Probiotics Composition Harbors Against Osteoarthritis and Inflammation through GABA in Mice

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Research Article

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Abstract

Background: Osteoarthritis (OA) is an age-related disease with multifactorial etiology and its prevalence growing globally. The role of Gut microbiota is inevitable concerning musculoskeletal disease and health. A method of controlling inflammation and cartilage destruction through changes in gut microbiota is proposed. Previously reported data lack the specific approach to microbial clusters and biomarkers in understanding the interactions between host and microbiome.

Method: We adopted a novel approach to elaborate the positive influence of *S. thermophilus* and *L. pentosus* to treat Anterior cruciate ligament transection (ACLT) induced OA in vivo. For in vitro analysis Human Chondrocyte Cell Line (C28/I2) was used to analyze chondrogenic effect of microbes and GABA. Tukey's multiple-comparisons test or Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli test were used to statistically analyze the data.

Results: The gut microbiota-joint axis promoted chondrogenesis and inhibited catabolism. Selected bacteria produced GABA as postbiotic. This study is the first to represent the chondrogenic and protective effects of γ-aminobutyric acid (GABA) on human chondrocytes and cartilage tissue in mice. Oral administration of it down-regulated cartilage degradation in OA-induced mice and decreased inflammation.

Conclusion: We speculated the positive results from GABA and probiotics producing GABA against OA. GABA may have functional roles in chondrocyte maturation /differentiation. This data provides a foundation for further studies to elucidate the role of GABA producing microbes and GABA in the regulation of cartilaginous cell proliferation. These findings open future horizons to understand the gut-joint axis and for the treatment of OA. Thus probiotic / GABA therapy could act as a nutraceutical modulator for OA.

Background

Osteoarthritis is described as a common age-related, heterogeneous group of disorders, pathologically characterized by pivotal areas of loss of articular cartilage in diarthrosis, subchondral bone change, synovitis, and associated with varying degrees of osteophyte formation (1). According to a report, arthritis was the most common cause of disability overall (19.0%; estimated population affected =8.6 million) and among which 24.3% are women (2) and the global prevalence of disease estimated to exceed 250 million (3). 25 million (9.3% of the adult population) are estimated to report activity limitations due to arthritis-attributable by the next two decades. More than 50 percent will be adults (above 65 years) and working-age adults (45–64 years) will comprise almost one-third of cases (4, 5).

Available pain treatments have limited efficacy with toxic effects. To address the increasing burden of OA no structural disease-modifying therapy is permitted by regulatory agencies. (3, 6). OA is an illness with multifactorial etiology and one single therapy is dubious to be effective uniformly and universally. Cellular inflammatory and humoral mechanisms, which play important role in the pathogenesis of OA, are

important in the development of pain via interactions between the immune and nervous systems (7, 8). Further work is an unmet need of time, to determine effective inhibitors of the low-grade inflammation in OA, and to identify whether therapies that target inflammation could prevent or slow the development and disease progression (8).

The intestinal microbiome, which also recognized as a human organ by many researchers (9, 10) and vice versa (11). It is generally accepted as a mutually beneficial relationship (11). The microbiome exists in both eubiosis and dysbiosis (9). There is a vital relationship between the healthy, functional intestine and the microbiome (12). The microbiome also produces key metabolites such as short-chain fatty acids and impacts host physiology and health (13). The microbiome is crucial to maintain balanced immunity and homeostasis (12, 13). It emerged as an integral part of the precision medicine approach as it contributes to all aspects of health and disease but is also potentially modifiable by therapeutics (14-16). Surprising links of the microbiome with distant and diverse organ systems and disease processes have been described such as periodontal disease(17), innate immune cells(18), the microglia(19), hematopoiesis(20), renal ischemia(21), bone homeostasis(22) and osteoarthritis (23, 24).

A common factor in many of these diseases is inflammation (chronic or systemic); the gut microbiota involves in the inflammatory mechanism by direct production of bacterial metabolites (also known as postbiotics) or inducing proinflammatory cytokines via host immune cells (62). The contribution of inflammatory markers is now understood to contribute to OA and as it is reported that the gut microbiome is a regulator of inflammation (25, 26). Given that there has been growing research interest in the microbiome, and studies have shown that the cartilage deteriorating during OA can be rescued by changes of the gut microbiota based on the concepts of the osteomicrobiology, gut–joint axis (12, 22, 27). A method of controlling chronic systemic low-grade inflammation by changing gut microbiota has been projected as a new nutraceutical treatment for OA (3, 23, 28, 29). Probiotics are extensively studied to deliver health benefits to the host by changes in the gut microbiota and by producing postbiotics.

The metabolites produced by gut microbiota had been causally related to the progress of cardiovascular diseases. This pathway can be used for drug treatments and it can prevent the development of atherosclerosis in mice revealed by studies (30). The direct influence of gut microflora on the drug metabolism capacity of the host expressed an interplay between bacterial and host metabolism (31). Recently the idea of using bacterial metabolites for the treatment of diseases is prevailing. Researchers around the globe focused on the impact of molecules produced by different bacteria within the human body as a result of metabolism called postbiotics, which can lead to protection against several diseases in humans (32).

γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter produced by the decarboxylation of glutamic acid (33) catalyzed by glutamic acid decarboxylase (GAD), play a crucial role in the immune system and GABA regulates communication between neurons (33-37). The concept of utilizing bacteria for GABA production is already established in the treatment of different diseases (32, 38, 39). In particular the T cell-mediated immunity, through activation of signal pathways and their influence on the production

of inflammatory cytokines (34). GABA is also associated with behavior, cognition, glucose tolerance, oxidative stress, and pathological conditions such as diabetes and Alzheimer's disease (33, 34, 36, 40, 41). Neurotransmitters bind to their receptors in T-cells and directly trigger or suppress T-cell functions. It plays a vital role in T-cell functions in a direct, specific, and potent manner (35). GABA regulates the release of a variety of cytokines in a concentration-dependent manner (42). Macrophage-mediated inflammation is considered to have a causal role in OA-related pain and severity. GABAergic components provide a new horizon for inflammatory and autoimmune diseases (34).

GABA levels in both skeletal and plasma muscles significantly increased on exercise in the mice model (43). Lower serum levels of GABA were observed in older women with osteoporosis (41). Local production of GABA in hypertrophic-zone (an important long bone growth site) chondrocytes of the rat tibial growth plate also reported and Activation of $GABA_A$ and $GABA_B$ receptors increases ATDC5 cell proliferation in culture. Acutely, knocked down of $GABA_BR1$ downregulates expression of aggrecan (AGCAN) and collagen type II (COL2), the early chondrogenic markers. Up-regulation of catabolic markers osteopathic and collagen type X (COLX) (44). The putative GABA effect differs with cell types. Existing literature suggests that GABA could be applied as an adjuvant treatment against osteoarthritis. Therefore, this study aimed to examine the possible effects of GABA as a postbiotic compound on the treatment of OA, whether oral administration of GABA producing bacteria and GABA can protect against pathological changes and cartilage loss during OA.

Methods

Bacterial Culture and Preparation of Conditioned Media

Streptococcus thermophilus (S. thermophilus, CICC 24202) and Lactobacillus pentosus (L. pentosus, CICC 6222) were purchased from the China Center of Industrial Culture Collection. S. thermophilus were anaerobically grown at 37C in MRS broth (Fluka, Heidelberg, Germany) containing 0.05% L-cysteine (Roth). L. pentosus cultured in a microaerophilic environment.

The overnight culture of S. thermophilus and L. pentosus were centrifuged at 4000g for 10 minutes and the bacterial pallet separated from the media. The media was discarded and bacterial pellets were resuspended in PBS washed and fixed with 5% formaldehyde for 3 hours at 4^oC, washed thrice with sterile PBS before incubating with cell culture media. Conditioned media (CM), bacteria (5×10^7 CFU/ml) from freshly grown cultures were transferred to Dulbecco's modified Eagle's medium (DMEM) and cultivated for 3 hours according to the requirements of bacterial species. Bacteria were separated from CM via centrifugation (4300 g, 15 min, RT). pH Adjusted to 7.6, filter-sterilized (0.22 mm) and stored at -80 (supplementary fig S1) (45).

Cell Culture

C28/I2 Human Chondrocyte Cell Line is widely used as a model cell line for studying normal and pathological cartilage repair mechanisms related to chondrocyte biology and physiology were purchased from Sigma-Aldrich (cat # SCC043). DMEM (GIBCO Invitrogen, Carlsbad, CA), 10% FCS (FCS Superior; Biochrom, Berlin, Germany), and 1% antibiotic antimycotic (GIBCO Invitrogen, Carlsbad, CA) in Cell tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) (5% CO2, 37^{o}C). Cells were grown in CM S.t & CM L.p, both media collectively and 0.1µl Gama Aminobutyric acid (GABA, Sigma# A5835). The results were compared with the no treatment group after 2 days of treatment.

Cells were stimulated with GABA inhibitor for 8 minutes then cultured in CM S.t & CM L.p, both media collectively and 0.1µl GABA for 2 days. The protein and mRNA were extracted to analyze the effect of $GABA_Br$.

