Probiotics Ameliorate Pioglitazone-associated Bone Loss in Diabetic Rats

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Abstract

Background: Pioglitazone as a PPAR-g agonist are used for management of type 2 diabetes mellitus. Nevertheless, evidence showed that the therapeutic modulation of PPARg activity using Pioglitazone may be linked with bone mass reduction and fracture risk in type 2 diabetes mellitus patients. The objective of the current research was to inspect the preventive role of some types of probiotic strains including (*Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Bifidiobacterum longum* and *Bacillus coagulans*) against pioglitazone-induced bone loss.

Methods: Streptozotocin (60 mg/kg) was administered for diabetes induction. Diabetic rats were fed orally with pioglitazone (300 mg/kg) and probiotics (1×109 CFU/ml/day) alone and in combination of both for 4 weeks. Dual energy X-ray absorptiometry (DEXA) were used to asses BMD, BMC and area of the femur, spine and tibia at the experiment termination. Serum glucose, serum calcium, alkaline phosphatase, phosphorus, BUN, Creatinine, and urine calcium were also analyzed.

Results: Administration of pioglitazone and probiotics alone and in combination significantly improved elevated blood glucose. Pioglitazone treatment significantly increased urinary calcium and BUN, and decreased ALP and creatinine. Co-treatment of probiotics with pioglitazone significantly decreased urinary calcium, creatinine and ALP. Pioglitazone showed detrimental effects on femur-BMD whereas treatment with probiotics remarkably ameliorated these effects. Among the tested probiotics *Bifidiobacterum longum* displayed the best protective effects on pioglitazone-induced bone loss in diabetic rats.

Conclusion: This study suggests probiotic supplementation in diabetic patients on pioglitazone regime could be considering as a good strategy to ameliorate bone loss induced by pioglitazone.

Background

According to WHO, about 422 million people had diagnosed diabetes mellitus, while the number of diabetic patients in 1980 was 180 million (1).The occurrence of type 2 diabetes (T2DM) is growing accompanied by the extension of the obesity epidemic (2). In uncontrolled or poorly controlled conditions, T2DM triggers severe systemic problems, including visual loss, neuropathy, renal damage and a shortened life hope (3). Both types of diabetes (T1DM and T2DM) are accompanying with low bone mineral density (BMD) and a high risk of bone fracture, especially at the hip due to osteoblastic dysfunction (4).

The insulin-sensitizing thiazolidinediones (TZDs) have complemented to the conventional management approaches for diabetes, such as diet, exercise and metformin (5). Pioglitazone and Rosiglitazoneare two main peroxisome proliferator-activated receptor-r (PPAR-r) thiazolidinediones which are considered as oral glucose-lowering anti-diabetic agent (6). Long-term intake of both TZDs can lead to bone loss andthe

risk of bone fractures has increased significantly in women who take these medications during T2DM (7). Some meta-analyses of randomized clinical trials clearly revealed that pioglitazone and rosiglitazone treatment is connected to an increased risk of fractures in all T2DM patients, especially young women (8-10). Also, a longitudinal observational cohort study using data from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial bone ancillary study showed that use of rosiglitazone (or if available pioglitazone) is linked with increased bone fracture risk in women (but not in men) (11). It seems that TZDs trigger differentiation of some stem cells into adipocytes rather than osteoblasts, causing lowered bone formation and enhanced bone resorption (12).

Recent studies have shown that probiotic bacteria have anti-T2DM effect for controlling the glucose levels (13, 14). It is supposed that their beneficial effect is related to many internal mechanisms including the skeletal system beyond control of blood glucose (15). In a preceding study, we reported that some types of probiotics could improve the bone formation, reduced bone resorption, and changes in the microstructure of the femur in ovariectomized-induced bone loss rat model (16, 17). Dar et al. disclosed that administration of *lactobacillus acidophilus* for 12 weeks could attenuate the bone loss and improve the mechanical bone strength in streptozotocin-induced diabetic rats with no impacts on glucose concentration (18).

The aim of this study was to inspect the supportive role of some types of probiotic strains containing (*Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Bifidiobacterum longum* and *Bacillus coagulans*) on the bone mass, BMD, and bone turnover markers in pioglitazone-treated rats.

