Effects of *Lactiplantibacillus plantarum* W1, inulin, and their combination against dexamethasone-induced osteoporosis via modulation of microRNA and gut microbiome

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Key words: probiotics; prebiotics; synbiotics; bone health; osteoporosis; glucocorticoids.

Contributions: HG acquired funding, provided resources, wrote the original draft, reviewed, edited the draft and managed project administration; AN developed and validated the methodology; YG conceptualized the research, acquired funding, reviewed and edited the draft, and prepared the microbiome figures; AE developed and validated the methodology, conducted formal analysis, curated data, and revised the microbiome analysis; RH developed and validated the methodology, conducted formal analysis, curated data, reviewed and edited the draft, and visualized data; YG and AE analyzed the microbiome data. All authors revised the final draft of the manuscript and approved the content.

Funding: this research project was funded by the Academy of Scientific Research and Technology, Egypt (Research grant of SNG: FRM-SGO-22).

Conflict of interest: the authors declare that they have no competing interests

Ethical approval and consent to participate: the study procedure was reviewed and approved by the Animal Ethics Committee of Beni-Suef University (approval number: 019-87 dated 13/02/2022).

Availability of data and materials: the data that support the findings of this study are not openly available pending permission from the funder, meanwhile, the data are available from the corresponding author upon reasonable request

Acknowledgments: the authors would like to thank the Academy of Scientific Research and Technology (ASRT), Egypt for funding the study. The authors also acknowledge the support of the faculty of Postgraduate Studies for Advanced Sciences (PSAS) and the faculty of Pharmacy at Beni-Suef University, Egypt, for helping to conduct the research and providing the required materials. Authors would like to thank Camilla Veronica Tafuro, Università degli Studi di Milano, Italy, for support in Microbiome analysis using MicrobiomePhylo.

Received: 4 May 2025.
Accepted: 26 September 2025.
Early view: 29 October 2025.

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Journal of Biological Research 2025; 98:13586
doi:10.4081/jbr.2025.13586

Abstract

Glucocorticoid-Induced Osteoporosis (GIO) is a common secondary form of osteoporosis characterized by rapid bone loss. While probiotics and prebiotics modulate the gut microbiota and influence host metabolism, their specific impact on GIO and associated molecular mechanisms remain unexplored. *Lactiplantibacillus plantarum* W1 (LpW1) represents a promising probiotic with reported protective effect against different diseases. In this study, seventy Wistar rats were assigned to seven groups (n=10) as follows: Control (CON), *L. plantarum* W1 alone (Lp), inulin alone (In), dexamethasone alone (DX), (DX+Lp), (DX+In), and (DX+Lp+In). Treatments lasted for 12 weeks. Bone morphology, serum calcium, phosphorus, alkaline phosphatase, bone histopathology, expression of miRNA-29b and miRNA-133, and gut microbiota diversity were assessed. The co-treatment groups (DX+Lp, DX+In, DX+Lp+In) showed improved bone health, reduced alkaline phosphatase activity, and enhanced serum mineral levels. Inulin significantly reduced miRNA-29b and miRNA-133 expression compared to the DX group. The Lp, In, and DX+Lp+In groups showed improved gut microbial profiles with enhanced alpha and beta diversity, compared to DX group. We conclude that inulin, LpW1, and their combination mitigated the skeletal abnormalities induced by DX treatment. Remarkably, Inulin alone demonstrated strong protective effects, and the synbiotic formulation reduced DX-induced osteoporosis.

Introduction

Bone is an active organ that regulates mobility, safeguards vital organs, maintains mineral balance, regulates bone metabolism and hemostasis, and contributes to the body's overall shape, strength, and structure.[1] Both osteoblasts, cells in charge of bone

formation, and osteoclasts, cells in charge of bone resorption, regulate the remodeling process of the bone.[1,2] Imbalances between osteoblasts and osteoclasts can lead to bone diseases.[3] Osteoporosis is an example of a bone disease marked with diminishing bone mass, and a higher chance of fractures in areas like the hip, wrist, and spine.[1,2] Glucocorticoids are potent immunomodulating and anti-inflammatory drugs.[4] They are used to treat conditions such as arthritis and inflammatory bowel diseases, and are also utilized as adjuvants in chemotherapeutic therapies.[4] However, glucocorticoid use is known to have significant adverse effects, including Glucocorticoid-Induced Osteoporosis (GIO). [4]

The gut microbiota encompasses all microorganisms residing in the intestine. It has an essential function in supporting host health, aiding in the modulation of the immune system, digestion of food, and defense against pathogens.[5] Recent studies have proposed a solid cause-effect relation between the gut microbiota and bone health, making it possible to modulate bone health via the gut microbiome.[6-9] *Lactiplantibacillus plantarum* W1 (LpW1) is a probiotic with proven benefits for gastrointestinal health, including inhibition of colon cancer, colitis, and diarrhea.[10,11] It improves gut function and barrier integrity[12] and also shows neuroprotective and cardiometabolic effects.[13,14] Despite these diverse applications, its potential role in GIO remains unexplored.

Prebiotics are beneficial, non-digestible food components that can be obtained naturally from plants or through synthetic processes via modifying carbohydrates with specific enzymes.[15] Prebiotics have been shown to enhance the growth and activity of the microbiome, potentially alleviating inflammatory conditions and influencing bone health and mineral metabolism.[15,16] Commonly effective prebiotics include inulin, fructooligosaccharides, glucooligosaccharides, and lactulose.[17] Studies have demonstrated that the use of synbiotics, combinations of beneficial bacteria (probiotics) and beneficial nutrients (prebiotics), can yield a synergistic effect.[16,18-20] The extent of this synergy may vary depending on the specific types of probiotics or prebiotics used.[16,18-20] Remarkably, the combination of *L. plantarum* with inulin has been proposed as a novel adjuvant therapy in type 2 diabetes[14]

