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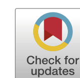
Gut-joint axis: Oral Probiotic ameliorates Osteoarthritis



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ABSTRACT

Osteoarthritis (OA) etiology is multifactorial, and its prevalence is growing globally. The Gut microbiota shapes our immune system and impacts all aspects of health and disease. The idea of utilizing probiotics to treat different conditions prevails. Concerning musculoskeletal illness and health, current data lack the link to understand the interactions between the host and microbiome. We report that *S. thermophilus, L. pentosus* (as probiotics), and γ-aminobutyric acid (GABA) harbour against osteoarthritis in vivo and alleviate IL-1β induced changes in chondrocytes in vitro. We examined the increased GABA concentration in mice's serum and small intestine content followed by bacterial treatment. The treatment inhibited the catabolism of cartilage and rescued mice joints from degradation. Furthermore, the anabolic markers upregulated and decreased inflammatory markers in mice knee joints and chondrocytes. This study is the first to represent GABA's chondrogenic and chondroprotective effects on joints and human chondrocytes. This data provides a foundation for future studies to elucidate the role of GABA in regulating chondrocyte cell proliferation. These findings opened future horizons to understanding the gut-joint axis and OA treatment. Thus, probiotic/GABA therapy shields OA joints in mice and could at least serve as adjuvant therapy to treat osteoarthritis.
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1. Introduction

Osteoarthritis (OA) is an age-related, heterogeneous group of disorders, pathologically characterized as pivotal area loss of cartilage in diarthrosis, subchondral bone changes, synovitis, and varying degrees of osteophyte formation.[1] In 2019, the OA was the 15th highest cause of disability worldwide, and the number of affected people has risen globally by 48% since 1990, with 500 million affected population.[2] Current pain treatments have limited efficacy with toxic effects. Regulatory agencies permit no modifying therapy to address the increasing burden of OA.[3,4] OA is multifactorial, and one treatment is insufficient for uniformly and universally effective. Cellular inflammatory and humoral mechanisms, which play an essential role in the pathogenesis of OA, are critical to the development of pain through interactions between the immune and nervous systems. Identifying therapies targeting inflammation could slow or prevent OA progression and development is an unmet need for time.[5]

The Gut microbiome (GM) contributes to the health and disease of the host and modifies the different therapeutic drugs.[6,7] Studies have shown that the cartilage deterioration during the onset of OA was rescued by gut microbiota and probiotics.[8–13] Based on the gut–joint axis, a method of inhibiting chronic or systemic low-grade inflammation by changing gut microbiota is predicted as a new nutraceutical treatment for osteoarthritis.[3,14,15]

GM emerged as a vital part of the precision medicine approach

Abbreviations: OA, Osteoarthritis; GM, Gut microbiome; GABA, γ-aminobutyric acid; TCA, tricarboxylic acid cycle; ABAT, 4-aminobutyrate aminotransferase; LAB, Lactobacillus strains; CM, Conditioned media; ST, conditioned media S. thermophilus; LP, conditioned media L. pentosus; GABABr, GABA B receptor; SO, Safranin O; ECM, Extracellular matrix; MMPs, Matrix metalloproteinases; ACAN, Aggrecan; ACLT, Anterior cruciate ligament transection.

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but is possibly modifiable by therapeutics; probiotics are one of them.[7,16,17] Researchers around the globe extensively studied probiotics to deliver health benefits to the host by microbial shift or by producing postbiotics/gut metabolites.[18,19] The bioactive molecules metabolized by different bacteria within the human body is called postbiotics, which could protect against several human diseases.[19–21] Recently, the idea of using postbiotics to treat diseases has prevailed, and extensive research is needed to understand it deeply.[22,23] Recognizing gut metabolites as participants in osteoarthritis pathogenesis suggests interventions aimed at "drugging the microbiome" may serve as a therapeutic approach for the treatment.

The GM modulates host γ-aminobutyric acid (GABA) levels and influences the host immune system.[24,25] GABA is one of the compounds produced by bacteria, which act as a potential mediator between host and gut microbiota.[26] Germ-free mice have decreased GABA levels in blood serum.[27] In a mice study decreased GABA level were observed upon antibiotic exposure.[28] In particular, GM modulates abdominal pain and visceral sensitivity via GABA production.[29] Increased GABA levels exhibited in the human plasma on transplanting a fecal microbiome.[25] Several bacteria and fungi produce GABA, among which lactic acid bacterial strains (LAB) are more prominent.[30,31] *Streptococcus thermophilus* and *Lactobacillus pentosus* are also included in GABA-producing LAB bacteria.[31–34] *S. thermophilus* and *L. pentosus* both can be used as probiotics and have the capacity to produce GABA in vitro and in vivo.[35–38] *S. thermophilus* was reported to improve OA severity in humans and mice.[39,40] Similarly, GABA-rich fermented milk produced by *S. thermophilus* effectively improved sleep.[32] But there is an unfortunate gap in developing therapies that leverage this appreciation by selectively targeting microorganisms to improve human health.

GABA is also associated with cognition, behaviour, oxidative stress, glucose tolerance, and pathological conditions such as Alzheimer's and diabetes.[41–43] At the same time, GABA also acts as an intermediate in an extension of the tricarboxylic acid cycle (TCA), which involves the oxidation of α-oxoglutarate to succinate through glutamate, GABA, and succinic semialdehyde. GABA is catabolized by GABA transaminase (encoded by 4-aminobutyrate aminotransferase, ABAT) into succinic semialdehyde (SSA).[44]

GABA concentration regulates the release of various inflammatory cytokines in a concentration-dependent manner.[45] Macrophage-mediated inflammation is considered to play a role in osteoarthritis severity and pain. GABAergic components are a new horizon for autoimmune and inflammatory diseases.[41] In the mice model, GABA levels in skeletal and plasma muscles significantly increased during exercise. A lower serum level of GABA was observed in older women with osteoporosis.

Activation of $GABA_A$ and $GABA_B$ receptors ($GABA_Br$) increases ATDC5 cell proliferation in culture. Acutely, knocked down of $GABA_Br1$ down-regulates the expression of the early chondrogenic markers, aggrecan (AGCAN) and collagen II (COL2), and upregulation of catabolic markers, osteopathic and collagen X (COLX).[46] Vigabatrin (clinically used to increase GABA levels in neural cells) protects against injury-induced OA in mice, and increased GABA levels in articular chondrocytes.[47] The putative GABA effect changes with cell types. Analyzing existing data, we hypothesize that GABA could protect against osteoarthritis. GABA-producing probiotics are utilized in treating different diseases.[48,19,25] Chondrocytes are the main cellular population that maintains and degrades articular cartilage structure.[49] The presence of the $GABA_B$ receptor and its vital role in chondrogenesis has been described previously,[50,51] suggesting the involvement of GABA in chondrocyte physiology. Although GABAergic machinery could regulate endochondral bone formation by directly regulating chondrogenesis and its role in the TCA cycle (GABA shunt), little attention has been given to exploring the potential direct role of GABA in chondrocyte proliferation. This is the first study investigating the gut-joint axis mechanism via GABA.

Therefore, this study aimed to examine the potential effects of GABA as a postbiotic/bioactive compound on OA treatment, and whether oral administration of GABA-producing bacteria and GABA can protect against pathological changes, cartilage loss, and inflammation during Anterior Cruciate Ligament Transection (ACLT) induced OA. We designed this study to examine GABA-producing probiotics' protective/regenerative role in vivo (mice knee joints) and in vitro (c28/I2, human chondrocytes).

2. Material and methods

2.1. Preparation of conditioned media

Previously reported GABA-producing probiotic strains, *Streptococcus thermophilus* (*S. thermophilus,* CICC 6222/ATCC 19258)[31,33] and *Lactobacillus pentosus*[26,31,34] (*L. pentosus,* CICC 24202), bought from the China Center of Industrial Culture Collection (CICC). *S. thermophilus and L. pentosus* were cultivated.[52]

The overnight culture of*L. pentosus and S. thermophilus* was centrifuged (4000 g; 10 min), media discarded, and bacterial pellets resuspended, PBS washed and fixed in 5% formaldehyde (3hrs; 4 °C), thrice washed before incubation to Dulbecco's Eagle's medium (DMEM). Bacteria (5×10^7 CFU/ml) were transferred to DMEM and cultivated for 3 h. Media separated from Bacteria by centrifugation (4300 g; 15min), pH Adjusted, and filter-sterilized (0.22 mm). Media now referred as conditioned media (CM) stored at −80 (Supplementary Fig. S1a).[53]

2.2. Cell culture & proliferation

C28/I2 Human Chondrocyte Cell Line purchased from Sigma-Aldrich (cat SCC043). It was cultured in DMEM (Gibco, California, USA), 10% FBS (BI, C04001050X10) and 1% penicillin-streptomycin (Beyotime, C0222), and under 5% CO_2, 37 °C. Cells were grown in CM ST (conditioned media *S. thermophilus*) & LP (conditioned media *L. pentosus*), ST + LP, and 0.1 μl Gama Aminobutyric acid (GABA, Sigma# A5835). The results were compared with the Control group (no-treatment, CN) after two days of treatment.

Cell Counting Kit-8 (CCK8; HY-K0301-500T) was used to determine viable cell numbers and proliferation based on detecting dehydrogenase in living cells in 96-well plates (5 × 103 cells/well). The control group with DMEM medium only and CM ST, LP, ST + LP, and 0.1 μl GABA for 24 h and 48 h. A microplate reader measured the absorbance value at 450 nm after incubation (4hrs) with CCK8 in the dark at 37 °C.

