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DESCRIPTION CN115141778A

A method for specific screening of *Akkermansia muciniphila* using a composite regulator

[0001]

Technical Field

[n0001]

This invention belongs to the field of microbial technology, specifically relating to a method for specifically screening *Akkermansia myxophilus* with a compound regulator.

[0003]

Background Technology

[n0002]

Akkermansia muciniphila (Akk bacteria) exists in the human gut and is a resident of the human gut, accounting for 3-5% of the human microbiome. Its standard strain, MucT, was isolated by Derrien in 2004. It is an oval, Gram-negative anaerobic bacterium and a representative of the Verrucous microbe.

Akk bacteria are mucin-degrading bacteria that use mucin as their sole source of carbon, nitrogen, and energy. They feed on mucin secreted by goblet cells in the intestinal epithelium, thereby promoting the proliferation of intestinal epithelial stem cells and maintaining the integrity of the intestinal barrier function.

In recent years, numerous human and animal studies have resolved the relationship between Akk bacteria abundance and various diseases.

Akk bacteria abundance is considered to be negatively correlated with metabolic disorders

and inflammatory diseases, including obesity, type II diabetes, inflammatory bowel disease (IBD), ALS, epilepsy, autism, and atopic dermatitis.

[n0003]

Akk bacteria were originally isolated by Derrien of Wageningen University in the Netherlands. Professor Peng Yongzheng's team at Southern Medical University in China used Derrien's team's method to isolate 22 Akk bacteria strains from the population in southern China.

Professor Zhai Qixiao's team at Jiangnan University has invented a specific screening medium for Akk bacteria by improving the method of the Derrien team, which can achieve a positive screening rate of up to 70% for Akk bacteria. All three teams used an enrichment culture medium containing only purified porcine gastric mucin for enrichment culture, and then selected the enrichment culture medium with the lowest turbidity for separation culture. This involved steps such as preparing and adding alkaline solutions, acidic solutions, and inorganic salt solutions, as well as purifying the mucin. Not only were the experimental steps cumbersome, but there were also problems such as the scarcity and high cost of some reagents, which were not conducive to the rapid and accurate screening and isolation of Akk

bacteria in the laboratory. Another team led by Professor Yang Yongjun of Jilin University used vancomycin solid culture medium to screen and isolate two Akk bacteria from the feces of healthy mice. Their plate isolation method simplifies the screening and isolation steps of Akk bacteria. However, in the actual implementation, the inventors found that many miscellaneous bacteria grew on the vancomycin solid culture medium, mainly odor-producing strains, making it difficult to screen Akk bacteria from these strains. Analysis revealed that vancomycin only inhibits Gram-positive bacteria and has no inhibitory effect on Gram-negative bacteria that are not Akk bacteria. Therefore, there are currently no reports of antibiotic modulators that can inhibit these Gram-negative bacteria without inhibiting Akk bacteria.

[n0004]

Chinese Patent No. CN201910721005.6 discloses an anaerobic culture method for Akkella myxophilus. The culture medium is a modified BHI medium supplemented with Mucin and Yeast Extract, which enables Akkella to grow on the plate surface and reduces the growth rate of Akkella from 3-4 days using ordinary mucin medium (BHI + mucin) to 24-36 hours, greatly shortening the culture time and meeting the requirements for rapid isolation and screening.

Because this method enables Akk bacteria to grow rapidly, the modified BHI culture medium involved in this invention is based on this patented method. In actual implementation, any culture medium capable of culturing Akk bacteria can be used, and the growth rate of Akk bacteria is determined by the characteristics of the culture medium. Therefore, in order to improve the success rate of screening, a method for specifically screening Akk bacteria was invented by combining compound regulators with modified BHI culture medium, enabling researchers to quickly and accurately screen Akk bacteria for in-depth research.

[0007]

Summary of the Invention

[n0005]

To address the aforementioned shortcomings of existing technologies, this invention provides a method for specifically screening *Akkermansia bacilli* using a composite regulator.

[n0006]

A method for specifically screening *Akkermansia myxophilus* with a composite regulator, wherein the composite regulator is regulator A, regulator B, and regulator C.

Regulator A is a narrow-spectrum antibiotic, effective only against Gram-positive bacteria.

Regulator B is a broad-spectrum aminoglycoside antibiotic that inhibits Gram-negative bacteria, staphylococci, and other Gram-positive bacteria.

Regulator C can inhibit both Gram-negative and Gram-positive bacteria.

[n0007]

Furthermore, the composite regulator is regulator A, regulator B, and regulator C.