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs comprising 18-22 nucleotides that play pivotal roles in pathogenesis, cell differentiation, and genetic control;[21] miRNAs can either activate or inhibit the transcription of target genes.[21] Remarkably, several miRNAs have been reported to regulate bone homeostasis, and their activity is closely tied to metabolism and bone development.[21-23] Among these miRNAs, miRNA-133 has been found to prevent osteogenesis *in vitro* and induce bone loss *in vivo*.[21, 23] A prior study examining miRNA-133 in bone marrow mesenchymal stem cells before and after osteoblast development revealed that its overexpression was inversely linked to osteoblast differentiation by targeting the Solute Carrier Family 39 member 1 (SLC39A1) mRNA.[21,23] This target is essential for promoting alkaline phosphatase (ALP) activity and mineralization.[21,23] In addition, miRNA-29b is another miRNA that inhibits fibrosis, promotes mineral deposition, and reduces the levels of Collagen type I, alpha 1(COL1A1), COL5A3, and COL4A2, and facilitates osteoblast differentiation. [21]

In the current study, we tested *L. plantarum* W1 (LpW1), inulin, and their combination (synbiotic) to protect against dexamethasone-induced osteoporosis in rats. We aimed to elucidate their effects on physical and biochemical bone parameters, bone histoarchitecture**,** miRNA-29b and miRNA-133 expression, and gut microbiome composition.

Materials and Methods

Materials

Dexamethasone disodium phosphate (Epidron® 4 mg/mL) was purchased from EPICO, 10th Ramadan City, Egypt. Inulin was purchased from Advent Chem Bio Pvt. LTD, Mumbai, India. DeMan, Rogosa, and Sharp (MRS) broth was purchased from Oxoid, Basingstoke, Hampshire, UK. *Lactiplantibacillus plantarum* W1 was obtained from Winclove Probiotics, Amsterdam, the Netherlands.

Animals

Seventy female Wistar rats were purchased from the animal breeding house at a Nahda University, Beni-Suef, Egypt. All animals were confirmed healthy at the start of the experiment and weighing an average of 200 g and 8–10 weeks old. No exclusion criteria were pre-established, and all data points were included in the analysis. The rats were randomly allocated to the experimental groups by a simple randomization method. The rats were kept in plastic cages with controlled standard environmental conditions, which included 12-12 hours of light and dark cycles, 25±2°C temperature, and 48±10% humidity. The rats were provided with an unlimited supply of water and food. The biochemical, histological and genetic analyses were performed by experimentally blinded researchers to the group identity.

All experimental procedures involving animals followed the guidelines for the care and use of experimental animals published by the National Institutes of Health (NIH), USA. The study procedure was reviewed and approved by the Animal Ethics Committee of Bnei-Suef University, Egypt (approval number: 019-87).

Bacterial culture and probiotic preparation

Lactiplantibacillus plantarum W1 underwent two consecutive cultures in MRS broth at 30°C for 48 hours. Subsequently, it was sub-cultured in MRS agar. One colony of *L. plantarum* W1 from the MRS agar was then reinoculated into MRS broth. Cells were harvested from the culture by centrifugation at 3000 × g for 10 minutes at 4°C. The bacterial cells were suspended in Phosphate Buffered Saline (PBS) and were adjusted to 2.4×10^9 colony forming unit (CFU)/mL.

Experimental design

Upon acclimatization for seven days, the rats were divided into 7 groups (n=10/group). The detailed treatments were as follows: i) CON (Control) group: Rats received 1 mL of Phosphate Buffered Saline (PBS) by oral gavage daily for 12 weeks; ii) Lp group: Rats received *Lactiplantibacillus plantarum* W1 at the dose of 2.4×10^9 CFU/mL suspended in PBS by oral gavage daily for 12 weeks; iii) In group: Rats received 1 mL of inulin dissolved in PBS at the dose of 400 mg/kg body weight (BW) by oral gavage daily for 12 weeks;[24] iv) DX group: Rats were injected intramuscularly with Epidron (dexamethasone disodium phosphate 4 mg/mL) at a dose of 7 mg/kg once a week for the last five weeks of the experimental period;[25,26] v) DX+Lp group: Rats received 1 mL of *L. plantarum* W1 at a dose of 2.4×10^9 CFU/mL suspended in PBS by oral gavage daily for 12 weeks, in addition to being intramuscularly injected with Epidron (dexamethasone disodium phosphate 4 mg/mL) at a dose of 7 mg/kg once a week for the last five weeks of the exper-

imental period;[25,26] vi) DX+In group: Rats received 1 mL of inulin dissolved in PBS at a dose of 400 mg/kg by oral gavage daily for 12 weeks,[24] along with intramuscular injection of Epidron (dexamethasone disodium phosphate 4 mg/mL) at a dose of 7 mg/kg body weight once a week for the last five weeks of the experimental period;[25,26] vii) DX+Lp+In group: Rats received 1 mL of *L. plantarum* W1 at a dose of 2.4×10^9 CFU/mL suspended in PBS by oral gavage daily for 12 weeks, along with 1 mL of inulin dissolved in PBS at a dose of 400 mg/kg by oral gavage daily for 12 weeks.[24] Additionally, rats in this group were intramuscularly injected with Epidron (dexamethasone disodium phosphate 4 mg/mL) at a dose of 7 mg/kg once a week for the last five weeks of the experimental period.[25,26]

At the end of the experiment, animal feces were collected and stored at -80°C for microbiota investigation. Rats fasted overnight, and blood samples were obtained from the eye's optic retro orbital sinus, left to coagulate for 30 minutes at 37°C, and then centrifuged at 3000 × g for 10 minutes. The resulting supernatants were collected and stored at -80°C for subsequent biochemical analysis. Rats were then anaesthetized using diethyl ether before sacrificing by cervical decapitation. The femur bones were removed and washed with isotonic saline and allocated as follows: the left femur bone's wet weight and length were measured; then they were fixed in 10% formalin-buffered saline for histopathological analysis, and the right femur was preserved at -80°C for genetic analysis.