2.3. Treatment effects on IL-1β stimulated chondrocytes

The control group was cultured with 10 ng/ml IL-1β (Proteintech, USA) for 48 h, and CM ST, LP, ST + LP, and LP and 0.1 μl GABA groups were cultured. Total mRNA was extracted, and shifts in genetic markers were observed via qPCR.

Cells were stimulated with GABA B receptor ($GABA_Br$) antagonist CGP 52432 for 8 min, then cultured in 10 ng/mL IL-1β and CM ST, LP, ST + LP, and 0.1 μl GABA for two days. The change in mRNA expression was compared.

2.4. Mice

We purchased 6–8 weeks old C57BL mice; Mice were assigned to five groups according to the difference in the treatment regime.

Normal mice were administered PBS (PBS), *S. thermophilus* (G1); *L. pentosus* (G2), and combined treatment of *S. thermophilus* & *L. pentosus* (G3); γ aminobutyric acid (G4) via oral gavage. Mice had free access to water and food and were kept in a sterile environment, with five or no less than five mice per cage. Mice anesthesia and euthanasia were performed according to best practice ARRIVE guidelines.[54] OA was surgically induced by Anterior Cruciate Ligament Transection (ACLT) injury to mice after two weeks of gavage treatment and continued till sacrifice. Weight was monitored throughout the treatment period.[55] Mice were sacrificed, whole blood was drained via eye rupture, and tissue was collected for histological assessment, protein, and mRNA quantification.

2.5. Gavage protocol

Overnight culture of *S. thermophiles* and *L. pentosus* harvested (4000 rpm; 10min), washed, and re-suspended PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). OD was adjusted, and bacteria were stored in Aliquots for gavage treatment. A viable Count was adjusted for the desired concentration of bacteria using OD Values (600 nm). Aliquots of bacterial suspensions were frozen (−80 °C) and stored till use. One fresh aliquot was thawed for every gavage treatment to minimize the change in bacterial viability (Supplementary Fig. S1b).

After freezing and thawing, the number of viable cells was analyzed by colony-forming unit (CFU) counting on MRS agar after 48 h incubation. More than 90% of cells were alive on freeze-thawing for both tested strains. The number of viable cells was determined several times in a period of two months, and no significant difference in bacterial viability was found during storage time. A Safety Review of Gamma-Aminobutyric Acid (GABA) explains its dosage, mode of action, and metabolism.[56]

Oral feeding started 2 weeks before ACLT and sustained up to 6 weeks. In feeding protocols, individual or combination bacteria were suspended in PBS, and the turbid bacterial suspension, GABA, or PBS, was orally administered using gavage needles thrice a week. The treatment regime according to body weight was: PBS (phosphate buffer saline), *S. thermophilus* (G1; 2×10^{10} CFU/kg, 500 mg/kg), *L. pentosus* (G2; 2×10^{10} CFU/kg, 500 mg/kg), Mixture (G3; combined treatment; 2×10^{10} CFU/kg, 500 mg/kg), GABA (G4; 50 mg/kg). Using the body surface area-based formula, GABA effective dose for mice (4 mg/20 g) was calculated based on previous recommendations.[57] We also fed GABA (0.4 mg/20 g/day) via drinking water to mice to mimic a continuous GABA supply identical to probiotics. For the optimal dose of *S. thermophilus* + *L. pentosus*, we used an effective probiotic dose showing a suppressive effect against OA based on previous studies.[11,13]

2.6. Tissue fixation and histological analysis of cartilage

Mice intestine and knee joints were collected upon euthanization and fixed in 4% formaldehyde (72hrs; 4°c). EDTA was used for the decalcification of joints for 14 days, and all samples were processed using a tissue processor and embedded in paraffin. 5 μm thick sections were cut from each paraffinized tissue. Safranin O (SO; catalog G1371; Solarbio life sciences) and Alcian Blue Hematoxylin (catalog G1121; Solarbio life science) were stained to analyze knee joints. Safranin O stained knee sections were used for OARSI scoring and histomorphometric analysis. Unstained knee sections were used for immunohistochemistry (IHC) and immunofluorescence (IF) staining.

A blinded observer quantified the Cartilage area (Tibial and femoral), Safranin O^{+} chondrocytes, and total chondrocyte numbers calculated. As described previously, for each joint slide, measurements were taken from one section at each of the three levels (50 μm apart) on both the femoral condyle and the tibial plateau in a 200 μm wide centered area on the joint. Three measurements were obtained along the knee joint for each specimen, and the average was calculated.

2.7. OARSI scoring

To evaluate the zone of cartilage restoration or degeneration, a semiquantitative histopathologic grading system of the Osteoarthritis Research Society International (OARSI) scoring system was used by two blind observers.[58,59] Briefly, utilizing the OARSI system, joint sections stained by Safranin O were graded on the following scale. 0: normal cartilage, 0.5: no cartilage fibrillation with loss of proteoglycan stain. 1: superficial/mild damage without loss of cartilage loss, 2: fibrillation/cliffing extending below the external zone along partial loss of surface lamina, 3: vertical erosion/clefts of the cartilage to the calcified zone and over 75% of the joint surface.

2.8. Immunohistochemistry (IHC)

Knee joint sections of mice were deparaffinized and moved in 2 changes of xylene each for 10 min, hydrated in a series of ethanol (100% ethanol, 95% ethanol, followed by one shift of 70% ethanol), and rinsed twice in PBS. The antigen was retrieved by retrieval kit (AR0022; Boster) for the sections (30min; 37 °C). H_2O_2 (0.5%) was used to quench endogenous peroxidases for 10 min. The tissue sections were incubated with selected primary antibodies (overnight; 4 °C). Slides were rinsed in PBS containing 0.1% tween 20 (PBST, catalog P1397, Millipore Sigma) and antigen blocked with 5% normal goat serum. Next, overnight incubation at 4 °C with rabbit anti-mouse ACAN (1:200 dilution, catalog DF7561, Affinity), mouse anti-mouse Runx2 (1:200 dilution, Affinity; AF5186), rabbit anti-mouse MMP-13 (1:200 dilution, catalog 18165-1-AP, Proteintech), HIF-1α (1:200 dilution, Affinity; AF1009) and ATF4 (1:200 dilution, Affinity; DF6008). Slides were incubated with anti-rabbit secondary antibody for 30 min at room temperature, 3 times PBS rinsed for 5 min each, followed by two washes in deionized water for 5 min each. A 3-min incubation detected antibody binding to ACAN, MMP13, Runx2, HIF-1α, and ATF4 antigen with DAB peroxidase substrate (catalog zli90181, OriGene). Mayer's hematoxylin solution (catalog AR0005, BOSTER) was used to counterstain nuclei for 10–15 s.

2.9. Immunofluorescence (IF)

The intestine and joint samples of mice were deparaffinized, as mentioned earlier. Permeabilized with PBS containing 0.5% Triton for 10 min and blocked with 4% BSA (30min 37 °C). The tissue slides were incubated (overnight at 4 °C) with primary antibodies against IL4 (Catalog 66142-1-lg), IL6 (1:200 dilution, catalog DF6087), IL10 (Catalog 60269-1-lg), and IL-1β (1:200 dilution, catalog AF5103) and COL2 (1:200 dilution, catalog bs-0709R). The PBS-washed slides were incubated with a secondary antibody. After rinsing, the nuclei were counterstained with DAPI (5min). Slides were imaged by fluorescence microscope.

2.10. Real-time PCR

The knee joints that underwent ACLT surgery and controls (no surgery) and the intestine were immediately transferred to liquid nitrogen on dissection to extract RNA. Tissues were pulverized with the help of a mortar and pestle. Total RNA was extracted from the intestine and knee joints using Cell/Tissue Total RNA Isolation Kit (FastPure; RC101) and was reverse transcribed to cDNA using

SuperScript-II reverse transcriptase according to the manufacturer's protocol.

RT-PCR reactions were carried out utilizing the PowerUp SYBR Green Master Mix Kit by Applied Biosystems using the BIO-RAD Real-Time PCR System with SYBR Green (Applied Biosystems by Life Technologies, 4367659). The protocol: 50 °C for 30 min, 40 cycles for 95 °C, and 65 °C for 45s. The RT-PCR primer sequences are enlisted in Supplementary Table S1.

Real-time PCR for ACAN, Adamts4, Adamts5, COL2, SOX9 COLX, IL4, IL6, IL10, TNF-α, and HIF-1α. All signals normalized to GAPDH. ΔΔCT method was used to calculate relative gene expression, and GAPDH was used as a reference gene for computing ΔCT. ΔΔCT was calculated relative to the control samples. Total extracted RNA from cells, compared between treatment and no-treatment groups.

2.11. Western blot

SDS–PAGE was used for Protein separation and then membrane transferred. The membranes were blocked for 1 h with 5% BSA and incubated overnight at four °C with primary antibodies specific for GAPDH (1:2000, catalog AF5009), COLX (1:1000, catalog A6889), MMP-13 (1:2000), IL6 (1:500), and β-actin (1:2000, catalog AF7018). After washing away, the membranes were incubated with a secondary antibody (1:10,000, catalog SA00001), and ECL detection system was used to detect protein bands.

2.12. ELISA for GABA

GABA assay was performed on the fecal sample, serum samples, and small intestine content of mice to ensure in vivo GABA production by *S. thermophilus* and *L. pentosus*. Quantitative determination of GABA was carried out by a commercially available enzyme immunoassay (ELISA, catalog; MM-0442M2) was used.