Methods

Bacterial isolation and formulation

The probiotics were isolated from traditional fermented dairy products, produced in Iran according to Montazeri et al. (16). Briefly, 10 g of each dairy samples was homogenized, serially diluted and cultured on TSA medium. Then, for counting the number of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*, the LS Differential medium was used, and for specific separation of Lactobacilli the MRS agar, and for Bifidobacteria BFM agar was applied. After 3 days of cultivation at 37°C under anaerobic conditions, final characterization was done based on Bergey's Manual of Systematic Bacteriology. Their cultural, morphological and biochemical features of each bacterium were joined to each isolate. For formulation, the strains were sub-cultured in TSA medium, maintained overnight at 37°C and freeze dried. Before use, the strains were suspended in PBS (pH 7.4) and stirred for 20 min. This probiotic solution was made ready for animal feeding. The final concentration of probiotic supplement in each interval was 1*109 CFU/mL.

Experimental design

56 adult female Sprague-Dawley rats (12-14 weeks old and weighing 200±20 g) were obtained from the Laboratory Animal Center of Shiraz University of Medical Sciences. The animals were retained under regular housing laboratory settings (room temperature with the relative humidity of 60±5%, temperature of 23±2 ºC, and 12 hr/12 hr light/dark cycles) and were nourished a normal pellet diet and water ad libitum. animals were adopted to the room for one week. Type 1 diabetes was prompted with streptozotocin inoculation (60 mg/kg). A sterile solution of streptozocin (Sigma-Aldrich Company, Germany) in a 0.1 M solution of sodium citrate (pH= 4.5) was made and inserted peritoneally. The healthy control group was nourished the equal volume of normal saline. Then, the stimulation of diabetes was confirmed, using fasting blood glucose samples. After that, the rats were casually distributed into eight groups (n=7 per group) as follow: group 1; Control, group 2; Diabetic, group 3; pioglitazone (30 mg/ kg/day), group 4; pioglitazone (30 mg/ kg/day) + *Lactobacillus acidophilus*, group 5; pioglitazone (30 mg/ kg/day) + *Lactobacillus casei*, group 6; pioglitazone (30 mg/ kg/day) + *Bacillus coagulans*, group 7; pioglitazone (30 mg/ kg/day) + *Bifidobacterium*, group 8; pioglitazone (30 mg/ kg/day) + *Lactobacillus reuteri*. The rats in groups 3, 4, 5, 6 and 7 were fed orally with 1 mL (1×109 CFU/ml/day) of probiotics for 4 weeks. The rats in groups 1 and 2 were nourished with PBS. Food and water intakes were observed and there was no difference between groups. All animal experiments were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by Ethical Committee of Shiraz University of Medical Sciences (97-01-33-18906).

Oophorectomy

The ovary of adult female rats was removed bilaterally under anesthesia by ketamine 10% (100 mg/kg, Alfasan, Netherlands) and xylazine 2% (10 mg/kg, Alfasan, Netherlands). Both ovaries were detached in all groupswith surgery, except for the control group after joining of the uterine horn through a midline longitudinal incision.

Dual energy X-ray absorptiometry parameters measurements

In order to assess the area, BMC, and BMD of femur, spine and tibia, Dual energy X-ray absorptiometry (DEXA) scans were applied on a Discovery QDR, USA device, via the particular software for small animals at the experiment termination.

Biochemical parameters measurement

All the rats were sedated with ketamine and xylazine solution intraperitoneally and forfeited using thiopental (100 mg/kg) At the experiment termination. Blood samples were collected in chilled non-heparinized tubes to clot at room temperature by cardiocentesis. The blood samples were centrifuged (3500 rpm at 4˚C for 20 min) and the separated sera were evaluated for biochemical indicators, comprising serum glucose, serum calcium (Ca), serum phosphorus (P), alkaline phosphatase (ALP), BUN, creatinine and urinary calcium.

Statistical analysis

Statistical tests were conducted using IBM© SPSS© Statistics v 22.0 for Windows. Data are displayed as mean ± SD. Variation of biochemical parameters (serum glucose, Ca, P, ALP, BUN, creatinine and urinary calcium) and bone densitometry parameters (BMD, BMC and Area) between groups was analyzed using one-way ANOVA. Tukey post hoc analysis was executed when the outcomes of ANOVA indicated significance ($P \leq 0.05$).

Results

Isolation, identification and characterization of bacterial strains

In spite of the isolation of more than 36 bacterial strains, in this research five bacterial strains which have appropriate probiotic properties were selected. Their physiological and biochemical characteristics were mentioned in supplementary data 1. Based on the standard references and the morphological characteristics of isolated strains, the probiotics were recognized and characterized as *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Bifidiobacterum longum* and *Bacillus coagulans* (Supplementary table s1).

Effects of probiotics on biochemical parameters

Biochemical parameters considered for testing probiotics in this study were serum glucose, Ca, P, ALP, BUN, creatinine and urinary calcium (Fig. 1).