Biochemical analysis

Calcium, phosphorus, and ALP activities were assessed in the serum samples using commercial kits from Spinreact, Sant Esteve de Bas, Spain.

Histopathology examination

The left femur of each rat was carefully extracted, precisely cleaned, and promptly fixed in a preservation solution (10% neutral-buffered formalin). Subsequently, the femur was immersed in 10% ethylenediaminetetraacetic acid (EDTA) (pH 7.4) for three weeks for decalcification, with solution changes every 2–3 days. Following decalcification, bones were dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin. Sections (4 µm thickness), that were fixed onto glass slides, were then cut and stained with hematoxylin and eosin. The investigator who carried out the histological examination and scoring was blinded to the experimental groups. All histological assessments were performed on the distal metaphyseal region of the femur to ensure consistency across samples.

Expression analysis of miRNA-29b and miRNA-133 using SYBR Green quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Bone processing

Every bone sample was fragmented into small pieces and transferred from storage at -80°C to liquid nitrogen. Subsequently, the femurs were cut using diagonal pliers. After the bone was placed in a rack of liquid nitrogen and cooled, one mL of Tri-Reagent, (Molecular Research Center, Inc., Cincinnati, OH, USA). was added, after homogenizing and centrifuging the bone for five minutes to allow the separation the solubilized bone extract from the beads and crushed bone material, which required fifteen seconds of centrifugation at 8,600 × g at room temperature.

Total RNA extraction

RNeasy Plus Micro Kit (ID. 74034; QIAGEN, Hilden, Germany) was used to extract total RNA from samples after centrifugation, according to the manufacturer's guidelines. Briefly, 150 µL of each sample underwent RNA extraction, yielding 100 µL of RNA extraction solution.

cDNA synthesis

Five µL of RNA was reverse transcribed for cDNA synthesis using the ReverAid RT Kit (Thermo Fisher, Massachusetts, United States) following the manufacturer's instructions. Reverse transcriptase was activated by incubating the RNAs for 10 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 85°C. The reaction conditions included 10 U of RNase inhibitor, 1 µL of RT-Sensiscript, 5 µL of template-RNA, 1 µM random primer, 0.5 mM concentrations of each deoxynucleoside triphosphate, 1× RT-RevertAid buffer; the final reaction volume was 20 µL.

Quantitative RT-PCR

Qiagen Rotor-Gene Q real-time PCR was used to measure the expression of miRNA-133 and miRNA-29bMiRNA-U6 was employed as a housekeeping gene control. The reaction mixture's final volume was 20 µL, consisting of the cDNA template and SYBR Green Master Mix (Qiagen, Valencia, CA, USA). The PCR program began with an initial denaturation at 94°C for 5 minutes, followed by 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds. Each experiment was performed in triplicate as previously mentioned in Li *et al*. [27] The following primers were used:

hsa-miRNA-29b: forward primer 5′-UAGCACCAUUUGAAAUCAGUGUU-3′, reverse primer 5′-CACUGAUUUCAAAUGGUGCUAUU-3′.
hsa-miRNA-133: forward primer 5′-CCGGGTTTGGTCCCCTTCAAC-3′, reverse primer 5′-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCAGCTG-3′.
miRNA-U6: forward primer 5′-CGCTTCGGCAGCACATATACTA-3′, reverse primer 5′-CGCTTCACGAATTTGCGTGTCA-3′.

Cycle threshold (Ct) values were recorded, which indicate the number of cycles required for the fluorescent signal in RT-PCR to achieve the threshold. The Ct values of the target miRNAs were subtracted from the Ct values of miRNA-U6 to obtain the ΔCt value, which was then subtracted from the ΔCt of the control samples to calculate ΔΔCt values. The $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative quantitative levels of miRNAs.

Microbiome analysis

DNA extraction

Fecal pellets were collected from each animal at the end of the experiment and stored in a -80°C freezer ready for DNA extraction. The DNA extraction was done using QIAamp® Fast DNA stool kit (Qiagen, Hilden, Germany). Quantification and quality checks were performed using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) to ensure successful extraction.

Library preparation, 16S rRNA amplification and Illumina sequencing

The DNA amplification and the subsequent DNA sequencing process were done by Dalhousie University (Halifax, Canada) Integrated Microbiome Resource (IMR). To amplify the bacteria 16s rRNA gene, the primers 341F (5'- CCTACGGGNGGCWGCAG -3') and 805R (5'- GACTACHVGGGTATCTAATCC -3') were used, that are targeting the V3-V4 in the DNA samples. The paired-end 300 bp sequencing on an Illumina MiSeq platform was applied, and the amplicons were sequenced.

The multiplexed samples were treated to equal volumes of 16S fusion primers; Illumina Nextera adapters and barcodes were integrated into the fusion primers for dual labeling at both ends of the amplicons. The reaction mixture's final volume was 25 µL and was composed of 5 µL forward and 5 µL reverse primer (1 µM), 5 µL of High Fidelity (HF) buffer, 0.5 µL of Deoxynucleotide Triphosphates (dNTPs) (40 mM), 0.25 µL Phusion polymerase (2 U/µL), 2 µL template, and 7.25 µL water; all reagents were from Thermo Fisher Scientific, Waltham, MA, United States. The PCR reaction started with an initial step of denaturation (98°C for 30 s), and then 30 cycles of 98°C (10 s), 55°C (30 s), and 72°C (30 s). At 72°C, the last extension lasted 4.5-6 minutes. After combining the samples and negative controls to produce a single library, the Illumina MiSeq platform (Illumina, San Diego, CA, USA) was used to process the samples using 2 × 300 bp Pair-End v3 chemistry, following the guidelines provided by the manufacturer.