Animal study

We purchased young (6-8 weeks) C57BL mice from Chongqing Medical University. All mice were kept under sterile conditions with five or less than 5 mice per cage. Mice had free access to food and water. The mice used for all experiments were assigned randomly to control or treatment groups. For treatments, normal C57BL mice were administered PBS, S. thermophilus, L. pentosus, both or GABA (γ aminobutyric acid) via oral gavage for 2 months until the sacrifices. Gavage treatment was initiated 2 weeks before ACLT.

OA was Surgically induced by an Anterior cruciate ligament transection (ACLT) injury to C57BL mice at 9-10 weeks of age. Animals were anesthetized using a mixture of 10% chloral hydrate 400ul/100g administered by a single intraperitoneal injection, and hind limbs were shaved and prepared for surgery, aseptically. For the ACLT surgery, the ACL was transected with k-wire after opening the joint cavity, under a surgical microscope. After rinsing with saline to remove tissue debris, the skin incision was closed with 2-3 sutures (46). After 2 months of the gavage treatment, mice were euthanized and samples were collected for mRNA, protein quantification, and histological assessment of tissues. Whole blood was drained via eye rupture (supplementary fig-S2).

Protocol for oral administration

Oral feeding was initiated 14 days before ACLT and continued up to 6 weeks. In feeding protocols, individual or combination bacteria was suspended in 1 ml PBS and the turbid suspension was administered orally using gavage needles thrice a week. Mice were into five groups according to the difference in feeding regime (Fig-2 a, b, supplementary Fig.S2).

Culture S. thermophiles and L. pentosus at 37°C for 24 h. Cells were harvested by centrifugation (4000 rpm for 10 min), washed, and re-suspended in phosphate buffer saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), for oral administration to mice. A Viable Count for Culture and desired

concentration of bacteria was obtained Using OD Values (600 nm). Od was adjusted and bacteria were stored in Aliquots. Aliquots of these suspensions were frozen at -80^{o}C and stored until used. One fresh aliquot was thawed for every new gavage treatment to reduce the changes in bacterial viability.

The number of live cells after freezing and thawing was determined by colony-forming unit (CFU) counting on MRS-C agar after 48 h incubation. For both tested strains, more than 90% of cells were alive upon thawing and no significant differences were found during storage time. The number of vial cells was determined several times throughout the gavage period of two months,

The amount of each treatment according to body weight was as follows: (G1) S. thermophilus (2×10^{10} CFU/kg, 500 mg/kg); (G2) L. pentosus (2×10^{10} CFU/kg, 500mg/kg) (G3)Mixture (S. thermophilus + L. pentosus; 2×10^{10} CFU/kg, 500 mg/kg); (4) GABA (50 mg/kg); (5) PBS (phosphate buffer saline). Using body surface area-based formula (47), the dose of GABA for mice (4 mg/20g) was determined based on the amount recommended previously. We also fed 0.4 mg/20g/day of GABA via drinking water to mice to mimic continuous GABA supply identical to postbiotics. Since the optimal dose of S. thermophilus + L. pentosus was not reported, we used an effective dose showing suppressive effect against OA based on previous studies (15, 28).

Real-time Q–PCR

To extract RNA, the intestine and knee joints that underwent surgery and no-surgery controls were immediately transferred to liquid nitrogen on dissection. Tissues were pulverized with help of mortar and pestle. Total RNA was extracted from knee joints using BioTeke (RP1202) RNA Rapid extraction kit and was reverse transcribed to cDNA using SuperScript II reverse transcriptase following the manufacturer's protocol. RT-PCR reactions were carried out using the PowerUp SYBR Green Master Mix Kit (Applied Biosystems) following the cycling protocol: 50°C for 30 min, followed by 40 cycles at 95°C for 15 min, 94°C for 15 s, and 60°C for 45 s. The primer sequences used for RT-PCR are enlisted in supplementary table S1.

Real-time PCR for Aggrecan, Adamts-5 COL2, IL4, Il6, IL10, TNF-α, and SOX9, was performed using the BIO-RAD Real-Time PCR System with SYBR Green RT supper Mix for qPCR (Applied Biosystems by Life Technologies, 4367659). All signals were normalized to that for GABDH. Relative gene expression was calculated by the ΔΔCT method, ΔCT was calculated using the GABDH as a reference gene. ΔΔCT was calculated relative to the non-surgery control samples.

In the in vitro studies total RNA was extracted from cells were compared between treatment and no treatment groups like mentioned above. See Supplementary Table S1 for a list of primers.

Tissue fixation and histologic preparation

Mice were euthanized using Chongqing Medical university-approved method. Both knee joints and intestine were removed and fixed for 72 hours at 4^{o}c in 4% formaldehyde (neutral PH). Knee joints were decalcified in EDTA for 14 days, and all tissues were processed using a microwave processor and were embedded in paraffin. Tissue blocks were sectioned in the midsagittal plane through the medial compartment of the joint. 5-µm thick sections were cut from each paraffinized tissue. Safranin-O/Fast Green (catalog G1371, Solarbio life sciences) and Alcian Blue Hematoxylin/Orange (catalog G1121, Solarbio life science) staining's were performed to analyze knee joint tissue. Knee tissue sections were stained with Safranin O/Fast Green for OARSI scoring and histomorphometric analysis. Intestine sections were used for Immunofluorescence (IF) staining. Unstained knee sections were employed for Immunohistochemistry (IHC) and IF staining.

Histomorphometric analysis of cartilage

Tibial and femoral cartilage area, total chondrocyte number, and Safranin O+ chondrocytes were quantified by a blinded observer via histomorphometry as previously described. Briefly, using Safranin O-stained sections, the articular cartilage area was measured using the ImageJ software. For each specimen, measurements were taken from one slide at each of the 3 levels (50µm apart) on both the tibial plateau and the femoral condyle in a 200-µm wide area of interest centered on the joint. Total chondrocyte and Safranin O+ chondrocytes were calculated. Three measurements were obtained across the joint for each specimen and the average was calculated.

Mouse OARSI scoring

To estimate the amount of cartilage repair or degeneration, a semiquantitative histopathologic grading system was employed through blinded graded observations by two observers who followed the Osteoarthritis Research Society International (OARSI) scoring system (48, 49). Utilizing this system, Safranin O-stained joint sections were graded based on the following scale: 0, normal cartilage; 0.5, loss of proteoglycan stain without cartilage damage; 1, mild superficial fibrillation without cartilage loss; 2, fibrillation/cliffing extending below the superficial zone coupled with partial loss of surface lamina; 3, vertical clefts/erosion of the cartilage to the calcified zone over 75% of the cartilage surface.

Immunohistochemistry (IHC)

Mouse knee joint sections evaluated by IHC were deparaffinized in 2 changes of xylene for 10 minutes each, rehydrated in a series of ethanol (100% ethanol, followed by 95% ethanol, followed by 1 change of 70% ethanol), and rinsed twice in deionized water. An antigen retrieval kit (AR0022; Boster) was prepared for the sections for 1 minute at 37°C. 0.5% percent H_2O_2 in ddH_2Owere used to quench endogenous peroxidases for 10 minutes. The slices were incubated with selected primary antibodies at 4℃ overnight. sections were rinsed in phosphate-buffered saline containing 0.1% tween 20 (PBST,

catalog P1397, Millipore Sigma). Next, slides were blocked with 5% normal goat serum followed by overnight incubation at 4°C with rabbit anti-mouse AGCAN (1:200 dilution, catalog DF7561, Affinity), rabbit anti-mouse MMP-13 polyclonal antibody (1:200 dilution, catalog 18165-1-AP, Proteintech), mouse anti-mouse Runx2 polyclonal antibody (1:200 dilution, catalog AF5186 Affininty), slides were incubated with biotinylated anti-rabbit secondary for 30 minutes at room temperature. Slides were once again rinsed in PBS 3 times for 5 minutes each, followed by 2 washes in deionized water for 5 minutes each. Antibody binding to Aggrecan Mmp-13 and Runx2 antigen was detected by a 3-minute incubation with DAB peroxidase substrate (catalog zli90181, OriGene). 3-minute application of DAB peroxidase substrate. Nuclei were then counterstained with Mayer's hematoxylin solution (catalog AR0005, BOSTER) for 15 seconds.

Immunofluorescence

Intestine and joint samples of mice fixed in 4% paraformaldehyde, were deparaffinized as mentioned above. Permeabilized with PBS containing 0.5% Triton X-100 for 10 min and then blocked with 4% BSA containing 0.25% Triton X-100 for 30 min at room temperature. The intestine tissue slides were incubated with primary antibodies against IL-6 (1:200 dilution, catalog DF6087) and IL-1β (1:200 dilution, catalog AF5103) overnight at 4 °C. The knee joints were incubated with IL-6 (1:200) and COL2 (1:200 dilution, catalog bs-0709R). The cells were rinsed three times with PBS and incubated with goat anti-rabbit IgG (H&L). After washing, the nuclei were counterstained with DAPI for 5 min. Nuclear against IL-6 and IL-1β were imaged by fluorescence microscopy.

