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DESCRIPTION CN113322202A

Akkermansia, its culture method and its application

[0001]

Technical Field

[n0001]

This invention belongs to the field of biomedicine, and more specifically, relates to an Akkermansia bacterium, its culture method, and its application.

[0003]

Background Technology

[n0002]

Akkermansia muciniphila (*A. muciniphila*, referred to as Akk bacteria in this article) is a novel mucus-degrading bacterium identified in human feces in 2004 by researchers at the Microbiology Laboratory of Wageningen University in the Netherlands.

It is an oval-shaped Gram-negative bacterium that is a resident of the human gut, accounting for 3-5% of the human microbiome.

It can grow in the intestinal mucus layer and "feed" on the mucin secreted by the host, thereby settling in the intestine and protecting the intestine from pathogens through competitive exclusion.

[n0003]

Currently, Akk bacteria have been reported to have a negative correlation with diseases such as obesity, IBD, ALS, autism, epilepsy, and hypertension.

In recent years, cancer immunotherapy studies have also found that PD-1 blockers are involved in the degree of patient response to PD-1 blockers.

[n0004]

However, the Akk strain is a strictly anaerobic intestinal strain with demanding culture requirements. It was first isolated in 2004 and typically requires a culture medium with porcine gastric mucin as the sole carbon and nitrogen source for good growth. Subsequently, there have been some reports on the optimization of Akk culture medium or culture conditions. Chinese patents CN201910721005.6, CN201910865365.3, and CN202010687713.5 also promote the screening, isolation, and industrial application of Akk strains by optimizing culture conditions and formulations.

[0007]

Summary of the Invention

[n0005]

In view of the above-mentioned defects or improvement needs of the existing technology, the present invention provides an Akkermansia bacterium, its culture method and its application. The purpose is to obtain an Akkermansia bacterium through strain screening, which grows rapidly under normal culture conditions, has a large total biomass and has relatively good oxygen tolerance. At the same time, many new applications have been discovered, thereby solving the technical problems of the existing AKK strains having harsh culture conditions and slow growth.

[n0006]

To achieve the above objectives, according to one aspect of the present invention, an Akkermansia bacterium, classified as Akkermansia muciniphila JWA48, was deposited on April 16, 2021, at the China Center for Type Culture Collection (CCTCC) in Wuhan, China, with accession number CCTCC M 2021388.

[n0007]

According to another aspect of the invention, a method for culturing the Akkermansia bacteria is provided, wherein the oxygen concentration in the culture atmosphere of the Akkermansia bacteria is controlled to be below 0.5%.

[n0008]

According to another aspect of the invention, the application of the aforementioned Akkermansia is provided, which is used in the preparation of foods, food additives, or pharmaceuticals with weight loss and lipid-lowering effects.

[n0009]

According to another aspect of the invention, the use of the aforementioned Akkermansia is provided for the preparation of neuroprotective agents.

[n0010]

Preferably, the application of Akkermansia is specifically used in the preparation of neuroprotective agents with anti-anxiety or antidepressant effects.

[n0011]

Preferably, the application of Akkermansia is specifically used in the preparation of neuroprotective agents that have an anti-alcohol craving and dependence-induced anxiety or alcoholism.

[n0012]

According to another aspect of the invention, the application of the aforementioned Akkermansia is provided for the preparation of antidepressant foods, food additives, or pharmaceuticals.

[n0013]

In summary, compared with the prior art, the above-described technical solutions conceived by this invention can achieve the following beneficial effects:

[n0014]

This invention obtained an Akkermansia strain through strain screening, which has the advantages of rapid growth, high yield, and relatively low requirements for culture conditions.

Compared to commercially available Akkermansia, it has approximately 50% higher maximum biomass and can tolerate O₂ concentrations below 0.5%.

[n0015]

Meanwhile, this invention also discovered that Akkermansia has effects in regulating lipid metabolism, neuroprotection, and antidepressant activity.

