USE OF *LACTOBACILLUS PLANTARUM* GKD7 IN MANUFACTURE OF MEDICAMENT FOR TREATING OSTEOARTHRITIS AND DEGENERATIVE JOINT DISEASE

BACKGROUND

Field of Invention

[0001] The present invention relates to the use of *Lactobacillus plantarum* GKD7. More particularly, the present invention relates to the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament of improving osteoarthritis and degenerative joint disease.

Description of Related Art

[0002] Osteoarthritis (OA), or so-called degenerative joint disease, is a chronic, degenerative, inflammatory disease developed in the joint. OA is commonly accompanied by pain which is relieved by rest but worsens with activity. Besides, other symptoms of OA also include stiffness that is relieved with activity but occurs again by rest. As analyzed by optical microscopy, the cartilage tissue, the synovial tissue and/or the subchondral bone of an OA-affected part often developed OA-related damages such as joint space narrowing, osteophytes, subchondral sclerosis, subchondral cysts, bone lesion, cartilage damage, cartilage inflammation and/or synovial inflammation). Moreover, as analyzed by molecular biotechnology, a patient with OA indeed has higher levels of pro-inflammatory cytokines in serum, synovial tissue and cartilage tissue compared to a normal person.

[0003] The aforementioned pro-inflammatory cytokines can include but not limited to interleukin (IL)-1β and tumor necrosis factor (TNF)-α. In the study focusing on OA, reducing pro-inflammatory cytokines production is some of the most important therapeutic aims in OA, as these pro-inflammatory cytokines play important roles in causing the cartilage destructive changes and modifying the subchondral bone structural changes. For example, the pro-inflammatory cytokines secreted from the synovial fibroblasts and the chondrocytes of OA-affected joints promote the synthesis of proteolytic enzymes that degrade the extracellular matrices in the joint, thereby worsening OA.

[0004] The administration of non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids are the mainstays of OA treatment nowadays for reducing OA-caused joint pain, joint swelling, and stiffness. However, NSAIDs or corticosteroids have undesirable side effect profiles, some of which can even worsen OA. When the pain or the functional disorders of a joint is too serious to be relieved by drugs, the patient can only decide to replace an artificial joint.

[0005] Probiotics are live cells that promote the ecosystem equilibrium of the microbiota of a host and thus can promote and benefit host health. Previous study has shown that some probiotics can inhibit inflammation. However, probiotics should be manufactured and preserved under specific conditions to maintain the growth activities of the live cells of probiotics, thereby limiting the form and/or type of the related products.

[0006] In view of this, it is urgent to provide a medicament including dead cells of probiotics to solve the aforementioned problems.

SUMMARY

[0007] Therefore, one aspect of the present invention is to provide a use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis. The medicament includes the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, and the medicament is to be administered to relieve osteoarthritis-caused joint pain of a subject.

[0008] Another aspect of the present invention is to provide a use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating degenerative joint disease. The medicament includes the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, and the medicament is to be administered in a form of an oral composition.

[0009] The other aspects of the present are to provide a pharmaceutical and/or a food composition for treating osteoarthritis. The pharmaceutical and/or the food composition include the aforementioned *Lactobacillus plantarum* GKD7 as an active ingredient.

[0010] According one aspect of the present invention, the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis is provided. The medicament includes the effective dosage of the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, the *Lactobacillus plantarum* GKD7 is deposited in National Institute of Technology and Evaluation (NITE), Patent Microorganisms Depositary on November 6, 2020 under an accession number of NITE BP-03313, and the medicament is to be administered to relieve the osteoarthritis-caused joint pain of a subject.

[0011] In some embodiments of the present invention, the dead cells of *Lactobacillus plantarum* GKD7 is to be obtained by subjecting the *Lactobacillus plantarum* GKD7 to a fermentation step and an inactivation step. In some embodiment, the fermentation step is performed at 25°C to 40°C in an anaerobic atmosphere on a fermentation substrate for 16 hours to 24 hours.

[0012] In some embodiments of the present invention, the inactivation step includes a heat inactivation step, a chemical inactivation step and/or a radiation inactivation step.

[0013] In some embodiments of the present invention, the heat inactivation step is performed by using saturated vapor pressure of 110°C to 120°C for 10 minutes to 20 minutes.

[0014] In some embodiments of the present invention, the dead cells of the *Lactobacillus plantarum* GKD7 are to be subjected to a lyophilization step.

[0015] In some embodiments of the present invention, when the subject is a rat, the effective dosage is 10 mg/kg weight/day to 1000 mg/kg weight/day.

[0016] In some embodiments of the present invention, when the subject is a human, the effective dosage is 1 mg/kg weight/day to 165 mg/kg weight/day.

[0017] According to another aspect of the present invention, the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating degenerative joint disease is provided. The medicament includes the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313, the

medicament is to be administered to relieve joint pain of a subject, and the medicament is to be administered in a form of an oral composition.

[0018] In some embodiments of the present invention, the dead cells of the *Lactobacillus plantarum* GKD7 is obtained by subjecting the *Lactobacillus plantarum* GKD7 to a fermentation step and a heat inactivation step.

