JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research Article
Article Title (within 20 words without abbreviations)	*Weissella confusa* WiKim51 (Wilac D001) fermented mycelium extract attenuates joint inflammation through PGE2 regulation in collagen-induced arthritis rats
Running Title (within 10 words)	Joint inflammation-attenuating effects of *Weissella confusa* WiKim51 fermented mycelium extract
Author	Jae-Young Kim[1,2], Yi Hyun Choi[1], Sang Min Park[3], Ji Ye Mok[3], Jeong Hyun Seo[3] and Sae Hun Kim[1,2*]
Affiliation	[1] College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea [2] Institute of Life Science and Natural Resources, Korea University, Seoul 02841, Republic of Korea [3]Pharmsville Co., Ltd., Seoul 06642, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Jae-Young Kim (https://orcid.org/0000-0003-1937-9535) Yi Hyun Choi (https://orcid.org/0009-0006-0513-1503) Sang Min Park (https://orcid.org/0000-0002-5599-233X) Ji Ye Mok (https://orcid.org/0009-0009-2542-0785) Jeong Hyun Seo (https://orcid.org/0000-0002-9197-4550) Sae Hun Kim (https://orcid.org/0000-0002-0990-2268)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was supported by grants from Korea University and the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (iPET) through the High Value-Added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (1545027002, RS-2021-IP321036).
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Kim SH, Kim JY, Park SM, Mok JY, Seo JH. Data curation: Kim JY. Formal analysis: Kim JY. Methodology: Kim SH, Choi YH. Software: Kim JY, Choi YH. Validation: Kim JY. Investigation: Choi YH. Writing - original draft: Kim JY. Writing - review & editing: Kim JY, Choi YH, Park SM, Mok JY, Seo JH, Kim SH.

Ethics approval and consent to participate	Animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2023-0076).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Sae Hun Kim
Email address – this is where your proofs will be sent	saehkim@korea.ac.kr
Secondary Email address	saehkim@gmail.com
Address	College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea
Cell phone number	82-10-9071-3055
Office phone number	82-2-3290-3055
Fax number	-

Weissella confusa WiKim51 (Wilac D001) fermented mycelium extract attenuates joint inflammation through PGE2 regulation in collagen-induced arthritis rats

Running Title: Joint inflammation-attenuating effects of *Weissella confusa* WiKim51 fermented mycelium extract

Jae-Young Kim[1,2], Yi Hyun Choi[1], Sang Min Park[3], Ji Ye Mok[3], Jeong Hyun Seo[3], and Sae Hun Kim[1,2*]

[1] College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea.

[2] Institute of Life Science and Natural Resources, Korea University, Seoul 02841, Republic of Korea.

[3] Pharmsville Co., Ltd., Seoul 06642, Republic of Korea

Author ORCIDs: Jae-Young Kim (0000-0003-1937-9535), Yi Hyun Choi (0009-0006-0513-1503), Sang Min Park (0000-0002-5599-233X), Ji Ye Mok (0009-0009-2542-0785), Jeong Hyun Seo (0000-0002-9197-4550), Sae Hun Kim (0000-0002-0990-2268)

Corresponding author

Prof. Sae Hun Kim, Ph.D.

Mailing address: College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea

Phone number: +82-2-3290-3055

Email address: saehkim@korea.ac.kr

Abstract

Rheumatoid arthritis (RA) is the state of joint inflammation, leading to cartilage and bone destruction. During rheumatoid arthritis progression, cyclooxygenase (COX) activity and its downstream production of prostaglandin E2 (PGE2) contribute to bone and cartilage erosion**.** WCF-ME was selected because its anti-inflammatory properties were confirmed *in vitro*. Therefore, this study aimed to evaluate the joint inflammation regulating capacities of *Weissella confusa* WiKim51 (Wilac D001) (WC), mycelium extract (ME), and WC-fermented ME (WCF-ME). In a collagen-induced arthritis (CIA) model, CIA-induced serum TNFα was normalized by WCF-ME treatment, while PGE2 was reduced by WC and WCF-ME treatment. ME had no effect in both serum markers. CIA-induced paw swelling and deformation was significantly lowered by WC and WCF-ME from the 21st day after immunization, while ME had no effect. Furthermore, knee joint gene expressions of *Il6* and *Mmp3* gene expressions were normalized by all treatments, while *Il1b* gene expression was normalized only in the WC group. *Mmp13* gene expressions were normalized by WC and WCF-ME, while ME had no significant difference. Spearman's correlation analysis showed that serum PGE2 was proportional to *Il6*, *Il1b*, and *Mmp13*. Overall, these results indicate that WC and WCF-ME ameliorated CIA-induced RA symptoms through inhibition of PGE2 activity, leading to reduced *Il6*, *Mmp3*, and *Mmp13* expressions in the knee. Therefore, WC and WCF-ME has potential as a novel RA treatment.

Keywords: Rheumatoid Arthritis, *Weissella confusa*, Mycelium, Anti-inflammatory, PGE2, joint

Introduction

Rheumatoid arthritis (RA) is the state of acute or chronic joint inflammation, resulting in cartilage and bone destruction. RA symptoms are treated through usage of drugs such as methotrexate (MTX), in which inhibit purine synthesis and subsequently induce cell apoptosis [1]. However, long term MTX usage may cause adverse effects such as nausea, mucosal ulcers, and hepatotoxicity [2]. Thus, studies on treatments targeting different mechanisms are on-going. Nonsteroidal anti-inflammatory drugs (NSAIDs) treat RA symptoms through inhibition of cyclooxygenase (COX) activity and their downstream production of prostaglandin E2 (PGE2) [3]. PGE2 and tumor necrosis factor alpha (TNFα) are released by macrophages participating in bone and cartilage erosion [4]. Thus, various studies are being conducted to develop treatments that regulate the production of these inflammatory markers.

Mycelia have been used in folk medicine for their nutritional and medicinal properties. Recent studies support their therapeutic effects, suggesting that this may be due to their high levels of polysaccharides, proteins, and bioelements etc. In a previous report, supplementation of *Auricularia auricula-judae*, a saprophytic fungus that grows on rotted wood in forests, reduced levels of inflammatory mediators such as interleukin 6 (*Il6*), interleukin 1 beta (*Il1β*), and *Tnfα* in a mouse obesity model [5]. Supplementation of *Sparassis crispa*, a brown-rot fungus that grows on coniferous tree stumps, also showed inhibitory effects on pro-inflammatory mediators [6]. Thus, results suggest that mycelia have potential as immunoregulating agents. Mycelia refer to the fungal biomass, including hyphal structures, while mycelium extracts are concentrated forms obtained via solvent extraction, which enhance the accessibility and potential bioactivity of key compounds. In this study, we focused on mycelium extracts due to their demonstrated anti-inflammatory potential in preliminary in vitro tests.