[n0008]

Furthermore, the compound regulator formulation consists of 6ug/ml regulator A, 10ug/ml regulator B, and 12ug/ml regulator B.

[n0009]

Furthermore, the modified BHI culture medium formula consists of 38.5 g/L brain and heart

extract broth, 5 g/L yeast extract, 2.5 g/L mucin, 1 mg/L resazurin, 0.5 g/L L-cysteine, and 0.3 g /L sodium bicarbonate.

[n0010]

Furthermore, the method for preparing the BHI culture medium modified by the composite regulator is as follows:

[n0011]

S1: Preparation of regulator solution: Dissolve regulator A in sterile ultrapure water to prepare a stock solution of 6 ug/mL; dissolve regulator B in sterile ultrapure water to prepare a stock solution of 10 ug/mL; dissolve regulator C in sterile ultrapure water to prepare a stock solution of 12 ug/mL; filter and sterilize, then store at 4°C for later use.

[n0012]

S2: Preparation and purification of mucin solution: Dissolve mucin in PBS buffer at pH 7.4 at a concentration of 1% (W/V), stir magnetically for 2 hours at room temperature, and centrifuge at 8000 rpm for 15 minutes after it is fully dissolved. Take the supernatant and cool it to 0-2°C to obtain the supernatant.

Add ethanol pre-cooled to 4°C to the supernatant until the final ethanol concentration is 60%. Gently invert and mix well. After standing at 4°C for 2 hours, discard the supernatant. Redissolve the precipitate with distilled water to the volume before purification. Autoclave at 121°C for 20 minutes. After cooling, aliquot into 50mL sterile centrifuge tubes and store in a -20°C refrigerator for later use.

[n0013]

S3: Preparation of razor blue: Dissolve razor blue in distilled water to obtain a 0.005% (W/V) razor blue stock solution, and store it in a refrigerator at 4°C for later use;

[n0014]

S4: Preparation of L-cysteine: Dissolve L-cysteine in distilled water to obtain a 10% (W/V) L-cysteine solution stock solution, filter and sterilize for later use.

[n0015]

S5: Preparation of sodium bicarbonate solution: Dissolve NaHCO_3 in distilled water to obtain a 3% (w/v) NaHCO_3 stock solution, filter and sterilize for later use;

[n0016]

S6: Take 38.5g of brain and heart extract broth, 5g of yeast extract powder, and 4mL of 0.005% (w/v) resveratrol stock solution and dilute to 1L of distilled water. After mixing evenly, adjust the pH of the culture medium to 6.5 ± 0.2 , autoclave at 121°C for 20min, then take 150mL of the sterilized solution and add 50mL of the mucin solution from step S1. After mixing evenly, add 1mL of the L-cysteine solution from step S3 and 200uL of the NaHCO₃-NER3 stock solution from step S4 to obtain the modified BHI medium (if a solid medium is required, add 1-1.5% agar before sterilization).

[n0017]

S7: After the above-mixed modified BHI medium has cooled to about 40-50°C, add 200uL of the stock solution of regulator A, regulator B, and regulator C to the modified BHI medium, shake well, and pour into plates before solidification to obtain the modified BHI medium with compound regulator.

[n0018]

Furthermore, the screening culture was conducted at a temperature of 37°C.

[n0019]

Furthermore, the mixed gas composition used in the screening culture is 90% N₂, 5% H₂, and 5% CO₂.

[n0020]

The technical solution of this invention has the following beneficial effects:

[n0021]

1. The composite regulator method of the present invention can effectively inhibit miscellaneous bacteria in fecal samples. Regulator A inhibits Gram-positive bacteria, regulator B inhibits Gram-negative bacteria that regulator A cannot inhibit, and regulator C inhibits both Gram-positive and Gram-negative bacteria. The combined use of the three regulators forms an effective complementary effect, reducing the difficulty of screening and isolating Akk bacteria in fecal samples. Furthermore, during the purification and culture of Akk bacteria, the use of the three composite regulators can effectively promote the growth of Akk bacteria, which is of great significance for the large-scale culture and in-depth research of Akk bacteria.

[n0022]

2. The screening and culture method of the present invention eliminates the need for enrichment culture using enrichment medium containing mucin. Instead, fecal samples can be serially diluted and then plated. Typical colony morphology can be formed on the surface of the solid medium, making it easy to select colonies for isolation and identification. It is fast, accurate, and simple to operate, and is suitable for the specific screening of Akk bacteria.