[0019] In some embodiments of the present invention, comparing to a control subject that the medicament is not to be administered to, the subject that the medicament is to be administered to has a lesser difference between weights borne by left and right limbs.

[0020] In some embodiments of the present invention, comparing to a control subject that the medicament is not to be administered to, the subject that the medicament is to be administered to has a lower expression level of interleukin (IL)-1β in a cartilage tissue and/or a synovial tissue.

[0021] In some embodiments of the present invention, comparing to a control subject that the medicament is not to be administered to, the subject that the medicament is to be administered to has a lower expression level of tumor necrosis factor (TNF)-α in a cartilage tissue and/or a synovial tissue.

[0022] In some embodiments of the present invention, the medicament is to be administered to the subject in a frequency of 3 times to 7 times per week.

[0023] In some embodiments of the present invention, the medicament is to be administered to the subject for 2 weeks to 8 weeks.

[0024] According to another aspect of the present invention, a pharmaceutical composition for treating osteoarthritis is provided. The pharmaceutical

composition includes the effective dosage of the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, in which the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313, the dead cells of *Lactobacillus plantarum* GKD7 is to be obtained by subjecting the *Lactobacillus plantarum* GKD7 to an inactivation step.

[0025] In some embodiments of the present invention, the pharmaceutical composition further includes medical-acceptable carriers, excipients and/or additives.

[0026] In some embodiments of the present invention, the pharmaceutical composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule, liquid spray or suppository.

[0027] According to another aspect of the present invention, a food composition for treating osteoarthritis is provided. The food composition includes the effective dosage of the lyophilized powder of the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, in which the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313.

[0028] In some embodiments of the present invention, the food composition further includes food-acceptable carriers, excipients and/or additives.

[0029] In some embodiments of the present invention, the food composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule, liquid spray or suppository.

[0030] By applying the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease, in

which the medicament includes the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, the medicament can relieve joint pain and decrease the expression level of IL-1β in the cartilage tissue and/or the synovial tissue, and thus can be used to improve osteoarthritis and degenerative joint disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The invention can be more fully understood by reading the following detailed description of the embodiment, with reference made to the accompanying drawings as follows:

[0032] FIG. 1 is a curve chart illustrating the change of the difference between the weights borne by left and right limbs of rats in different groups 6 weeks after the surgery according to an embodiment of the present invention.

[0033] FIG. 2 is a curve chart illustrating the change of the difference between the weights borne by left and right limbs of rats in the different group 6 weeks after the surgery according to an embodiment of the present invention.

[0034] FIGs. 3A to 3G are box charts respectively illustrating the bone mineral density, the bone mineral content, the bone volume fraction, the bone area fraction, the trabecular thickness, the trabecular number and the trabecular separation of rats in different groups according to an embodiment of the present invention.

[0035] FIGs. 4A to 4C are box charts respectively illustrating the OARSI score, the cartilage degeneration score and the synovium inflammation score of different groups according to an embodiment of the present invention.

[0036] FIGs. 5A to 5B are box charts respectively illustrating the IHC scores of IL-1β and TNF-α of the cartilage tissue of rats in different groups according to an embodiment of the present invention.

[0037] FIGs. 6A to 6B are box charts respectively illustrating the IHC scores of IL-1β and TNF-α of the synovial tissue of rats in different groups according to an embodiment of the present invention.

DETAILED DESCRIPTION

[0038] If the definition or usage of a term in a cited document is inconsistent with or contrary to the definition of the term here, the definition of the term here applies instead of the definition of the term in the cited document. Moreover, a singular term can be plural and the plural term can be singular unless it is defined in the context.

[0039] As mentioned above, the present invention provides the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis, in which the medicament includes the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient. The aforementioned *Lactobacillus plantarum* GKD7 is deposited in National Institute of Technology and Evaluation (NITE), Patent Microorganisms Depositary (address: #122, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan) on November 6, 2020, under an accession number of NITE BP-03313. *Lactobacillus plantarum* GKD7 is also deposited in Bioresource Collection and Research Center (BCRC) of Food Industry Research and Development Institute (FIRDI, address: No.331 Shih-Pin

Road, Hsinchu, Taiwan 300) on July 18, 2019, under BCRC 930210. The deposit has been made under the terms of the Budapest Treaty.

[0040] The term “dead cells” of *Lactobacillus plantarum* GKD7 in the content are the cells of *Lactobacillus plantarum* GKD7 without growth activities. In some embodiments, the dead cells of *Lactobacillus plantarum* GKD7 are obtained by subjecting the *Lactobacillus plantarum* GKD7 to a fermentation step and an inactivation step. In some embodiments, the fermentation step is performed at 25°C to 40°C, or 35°C to 40°C, or 37°C, so that *Lactobacillus plantarum* GKD7 had a better growth activity, thereby obtaining more cells of *Lactobacillus plantarum* GKD7. In some embodiments, the fermentation step is performed for 16 hours to 24 hours, or 18 hours, so that the cells of *Lactobacillus plantarum* GKD7 enter into the exponential phase or the stationary phase of a growth curve. In some embodiments, the fermentation step is performed under an anaerobic atmosphere. There is no specific limitation on the air content of an anaerobic atmosphere as the oxygen content is substantially zero. In a specific embodiment, the anaerobic atmosphere can include but not limited to 75% to 85% of nitrogen, 5% to 15% of hydrogen and 5% to 15% of CO_2.