The fecal sample was used to determine the shift in GABA levels. The fecal samples were mixed with distilled water/PBS and vortex until a slurry was made. The supernatant was used for GABA assay. The small intestinal content was diluted in PBS (10%, w/v), homogenized by vortex, and centrifuged for 20 min at 3000 rpm. A commercially available enzyme immunoassay was then used to determine GABA quantitatively. The whole drained-out blood was allowed to coagulate naturally and centrifuged at 3000 rpm for 20 min, the supernatant used for ELISA.

Standard curve prepared with known standards for the quantification of unknown samples and results standardized to individual sample weights.

2.13. Statistics

All statistical analyses were completed with Prism 7 software from GraphPad (San Diego, CA, USA). Statistical significance was determined to be $P < 0.05$, 0.01, and 0.001. To determine the average, standard deviation, and characterization methods, all in vivo data are expressed as the mean. Each data point signifies an individual mouse. All in vitro data is expressed as mean ± standard deviation. Statistical significance (P values) is determined by ANOVA (Dunnett's multiple comparisons) for grouped data.

3. Results

3.1. Probiotics and GABA increase the proliferation and anabolism of chondrocyte

We first cultured the C28/I2 Human Chondrocyte Cell Line with conditioned media (Fig. S1a), *S. thermophilus* (ST), CM *L. pentosus* (LP), combined group (ST + LP), and GABA (γ aminobutyric acid; 1 μl) for 24hr and 48 h[53] GABA was included in the study for comparison and ensured the similar effect of GABA produced by probiotic strains. Cell viability and proliferation were examined by CCK8 assay (Fig. 1A). The number of cells increased in treatment groups compared to placebo (control). However, the effect was profound after 48hrs culture. Furthermore, we elaborated on the functional expression of chondrogenic markers in C28/12 cells.

The primary inhabitants of articular cartilage, the Chondrocytes, are responsible for the synthesis, preservation, and repairing of ECM.[49] The expression profile of various proliferation markers was observed through RT-qPCR. In vitro, human chondrocytes were cultured for 48hrs in the presence of CM ST & CM LP, CM ST + LP, and GABA 0.1 μm. Proliferation markers such as Cyclin B1 and proliferating cell nuclear antigen (PCNA) and Cyclin D1, including ACAN, COL2, and cdk1 highly expressed as compared to placebo Fig. 1B. The slicing of cyclin D1 was reported to suppress the proliferation of chondrocytes and apoptosis.[60,61]

Increased mRNA expression of all observed chondrogenic markers examined; as represented in Fig. 1B, treatment of ST and LP increased the expression of ACAN, COL2, CyclinB1, CyclinD1, PCNA, and cdk1. Although co-administration of ST and LP decreased expression slightly less than independent treatment groups, all data was statistically significant ($p < 0.05$).

3.2. IL-1β-induced genetic expression shift attenuated by probiotics and GABA treatment

Chondrocytes' mechanical stress overburdened during the onset of OA, chemokines, oxidants, pro-inflammatory cytokines, and catabolic factors in OA leads to poor autophagy and cell death.[62] To mimic the pathological process of the OA in vitro, we incubated chondrocytes with 10 ng/ml IL-1β for 48 h. Subsequently, we examined the expression of several genes reported to upregulate in OA cartilage.

Matrix metalloproteinases (MMPs) have been studied extensively and directly linked to cartilage degradation during the onset of OA. MMPs Inhibition is proposed as a promising therapeutic strategy to protect cartilage. The RT-PCR expression data indicated that an optimum concentration of GABA, ST, LP, and co-treatment downregulated the expressions of catabolic markers and inflammatory factor IL6 compared to placebo (control), such as COLX, MMP13, ADAMTS4, ADAMTS5 and IL6 reduced (Fig. 2A,C,D). The expressions of MMP13, COLX and IL6 were also confirmed by western blotting in chondrocytes (Fig. 2D,E,F). We also examined the expression of anabolic genes (ACAN, COL2 & SOX9) and anti-inflammatory factor IL10, which were previously reported to suppress the onset of OA and progression. The treatment reversed the IL-1β induced shift in these markers, as represented in Fig. 2B,C. Together, these results indicated that GABA decreased catabolism and promoted the expressions of SOX9, COL2, ACAN and IL10.

We further confirmed that the activity of these markers depends on GABA. We stimulate the cells with GABA inhibitor (GI), culture them in the presence of IL-1β, and examine their influence on anabolic, catabolic, and inflammatory markers via RT-qPCR. The GI reversed all the genetic shifts induced by GABA.

3.3. Protection against ACLT-induced OA progression by probiotics and GABA in mice

Mice were divided into five groups randomly according to the treatment regime difference as PBS, G1, G2, G3, and G4. Gavage treatment started from day 1, as shown in (Fig. S1b & Fig. 3A). To model the pathological process of OA, we employed the ACLT mouse injury to induce OA on day 14. Mice were sacrificed, and tissues were collected for processing 56th day (Fig. 3A). There was

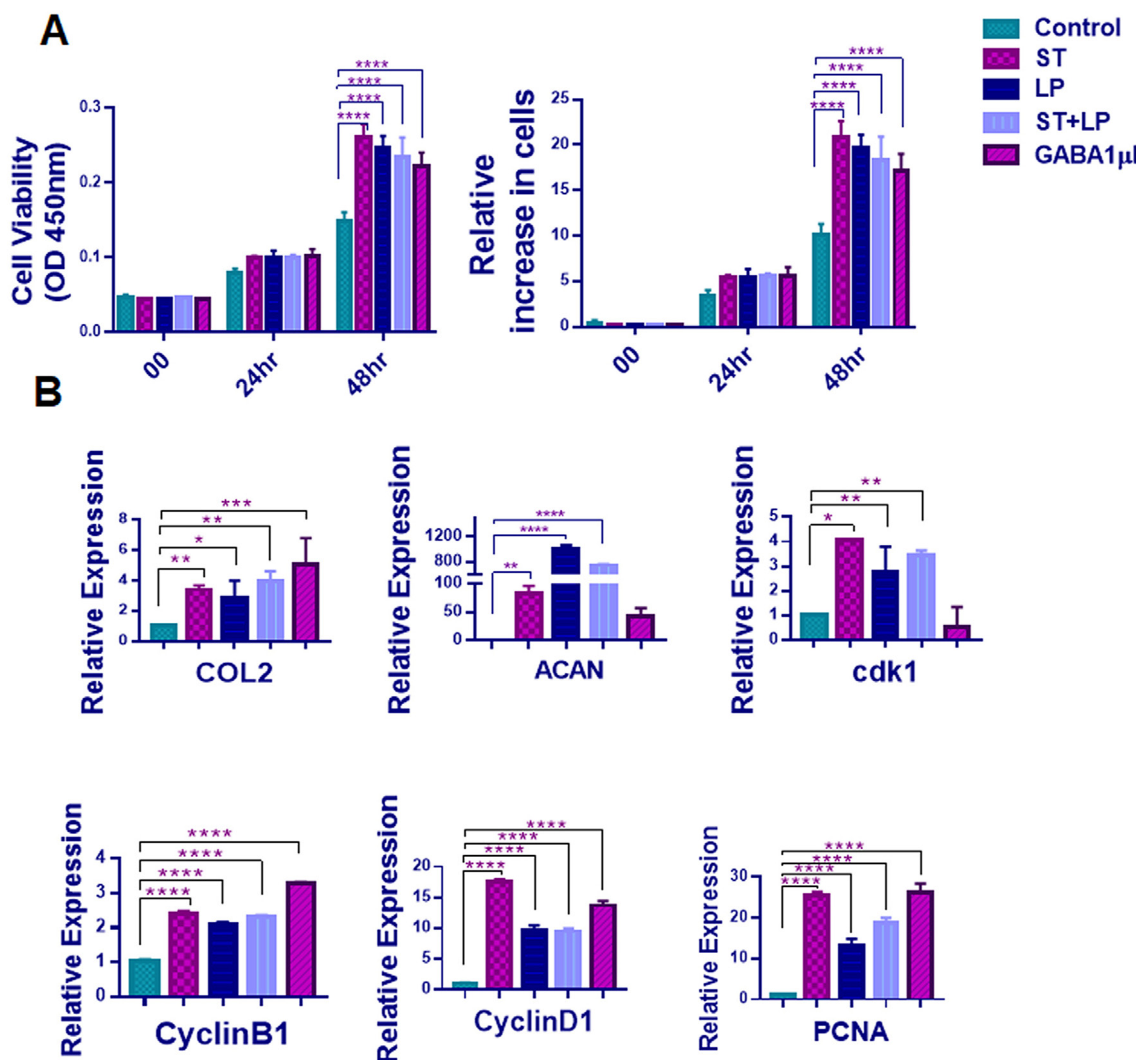

Fig. 1. Probiotics and GABA increase the proliferation and anabolism of chondrocytes. A- The viability and proliferation via CCK8. **B-**The increased expression of anabolic markers COL2, CyclinB1. CyclinD1, PCNA, cdk1, and ACAN (Aggrecan) by Real-time qPCR in the human chondrocyte cell line followed by treatment; ST (conditioned media *S. thermophilus*) & LP (conditioned media *L. pentosus*), ST + LP, (Combined treatment) and GABA (0.1 µl γ-Aminobutyric acid). The mRNA levels were normalized to that of GAPDH and then normalized to the control group. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; *$p < 0.05$, **< 0.01, ***< 0.001, ****< 0.0001).

no significant difference in mice weight among all treatment groups (Fig. 3B). ELISA for GABA was conducted to confirm whether selected bacteria produced GABA in vivo fecal small intestine content and serum samples of mice (Fig. 3C).

