

Probiotic *Lactobacillus Acidophilus* prevents bone loss in aged osteoporosis in rats; the possible implication of NLRP3 Inflammasome

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Abstract

Senile osteoporosis (SOP) is a degenerative bone disease associated with increasing susceptibility to fractures and mortality in the elderly. Innate immunity and specifically the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, with its subsequent mediators caspase-1 and interleukin-1b (IL-1b), have recently been linked to osteoporosis. Probiotic *Lactobacillus acidophilus* (L.A) was reported to exert favorable effects on osteoporosis. The aim of this study was to identify the protective effects of probiotic L.A in aged osteoporotic rat model and to evaluate the possible underlying mechanisms focusing on NLRP3 inflammasome and its effectors caspase-1 and interleukin -1b. Thirty-two adult male albino rats were designated to four equivalent groups. Group I; control, group II; probiotic L.A, group III; osteoporotic group, and group IV; probiotic L.A+ osteoporosis group. Osteoporotic rats pretreated with L.A in a dose of 10^9 CFU/ml / day for 8 weeks revealed a significantly lower oxidative stress state, increased bone mineral density (BMD), enhanced bone histological architecture, lower serum calcium, higher bone formation markers associated with lower bone resorption marker, lower serum receptor activator of nuclear factor kappa-B ligand (RANKL), decreased bone NLRP3 inflammasome as well as caspase-1 expression levels and lower serum IL-1b. Osteoprotective effects of probiotic L.A in SOP rat model mediated even in part via its anti-inflammatory effects that was represented by decreased NLRP3 inflammasome and its subsequent mediators caspase-1 and IL-1b, that resulted in enhancement of bone formation and reduction of bone resorption.

1. Introduction:

Aging is an inevitable physiological process, that entails the progressive accumulation of changes over time, typically associated with an increased susceptibility to numerous degenerative disorders [1]. Senile osteoporosis (SOP) is a systemic degenerative skeletal pathology that is characterized by decreased bone mass, higher fracture risk, and micro- architectural bone deterioration and increased risk of fracture[2].

Studies have shown the association between chronic systemic inflammation and aging, which is also known as “inflammaging”[3][4]. The elderly typically have greater levels of systemic inflammatory cytokines[3]. The innate immune system has been linked to both aging and inflammaging [1]. The nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, is a supramolecular cytoplasmic complex that senses endogenous stress signals that result from the process of aging[5]. The NLRP3 inflammasome is linked to many age-dependent diseases[6], moreover, studies revealed that NLRP3 inflammasome was associated with bone loss and osteoporosis[7], however the role of NLRP3 inflammasome in SOP has yet to be understood.

Currently, several osteoporosis treatment modalities are aimed at reducing bone resorption or enhancing bone formation [8]. However, these agents demonstrated controversies due to their higher cost, side effects, and poor patient compliance[9]. Therefore, alternative treatment modalities are thought ,Probiotics are live microorganisms when administered in adequate amounts it confer a health benefit on the

host[10], moreover, Probiotics were reported to exert favorable effects on the skeletal system [11].

Few studies till date have fully discussed the different underlying molecular mechanisms by which probiotics affect bone health. Probiotics were found to affect bone homeostasis by endocrinal factors that are also involved in the maintenance of the skeletal system (eg. incretins and serotonin)[12]. Anti-inflammatory effects are also among the underlying mechanisms by which probiotics benefit bone metabolism. Previous studies stated that probiotic supplementation reduces tumor necrosis factor- α (TNF- α), IL - 17 and receptor activator of nuclear factor kappa-B ligand (RANKL) expression levels in ovariectomized mice [13].

Lactobacillus acidophilus (L.A); probiotic used in our study, a former study revealed its immunomodulatory and anti- inflammatory effect on ovariectomized osteoporosis mice model via modulating Treg-Th17 cell balance resulted in preventing bone loss [14]. Moreover, a recent study have reported anti-inflammatory effects of L.A via modulating NLRP3 inflammasome in a rat model of ulcerative colitis [15]. The present study aimed at clarifying the potential protective mechanisms of probiotic L.A in aged osteoporotic rat model thus it can serve as a targeted intervention to delay osteoporosis. The specific focus was on NLRP3 inflammasome and its main effectors caspase-1 and IL-1 β in mediating such effect.

2. Materials and Methods:

The experiment was carried out at the Physiology department in collaboration with the medical Biochemistry department and molecular

biology unit, at the Faculty of Medicine, Benha University, following its approval by the local ethical committee of the university (Approval No.RC.38.10.2022).

