USE OF CLOSTRIDIUM BUTYRICUM GKB7 IN MANUFACTURE OF MEDICAMENT FOR TREATING OSTEOARTHRITIS AND DEGENERATIVE JOINT DISEASE

BACKGROUND

Field of Invention

[0001] The present disclosure relates to a use of a probiotic in manufacture of a medicament for treating musculoskeletal diseases, in particular to *Clostridium butyricum* GKB7 in manufacture of a medicament for treating osteoarthritis.

Description of Related Art

[0002] Osteoarthritis (OA), or called degenerative joint disease, is a disease which chronic degenerative inflammation occurs in a joint. Osteoarthritis often occurs in hands, hips and knee joints. Symptoms such as bone damage, cartilage damage, subchondral remodeling, inflammation of a cartilage tissue and a synovial tissue may occur in an affected part of osteoarthritis, resulting in pain, stiffness and/or swelling in the affected part. Osteoarthritis is extremely common, affecting one-third of the world's population over the age of 65, according to statistics published in 2021. In countries with aging populations, osteoarthritis has become a serious problem.

[0003] Osteoarthritis cannot be cured at present, and severe cases can only be intervened with surgery. Before surgical intervention, conservative treatment can be used to relieve joint pain and/or improve joint mobility caused by osteoarthritis. Among them, non-steroidal anti-inflammatory drugs are common

drugs for conservative treatment, which can inhibit action of cyclooxygenase (COX), thereby reducing production of prostaglandin E2 (PGE2) to relieve osteoarthritis. However, NSAIDs can only relieve osteoarthritis, not only fail to treat osteoarthritis, but may also inhibit synthesis of proteoglycans, thereby accelerating deterioration of osteoarthritis. Furthermore, long-term use of NSAIDs may cause side effects such as peptic ulcer, lower extremity edema, damage to kidney and heart functions.

[0004] The intestinal microbiota ecosystem includes bacteria, fungi, viruses, phages and parasites from the mouth to the anus. The microbiota is involved in many important functions including host immune function, regulation of intestinal permeability, nutrient absorption, pathogen resistance, neurotransmitter synthesis and secretion, and metabolic homeostasis. Intestinal dysbiosis increases intestinal mucosal permeability and interferes with metabolic balance, leading to systemic inflammation, degradation in cartilage, and gut-brain axis imbalance, which is considered to be one of key causes of osteoarthritis. However, there are few studies on whether *Clostridium butyricum* can improve musculoskeletal diseases.

SUMMARY

[0005] One aspect of the present disclosure is to provide a use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating osteoarthritis, in which the medicament includes *Clostridium butyricum* GKB7 as an active ingredient.

[0006] Another aspect of the present disclosure is to provide a use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating degenerative joint disease, in which the medicament includes *Clostridium butyricum* GKB7 as an active ingredient, so as to improve bone damage, cartilage damage, inflammation of a cartilage tissue and/or a synovial tissue in a subject.

[0007] The other aspect of the present disclosure is to provide a pharmaceutical composition or a food composition for treating osteoarthritis, in which the pharmaceutical composition or the food composition include live cells and/or dead cells of *Clostridium butyricum* GKB7 as an active ingredient.

[0008] According to the above aspects of the present disclosure, a use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating osteoarthritis is provided. The medicament includes an effective dose of the *Clostridium butyricum* GKB7 as an active ingredient, and the *Clostridium butyricum* GKB7 is deposited in NITE National Institute of Technology and Evaluation, NITE Patent Microorganisms Depositary with an accession number of NITE BP-03889 on May 22, 2023, and the medicament is to be administered to relieve osteoarthritis of a subject.

[0009] According to some embodiments of the present disclosure, the *Clostridium butyricum* GKB7 is live cell that are to be obtained by subjecting the *Clostridium butyricum* GKB7 to a fermentation step, and the fermentation step is performed on the *Clostridium butyricum* GKB7 by using a fermentation medium in an anaerobic environment of 25°C to 40°C for 16 hours to 24 hours.

[0010] According to some embodiments of the present disclosure, the *Clostridium butyricum* GKB7 is dead cell that are to be obtained by subjecting the *Clostridium*

butyricum GKB7 to a fermentation step and an inactivation step, and the fermentation step is performed on the *Clostridium butyricum* GKB7 by using a fermentation medium in an anaerobic environment of 25°C to 40°C for 16 hours to 24 hours.

[0011] According to some embodiments of the present disclosure, the inactivation step comprises a heat inactivation step, a radiation inactivation step, and/or a chemical inactivation step.

[0012] According to some embodiments of the present disclosure, the heat inactivation step performed at a saturated vapor pressure of 110°C to 120°C for 10 minutes to 20 minutes.

[0013] According to some embodiments of the present disclosure, when the subject is a rat, the effective dose is 50 mg/kg body weight/day to 2,000 mg/kg body weight/day.

[0014] According to some embodiments of the present disclosure, when the subject is a human being, the effective dose is 1 mg/kg body weight/day to 200 mg/kg body weight/day.

[0015] According to another aspect of the present disclosure, a use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating degenerative joint disease, in which the medicament includes the *Clostridium butyricum* GKB7 as an active ingredient, and the *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889, and the *Clostridium butyricum* GKB7 is to be administered to improve bone damage, cartilage damage, inflammation of a cartilage tissue and/or a synovial tissue in a subject.