[0019]

Attached Figure Description

[n0016]

Figure 1 is an agarose gel electrophoresis image of the AKK strain isolated in Example 1;

[n0017]

Figure 2 shows the morphology of the AKK-JWA strain isolated in Example 1 on solid culture medium;

[n0018]

Figure 3 shows the growth activity of the Akk-JWA strain in Example 2 and a commercially available strain. Figure 3(A) is a growth curve of the Akk-JWA strain and a commercially available strain, and Figure 3(B) is a comparison of the growth activity of the Akk-JWA strain and a commercially available strain under different oxygen concentration conditions.

[n0019]

Figure 4 shows the weight changes in obese mice orally administered Akk-JWA in Example 3;

[n0020]

Figure 5 shows the fasting blood glucose levels in obese mice orally administered Akk-JWA in Example 3.

[n0021]

Figure 6 shows the glucose tolerance of Akk-JWA obese mice orally administered in Example 3;

[n0022]

Figure 7 shows the HE staining results of the liver (left) and gonadal fat (right) of Akk-JWA obese mice orally administered in Example 3;

[n0023]

Figure 8 is a statistical result of the total distance traveled by mice in the open field experiment of Example 4;

[n0024]

Figure 9 is a statistical result of the activity time of mice in the middle area in the open field experiment of Example 4;

[n0025]

Figure 10 is a diagram of the activity path of mice in the open field experiment of Example 4;

[n0026]

Figure 11 is a statistical result of the mouse resting time in the tail suspension experiment of Example 4;

[n0027]

Figure 12 shows the results of the three-compartment social experiment in Example 5. Figure 12A shows the results of the first stage preference recording, which shows no preference between the two sides. Figure 12B shows the results of the second stage mouse interaction time recording, which shows a significant increase in social interaction between the experimental mice and the unfamiliar mice. Figure 12C shows the results of the third stage interaction time with the newly introduced mice, which shows an increase in social interaction between the experimental mice and the newly introduced mice.

[n0028]

Figure 13 shows the results of the stone burial experiment in Example 5.

[0033]

Detailed Implementation

[n0029]

To make the objectives, technical solutions, and advantages of this invention clearer, the invention will be further described in detail below with reference to embodiments.

It should be understood that the specific embodiments described herein are merely illustrative of the invention and are not intended to limit the invention.

Furthermore, the technical features involved in the various embodiments of the present invention described below can be combined with each other as long as they do not conflict with each other.

[n0030]

Unlike previous techniques that optimized culture conditions to increase Akkermansia biomass, this invention increases Akkermansia biomass for industrial production by screening strains with less demanding culture requirements or those with growth advantages. This is an important pathway for the industrialization of Akk strains.

A strain of Akkermansia was selected that is more tolerant to low concentrations of oxygen, which is beneficial for industrial applications, and has a 50% increase in biomass under the same culture conditions.

Meanwhile, this strain has good oxygen tolerance and still maintains good growth viability in an environment with an oxygen concentration below 0.5%.

[n0031]

In addition, this invention also discovers the application of the Akkermansia bacteria in the neuroprotection of alleviating mental illnesses such as anxiety and alcoholism caused by alcohol craving and dependence.

[n0032]

Patients with neurological damage such as anxiety disorders and alcoholism have increased levels of innate immune signaling molecules in their brains, leading to neuroinflammation. Studies have shown that the microbiome plays a key role in this process and affects central neurochemistry and behavior.

Disruptions or alterations to the close interaction between the microbiome and the brain may be a significant factor in many neurological disorders.

[n0033]

It is well known that stress, anxiety, and depression are linked to gastric secretions and may affect the gut microbiome.

On the other hand, changes in the composition of the microbiome that regulates the hypothalamic-pituitary-adrenal (HPA) axis may be associated with the development of mental illnesses such as alcohol cravings and dependence.

Therefore, the modulation of the gut-brain axis is increasingly considered a suitable target for developing new therapies.

[n0034]

The following is an example:

[n0035]

Example 1: Screening and Isolation of Akkermansia

[n0036]

We collected healthy, fresh fecal samples from participants and obtained serially diluted solutions. These solutions were then inoculated onto mucin liquid culture medium for initial screening and enrichment of Akk in the fecal samples. Using these bacterial solutions as templates, PCR was performed using primers for the Akkermansia genus. The bacterial solution with the highest dilution in the PCR positive tube was selected to repeat the enrichment steps. After several rounds of enrichment, the enriched solution with the highest dilution was selected for plate plating and isolation. Individual colonies on the plates were identified to obtain Akk strains.