Lactic acid bacteria are gram-positive, lactic acid-producing organisms that possess various health benefits. Major strains include probiotics—live microorganisms that confer health benefits to the host when administered in adequate amounts [7]. Among various probiotics, the *Lactobacillaceae* family is the most well-known and used in the probiotic industry [8]. *Lactobacillaceae* family strains

are involved in fermentation; they regulate microbial growth and the enzymatic conversion of food constituents, which in turn produces beneficial end-products, such as organic acids, bacteriocins, and peptides [9]. *Weissella*, a genus belonging to the *Lactobacillaceae* family, are known to exert host-beneficial effects such as anti-microbial, anti-inflammatory, and anti-cancer activity [10]. Moreover, *Weissella*-fermented *Asparagus cochinchinensis* showed higher anti-inflammatory effects than the non-fermented *Asparagus cochinchinensis*, indicating the potential of *Weissella*-fermentation in enhancing immunomodulatory effects of natural products [11]. Thus, *Weissella*-fermentation products may be a potent candidate for RA treatment through the regulation of inflammatory responses. Therefore, this study aimed to compare the RA attenuating effects of *Weissella confusa* WiKim51 (Wilac D001) (WC), mycelium extract (ME), and *Weissella confusa* WiKim51 (Wilac D001)-fermented mycelium extract (WCF-ME) in RA models. Our study introduces a novel perspective on the utilization of mycelium extracts as a treatment for RA, which has not been frequently addressed in previous studies. Moreover, our study aims to enhance functional properties of mycelium extracts through fermentation, and ultimately heighten the value of mycelium extracts as a food product.

Materials and Methods

Materials

Bacterial strain *Weissella confusa* WiKim51 (Wilac D001) (*W. confusa*), (99.73 % identity; accession no. LC063164.1) were obtained from Pharmsvile Co., Ltd. (Seoul, South Korea). 16s rRNA sequencing was performed by Macrogen (Seoul, South Korea) for strain identification. *W. confusa* WiKim51 was cultured for 24 h at 37 °C in Man, Rogosa, and Sharpe (MRS) broth (BD Co., Franklin Lakes, NJ, USA). All strains were subcultured three times prior use.

Sample preparation

Auricularia auricula-judae mycelium powder (Mush&, Jeonju, South Korea) and *Sparassis crispa* mycelium powder (Mush&, Jeonju, South Korea) were mixed [1:3 w/w], dissolved in distilled

water [1:10 w/v], and used for reflux extraction at 100 ℃ for 6 hr. The resulting extracts were added with minimal broth (glucose [1:25 w/v], peptone [1:100 w/v], sodium acetate $3H_2O$ [1:200 w/v], magnesium sulfate $7H_2O$ [1:10,000 w/v], manganese sulfate $4H_2O$ [1:20,0000 w/v], 5 mL tween 80 [1:100 v/v], diammonium citrate [1:500 w/v], dipotassium phosphate [1:500 w/v]), and autoclaved (121 ℃, 15 min). The resulting mixture was termed Mycelium Extract (ME). Thereafter, *W. confusa* WiKim51 (WC) culture was centrifuged at 10,800 × g for 3 min (VS-180Cfi, Vision Scientific Co., Daejeon, Korea) and washed twice with phosphate-buffered saline (PBS). Next, the optical density of the harvested bacterial pellets at 600 nm was adjusted to 0.3; the pellets were added to ME (1:50 v/v) and incubated for 48 h. Sample preparations were done at 0 h, 24 h, and 48 h fermentation and spread on MRS agar plates (Kisan Bio) to assess bacterial growth. ME fermented with WC for 48 h was termed WCF-ME. Subsequently, WCF-ME was filtered using Whatman filter paper–Grade 3 (6μm) (Whatman™, Maidstone, United Kingdom), freeze dried, and kept at − 80 °C until use.

Gas chromatography/mass spectrometry (GC/MS) instrumentation and chromatographic condition

Short chain fatty acid (SCFA) contents of ME and WCF-ME were determined using GC/MS, per the method described by Kim, Jang [9] with slight modifications. Briefly, 50mg of ME or WCF-ME samples were combined with 100 μL crotonic acid, 50 μL HCl, and 200 μL ether, and then homogenized and centrifuged at 1000 ×*g* for 10 min. Supernatants were transferred to vials and 16 μL N-tert-butyldimethylsilyl-N-methylttrifluoroacetamide (Sigma–Aldrich, USA) was added. After mixing, vials were sealed and heated at 80 ℃ for 20 min, and then kept in room temperature for 48 h. Samples were placed in a 6890N Network GC System (Agilent Technologies, California, USA) with a HP-5MS column (30 m, 0.25 mm, 0.25 μm) and 5973 Network Mass Selective Detector (Agilent Technologies, USA). Helium (99.9999% purity) was used as a delivery gas at a flow rate of 1.2 mL/min. The head pressure was 97 kPa and the split was 20:1. The inlet and transfer line temperatures were 250 and 260 ℃, respectively. The following temperature program was used: 60 ℃ (3 min), 60–120 ℃ (5 ℃

min), 120–300 ℃ (20 ℃ min). One microliter of sample was injected with 30 min of run time. SCFA concentrations were qualified by comparing their peak areas with those of the standards.

Cell culture

MH7A human rheumatoid fibroblast-like synoviocytes were obtained from the RIKEN BioResource Research Center (BRC, Tsukuba, Japan). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose (Gibco, Dublin, Ireland) with 10% fetal bovine serum (Hyclone, MA, USA), and 1% P/S (GE Healthcare). All cultures were incubated in a humid atmosphere (37 °C, 5% CO_2).

Cell cytotoxicity assessment using the CCK-8 assay

Cell cytotoxicity was measured by the cell counting kit-8 assay (CCK-8) (K1018, APExBIO Corporation, USA) according to the manufacturer's instructions. MH7A cells were seeded at a density of 3×10^4 cells/well in 48-well plates. After 24 h incubation, the cells were pre-treated with ME or WCF-ME dose-dependently (100, 200, 300, 500, 750, and 1000 µg/mL) and incubated for 24 h (37 °C, 5 % CO_2). Subsequently, the cells were treated with 200 µL of 10 % CCK-8 solution and incubated in the dark for 2 h. Eventually, absorbance was measured at 450 nm using the Epoch microplate spectrophotometer (BioTek, VT, USA), and the relative percentage of cell proliferation was calculated.

Anti-inflammatory activity evaluation in MH7A Cells

The gene expression of inflammation-related biomarkers was measured by RT-qPCR [12]. MH7A cells were each seeded at a density of 2×10^5 cells/well in 6-well plates. After 24 h incubation, cells were pre-treated with ME (750 µg/mL) and WCF-ME (750 µg/mL) diluted in DMEM and incubated for 18 h (37 ℃, 5 % CO_2). To stimulate inflammatory responses, 10 ng/mL of TNFα (PeproTech, USA) diluted with DMEM was added and incubated for 6 h (37 ℃, 5 % CO_2). RNA was extracted from the cells using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol. The final RNA concentration and purity were determined by ultraviolet absorbance using a

NanoDrop spectrophotometer (BioTek, VT, USA). cDNA was synthesized using a cDNA reverse transcription kit (Thermo Fisher Scientific). Reverse transcription quantitative real-time PCR (RT-qPCR) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Targeted gene expression was quantified using 2X Real-Time PCR Smart Mix (SolGent, Daejeon, South Korea). The mRNA expression levels of each target gene were analyzed and normalized to that of the housekeeping gene GAPDH. The primer sequences used in this study are listed in Table 1.