[0016] According to some embodiments of the present disclosure, compared with a control subject that the medicament is not to be administered to, an expression level of interleukin (IL-1β) and/or an expression level of tumor necrosis factor (TNF)-α in the cartilage tissue and/or the synovial tissue in the subject administered is to be administered to decreases.

[0017] According to some embodiments of the present disclosure, compared with a control subject that the medicament is not to be administered to, joint pain of the subject that the medicament is to be administered to relieves.

[0018] According to some embodiments of the present disclosure, compared with 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.

[0019] According to some embodiments of the present disclosure, the osteoarthritis is early osteoarthritis.

[0020] According to some embodiments of the present disclosure, the medicament is to be administered to the subject at a frequency of 3 times to 7 times per week.

[0021] According to some embodiments of the present disclosure, the medicament is to be administered to the subject for 2 weeks to 8 weeks.

[0022] According to the other aspects of the present disclosure, a pharmaceutical composition for treating osteoarthritis is provided. The pharmaceutical composition for treating osteoarthritis includes live cells and/or dead cells of

Clostridium butyricum GKB7 as an active ingredient. The *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889.

[0023] According to some embodiments of the present disclosure, the pharmaceutical composition can selectively include medical-acceptable carriers, excipients and/or additives.

[0024] According to some embodiments of the present disclosure, the pharmaceutical composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule or liquid spray.

[0025] According to the other aspects of the present disclosure, a food composition for treating osteoarthritis is provided. The food composition for treating osteoarthritis includes live cells and/or dead cells of *Clostridium butyricum* GKB7 as an active ingredient. The *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889.

[0026] According to some embodiments of the present disclosure, the pharmaceutical composition can selectively include food-acceptable carriers, excipients and/or additives.

[0027] According to some embodiments of the present disclosure, the pharmaceutical composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule or liquid spray.

[0028] By applying the use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease, in which the medicament includes the *Clostridium butyricum* GKB7 as an active ingredient, thereby improving bone damage, cartilage damage, inflammation of a cartilage

tissue and/or a synovial tissue, as well as decreasing an expression level of IL-1β and/or an expression level of TNF-α in the cartilage tissue and/or the synovial tissue, and so as to relieve osteoarthritis-caused joint pain.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] In order to allow the above-mentioned and other objects, features, advantages and embodiments of the present disclosure to be more clearly understood, the detailed description of the accompanying drawings is as follows:

[0030] Fig. 1 is a curve chart showing differences in weights borne by rats in different groups within 6 weeks after surgery according to an embodiment of the present disclosure.

[0031] Fig. 2 is a curve chart showing differences in weights borne by rats in different groups within 6 weeks after surgery according to an embodiment of the present disclosure.

[0032] Fig. 3A to Fig. 3G are box plots respectively showing bone mineral density, bone mineral content, bone volume fraction, bone area fraction, trabecular thickness, trabecular number, and trabecular separation of different groups according to an embodiment of the present disclosure.

[0033] Fig. 4A to Fig. 4C are diagrams respectively showing different groups of OARSI scores, cartilage degeneration scores and synovial inflammation scores according to an embodiment of the present disclosure.

[0034] Fig. 5A to Fig. 5B are box plots respectively showing IHC scores of IL-1β and IHC scores of TNF-α in different groups of cartilage tissues according to an embodiment of the present disclosure.

[0035] Fig. 6A to Fig. 6B are box plots respectively showing IHC scores of IL-1β and IHC scores of TNF-α in different groups of synovial tissues according to an embodiment of the present disclosure.

DETAILED DESCRIPTION

[0036] If the definition or usage of a term in the 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. Secondly, unless otherwise defined by context, the singular term may include plural, and the plural term may include singular.

[0037] As mentioned above, the present disclosure provides a use of *Clostridium butyricum* for preparing a medicament of improving musculoskeletal diseases. The musculoskeletal diseases, as used herein, refer to impairment of the locomotor system (including muscles, bones, joints, and adjacent connective tissues), resulting in temporary or lifelong functional limitations. The musculoskeletal diseases are often characterized by pain and limitations in mobility and flexibility. The musculoskeletal diseases include joint problems such as osteoarthritis, rheumatoid arthritis, psoriatic arthritis, gout, ankylosing spondylitis, etc., bone problems such as osteoporosis, osteopenia, fragility fractures, traumatic fractures, etc., muscle problems such as sarcopenia, etc.,

spinal problems such as back and neck pain, etc., and problems with multiple body parts or systems.

[0038] In some embodiments, the musculoskeletal disease is osteoarthritis. Clinical symptoms of osteoarthritis include pain and stiffness, in which the pain increases after activity but relieves after rest, and the stiffness improves after activity but recurs after rest. Using optical imaging tools to detect, the affected part of osteoarthritis often has joint cavity narrowing, bone spur formation, subchondral bone sclerosis or subchondral bone cyst, bone damage, cartilage damage, inflammation of the cartilage tissue and/or the synovial tissue.

