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The relationship between *Spirulina platensis* and selected biomechanical indicators of tibiae in rats

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Abstract: There are several dietary supplements, particularly herbal foods, that have been used in an attempt to improve bone growth. In this study, we aim at determining the effects of low- and high-doses of *Spirulina platensis*, a "Superfood", on the bone growth and biomechanical indicators. Thirty Wistar rats, weighing 250 g, at the age of 7–8 weeks were assigned to three groups: The Control group (basal diet), Low-dose group (LDG; 500 mg/kg) and High-dose group (HDG; 1 000 mg/kg) of *S. platensis*. *S. platensis* was given daily by oral gavage in a 45-day-trial. At the end of the study, the right tibiae were collected and subjected to bone biomechanical tests (bone weight, bone length, maximum load, stiffness, breaking deflection, fracture toughness, post-yield displacement and yield load). Serum samples were also analysed for the calcium and phosphorus concentrations. There were significant increases in bone weight, bone length, maximum load, breaking deflection, work to fracture, post-yield displacement and yield load (P = 0.025, P = 0.019, P = 0.030, P = 0.015, P = 0.031, P = 0.028, P = 0.049, respectively), whereas stiffness non-significantly increased. However, there were no significant differences (P > 0.05) for any variables between the LDG and the HDG. Although the serum phosphorus concentrations showed no differences among any of the groups, the serum calcium concentration increased significantly in LDG compared to Control group (P = 0.009; 7.14 ± 0.47 and 9.45 ± 0.67, respectively). However, no differences were observed in HDG in terms of serum calcium. In conclusion, *S. platensis* had positive effects on the bone growth and biomechanical bone features. Therefore, our study supports the use of *S. platensis* as an alternative food additive for bone growth and health in growing animals.

Keywords: bone biomechanics; serum; calcium; phosphorus; natural foods

Nutrition is critical for both health and wellness. The ability of a food to strengthen the immune system, maintain growth, and provide antioxidants and protein are all selection criteria for both humans and animals. The most popular alga, *Spirulina platensis*, is approved as a healthy food by the World Health Organization (Karkos et al. 2011; Liu et al. 2016). *S. platensis* contains protein, vitamins, pigments and many minerals and has been investigated by researchers attempting to understand its effects on the growth, antioxidant mechanisms and immunity (Simpore et al. 2005;

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Rasool et al. 2006; Mayer et al. 2009; Seyidoglu et al. 2017). These studies have recommended the safe daily use of *S. platensis*, particularly due to its beta carotene, phycocyanin, high protein and mineral content. No side effects of the use of *S. platensis* were reported, and the literature suggests that it is a safe food supplement but its role as a drug remains to be seen (Karkos et al. 2011).

Many nutrients and supplements act by mechanisms, such as the alteration of the bone structure, bone metabolism rate, endocrine system and calcium homeostasis. Muhlbauer et al. (2003) observed that some herbal foods, such as peeled oranges, affect bone resorption. Other researchers indicated that some herbals, such as seaweed and Chinese herbals, have a protective role against bone loss (Craig and Mangels 2009; Moorhead et al. 2012). The mineral contents of *S. platensis*, especially calcium and phosphorus, have a positive effect on bone calcification and health due to their stimulating effect on the mineral absorption in the intestinal microflora. Craig and Mangels (2009) indicated that the calcium in *Spirulina* and its high absorption are associated with bone health balance. Additionally, Moorhead et al. (2012) explained that *Spirulina* contains about 26 times more calcium than milk, which supports bone and teeth development, as well as phosphorus, which affects the remineralisation of teeth. It was also indicated that the high mineral and protein content of *Spirulina* can help to avoid bone loss by preventing a release of minerals in the kidneys (Ishimi et al. 2006).