Immunoblotting

Proteins were separated by SDS–PAGE and then transferred on membranes. The membranes were blocked for 1 h with 5% BSA and incubated overnight at 4 °C with primary antibodies specific for MMP-13 (1:2000), COLX (1:1000, catalog A6889), IL6(1:500), ß-actin (1:2000, catalog AF7018), and GAPDH (1:2000, catalog AF5009). After washing away unbound primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:5,000; Bio-Rad, 1706515) and anti-rabbit secondary antibodies (1:10,000, catalog SA00001-2) and protein bands were detected using an ECL detection system.

GABA Assay

The frozen samples of small intestinal content were diluted (10%, (w/v)) in PBS and homogenized by vertex. The samples were then centrifuged for 20 minutes at 3000rpm. A commercially available enzyme immunoassay was then used for the quantitative determination of GABA by ELISA (MM-0442M2). Quantification of unknown samples was calculated by comparing their absorbance with a standard curve prepared with known standards and results were standardized to individual sample weights.

The whole blood was drained out via eye rapture and allowed to coagulate naturally. Centrifuged at 3000rpm for 20 minutes, the supernatant was collected and used for ELISA. The fecal water was also used to determine GABA levels. The fecal samples were collected and mixed with distilled water and vertex until slurry was made. Centrifuged like above and the supernatant was used for GABA assay (supplementary figure S3B).

Statistics

All statistical analyses were performed with Prism 7 software from GraphPad (San Diego, CA, USA). Statistical significance was determined to be $P < 0.05$. To determine the average and standard deviation in the model and characterization methods; all in vivo data expressed as the mean and each data point signifies an individual mouse. All in vitro data expressed as averages ± standard deviation. Statistical significance (P values) was determined by or one-way ANOVA (Tukey's multiple-comparisons test or Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli).

Results

1. S. thermophilus and *L. pentosus* increases anabolism and cell proliferation in chondrocyte

The therapeutic strategies that support and encourage the anabolic activities of chondrocytes are, need of time. We first examined the expression profile of a variety of chondrocyte differentiation markers in the C28/I2 Human Chondrocyte Cell Line. Cultured with conditioned media *S. thermophilus (*CM *S.t)* and CM *L. pentosus* (CM *L.p)*, and GABA (γ aminobutyric acid) for different periods(45). A drastic increase was seen in the mRNA expression of all chondrogenic marker genes examined. We analyzed the expression levels of anabolic and inflammatory markers in chondrocytes cultured in CM *S.t,* CM *L.p,* both collective. GABA was included in the study to compare and ensure effect with bacterial strains which produced GABA Fig.1a.

Chondrocytes are the main cellular population involved in the maintenance and degradation of articular cartilage structure(50, 51). The presence of $GABA_BR$ (GABA B receptor) and its important role in chondrogenesis and local production of GABA in hypertrophic-zone chondrocytes of the rat growth plate are reported previously (52, 53) suggesting the involvement of GABA in chondrocyte physiology. Although GABAergic signaling machinery could regulate the endochondral bone formation through directly regulating chondrogenesis, little attention has been paid to the possible direct effect of GABA in chondrocytes to date.

Chondrocytes are the primary inhabitants of articular cartilage and are liable for the synthesis and preservation of ECM(50). As represented in Fig.1 a, b, and c treatment of *S. thermophilus* and *L.p* increased the expression of anabolic markers (COL2 and Aggrecan), cell proliferation markers (CyclinB1,

CyclinD1, PCNA & cdk1). Although co-administration of *S.t* and *L. pentosus* decreased expression slightly than independent treatment groups, these effects were statistically significant ($p < 0.05$). Furthermore, we have attempted to elaborate on the functional expression of particular chondrogenic markers in c28/12 cells. GABA may have functional roles in chondrocyte maturation /differentiation. This data provides a foundation for further studies to elucidate the role of GABA and GABA producing microbes in the regulation of cartilaginous cell proliferation.

2. S. thermophilus and *L. pentosus* inhibits catabolism in chondrocyte

The mechanical stress of chondrocytes overburdened during OA. Chondrocytes are being exposed to pro-inflammatory cytokines, chemokines, oxidants, and catabolic factors in OA which lead to poor autophagy and cell death (54). Matrix metalloproteinases (MMPs) have been implicated in cartilage degradation in OA. Inhibition of MMPs has been proposed as a therapeutic strategy to prevent cartilage. The RT-qPCR expression data indicated that an optimum concentration of GABA, CM *S.t,* CM *L.*p, and both collective downregulate the expressions of cartilage matrix-degrading protein compared to no treatment control, such as MMP-13 and ADAMTS-4 was reduced (Fig.1d). The expression of MMP-13 was confirmed by western blotting in chondrocytes (Fig.1, e, & f). Taken together, these results indicated that GABA decreased the expressions of MMP-13, and ADAMTS-4 and induced expressions of COL2 and aggrecan, which led to reduced cartilage extracellular matrix (ECM) degradation and promotion of cartilage anabolism (Fig.1).

3. S. thermophilus and *L. pentosus* harbor against cartilage loss in OA mice

We then divided C57BL mice into five groups randomly according to the difference in the treatment regime. To model the pathological process of the OA we employed the standard (ACLT) mouse injury to initiate OA. Mice were sacrificed 6 weeks after ACLT and tissue were collected for processing (Fig.2a & b). Mice weight was monitored and the weight of every treatment group has no significant difference (Fig.2c). We examined the impact of GABA and bacterial treatments on the progression of joint degeneration (Fig.2). In this context, the OA was more progressed in PBS treated mice than other treatment groups, with most joints terminally degenerated (Figure 2d) Cartilage and chondrocyte loss were more significant in every metric examined. All treatment groups affect the degenerative process in mice. Remarkably, GABA and microbial-treated mice were rescued from the degenerative effect of OA (Fig.2d and Safranin O+ staining). The histomorphometric outcome was significantly improved, with Osteoarthritis Research Society International (OARSI) scores also trending toward improvement (Fig.2e, & f) (49).

The knee joint of the PBS group showed loss of cartilage tissue and infiltration of mononuclear cells result in an increased loss of the articular cartilage. PBS-treated mice exhibited uneven and rough cartilage surfaces, and their superficial fibrils were visible. ACLT induced distortion of surface and deep. Group 3 also showed destruction of cartilage, similar to the PBS-treated group, representing independent bacterial treatment more effective than collective treatment. However, throughout all the tested results, coadministration of *S. thermophilus* and *L. pentosus* was less effective in protecting arthritic changes and suppressing cartilage destruction than separate treatments (Fig.2). Based on results, GABA and GABA producing microbes provide nearly complete protection against trauma-induced OA, suggesting its potential as a nutraceutical disease modifier in this context.

4. S. thermophilus and *L. pentosus* promotes anabolism and reduce catabolism in OA mice

We next investigated the chondrogenic markers, aggrecan, SOX9, and COL2, which were significantly increased (Fig.3). The increasing trend of aggrecan positive cells was observed in Immunohistochemical (IHC) evaluation of G1 (*S. thermophilus* treated mice), G2 (*L. pentosus* treated mice), G3 (treated by both *S. thermophilus* and *L. pentosus),* and GABA treated mice as compared to PBS treated (Fig.3a, c). Furthermore, knees were exposed to immunofluorescence detection of COL2, with representative staining Fig-3b. Nuclei are counterstained with DAPI. The expression of COL2 also increased in G1 (*S. thermophilus* treated mice), G2 (*L. pentosus* treated mice), G3 (treated by both *S. thermophilus* and *L. pentosus),* and GABA treated mice as compared to PBS treated (Fig.3b, d). The increasing trend of SOX9 and COL2 was analyzed by RT-PCR (Fig.3e & f).

Matrix metalloproteinase-13 (MMP-13) and ADAMTS-5, RunX2, and COLX which are widely studied in OA play a crucial role in cartilage ECM degradation (Fig.4). IHC analysis showed that the proportion of MMP-13 and RunX2 positive cells were significantly lower in G1, G2, G3, and GABA-treated mice compared with the PBS group (Fig.4a & b). The reducing expression of ADAMTS-5, MMP-13, and COLX was also confirmed by RT-PCR and western blot compared with the PBS group (Fig.4c, d, & e).

Taken together, these results indicated the chondroprotective effect of both GABA producing *S. thermophilus* and *L. pentosus.* Decreased expressions of MMP-13, RunX2, ADAMTS-5, and COLX, and increased expressions of aggrecan, COL2, and SOX9 were observed (Fig.3, Fig.4), which led to rescue mice from ACLT induced OA via suppressing cartilage ECM degradation and promotion of cartilage anabolism.