We examined the impact of GABA and GABA-producing bacteria on the progression/protection of joint degeneration after ACLT. The OA progressed degree in PBS-treated mice more than in treatment groups. Cartilage and chondrocyte loss was significant and severe in the PBS-treated group of every examined metric. Remarkably, probiotic and GABA-treated mice were rescued from OA's degenerative effect (Fig. 3D; HE and SO staining). The histomorphometric outcome significantly improved, with less OARSI scores, and safranin O-positive cells increased (Fig. 3D).[58]

PBS-treated mice exhibited uneven/rough cartilage surfaces, their superficial fibrils were visible, and ACLT-induced distortion of the surface was profound. G3 also showed cartilage destruction, though lesser than the PBS-treated group, revealing that independent bacterial treatment was more effective than co-treatment. However, coadministration of *S. thermophilus* and *L. pentosus* was effective compared to PBS in protecting against arthritic changes and suppressing cartilage destruction, but slightly less than individual treatments (Fig. 3). Based on the results, GABA and GABA-producing microbes provide nearly complete protection against ACLT-induced OA, suggesting its potential as a nutraceutical modifier in osteoarthritis treatment.

3.4. Probiotics and GABA treatment suppresses OA-induced catabolic and anabolic changes in mice

We next investigated the chondrogenic markers ACAN, COL2, and SOX9. The increasing trend of aggrecan-positive cells was detected via IHC as compared to PBS treated (Fig. 4A,B). Furthermore, mice knee sections were exposed to IF detection of COL2 (representative staining Fig. 4C,D). The observed expression of COL2 also increased in G1, G2, G3, and G4 mice compared to PBS group (Fig. 4C,D). The increasing trend of COL2 and SOX9 was evaluated by RT-qPCR (Fig. 4E,F).

Catabolism markers such as MMP13 and ADAMTS5, RunX2, and COLX play a crucial role in cartilage ECM degradation and are widely studied regarding OA. IHC analysis showed that the

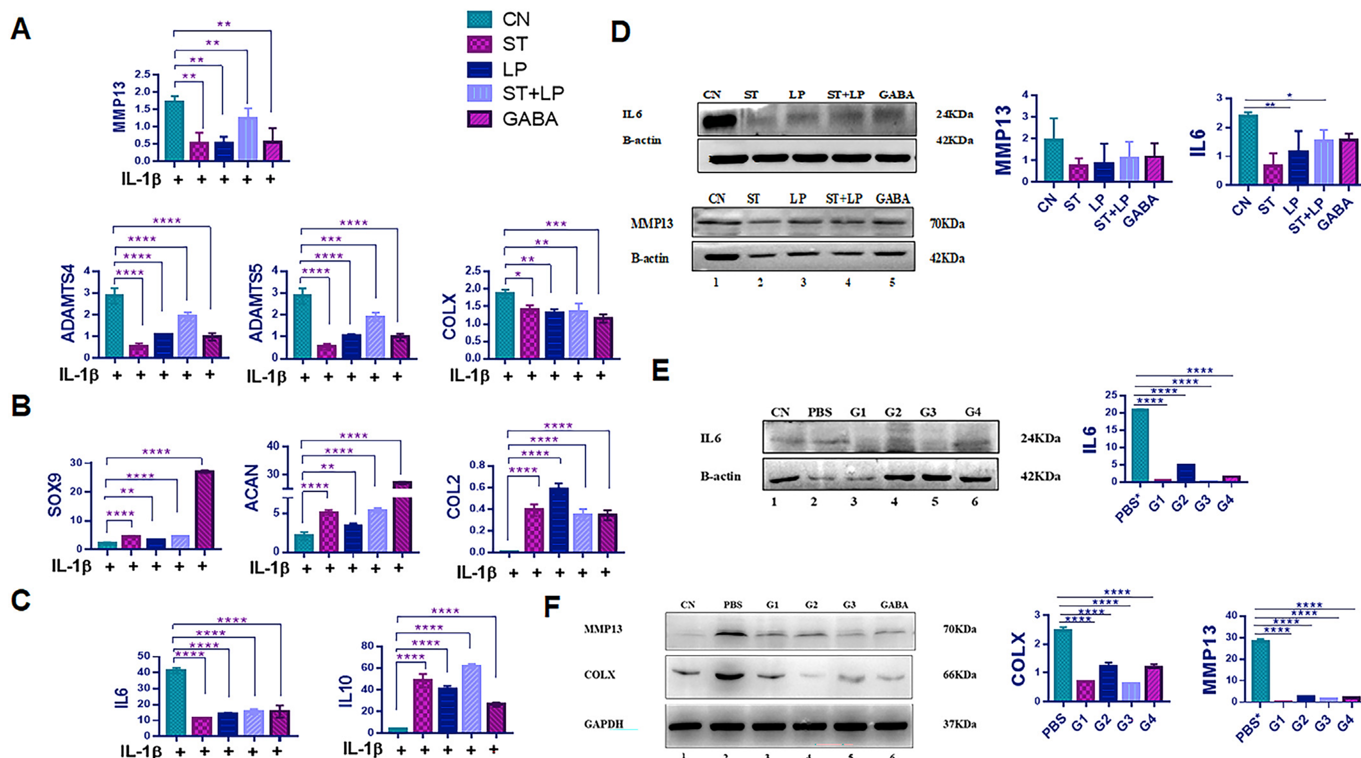

Fig. 2. Effect of *S. thermophilus* and *L. pentosus* on anabolism and catabolism of chondrocytes. The impact of ST (conditioned media *S. thermophilus*) & LP (conditioned media *L. pentosus*), ST + LP (Combined treatment), and GABA (0.1 μl Gama Aminobutyric acid) on human chondrocytes in the presence of IL-1β. **A-** The decreased expression of catabolic markers of osteoarthritis by RT qPCR. **B-** The increased expression of anabolic markers in chondrocytes treated with conditioned media/GABA. **C-** Real-time qPCR evaluation of inflammatory markers for OA tested against all treatment groups. **D-** Western Blot Comparative expression of IL6 and MMP13 in chondrocytes **E-** Comparative expression of IL6 in mice joints. **F-** Comparative expression of COLX and MMP13 in mice joints. The mRNA levels were normalized to that of GAPDH and then normalized to the control group. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; *p < 0.05, **< 0.01, ***< 0.001, ****< 0.0001).

proportion of MMP13 and RunX2 positive cells was significantly lower in treatment groups compared to the no treatment/PBS group (Fig. 4A,B). RT-qPCR also confirmed the reduction of ADAMTS5 compared to PBS group (Fig. 4E).

Decreased expressions of MMP13, Runx2, ADAMTS5 and increased expressions of ACAN, COL2, and SOX9 were observed (Fig. 4), which led to rescue mice from ACLT-induced OA via promotion of cartilage anabolism and suppressing cartilage ECM degradation. These results indicate the chondroprotective effect of both GABA-producing *S. thermophilus* and *L. pentosus*.

3.5. Probiotics and GABA mitigate inflammation in mice

Inflammatory markers are now understood to contribute to OA; as reported, GM is a regulator of inflammation.[8,63,64] During the onset of OA, inflammatory cytokines modulate the microenvironment and play a crucial role in tissue destruction. To investigate the treatment effect on the inflammatory cytokines, immunofluorescence (IF) staining was performed on the intestine and knee joint sections of all groups against IL4, IL10, IL6, and IL1β. The downregulated expression of IL6 and IL1β, while upregulation of anti-inflammatory maker IL4 and IL10 was observed (Fig. 5A and D). The mRNA expression levels of IL6 and TNFα in the knee joint and intestine decreased (Fig. 5B and C). The probiotic treatment showed suppression of inflammatory influx into cartilage tissues compared to PBS, also confirmed by western blot (Fig. 2E). The IL6 and IL10 levels were observed in chondrocytes and coordinated with in vivo experiments (Fig. 2C,D,E).

We also examined whether the therapeutic effect of probiotics is linked to the downregulation of inflammatory cytokines in the intestine (TNFα, IL6 & IL-1β). Interestingly, oral administration of the GABA-producing probiotics significantly decreased inflammatory cytokines, TNFα, IL6, and IL-1β, compared to PBS. Upregulation of anti-inflammatory makers IL4 and IL10 was observed by immunofluorescence (IF). IL6 and IL-1β were also detected via immunofluorescence (Fig. 5D). Intestine IL6 and TNFα mRNA changes were further assessed (Fig. 5C). Collectively, the level of the inflammatory cytokine was downregulated in the intestine and joints (Fig. 5).