[0039] In some embodiments, evaluation items of osteoarthritis may include but not limited to the level of pain, the degree of bone damage, the degree of cartilage damage, the expression levels of pro-inflammatory cytokines in the cartilage tissue and the synovial tissue. In some specific examples, since osteoarthritis is generally asymmetrical, the level of pain can be assessed using differences between weights borne by limbs.

[0040] In some specific examples, the degree of bone damage can be determined 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 degree of cartilage damage can be scored using a rat joint histological scoring system recommended by the Osteoarthritis Research Society International (OARSI).

[0041] In some specific examples, the above-mentioned pro-inflammatory cytokines may include but not limited to interleukin (IL)-1β and tumor necrosis factor (TNF)-α. It is supplemented that IL-1β and TNF-α can promote synthesis

of proteolytic enzymes, thereby degrading the extracellular matrix in joints, leading to more serious osteoarthritis.

[0042] Experiments have confirmed that *Clostridium butyricum* GKB7 can improve osteoarthritis. Specifically, compared with control subjects not administered with *Clostridium butyricum* GKB7, the subjects administered with *Clostridium butyricum* GKB7 have lower level of pain, lower degree of bone damage, lower degree of cartilage damage, and lower expression levels of pro-inflammatory cytokines in the cartilage tissue and the synovial tissue. The above-mentioned *Clostridium butyricum* GKB7 was deposited in National Institute of Technology and Evaluation, NITE Patent Microorganisms Depositary with an accession number of NITE BP-03889 on May 22, 2023.

[0043] Therefore, *Clostridium butyricum* GKB7 can be applied to the manufacture of a medicament for treating osteoarthritis and degenerative joint disease, in which the medicament may include but not limited to an effective dose of *Clostridium butyricum* GKB7 as an active ingredient. In some embodiments, *Clostridium butyricum* GKB7 is live cells obtained by subjecting *Clostridium butyricum* GKB7 to a fermentation step. In some embodiments, the fermentation step is carried out at 25°C to 40°C, or 35°C to 40°C, or 37°C, so that *Clostridium butyricum* GKB7 has a better growth activity and/or a better activity to improve osteoarthritis. In some embodiments, the fermentation step is carried out for 16 hours to 24 hours, or 18 hours, so as to obtain more live cells of *Clostridium butyricum* GKB7. In some embodiments, the fermentation step is performed in an anaerobic environment. The air content of the anaerobic environment is not particularly limited, but the oxygen content should be 0% or

close to 0%. In one specific example, the anaerobic environment may include but not limited to 75% to 85% of nitrogen, 5% to 15% of hydrogen, and 5% to 15% of carbon dioxide.

[0044] A fermentation medium of the fermentation step is not particularly limited, and may be, for example, a commercially available fermentation medium. In some embodiments, the fermentation medium may be a reinforced clostridial medium (RCM). In some embodiments, the fermentation medium may include but not limited to 1 wt% to 10 wt% of carbohydrates, 0.1 wt% to 5.0 wt% of yeast extract, 0.1 wt% to 5.0 wt% of protein peptone, 0.01 wt% to 2.00 wt% trace elements, 0.01 wt% to 0.10 wt% cysteine, 0.05 wt% to 1.00 wt% TWEEN-80 and a balanced amount of water. The above carbohydrates may include but not limited to monosaccharides and/or disaccharides. In some embodiments, the carbohydrates may be selected from the group consisting of glucose, galactose, fructose, maltose, lactose, sucrose and any combination thereof. The trace elements may be selected from the group consisting of magnesium ions, potassium ions, sodium ions, chloride ions and any combination thereof.

[0045] In some embodiments, *Clostridium butyricum* GKB7 is dead cells obtained by subjecting *Clostridium butyricum* GKB7 to the above-mentioned fermentation step and an inactivation step. The method of the inactivation step is not particularly limited, as long as *Clostridium butyricum* GKB7 loses its growth activity. In some embodiments, the inactivation step may include but not limited to a heat inactivation step, a radiation inactivation step, and/or a chemical inactivation step. In some specific embodiments, the inactivation step is a heat inactivation step performed at a saturated vapor pressure of 110°C to 120°C for

10 minutes to 20 minutes. It is worth noting that although the dead cells of *Clostridium butyricum* GKB7 lose the growth activity, experiments have proved that the dead cells of *Clostridium butyricum* GKB7 still have the activity to improve osteoarthritis. Secondly, compared with the live cells of *Clostridium butyricum* GKB7, the preservation and/or dosage forms of the dead cells of *Clostridium butyricum* GKB7 are less restricted, and thus the dead cells of *Clostridium butyricum* GKB7 can be applied to more types of products.

[0046] In some embodiments, after the fermentation step, or after the fermentation step and the inactivation step, a solid-liquid separation step may be optionally performed on *Clostridium butyricum* GKB7 to remove the fermentation medium. The solid-liquid separation step is not particularly limited, but it is necessary to remove the fermentation medium and retain the live or dead cells of *Clostridium butyricum* GKB7. In some embodiments, the solid-liquid separation step may include but not limited to a filtration step and/or a centrifugation step. It is supplemented that the sequence of the heat inactivation step and the solid-liquid separation step is not particularly limited. In some embodiments, after the fermentation step, *Clostridium butyricum* GKB7 may be subjected to a solid-liquid separation step and then an inactivation step.