5. S. thermophilus and *L. pentosus* effects on the inflammatory factors of cartilage and intestine in mice

In OA inflammatory cytokines play a key role in tissue destruction by modulating the microenvironment. To investigate the role of *S. thermophilus* and *L. pentosus,* we detected the inflammatory cytokines affected by both probiotics or GABA in the joint and intestine (Fig.5, & 6). The Immunofluorescence (IF) staining, western blot, and RT-PCR analysis were performed on G1 (*S. thermophilus* treated mice), G2 (*L. pentosus* treated mice), G3 (treated by both *S. thermophilus* and *L. pentosus),* and GABA treated mice and compared to PBS treated mice group. The downregulated expression of IL6 was observed by Immunofluorescence (IF) staining (Fig.5a, b). The real-time qPCR expression levels of IL-6, and TNF-α decreased (Fig.5c, d). Co-treatment of *S. thermophilus* and *L. pentosus* showed decreased suppression of influx of inflammatory cells into cartilage tissues than separate. The IL6 and IL10 levels were observed in chondrocytes and were coordinated with in vivo experiments (Fig.5g, h) and western blot analysis of IL6 in cells (Fig-5e, f).

We also examined whether the therapeutic effect is linked with changes in the expression level of inflammatory cytokines (TNF-α, IL-6 & IL-1β) in the intestine. Interestingly, oral administration of the probiotics significantly decreased the levels of inflammatory cytokines, TNF-α, IL-6, and IL-1β compared to PBS. IL-6 and IL-1β were detected via immunofluorescence, with DAPI counterstaining of nuclei (Fig.6a, b, c). IL-6 and TNF-α mRNA levels were further assessed via RT-PCR analysis (Fig.6d, e). Collectively the level of inflammatory cytokines was downregulated in the intestine also (Fig.6).

6. The harbor effect of Probiotics composition relies on GABA

GABA assay was performed on small intestine content and serum samples of mice to ensure that selected microbes produce GABA in vivo. A commercially available enzyme immunoassay was used for the quantitative determination of GABA. The ELISA results assured the in vivo secretion of GABA by *S. thermophilus* and *L. pentosus* (Fig.6 f, g).

To strengthen our results further $GABA_BR$ inhibitor (CGP 52432) was included in the experiment model to analyze whether it blocks the chondrogenic effect or not. Chondrocytes were stimulated with a 10nM GABA inhibitor (CGP 52432) for 8 minutes to block the GABA receptor as previously described (55). After blocking GABA receptor ($GABA_Br$) cells were treated with conditioned media *S. thermophilus (*CM *S.t)* and CM *L. pentosus* (CM *L.p)*, and GABA (0.1μM). RT-qPCR expression of anabolic and chondrogenic markers significantly decreased (Fig-7).

The expression of different androgenic markers was observed in cells. The RT qPCR expression of Aggrecan, COL2, cdk-4, Cyclin D1, Cyclin B1, and PCNA was masked by GABA inhibitor and the effect of treatments was neutralized (Fig.7a, b & c). while the expression of Inflammatory markers IL10 and IL6 changed lightly (Fig.7d). The possible mechanism of GABA and GABA inhibitors' effect on chondrocytes is shown (Fig.7e).

Discussion

OA disease-modifying therapy is a critical unmet need due to the growing global prevalence of OA. The absence of clinical trials to address the current and future burden of OA is Noteworthy (56). Joint degradation and inflammation are inevitable with the progression of OA (57). There is a distinct role of Gut microbiota in shaping our immune system and its role in chronic and systematic inflammation is also evident (27). The role of key microbial species outside the gastrointestinal tract (GIT) can control unique physiological niches and potentiate implications on the establishment of immune homeostasis and protective response (26). In addition, the role of postbiotics in shaping the immune system is evident now that unique bacterial groups could dominantly impact immune system development and function under normal and inflammatory conditions (58, 59). Species that downregulate inflammation has been focused on as a future strategy as a disease-modifying agent in pathologic conditions (60, 61). There are fewer studies, which discussed the role of gut microbiota in OA of obesity. This is the first study to elaborate the positive influence of *S. thermophilus* and *L. pentosus* (as probiotics) to treat ACLT induced OA, with a focus on GABA secretion as Postbiotics (in vivo) and in vitro. The GABA secretion was confirmed by GABA assay in small intestine content and serum levels (39).

Aerobic exercise not only reduced arthritis pain and improved function but also the expression of proinflammatory cytokines declined on exercise (62). GABA levels were also reported to increase 20 times on exercise (63). GABA is an important part of the tricarboxylic acid (TCA) cycle, Abat is an enzyme that catabolizes GABA into succinate acid during the TCA cycle (Fig.7e). In a recent study lentiviral-based knockdown of the enzyme (Abat) that catalyzes GABA protects against cartilage degradation. In contrast, the Overexpression of Abat in murine knee joints through lentiviral injection increased the degradation of cartilage in a surgical induced model of OA (64).

Many bacterial metabolites are absorbed into the blood circulation, where they can act directly or be further processed by the host system, which leads to bioactive compounds that can act on tissues and affect the host metabolism (31) and there are many gut metabolites testified to change in a metabolic syndrome (12) The GABA modulating bacteria in the human gut was identified previously (38, 39). By keeping this in view, the *S. thermophilus* and *L. pentosus* were selected due to their increased GABA production in vitro and potential for probiotic treatment. The in vivo production of GABA was confirmed by ELISA *(*Fig.6f & g). However, oral administration of probiotic mixture (G3) in this study leads to downregulating the GABA level in the plasma (fig 6g). Which exhibit the antagonistic effect of combined treatment (*S. thermophilus* and *L. pentosus*). The same trend follows throughout the study, separate treatment was more effective as compared to combined treatment of microbes (65).

First, we examined the expression profile of a variety of chondrocyte differentiation markers in Human chondrocyte cell lines cultured with conditioned medium (CM *S.t* & CM *L.p*) and GABA. Different concentrations of GABA were tested to find the EC_{50}. A drastic acceleration was observed in the mRNA expression of all examined chondrogenic marker genes. In vitro, human chondrocytes were cultured for 2 days, in presence of CM *S.t* & CM *L.p* and GABA 0.1µm. proliferation markers such as Cyclin D1 and

proliferating cell nuclear antigen (PCNA) and Cyclin D1, cartilage matrix secretion, including COL2 and Aggrecan, PCNA were highly expressed as compared to no treatment group fig-1. The slicing of cyclin D1 is reported to suppress the proliferation of chondrocytes and initiate apoptosis (66, 67). The expression of $GABA_Br$ on chondrocytes is reported (24), $GABA_Br$ knockout mice reported to have decreased body size and delayed calcification as testified previously (52, 55). GABA could act as an endocrine or paracrine factor in distinct cell types such as chondrocytes, osteocytes, and osteoblasts (68). The presence and importance of the GABAergic system on chondrocytes was revealed by decreased body size and delayed calcification in $GABA_BR1$-KO mice (52). More severe OA pain is related to low GABA(69). However, the role of GABA in chondrocytes maturation needs to be further explored.

The chondrocytes lack the potential for cartilage repair during OA. The most striking finding in this study is that CGP 52432 significantly blocked the chondrogenic effect of CM *S.t*, CM *L.p* & GABA on cells (Fig.7a, b). CGP 52432 in a potent $GABA_BR$ inhibitor (47). Which further assured the important role of GABA in the normal function of chondrocytes Inhibition of Abat via vigabatrin is (an irreversible inhibitor of ABAT) also prevents the development of OA in mice (64).

The genetic expression of major cartilage markers (COL2, Aggrecan, SOX9) there was a distinct increase in both in vitro and in vivo (Fig.1, Fig.3) (28, 56, 70). In vivo, the expression of aggrecan was confirmed via IHC (Fig.3a), while the expression of COL2 was analyzed by IF (Fig.3b) This effect of the microbiota is also linked to its capacity to promote anti-inflammatory markers such as IL-10. RT-qPCR expression of IL10 was increased compared to the no treatment group (Fig.5e). The expression of Inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) was reduced significantly in mice joints and intestines (Fig.5, Fig.6) (28). TNF-α and IL6 known to have a catabolic effect on chondrocytes were downregulated in treatment groups. The expression level of IL6 and IL-1β was assessed by IF of joint sections. The results showed a significant decrease in IL6 positive cells in treatment groups (Fig.5 a & b). The reduced protein expression of IL6 in vivo and in vitro was confirmed by western blot (Fig.5i & j).

IL-1β and TNF- α stimulate the production of matrix metalloproteinases (MMPs) (71). The MMP-13, have predominant roles in OA because they rise in the collagen degradation process. MMP-13 is produced by chondrocytes that reside in the cartilage. The MMP-13 expression was decreased by treatment groups in vivo as evaluated by IHC on mice joint sections (Fig.4a). The percentage of Runx2 positive cells in joints downregulated in treatment groups as compared to the PBS group as shown via IHC (Fig.4b). Deletion of Runx2 in chondrocytes of 8 weeks old mice results in a decrease in OA progression. The downstream target of Runx2 is Mmp-13. Runx2 plays a crucial role in conciliating Mmp-13 and Adamts-5 expression during the development of OA (72). The expression of Adamts-5 was reduced as expressed by RT qPCR expression of joints (Fig.4c). The protein expression of COLX and MMP-13 was reduced as well, representing a similar trend to IHC (AGCAN) (Fig.2). and IF (IL6) results (Fig.5).