[0041] There is no specific limitation on the fermentation substrate which can be a commercial fermentation substrate, such as MRS culture solution. In some embodiments, the fermentation substrate can include but not limited to 1 wt% to 10 wt% of sugar, 0.1 wt% to 5.0 wt% of yeast extract, 0.1 wt% to 5.0 wt% of peptone, 0.01 wt% to 2.00 wt% of trace elements, 0.01 wt% to 0.10 wt% of cysteine, 0.05 wt% to 1.00 wt% of TWEEN-80 and water in balance. The aforementioned sugar can include but not limited to monosaccharide and/or

disaccharide. In some specific embodiments, the sugar can be selected from a group consisting of glucose, galactose, fructose, maltose, lactose, sucrose and any combination thereof. The aforementioned trace elements can be selected from a group consisting of magnesium ions, potassium ions, sulfate ions, hydrogen phosphite ions and any combination thereof.

[0042] In some embodiments, after the fermentation step, a solid-liquid separation step can be performed to remove the fermentation substrate. There is no specific limitation on the solid-liquid separation step that can remove the fermentation substrate. In some specific embodiment, the solid-liquid separation step can include but not limited to a filtration step and/or a centrifuge step.

[0043] There is no specific limitation on the inactivation step that can make the cells of *Lactobacillus plantarum* GKD7 lose their growth activity. In some embodiments, the inactivation step can include but not limited to a heat inactivation step, a chemical inactivation step and/or a radiation inactivation step. In some specific embodiments, the heat inactivation step is performed by using saturated vapor pressure of 110°C to 120°C for 10 minutes to 20 minutes. It should be noted that with experimental support, the dead cells of *Lactobacillus plantarum* GKD7 still have the effect to improve osteoarthritis although they lose their growth activity.

[0044] In some embodiments, the dead cells of the aforementioned *Lactobacillus plantarum* GKD7 can be further subjected to a lyophilization step, thereby obtaining a lyophilized powder, so as to extend the storage life. In some

embodiments, the lyophilized powder includes *Lactobacillus plantarum* GKD7 with an amount equivalent to 5.5×10^7 CFU/g.

[0045] The dead cells of *Lactobacillus plantarum* GKD7 can improve osteoarthritis. The term “osteoarthritis” in the content is a degenerative joint disease that will lead to the degradation of joint cartilage and a subchondral bone, resulting in joint pain and even structural changes in the cartilage and the bone. “To improve (or treat) osteoarthritis” in the content indicates to relieve joint pain, decrease the cartilage damage, the bone lesions and/or to decrease pro-inflammatory cytokines. In some embodiments, the evaluation items to evaluate whether osteoarthritis is improved can include but not limited to levels of joint pain, the expression levels of pro-inflammatory cytokines in the cartilage tissue and the synovial tissue, the levels of bone lesions and/or cartilage damage. In some specific embodiments, since osteoarthritis is often asymmetric, the levels of joint pain can be evaluated by the difference between weights borne by left and right limbs. A smaller difference between weights borne by left and right limbs indicates a lower level of pain in the osteoarthritis-affected part. In some specific embodiments, the aforementioned pro-inflammatory cytokines can include but not limited to interleukin (IL)-1β and tumor necrosis factor (TNF)-α.

[0046] In some specific embodiments, the level of bone damage can be evaluated by bone mineral density (BMD), bone mineral content (BMC), bone volume fraction, bone area fraction, trabecular thickness, trabecular number and trabecular separation. In some specific embodiments, the level of cartilage damage can be evaluated by the evaluating system of the joint histology of rats recommended by Osteoarthritis Research Society International (OARSI).

[0047] With experimental support, the live cells and/or the dead cells of *Lactobacillus plantarum* GKD7 can improve osteoarthritis by oral administration. Specifically, compared to the control object not administering *Lactobacillus plantarum* GKD7, a subject administered with *Lactobacillus plantarum* GKD7 has a lower pain level, a lower level of bone damage, a lower level of cartilage damage and lower expression levels of pro-inflammatory cytokines in the cartilage tissue and the synovial tissue. Thus, *Lactobacillus plantarum* GKD7 can be used to manufacture a medicament for treating osteoarthritis and degenerative joint disease. In the aforementioned embodiment, the medicament can include but not limited to the effective dosage of the dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient.