2.1. Animals

Thirty-two adult male Wistar albino rats were obtained from the Faculty of Agriculture, Moshtohor, Egypt and involved in this trial. Rats aged 8-10 weeks and weighing 180 ± 20 g. Animals were designated to four equivalent groups (8 rats each), each group was placed in a separate cage. The rats were housed in cages maintained at 25°C with a 12-hour light-dark cycles. Animal was fed a standard diet and had free access to water ad libitum. After study completion animals were disposed of using the incinerator.

2.2. Drugs and chemicals:

D- galactose (D- gal) was provided in the form of a white powder extra pure <99% (LOBA chemie Pvt.Ltd, Mumbai, India). Probiotics *Lactobacillus Acidophilus* provided as capsules. Each capsule contains 10^9 active *Lactobacillus Acidophilus* bacteria (Natrol LLC, USA).

2.3. Study design

After one week acclimatization period based on laboratory settings, the rats were categorized into four groups of 8 ($n = 8$ rats per group) as follow:

Group I:(Control). Animals were administered normal saline via an intraperitoneal injection. They also received distilled water using an oral gavage daily for a duration of eight weeks.

Group II:(Probiotic L.A group). Rats received *Lactobacillus acidophilus* by oral gavage in a dose of 10^9 CFU/ml / day for 8 weeks. Capsules were dissolved in distilled water [14][16].

Group III: (Osteoporosis group). Rats were intraperitoneally injected by D-gal dosage of 200 mg/kg/day dissolved in saline, for eight weeks [17].

Group IV: (Probiotic LA+ Osteoporosis group). Rats received probiotics L.A and D-gal that were given the same doses in the group II and group III, respectively.

2.4. Determination of bone densitometry using a DXA scan:

The day following the final treatment day, all rats underwent a non-invasive dual energy X-ray absorptiometry scan (DXA) (DXA-Hologic model 4500A, Waltham, MA, USA) using a software program (Version 610-0691 for QDR XP). Prior to performing the DXA scan, the rats underwent anesthesia using ketamine/xylazine (40/20 mg/Kg. B.W.). The bodies were repositioned three times, the device estimates bone mineral content (BMC) in grams and area in cm^2 to detect bone mineral density (BMD) in g/cm^2 by dividing BMC over area of bone measured [18].

2.5. Blood and tissue sampling

After DXA assessment, rats were housed for another three days and subsequently underwent anesthesia using urethane (1.5 g/kg; i.p.) to obtain tissue samples. Blood was drawn by cardiac puncture in a dry clean test tubes to separate the serum. The blood was left to clot for ~ 30 min, then serum was divided using centrifugation at $3000 \times g$ for 15 min then was stored at -20°C for further biochemical analysis. Bone dissection took place immediately after euthanization by decapitation. Right and left femora bone specimens were detached and washed off soft tissues. The right femora specimens were kept at -80°C for western blot analysis, while the left

femora specimens were kept in formalin for histopathological examination.

2.6. Biochemical analysis

Collected sera were used for assessment of the following parameters:

-Serum Calcium

Calcium was determined using colorimetric method using kits supplied by (Bio-diagnostic, Cairo, Egypt).

-Biomarkers for bone turnover:

Using kits supplied by Sigma Co. (St Louis, Missouri, USA), the concentration of alkaline phosphatase (ALP) was measured by colorimetric method, enzyme-linked immunosorbent assay (ELISA) technique used to measure serum osteocalcin (OCN) concentration using Rat Mid, Osteocalcin ELISA kit (IDS Inc., Fountain Hills, Arizona, USA), serum osteoprotegrin (OPG) was determined by ELISA technique using (Rat Elisa kits, R&D Systems, Minnesota, USA) kits, tartrate-resistant acid phosphatase (TRAP), was evaluated by sandwich ELISA kit (Biotang Inc., Waltham, Massachusetts, USA), and Serum RANKL level was determined by ELISA technique using ELISA kits supplied by (Rat Elisa kits, R&D Systems, Minnesota, USA).

-The oxidative stress and pro-inflammatory biomarkers

The oxidative stress markers: Malondialdehyde (MDA) indicating lipid peroxidation was determined using MDA colorimetric assay kit (Abcam, Cambridge, UK) and superoxide dismutase activity (SOD) was determined using SOD colorimetric kit (Bio-diagnostic, Cairo, Egypt). The pro-inflammatory cytokines: interleukin 1beta (IL-1b) levels using rat ELISA colorimetric assay kit (Ray Biotech, Inc., USA).