Animal model and design

Animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2023-0076) and performed in accordance with the guidelines from the NIH Guide for the Care and Use of Laboratory Animals and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Seven-week-old male Wistar rats (ORIENTBIO Inc., Sungnam, South Korea) were acclimated for one week at 25 °C under a 12 h light/dark cycle and given *ad libitum* access to food and water. Forty-two rats were randomly divided into six groups: Con [vehicle (phosphate buffered saline, PBS) treatment], CIA [immunization + vehicle (PBS) treatment], MTX [immunization + Methotrexate (0.2 mg/kg; Korea United Pharm. Inc., Seoul, South Korea)] treatment], WC [immunization + *W. confusa* WiKim51 (10^{10} CFU/kg) treatment], ME [mycelium extract (600 mg/kg) treatment, and WCF-ME [immunization + *W. confusa* WiKim51 fermented-mycelium extract (600 mg/kg) treatment]. ME and WCF-ME dosages were selected based on previous studies reporting effective anti-inflammatory or metabolic regulatory activity of mycelium-derived substances in rodent models [5, 6]. After acclimatization, all groups except the Con group were immunized twice. Emulsions for immunization were prepared by mixing bovine type II collagen in 0.05 M acetic acid (2 mg/mL) (20022, Chondrex. Inc, Woodinville, USA) and Freund's Incomplete Adjuvant (IFA) (7002, Chondrex. Inc, Woodinville, USA) at a ratio of 1:1. The first immunization was conducted on the 7th day after acclimatization through a subcutaneous tail injection of 0.2 mL emulsion. The second immunization was conducted on the 14th day after acclimatization through a subcutaneous tail injection of 0.1 mL

emulsion. On the 21st day after acclimatization, rats in which the immunization model was not successfully established were eliminated, guaranteeing at least 5 rats in each group. On the 22nd day after acclimatization treatments were administered via oral gavage for four weeks. All treatment suspensions were prepared daily using PBS as the vehicle. Body weight, food intake, water intake, paw thickness, and arthritis scores were measured during this period. Arthritis severity was scored based on the criteria listed in Table 2 [13].

After all treatments, mice were fasted for 24 h, anesthetized by isoflurane–oxygen mixture inhalation (1.5-2.5 %; Hana Pharm, Sungnam, South Korea), and euthanized under isoflurane anesthesia by excising the heart. Blood samples were collected via cardiac puncture and centrifuged at 13000 ×*g* for 15 min at 4 °C to extract serum, which was subsequently stored at -20 °C for a maximum of two weeks for further analysis. After excision, the knee joint and hind paw tissues were washed with phosphate buffered saline (PBS). One piece was fixed in 10 % neutral-buffered formalin (Sigma-Aldrich) for hematoxylin and eosin (H&E) staining, and the other was stored in RNA*later* solution (Invitrogen, California, USA) at -80 °C for further analysis.

Serum analysis

ELISA kits were used to measure serum TNFα (K0331196, Komabiotech, Korea) and PGE2 (KGB004B, R&D systems, USA) according to the manufacturer's instructions.

Histological analysis

At the end of the experiment, a histological analysis was performed on the hind paw joints of each mouse. Hind paw joints from each group were fixed in 10 % (v/v) formaldehyde, decalcified (RDO Gold, Apex Engineering Products Corporation, USA), and embedded in paraffin blocks using standard techniques. Further, 4–5 μm sections were sliced and stained with H&E. Digital photomicrographs were taken from representative areas using a slide scanner (Easyscan Pro6, Motic, Hong Kong).

RNA extraction and RT-qPCR

The knee joint tissues were homogenized using a tissue tear homogenizer (BioSpec, Oklahoma, USA). Total mRNA from the homogenized tissues was extracted using TRIzol reagent (15596018, Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA extracts mixed with 30 μL lithium chloride (LiCl) (AM9480, Invitrogen, USA) and incubated at -20 ℃ for 30 min for purification. Solutions were centrifuged at 13000 ×*g* for 15 min at 4 °C to collect pellets, which were subsequently washed three times with 75 % ethanol to remove residual salts. RNA pellets were resuspended in diethyl pyrocarbonate (DEPC, Sigma-Aldrich, USA) for cDNA synthesis. The final RNA concentration and purity were determined by ultraviolet absorbance using a NanoDrop spectrophotometer (BioTek, VT, USA). Subsequent procedures were similar to those used to evaluate the anti-inflammatory activity in MH7A cells. The primer sequences used in this study are listed in Table 1.

Statistical Analysis

Statistical analyses were performed using the SPSS Statistics software (version 25.0; IBM, Armonk, NY, USA). One-way analysis of variance with Duncan's or Student's t-test was used to quantify the statistical differences between the mean values of the samples. $P < 0.05$ was considered statistically significant. Correlation strength was interpreted based on the absolute value of Spearman's ρ (rho), with $|\rho| > 0.6$ considered a moderate to strong correlation. All figures were generated using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). Correlation-based analyses and visualizations were performed using R Studio (RStudio, Boston, MA, United States) and related packages such as dplyr, tibble, tidyr, magrittr, psych, corrplot, RcolorBrewer, and ggcorrplot.

Results

In vitro assessment of ME and WCF-ME based on secondary metabolite production and anti-inflammatory activity

In vitro assessments of ME and WCF-ME were conducted to compare their compositional and functional characteristics (Fig. 1.). ME supplementation in bacterial growth medium had no effect on WC growth, indicating non-toxicity (Fig. 1a) ($p < 0.05$). Although the most rapid bacterial growth was observed at 24 h, WC bacterial growth remained elevated at 48 h and exhibited significantly increased acetate concentration at this time point (Fig. 1b) ($p < 0.05$), suggesting enhanced metabolic activity. Therefore, 48 h was selected as the optimal fermentation time to ensure both microbial activity and functional enhancement. Thus, WC-fermented ME at 48 h was selected as WCF-ME for further experiments.

The anti-inflammatory properties of WCF-ME were further evaluated with a TNFα-induced inflammatory cell model. The optimal dosage for in vitro assays was selected based on cell viability measurements using the CCK-8 assay (Figs. 1c and d). ME and WCF-ME treatments of 1000 μg/mL reduced cell viability to levels lower than the control (0 μg/mL) ($p < 0.05$). Treatments of 750 μg/mL ME and WCF-ME maintained cell viability at 95 % ($p < 0.05$). As a healthy cell culture is considered to show 80–95 % cell viability [14], 750 μg/mL was selected as the optimal dosage for in vitro anti-inflammatory assessments.