As summarized in the model of Fig.8, the gut microbiome (*S. thermophilus* & *L. pentosus*) produces GABA and control inflammation in the intestine through diminishing the inflammatory factors, then these

probiotics compositions pass the gut-blood barrier, and protect the cartilage tissue through upregulation of anabolic markers such as AGCAN, COL2, SOX9 and downregulation of catabolic molecular markers including MMP-13, COLX, Runx2, and ADAMTS-5. Intestinal probiotics are very important in maintaining the balance of gut and bone metabolism. Treatment of chondrocytes with vigabatrin and FDA-approved drug, which increase the level of GABA in the cell environment also suppressed the expression of catabolic genes Runx2, MMP-13, and COLX in articular chondrocytes (64). However, our study is the first of its kind for utilizing GABA producing microbes and analyzing the direct effect of GABA on chondrocytes and in OA mice.

GABA and microbes stimulate chondrogenesis by upregulation of anabolic genes, possibly via the activation of $GABA_B$ receptor as the effect of GABA diminished by $GABA_BR$ inhibitor (CGP 52432) and by inhibiting the activity of inflammatory cytokines and reactive oxygen species. The suppression of anabolism and proliferation by GABA inhibitor further support and strengthen our hypothesis (Fig.7). Our results exhibit upregulation of Anabolic markers in vivo and in vitro. Also, represent the rescue of mice knee joints from cartilage degeneration on treatment with GABA and GABA producing microbes.

Conclusion

GABA-producing microbes could be used effectively in the management of osteoarthritis. Hence, we report the positive influence of microbes producing GABA, but we do not take into account the effect of other possible gut metabolites / bacterial metabolites and their influence on joints. This study is confined to elaborate on the individual effect of selected microbial strains in the treatment of OA. The effect of the low level of GABA at the cellular level and its influence on the Abat and TCA cycle need to be analyzed deeply to understand the molecular mechanism. From the data presented here, we speculate that positive results from GABA and probiotics produce GABA against OA. GABA may have functional roles in chondrocyte maturation /differentiation. This data provides a foundation for further studies to elucidate the role of GABA and GABA producing microbes in the regulation of cartilaginous cell proliferation and treatment of OA.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved and performed according to protocols from the Institutional Animal Care and Use Committee at Chongqing Medical University. The species strains, grade, specification, and the number of animals used are justified. Appropriate animal care was carried out throughout the experiment. Mice sacrifice criteria and study methods were under the code of ethical practice for the care and use of animals for scientific purposes.

Consent for publication

Not applicable.

Availability of data and materials

All the analysed or generated data in this study are included in this article and supplementary files.

Competing interests

All authors declared that they have no conflict of interest.

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Authors' contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. F.J.G. and UZMA designed experiments; Uzma Amin, R.J., L. L., F.N.B., Y.Y.Y. carried out experiments; Uzma Amin, Shahid Masood Raza, F.J.G. analyzed data.

Prof. Guo FJ designed the manuscript and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final manuscript prior to submission.

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Figures

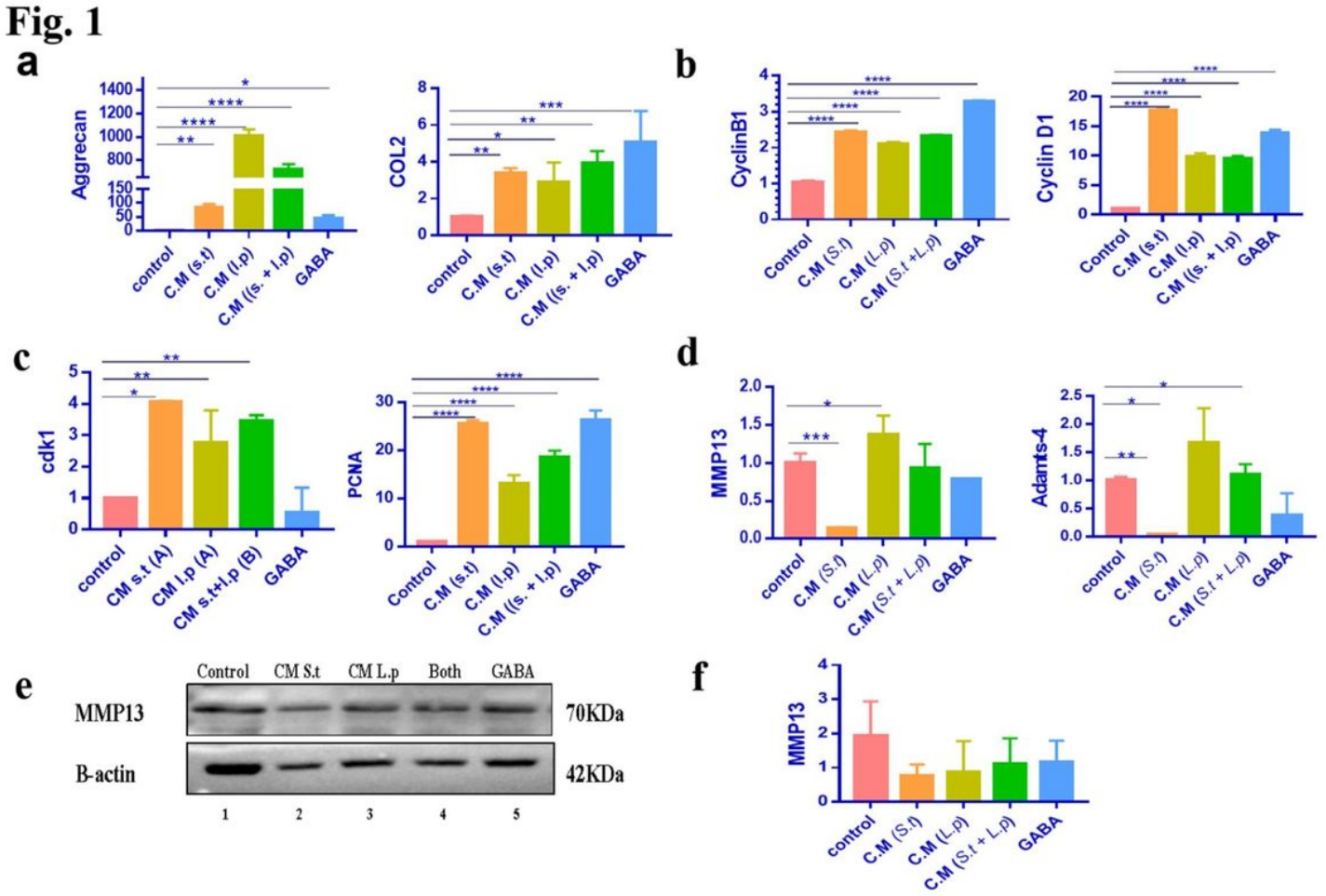

Figure 1

Effect of *S. thermophilus* and *L. pentosus* on anabolism and catabolism of chondrocytes. A- The increased expression of anabolic markers Aggrecan and COL2 by Real-time qPCR. cell proliferation, and inflammatory cytokines in the human chondrocyte cell line (c28/I2). **B, C-**Real-time qPCR analyses, CyclinB1, CyclinD1, PCNA, and cdk1. **D-The decreased expression of catabolism,** Adamts-4 & MMP-13 the catabolic markers for OA was tested via real-time qPCR against all treatment groups. **E-** Western blot for catabolic marker MMP-13. **F-** Western blot quantification. The mRNA levels were normalized to that of GABDH and then normalized to the control group. *$p < 0.05$ p**< 0.01, ***< 0.001, ****< 0.0001 compared with control by the Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli.