[n0037]

Specific process: Select bacterial culture from PCR positive tubes and perform flow-line analysis on Mucin basic salt solid plates (3 plates). Scrape off single colonies for PCR verification. Select positive samples at the maximum dilution and scrape off all colonies from the corresponding parallel plates with an inoculation loop. Spread the samples on plates to allow 40-100 colonies to grow (5 plates). Pick all single colonies according to region or colony size and morphology and inoculate them into 96-well plates (BHI+Mucin medium). Combine the colonies in two rows and extract DNA for PCR verification. Select positive samples after narrowing down the range for single bacterial culture verification. Finally, screen to obtain Akk strain.

[n0038]

Mucin medium formulation: basal medium + 0.25% mucin

[n0039]

The components of the basal culture medium are shown in the table below:

[n0041]

step:

[n0042]

1.

Separation and Filtering

[n0043]

a.

Preparation of bacterial culture: Feces and physiological saline were mixed and diluted to obtain a diluted solution with a fecal concentration of 10^{-6} g/ml. The diluted solution was inoculated into mucin liquid culture medium at a volume ratio of 1:9. After incubation, the bacterial culture was obtained and anaerobic cultured at 37°C for 5 days.

[n0044]

b.

PCR amplification: Using the above bacterial culture as a template, PCR was performed using primers for the genus *Akkermansia*.

[n0045]

C.

Select bacterial suspensions from PCR positive tubes, serially dilute the bacterial suspensions of the positive samples with the highest dilution, and then inoculate them again into mucin liquid medium at a volume ratio of 1:9. Continue enrichment culture to obtain bacterial suspensions, and then anaerobic culture at 37°C for 5 days.

Repeat this step until Akk is isolated and purified.

[n0046]

Akkermansia identification:

[n0047]

Specific primers for the genus Akkermansia:

[n0048]

F:5' CAGCACGTGAAGGTGGGGAC 3'

[n0049]

R:5' CCTTGCGGTTGGCTTCAGAT 3'

[n0050]

PCR amplification steps:

[n0051]

Bacterial culture was diluted with PBS (pH 7.4) - genome extraction - nucleic acid electrophoresis identification. DNA extraction results were obtained using the above genome as a template, with F: 5' CAGCACGTGAAGGTGGGGAC 3'

[n0052]

Using primers R:5' CCTTGCGGTTGGCTTCAGAT 3' , PCR amplification was performed.

[n0053]

The amplification system is as follows (20 µL):

[n0054]

Template 1uL, Primer F 1uL, Primer R 1uL, Mix 7uL, DDH2O 10uL

[n0055]

Positive controls used Akk DNA as a template, and negative controls used H₂O as a template.

[n0056]

The PCR procedure is as follows:

[n0057]

1 95°C 10min 2 95°C 30s 3 55°C 30s 4 72°C 1min 5 Goto step2 30× 6 72°C 10min 7 4°C ∞

[n0058]

Collect the PCR products and run nucleic acid electrophoresis.

Using the DL5000 DNA ladder as a marker, the target band was identified, and positive samples were selected for subsequent operations.

[n0059]

Select bacterial cultures from PCR-positive tubes and perform flow-line assays on Mucin basal salt solid plates (3 plates). Scrape off single colonies for PCR verification. Select positive samples at the highest dilution and scrape off all colonies from the corresponding parallel plates with an inoculation loop. Spread the samples on plates to allow 40-100 colonies to grow (5 plates). Pick all single colonies according to region or colony size and morphology and

inoculate them into 96-well plates (BHI+Mucin medium). Combine the colonies in two rows for DNA extraction and PCR verification. Select positive samples from the narrowed-down range for individual bacterial culture verification. Finally, screen to obtain the Akk strain.