ME and WCF-ME were pre-treated in TNFα-induced inflammatory MH7A cells to determine their in vitro anti-inflammatory potential. Both treatments showed lower gene expressions of *MMP3*, *MMP13*, and *IL1B* than the TNFα only-treated group ($p < 0.05$) (Figs. 1e–g). Notably, TNFα-induced gene expression of *IL6* was lowered only by WCF-ME, while ME had no effect ($p < 0.05$) (Fig. 1h). These results indicate that both ME and WCF-ME have anti-inflammatory potential, while WC-fermentation may enhance the anti-inflammatory abilities of WCF-ME to those greater than ME.

Effects of WC, ME, and WCF-ME in growth parameters and serum biomarkers of an CIA-induced arthritis rat model

The in vivo effects of ME, WC, and WCF-ME treatments were evaluated in an CIA-induced arthritis rat model. CIA treatment reduced body weight and food intake, indicating induction of inflammation ($p < 0.05$) (Figs. 2a and b). However, all treatments did not ameliorate CIA-induced loss

of body weight and food intake. All groups showed no difference in water intake (Figs. 2c). Moreover, CIA-injections elevated levels of serum inflammatory markers TNFα and PGE2 to those higher than the Con group ($p < 0.05$) (Figs. 2d and e). Serum TNFα was normalized by MTX and WCF-ME treatment, while WC and ME had no effect ($p < 0.05$). Serum PGE2 was normalized by MTX and WC ($p < 0.05$), while WCF-ME showed reducing effects ($p < 0.05$). Thus, results indicate that CIA-induced inflammation may be attenuated by WC and WCF-ME treatment.

Effects of WC, ME, and WCF-ME on CIA-induced joint destruction

Severity of joint destruction was evaluated through gross morphology and H&E-stained hind paw joints. CIA induced severe swelling and deformation in gross images (Fig. 3a). Furthermore, CIA induced cartilage destruction, synovium hyperplasia, and pannus formation in H&E-stained tissues, in which are evident symptoms of rheumatoid arthritis (Figs. 3b and c). MTX, WC, and WCF-ME attenuated these changes, suggesting protective effects on rheumatoid arthritis. Moreover, measurements of paw thickness and arthritis scores were conducted every three days after immunization to determine arthritis development. CIA-induced paw swelling was reduced by WC and WCF-ME from the 21st day after immunization. Arthritis scores of MTX, WC, and WCF-ME groups were significantly lower than the CIA group from the 21st day after immunization. Therefore, results indicate that WC and WCF-ME may have protective effects against CIA-induced rheumatoid arthritis.

Effects of WC, ME, and WCF-ME on knee joint inflammation

The effects of WC, ME, and WCF-ME on knee joint inflammation were evaluated using RT-qPCR (Fig. 4). Gene expression of *Il6, Il1b, Mmp3, and Mmp13* was increased in the CIA group ($p < 0.05$) (Figs. 4a–d). *Il6* and *Mmp3* gene expressions were normalized by all treatments ($p < 0.05$). *Il1b* gene expression was normalized only in the WC group ($p < 0.05$). *Mmp13* gene expressions were normalized by WC and WCF-ME, while ME had no significant difference ($p < 0.05$). Thus, results indicate that WC and WCF-ME may ameliorate CIA-induced knee joint inflammation.

Spearman's correlation analysis was conducted to determine the relationship between serum ELISA and knee joint RT-qPCR results (Fig. 4e). Serum TNFα was positively correlated with knee joint *Il6* ($\rho = 0.3717$, $p < 0.05$). Serum PGE2 was proportional to *Il6* ($\rho = 0.4557$, $p < 0.05$), ($\rho = 0.4562$, $p < 0.05$), and *Mmp13* ($\rho = 0.4938$, $p < 0.05$). These results indicate that WC and WCF-ME may have altered levels of serum inflammation markers, thereby subsiding knee joint inflammation marker expressions in the host.

Discussion

In this study, we investigated the potential benefits of WC, ME, and WCF-ME as RA treatments in a CIA-induced rat model. Pathogenesis of RA remain unclear. However, major RA treatments such as NSAIDs exert their effects through COX inhibition, which leads to the reduction of PGE2 [3]. Inflammatory stimuli such as TNFα can activate PGE2-mediated inflammatory responses, in which induces MMP3 and MMP13 expression [15]. MMPs are zinc-dependent endopeptidases that can cause cartilage extracellular matrix degradation when existing in excess amounts. In previous reports, MMP3 and MMP13 increase in arthritis patients, suggesting that MMPs play a critical role in cartilage destruction [16]. Therefore, in this study, we aimed to select treatments with anti-inflammatory potential to attenuate PGE2-mediated cartilage destruction.

Our in vitro data showed that *Weissella*-fermentation of ME increased acetate concentrations (Fig. 1b). In a previous report, administration of acetate prior to the onset of CIA-induced arthritis in mice improved arthritic symptoms [17]. Acetate, a short chain fatty acid, suppresses inflammation through inhibition of NLRP3 inflammasome activity, consequently leading to NLRP3 degradation [18]. NLRP3 expression is positively correlated with arthritis severity in various arthritis models such as CIA or antigen-induced arthritis models, implying its involvement in RA pathogenesis [19]. Therefore, fermentation may enhance the bioactivity of ME by increasing acetate content, thereby improving its therapeutic potential.

Consistently, only WCF-ME suppressed *IL6* gene expression, while both ME and WCF-ME reduced gene expressions of *MMP3*, *MMP13*, and *IL1B* in TNFα-induced inflammatory MH7A cells

(Figs. 1e-h). IL6 has a crucial role in RA pathogenesis that promotes osteoclast activation, neutrophil recruitment, and B cell-mediated autoimmunity [20]. Thus, the inhibitory effects of WCF-ME on *IL6* gene expression highlights its superior potential in modulating core pathogenic pathways in RA.

Our in vivo study showed that MTX and WCF-ME suppressed systemic TNFα and PGE2 levels, whereas WC selectively reduced PGE2 (Figs. 2d and e). These findings imply that WC and WCF-ME exert anti-inflammatory effects via distinct mechanisms. Given that ME alone showed no significant effect, this supports the enhanced bio-efficacy imparted by fermentation. Furthermore, clinical indicators such as paw thickness and arthritis scores were significantly improved by WC and WCF-ME, but not by ME, indicating therapeutic enhancement throughout fermentation (Fig. 3). Histological analysis revealed that MTX, WC, and WCF-ME alleviated CIA-induced joint destruction, including pannus formation and cartilage erosion. Molecular analyses further confirmed that CIA-induced expression of *Il1b*, *Il6*, and *Mmp13* in knee joints was reduced by WC and WCF-ME. Notably, correlation analysis revealed that serum PGE2 was positively correlated with *Il1b*, *Il6*, and *Mmp13* expression, implying that the therapeutic effects of WC and WCF-ME may be mediated through systemic regulation of PGE2 [21, 22].

Taken together, our findings suggest that WC and WCF-ME mitigate RA progression by inhibiting PGE2 and downstream inflammatory mediators, thereby protecting joint integrity. This is the first report demonstrating the enhanced anti-inflammatory activity of ME via *Weissella*-fermentation in the context of RA.