[0048] There is no specific limitation on the subject, which can be a mammal such as a rat, a mouse, a rabbit, a cat, a dog or a human, for example. In some embodiments, to achieve a better effect with a lower dosage, the effective dosage of *Lactobacillus plantarum* GKD7 can be 10 mg/kg weight/day to 1000 mg/kg weight/day, such as 10 mg/kg to 500 mg/kg, or 10 mg/kg to 100 mg/kg when the subject is a rat. In some embodiments, the effective dosage of *Lactobacillus plantarum* GKD7 can be 1 mg/kg weight/day to 165 mg/kg weight/day, such as 1 mg/kg weight/day to 100 mg/kg weight/day, or 1 mg/kg weight/day to 50 mg/kg weight/day when the subject is a human. It should be noted that with experimental support, the administration of 100 mg/kg to 1000 mg/kg of *Lactobacillus plantarum* GKD7 can indeed improve osteoarthritis. However, in the abovementioned animal experiment, osteoarthritis is induced by an anterior cruciate ligament transaction, so that course of osteoarthritis is very fast. Thus,

the same effect can achieve with a lesser dosage of *Lactobacillus plantarum* GKD7 clinically. In some embodiments, when the subject is a human, the effective dosage of *Lactobacillus plantarum* GKD7 can be 1 mg/kg weight/day to 10 mg/kg weight/day, for example. In the aforementioned embodiment, the subjects are those who want to prevent osteoarthritis or those who are diagnosed with early osteoarthritis, that is, the subject has at most 1 and at least 4 joints that are inflamed and the osteoarthritis is onset within two years.

[0049] *Lactobacillus plantarum* GKD7 is required to be administered to a subject for a period. In some embodiments, *Lactobacillus plantarum* GKD7 can be administered to a subject in a frequency of 3 times to 7 times per week, such as 5 times per week. In some embodiments, *Lactobacillus plantarum* GKD7 can be administered to a subject for 2 weeks to 8 weeks and can be shorter or longer.

[0050] As applied, the aforementioned medicament can be an oral composition. In some embodiments, the aforementioned oral composition can be a medical composition or a food composition. In one embodiment, the oral composition can selectively include a food- or medical-acceptable carriers, excipients, diluents, adjuvants, preservatives, fillers and/or additives.

[0051] Although the present invention has been described in considerable detail with reference to certain embodiments thereof, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the embodiments contained herein.

1. Preparation example 1

1.1. Origin of strain

[0052] *Lactobacillus plantarum* GKD7 (abbreviated as strain GKD7 herein) was deposited at NITE on November 6, 2020, under the accession number of NITE BP-03313. Please refer to Taiwan patent TW I733207B for the microorganism traits and the culture method of strain GKD7. The patent was cited as a reference herein.

1.2. Preparation of lyophilized powder

[0053] The bacterial solution of strain GKD7 was spread on the MRS medium and was incubated at 37°C in an anaerobic atmosphere (80% of N_2, 10% of H_2 and 10% of CO_2) for 18 hours, so as to obtain single colonies. Then, the single colonies were inoculated in a broth and were incubated at 37°C in an anaerobic atmosphere for 18 hours, so as to obtain a liquid-state culture. The broth contained 1% to 10% of glucose, 0.1% to 5% of yeast extract, 0.1% to 0.5% of peptone, 0.01% to 2.00% of trace element, 0.01% to 0.10% of cysteine, 0.05% to 1.00% of TWEEN-80 and water in balance. The trace elements were selected from a group consisting of magnesium ions, potassium ions, sulfate ions, hydrogen phosphite ions and any combination thereof. It should be noted that the kinds of medium did not affect the effect of strain GKD7 on improving osteoarthritis, and that the aforementioned media listed herein were not intended to limit the present invention.

[0054] Next, the liquid-state culture was added with 15 wt% of skim milk powder, followed by a centrifuge step at 1000 rpm to 15000 rpm to obtain the active cells of strain GKD7. Before the centrifuge step, the liquid-state culture was mixed with 15 wt% of skim milk powder to help the cells of strain GKD7 to form aggregates, thereby increasing the recycling rate of the cells. Then, after mixing

the cells with 6 wt% to 50 wt% of skim milk powder, the lyophilization step was performed to obtain the lyophilized powder of the active cells of strain GKD7. It should be noted that the lyophilization step was performed to make administration, quantization and preservation more easily and did not intend to limit the present invention.

2. Preparation example 2

[0055] The liquid-state culture was subjected to a heat inactivation step by using a saturated vapor pressure of 110°C, so as to obtain a heat-inactive liquid-state culture. Further, the heat-inactive liquid-state culture was subjected to the centrifuge step to obtain the dead cells of strain GKD7. Next, the dead cells of strain GKD7 were subjected to a lyophilization step to obtain the lyophilized powder of the dead cells of strain GKD7.

3. Methods of evaluation and results

3.1. Animal model

[0056] Eight-week-old male Sprague Dawley (SD) rats were maintained in stainless mouse cages. The feeding environment was at a temperature of 22±2°C, a humidity of 60% to 80%, and the light period and the dark period was 12 hours for each. During the test, the rats were *ad libitum* feeding with standard chow and distilled water. The rats were weighed 300 g to 350 g, and the bones of the rats were well-developed.

[0057] The rats were grouped into the sham-operated control group (n=6), the operated control group (n=6) and the experimental group 1 (n=6). The right hind limbs of rats in the sham-operated control group, the operated control group and

the experimental group 1 were subjected to the corresponding surgery. In detail, the right hind limbs of rats in the operated control group and the experimental group 1 were subjected to anterior cruciate ligament transaction (abbreviated as ACLT) including an arthrotomy and the transaction of the anterior cruciate ligament, so as to induce osteoarthritis at the right hind limbs of the rats. The right hind limbs of rats in the sham-operated control group were subjected to arthrotomy without the transaction of anterior cruciate ligaments.