[n0060]

PCR verification (Figure 1) confirmed that Akk strain was successfully enriched in serially diluted fecal samples. A new Akk strain was obtained and named *Akkermansia muciniphila* JWA48 (abbreviated Akk-JWA), with accession number CCTCC M 2021388. The plate morphology of this strain is shown in Figure 2.

The strain sequence was analyzed using 16SRNA and is SEQ NO.1.

After comparison with NCBI, it was confirmed to be a novel *Akkermansia muciniphila*.

[n0061]

Example 2: Cultivation Experiment

[n0062]

After activating the frozen Akk and Akk-JWA strains, they were inoculated into BHI+M liquid medium as seed culture and anaerobic cultured at 37°C for 64 hours. The OD value was measured, and after adjusting the seed culture concentration to be consistent, they were inoculated into the fermentation broth. The fermentation broth was transferred at a 2% inoculation rate (100 ml), and 200 µl of fermentation broth was taken at regular intervals to measure the bacterial concentration (OD600).

[n0063]

The growth curves are shown in Figure 3(A). As can be seen from the growth curves, the Akk-JWA strain has certain advantages in growth. When it reaches the stationary phase, the biomass increases to 145% of the control strain, the OD value increases from 0.59 to 0.78, and the corresponding maximum biomass increases from 6.7×10^7 CFU/ml to 9.7×10^7 CFU/ml. That is, the amount of cells recovered per milliliter of fermentation broth increases by 3×10^7 CFU/ml, and the biomass increases by about 50%, which is very beneficial for industrial applications.

[n0064]

With the culture medium unchanged, the gas composition in the culture conditions was adjusted (different amounts of oxygen were injected into anaerobic test tubes). It can be seen (Figure 3(B)) that under the conventional anaerobic conditions with CO_NER6, both Akk-JWA and Akk strains can grow. However, under low oxygen conditions, at concentrations of 0.1% and 0.5% O_NER7, Akk-JWA strain can still survive, while Akk cannot grow. This indicates that Akk-JWA strain has a good tolerance to oxygen, which is crucial for controlling conditions in industry. Because it is extremely difficult to maintain strictly anaerobic conditions in

production, occasional O₂ leakage is inevitable. Strains that are more tolerant to oxygen will have more application value in production.

[n0065]

Example 3: Application of *Akkermansia bacillus* provided in Example 1 in weight loss and lipid reduction

[n0066]

Eight-week-old male C57BL/6 mice were used. The experimental mice were housed under specific pathogen-free (SPF) conditions. After one week of acclimatization feeding, the mice were placed in a constant temperature of $25 \pm 2^{\circ}\text{C}$ and a relative humidity of $50 \pm 5\%$, with a 12 /12h diurnal cycle.

Twenty mice were used in two groups of six, with three mice per cage. The healthy control group was fed a maintenance diet (ND), while the other mice were fed a high-fat diet (HFD (Research Diets D12492)). After seven weeks, mice that did not reach the target weight were removed. The acclimatized mice were then assigned to different groups based on their weight to ensure a common starting point. They were administered PBS (obese control group) and Akk bacterial suspension (resuspended in PBS and administered by gavage every three days) by gavage. The probiotic group received a dose of 1×10^8 CFU/ml. During the feeding period, the mice had free access to food. The ND and HFD groups were administered 200 μ L by gavage at the same time every day. The weight and food intake of the mice were measured weekly. After 15 days of gavage, obesity-related indicators were measured to evaluate the weight loss and lipid-lowering effects of Akk in the mice.

[n0067]

Obese mice given Akk-JWA by gavage showed a significant reduction in body weight over 15 days (Figure 4), lower fasting blood glucose levels (close to normal values) (Figure 5), and

better glucose tolerance (Figure 6), demonstrating that Akk-JWA can improve insulin resistance in mice and has the potential to treat diabetes.

[n0068]

HE staining results (Figure 7) show that Akk-JWA administration significantly improves liver fat deposition in mice, reduces the size of gonadal adipocytes, and improves the regularity of gonadal adipocyte arrangement. Mid-oval vacuoles are liver fat deposits; the more and larger the vacuoles, the more severe the fat deposition. In mice administered Akk-JWA, liver vacuoles were reduced and smaller, and adipocytes were uniform in size, neatly arranged, and smaller in size, all of which are markers of healthier and lower-fat mice.