Bioinformatic analysis

Microbiome analysis utilized the web platform Microbiomephylo.com, tailored explicitly for 16S rRNA microbiome analysis.[28] Supplementary to this platform is the qiime2_pipeline_wrapper.py script (provided upon reasonable request from the corresponding author), designed to process sequence all-quality files (FASTQ) from sequencing providers. The script generated the necessary files for the further analysis using the software Quantitative Insights Into Microbial Ecology 2 (QIIME2).[29] The script is optimized for handling paired-end reads targeting the V3V4 hypervariable regions of the 16S rRNA gene, sequenced using Illumina MiSeq technology kit v3 (Illumina, San Diego, CA, United States). It integrates functions for merging paired-end reads, conducting quality control, denoising with Divisive Amplicon Denoising Algorithm 2 (DADA2)[30], and taxonomic classification. Raw paired-end 16S rRNA gene sequencing data (FASTQ files) were initially imported into QIIME 2 (version 2024.5). The imported sequences were demultiplexed and summarized to assess data quality and quantity. Paired-end reads were merged using the "vsearch" plugin with the default parameters. Subsequently, reads were denoised using the DADA2 plugin, with a truncation length set to 300 bases to remove low-quality tails, generating a feature table and representative sequences. A phylogenetic tree was constructed from the representative sequences using the "align-to-tree-mafft-fasttree" pipeline. Taxonomic classification was conducted using a pre-trained classifier from the Greengenes 13_8 database. Specifically, the Weighted Greengenes 13_8 99% Operational Taxonomic Units (OTUs) full-length sequences classifier (".qza" file) was downloaded from the Weighted Taxonomic Classifiers section, and the corresponding Greengenes (16S rRNA) 13_8 reference database (".tar" file) was retrieved from the Marker Gene Reference Databases section. These files were placed in the same folder as "qiime2_pipeline_wrapper.py". Taxonomic classification was performed using the "classify-hybrid-vsearch-sklearn" method, leveraging the reference OTUs and taxonomy data to assign taxonomy to the representative sequences.

Unless specified otherwise, all analyses were conducted with default settings, and outputs were saved for downstream analysis. Visualization of the artifacts produced by QIIME 2 and statistical analyses was performed using the MicrobiomePhylo web application. This application is based on several visualization and statistical analysis packages, such as Phyloseq.[31] The analysis process started with the QIIME 2 artifacts produced by the wrapper script ("filtered_seqs.qza", "dada2_feature_table.qza", and "rooted_tree.qza") and the metadata file describing the sample criteria. The analytical workflow included rarefaction abundance analysis, correlation analysis, alpha diversity analysis, beta diversity analysis, statistical tests, and effect size analysis. A Wilcoxon test was performed to assess the significance of differences in abundance between groups for the top four taxa. Kruskal-Wallis tests were conducted to identify differences in alpha diversity metrics across groups, followed by Dunn tests for post-hoc analysis. Effect sizes were calculated to quantify the magnitude of observed differences. One-way Analysis Of Variance (ANOVA) tests for dispersions and permutation tests for pairwise comparisons, followed by a Tukey test, were conducted to explore beta diversity further.

Data analysis

The data were statistically analyzed with GraphPad Prism 10.1.2. The statistical tests of ANOVA and Tukey's multiple comparisons were used for group comparison. The results were shown as mean ± Standard Error (SE), with significance levels set at $p<0.05$.

Results

Effect on physical bone parameters

The DX group showed a significant decrease in femur length compared to the CON group. However, the DX+Lp, DX+In, and DX+Lp+In groups demonstrated a highly significant increase in femur length compared to the DX group ($p<0.01$, Table 1). The femur weight showed no significant difference between the DX and DX+Lp groups. In contrast, the femur weight showed a highly significant increase in the DX+In group compared to the DX group ($p<0.01$). Moreover, there was a highly significant increase in femur weight in the DX+Lp+In group compared to both the DX and CON groups ($p<0.01$, Table 1). Throughout the experimental period, no abnormal behavior, mortality, or visible signs of distress were observed in any of the treatment groups, indicating that LpW1, inulin, and their combination were well tolerated.

Effect on biochemical parameters

The DX and DX+Lp groups showed significantly higher ALP activities than the CON group ($p<0.01$). However, the ALP levels were significantly reduced in the DX+In and DX+Lp+In groups compared to the DX group but still significantly increased compared to the CON group ($p<0.05$). However, the DX+Lp group exhibited a non-significant reduction in ALP compared to the DX group (Table 1). Calcium levels in the DX group were significantly decreased compared to the CON group. However, calcium levels showed a highly significant increase in the DX+Lp, DX+In, and DX+Lp+In groups compared to the DX group, and they did not differ significantly from the CON group ($p<0.01$, Table 1).

The phosphorus levels did not vary significantly between the DX and CON groups. A significant increase was observed in the In

Table 1. Effect of treatments on the physical and chemical bone parameters.

Group	Femur length (cm)	Femur weight (g)	Serum alkaline phosphatase (U/L)	Serum calcium (mg/dL)	Serum phosphorus (mg/dL)
CON	29.50±0.68	0.84±0.06	195.28±13.70	13.64±0.65	5.61±0.27
Lp	30.81±0.54	0.92±0.03	175.82±9.96	14.17±0.77	5.71±0.60
In	30.20±0.36	0.96±0.03	232.30±16.84	14.91±0.89	7.39±0.63[a]
DX	26.12±0.29[a]	0.73±0.01	412.63±30.93[a]	11.67±0.43[a]	4.61±0.28
DX+Lp	29.11±0.22[b]	0.83±0.04	333.52±22.60[a]	16.28±0.70[b]	9.03±0.21[a,b]
DX+In	30.10±0.54[b]	0.91±0.46[b]	285.71±40.55[a,b]	14.67±0.32[b]	5.36±0.40
DX+Lp+In	29.63±0.77[a,b]	1.06±0.04[a,b]	275.96±29.36[a,b]	15.00±0.38[b]	4.48±0.33

Data are expressed as mean±standard error (SE). CON, control; Lp, *L. plantarum* W1; In, Inulin; DX, Dexamethasone; DX+Lp, Dexamethasone + *L. plantarum*; DX+In, Dexamethasone + Inulin; DX+Lp+In, Dexamethasone + *L. plantarum* W1 + Inulin. [a]Significant difference ($p<0.05$) compared to the CON group; [b]significant difference ($p<0.05$) compared to the DX group.

group compared to the CON group, and a highly significant increase was noted in the DX+Lp group compared to the DX group or the CON group (Table 1).