[0058] Two days after surgery, 1 mL of distilled water was added to the chow in each group. Five days were selected to add the lyophilized powder of the active cells of strain GKD7 (preparation example 1) to the distilled water in the experimental group 1 each week, so that the rat in the experimental group 1 obtained 100 mg/kg bw/day (equivalent to 5×10^{11} CFU/g, which could convert to a human dosage of 16.1 mg/kg weight/day) of the lyophilized powder of the active cells of strain GKD7.

[0059] Subsequently, the lyophilized powders of the active cells of strain GKD7 and the dead cells of strain GKD7 with different dosages were used for the evaluation. The eight-week-old rats were grouped into six groups, which were the sham-operated control group 2, the operated control group 2, the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group. Eight rats were in each group. The right hind limbs of rats in the sham-operated control group 2 were subjected to arthrotomy without the transaction of the anterior cruciate ligaments. The right hind limbs of rats in the operated control group 2, the experimental group 2, the experimental group

3, the experimental group 4 and the positive control group were subjected to ACLT surgery.

[0060] After one to two days of recovery, five days were selected every week to add 1 mL of distilled water to the chow of each group. The lyophilized powders of the active cells of strain GKD7 and the dead cells of strain GKD7 with different dosages were added to the distilled water in different groups, so that the rats in the experimental group 2 could obtain a low dosage (100 mg/kg bw/day, equivalent to the dosage of the experimental group 1) of the lyophilized powder of the active cells of strain GKD7 (preparation example 1), the rats in the experimental group 3 could obtain a high dosage (1 g/kg bw/day, equivalent to a human dosage of 161.3 mg/kg weight/day) of the lyophilized powder of the active cells of strain GKD7 (preparation example 1), and the rats in the experimental group 4 could obtain the lyophilized powder of the dead cells of strain GKD7 (preparation example 2) with a dosage of 1 g/kg bw/day. The distilled water of the positive control group was mixed with 50 mg/kg of celecoxib, which is a commercial analgesic drug that had the effect to inhibit the activity of cyclooxygenase (COX), thereby inhibiting the synthesis of prostaglandin E2, (PGE_2) to relief an inflammatory pain.

3.2 Static weight-bearing test (or so-called incapacitance test)

[0061] Before the surgery, the left and right hind limbs of rats in the sham-operated control group, the operated control group and the experimental group 1 were respectively placed on separate sensor boards for 10 seconds to measure the difference between weights borne by the left and right hind limbs of rats (unit: gram weight, normalized by the weights of rats)when they stood on the hind limbs.

After the surgery, the aforementioned static weight-bearing test was done every week. Generally speaking, a normal rat would distribute the weight borne evenly by the left and right hind limbs. Thus, a bigger difference between weights borne by the left and right limbs represented a higher level of pain in the right hind limb of the rat. The results between different groups were analyzed by using paired t-test.

[0062] Referred to FIG, 1, which was a curve chart illustrating the change of the difference between weights borne by the left and right limbs of rats in different groups 6 weeks after the surgery according to an embodiment of the present invention. The x-axis represented time (unit: week), the y-axis represented the difference between weights borne by the left and right limbs of rats (unit: g), curve 110, curve 120 and curve 130 respectively represented the sham-operated control group, the operated control group and the experimental group 1, the symbol "*" represented a statistically significant difference ($p<0.05$) comparing to the sham-operated control group, and the symbol "#" represented a statistically significant difference ($p<0.05$) comparing to the operated control group.

[0063] As shown in FIG, 1, the difference between the weights borne by the left and right limbs of rats in the operated control group (curve 120) was significantly higher than that of rats in the sham-operated control group (curve 110). Noted that the difference between weights borne by the left and right limbs got higher as time went on, indicating that ACLT indeed induced osteoarthritis, thereby resulting in joint pain. On the other hand, the difference between the weights borne by the left and right limbs of rats in the experimental group 1 significantly decreased 2 weeks after the surgery. Moreover, the difference between the

weights borne by the left and right limbs of rats in the experimental group 1 was only 50% of that of rats in the operated control group 6 weeks after the surgery. As shown by the results, the lyophilized powder of the active cells of strain GKD7 was able to significantly relieve osteoarthritis-caused joint pain after the administration for 14 continuous days (2 weeks). It should be noted that there was no statistically significant difference between the rats in each group (not shown) in the weight measured before the surgery and once 6 weeks after the surgery, indicating that ACLT did not affect the weight of rats.

[0064] The static weight-bearing test was also performed on the sham-operated control group 2, the operated control group 2, the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group. The method of the static weight-bearing test was mentioned above and would not be elaborated herein.

[0065] Referred to FIG, 2, which was a curve chart illustrating the change of the difference between the weights borne by the left and right limbs of rats in the different groups 6 weeks after the surgery according to an embodiment of the present invention. The x-axis represented time (unit: week), the y-axis represented the difference between weights borne by the left and right limbs of rats (unit: g), curve 210, curve 220, curve 230, curve 240, curve 250 and curve 260 respectively represented the sham-operated control group 2, the operated control group 2 and the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group. The symbol “#” represented a statistically significant difference (p<0.05) compared to the sham-operated control group 2 (curve 210), and the symbol “*” represented a

statistically significant difference (p<0.05) compared to the operated control group 2 (curve 220).