2.7. Western blot analysis of bone NLRP3 inflammasome and Caspase -1 Protein

NLRP3 inflammasome and Caspase -1 Proteins expression in bone was determined using Western blot procedure. Femora bones were used for protein extraction. After bone marrow collection, the femora of rats were crushed using a mortar then an appropriate volume of RIPA lysis buffer was added. Cell lysates were placed in a centrifuge running at 14,000 rpm for 15 minutes at 4°C. Using the Folin Lowry method (with bovine serum albumin), the overall protein content was determined. The same exact quantity (20 µg) of protein samples were thoroughly mixed and boiled with a SDS loading buffer, which was subsequently cooled on ice. This mixture was then loaded into an SDS-polyacrylamide gel and separated using a Cleaver electrophoresis unit (Cleaver, UK), and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad) using a Semi-dry Electroblotter (Biorad, USA).

Using 5% nonfat dry milk in Tris-buffered saline-Tween-20 (TBS-T), the membrane was blocked and incubated overnight with the primary antibodies for NLRP3 inflammasome, Caspase -1, and β-actin (Santa Cruz Biotechnology) diluted at 1:1000, 1:500, and 1:500 respectively.

The membrane was washed and incubated using the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) diluted at 1:5000 in the blocking solution. Based on the manufacturer manual, the chemiluminescent Western ECL substrate (Perkin Elmer, Waltham, MA) was applied to the blot. Chemiluminescent signals were taken using the Chemi Doc imager (Biorad, Hercules, CA, USA), and analyzed using

Bio-Rad Image Lab software with normalization to β actin.

2.8. Histopathological examination.

Following the dissection of the left femora, specimens were fixed in 10% buffered formalin for a duration of 48 hours, and then decalcified in an EDTA solution for a duration of six weeks. Paraffin was used to embed the decalcified specimens. Overall, 5- μ m-thick sections were stained with hematoxylin and eosin (H&E) [19]. Using the ImageJ analysis program (version 1.36, NIH, USA), histomorphometric analyses were performed. All animals in each group underwent morphometric analyses of the femur via 3 stained sections/rat (ten fields/section). Mean outer cortical bone thickness (CBT) and mean trabecular bone area (TBD%) were determined [20].

2.9. Statistical analysis.

Data was demonstrated as mean \pm standard deviation (SD) ranges, ratios, and numbers. Variations among the groups were assessed using one-way ANOVA with post hoc test (LSD) using the Software, Statistical Package for Social Science, (SPSS Inc. Released 2009-PASW Statistics for Windows Version 19 Chicago: SPSS Inc.). A value $p < 0.05$ was significant.

3. Results:

3.1. Probiotic L.A pretreatment decreased serum Ca^{2+} and ameliorated the bone turnover markers in aged osteoporotic rats:

Significant higher ($p < 0.05$) serum calcium level was detected in rats given D-gal for eight weeks (Osteoporosis group) compared to control group. Moreover, administration of probiotic L.A for 8 weeks in probiotic L.A group resulted in its significant

increase ($p < 0.05$) compared to control group, while Probiotic administration of probiotic L.A in L.A + osteoporosis group showed a significant increase ($p < 0.05$) compared to control group and a significant decrease ($p < 0.05$) compared to osteoporosis group.

Significant lower ($p < 0.05$) serum levels of bone formation markers; ALP and OCN, accompanied by significant higher ($p < 0.05$) serum levels of bone resorption marker; TRAP, moreover, significant lower ($p < 0.05$) serum levels OPG accompanied by significant higher ($p < 0.05$) levels of serum RANKL all were detected in rats which were given D-gal for 8 weeks (Osteoporosis group) compared to control group. However, administration of probiotic L.A for eight weeks in Probiotic L.A + osteoporosis group resulted in a significant increase ($p < 0.05$) in serum ALP, OCN and OPG levels with a significant decrease ($p < 0.05$) in serum TRAP and RANKL levels compared to osteoporosis group (Table 1).

3.2. Probiotic L.A pretreatment reduced oxidative stress state and serum pro-inflammatory cytokine levels in aged osteoporotic rats:

Serum MDA was much greater ($p < 0.05$), while serum SOD was significantly reduced ($p < 0.05$) in rats that received D-gal for eight weeks (Osteoporosis group) compared to control group. Interestingly, probiotic L.A pretreatment for eight weeks in Probiotic L.A + osteoporosis group resulted in significantly lower levels of MDA and significantly higher ($p < 0.05$) levels of SOD compared to osteoporosis group.

Serum IL-1 β levels were significantly greater ($p < 0.05$) in rats which were given D-gal for eight

weeks (Osteoporosis group). On contrary, pretreatment with probiotic L.A for eight weeks in the Probiotic L.A + osteoporosis group caused a

significant decrease ($p < 0.05$) in this pro-inflammatory cytokine compared to osteoporosis group (Table 2).