Effect on bone histoarchitecture

The hematoxylin and eosin-stained sections of femur bones from the CON rats revealed no osteoporotic lesions and a typical normal histological structure of the condyle cartilaginous surface, bone marrow, as well as the cortical and bony trabecular lamellae. In contrast, the DX group exhibited marked histopathological changes characterized by thinning in the cortical and trabecular bones, dilation of the bone marrow cavity, and increased osteoclastic activity. However, rats treated with inulin (In group) and those treated with LpW1 (Lp group) showed no histopathological changes in the femurs. Interestingly, the DX+In group displayed a marked improvement in osteoporotic pathology compared to the group of DX+Lp, which showed a moderate improvement. Meanwhile, the DX+Lp+In group revealed no features of osteoporotic pathology (Figure 1A). Quantification of the femur cortical thickness showed that all treatment groups (DX+Lp, DX+In, DX+Lp+In) exhibited a significant increase in the femur cortical thickness when compared to the DX group. ($p<0.05$, Figure 1B).

Effect on miRNA-29b and miRNA-133 expression

The DX and DX+Lp groups showed significant increases in miRNA-29b expression when compared to the CON group. However, miRNA-29b levels exhibited a significant decrease in both DX+In and DX+Lp+In groups compared to the DX group ($p<0.01$, Figure 2).

There was a significant increase in miRNA-133 expression in the Lp, DX, and DX+Lp groups when compared to the CON group. There was a significant decrease in miRNA-133 expression observed in the DX+In group when compared to the DX group. No other significant differences were detected among groups regarding miRNA-133 expression (Figure 2).

Microbiome analysis

Determination of the alpha diversity metrics for the microbial communities

To evaluate the effect of treatments on gut microbial diversity, sequencing data were analyzed for alpha (α)-diversity. *Supplementary Figure S1* shows the curves of species richness in relation to sequencing depth and the overall library sizes across the groups. The α-diversity analysis revealed a reduction in gut microbiota diversity in animals treated with dexamethasone (DX group) (Figure 3). The calculated α-diversity metrics included observed species, Chao1, Shannon, and Simpson indices. The data showed improvement in the α-diversity metrics in the (In) and DX+Lp+In groups compared to both the DX groups. Administration of *L. plantarum* W1, inulin, or their combination appeared to counteract the dexamethasone-induced reduction in gut microbial diversity. The measured microbiome alpha diversity metrics showed differences among the tested groups; however, they did not reach significant levels based on samples tested in our experiments.

4.2. Microbiome beta diversity analysis

The beta diversity analysis, visualized through Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) based on weighted UniFrac, Jaccard, and Jaccard NMDS metrics, reveals distinct clustering patterns among the different treatment groups (Figure 4). In the weighted UniFrac PCoA plot (Fig 4A), the DX group forms a separate cluster, indicating differences in microbial community composition compared to the In, DX+In, and DX+Lp+In groups. This suggests that the treatments with inulin and the combination of dexamethasone, LpW1, and inulin altered the gut microbiota composition. The Jaccard PCoA plot shows separation between the DX, In and DX+In groups (Figure 4B). Similarly, the Jaccard NMDS plot shows distinct clustering of the DX, In, and DX+In groups (Figure 4C).

Discussion

Administration of glucocorticoids is the most prevalent secondary cause for osteoporosis, which raises the risk of fractures and morbidity rates in a dose-dependent manner.[4] Osteoporosis is treated with a variety of drugs, including hormone replacement therapy and bisphosphonates.[32] Nevertheless, adverse effects from these medications include ovarian and breast cancer as well as jaw osteonecrosis.[33] Therefore, new treatments for osteoporosis are desperately needed.[34] The gut microbiota may be a target for osteoporosis treatment as recent research suggested its link with bone health.[5,8,35] In this study, the effects of a probiotic (*L. plantarum* W1), a prebiotic (inulin), and a synbiotic (combination) on the enrichment of gut microbiome bacteria and the prevention of GIO were assessed.

The present findings revealed that rats treated with DX experi-

enced a significant reduction in the femur length and exhibited notable histopathological alterations confirming osteoporosis induction. Furthermore, the DX group demonstrated a significant elevation in ALP activity but a significant decrease in serum calcium concentration compared to the CON group. It was previously noted that elevated ALP activity increased bone turnover and fracture risk.[36] Our findings are consistent with a previous research demonstrating that glucocorticoids primarily affect bone by promoting marrow adiposity, suppressing osteoblast development, and increasing osteocyte and osteoblast death.[2] The excessive administration of glucocorticoids has negative impacts on both muscle mass and function, resulting in myopathy and a higher chance of falling and fractures.[4]

Our findings indicated that the groups DX+Lp, DX+In, and DX+Lp+In showed significant improvements in physical (femur weight and length), biochemical markers, and histological characteristics of bone. For instance, the DX+Lp group showed significantly increased phosphorus concentration compared to the DX