[0066] As shown in FIG, 2, compared to the operated control group 2 (curve 220), the differences between weights borne by left and right limbs of rats in the experimental group 2 (curve 230), the experimental group 3 (curve 240), the experimental group 4 (curve 250) and the positive control group (curve 260) were smaller, indicating that the lyophilized powder of the active cells and the dead cells of strain GKD7 could indeed relieve osteoarthritis-caused joint pain. Noted that curve 240 representing the experimental group 3 (the lyophilized powder of the active cells of strain GKD7 with a high dosage) and curve 260 representing the positive control group almost overlapped on the 4th to the 6th week after the surgery. The results indicated that the effect to relieve joint pain of the lyophilized powder of the active cells of strain GKD7 was the same as that of a commercial analgesic drug (celecoxib). Moreover, curve 230 representing the experimental group 2 (the lyophilized powder of the active cells of strain GKD7 with a low dosage) and curve 250 representing the experimental group 4 had the same trend. The results indicated that the effect to relieve joint pain of the lyophilized powder of the active cells of strain GKD7 was the same as that of the lyophilized powder of the dead cells of strain GKD7. Although the subsequent evaluation was performed by the active cells lyophilized powder of strain GKD7 with a low dosage, a person skilled in the art should understand that the same effect could be achieved by administering the lyophilized powder of the dead cells of strain GKD7.

3.3 Bone image analysis

[0067] Six weeks after ACLT, the rats in the sham-operated control group, the operated control group and the experimental group 1 were sacrificed, the knee joints of the right hind limbs of rats were analyzed by using Micro-computerized tomography (Micro-CT, or μ-CT) to obtain the bone parameters including the bone mineral density, the bone mineral content, the bone volume fraction, the bone area fraction, the trabecular thickness, the trabecular number and the trabecular separation. The μ-CT images showed that the bone lesions were indeed found in the rats in the operated control group, and the bone lesions included the bone mineral loss of subchondral bone (not shown). However, the rats in the experimental group 1 had lower levels of bone lesions compared to the rats in the operated control group (not shown).

[0068] Referred to FIGs. 3A to 3G, which were box charts respectively illustrating the bone mineral density (FIG, 3A), the bone mineral content (FIG, 3B), the bone volume fraction (FIG, 3C), the bone area fraction (FIG, 3D), the trabecular thickness (FIG, 3E), the trabecular number (FIG, 3F) and the trabecular separation (FIG, 3G) of rats in different groups according to an embodiment of the present invention. The x-axis represented groups, which were the sham-operated control group, the operated control group and the experimental group 1 from left to right. The y-axis represented the bone mineral density (unit: g/cm^3), the bone mineral content (unit: mg), the bone volume fraction (i.e., the ratio value of bone volume to total volume, abbreviated as BV/TV, unit: %), the bone area fraction (i.e., the ratio value of bone surface to total volume, abbreviated as BS/TV, unit: 1/mm), the trabecular thickness (abbreviated as Tb.Th, unit: mm), the trabecular number (abbreviated as Tb.N, unit: 1/mm) and the trabecular

separation (abbreviated as Tb.Sp, unit: mm). The symbol "*" represented a statistically significant difference (p<0.05) compared to the sham-operated control group, and the symbol "#" represented a statistically significant difference (p<0.05) compared to the operated control group.

[0069] As shown in FIGs. 3A to 3G, compared to the sham-operated control group, the knee joint of the right hind limb of rats in the operated control group had significantly decreased bone mineral densities, bone mineral contents, bone volume fractions, bone area fractions, trabecular thicknesses and the trabecular numbers but significantly increased trabecular separations. The results indicated that ACLT-induced osteoarthritis could indeed cause the bone lesions such as bone mineral loss and bone destruction. On the other hand, the knee joint of the right hind limb of rats in the experimental group 1 had a significant improvement in the aforementioned bone parameters. The results indicated that the lyophilized powder of the active cells of strain GKD7 could indeed improve the bone lesions caused by osteoarthritis.

3.4 Histological analysis

[0070] After the bone image analysis, the knee joint tissues of the right hind limbs of rats were fixed by phosphate-buffered saline (PBS) with 4% paraformaldehyde for 24 hours. After that, decalcification was performed with 10% ethylenediaminetetraacetic acid (EDTA) for 2 weeks. After dehydration by ethanol with increased concentrations, the knee joint tissues were embedded into paraffin blocks, followed by sectioning to obtain tissue slices that were 5-μm in thickness. Next, the tissue slices were subjected to hematoxylin and eosin (H&E) and Safranin-O/Fast Green staining. The histopathological changes of knee

joint tissues were observed under an optical microscope. It could be seen from the tissue slices that the pathological changes and the cartilage damage such as synovial hyperplasia indeed existed in the knee joint tissues of rats in the operated control group (not shown), but the levels of the cartilage damage of rats in the experimental group 1 were lower than that of rats in the operated control group (not shown).