Table (1): Serum calcium and bone turnover markers among the experimental groups:

	Control group	Probiotic L.A group	Osteoporosis group	Probiotic L.A +Osteoporosis group
Serum Calcium (mg/dl)	9.71±0.53	10.53±0.69*	13.82±0.88*	10.69±0.67*#
Serum ALP (U/L)	71.01±2.10	70.26±1.94	58.76 ± 5.54*	69.45±7.66*#
Serum OCN (ng/ml)	60.31±2.45	60.72±2.33	44.97 ± 2.63*	63.54±2.48*#
Serum OPG (pg/ml)	13.13±1.55	13.80±1.79	7.68 ±1.00*	12.65 ±1.41#
Serum TRAP(U/L)	10.06±1.10	9.71±1.13	31.04±1.96*	17.14±2.23*#
Serum RANKL (pg/ml)	74.89±0.91	73.75±1.94	124.38±3.12*	88.93±7.30*#

Data are expressed as mean ± standard deviation ($n = 8$). $P < 0.05$ is significant tested by using one-way analysis of variance (ANOVA) and post hoc multiple comparisons (LSD). * $P < 0.05$ vs. control group; # $P < 0.05$ vs. osteoporosis group. ALP, Alkaline phosphatase; OCN, Osteocalcin; OPG, osteoprotegerin; TRAP, tartrate resistant acid phosphatase; RANKL, receptor activator of nuclear factor-kappa B ligand.

Table (2): Serum oxidative stress markers and pro-inflammatory cytokines among the experimental groups:

	Control group	Probiotic L.A group	Osteoporosis group	Probiotic L.A +Osteoporosis group
Serum MDA(μmol/L)	1.02±0.11	1.03±0.10	2.09±0.16*	1.29±0.08*#
Serum SOD (mmol/L)	390.87±6.85	395.00±6.67	304.50±7.34*	367.62±6.69*#
Serum IL-1b (pg/ml)	18.76±1.07	19.11±1.22	25.68±1.46*	21.39±3.11*#

Data are expressed as mean ± standard deviation ($n = 8$). $P < 0.05$ is significant tested by using one-way analysis of variance (ANOVA) and post hoc multiple comparisons (LSD). * $P < 0.05$ vs. control group; # $P < 0.05$ vs. osteoporosis group. IL-6, (Interlukin-6); IL-1b, (Interlukin 1beta), MDA, (Malondialdehyde); SOD, (Superoxide

3.3. Probiotic L.A pretreatment enhanced the bone mineral density (BMD) in aged osteoporotic rats:

For all study groups, the whole-body BMD was determined using the DXA scan. These scans revealed that rats which were given D-gal for eight weeks (Osteoporosis group) showed a significantly lower ($p < 0.05$) BMD compared to control group. On contrast, administration of probiotic L.A for 8 weeks in Probiotic L.A + osteoporosis group caused a significant elevation($p < 0.05$) in BMD compared to osteoporosis group (Figure 1).

3.4. Probiotic L.A pretreatment reduced the NLRP3 inflammasome and Caspase -1 Protein in bone of aged osteoporotic rats:

By comparing NLRP3 inflammasome and Caspase -1 expression levels in bone tissue using western blot analysis, significant higher levels($p < 0.05$) of NLRP3 inflammasome and caspase -1 were detected in the rats which were given D-gal for eight weeks (Osteoporosis group) when compared to the control. Administration of probiotic L.A for eight weeks in Probiotic L.A + osteoporosis group resulted in a significant reduction in the osteoporosis group($p < 0.05$)(Figure 2).

3.5. Probiotic L.A pretreatment increased CBT and TBD% of aged osteoporotic rats:

Microscopic examination of the bone specimens of different groups revealed the classical characteristics of cortical and trabecular

bone. The endosteal surface appeared smooth in both the control and probiotic L.A groups (Figure 3 A&B). D-gal treatment for eight weeks demonstrated visible histological alterations that was seen as distorted architecture with a significant reduction ($p < 0.05$) in both CBT and TBD% in osteoporosis group when compared to

control rats (Figure 3 C,E&F). Where as probiotic L.A pretreatment for 8 weeks in Probiotic L.A + osteoporosis group resulted in histological improvement of the bone architecture demonstrated by the significantly higher ($p < 0.05$) CBT and TBD% compared to osteoporosis group (Figure 3 D,E&F).