The European Prospective Investigation into Cancer and Nutrition described that bone fractures occur with a lower calcium intake (Appleby et al. 2007). It was also reported that the calcium-phosphorus ratio is important for measuring the risk of a reduced decalcification capability (Li et al. 2012). Through a study conducted with rats, it was demonstrated that the bioavailability of the calcium in *S. platensis* can maintain bone integrity (Ekantari et al. 2017). Kumar et al. (2009) assessed the protective effect of *S. platensis* against collagen-induced arthritis in rats. They reported the protective role of *S. platensis* on the modulation of arthritis with biochemical and histological indicators. However, there is a need to clarify the facts about the effects of *S. platensis* on the bone features which can also be associated with the biochemical indicators. We aimed at evaluating the effects of the different doses of *S. platensis* on some bone biomechanical indicators via serum calcium and phosphorus concentrations important for bone growth and health. Our hypothesis is that *S. platensis* will have positive effects on the bone material and mechanical properties due to its high protein and mineral content.

MATERIAL AND METHODS

Animals and feeding

The experimental protocols for this study were approved by the Animal Care and Use Committee of Tekirdag Namik Kemal University and are in accordance with the Institute´s Health Guide for the Care and Use of Laboratory Animals. The study was carried out with the permission of the University Animal Experimentation Local Ethics Committee (Approval No. 2017/04-4).

Thirty male Wistar albino rats weighing 200–250 g and aged 7–8 weeks were included in this study and allocated to three groups (n = 10). The groups were the Control group (basal diet, C), the Low-dose *Spirulina* group (500 mg/kg, LDG) and the High-dose *Spirulina* group (1 000 mg/kg, HDG). Food and water were offered *ad libitum* and *S. platensis* (Egert, Izmir, Turkey) was given once daily by oral gavage throughout the 45-day trial. The basal diet contained 2 000–2 500 kcal/kg of energy and the content of the raw protein, crude oil, crude cellulose and ash was 23%, 3%, 7% and 8%, respectively, which was prepared by a commercial company (NRC 1995).

Measurements

The blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes by heart puncture of the overnight-fasted rats under isoflurane anaesthesia at the end of the trial. The serum calcium and phosphorus were determined using the spectrophotometric method (Shimadzu UV-VIS Spectrophotometer 2600 device; Shimadzu, Canby, OR, USA).

Bone preparation and bone biomechanical tests

The rats were euthanised by an anaesthetic overdose of isoflurane. The right tibiae were collected

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from the euthanised rats and cleaned from the surrounding soft tissues and then frozen in plastic bags at −20 °C until the mechanical tests were conducted (Swiatkiewicz and Arczewska-Wlosek 2012). The tibiae were weighed with a Precisa XB4200C digital scale (Precisa Instruments Ltd., Dietikon, Switzerland) and the lengths were measured with a Mitutoyo CDN-20C digital calliper (Mitutoyo Corp., Kawasaki, Japan).

The three-point bending tests were carried out on a custom-made testing machine designed for low strength materials (Tufekci et al. 2014). To measure the force and corresponding deflection during the tests, a load-cell (50 N; Tedea Huntleigh, Malvern, USA) and a Linear Variable Differential Transformer (LVDT) (10-mm stroke; Novotechnik Tr10, Ostfildern, Germany) were used. An oscilloscope (Nicholet-Oddysey XE, Madison, USA) at the rate of 100 data/sec recorded the maximum load, stiffness, breaking deflection, work to fracture, post-yield displacement and yield load. The results could be affected by the displacement rate; therefore, all the tests were performed at a constant displacement rate of 10 mm/min (An and Draughn 1999). The distance between the supports was set to 20 mm, and the force was applied at the middle of the distance.

Statistical analysis

The statistical analyses were performed in SPSS (Version 20.0). All the values were grouped and calculated as means ± standard errors. The data were tested for normality distribution and variance homogeneity. A one-way ANOVA (analysis of variance) was applied to all the indicators for statistical evaluation of the homogenous parameters. If the differences were accepted as being significant ($P < 0.05$), Tukey's test was used (Dowdy and Wearden 1981). In the non-homogenous groups, the differences between groups were analysed by the Kruskal Wallis method followed by the application of the Mann-Whitney *U* test between the groups, one by one (Dawson and Trapp 2004).