In conclusion, WC and WCF-ME ameliorated CIA-induced RA symptoms by modulating key inflammatory pathways, notably through PGE2 suppression and downstream cytokine regulation. These findings suggest that fermentation enhances the functional properties of ME and that WC and WCF-ME hold promise as functional food ingredients or complementary therapies for RA. Nonetheless, limitations such as the use of a single animal model and lack of human validation must be addressed. Future studies should include long-term safety evaluation, mechanistic exploration of bioactive metabolites, and clinical validation.

337

338

Competing Interests

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was supported by grants from Korea University and the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (iPET) through the High Value-Added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (1545027002).

Ethics approval

Animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2023-0076).

References

1. Friedman B, Cronstein B. Methotrexate mechanism in treatment of rheumatoid arthritis. Joint Bone Spine. 2019;86(3):301-7. https://doi.org/10.1016/j.jbspin.2018.07.004

2. Wang W, Zhou H, Liu L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. European Journal of Medicinal Chemistry. 2018;158:502-16. https://doi.org/10.1016/j.ejmech.2018.09.027

3. McCoy JM, Wicks JR, Audoly LP. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. Journal of Clinical Investigation. 2002;110(5):651-8. https://doi.org/10.1172/JCI15528

4. Shabbir A, Shahzad M, Ali A, Zia-ur-Rehman M. Anti-arthritic activity of N′-[(2,4-dihydroxyphenyl)methylidene]-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-c][1,2]benzothiazin-1(4H)-yl)acetohydrazide. European Journal of Pharmacology. 2014;738:263-72. https://doi.org/10.1016/j.ejphar.2014.05.045

5. Liu Q, Ma R, Li S, Fei Y, Lei J, Li R, et al. Dietary Supplementation of Auricularia auricula-judae Polysaccharides Alleviate Nutritional Obesity in Mice via Regulating Inflammatory Response and Lipid Metabolism. Foods. 2022;11(7). https://doi.org/10.3390/foods11070942

6. Wang Z, Liu J, Zhong X, Li J, Wang X, Ji L, et al. Rapid characterization of chemical components in edible mushroom sparassis crispa by UPLC-Orbitrap MS analysis and potential inhibitory effects on allergic rhinitis. Molecules. 2019;24(16). https://doi.org/10.3390/molecules24163014

7. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology. 2014;11(8):506-14. https://doi.org/10.1038/nrgastro.2014.66

8. Lee CS, Kim BK, Lee IO, Park NH, Kim SH. Prevention of bone loss by using Lactobacillus-fermented milk products in a rat model of glucocorticoid-induced secondary osteoporosis. Int Dairy J. 2020;109. https://doi.org/10.1016/j.idairyj.2020.104788

9. Kim JY, Jang S, Song HJ, Lee S, Cheon S, Seo EJ, et al. Sargassum horneri extract fermented by Lactiplantibacillus pentosus SH803 mediates adipocyte metabolism in 3T3-L1 preadipocytes by regulating oxidative damage and inflammation. Scientific Reports. 2024;14(1). https://doi.org/10.1038/s41598-024-65956-8

10. Ahmed S, Singh S, Singh V, Roberts KD, Zaidi A, Rodriguez-Palacios A. The Weissella Genus: Clinically Treatable Bacteria with Antimicrobial/Probiotic Effects on Inflammation and Cancer. Microorganisms. 2022;10(12). https://doi.org/10.3390/microorganisms10122427

11. Lee HA, Song BR, Kim HR, Kim JE, Yun WB, Park JJ, et al. Butanol extracts of asparagus cochinchinensis fermented with weissella cibaria inhibit iNOS-mediated COX-2 induction pathway and inflammatory cytokines in LPS-stimulated RAW264.7 macrophage cells. Experimental and Therapeutic Medicine. 2017;14(5):4986-94. https://doi.org/10.3892/etm.2017.5200

12. Kim JY, Lee S, Kim G, Shin HJ, Lee EJ, Lee CS, et al. Ameliorating effect of 2'-Fucosyllactose and 6'-Sialyllactose on lipopolysaccharide-induced intestinal inflammation. J Dairy Sci. 2024. https://doi.org/10.3168/jds.2024-24325

13. Poutoglidou F, Pourzitaki C, Manthou ME, Samoladas E, Saitis A, Malliou F, et al. Infliximab prevents systemic bone loss and suppresses tendon inflammation in a collagen-induced arthritis rat model. Inflammopharmacology. 2021;29(3):661-72. https://doi.org/10.1007/s10787-021-00815-w

14. Segeritz CP, Vallier L. Cell Culture: Growing Cells as Model Systems In Vitro. Basic Science Methods for Clinical Researchers. 2017. p. 151-72. https://doi.org/10.1016/B978-0-12-803077-6.00009-6

15. Jiang X, Renkema H, Pennings B, Pecheritsyna S, Schoeman JC, Hankemeier T, et al. Mechanism of action and potential applications of selective inhibition of microsomal prostaglandin E synthase-1-mediated PGE2 biosynthesis by sonlicromanol's metabolite KH176m. Scientific Reports. 2021;11(1). https://doi.org/10.1038/s41598-020-79466-w

16. Sirikaew N, Chomdej S, Tangyuenyong S, Tangjitjaroen W, Somgird C, Thitaram C, et al. Proinflammatory cytokines and lipopolysaccharides up regulate MMP-3 and MMP-13 production in Asian elephant (Elephas maximus) chondrocytes: Attenuation by anti-arthritic agents. BMC Veterinary Research. 2019;15(1). https://doi.org/10.1186/s12917-019-2170-8

17. Yao Y, Cai X, Zheng Y, Zhang M, Fei W, Sun D, et al. Short-chain fatty acids regulate B cells differentiation via the FFA2 receptor to alleviate rheumatoid arthritis. British Journal of Pharmacology. 2022;179(17):4315-29. https://doi.org/10.1111/bph.15852

18. Xu M, Jiang Z, Wang C, Li N, Bo L, Zha Y, et al. Acetate attenuates inflammasome activation through GPR43-mediated Ca2+-dependent NLRP3 ubiquitination. Experimental and Molecular Medicine. 2019;51(7). https://doi.org/10.1038/s12276-019-0276-5

19. Yin H, Liu N, Sigdel KR, Duan L. Role of NLRP3 Inflammasome in Rheumatoid Arthritis. Frontiers in Immunology. 2022;13. https://doi.org/10.3389/fimmu.2022.931690

20. Srirangan S, Choy EH. The role of Interleukin 6 in the pathophysiology of rheumatoid arthritis. Therapeutic Advances in Musculoskeletal Disease. 2010;2(5):247-56. https://doi.org/10.1177/1759720X103783

420 21. Jenei-Lanzl Z, Meurer A, Zaucke F. Interleukin-1β signaling in osteoarthritis – chondrocytes in
421 focus. Cellular Signalling. 2019;53:212-23. https://doi.org/10.1016/j.cellsig.2018.10.005