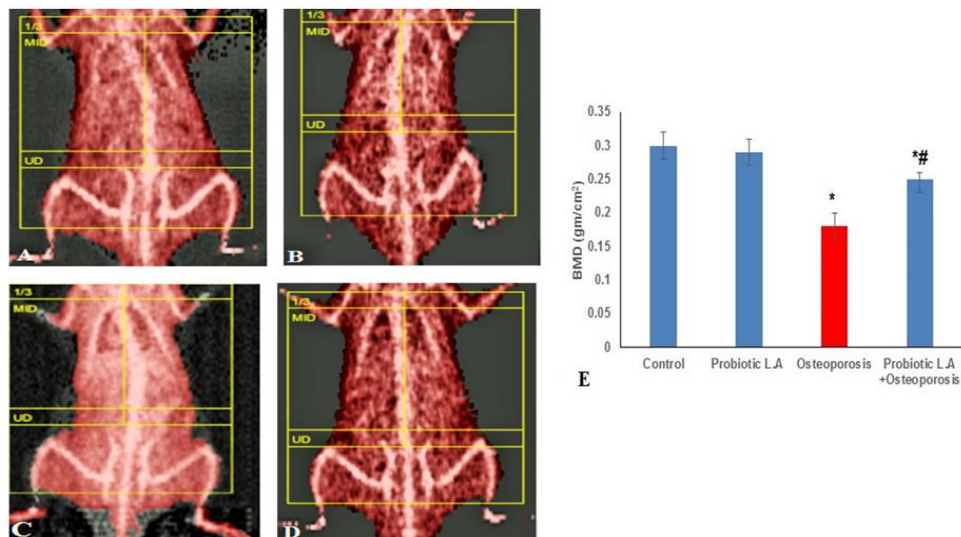


Figure (1): DXA scan for (A) Control group, (B) Probiotic L.A group, (C) Osteoporosis group, and (D) Probiotic L.A + Osteoporosis group. (E) Bone mineral density (BMD) in different experimental groups, data are expressed as mean \pm standard deviation ($n = 8$). $P < 0.05$ is significant tested by using one-way analysis of variance (ANOVA) and post hoc multiple comparisons (LSD). * $P < 0.05$ vs. control group; # $P < 0.05$ vs. osteoporosis group.

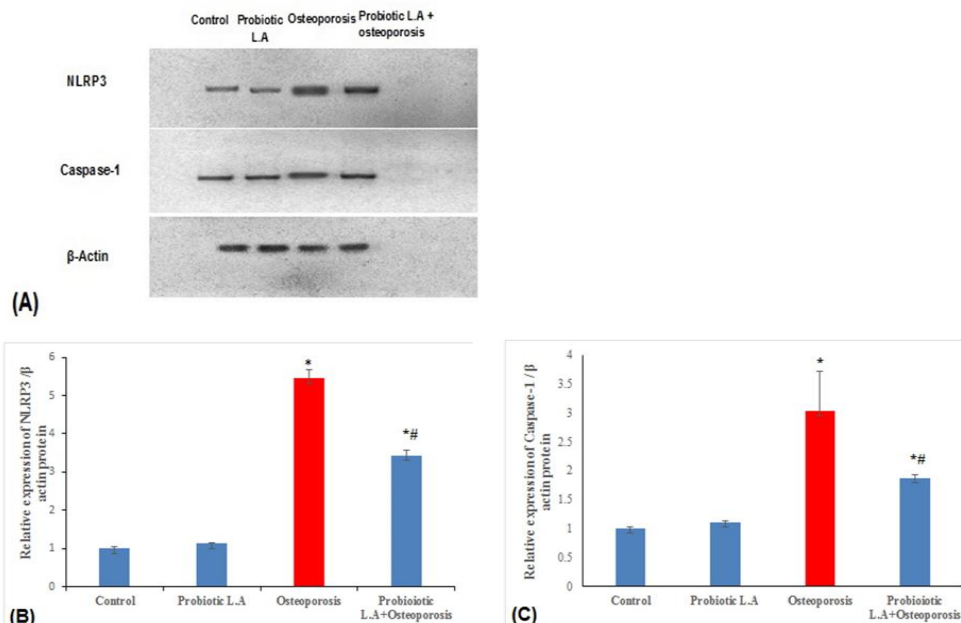


Figure (2)(A) Relative protein expression levels of NLRP3 inflammasome and Caspase-1 were analyzed by Western Blotting, **(B)** The ratio of NLRP3 inflammasome / β-actin expression in different experimental groups **(C)** The ratio of Caspase-1 / β-actin expression in different experimental groups, data are expressed as mean \pm standard deviation ($n = 8$). $P < 0.05$ is significant tested by using one-way analysis of variance (ANOVA) and post hoc multiple comparisons (LSD). * $P < 0.05$ vs. control group; # $P < 0.05$ vs. osteoporosis group.