[0071] The levels of cartilage damage in the rats were quantified by the OARSI histopathology initiative recommendations for histological assessments of osteoarthritis in the rats including Osteoarthritis Research Society International (OARSI) score, the cartilage degeneration score and the synovial inflammation score. The OARSI score, the cartilage degeneration score and the synovial inflammation score were well-known by those skilled in the art and would not be elaborated herein once again.

[0072] Referred to FIGs. 4A to 4C, which were box charts respectively illustrating the OARSI score, the cartilage degeneration score and the synovium inflammation score of different groups according to an embodiment of the present invention. The x-axis represented groups, which were the sham-operated control group, the operated control group and the experimental group 1 from left to right. The y-axis represented the OARSI score, the cartilage degeneration score and the synovium inflammation score (unit: score). The symbol "*" represented a statistically significant difference (p<0.05) compared to the sham-operated control group, and the symbol "#" represented a statistically significant difference (p<0.05) compared to the operated control group.

[0073] As shown in FIGs. 4A to 4C, the OARSI score, the cartilage degeneration score and the synovium inflammation score of rats in the operated control group significantly increased compared to that of the rat in the sham-operated control group, indicating that ACLT-induced osteoarthritis indeed led to cartilage damages. However, the OARSI score, the cartilage degeneration score and the synovium inflammation score of rats in the experimental group 1 significantly decreased compared to that of the rats in the operated control group, indicating that the lyophilized powder of the active cells of strain GKD7 could indeed improve the osteoarthritis-caused cartilage damages.

3.5 Immunohistochemistry (IHC) analysis

[0074] The aforementioned tissue slides were sunk in 3% hydrogen peroxide solution to inhibit the activities of endogenous catalase and then sunk in PBS solutions containing 3% bovine serum albumin (BSA) to decrease the formation of background formed by nonspecific bindings. After a first antibody incubation with IL-1β antibody and TNF-α antibody at 4°C, the tissue slides were subjected to a secondary antibody incubation at room temperature for 1 hour, followed by diaminobenzidine (DAB) staining. The tissue slides were observed under an optical microscope. The IHC score represented the signals for measuring immune response of IL-1β and TNF-α. A higher IHC score represented a stronger signal for measuring immune response, i.e., a higher expression level of IL-1β or TNF-α.

[0075] Referred to FIGs. 5A to 5B, which were box charts respectively illustrating the IHC scores of IL-1β (FIG. 5A) and TNF-α (FIG. 5B) of the cartilage tissue of rats in different groups according to an embodiment of the present invention.

The x-axis represented groups, which were the sham-operated control group, the operated control group and the experimental group 1 from left to right. The y-axis represented the IHC scores of IL-1β and TNF-α (unit: score). As shown in FIGs. 5A to 5B, the IHC scores of IL-1β and TNF-α of rats in the operated control group significantly increase compared to that of rats in the sham-operated control group, indicating that the expression levels of pro-inflammatory cytokines such as IL-1β and TNF-α were higher in the cartilage tissue with ACLT-induced osteoarthritis. On the other hand, the IHC scores of IL-1β and TNF-α of rats in the experimental group 1 significantly decreased compared to that of rats in the operated control group, indicating that the lyophilized powder of the active cells of strain GKD7 could indeed decrease the expression level of pro-inflammatory cytokines in the cartilage tissue with osteoarthritis.

[0076] Referred to FIGs. 6A to 6B, which were box charts respectively illustrating the IHC scores of IL-1β (FIG. 6A) and TNF-α (FIG. 6B) of the cartilage tissue of rats in different groups according to an embodiment of the present invention. The x-axis represented groups, which were the sham-operated control group, the operated control group and the experimental group 1 from left to right. The y-axis represented the IHC scores of IL-1β and TNF-α (unit: score). As shown in FIGs. 6A to 6B, the IHC scores of IL-1β and TNF-α of the rats in the operated control group significantly increased compared to that of rats in the sham-operated control group, indicating that the expression levels of pro-inflammatory cytokines in cartilage tissue with ACLT-induced osteoarthritis were indeed higher. On the other hand, the IHC scores of IL-1β and TNF-α of rats in the experimental group 1 significantly decreased compared to rats in the operated control group,

indicating that the lyophilized powder of the active cells of strain GKD7 could indeed decrease the expression levels of pro-inflammatory cytokines in the synovial tissue with osteoarthritis.

[0077] In conclusion, the specific dosage, the specific form, the specific test or the specific evaluation method are only intended to illustrate and describe the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease of the present invention. However, those skilled in the art should understand that other dosages, other forms, other tests or other evaluation method can also be used for describing the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease of the present invention without departing from the spirit and scope of the present invention and thus are not limited. For example, the medicament can be administered in a form of medical composition or a food composition and can selectively include medical and food-acceptable carriers, excipients and/or additives. Moreover, the medicament can be administered in the form of powder, lozenge, granule, suppository, microcapsule, ampule, liquid spray or suppository, etc. In other examples, *Lactobacillus plantarum* GKD7 can also be incubated in other appropriate medium or other incubation environments, and the obtained cells of *Lactobacillus plantarum* GKD7 can also have the aforementioned effect to improve osteoarthritis. In other embodiments, the effect to improve osteoarthritis-caused bone lesions, cartilage damages, cartilage inflammation and/or synovial inflammation, etc., of the active cells with a low dosage (the experimental group

1) can also be achieved by the active cells with a high dosage (the experimental group 2) and the dead cells with a high dosage (the experimental group 4).