[n0069]

Example 4: Application of Akkermansia provided in Example 1 in improving neurological diseases

[n0070]

Eight-week-old male C57BL/6 mice were used. The experimental mice were housed under specific pathogen-free (SPF) conditions. The mice were kept at a constant temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$ for a 12/12h day/night cycle.

[n0071]

Specific experimental steps: 24 mice were divided into 4 groups: control group 1: normal liquid diet + PBS; control group 2: normal liquid diet + Akk; experimental group 1: liquid diet

(containing 4% alcohol) + PBS; experimental group 2: liquid diet (4% alcohol) + Akk; 6 mice in each group.

After a week of acclimatization, mice in each cage were subjected to subsequent experiments. The experimental group was fed a liquid diet with 4% alcohol for 31 days, while the control group was fed a normal liquid diet. During this period, mice were gavaged every two days with 200ul PBS (control group 1, experimental group 1)/ 1×10^8 CFU/200ul Akk (control group 2, experimental group 2). On days 28 and 29, all mice were gavaged with 20mg/kg LPS. On day 31, open field test and tail suspension test were performed to evaluate the behavior and examine the effect of Akk on improving nerve damage in mice.

[n0072]

The open field test is one of the most commonly used behavioral testing methods for assessing anxiety behavior in rodents.

When rodents enter an open enclosure, they tend to avoid the central area of the enclosure

due to their fear of the new environment and instead stay in the area around the enclosure walls.

However, the exploratory nature of rodents also creates an impulse to explore the central area, thus forming a contradiction and conflict.

Substances that alleviate anxiety can resolve this conflict state by increasing the number of times rodents enter the central area of the open field and the length of time they stay there, without changing the total amount of activity.

Therefore, changes in activity in the central region are used as an indicator for assessing anxiety in rodents.

It is worth noting that anti-anxiety treatments themselves do not increase exploration of the open field, but they do reduce the inhibitory effect of anxiety on exploratory behavior.

[n0073]

The open field consists of two parts: an open field box and a video acquisition system. The open field box is 40cm high and has a base of 40cm x 40cm.

The experiment was conducted 30 minutes after the last gavage in a quiet environment.

Before the experiment, the mice were placed in a box lined with bedding and allowed to explore freely for 5 minutes.

At the start of the experiment, the mice were quickly placed in the center of an open field, and video recording and timing were started simultaneously to record the mice's activities over 10 minutes.

After collection, the open area is cleaned and wiped to remove feces and urine and eliminate the odor left by the mice before testing the next mouse to avoid the influence of odor and residue on the next mouse.

The total distance traveled within 10 minutes, the number of times the central area was entered, and the dwell time were used as indicators for data analysis using EthoVision XT 11 software.

[n0074]

The tail suspension test (TST) is a classic and rapid method for evaluating the efficacy of antidepressants, stimulants, and sedatives.

The principle is to use the mouse's tail to hang and attempt to escape but fail to do so, thus giving up the struggle and entering a unique state of depression and immobility. During the experiment, the time the animal remains still is recorded to reflect the state of depression. Antidepressants and stimulants can significantly shorten and change this state.

[n0075]

Secure the mouse to the rear third of its tail with tape and suspend it on a stand with its head

15cm above the table for filming. The background of the filming should have a clear contrast with the color of the mouse's fur; use a black background for white mice.

Stop timing after 6 minutes and use small animal behavior analysis software: Smart 2.5 software to count the immobility time of the mice in the last four minutes (3-6 minutes).

[n0076]

The neuroprotective effect of the Akk-JWA strain on mice was investigated behaviorally using open field and tail suspension experiments.

[n0077]

As shown in Figures 8, 9, 10, and 11, in the open field experiment, mice orally administered Akk-JWA covered a greater total distance and spent more time in the central region compared

to the treatment group, exhibiting behavior more similar to the untreated normal group. Simultaneously, in the tail suspension experiment, mice orally administered Akk-JWA showed a significantly shorter immobility time compared to the treatment group, again exhibiting behavior more similar to the untreated normal group. These combined results indicate that the Akk-JWA strain can alleviate anxiety and alcoholism caused by alcohol craving and dependence in mice, demonstrating a significant neuroprotective effect.