3.6. The role of probiotics relies on GABA

Microbiota produces gut metabolites that fundamentally function as a dynamic endocrine organ that can enter the blood circulation and elicit biological effects at distant sites within the human body[65] as Fig. 3C represents the increased GABA level in feces, small intestine content and serum in all treatment groups as compared to control. Recent studies also revealed that specific taxa among residents of the human gut could modulate the gut-brain axis by GABA production. The role of GABA-producing lactobacillus on stress and GABA-modulating bacteria in the human gut has been reported previously.[25,48] To further ensure that the treatment effect depends on GABA, we utilize the $GABA_B$ receptor inhibitor (CGP 52432) in vitro.[66]

Cells were stimulated and cultured separately in conditioned media ST, LP, combined group (ST + LP), and GABA for 48hrs, and mRNA was extracted. The shift in relative mRNA expression of ACAN, COL2, CyclinD1, CyclinB1, PCNA, and cdk1 was analyzed in

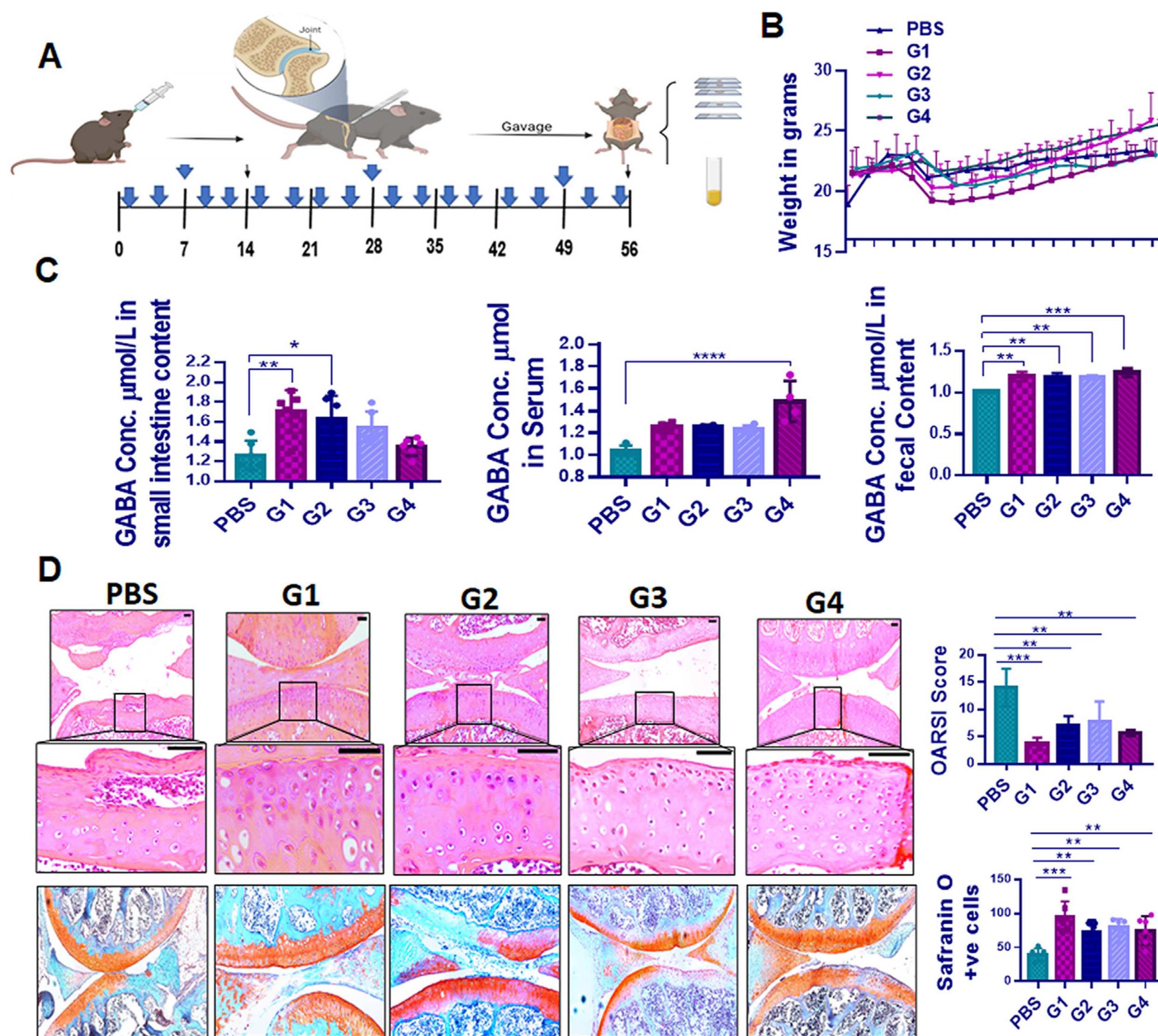

Fig. 3. Effect of *S. thermophilus* and *L. pentosus* on the cartilage of OA mice. A-study design**,** treatment was initiated 14 days before ACLT; Mice were treated with bacteria and GABA thrice a week starting day one of treatment via gavage. OA was induced via ACLT rupture on the 14th day of treatment. Samples were collected on the 56th day of treatment on sacrifice. **B-** weight monitored 2 times a week throughout the experimental period **C-** GABA assay, Increased GABA concentration detected by ELISA in small intestine content and serum on a probiotic treatment. **D-** Histopathological analysis of cartilage tissue. Representative images of H & E staining and Safranin O staining of mice knee joints. OARSI score grading for different treatment groups. The five-treatment group is the following: (PBS: Control; G1: Mice treated by *S. thermophilus* mice; G2: Mice treated by *L. pentosus*; G3: Mice treated by both bacteria; G4: treated by GABA). (n = 5) in each group of mice (the black scale bar represents 50 μm). Percentage of safranin O positive cells. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; *p < 0.05, **< 0.01, ***< 0.001, ****< 0.0001).

Fig. 6A. The increase in proliferation markers diminished after stimulation with GABA inhibitor. This data provides a foundation for further studies to explore the role of GABA and GABA-producing microbes in regulating cartilaginous cell proliferation. GABA may have active roles in chondrocyte maturation/differentiation.

As represented in Fig. 6, the GABA inhibitor alleviated the treatment effect, the comparative impact of the *S. thermophilus* (ST), CM *L. pentosus* (LP), combined group (ST + LP), and GABA (γ aminobutyric acid; 1 μl) stimulated by IL-1β with or without of GABA inhibitor examined (Fig. 6B, C & D). The negative regulation of catabolic and inflammatory markers fades away after utilizing GI. Thus, different microbial species outside the gut potentiates implications for establishing and protective response and could control unique physiological and immunological homeostasis.[63,67]

3.7. The harbour effect of probiotics composition activates HIF-1α

HIF-1α maintains glycolytic and TCA cycle flux, and its function is linked to both anabolic and catabolic pathways of OA.[68] HIF-dependent pathways are assessed to contribute to cellular adaptation to hypoxic stress. Activating transcription factor-4 (ATF4) acts regulator of metabolic and redox processes under normal cellular conditions and as a master transcription factor during the integrated stress response. ATF4 activates the Indian hedgehog (Ihh) in chondrocytes and regulates osteogenesis.[69] The upregulation of ATF4 and HIF-1α was observed vis IHC in knee joint sections of mice, as represented in Fig. 7A. The mRNA expression of HIF-1α was observed in chondrocytes as well (Fig. 7B). The GABA confers adaptive responses in both the hypoxia-inducible factor (HIF) and ATF4 systems recruited.[70] The possible mechanism of action of GABA is elaborated in Fig. 7C,D.