Serum glucose

As demonstrated in Fig. 1A, glucose levels significantly reduced after administration of STZ compared to the control group. Blood glucose concentration in the pioglitazone group were significantly lower than the STZ group. *Bifidobacter* sp. and *Bacillus coagulans* significantly decreased the glucose concentrations in rats compared to the STZ group. *Bifidobacter* sp. significantly reduced the serum glucose concentrations more than pioglitazone in diabetic rats. No significant changes in serum glucose concentrations were detected for other probiotic strains compared to STZ and pioglitazone groups.

Serum calcium

As demonstrated in Fig. 1B, no significant differences in terms of serum calcium concentration between treated groups and control were found.

Serum phosphorus

Similar to calcium, no changes were seen in the serum phosphorus concentration between treated groups and control (Fig. 1C).

Serum alkaline phosphatase (ALP)

Pioglitazone meaningfully declined the serum ALP in comparison with the control group. Interestingly, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bifidiobacter* and *Bacillus coagulans* reduced the serum ALP in comparison to the STZ group. Among the groups treated with probiotics, only *Bacillus coagulans* significantly decreased ALP concentration compared to pioglitazone group (Fig. 1D).

Blood urea nitrogen (BUN)

In the pioglitazone group, BUN concentration was significantly elevated in comparison with the control group. Again, BUN values was significantly augmented in all groups in comparison with the STZ group, except for the *Bacillus coagulans* group (Fig. 1E).

Serum creatinine

The STZ group showed significantly greater serum creatinine level in comparison with the control group. The high level of serum creatinine was diminished in the pioglitazone, *Bifidiobacter*, *Bacillus coagulans*, and *Lactobacillus casei* groups. However, there was no change in the serum creatinine concentration in the *Lactobacillus acidophilus* and *Lactobacillus reuteri* groups compared to the STZ group (Fig. 1F).

Urine calcium

STZ, pioglitazone and combination of pioglitazone and *Lactobacillus acidophilus* significantly increased the urinary calcium level in contrast the control group. *Bifidiobacter*, *Bacillus coagulans*, *Lactobacillus casei*, and *Lactobacillus reuteri* improved the urinary calcium concentration to the normal level which was as the same as control group.

Effects of probiotics on DEXA parameters

Besides biochemical parameters, the impacts of probiotic strains on each DEXA outputs (BMD, BMC, and bone area) of global, femur, spine, and tibia were distinctly examined.

Bone area

The probiotics impacts on the global bone area of global, spine, femur and tibia are revealed in Figure 2. The outcomes exhibited that global area was significantly reduced in the pioglitazone in contrast to the control group (Figure 2A). In addition, probiotics did not significantly improve the global area in the pioglitazone groups in contrast to the control group. In the case of spine-area (Figure 2B), femur-area (Figure 2C) and tibia-BMD (Figure 2D), there were no significant variances between all groups.

BMC

The impact of probiotics on the BMC of global, spine, femur and tibia are displayed in Figure 3. The pioglitazone group and STZ group exhibited significantly minor global BMC in contrast to the control group. However, global BMC was notably ameliorated in all probiotics-treated groups in contrast to the pioglitazone group. The global-BMC in the *Bifidiobacter* group was equal to the control group (Figure 3A).

In the case of spine-BMC (Figure 3B), no substantial variations were detected in STZ, pioglitazone and probiotics-treated groups compared to the control group. In respect to the femur- BMC (Figure 3C), similar to global BMC, pioglitazone-treated group and STZ group displayed remarkably low global BMC compared to the control group, but all the probiotics significantly improved the BMC in comparison with the pioglitazone groups and return it to normal level. In terms of tibia BMC (Figure 3D), there was a significant decreased level in the pioglitazone treated group. All probiotic strains ameliorated tibia BMC compared to the untreated pioglitazone group which was only significant in *Bifidiobacter* group. No significant changes were observed in terms of tibia BMC after probiotic supplementation in contrast to control group however, probiotics were capable to return the tibia BMC value to the normal level after bone loss induced by pioglitazone.

BMD

The impact of probiotics on the BMD of global, spine, femur and tibia are reported in Figure 4. Pioglitazone significantly decreased the global-BMD compared to the control group but all probiotic strains significantly improved global-BMD compared to the STZ and pioglitazone groups (Figure 4A). For spine BMD (Figure 4B), similar trend was observed. No significant variance was spotted between probiotics-treated groups and the control group. In regard to femur BMD (Figure 4C), all probiotics used in this study significantly improved the BMD compared to pioglitazone. In tibia BMD (Figure 4D), despite the significant effect of probiotics on the BMD compared to the pioglitazone group, only *Lactobacillus acidophilus* and *Bifidobacter* sp. revealed normal BMD among the probiotics.