[0047] In some embodiments, the live and/or dead cells of the above-mentioned *Clostridium butyricum* GKB7 may be further subjected to a freeze-drying step to obtain a lyophilized powder, thereby prolonging its storage time. In some embodiments, the lyophilized powder includes 5.5×10^7 CFU/g of *Clostridium butyricum* GKB7.

[0048] The subjects are not particularly limited, and may be mammals such as rats, mice, rabbits, cats, dogs or humans. In some embodiments, when the subject is a rat, the effective dose of *Clostridium butyricum* GKB7 may be, for example, 50 mg/kg body weight/day to 2,000 mg/kg body weight/day, so as to achieve optimal efficacy with a relatively small dose, such as 50 mg/kg to 500 mg/kg, or 80 mg/kg to 100 mg/kg. In some embodiments, when the subject is a human, the effective dose of *Clostridium butyricum* GKB7 may be, for example, 1 mg/kg body weight/day to 200 mg/kg body weight/day, such as 1 mg/kg body weight/day to 100 mg/kg body weight/day, or 1 mg/kg body weight/day to 50 mg/kg body weight/day. It is worth noting that animal experiments have proven that the administration of *Clostridium butyricum* GKB7 at 100 mg/kg to 1,000 mg/kg can indeed improve osteoarthritis, but in the animal experiments, osteoarthritis was induced by anterior cruciate ligament transaction, so course of osteoarthritis was rapid. In clinical application, it should be possible to use less dosage to achieve the same effect. In some embodiments, when the subject is a human, the effective dose of *Clostridium butyricum* GKB7 is 1 mg/kg body weight/day to 10 mg/kg body weight/day. In the above embodiments, the subject desires to prevent osteoarthritis or suffers from early osteoarthritis (inflammation of at least one and less than four joints, and within two years of onset).

[0049] *Clostridium butyricum* GKB7 needs to be continuously administered to the subject for a period of time. In some embodiments, *Clostridium butyricum* GKB7 is administered to the subject at a frequency of 3 times to 7 times per week, such as 5 times per week. In some embodiments, *Clostridium butyricum* GKB7 is administered to the subject for 2 weeks to 8 weeks, or longer or shorter.

[0050] In application, the above-mentioned medicament may be, for example, an oral composition. In some embodiments, the aforementioned medicament may be, for example, a pharmaceutical composition or a food composition. In one embodiment, the medicament may optionally contain food or pharmaceutically acceptable carriers, excipients, diluents, adjuvants, preservatives, fillers and/or additives.

[0051] Several embodiments below are utilized to illustrate the application of the present disclosure, but those are not in order to limit the present disclosure, and those skilled in the art of the present disclosure may make various changes and modifications without departing from the spirit and scope of the present disclosure.

1. Preparation Example 1

1.1. Source of strain

[0052] The strain GKB7 was isolated from feces of healthy humans. After DNA of the strain GKB7 was purified, gene sequencing was performed to obtain a nucleic acid fragment sequence of 16s rRNA gene of the strain GKB7, as shown in a sequence identification number (SEQ ID NO): 1. After sequence comparison, the strain GKB7 was identified as *Clostridium butyricum*. The above-mentioned DNA purification, gene sequencing and sequence comparison were performed using conventional methods, and did not affect subsequent evaluation the activity to improve osteoarthritis of the strain GKB7, so details would not repeated here. *Clostridium butyricum* strain GKB7 is Gram-positive, absolutely anaerobic, motile, with spores and flagella. *Clostridium butyricum* strain GKB7 was deposited in National Institute of Technology and Evaluation, NITE Patent Microorganisms Depositary with an accession number of NITE BP-

03889 on May 22, 2023, and the viability of strain GKB7 was tested on May 24, 2023. Noted that the address of the depositary institution was #122, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan, and the deposit was made under the terms of the Budapest Treaty.

1.2. Preparation of lyophilized powder

[0053] The strain GKB7 was spread on RCM agar and incubated in an anaerobic environment (80% of N_2, 10% of H_2 and 10% of CO_2) at 37°C for 24 hours to obtain a single colony. Next, the single colony was inoculated in a culture medium, and a fermentation step was then performed in the anaerobic environment at 37°C for 24 hours, so as to obtain a liquid culture. The culture medium contained 5% of glucose, 1.5% of yeast extract, 0.5% of protein peptone, 0.1% of $MgSO_4$, 0.2% of K_2HPO_4, 0.1% of sodium acetate, 0.2% of NaCl and a balanced amount of water. Noted that the type of the culture medium would not affect the effect of the strain GKB7 on improving osteoarthritis, and the culture medium listed above was not intended to limit the present disclosure.

[0054] Next, a centrifugation step of 1,000 rpm to 15,000 rpm was performed on the liquid culture to obtain the live cells of the strain GKB7. Before the centrifugation step, the liquid culture and 15 wt% of skimmed milk powder could be mixed to help the bacteria aggregate into agglomerates, thereby increasing a recovery rate of the bacteria. Next, the bacteria and 6 wt% to 50 wt% of skim milk powder were mixed, and a freeze-drying step was then performed to obtain a live cell-lyophilized powder of the strain GKB7. Noted that the purpose of freeze-drying was only to facilitate administration, quantification and storage, and was not intended to limit the present disclosure.