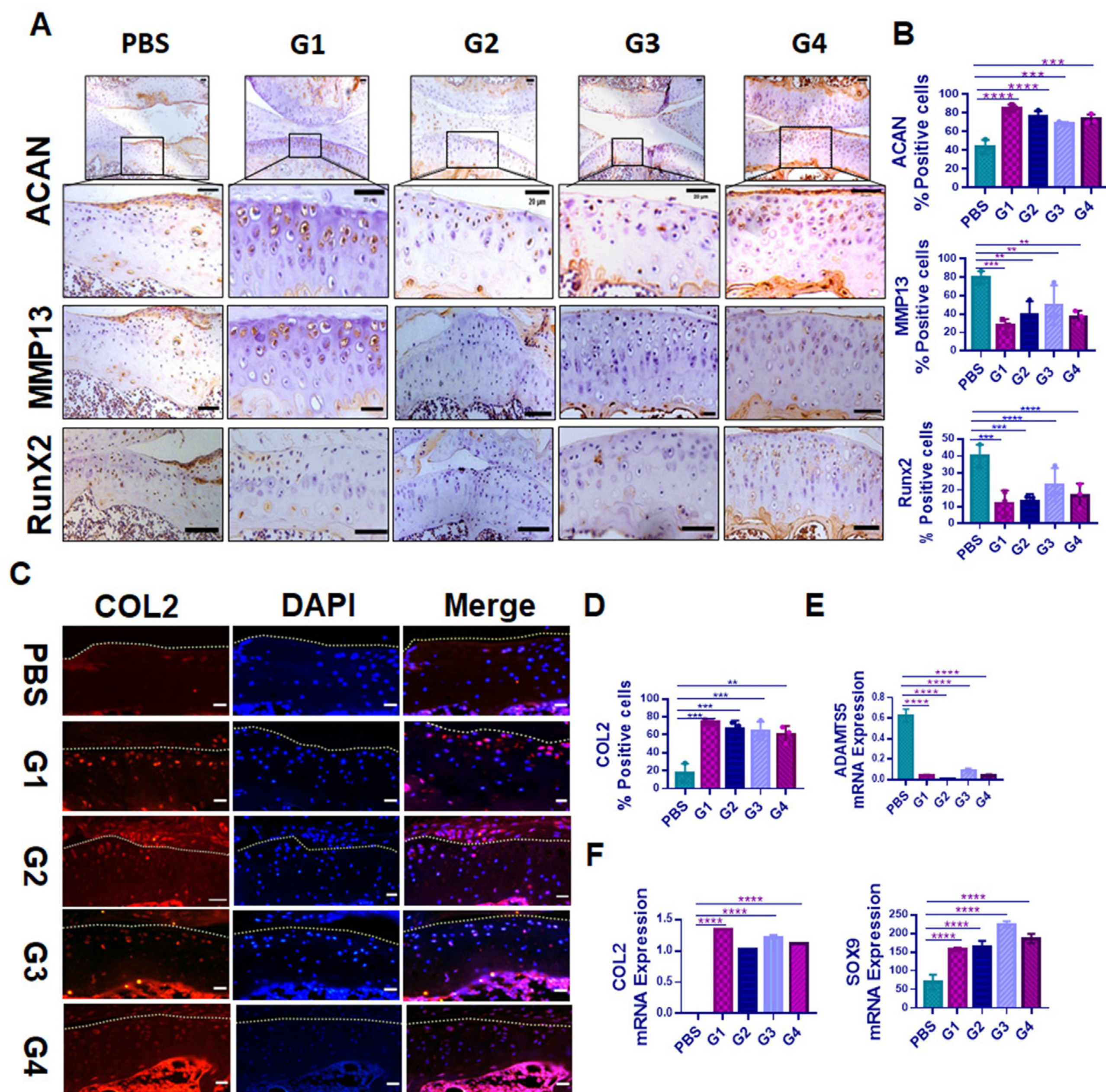

Fig. 4. Probiotic effect on Expression of anabolism and catabolism in vivo. A-Immunohistochemistry (PBS: Control; G1: Mice treated by *S. thermophilus* mice; G2: Mice treated by *L. pentosus*; G3: Mice treated by both bacteria; G4: treated by GABA). The mice knee joints were examined via IHC on the joint section of different treatment groups by ACAN, MMP13, and RunX2. Dark brown represents positive cells, and blue represents negative cells. To evaluate articular chondrocyte hypertrophic differentiation and cartilage matrix changes, Runx2 and MMP-13 expression were examined via IHC on the joint section of different treatment groups of mice. **B-** The increased percentage of ACAN positive cells compared to the control and decreased MMP-13 and RunX2 positive cells compared to the control group (scale bars represent 20 μm). **C-D-** The increased COL2 in the knee tissue section was evaluated by Immunofluorescence. The nucleus was stained by DAPI staining. The white scale bars represent 20 μm, and the yellow dotted line represents the cartilage layer. **E-F-** RT-PCR analysis of COL2 and SOX9, and Adamts5. The mRNA levels were normalized to that of GAPDH and then normalized to the control group. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; $*p < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$).

Furthermore, the interactions between $GABA_{B}r$, ATF4, and HIF-1α were confirmed through protein-protein interactions (PPI enrichment; p-value:0.0126). The PPI network consisting of all these proteins generated by STRING indicates that the proteins are biologically connected at least partially (Fig. 8). As exhibited Fig. 7C, proteins have experimentally proven interactions among themselves.[71]

4. Discussion

Modifying therapy for OA disease is a critical unmet need because of the growing global prevalence of OA. The absence of clinical trials to address OA's current and future burden is noteworthy.[72] Joint inflammation and degradation are inevitable factors in the progression of OA.[73] The distinct role of GM in shaping the host immune system and its role in inflammation (chronic and systematic) is also evident.[10] The critical microbial species can control unique physiological niches outside the gastrointestinal

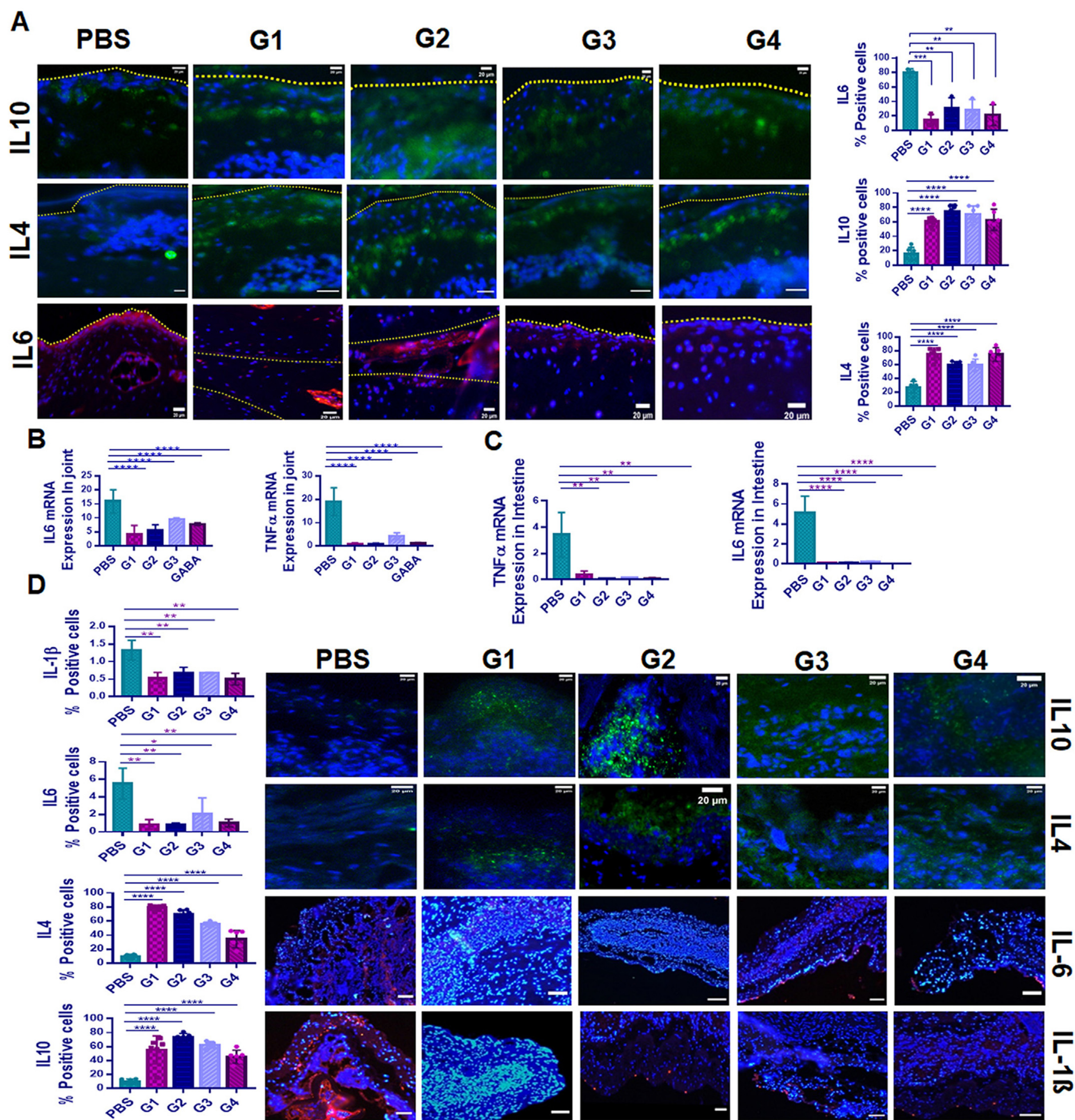

Fig. 5. Decreased expression of inflammatory cytokine in mice joints & intestines. A- (PBS: Control; G1: Mice treated by *S. thermophilus* mice; G2: Mice treated by *L. pentosus*; G3: Mice treated by both bacteria; G4: treated by GABA). IL6, IL4 & IL10 evaluated joint sections of mice. Downregulation of IL6 positive cells while upregulation of anti-inflammatory markers IL4 & IL10 by Immunofluorescence. The nucleus was stained by DAPI staining. The white scale bars represent 20 μm, and the yellow dotted line represents the cartilage layer. Positively stained cells were counted. **B-** Real-time qPCR analyses of the relative expression of inflammatory markers IL6 & TNFα in the mice joints. **C-** Real-time qPCR analyses the relative expression of inflammatory markers IL6 & TNFα in the mice intestines. **D-** Immunofluorescence in the intestine and quantitative analysis. (PBS: Negative control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes; G4: treated by GABA). Intestine sections of mice were evaluated by IL4, IL6, IL10 & IL-1β positive cells. The nucleus was stained by DAPI staining. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; *$p < 0.05$, **< 0.01, ***< 0.001, ****< 0.0001).

tract (GIT) and potentiate implications for establishing immune homeostasis and protective response.[63] Besides, the post-biotics' role in shaping the immune system is clear now that unique bacterial groups could dominantly influence the development and function of the immune system under normal and inflammatory conditions.[74] Fewer studies have conferred the role of GM in the OA of obesity. Decreased GABA levels have been reported in the feces and serum of germ-free mice.[27] Antibiotics also modify the fecal