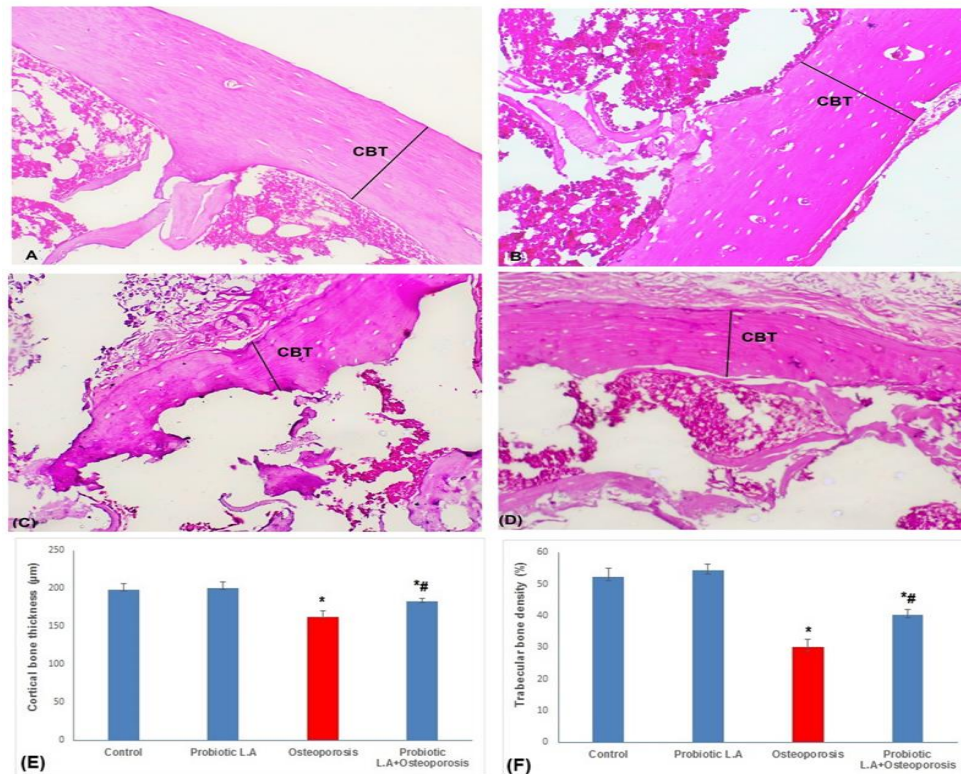


Figure (3) (A-D) Photomicrograph of bone tissue sections in the femur stained with (H&E), magnification $\times 100$, showing mean cortical bone thickness (CBT) and mean trabecular bone density (TBD)%. (A) Control group, (B) Probiotic L.A group, (C) Osteoporosis group, and (D) Probiotic L.A + Osteoporosis group. Histomorphometric analysis of (E) cortical bone thickness (CBT) (μm) and (F) trabecular bone density (TBD) %, data are expressed as mean \pm standard deviation ($n = 8$). $P < 0.05$ is significant tested by using one-way analysis of variance (ANOVA)

4. Discussion:

With the increasing elderly population, SOP is becoming a greater challenge for physicians and health care workers globally. SOP negatively impacts the quality of life by weakening the skeletal system making it more susceptible to fractures, which subsequently increases the mortality rate [21][22]. Therefore, prevention or slowing the progression of SOP is favorable for aged populations.

Probiotics being complementary therapies gained much attention especially since they present with fewer side effects and are more affordable compared to the major osteoporotic treatment modalities. Evidence has shown that dietary probiotic supplementation can slow bone resorption [10], however, possible underlying

mechanisms for such effects are not completely clarified. Our present study demonstrated the osteoprotective effects of probiotic L.A against D-gal induced SOP in adult male albino rats. Our results revealed that pretreatment with probiotic L.A in aged osteoporotic rats prevented osteoporosis development even in part via anti-inflammatory effect by reducing bone NLRP3 inflammasome and caspase-1 protein expression levels.

In this study, D-gal (a reducing sugar) intraperitoneal injection for 8 weeks resulted in SOP development in rats. Injection of D-gal in animals have been shown to accelerate aging, consistent with the symptoms of natural aging after a period of time [23]. The mechanisms underlying D-gal associated aging involve the accumulation

of reactive oxygen species (ROS), elevated DNA damage, as well as mitochondrial dysfunction[24][25]. Our results revealed that D-gal injection, resulted in oxidative stress state that was represented by significant higher serum MDA levels associated with lower serum levels of SOD in the D-gal injected group compared to the control one, moreover, DXA scan of the rats in D-gal group showed significantly lower BMD levels compared to the control rats. Previous studies reported that low BMD is a crucial sign for determining osteoporosis, and that the DXA scan is a current gold standard radiological examination used to confirm the diagnosis of osteoporosis[26][27]. Furthermore, the D-gal injected group showed bone tissue deterioration, disturbed bone architecture, and significant reduction of CBT and TBD% presented by histopathological examination, all these results indicate that oxidative stress induced aged osteoporosis model in rats was successful. Interestingly, our results are similar to that in other previous reports, Mahmoud et al. [17] and Wang et al. [28] who reported that D-gal injection resulted in a SOP rat and mice models respectively that mimic the human bone aging. Development of osteoporosis in aged rats could be attributed to the loss of balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption during aging (a process that favors more bone resorption and less bone formation)[29].