Discussion

This study, for the first time, provides some evidence about the protective effect of probiotic bacteria against TZD-associated bone loss. In this research, we assessed the protective effects of *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Bifidiobacterum longum* and *Bacillus coagulans*

on pioglitazone-induced bone loss in diabetic male rats. The results show that these probiotics in combination with pioglitazone can control blood glucose and improve the BMD and bone quality.

Previously, a lot of clinical and preclinical researches have revealed that pioglitazone decreases the trabecular bone volume, BMD and BMC (19, 20). Therefore, it seems that this antidiabetic medicine (and the other insulin-sensitizing TZDs) can increase the bone resorption and decrease the bone formation by inhibiting osteoblast differentiation (21), specifically in postmenopausal women (22, 23). Consistent with large previous works, this *in-vivo* animal study indicated that pioglitazone had such effect on the BMD and trabecular bone volume which is associated with elevation of bone resorption and reduction of bone formation.

In a previous study, we revealed that some probiotic strains could increase the BMD, improve bone formation, reduce bone resorption, and change the microstructure of the femur (16). It appears that certain types of probiotic strains can play a role in GI microbiota restoration (24), enhancing the epithelial barrier (25), normalizing the immune responses (26) and facilitating the GI calcium absorption (27). Therefore, these strains are effective in prevention of bone resorption and maybe useful for post-menopausal osteoporosis treatment (16). In this study we found declined BMD in the diabetic and pioglitazone-treated diabetic rats which was in agreement with the earlier literature (28). In the existing research, we also detected lower BMD in the diabetic groups in contrast to normal rats. Pioglitazone reduced BMD, especially in femur and tibia. The adverse effects of pioglitazone on femur-BMD was reversed completely after treatment with all probiotics. The tibia-BMD loss caused by STZ and pioglitazone were returned to normal level which was significant for *Bifidiobacterum longum* and *Lactobacillus acidophilus*.

On the other hand, some clinical trials and meta-analysis studies indicated the beneficial effects of probiotic strains in T2DM (29-31). Probiotics can ameliorate the glucose control in T2DM patients and glycated hemoglobin level (which is a diagnostic indicator of blood glucose over the past 2 to 3 months) was decreased, representing improvement in insulin resistance (32).

It has been suggested that total body weight of diabetic patients is reduced due to proper blood glycemic control caused by probiotic intake. In addition, a general metabolic condition improvement was detected in the diabetic person following probiotic consumption indicated by reduced triglyceride and C-reactive protein (33, 34). Overall, probiotics supplementation significantly reduced the fasting plasma glucose, glycated hemoglobin, fasting insulin, and homeostasis model assessment of insulin resistance in diabetic cases (29, 35, 36).

In the current study we found that co-administration of probiotics and pioglitazone improved the high glucose levels caused by STZ. The overall best combination therapy in controlling blood glucose without reducing calcium bone resorption has been shown in the animal group which received *Bifidiobacterum longum* and pioglitazone. Previous study showed that pioglitazone treatment did not affect serum calcium levels (37). Similarly, no changes in serum calcium concentration in pioglitazone group compared to control group were found. Calcium excretion into urine was increased with pioglitazone

which was in accordance with Zanchi et al. study in which they examined the effects of Pioglitazone on renal calcium excretion (38). In addition, the results displayed that elevated urinary calcium excretion caused by STZ and pioglitazone was ameliorated after treatment with probiotics.

The mechanism by which probiotics exert their beneficial effect on bone health is not fully understood and seems to be very complex because of the nature of these organisms. Also, each probiotic strain produces different components affecting different pathways in the host's body. However, it is suggesting that there are some possible mechanisms for these bone protecting effects. First, their impact on the gut composition and media via adjustment of the intestinal pH; production of biologically effective peptides and modification of the gut microflora have been shown (15, 39, 40).

Second, the immunological aspect of probiotics has been mentioned in several studies. Many studies have reported that probiotic had anti-inflammatory effects and they were already demonstrated to increase the regulatory T cells (41, 42) along with modulating Th17 cells differentiation and production (43). It is proved that both Treg cells (CD4+ and CD8+) are correlated to mechanisms of bone protection (18). Also, there is another amazing nutritional possibility. Intestinal microbiota (e.g. probiotic bacterial strains) synthesize many proteins, enzymes and vitamins which are necessary for bone formation and growth, such as folic acid, vitamin D, K and C (44). It is especially observed after consumption of *Bifidobacteria* species (45). Additionally, the genus *Bifidobacteria* can synthesize and secret short chain fatty acids that can lower the luminal pH and helps the minerals absorption (46).