2. Preparation Example 2

[0055] A heat inactivation step was performed on the liquid culture at a saturated vapor pressure of 110°C to obtain a heat-inactivated liquid culture. Next, a centrifugation step was performed on the heat-inactivated liquid culture to obtain dead cells of the strain GKB7. Next, a freeze-drying step was performed on the dead cells of the strain GKB7, so as to obtain a dead cell-lyophilized powder of the strain GKB7.

3. Evaluation method and results

3.1. Animal model

[0056] Male Sprague Dawley (SD) rats aged 8 weeks were raised in stainless steel mouse cages, and temperature of raising environment was 22±2°C, humidity was 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 feed (standard chow diet) and distilled water. The above-mentioned rats weighed 300 g to 350 g and had mature bones.

[0057] The rats were divided into a sham operated control group (n=6), a surgical control group (n=6) and an experimental group 1 (n=8). Corresponding operations were carried out on right hind limbs of the rats in the sham operated control group, the surgical control group and the experimental group 1, in which anterior cruciate ligament transection (abbreviated as ACLT) was performed on the right hind limbs of the rats in the surgical control group and the experimental group 1, which included arthrotomy and transection of fibers of the anterior cruciate ligament, to induce osteoarthritis in the right hind limbs of the rats by ACLT, and the right hind limbs of the rats in the sham operated control group

were subjected to arthrotomy without the transaction of anterior cruciate ligaments .

[0058] After 2 days of operation, 1 mL of distilled water was added to the feed of each group. The live cell-lyophilized powder of the strain GKB7 (Preparation Example 1) was added to the distilled water of the experimental group 1 for 5 selected days per week, so that the rats in the experimental group 1 obtained 100 mg/kg bw/day (equivalent to 5×10^{11} CFU/g, which could be converted to a human dose of 16.1 mg/kg body weight/day).

[0059] Subsequently, tests were carried out with the live cell-lyophilized powder and the dead cell-lyophilized powder of the strain GKB7 with different doses, respectively. The 8-week-old rats were divided into 6 groups, namely a sham operated control group 2, a surgical control group 2, an experimental group 2, an experimental group 3, an experimental group 4 and a positive control group. There were 8 experimental animals in each group. Anterior cruciate ligament transaction was performed on right hind limbs of the rats in the surgical control group 2, the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group.

[0060] After a recovery period of 1 day to 2 days in rats, 1 mL of distilled water was added to the feed of each group, and the live cell-lyophilized powder of GKB7 and the dead cell-lyophilized powder of GKB7 of corresponding doses were respectively added in the distilled water of different groups for 5 selected days per week, so that the rats in the experimental group 2 obtained a high dose (1 g/kg bw/day, which could be converted to a human dose of 161.3 mg/kg body weight/day) of the live cell-lyophilized powder of the strain GKB7 (Preparation

Example 1), and the rats in the experimental group 3 obtained a low dose (100 mg/kg bw/ day, identical with the dose of the experimental group 1) of the live cell-lyophilized powder of the strain GKB7 (Preparation Example 1), and the rats in the experimental group 4 obtained 1 g/kg bw/day of the dead cell-lyophilized powder of the strain GKB7 (Preparation Example 2). 50 mg/kg of Celecoxib was added to the distilled water of the positive control group, where Celecoxib was a commercially available analgesic, which had the effect of inhibiting activity of cyclooxygenase (COX), so that inflammatory pain could be reduced by inhibiting synthesis of prostaglandin E2 (PGE2).

3.2. Static weight bearing test (or called incapacitance test)

[0061] Before surgery, left and right hind limbs of the rats in the sham operated control group, the surgical control group and the experimental group 1 were placed on separate sensor plates for 10 seconds to measure the difference weights borne by left and right hind limbs (unit: gram weight, normalized by the rat body weight) when the rat stood on the limbs. After the surgery was performed, the above-mentioned tests were performed weekly. Generally, a normal rat would distribute their body weight equally to left and right hind limbs, so the greater the difference between weights borne by the left and right hind limbs, the higher the level of pain in the right hind limb of the rat. The results of different groups were compared using a paired t-test.

[0062] Please refer to Fig. 1, which was a curve chart showing differences between weights borne by the left and right limbs of rats in different groups within 6 weeks after the surgery according to an embodiment of the present disclosure, in which the horizontal axis represented time (unit: week), and the

vertical axis represented the difference between weights borne by the left and right limbs of rats (unit: g), and curve 110, curve 120 and curve 130 represented the sham operated control group, the surgical control group and the experimental group 1, respectively, and the symbol "*" indicated that there was a statistically significant difference (p<0.05) between the surgical control group and the sham operated control group, and the symbol "#" indicated that there was a statistically significant difference (p<0.05) between the experimental group 1 and the surgical control group.

[0063] As shown in Fig. 1, difference between the weights borne by the left and right limbs of rats in the surgical control group (curve 120) was significantly higher than that of the sham operated control group (curve 110), and the weight bearing difference was more and more serious with time, which means that ACLT did induce osteoarthritis, resulting in joint pain. On the contrary, the difference between the weights borne by the left and right limbs of rats in the experimental group 1 was significantly reduced 2 weeks after ACLT surgery, and continued to decrease over time, and was only one-third of that of the surgical control group 3 weeks after ACLT surgery. The results showed that the live cell-lyophilized powder of the strain GKB7 could significantly relieve the joint pain caused by osteoarthritis after 14 days (2 weeks) of continuous administration, and could achieve a more ideal effect after 21 days (3 weeks) of continuous administration.