RESULTS

The bone weight, length and selected bone biomechanical traits (maximum load, stiffness, breaking deflection, work of fracture, post-yield displacement and yield load) are presented in Table 1. In terms of the bone weight, length, maximum load, breaking deflection, work of fracture, post-yield displacement and yield load, there were significant increases in the LDG and the HDG compared to the Control group ($P < 0.05$). However, no significant differences were observed in the bone biomechanical traits between the LDG and the HDG. Although it was not significant, the stiffness showed a numerical increase in both the LDG and the HDG compared to the Control group. The average strength/deformation curves of all the groups are presented in Figure 1.

The serum calcium and phosphorus for all the groups are shown in Figure 2. There were no differences in serum phosphorus among any of the groups ($P > 0.05$). However, the serum calcium value in the LDG (500 mg/kg) was higher than the Control group ($P = 0.009$) and the HDG ($P = 0.005$).

Table 1. The effects of *Spirulina platensis* on the bone weight, length and some bone biomechanical indicators of the rat tibiae (mean ± SEM, $n = 30$)

Indicators	Groups		
	Control (C)	Low dose *Spirulina* (LDG) (500 mg/kg)	High dose *Spirulina* (HDG) (1 000 mg/kg)
Bone length (mm)	34.84 ± 0.49^a	36.68 ± 0.27^b	36.75 ± 0.31^b
Bone weight (g)	0.44 ± 0.02^a	0.52 ± 0.02^b	0.51 ± 0.02^b
Breaking deflection (mm)	0.67 ± 0.05^a	0.95 ± 0.05^b	0.94 ± 0.09^b
Maximum load (N)	68.85 ± 4.31^a	81.52 ± 3.53^b	82.96 ± 3.00^b
Stiffness (N/mm)	155.50 ± 9.00^a	170.00 ± 11.62^a	173.33 ± 13.08^a
Post-yield displacement (mm)	0.39 ± 0.01^a	0.47 ± 0.03^b	0.43 ± 0.01^b
Yield load (N)	65.05 ± 1.10^a	72.28 ± 3.76^b	78.08 ± 2.99^b
Work to fracture (N/mm)	33.28 ± 3.33^a	47.28 ± 2.26^b	46.59 ± 3.97^b

^{a,b}The different superscripts show differences: $^aP < 0.05$, LDG versus the C group; $^bP < 0.05$, HDG versus the C group

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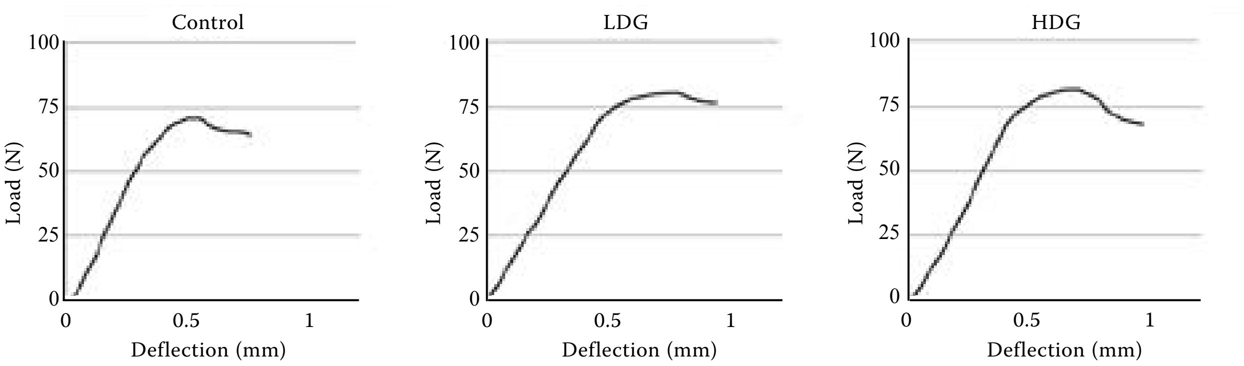

Figure 1. The load-deflection curves during the 3-point bending tests for the tibiae from the rats in the Control and experimental (Low-dose and High-dose *Spirulina*) groups