Among all probiotic strains used in this study, *Bifidobacterium longum* showed the best protective effect against osteoporosis induced by pioglitazone. It may be due to their additional protective effect on the bone health in comparison to other probiotics. *Bifidobacterium longum* can decrease periodontal oxidative stress by modifying the NF-κB gene expression (47). Reactive oxidative species can actually suppress the osteoblast differentiation while enhancing osteoclast differentiation (48). Thus, because of the potential effect of *Bifidobacterium longum* in stimulating osteoblastogenesis and inhibiting osteoclastogenesis, together with our experimental data, it is suggested that this strain can be used in combination with pioglitazone to prevent its bone loss effects in Type 2 diabetic patient. Further in vivo studies and clinical trials are recommended to be conducted to discover the vast aspects of this combination therapy and their mechanism.

Conclusion

Some probiotic strains, especially *Bifidobacterium longum,* increased the bone mass in diabetes-induced rat model and co-supplementation of probiotic with pioglitazone improved the bone loss caused by pioglitazone. Therefore, co-administration of probiotic with pioglitazone as a clinical strategy is estimated to minimize bone loss and fracture risk in T2DM patients treated by pioglitazone.

Declarations

Ethics approval and consent to participate:

All animal experiments were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by Ethical Committee of Shiraz University of Medical Sciences (97-01-33-18906)

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests

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Author contributions:

AG contributed to conception and design, acquisition of data, drafting the manuscript and final approval of the manuscript; MHM contributed to conception and design and final approval of the manuscript, YG contributed to conception and design, and final approval of the manuscript; PT involved in acquisition of data and analysis of data; FK contributed to conception and design, acquisition of data and analysis of data and final approval of the manuscript; NMN contributed to conception and design, acquisition of data, drafting the manuscript and final approval of the manuscript.