[0064] The static weight bearing test was also performed on the sham operated control group 2, the surgical control group 2, the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group, and the method has been described above, and would not be repeated herein.

[0065] Please refer to Fig. 2, which was a curve chart showing differences between weights borne by the left and right limbs of rats in different groups within 6 weeks after surgery according to an embodiment of the present disclosure, in which the horizontal axis represented time (unit: week), and the vertical axis represented the difference between weights borne by the left and right limbs of rats (unit: g), and curve 210, curve 220, curve 230, curve 240, curve 250 and curve 260 represented the sham operated control group 2, the surgical control group 2, the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group, respectively, and the symbol "*" indicated that there was a statistically significant difference (p<0.05) between the surgical control group 2 and the sham operated control group 2, and the symbol "#" indicated that there was a statistically significant difference (p<0.05) between the experimental group 2, the experimental group 3, the experimental group 4 and the positive control group, and the surgical control group 2.

[0066] As shown in Fig. 2, compared with the surgical control group 2 (curve 220), the difference between weights borne by the left and right limbs of rats of 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, showing that the live cell-lyophilized powder of the strain GKB7 and the dead cell-lyophilized powder of GKB7 did significantly relieve the joint pain caused by osteoarthritis. Noted that the curve 230 representing the experimental group 2 and the curve 250 representing the experimental group 4 were almost overlapped, which meant that the live cell-lyophilized powder of the strain GKB7 and the dead cell-lyophilized powder of the strain GKB7 with the same doses exhibited the same effect on improving osteoarthritis, indicating that

there was the same effect on improving osteoarthritis and degenerative joint disease. The doses of the rats in the experimental group 2 and the experimental group 4 were different from that in the experimental group 3, but those all had the effect of relieving the joint pain. Therefore, although the following experiment investigated the effect of administering a low dose of the live cell-lyophilized powder of the strain GKD7 on the rats in the experimental group 1, those skilled in the art of the present disclosure should understand that the live cell-lyophilized powder and the dead cell-lyophilized powder of the strain GKD7 administered with a higher dose can achieve the same effect.

3.3. Bone image analysis

[0067] After 6 weeks of ACLT operation, the rats in the sham operated control group, the surgical control group and the experimental group 1 were sacrificed, and micro-computerized tomography (micro-CT, μ-CT) was used to analyze bone parameters of knee joints of the right hind limbs of the rats, which included bone mineral density, bone mineral content, bone volume fraction, bone area fraction, trabecular thickness, trabecular number and trabecular separation. Micro-CT showed that the rats in the surgical control group did have bone damage, including bone loss and bone destruction (not shown in the figure), but the degree of bone damage of the rats in the experimental group 1 was lower than that of the rats in the surgical control group (not shown in the figure).

[0068] Please refer to Fig. 3A to Fig. 3G, which were box plots respectively showing bone mineral density (Fig. 3A), bone mineral content (Fig. 3B), bone volume fraction (Fig. 3C), bone area fraction (Fig. 3D), trabecular thickness (Fig. 3E), trabecular number (Fig. 3F) and trabecular separation (Fig. 3G) of different

groups according to an embodiment of the present disclosure, in which the horizontal axis represented the groups, and from left to right were the sham operated control group, the surgical control group and the experimental group 1, respectively, and the vertical axis of Fig. 3A to Fig. 3G represented bone mineral density (unit: g/cm^3), bone mineral content (unit: mg), bone volume fraction (i.e., a ratio of bone volume to total volume, abbreviated as BV/TV, unit: %), bone area fraction (i.e., a ratio of bone surface area to total volume, abbreviated as BS/TV, unit: 1/mm), trabecular thickness (abbreviated as Tb.Th, unit: mm), trabecular number (abbreviated as Tb.N, unit: 1/mm) and trabecular separation (abbreviated as Tb.Sp, unit: mm), and the symbol "*" indicated there was a statistically significant difference (p<0.05) between the surgical control group and the sham operated control group, and the symbol "#" indicated that there was a statistically significant difference (p<0.05) between the experimental group 1 and the surgical control group.

[0069] As shown in Fig. 3A to Fig. 3G, compared to the sham operated control group, the bone mineral density, the bone mineral content, the bone volume fraction, the bone area fraction, the trabecular thickness, and the trabecular number of the knee joints of the right hind limbs of the rats in the surgical control group were decreased significantly, but the trabecular separation thereof was increased significantly, indicating that ACLT-induced osteoarthritis did cause bone damage such as bone loss and bone destruction. On the contrary, the above-mentioned bone parameters of the knee joints of the right hind limbs of the rats in the experimental group 1 were significantly improved, indicating that the live cell-lyophilized powder of the strain GKB7 did improve bone damage caused by osteoarthritis.