422 22. Wang P, Zhu F, Konstantopoulos K. Prostaglandin E2 induces interleukin-6 expression in human
423 chondrocytes via cAMP/protein kinase A- and phosphatidylinositol 3-kinase-dependent NF-κB
424 activation. American Journal of Physiology - Cell Physiology. 2010;298(6):C1445-C56.
425 https://doi.org/10.1152/ajpcell.00508.2009

426

Tables

Table 1. Primer sequences used in RT-qPCR

Model	Gene	Sequence	Tm (°C)
Human	*IL6*	F: 5′- CCT GAC CCA ACC ACA AAT GC -3′	64.5
		R: 5′- ATC TGA GGT GCC CAT GCT AC -3′	
	IL1B	F: 5′- CCT GTC CTG CGT GTT GAA AGA -3′	57
		R: 5′- GGG AAC TGG GCA GAC TCA AA -3′	
	MMP3	F: 5′- CTG GAC TCC GAC ACT CTG GA -3′	55
		R: 5′- CAG GAA AGG TTC TGA AGT GAC C -3′	
	MMP13	F: 5′- CAA GAT GCG GGG TTC CTG AT -3′	63.3
		R: 5′- AAT GCC ATCGTGAAGTCTGGT -3′	
	GAPDH	F: 5′- TCA CCA GGG CTT GCT TTT AAC -3′	55
		R: 5′- AAT GAA GGG GTC ATT GAT GG -3′	
Rat	*Il6*	F: 5′- TCC TAC CCC AAC TTC CAA TGC TC -3′	63.3
		R: 5′- TTG GAT GGT CTT GGT CCT TAG CC -3′	
	Il1b	F: 5′- CAC CTC TCA AGC AGA GCA CAG -3′	65
		R: 5′- GGG TTC CAT GGT GAA GTC AAC -3′	
	Mmp3	F: 5′- ATC CCT CTA TGG ACC TCC CAC -3′	59
		R: 5′- AAC AAG ACT TCT CCC CGC AG -3′	
	Mmp13	F: 5′- GCG GTT CAC TTT GAG GAC AC -3′	63.3
		R: 5′- TAT GAG GCG GGG ATA GTC TTT -3′	
	Actb	F: 5′- AAG TCC CTC ACC CTC CCA AAA G -3′	65
		R: 5′- AAG CAA TGC TGT CAC CTT CCC -3′	

Tm: Annealing Temperature; *IL6*, *Il6*: interleukin 6; *IL1B*, *Il1b*: interleukin 1 beta; *MMP3*, *Mmp3*: matrix metallopeptidase 3; *MMP13*, *Mmp13*: matrix metallopeptidase 13; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *Actb*: beta-actin

436 **Table 2.** Arthritis Severity Scoring Criteria

Score	Joint Involvement
0	No evidence of erythema or swelling
1	Minimal erythema and swelling affecting ankle joint or mid-foot
2	Minimal erythema and swelling extending from ankle joint to mid-foot
3	Moderate erythema and swelling affecting the ankle joint, the mid-foot and the metatarsal joints
4	Severe erythema and swelling affecting the ankle joint, the mid-foot, the metatarsal joints and the digits

437

Fig 1.

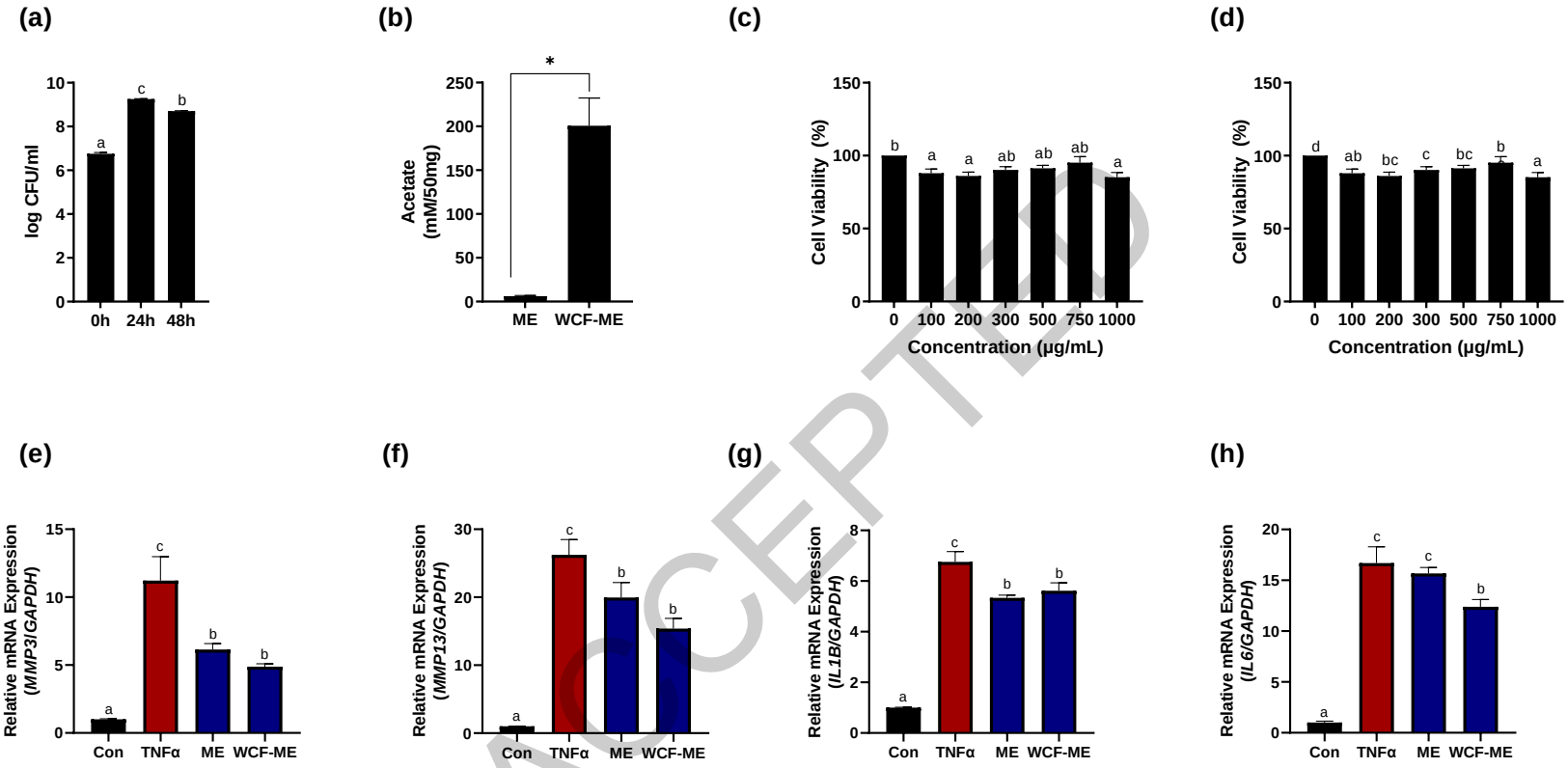

Fig 1. In vitro assessment of *W. confusa* WiKim51 (Wilac D001)-fermented Mycelium Extract. (a) Bacterial growth of *W. confusa* WiKim51 (Wilac D001) in growth media supplemented with ME for 48h. (b) Acetate content in ME and WCF-ME. Viability of MH7A cells pretreated with different concentrations (0, 100, 200, 300, 500, 750, 1000 μg/mL) of (c) ME and (d) WCF-ME. Effects of ME and WCF-ME on gene expressions of inflammation related markers (e) *MMP3*, (f) *MMP13*, (g) *IL1B*, and (h) *IL6* in TNFα-treated MH7A cells. Results are expressed as mean ± SE (n = 3). ^{abcd}Results in the same series with different lowercase superscript letters are significantly different ($p < 0.05$, one-way ANOVA); *$p < 0.05$ (t-test ME vs. WCF-ME).