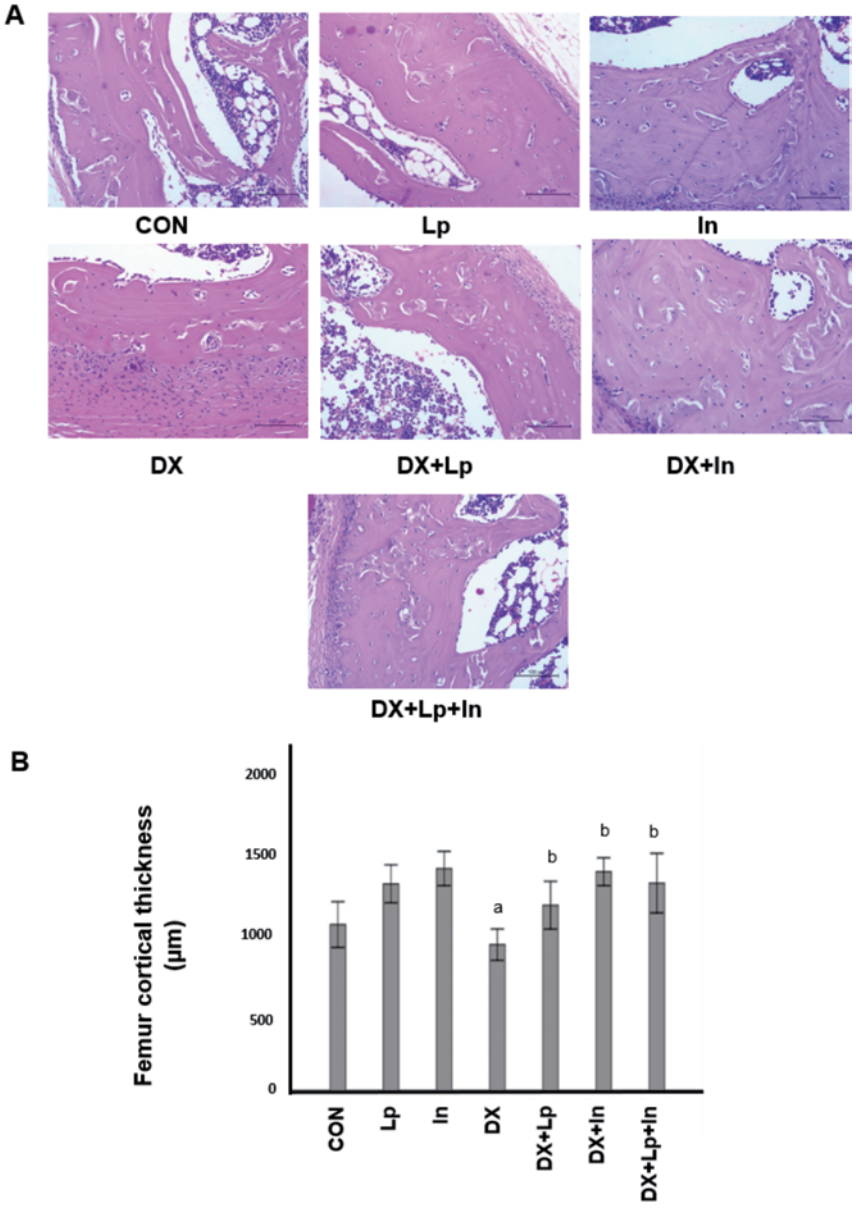

Figure 1. Histopathological analysis of bone tissues. A) Photomicrographs of hematoxylin and eosin-stained bone tissues revealed normal femur structure in the CON rats, while the DX group rats exhibited significant abnormalities. Treatment with In or Lp showed no histopathological changes. The group of DX+Lp+In demonstrated complete prevention of the osteoporotic features; B) Quantification of the femur cortical thickness among groups. Data are displayed as mean±Standard Error (SE); a indicates a significant difference ($p<0.05$) compared to the CON group, and b indicates a significant difference ($p<0.05$) compared to the DX group. Control (CON), *L. plantarum* W1 (Lp), Inulin (In), Dexamethasone (DX), Dexamethasone + *L. plantarum* W1 (DX+Lp), Dexamethasone + Inulin (DX+In), and Dexamethasone + *L. plantarum* W1 + Inulin (DX+Lp+In).

group. In addition, the DX+In and the DX+Lp+In groups demonstrated a reduction in ALP along with an increase in Ca concentration. Notably, Calcium is the primary mineral for healthy bones where severe skeletal abnormalities are caused by calcium deficiency.[37] Our results align with a previous research suggesting that inulin may enhance immune function, bone mineral density, and calcium absorption by acidifying the intestinal lumen after producing short-chain fatty acids, thus increasing calcium solubility.[17] In

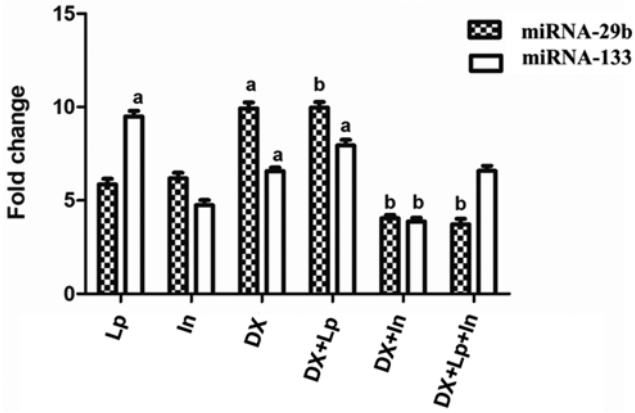

Figure 2. Effect of treatments on miRNA-29b and miRNA-133 expression among groups. Fold change of miRNA-29b and miRNA-133 expression relative to the control (CON) group. The data are plotted as mean±Standard Error (SE); a denotes a significant difference ($p<0.05$) compared to the CON group and b denotes a significant difference compared to the DX group. *L. plantarum* W1 (Lp), Inulin (In), Dexamethasone (DX), Dexamethasone + *L. plantarum* W1 (DX+Lp), Dexamethasone + Inulin (DX+In), and Dexamethasone + *L. plantarum* W1 + Inulin (DX+Lp+In).