Our osteoporosis model was emphasized by a significant alteration in serum calcium. Our study showed higher serum calcium levels in the osteoporotic group in comparison to the control

one. Similarly, Mahmoud et al. [17] and El-baz et al. [30] also reported higher serum calcium levels in SOP rat model. Changes in serum calcium homeostasis, common in elderly, may be caused by the reduction in intestinal calcium absorption that comes with age as well as associated vitamin D deficiency that subsequently cause secondary hyperparathyroidism, activating osteoclasts and directly contributing to greater bone resorption[31]. The reduced calcium absorption during aging correlates with the reduced intestinal calbindin-D9k expression [32] and intestinal resistance to $1,25(\text{OH})_2\text{D}_3$ [33]. Moreover, vitamin D deficiency in aged rats was explained by increased renal CYP24A1, which restricts the amount of $1,25(\text{OH})_2\text{D}_3$ produced via speeding up the catabolism of $1,25(\text{OH})_2\text{D}_3$ [34][35].

Interestingly, our osteoporotic aged rat model revealed a significant affection in the two aspects of bone metabolism; bone formation and resorption that were detected by measuring bone turnover markers and comparing them to the control. A significant reduction in serum ALP and OCN associated with a significantly elevated serum TRAP was demonstrated. ALP and OCN reflected bone formation ability, ALP was useful as a marker for osteogenic differentiation, while OCN was associated with the osteoblast mineralization due to its affinity with hydroxyapatite after carboxylation [36]. TRAP; lysosomal protease enzyme values reflected the bone resorption ability of osteoclasts, being found abundantly within the ruffled border of the osteoclast, which results in the degradation and demineralization of the bone matrix especially type-1 collagen [37]. Coinciding with our study, Zhu et al. [38], Xu et al. [39] and Huang et

al.[40] who reported lower serum levels of ALP and OCN accompanied with higher serum TRAP levels in SOP models. In contrast to our results Mahmoud et al. [17] and El-baz et al.[30] demonstrated higher serum OCN in SOP rat model, their explanation was that OCN embedded in bone matrix also be released during bone resorption suggesting that serum OCN should be considered as a marker of bone resorption rather than bone formation.

Activation of OPG/RANKL/RANK signaling has gained much attention concerning osteoporosis, RANKL/RANK signaling was highlighted as the main factor of bone resorption [41], such activation could be prevented by the osteoprotegerin (OPG) produced by osteoblasts, it acts as a decoy receptor, it binds to RANKL, thus preventing the interaction between RANKL and its receptor RANK expressed on osteoclasts, so OPG is thought to be a key inhibitory player in bone resorption[42]. Our study revealed a reduction in serum OPG level accompanied by a significant increase in RANKL level in the osteoporotic group compared to the control one. Similarly, Huang et al.[40] and Wang et al. [43] stated higher levels of RANKL associated with lower levels of OPG in SOP models. Higher serum RANKL levels in our study may be explained in part by the higher levels of NLRP3 inflammasome with its subsequent mediator caspase-1 that were detected in the femora of osteoporotic group associated with higher significant serum levels of IL-1b, as it was reported that inflammation is involved in bone remodeling through pro-inflammatory cytokines which disturb the balance of the OPG/RANKL/RANK system[44]. Oxidative damage occurs during aging induces stress signals

was reported to enhance the NLRP3 inflammasome upregulation [45], and activation that causes the activation of its subsequent Caspase-1 and IL-1b [46]. IL-1b released via activated NLRP3 inflammasome stimulates RANKL expression in bone marrow mesenchymal stem cells and in osteoblasts [47]. Moreover, IL-1b binds its receptors on T lymphocytes, B lymphocytes, and macrophages, stimulating the generation of RANKL, thereby enhancing the bone resorption [48]. In line with our explanation, it was reported that blocking NLRP3 inflammasome with MCC950 (a potent inhibitor of the NLRP3 inflammasome) decreased alveolar bone loss in aged rats as there was a significant inhibition of RANKL-activated caspase-1 osteoclast differentiation [49].