3.4. Histological analysis

[0070] After the bone image analysis, knee joint tissues of the right hind limbs of the rats were fixed at 37°C by phosphate-buffered saline (PBS) with 4% of paraformaldehyde for 24 hours, and decalcification was performed with 10% of ethylenediaminetetraacetic acid (EDTA) for 2 weeks. After dehydration using increasing concentrations of ethanol, the knee joint tissues were embedded in paraffin blocks and then cut into 5 μm thick tissue sections. Next, the tissue sections were stained with hematoxylin and eosin (H&E) and safranin-O/fast green, and histopathological changes of the knee joint tissues were observed with an optical microscope. It can be seen from the tissue sections that the knee joint tissues of the rats in the surgical control group did have cartilage damage such as pathological changes in cartilage tissues and synovial tissue hyperplasia (not shown in the figure), but the degrees of cartilage damage of the rats in the experimental group 1 were lower than those of the rats in the surgical control group (not shown in the figure).

[0071] The rat joint histological scoring systems recommended by the OARSI (including OARSI score, cartilage degeneration score and synovial inflammation score) were used to quantify the degree of cartilage damage in the knee joint tissue. The OARSI score, the cartilage degeneration score, and the synovial inflammation score were well known to those skilled in the art of the present disclosure, and would not be repeated here.

[0072] Please refer to Fig. 4A to Fig. 4C, which respectively showed different groups of OARSI scores, cartilage degeneration scores and synovial inflammation scores according to an embodiment of the present disclosure, in

which the horizontal axis represented the groups, and from left to right were the sham operated control group, the surgical control group and the experimental group 1, and the vertical axis represented the OARSI scores, the cartilage degeneration scores and the synovial inflammation scores (unit: point), and the symbol "*" indicated that there was a statistically significant difference (p<0.05) between the surgical control group and the sham operated control group, and the symbol "#" indicated that there was a statistically significant difference (p<0.05) between the experimental group 1 and the surgical control group.

[0073] As shown in Fig. 4A to Fig. 4C, compared with the sham operated control group, the OARSI scores, the cartilage degeneration scores and the synovial inflammation scores of the surgical control group were significantly increased, indicating that symptoms of ACLT-induced osteoarthritis did include cartilage damage. However, compared with the surgical control group, the OARSI scores, the cartilage degeneration scores, and the synovial inflammation scores of the experimental group 1 were significantly reduced, indicating that the live cell-lyophilized powder of the strain GKB7 did improve cartilage damage caused by osteoarthritis.

3.5. Immunohistochemistry (IHC)

[0074] The above-mentioned tissue sections were soaked in 3% of hydrogen peroxide solution to inhibit activity of endogenous catalase, and then soaked in PBS solutions containing 3% of bovine serum albumin to reduce the formation of background formed by nonspecific bindings. After a primary antibody reaction (incubation) with anti-IL-1β or anti-TNF-α antibody at 4°C, the tissue sections were subjected to a secondary antibody reaction at room temperature for 1 hour,

and then stained with diaminobenzidine (DAB). The tissue sections were observed under the optical microscope. The signals for measuring immune response of IL-1β and TNF-α were represented by IHC scores, and the higher IHC score indicated a stronger signal for measuring immune response, that is, a higher expression level of IL-1β or TNF-α.

[0075] Please refer to Fig. 5A to Fig. 5B, which were box plots respectively showing IHC scores of IL-1β (Fig. 5A) and IHC scores of TNF-α (Fig. 5B) in the cartilage tissues of rats in different groups of according to an embodiment of the present disclosure, in which the horizontal axis represented the groups, and from left to right were the sham operated control group, the surgical control group and the experimental group 1, and the vertical axis represented the IHC scores of IL-1β and TNF-α (unit: point). As shown in Fig. 5A and Fig. 5B, compared with the sham operated control group, the IHC scores of IL-1β and the IHC scores of TNF-α of the surgical control group were significantly increased, indicating that expression levels of pro-inflammatory cytokines such as IL-1β and TNF-α in the cartilage tissue of ACLT-induced osteoarthritis were higher. On the contrary, compared with the surgical control group, the IHC scores of IL-1β and TNF-α of the experimental group 1 were significantly decreased, indicating that the live cell-lyophilized powder of the strain GKB7 did reduce expression levels of pro-inflammatory cytokines in the cartilage tissue of osteoarthritis.

[0076] Please refer to Fig. 6A to Fig. 6B, which were box plots respectively showing IHC scores of IL-1β (Fig. 6A) and IHC scores of TNF-α (Fig. 6B) of synovial tissues of rats in different groups according to an embodiment of the present disclosure, in which the horizontal axis represented the groups, and from

left to right were the sham operated control group, the surgical control group and the experimental group 1, and the vertical axis represented the IHC scores of IL-1β and the IHC scores of TNF-α (unit: point). As shown in Fig. 6A and Fig. 6B, compared with the sham operated control group, the IHC scores of IL-1β and the IHC scores of TNF-α of the surgical control group were significantly increased, indicating that expression levels of pro-inflammatory in the synovial tissue of ACLT-induced osteoarthritis were higher. On the contrary, compared with the surgical control group, the IHC scores of IL-1β and the IHC scores of TNF-α of the experimental group 1 were significantly decreased, indicating that the live cell-lyophilized powder of the strain GKB7 did reduce expression levels of pro-inflammatory cytokines in the synovial tissue of osteoarthritis.