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Figures

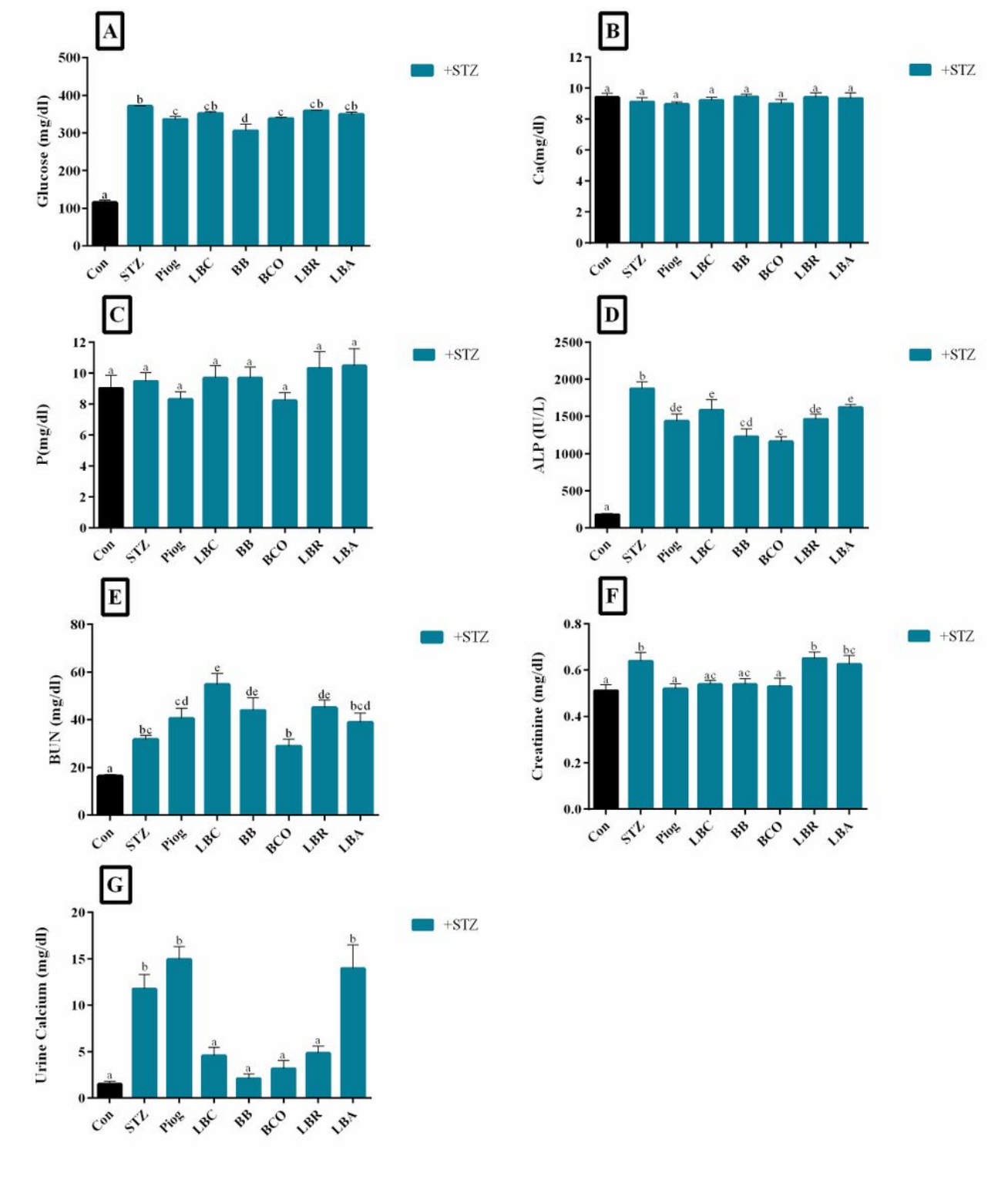

Figure 1

The effect of probiotics on serum glucose (a), serum calcium (b), phosphorus (c), alkaline phosphatase (d) phosphorus, BUN (e), Creatinine (f), and urine calcium (g) concentrations in rats during treatment with pioglitazone.