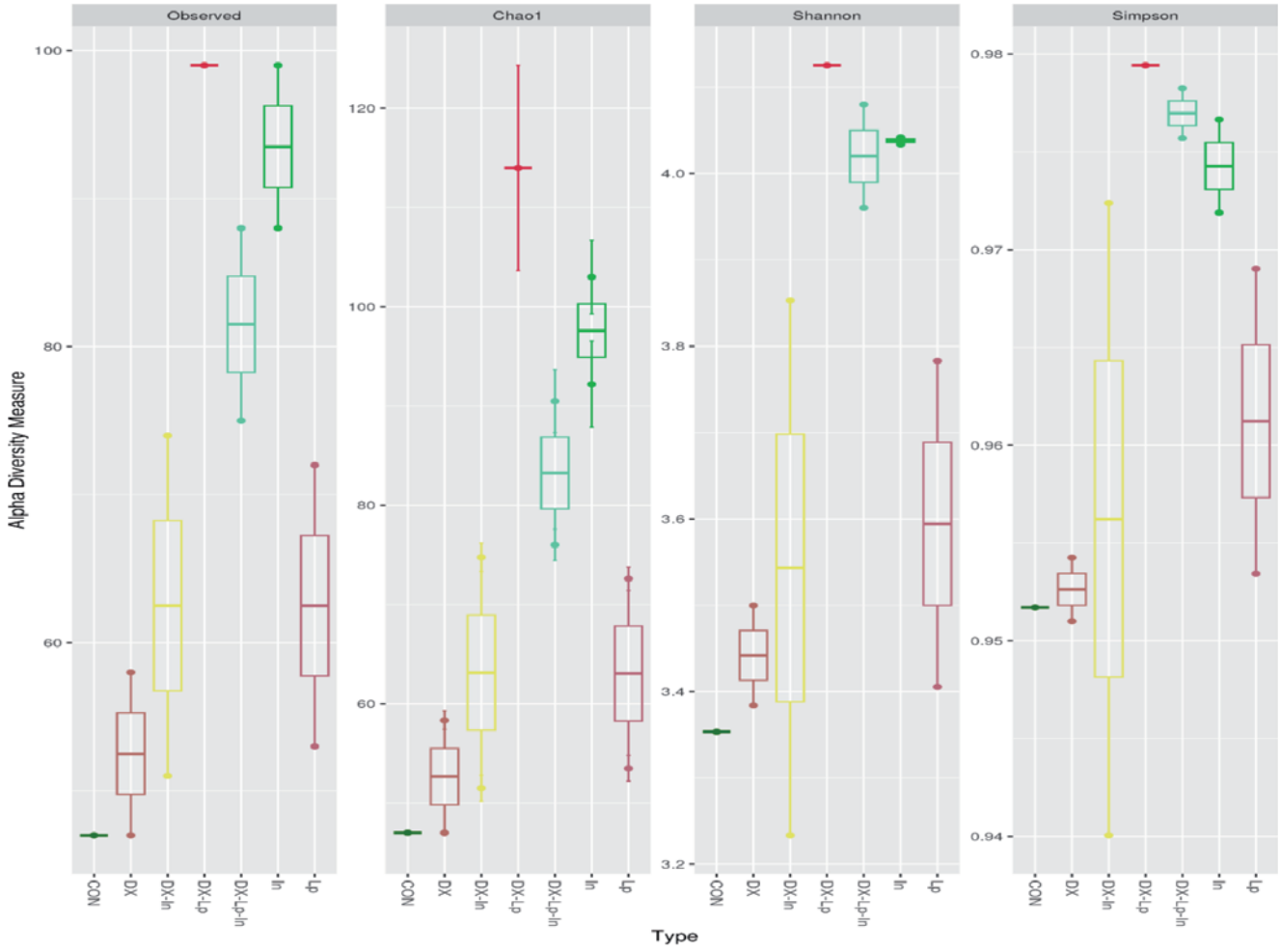

Figure 3. Alpha-diversity metrics of the microbial communities extracted from the seven animal groups. The α-diversity was assessed using the metrics: observed species, Chao1, Shannon, and Simpson indices. Control (CON), *L. plantarum* W1 (Lp), Inulin (In), Dexamethasone (DX), Dexamethasone + *L. plantarum* W1 (DX+Lp), Dexamethasone + Inulin (DX+In), and Dexamethasone + *L. plantarum* W1 + Inulin (DX+Lp+In).

addition, inulin promotes the enlargement of the intestinal surface area available for absorption, leading to elevated calcium-binding protein expression, particularly in the large intestine. Furthermore, stimulating beneficial microorganisms in the gut promotes greater calcium uptake by intestinal cells.[5-9] Earlier studies also highlighted the positive impact of probiotics, including *L. reuteri*, *L. helveticus*, *L. acidophilus*, *L. paracasei*, *L. casei*, *L. plantarum*, on bone health through modulating the immune system and improving mineral absorption, leading to enhanced osteogenesis.[22,35,38-41]

Remarkably, the DX+In group exhibited reduced expression of miRNA-133 compared to the DX group. Nevertheless, the miRNA-29b expression was reduced after the Inulin or synbiotic treatments along with DX. The alteration in the expression levels of miRNAs is linked to bone metabolism and can contribute to conditions such as osteoporosis.[42] Specifically, miRNA-133 serves as a negative regulatory factor for the Runt-related transcription factor 2 (Runx2)/Bone morphogenetic protein 2 (BMP2) axis, therefore inhibiting the osteogenesis process.[42] Our results are compatible with the previous study and confirm the beneficial effect of inulin administration along with DX.