[n0078]

Example 5: Application of Akkermansia in improving autism

[n0079]

Thirty specific pathogen-free (SPF) weaned 3-4 week old male C57BL/6J mice were housed at

a constant temperature of $25 \pm 2^{\circ}\text{C}$ and a relative humidity of $50 \pm 5\%$ for a 12/12h diurnal cycle.

A clinical stool sample from a 4-year-old child with ASD.

[n0080]

After one day of acclimatization, the mice were treated with antibiotics: a broad-spectrum antibiotic mixture consisting of vancomycin (0.1 g/L), neomycin (0.2 g/L), ampicillin (0.2 g/L), and metronidazole (0.2 g/L) was mixed in drinking water and fed for 14 days.

[n0081]

Intestinal cleansing treatment: Mice were given polyethylene glycol saline solution as drinking water for 3 consecutive days.

[n0082]

Fecal transplantation: After bowel preparation, mice were randomly divided into two groups: a normal group and an ASD model group (n=20).

The normal group was given saline by gavage, while the ASD model group was given fecal supernatant from children with autism (ASD) by gavage. 100 μ L of fecal supernatant was given to each mouse by gavage once, with a one-day interval, for 14 consecutive days.

[n0083]

Fourteen days after fecal microbiota transplantation, the ASD group was divided into two groups: one group was given normal saline by gavage, and the other group was given Akk-JWA by gavage. The normal group was still given normal saline by gavage.

Gavage was administered every other day for 14 consecutive days, followed by behavioral testing.

[n0084]

Behavioral studies mainly include three-compartment socialization: Phase 1: Familiarization phase. The experimental mice are placed in the middle compartment, with an empty cage in each of the two side compartments. The partitions are opened, and the mice explore on their own. Smart3.0 is used to record whether the mice show preference for the two side compartments. The experiment ends after ten minutes, and the mice are taken out to rest for 10 minutes.

Phase 2: A completely unfamiliar mouse, strange1, was randomly placed in one cage, and a wooden toy was placed in the other cage.

The experimental mice were placed in the laboratory, and Smart3.0 was used to record the time that the experimental mice interacted with strange1 within 10 minutes.

Phase 3: Keeping Strange1 still, remove the wooden block on the other side, put in a new mouse Strange2, and observe and record for 10 minutes.

The results are shown in Figures 12A, B, and C.

[n0085]

Buried Glass Beads (MB) Experiment: In a normal cage, fill the bottom with a 3-4 cm layer of fresh sawdust. First, let the mice get used to the experimental cage for 10 minutes, then transfer them to a new clean cage. Re-lay the bedding in the experimental cage and place 20 glass beads (4×5) on top. Put the mice back into the experimental cage. After 10 minutes, the mice are returned to their own cages and removed. Record and photograph the number of buried glass beads (if more than $\frac{2}{3}$ of the volume of the glass bead is covered, it is considered buried).

The results are shown in Figure 13.

[n0086]

The Akk-JWA strain was used to investigate the behavioral effects of the mouse three-compartment socialization test and the burial test on autistic mice.

[n0087]

As can be seen from Figures 12 and 13, after the mice were moved to the feces of autistic patients, they showed obvious autistic symptoms, which were reflected in a significant reduction in the time spent interacting with other mice and new mice, i.e., reduced social interaction, and an increase in burying behavior.

After gavage administration of the Akk-JWA strain, social behavior significantly increased, manifested as greater social behavior compared to toys and other mice, significantly

increased social interaction with new mice, and a significant decrease in repetitive behaviors, indicating a significant improvement in corresponding autism symptoms. This suggests that the Akk-JWA strain has a significant effect on improving autism.

[n0088]

Those skilled in the art will readily understand that the above description is merely a preferred embodiment of the present invention and is not intended to limit the present invention. Any modifications, equivalent substitutions, and improvements made within the spirit and principles of the present invention should be included within the scope of protection of the present invention.

[0094]

sequence list

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