[0077] In summary, the specific dosage, specific dosage form, specific detection method or specific evaluation method are only used to illustrate the use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease of the present disclosure. However, those skilled in the art of the present disclosure should understand that without departing from the spirit and scope of the present disclosure, other dosages, other dosage forms, other detection methods, or other evaluation methods can also be used to the use for preparing the medicament in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease, and those are not limited to the above. For example, the medicament may be, for example, a pharmaceutical composition or a food composition, which can optionally include pharmaceutically and food-acceptable carriers, excipients and/or additives, and can be made into other dosage forms, such as a powder, a lozenge, a granule, a suppository, a microcapsule, an ampoule, a liquid or a spray. In other examples,

the strain GKB7 can also be cultured using other suitable media or other culture environments, and the obtained bacteria should also have the above-mentioned effect of improving osteoarthritis. In other embodiments, the live cells at a low dose (the experimental group 1) can achieve the effects of improving bone damage, cartilage damage, inflammation of the cartilage tissue and/or the synovial tissue caused by osteoarthritis, and the live cell at a high dose (the experimental group 2) and the dead cell at a high dose (the experimental group 4) can also achieve such effects.

[0078] According to the foregoing embodiments, the advantage of the use of *Clostridium butyricum* GKB7 in the manufacture of a medicament for treating osteoarthritis and degenerative joint disease of the present disclosure is that the use of *Clostridium butyricum* GKB7 can not only relieve the joint pain caused by osteoarthritis but can also improve bone damage, cartilage damage, inflammation of the cartilage tissue and/or the synovial tissue caused by osteoarthritis, and in the future, it can be used as the active ingredient and used to prepare various compositions for improving osteoarthritis, and even other musculoskeletal diseases.

[0079] While the present disclosure has been disclosed above in terms of several specific embodiments, 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.

WHAT IS CLAIMED IS:

1. Use of *Clostridium butyricum* GKB7 in manufacture of a medicament for treating osteoarthritis, wherein the medicament comprises an effective dose of the *Clostridium butyricum* GKB7 as an active ingredient, and *Clostridium butyricum* GKB7 is deposited in National Institute of Technology and Evaluation, NITE Patent Microorganisms Depositary with an accession number of NITE BP-03889 on May 22, 2023, and the medicament is to be administered to relieve osteoarthritis of a subject.

2. The use according to claim 1, wherein the *Clostridium butyricum* GKB7 is live cells that are to be obtained by subjecting the *Clostridium butyricum* GKB7 to a fermentation step, and the fermentation step is performed on the *Clostridium butyricum* GKB7 by using a fermentation medium in an anaerobic environment of 25°C to 40°C for 16 hours to 24 hours.

3. The use according to claim 1, wherein the *Clostridium butyricum* GKB7 is dead cells that are to be obtained by subjecting the *Clostridium butyricum* GKB7 to a fermentation step and an inactivation step, and the fermentation step is performed on the *Clostridium butyricum* GKB7 by using a fermentation medium in an anaerobic environment of 25°C to 40°C for 16 hours to 24 hours.

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

5. The use according to claim 4, wherein the heat inactivation step performed at a saturated vapor pressure of 110°C to 120°C for 10 minutes to 20 minutes.

6. The use according to claim 1, wherein when the subject is a rat, the effective dose is 50 mg/kg body weight/day to 2,000 mg/kg body weight/day.

7. The use according to claim 1, wherein when the subject is a human being, the effective dose is 1 mg/kg body weight/day to 200 mg/kg body weight/day.

8. Use of *Clostridium butyricum* GKB7 in manufacture of a medicament for treating degenerative joint disease, wherein the medicament comprises the *Clostridium butyricum* GKB7 as an active ingredient, and the *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889, and the *Clostridium butyricum* GKB7 is to be administered to improve bone damage, cartilage damage, inflammation of a cartilage tissue and/or a synovial tissue in a subject.

9. The use according to claim 8, wherein compared with a control subject that the medicament is not to be administered to, an expression level of interleukin (IL)-1β and/or an expression level of tumor necrosis factor (TNF)-α in the cartilage tissue and/or the synovial tissue in the subject that the medicament is to be administered to decreases.

10. The use according to claim 8, wherein compared with a control subject that the medicament is not to be administered to, joint pain of the subject that the medicament is to be administered to relieves.

11. The use according to claim 8, wherein compared with 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.

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

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

14. A pharmaceutical composition for treating osteoarthritis, comprising live cells and/or dead cells of *Clostridium butyricum* GKB7 as an active ingredient, wherein the *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889.

15. The pharmaceutical composition according to claim 14, further comprises medical-acceptable carriers, excipients and/or additives.

16. The pharmaceutical composition according to claim 14, wherein the pharmaceutical composition is to be in a form of powder, lozenge, granule, suppository, microcapsule, ampule or liquid spray.

17. The use according to claim 14, wherein the osteoarthritis is early osteoarthritis.

18. A food composition for treating osteoarthritis, comprising live cells and/or dead cells of *Clostridium butyricum* GKB7 as an active ingredient, wherein the *Clostridium butyricum* GKB7 is deposited with an accession number of NITE BP-03889.

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 or liquid spray.