C = Control group; HDG = High-dose *Spirulina* group; LDG = Low-dose *Spirulina* group

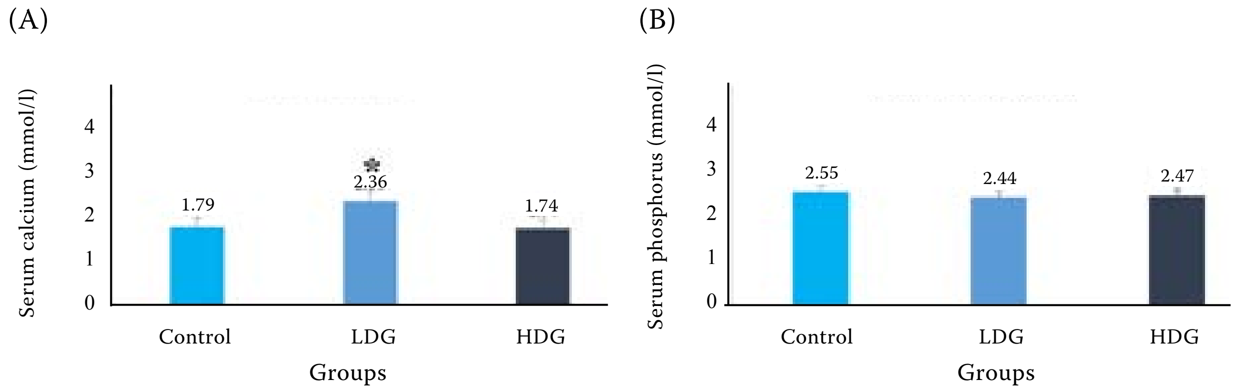

Figure 2. The serum indicators of the rats in the control and experimental (Low-dose and High-dose *Spirulina*) groups. (A) The serum calcium concentration of the rats. (B) The serum phosphorus concentration of the rats

C = Control; HDG = High dose *Spirulina*; LDG = Low dose *Spirulina*
*P < 0.05 LDG versus Control group

DISCUSSION

The morphological and material properties of bones affect the whole-bone properties. In the present study, some biomechanical traits of the tibiae, such as the maximum load, stiffness, breaking deflection, work of fracture, post-yield displacement and yield load, were measured. These terms can be explained as follows: the maximum load determines the highest load achieved before fracture, the stiffness is a measure of the elastic deformation when a structure is loaded, the breaking deflection is the displacement of a structure from the beginning of the process until fracture, the work of fracture measures the energy absorbed by the structure to deform and fail the structure, the post-yield displacement refers to the deformation that the structure experiences from the start of he failure until fracture, the yield load is a measure of how much load the bone can maintain before it suffers permanent damage. The tibial length has been used as an indicator of linear growth (Jepsen et al. 2015). In this study, the tibial length and weight in the *Spirulina* fed groups (LDG and HDG) increased compared to the Control group (P = 0.008 and P = 0.03, LDG and HDG, respectively), although there was no significant difference between the LDG and the HDG (P = 1.000). In accordance with these results, Sixabela et al. (2011) reported that the dietary supplementation of *S. platensis* significantly increased the tibial length in rats. Furthermore, Fournier et al. (2016) determined that *Spirulina* protects the bone geometry, such as length, diameter and bone mineral density, due to its protein contents. The rich mineral and protein content of *S. platensis* and its ability