Previous studies established the significant impact of the gut microbiome on various physiological and pathological processes, including its association with osteoporosis. The beneficial gut microbiome can modulate the immune system, host metabolism, and hormone secretion which altogether are associated with bone health.[5-9] The current results demonstrate the positive influence of *L. plantarum* W1, inulin and their combination on enhancing the beneficial gut microbiota. This effect has been demonstrated via the increased alpha diversity in the groups In and DX+Lp+In compared to the DX group. The relation of microbial diversity in the intestine to the beneficial health outcomes in the host was previously proven.[43] Notably, our library size demonstrates that the DX+Lp group showed the highest species richness, followed by the In group. Furthermore, a reasonably high species richness was shown by the DX+Lp+In and DX+In groups. Beta diversity analyses showed that DX samples clustered separately from In,

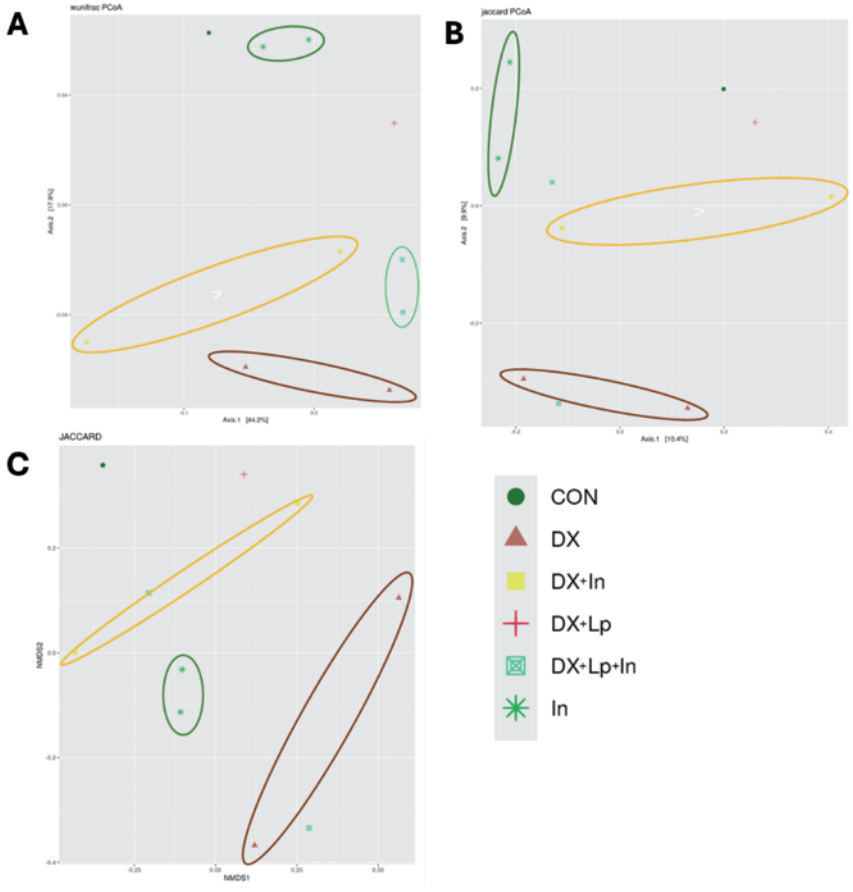

Figure 4. Beta diversity analysis of the tested animal groups Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) plots based on weighted UniFrac and Jaccard metrics illustrate the beta diversity among the different experimental groups. Each symbol represents an individual sample: circles for CON (control), triangles for DX (dexamethasone), squares for DX+In (dexamethasone + inulin), crosses for DX+Lp (dexamethasone + LpW1), hexagons for DX+Lp+In (dexamethasone+ LpW1+ inulin), and stars for In (inulin). A) Weighted UniFrac PCoA plot shows distinct clustering patterns, with the DX group forming a separate cluster from the CON group. B) Jaccard PCoA plot reveals separation between the DX group and other treatment groups, with the DX+Lp group clustering closely with the CON group. C) Jaccard NMDS plot shows clustering of the DX, DX+In, and DX+Lp+In groups, while the CON and DX+Lp groups form separate clusters. Control (CON), *L. plantarum* W1 (Lp), Inulin (In), Dexamethasone (DX), Dexamethasone + *L. plantarum* W1 (DX+Lp), Dexamethasone + Inulin (DX+In), and Dexamethasone + *L. plantarum* W1 + Inulin (DX+Lp+In).

DX+In, and DX+Lp+In groups, suggesting that inulin and the synbiotic treatment with LpW1 and inulin restored healthy microbial community structure away from the dysbiosis induced by DX. These results demonstrate the influence of treatments and experimental settings on microbiome richness. The current microbiome analysis encountered some limitations that relate to the sample size tested per each group, however, the overall conclusion of the beneficial effect of using LpW1, inulin or both are still valid. In the current study, while femur cortical thickness was quantified, future studies should include histomorphometric scoring of osteoblast and osteoclast populations to enhance cellular-level resolution.

Conclusions

Our study indicates that *Lactiplantibacillus plantarum* W1, inulin, and their combination exert a protective effect against dexamethasone-induced osteoporosis in rats via their effect on bone physical, histological, and biochemical parameters such as ALP, calcium and phosphorus levels. Moreover, they demonstrate the ability to modulate the gut microbiome towards the healthy status as well as affecting the miRNA29b and miRNA-133 expression in bones. Inulin seems to be a very attractive option in the prevention of dexamethasone-induced osteoporosis. Further investigations are required to explore the detailed mechanistic impacts of probiotics, prebiotics, and synbiotics in modulating the pathogenesis of osteoporosis, encompassing various strains and administration approaches.

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Online supplementary material:

Figure S1. Library Size and Microbiome Analysis Overview. CON (control), DX (dexamethasone), DX-Lp (dexamethasone+LpW1), DX-In (dexamethasone + inulin), and DX-Lp-In (dexamethasone + LpW1+inulin). Suffixes either a or b denote samples inside the group. (A) The library size overview shows read counts obtained for each group. For the DX+Lp group, sequencing resulted in only one successful sample. (B) The rarefaction curve illustrates species richness (y-axis) versus sequence sample size, indicating that the top group samples with the highest species richness (>65) are DX+Lp (one sample), inulin group (two samples), DX+Lp+In (two samples), DX+In (one sample), and Lp (one sample). Additionally, the two samples from the DX group showed an average species richness of 53. (C) The overview of the microbiome structure of the seven groups studied is represented by major phyla, with Bacteroidetes, Firmicutes, and Proteobacteria being predominant. TM7 refers to Saccharibacteria, a rare or uncultured phylum, NA refers unclassified bacterial sequences.