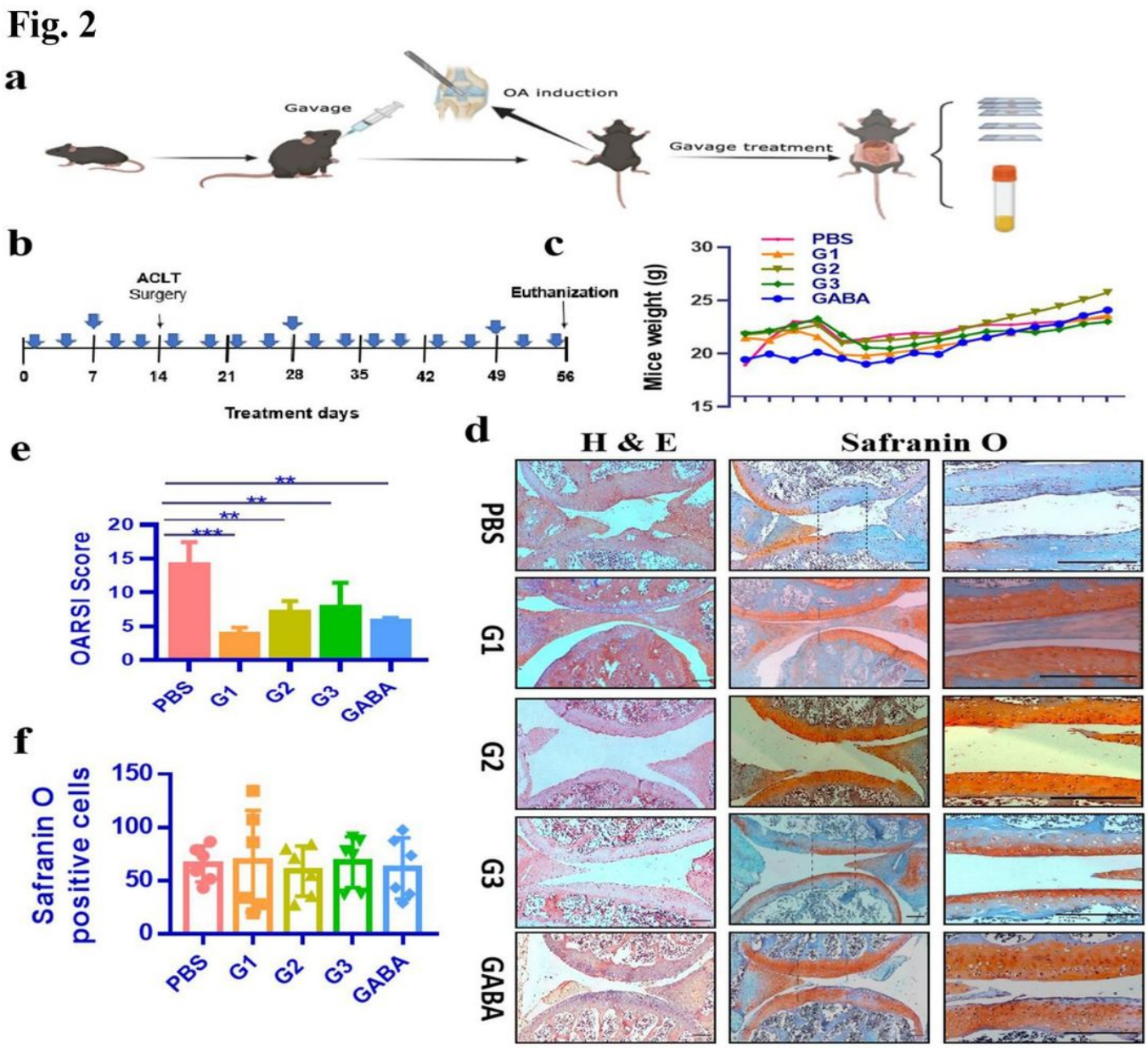

Figure 2

Effect of *S. thermophilus* and *L. pentosus* on the cartilage of OA mice. a, b 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. **c** -weight measurements 2 times a week throughout the study period. **d**- Representative images H & E staining and Safranin O staining mice knee joints. **e**- OARSI score grade for different probiotics. The five-treatment group is the following: PBS-treated, G1: mice treated with *S. thermophilus*; G2: *L. pentosus*; G3: treated with both microbes and GABA treated (n = 5) in each group mice (black scale bar represents 160 μm). **f**- Histopathological analysis of cartilage tissue. Percentage of

safranin O positive cells. *$p < 0.05$ p**< 0.01, ***< 0.001, ****< 0.0001 compared with control by one-way ANOVA by Tukey's multiple comparisons test.

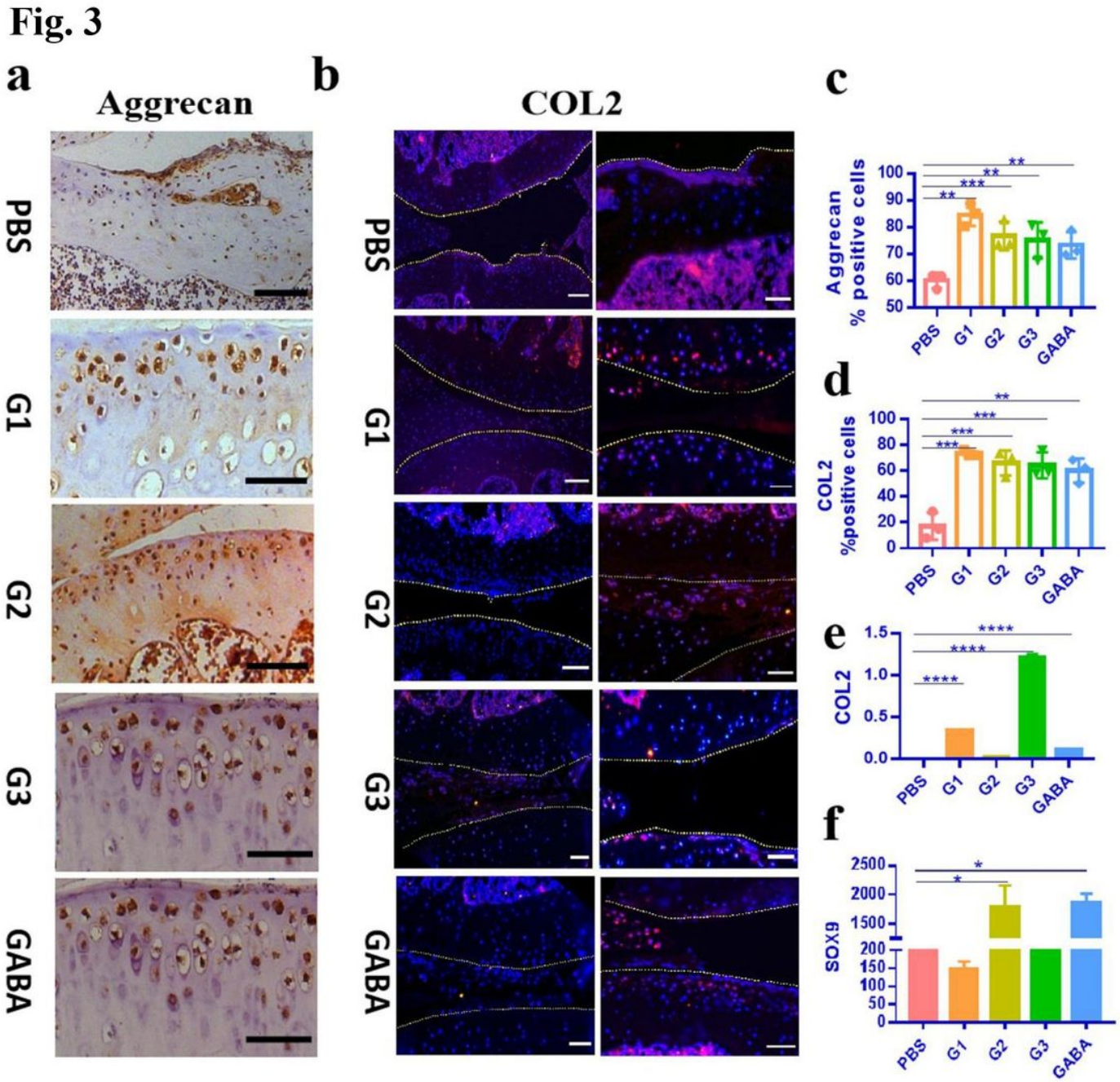

Figure 3

Increased expression of anabolism. a- (PBS: Negative control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes). Joint sections of mice were evaluated by COL2. The nucleus was stained by DAPI staining. The white scale bars represent 50μm, yellow dotes represent the cartilage layer. **b-** Aggrecan expression was examined via IHC on the joint section of different treatment groups of mice. The black scale bars represent 30μm, Dark brown represents positive cells and blue represent negative cells. **c-**Positive stained cells were counted. **d-**The percentage of aggrecan

positive cells in joint sections. **e, f-**RT-PCR analysis of chondrogenic marker COL2 and SOX9. *$p < 0.05$ p** < 0.01, ***< 0.001, ****< 0.0001 compared with control by one-way ANOVA by Tukey's multiple comparisons test.