Fig 2.

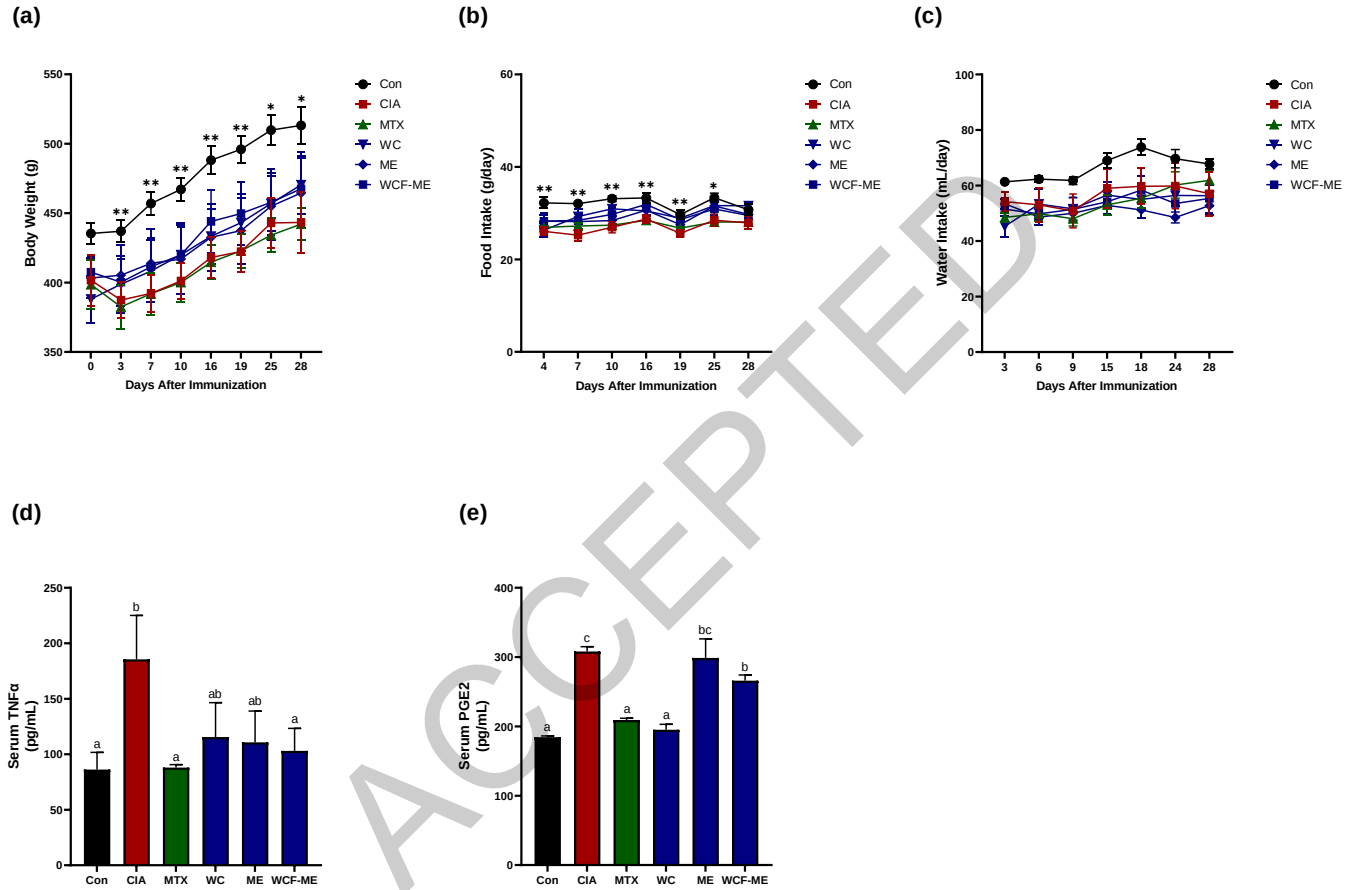

Fig 2. Effects of WC, ME, and WCF-ME in growth parameters and serum biomarkers of an CIA-induced arthritis rat model. (a) Body weight. (b) Food intake. (c) Water intake. Serum levels of (d) TNFα and (e) PGE2. Data are expressed as the mean ± SE (n = 5). ^{abc}Means in the same series with different lowercase superscript letters are significantly different ($p < 0.05$, one-way ANOVA); $^{**}P < 0.01$, $^{*}P < 0.05$ (t-test CIA vs. Each treatment).

Fig 3.