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to stimulate mineral absorption by affecting the intestinal microflora, suggest that *S. platensis* has the ability to stimulate bone development. In this study, it was clearly observed that the tibiae of the rats in the LDG and the HDG had better biomechanical traits than in the Control group (P < 0.05), although no significant difference was observed between the LDG and the HDG. The maximum load (P = 0.030), breaking displacement (P = 0.015), yield load (P = 0.049), post-yield displacement (P = 0.028) and work of fracture (P = 0.031) were observed to be significantly higher in both the LDG and the HDG compared to the Control group while the stiffness (P > 0.005) had a non-significant increase (Table 1, Figure 1). These results may be the results of the vitamin C, phycocyanin or collagen properties of *S. platensis*. As a natural composite material, bone contains a substantially hard mineral phase (mainly hydroxyapatite crystals) and a softer collagen matrix (Katz 1971). Collagen increases the bone toughness, and a decrease in the collagen crosslink concentration is associated with a reduced bone stiffness and capacity to absorb energy (Oxlund et al. 1995; Wang et al. 2002). It was reported that circulating physiological vitamin C concentrations are required to maintain an optimal collagen network which correlates with the maximum load, stiffness, work of fracture and post-yield displacement (Katz 1971; Currey et al. 1996; Boyera et al. 1998; Jepsen et al. 2015). In this study, the rats in the LDG and the HDG had significantly higher work of fracture value compared to the Control group (P = 0.009 and P = 0.049, LDG and HDG, respectively), which may be due to the vitamin C and protein contents of *S. platensis*. Similar to our results, Nakagawa et al. (2000) suggested that *Spirulina* protects and improves the vitamin C content, which is a co-factor for collagen synthesis against degradation, by which the muscle and bone structure can be maintained. Ishimi et al. (2006) also observed that *Spirulina* may improve the bone elastic properties of the bone collagen structure due to its protein content.

The bone tissue is a model which has a lifelong construction and destruction cycle. The most important factor that drives the cycles is calcium. In this study, there was a significant increase in the blood calcium in the LDG compared to the Control group, which was also in the reference value for the rats (P = 0.009; 7.14 ± 0.47 and 9.45 ± 0.67, Control and LDG, respectively). This result may be due to the calcium content of *S. platensis*, and furthermore, it can also be said that the low dose given in the study (LDG) may be the optimal dose for bone health (Goldstein 1990; Xu and Lawson 2004). Nevertheless, due to its rich mineral content and ability to stimulate mineral absorption by its effect on the intestinal microflora, *S. platensis* might cause an increase in the bone mineralisation (Hidaka et al. 2004). Gutierrez-Salmean et al. (2015) reported that the development of the mechanical and material properties of the bone are the result of the rich protein (60–70%), vitamin C and minerals like iron, calcium and phosphorus of *S. platensis.*

In this study, the post-yield displacement was higher in both the LDG and the HDG than in the Control group (P = 0.015 and P = 0.037, for LDG and HDG, respectively). Also, the bones were slightly stiffer in both the LDG and the HDG than in the Control group, although not significantly higher. These results may be due to the calcium content of *S. platensis*, because both an adequate blood and bone calcium concentration is essential for the bone stiffness and rigidity. A stiffer bone contains more inorganic components, and this makes the bone more rigid. Therefore, increased mineralisation causes an increase in stiffness, and ultimately, the bone can withstand more load at a given displacement. As previously reported by Katz (1971), bone has a collagen matrix, and 90% of it consists of type I collagen. Nakagawa et al. (2000) suggested that a dietary supplement of *Spirulina* could increase the vitamin C metabolism in young sea breams. Moreover, vitamin C stimulates type I collagen deposits (Boyera et al. 1998). Gunes et al. (2017) also reported that *S. platensis* led to a significant increase in the type I collagen in the skin cell cultures in humans. Thus, in the present study, *S. platensis* may have increased the collagen content of the bones by stimulating the vitamin C metabolism. Ultimately, the bones may have a higher post-yield displacement and elasticity in the *Spirulina*-fed groups. Furthermore, Ishimi et al. (2006) observed that bone elastic properties might be increased by the *Spirulina* protein due to its effect on the bone collagen structure.

Herbal foods containing rich minerals and growth factors may improve bone metabolism. *S. platensis* may be one of the best for bone health due to its rich protein, mineral and vitamin contents that have sometimes given it a special mention as a "Superfood".

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It is clear that *S. platensis* had positive effects on the bone development and the biomechanical properties in this study. In conclusion, it is recommended that *S. platensis* can be used as a food additive either in low- or in high-doses because of its positive effects on bone health, and thereby, the quality of life.

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Conflict of interest

The authors declare no conflict of interest.

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