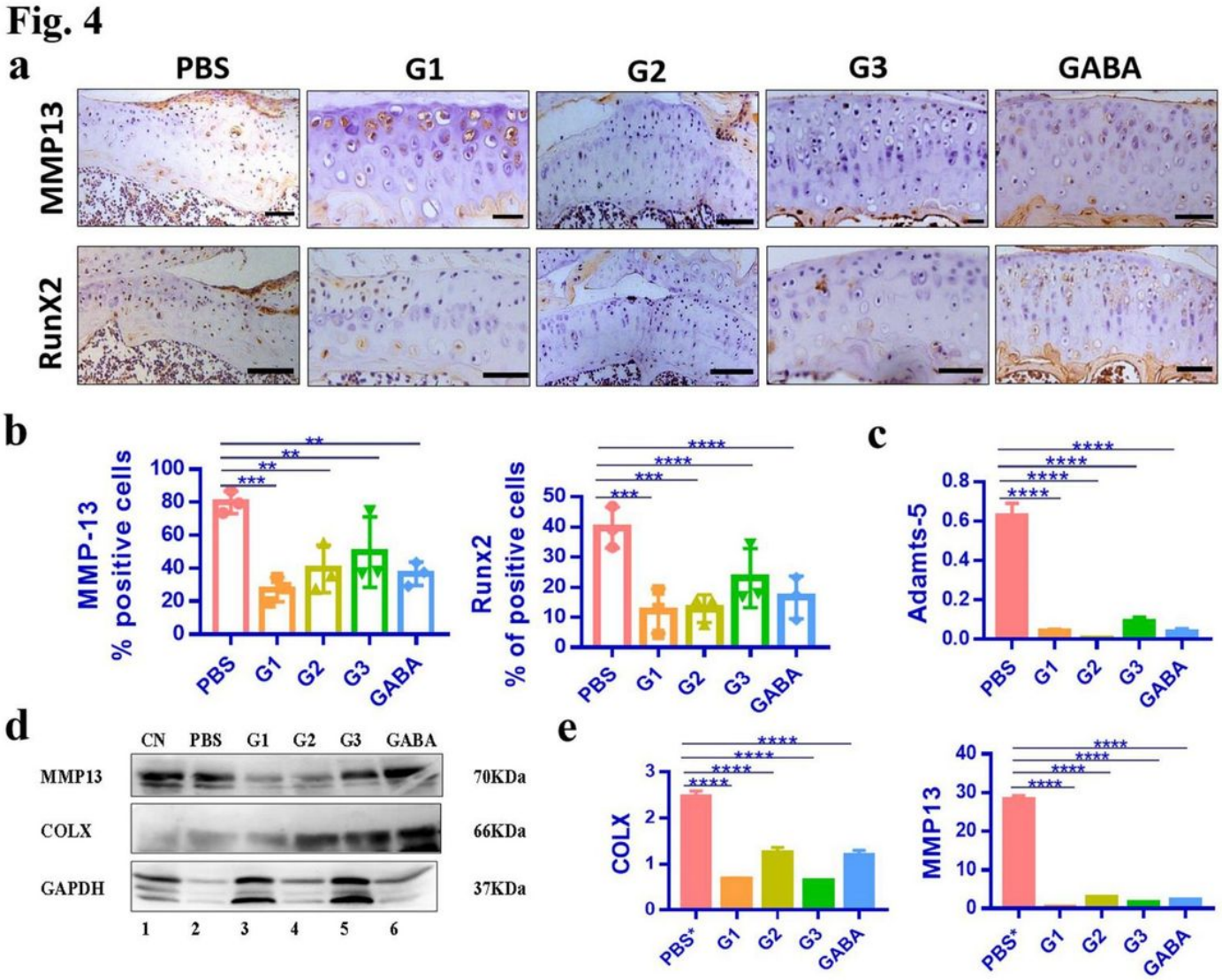

Figure 4

Decreased expression of catabolism. a- Immunohistochemistry. (PBS: Negative control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes). To evaluate articular chondrocyte hypertrophic differentiation and cartilage matrix changes, Runx2, and MMP-13 expression was examined via IHC on the joint section of different treatment groups of mice. The black scale bars represent 30 μm, Dark brown represents positive cells and blue represent negative cells. **b-** Positive stained cells were counted. **c-** RT-PCR analysis of catabolic marker ADAMTS-5. **d-**Western blot**. e-** quantitative analysis of western blot. *$p < 0.05$, **< 0.01, ***< 0.001, ****< 0.0001 compared with control by one-way ANOVA by Tukey's multiple comparisons test.

Figure 5

Decreased expression of inflammatory cytokine in mice. a-Downregulation of IL6 via Immunofluorescence. (PBS: Negative control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes). Joint sections of mice were evaluated by IL6. The nucleus was stained by DAPI staining. The white scale bars represent 50 μm, the yellow dotted line represents the cartilage layer. **b-**Positive stained cells were counted**. c-** Western blot quantification of IL6 in joints. **d-** Quantitative analysis of Western blot. **e, f-**RT-PCR analysis of inflammatory cytokines (IL-6, and TNF-α) in joints**. g, h**-RT-PCR analysis of inflammatory cytokines (IL-6, and IL-10) in cells. **i-** Western blot. **j-** quantification of IL6 Western blot in cells. Significant differences between groups were identified via 2-way ANOVA with a Tukey multiple comparison post-test (P values for the effect are reported; *$p < 0.05$ **< 0.01, ***< 0.001, ****< 0.0001).

Fig. 6

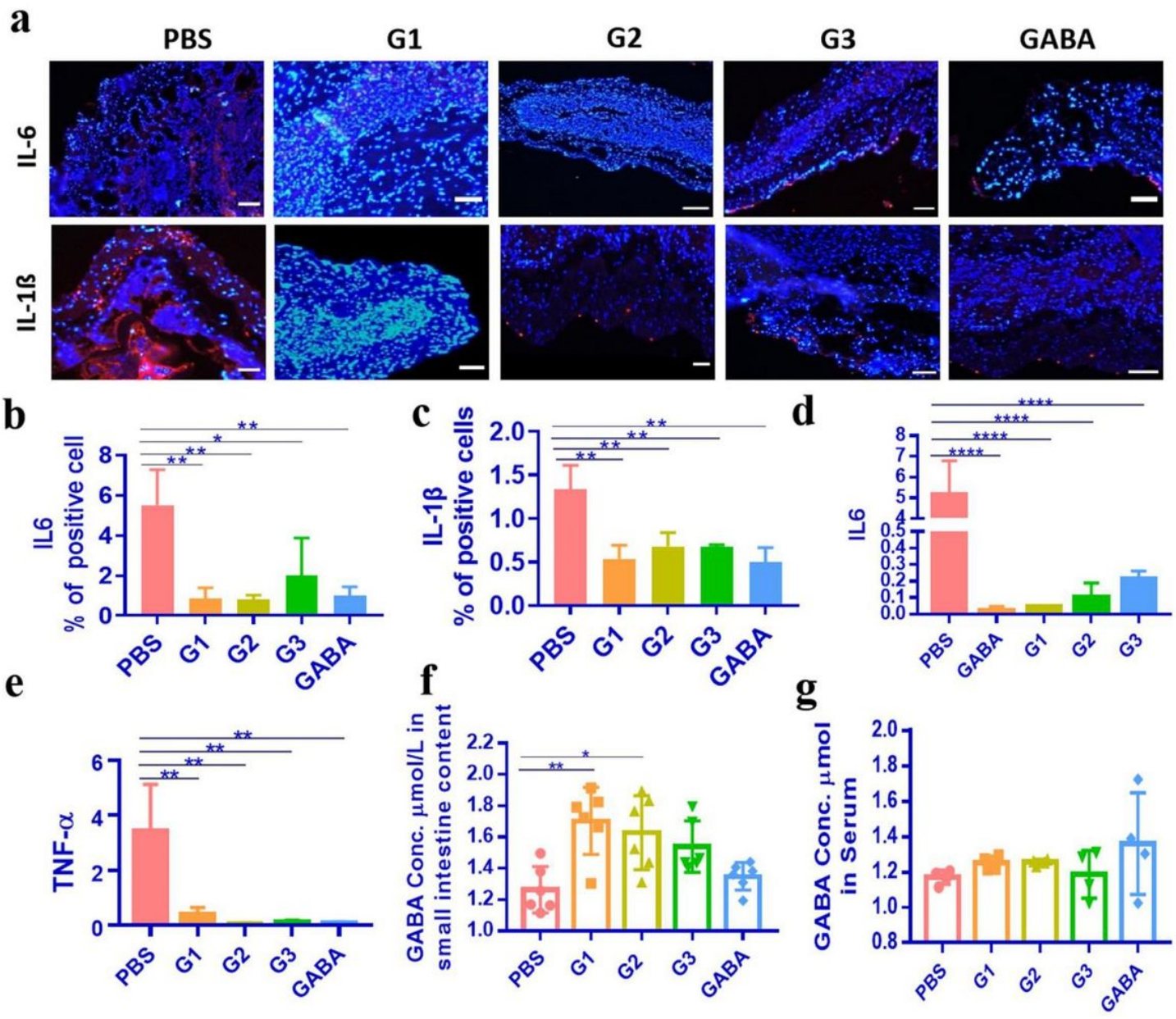

Figure 6

GABA and microbial treatment reduced intestinal inflammation. a-To assess the inflammatory signature on the intestine of mice supplemented with (PBS* Negative control; G1: *S. thermophilus* treated mice; G2: *L. pentosus* treated mice; G3: treated by both microbes & GABA). IL6 and IL-1β expression was detected via immunofluorescence, with DAPI counterstaining of nuclei. The white scale bars represent 30µm. **b-** Percentage of IL6 positive cells. **c-** Percentage of IL-1β positive cells. **d, e** Real-time qPCR analyses of the relative expression of inflammatory markers IL6 & TNFα in the intestine. **f, g- GABA assay,** GABA levels were detected by ELISA in small intestine content and serum. Significant differences between groups

were identified via 2-way ANOVA with a Tukey multiple comparison post-test (P values for the effect are reported; *$p < 0.05$, **< 0.01, ***< 0.001, ****< 0.0001).