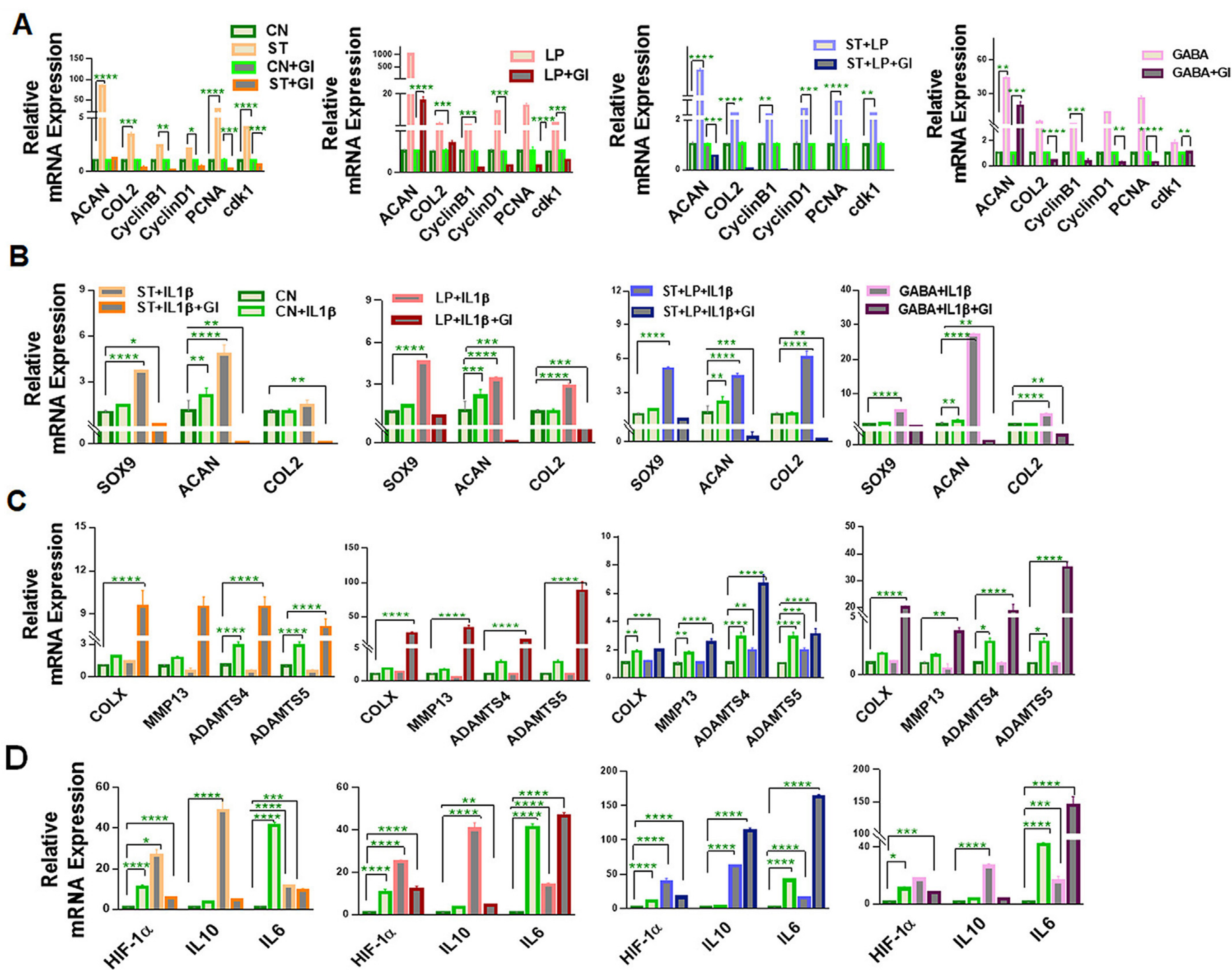

Fig. 6. GABA inhibitor (GI) alleviates the treatment effect in Human chondrocytes. A- Real-time qPCR analyses, the relative expression of anabolic and proliferative markers on human chondrocytes after treatment with GI. **B-** The increasing trend in anabolic markers in treatment groups thrash with GI. The treatment effect on human chondrocytes in the presence of IL-1β and IL-1β+ GABA inhibitor **C-D-** The compared treatment effect on human chondrocytes in the presence of IL-1β and IL-1β+ GABA inhibitor catabolic and Inflammatory mRNA expression by Real-time qPCR. The mRNA levels were normalized to that of GAPDH and then normalized to the control group. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; $*p < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$).

GABA content in specific germ-free mice.[28] Moreover, GABA produced by GM modulates abdominal pain and visceral sensitivity.[29] Importantly, a recent study confirmed increased GABA levels in the plasma on transplanting a fecal microbiome from lean to obese individuals.[25,75] Germ-free mice have decreased GABA levels in blood serum.[27] In a mice study decreased GABA level were observed upon antibiotic exposure.[28] In particular, GM modulates abdominal pain and visceral sensitivity via GABA production.[29] Increased GABA levels exhibited in the human plasma on transplanting a fecal microbiome.[25,75]

In this report, we elaborate for the first time on the positive influence of GABA and GABA-producing bacteria (S. *thermophilus* and *L. pentosus*) on the treatment of ACLT-induced OA in vivo and in vitro. The GABA assay confirmed the GABA secretion in fecal, small intestine content, and serum levels, as represented in Fig. 3B.[48] The probiotic treatment harbours calcified cartilage change, eliminating OA entirely or partially.[76] The expression of $GABA_{B}r$ and its importance for chondrocytes normal function and $GABA_{B}r$ knockout mice have decreased body size and delayed calcification.[50,66] GABA acts as an endocrine factor in distinct cell types, such as osteocytes, osteoblasts, and chondrocytes.[77] More severe OA pain related to low GABA further strengthens our hypothesis and utilizes GABA as a nutraceutical treatment against OA.[78] However, the role of GABA in chondrocyte maturation needs to be further explored.

GABA acts on the endocrine system and increases growth hormone, and serves as a neurotransmitter.[56] Reduced arthritis pain and improved function by aerobic exercise is evident,[79] and declined expression of proinflammatory cytokines.[80] GABA levels were also stated to elevate 20 times on exercise.[81] As Fig. 7D elaborated, GABA is essential to the tricarboxylic acid (TCA) cycle. This conversion is associated with considerable increases in energy demand and production.[82] In a recent study, lentiviral-based knockdown and overexpression of Abat (an enzyme that catabolizes GABA into succinate acid) in murine knee joints increased cartilage degradation in a surgical-induced model of OA.

Under the mechanical stress of OA, the chondrocytes lack the potential for cartilage repair. We examined the expression profile of

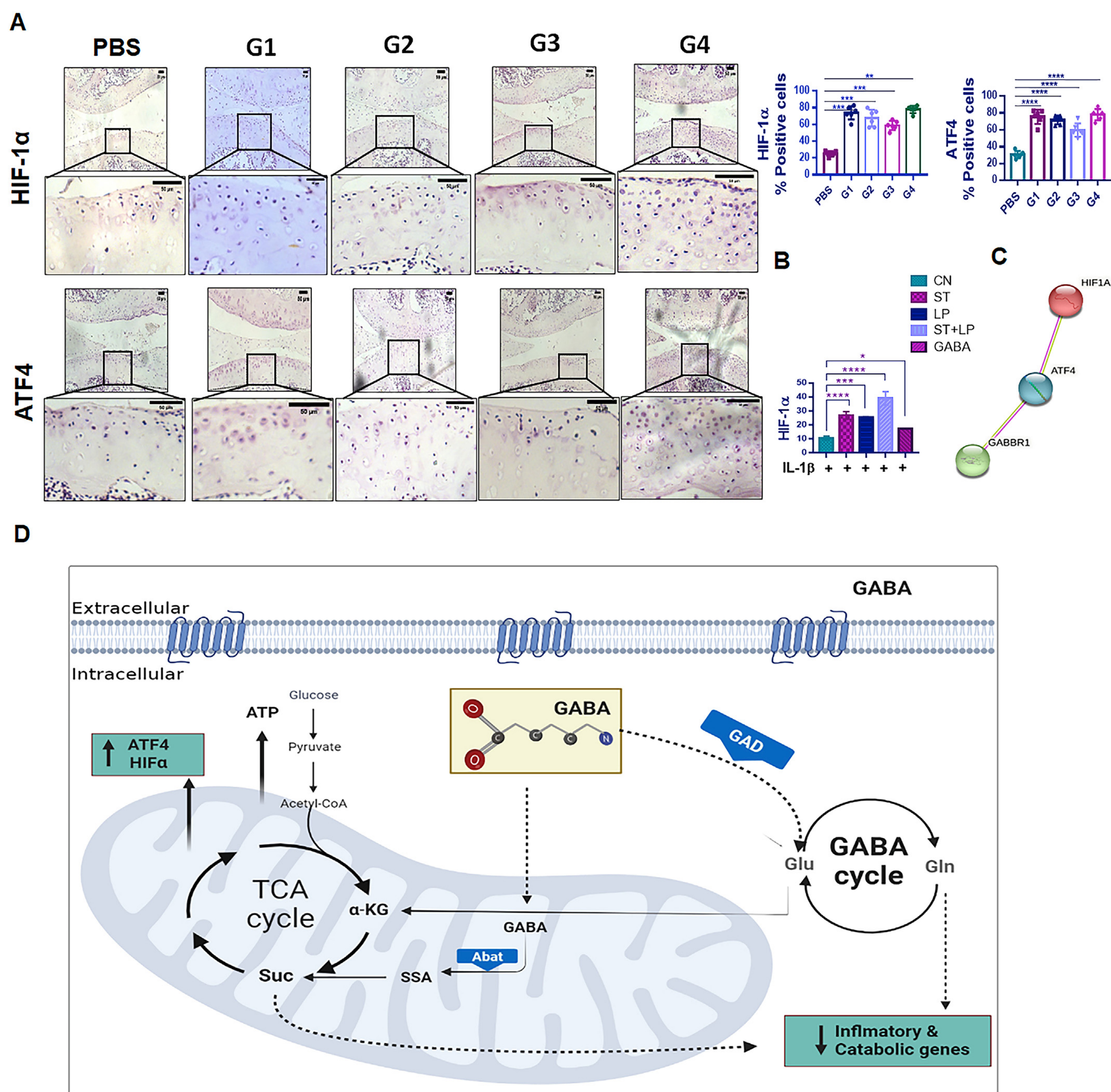

Fig. 7. GABA interacts with ATF4 & HIF-1α. A- Immunohistochemistry (PBS: Control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes; G4: treated by GABA). The mice joint sections of different treatment groups were examined for ATF4 & HIF-1α by IHC. Increased number of cells representing these cellular markers by GABA secreted by Probiotics. **B-** The compared treatment effect on human chondrocytes in the presence of IL-1β on HIF-1α via Real-time qPCR. **C-** STING experimentally proved an influence on these genetic markers by the STING database. The gold colour represents the theoretical, and the pink colour represents the experimental linkage of these genes. **D-** The possible mechanism of action of GABA, TCA cycle described in the existing literature. We confirmed via proliferation markers. Significant differences between groups were identified via ANOVA with a Dunnett's multiple comparison test (P values for effect are reported; *$p < 0.05$, **<0.01, ***<0.001, ****<0.0001).