Besides, high levels of IL-1b was reported to stop the osteogenic differentiation by activating NF- κ B [50], and to inhibit the osteoblastic differentiation by activating mitogen-activated protein kinase (MAPK) pathway [51]. This may in part explain the low levels of bone formation markers; ALP and OCN that were detected in our study. Thus, regarding the dual effects of NLRP3 inflammasome activation in the pathogenesis of osteoporosis, as it enhances bone resorption and reduces bone formation, its regulation may be an ideal target for management of osteoporosis.

Our present work explored that pretreatment of osteoporotic rats for eight weeks with probiotic L.A. The probiotic of our choice in the present study is one of the most recognized and commonly used species of probiotics[14]. It resulted in amelioration of the oxidative stress state as there was a significant reduction of serum MDA coupled with a significant elevation of

serum SOD in probiotic L.A+ osteoporosis group compared to osteoporotic one. Such antioxidant effect was reported previously in several models such as aging, ulcerative colitis and arthritis [52][53][54]. The antioxidant activity was attributed to their ROS radical scavenging ability and enhancement of Nrf2- HO-1 axis [55].

Interestingly, probiotic L.A significantly restored the BMD, improved the histological bone construction that was manifested by a significant elevation in the CBT and TBD% and lowered serum calcium in probiotic L.A+ osteoporosis group compared to osteoporotic group indicating an anti-osteoporotic activity of probiotic L.A. Our results are in line with Montazeri-Najafabady et al. [16] who found higher BMD in ovariectomized rats treated with L.A for four weeks. Also, Zhou et al. [56] who revealed that probiotic L.A pretreatment for eight weeks improved the BMD and increased the TBD% in dexamethasone induced osteoporosis rat model, they explained such improvement of BMD and bone microarchitecture by the increasing calcium absorption that contributed to the restoration of bone. Moreover, Dar et al. [14] described anti-osteoporotic effects of probiotic L.A given for six weeks following ovariectomy in postmenopausal osteoporosis mice model due to its immunomodulatory effect on host immune system that resulted in reducing the levels of osteoclastogenic cytokines indicating the role of probiotic L.A as a bone resorption inhibitor in the treatment of osteoporosis. In our study, the significant reduction of serum TRAP in the probiotic L.A+ osteoporosis group compared to osteoporotic group verified the inhibitory effect of probiotic L.A on osteoclasts and bone resorption

which being in line with Chen et al. [57] who revealed that L.A prevents the proliferation and differentiation of osteoclasts in osteoclast precursor cell line by regulating the expression levels of the genes controlling such processes. At the same time, serum ALP and OCN significantly were increased in the probiotic L.A+ osteoporosis group compared to osteoporotic group. This corresponds to the findings of Montazeri-Najafabady et al. [16] and Zhou et al. [56] who reported higher serum ALP and OCN respectively, in osteoporotic rat models that received L.A, indicating its ability to promote osteoblastic activity. Interestingly, L.A was reported to stimulate osteoblast proliferation, differentiation, and maturation via butyric acid in its metabolites in osteoblast precursor cell line, thereby improving bone quality [57]. Changes of our bone turnover markers suggested that probiotic L.A inhibits bone resorption while promoting bone formation.

The osteoprotective effect of the probiotic L.A informed in the present study was associated by lowering bone NLRP3 inflammasome with its subsequent mediators, caspase-1 and IL-1 β , that could attribute to the lower RANKL levels in the probiotic L.A + osteoporosis group. Several studies previously described that L.A reduced the expression of NLRP3 inflammasome signaling pathway in different organs such as jejunum, ileum and colon [58][15], such effect was explained by the inhibition at the mRNA level, moreover, the antioxidant effects of L.A could be involved in lowering NLRP3 inflammasome, since ROS was one of the main triggers of the NLRP3 inflammasome [15]. Interestingly, IL-1 receptor antagonist (anakinra) decreased serum RANKL

and reduced bone loss in arthritis rat model[59]. A clinical study revealed that anakinra was exhibited a resistance to bone resorption in postmenopausal women [60], which indicated the role of the downregulation of NLRP3 inflammasome with its subsequent mediators in the protection of bone. Besides, we also demonstrate that L.A could possibly improve the osteoporosis by upregulating the expression of OPG, which is known to inhibit osteoporosis, such increase indicates the ability of L.A to restore the balance between OPG/RANKL/RANK pathway.

5. Conclusion:

In conclusion, this study demonstrated the osteoprotective effect of L.A in a D-gal induced SOP rat model. Such effect was mediated in part via its anti-inflammatory effects that was represented by decreased NLRP3 inflammasome and its subsequent mediators caspase-1 and interleukin-1b that finally resulted in an increase of bone formation and a reduction of bone resorption.

Disclosure statement

The authors state that there is no conflict of interest.

Author contributions

All authors of this research paper have contributed equally in the planning, execution, or analysis of this study.

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