[0078] It can be seen from the aforementioned embodiments that the use of *Lactobacillus plantarum* GKD7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease has the advantage of using the dead cells of *Lactobacillus plantarum* GKD7, which can not only relieve osteoarthritis-caused joint pain but also improve osteoarthritis-caused bone lesions, cartilage damages, cartilage inflammation and/or synovial inflammation. Thus, the dead cells of *Lactobacillus plantarum* GKD7 can be used as an active ingredient and be applied in the medicament for treating osteoarthritis.

[0079] It will be apparent to those skilled in the art that various modifications and variations can be made to the structure of the present invention without departing from the scope or spirit of the invention. In view of the foregoing, it is intended that the present invention cover modifications and variations of this invention provided they fall within the scope of the following claims.

WHAT IS CLAIMED IS:

1. Use of *Lactobacillus plantarum* GKD7 in manufacture of a medicament for treating osteoarthritis, wherein the medicament comprises an effective dosage of dead cells of the *Lactobacillus plantarum* GKD7 as an active ingredient, the *Lactobacillus plantarum* GKD7 is deposited in National Institute of Technology and Evaluation (NITE), Patent Microorganisms Depositary on November 6, 2020 under an accession number of NITE BP-03313, and the medicament is to be administered to relieve osteoarthritis-caused joint pain of a subject.

2. The use according to claim 1, wherein the dead cells of the *Lactobacillus plantarum* GKD7 is to be obtained by subjecting the *Lactobacillus plantarum* GKD7 to a fermentation step and an inactivation step, the fermentation step is performed at 25°C to 40°C in an anaerobic atmosphere on a fermentation substrate for 16 hours to 24 hours.

3. The use according to claim 2, wherein the inactivation step comprises a heat inactivation step, a chemical inactivation step and/or a radiation inactivation step.

4. The use according to claim 3, wherein the heat inactivation step is performed by using saturated vapor pressure of 110°C to 120°C for 10 minutes to 20 minutes.

5. The use according to claim 2, wherein the dead cells of the *Lactobacillus plantarum* GKD7 are to be subjected to a lyophilization step.

6. The use according to claim 1, wherein when the subject is a rat, the effective dosage is 10 mg/kg weight/day to 1000 mg/kg weight/day.

7. The use according to claim 1, wherein when the subject is a human, the effective dosage is 1 mg/kg weight/day to 165 mg/kg weight/day.

8. Use of *Lactobacillus plantarum* GKD7 in manufacture of a medicament for treating degenerative joint disease, wherein the medicament comprises dead cells of the *Lactobacillus plantarum* GKD7 as an active ingredient, the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313, the medicament is to be administered to relieve joint pain of a subject, and the medicament is to be administered in a form of an oral composition.

9. The use according to claim 8, wherein the dead cells of the *Lactobacillus plantarum* GKD7 is obtained by subjecting the *Lactobacillus plantarum* GKD7 to a fermentation step and a heat inactivation step.

10. The use according to claim 8, wherein comparing to a control subject that the medicament is not to be administered to, the subject that the medicament is to be administered to has a lesser difference between weights borne by left and right limbs.

11. The use according to claim 8, wherein comparing to a control subject that the medicament is not to be administered to, the subject that the medicament

is to be administered to has a lower expression level of interleukin (IL)-1β in a cartilage tissue and/or a synovial tissue.

12. The use according to claim 8, wherein comparing to a control subject that the medicament is not to be administered to, the subject that the medicament is to be administered to has a lower expression level of tumor necrosis factor (TNF)-α in a cartilage tissue and/or a synovial tissue.

13. The use according to claim 8, wherein the medicament is to be administered to the subject in a frequency of 3 times to 7 times per week.

14. The use according to claim 8, wherein the medicament is to be administered to the subject for 2 weeks to 8 weeks.

15. A pharmaceutical composition for treating osteoarthritis, comprising an effective dosage of dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, wherein the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313, the dead cells of the *Lactobacillus plantarum* GKD7 is to be obtained by subjecting the *Lactobacillus plantarum* GKD7 to an inactivation step.

16. The pharmaceutical composition according to claim 15, further comprises medical-acceptable carriers, excipients and/or additives.

17. The pharmaceutical composition according to claim 15, wherein the pharmaceutical composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule, liquid spray or suppository.

18. A food composition for use in treating degenerative joint disease, comprising an effective dosage of a lyophilized powder of dead cells of *Lactobacillus plantarum* GKD7 as an active ingredient, wherein the *Lactobacillus plantarum* GKD7 is deposited under an accession number of NITE BP-03313.

19. The food composition according to claim 18, further comprises food-acceptable carriers, excipients and/or additives.

20. The food composition according to claim 18, wherein the food composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule, liquid spray or suppository.