5 g kg^{-1}. It was higher than the dose in the previous study[3]. It has been reported that at the dose of 30 g kg^{-1} (fresh) or 10 g kg^{-1} of dry biomass did not cause any pathological changes in the internal organs[16].

CONCLUSION

It is concluded that bioavailability of calcium from *S. platensis* was higher than calcium carbonate and high-calcium milk. *Spirulina platensis* can maintain the Ca level in serum of rat given vitamin D-deficient diet at the normal condition after 8 weeks of intervention. This study proved that *S. platensis* supplementation in the diet effectively maintain bone integrity in animals fed with vitamin D-deficient diet.

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