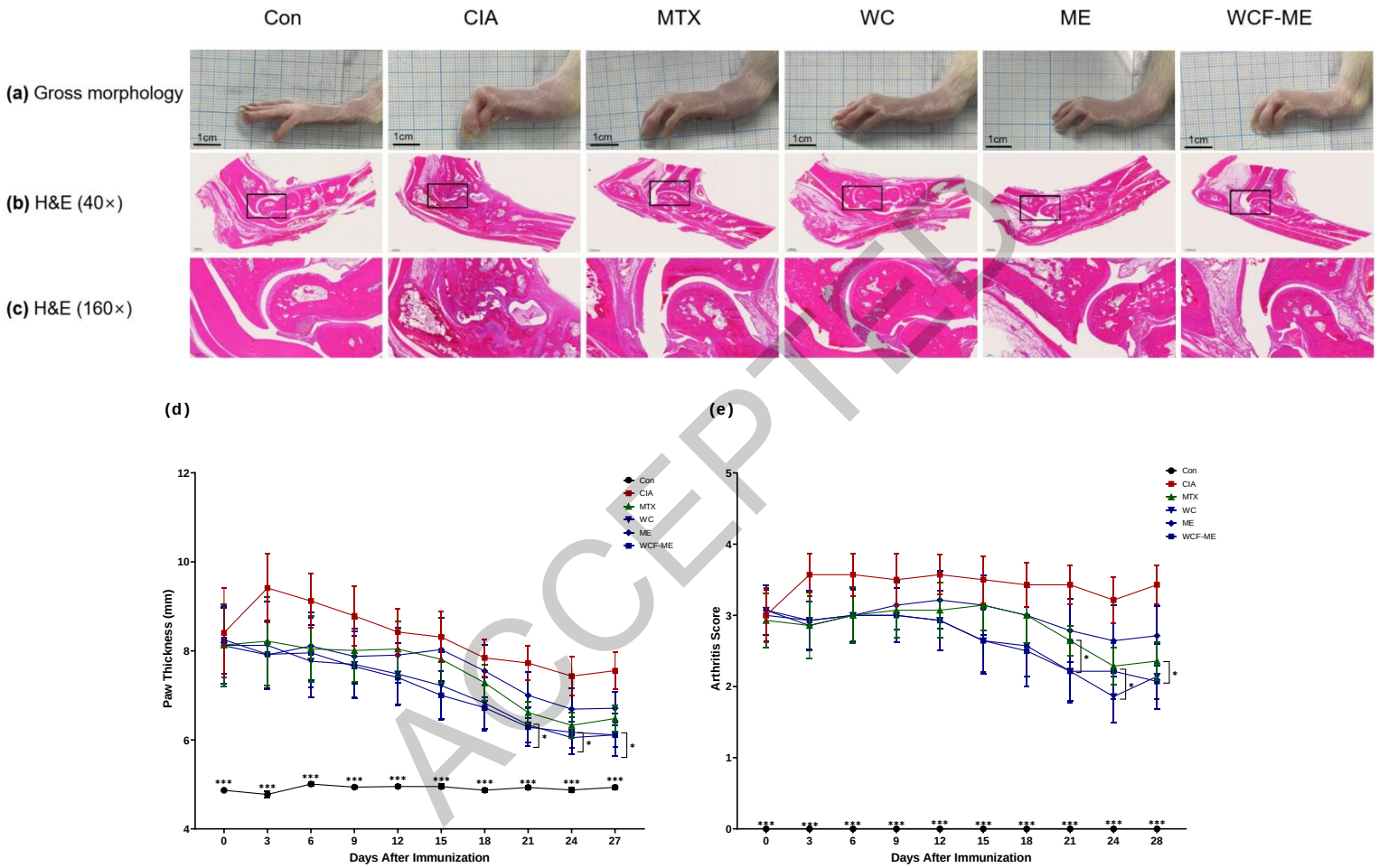

Fig 3. Effects of WC, ME, and WCF-ME on CIA-induced arthritis symptoms. Representative images of (a) gross morphology (scale bar: 1 cm) and H&E-stained hind paw joints (b) magnification: 40 ×, scale bar: 1000 μm; (c) magnification: 160 ×, scale bar: 1000 μm. The black box represents the area of magnification in the corresponding figure below. (d) Paw thickness. (e) Arthritis Scores. Data are expressed as the mean ± SE (n = 5). ***p < 0.001, *p < 0.05 (t-test CIA vs. Each treatment).

Fig 4.

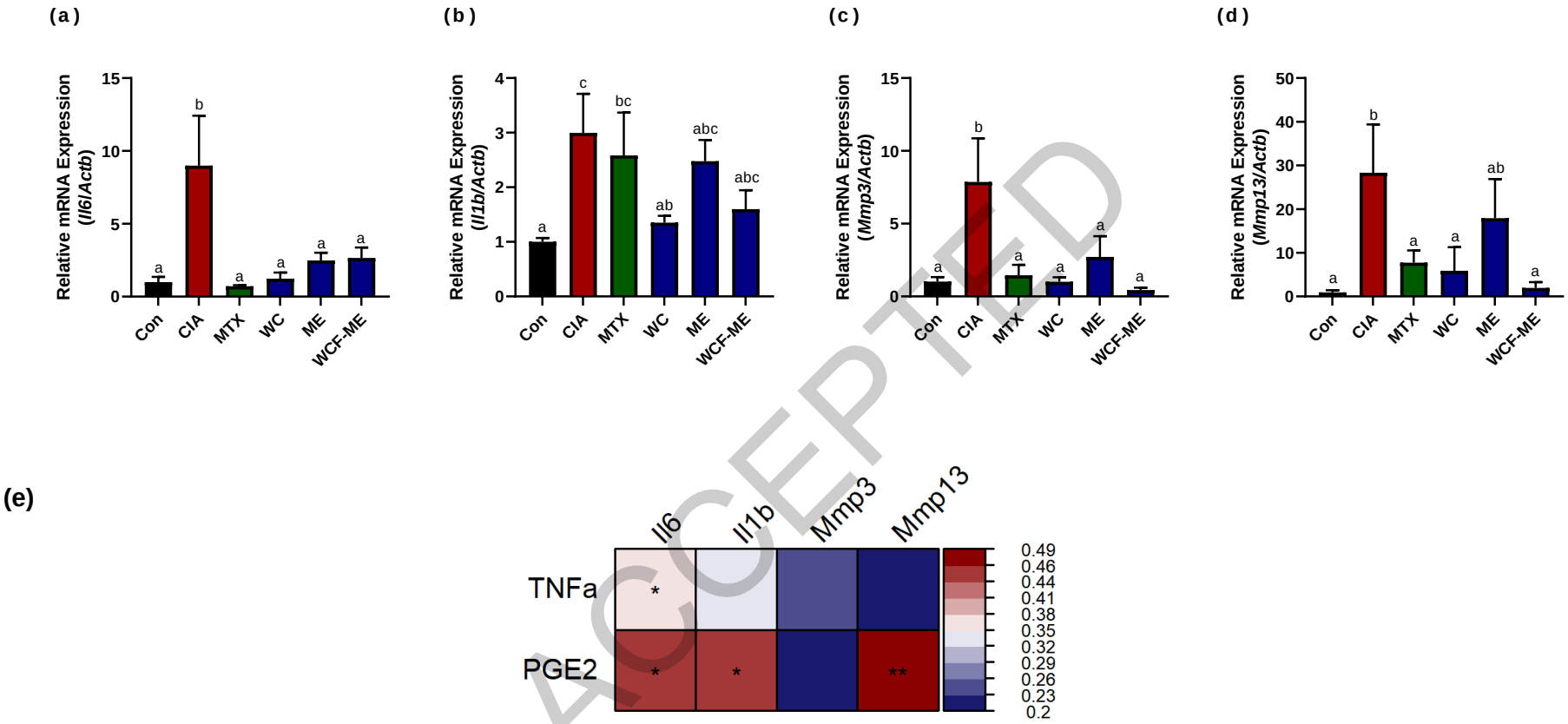

Fig 4. Effects of WC, ME, and WCF-ME on knee joint inflammation. Gene expressions of knee joint (a) *Il6*, (b) *Il1b*, (c) *Mmp3*, and (d) *Mmp13*. Spearman's correlation analysis of serum TNFα and PGE2 concentrations; and knee joint *Il6*, *Il1b*, *Mmp3*, and *Mmp13* gene expressions. Data are expressed as the mean ± SE (n = 5). [abc]Mean values in the same series with different lowercase letters are significantly different ($p < 0.05$, one-way ANOVA); ***$p < 0.001$, *$p < 0.05$).