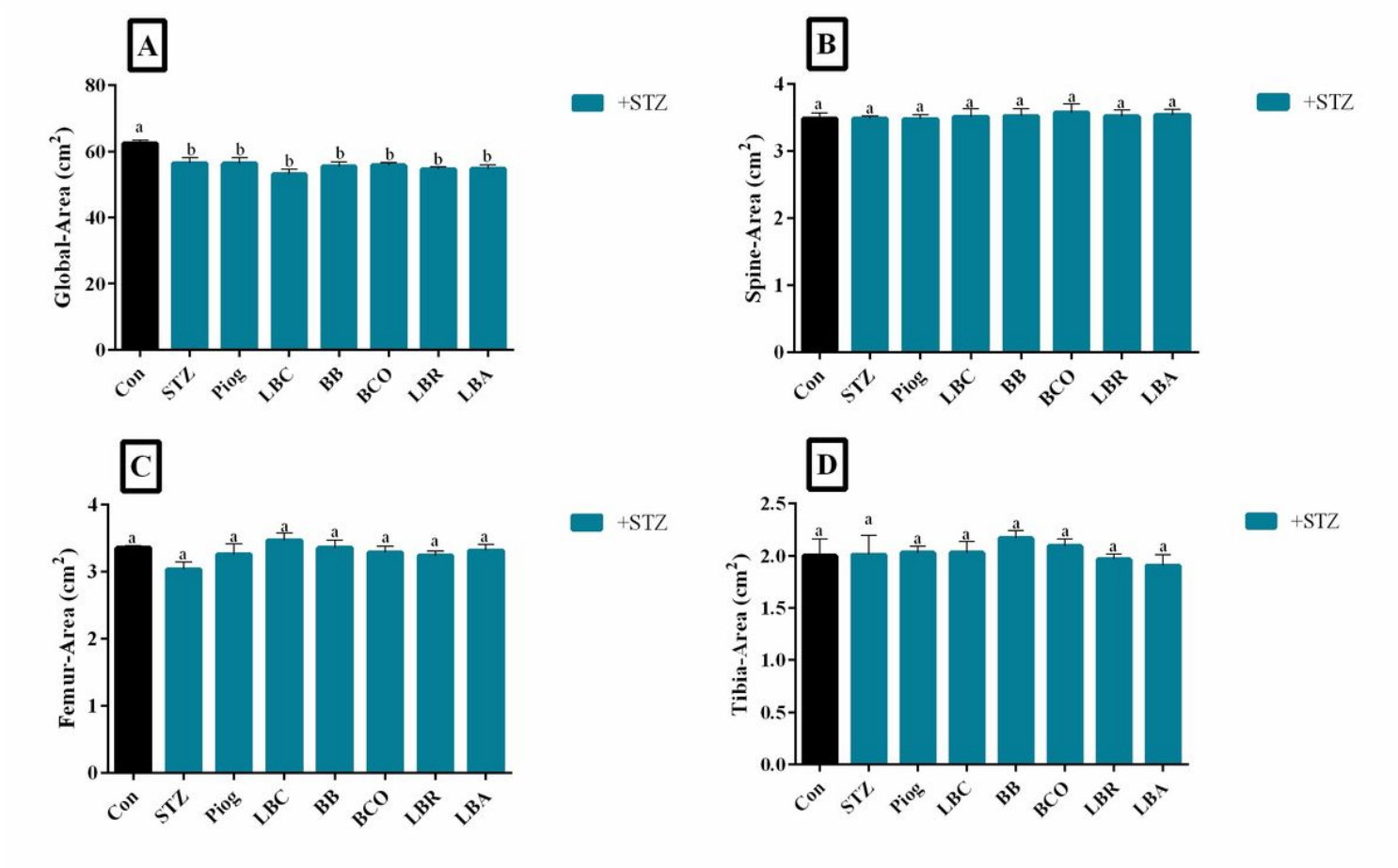

Figure 2

The effects of probiotics on the global area of spine, femur and tibia of pioglitazone treated rats 4 weeks after treatment.

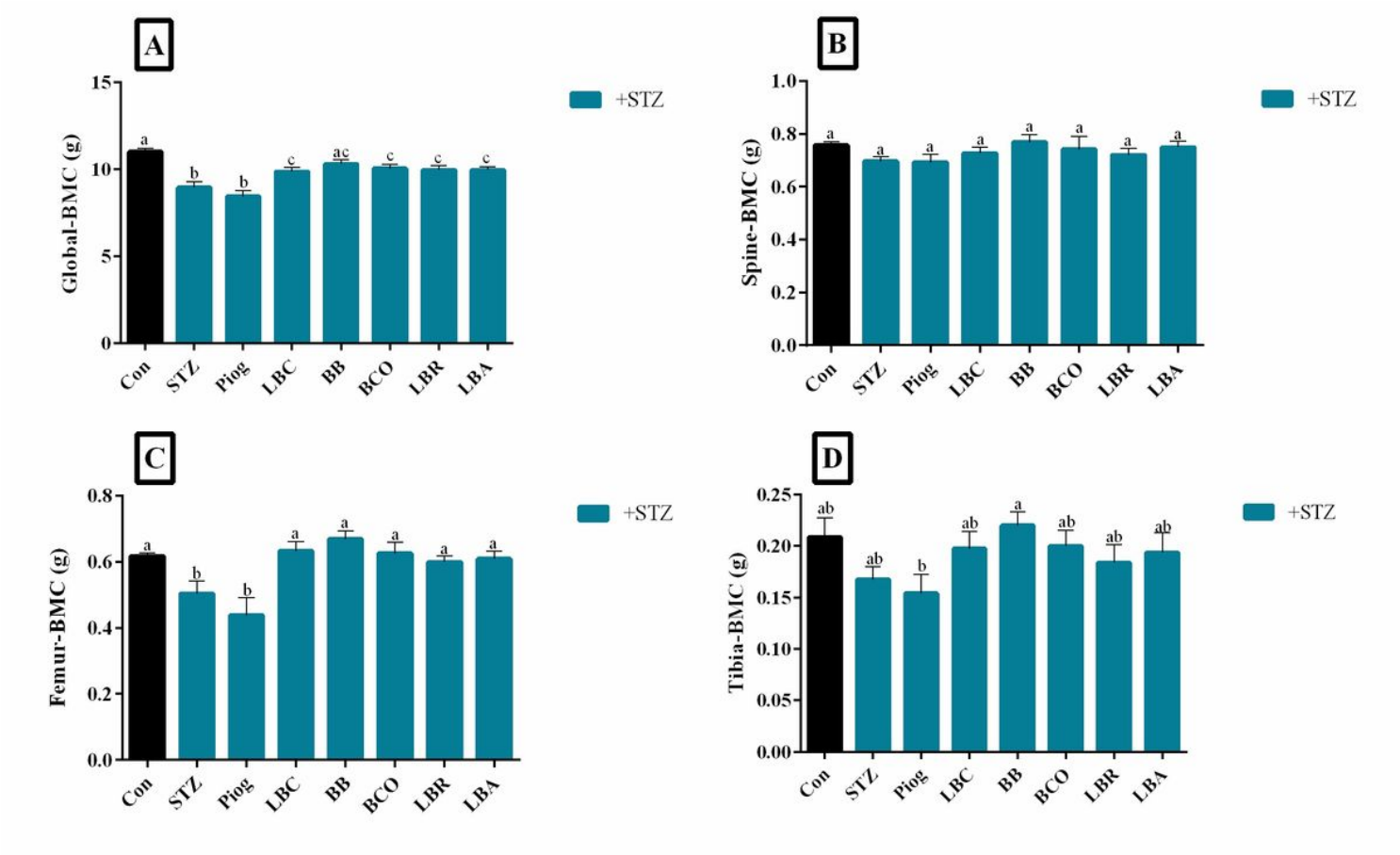

Figure 3

The effects of probiotics on the BMC of global, spine, femur and tibia of the pioglitazone-treated rats 4 weeks after treatment.