various proliferation markers in vitro. A drastic acceleration in cell number was observed by CCK8 assay, as Fig. 1 represents, and elevation in the expression of chondrogenic genes (Fig. 1B). The most striking finding in the study is that CGP 52432 (a potent $GABA_{B}r$ inhibitor) significantly blocked the chondrogenic effect of ST, LP & GABA on chondrocytes (Fig. 6). This further assured the critical role of GABA in the normal function of chondrocytes. In accordance with existing studies, inhibition of Abat via vigabatrin (an irreversible inhibitor of ABAT) leads to increased GABA levels. Also, it prevents the development of OA in mice.[47]

There was a distinct increase in both in vitro and in vivo gene expression of significant anabolic markers such as COL2, ACAN, and SOX9 (Figs. 1 and 4).[13,72,83] The effect of the microbiome is also linked to its capacity to reduce inflammatory markers such as IL6 and promote anti-inflammatory markers such as IL10 and IL4 (Fig. 5). RT-qPCR expression of increased IL10 compared to the PBS group (Fig. 5). The expression of inflammatory cytokines down-regulated significantly, such as interleukin-1 beta (IL-1β), IL6 and

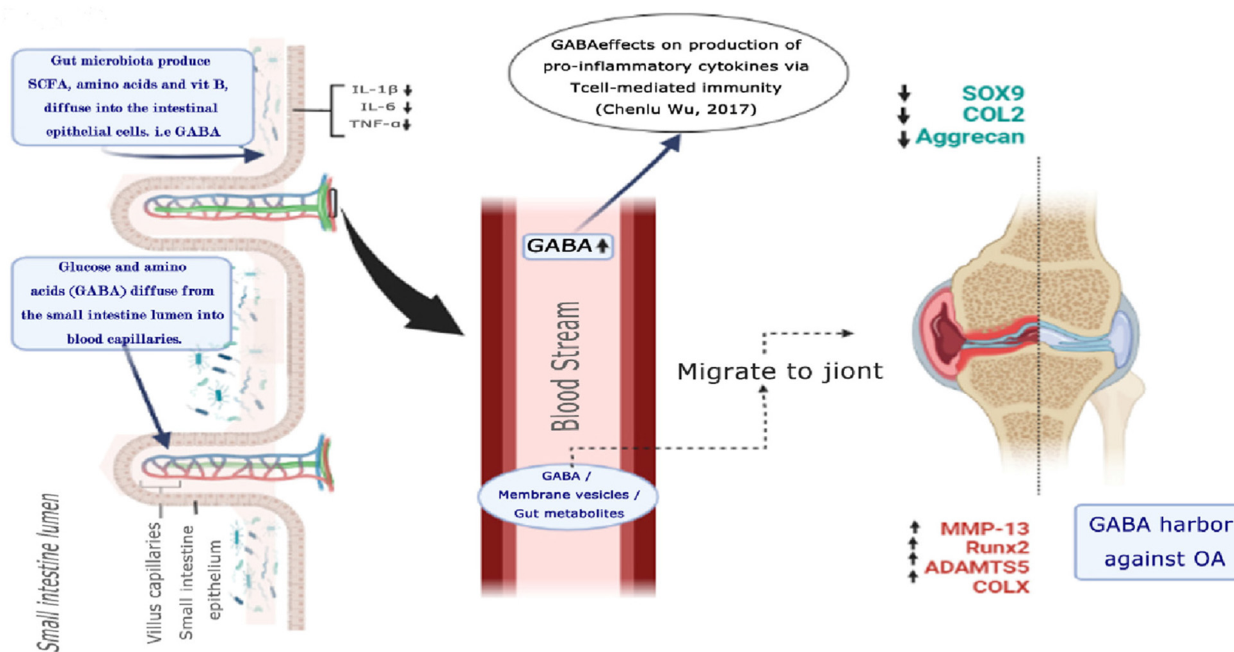

Fig. 8. Proposed hypothetical mechanism of gut-joint-axis, explaining the association between the gut microbiome (*S. thermophilus* & *L. pentosus*). Bacterial metabolites (i.e., GABA) could pass the gut-blood barrier and possibly either diminish or downregulate inflammation. Protect the knee joint by upregulating chondrogenic markers, such as AGCAN, COL2, and SOX9. Also, it down-regulates the catabolic markers of knee cartilage, i.e., MMP-13, COLX, Runx2, and ADAMTS5.

tumor necrosis factor-alpha (TNFα) in mice joints and intestines of treatment groups (Fig. 5A).[13] The expression level of IL10, IL4, IL6, and IL-1β assessed by the IF of joint and intestine sections. The results showed a significant decrease in IL6-positive cells in treatment groups (Fig. 5A and B).

TNFα and IL-1β stimulate the production of matrix metalloproteinases (MMPs).[84] The MMP13 has a predominant role in OA because they rise in the cartilage degradation process. MMP13 is produced by chondrocytes that reside in the cartilage. The percentage of MMP13 positive cells and Runx2 in mice joints downregulated by treatment compared to the PBS group as shown via IHC (Fig. 4A). Deletion of Runx2 from mice chondrocytes result in a decrease of OA progression. The downstream target of Runx2 is MMP13. Runx2 plays a crucial role in conciliating Mmp13 and Adamts5 expression during the development of OA.[85] The expression of Adamts5 was also downregulated, as represented in Fig. 4E. The upregulation of Adamts5 and Adamts4 was linked with the progression of OA.[86] The protein expression of COLX and MMP13 declined as well, representing a similar trend to IF (IL6) results (Figs. 2 and 5).

Treatment of chondrocytes with vigabatrin, an FDA-approved drug, increase GABA level in the cellular environment and also suppresses the expression of catabolic genes COLX, MMP13, and Runx2 in chondrocytes.[47] However, our study is the first to utilize GABA-producing microbes and analyze GABA's direct effect on chondrocyte proliferation and OA mice.

GABA stimulates chondrogenesis by upregulation of HIF-1α, ATF4, and the activation of $GABA_B$ receptor as the anabolic effect of GABA diminished by $GABA_{B}r$ inhibitor (CGP 52432) (Fig. 7A). The $GABA_{B}r$ is reported to activate ATF4 in different brain regions directly.[87,88] The suppression of proliferation and anabolism by GABA-inhibitor further supported and strengthened our hypothesis (Fig. 6). Our results represent in vivo and in vitro upregulation of anabolic markers. In addition, rescued cartilage degeneration of the mice's knee joints on treatment.

5. Conclusion

GABA-producing microbes could use nutraceutical treatment in the management of osteoarthritis. Hence, we report the positive influence of GABA-producing *S. thermophilus* & *L. pentosus*. We also analyzed the effect of blocking GABA receptors in vitro. However, the impact of low GABA on the TCA cycle needs further analysis for a deeper understanding of the molecular mechanism. We speculate from the data presented here harbour the effect of GABA and probiotics producing GABA against OA.

As summarized in Fig. 8, the probiotics (*S. thermophilus* & *L. pentosus*) produce GABA and control inflammation in the intestine by diminishing the inflammatory factors. GABA produced by these probiotics passes the gut barrier and is absorbed in the blood, increasing the GABA concentration in blood serum and protecting the joint cartilage. Upregulation of anabolic markers such as ACAN, SOX9, and COL2 and downregulation of catabolic molecular markers including MMP13, COLX, Runx2, ADAMTS4, and ADAMTS5 were observed in vivo and in vitro.

The intestinal microbiome is not only essential in maintaining the balance of the gut but also for healthy bones and joints. GABA may have functional roles in chondrocyte differentiation and maturation. This study serves as a foundation for further studies to elaborate on the role of GABA and GABA-producing microbes in regulating cartilaginous cell proliferation and treatment of OA.

Ethics approval

The Animal Care and Use Committee at Chongqing Medical University approved experiments. The number of used mice, species, strains, specifications, and grades were justified. All experiments were performed according to protocol. Apposite animal care was carried out throughout the experiment. Study methods and mice sacrifice criteria were according to the ethical practice of scientific purposes use and the respect for animals.

Availability of data and materials

This article and supplementary files include this study's analyzed or generated data.

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Authors contributions

All authors approved and consented to the final version of the manuscript. FJG and Amin U designed experiments; Amin U, RJ, R. SM, M. F, L. L, YYY, FNB. Carried out experiments; Amin U, Raza SM, and FJG analyzed data.

Prof. Guo FJ designed the manuscript, authorized all data, and was responsible for the data's integrity and the data analysis's accuracy. All authors approved the manuscript before submission.

Declaration of competing interest

The authors have no conflict of interest.

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Abbreviation list

OA	Osteoarthritis
GM	Gut microbiome
GABA	γ-aminobutyric acid
TCA	tricarboxylic acid cycle
ABAT	4-aminobutyrate aminotransferase
LAB	Lactobacillus strains
CM	Conditioned media
ACLT	Anterior cruciate ligament transection
ST	conditioned media *S. thermophilus*
LP	conditioned media *L. pentosus*
ST + LP	conditioned media *S. thermophilus* + *L. pentosus*
GABA$_{B}$r	GABA B receptor
SO	Safranin O
IHC	Immunohistochemistry
IF	Immunofluorescence
ECM	Extracellular matrix
MMPs	Matrix metalloproteinases
ACAN	Aggrecan
IL	Interleukin

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2023.06.002.

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