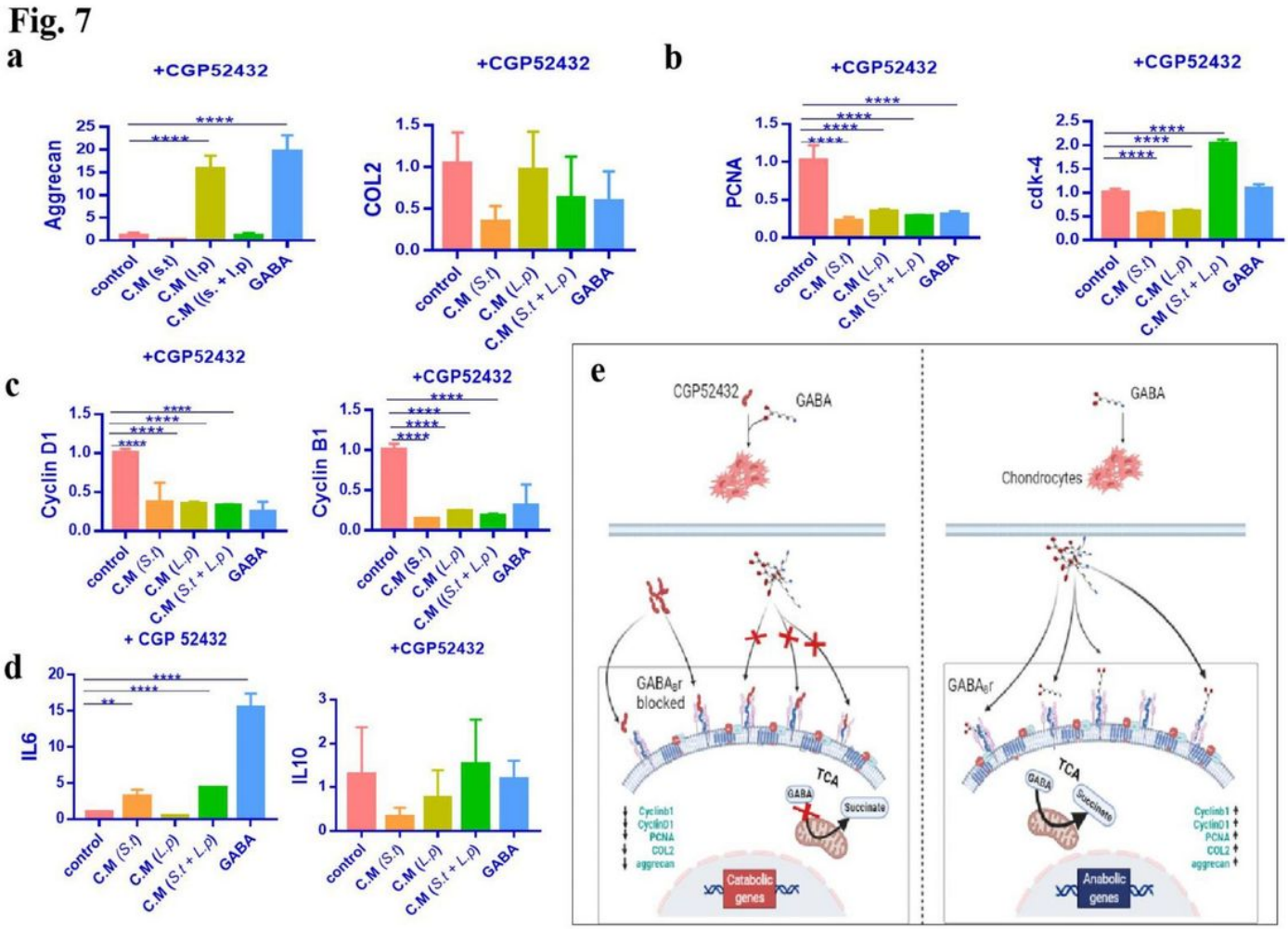

Figure 7

The effect of *S. thermophilus* and *L. pentosus* on cartilage is dependent on GABA. CGP52432 GABA$_B$r was used to block the GABA B receptor. **a, b, c, d-** Real-time qPCR analyses were performed to determine the relative expression of mRNA level of Aggrecan, COL2, cdk1, CyclinB1, CyclinD1, PCNA, IL6, and IL10. IL6 expression slightly increased in presence of GABA inhibitor. The mRNA levels were normalized to that of GAPDH and then normalized to the control group. **e-** The possible mechanism of action of GABA at cellular level, TCA cycle described in existed literature. we confirmed via proliferation markers. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared with control by one-way ANOVA with Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli.

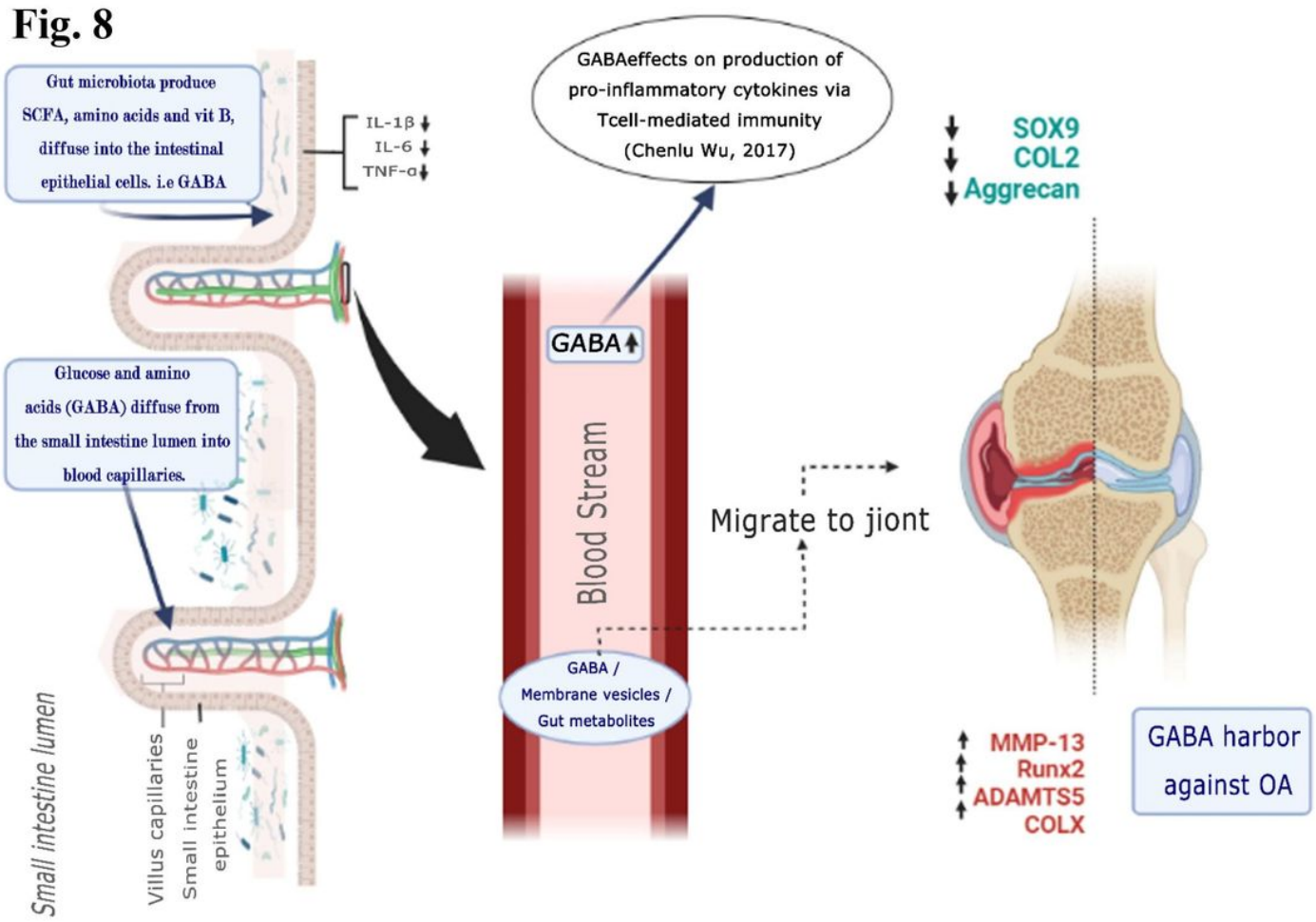

Figure 8

Proposed hypothetical gut-joint axis mechanism explaining the association between the gut microbiome (*S. thermophilus* & *L. pentosus*). These bacterial products can pass the gut-blood barrier, and possibly either diminish or downregulate inflammation. Protect the knee joint through upregulation of chondrogenic markers such as AGCAN, COL2, and SOX9. Also, it downregulates the catabolic markers of knee cartilage i.e., MMP-13, COLX, Runx2, and ADAMTS-5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.pdf