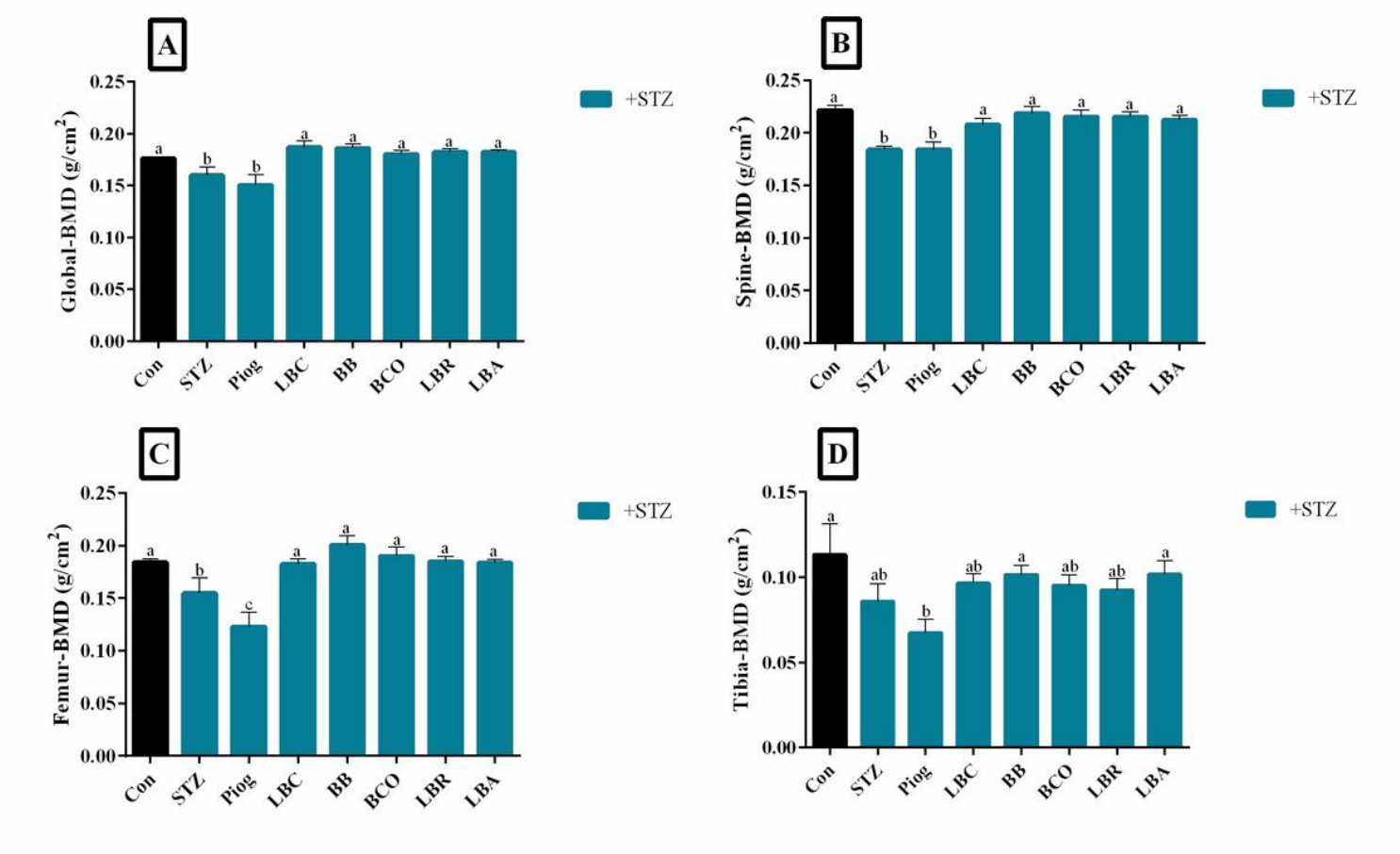

Figure 4

The effects of probiotics on the BMD of global, spine, femur and tibia of pioglitazone-treated rats 4 weeks after treatment.

Supplementary Files

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- TableS1.docx