[0026]

Attached Figure Description

[n0023]

The accompanying drawings, which are provided to further illustrate the invention and

constitute a part of this invention, are illustrative embodiments of the invention and their descriptions are used to explain the invention and do not constitute an undue limitation of the invention.

In the attached diagram:

[n0024]

Figure 1 shows the plate screening diagrams of the same fecal sample with and without the combination of regulators; where Figure A is the screening plate with regulators and Figure B is the screening plate without regulators.

[n0025]

Figure 2 shows the morphology of Akk colonies isolated in this invention; where Figure A is the bacterial suspension diluted and spread, and Figure B is the colony streak on a plate.

[n0026]

Figure 3 is a Gram staining image of Akk bacteria isolated in this invention under a 1000x ordinary optical electron microscope;

[n0027]

Figure 4 is a molecular identification band diagram of Akk bacteria isolated by PCR amplification in Example 2 of the present invention; wherein, from left to right, the electrophoresis lanes are Marker with a molecular weight of 500bp, suspected colonies 1-8 selected on the plate, and blank control CK.

[n0028]

Figure 5 is a growth curve of Akk bacteria in Example 4 of the present invention with and without the compound regulator.

[0033]

Detailed Implementation

[n0029]

The technical solution of the present invention will be clearly and completely described below with reference to the accompanying drawings. Obviously, the described embodiments are only some embodiments of the present invention, and not all embodiments.

Based on the embodiments of the present invention, all other embodiments obtained by those skilled in the art without inventive effort are within the scope of protection of the present invention.

[n0030]

The mucin used in the following examples was purchased from Sigma-Aldrich (Mucin from porcine stomach, type II, Sigma, M2378); the brain heart broth (BHI) was purchased from Qingdao Haibo Biotechnology Co., Ltd.; yeast extract, L-cysteine, sodium bicarbonate, soybean peptone, and N-acetylglucosamine were purchased from Beijing Aoboxing Biotechnology Co., Ltd.; and the feces were obtained from a centenarian in a county in Hechi City, Guangxi Zhuang Autonomous Region.

[n0031]

Example 1

[n0032]

Compound regulators improve the preparation of BHI culture medium

[n0033]

Although Chinese patent CN201910721005.6 discloses an anaerobic culture method for

Akkermansia myxophilus, its method of using the mucin solution is inaccurate and lacks a step for purifying the mucin. This makes it difficult to eliminate interfering substances even after autoclaving, often causing the modified BHI liquid medium to change from a clear state to a turbid state after adding the mucin solution (both of which have been autoclaved). This phenomenon will seriously affect the subsequent isolation and culture effect. Therefore, the turbidity of the medium severely hinders the screening culture experiment. Thus, the mucin solution must be purified first, then sterilized, and then added to the modified BHI medium.

[n0034]

1. Preparation of regulator solution: Dissolve regulator A in sterile ultrapure water to prepare a stock solution of 6 ug/mL; dissolve regulator B in sterile ultrapure water to prepare a stock solution of 10 ug/mL; dissolve regulator C in sterile ultrapure water to prepare a stock solution of 12 ug/mL; filter and sterilize, then store at 4°C for later use.

[n0035]

2. Preparation and purification of mucin solution: Dissolve mucin in PBS buffer at pH 7.4 at a concentration of 1% (W/V), stir magnetically for 2 hours at room temperature until fully dissolved, then centrifuge at 8000 rpm for 15 minutes, collect the supernatant, and cool it to 0-2°C to obtain the supernatant.

Add ethanol pre-cooled to 4°C to the supernatant until the final ethanol concentration is 60%. Gently invert and mix well. After standing at 4°C for 2 hours, discard the supernatant. Redissolve the precipitate with distilled water to the volume before purification. Autoclave at 121°C for 20 minutes. After cooling, aliquot into 50mL sterile centrifuge tubes and store in a -20°C refrigerator for later use.

[n0036]

3. Preparation of razor blue: Dissolve razor blue in distilled water to obtain a 0.005% (W/V) razor blue stock solution, and store it in a refrigerator at 4°C for later use;

[n0037]

4. Preparation of L-cysteine: Dissolve L-cysteine in distilled water to obtain a 10% (w/v) L-cysteine solution stock solution, filter and sterilize for later use.

[n0038]

5. Preparation of sodium bicarbonate solution: Dissolve NaHCO₃-NER7 in distilled water to obtain a 3% (w/v) NaHCO₃-NER8 stock solution, filter and sterilize for later use;

[n0039]

6. Take 38.5g of brain and heart extract broth, 5g of yeast extract powder, and 4mL of 0.005% (w/v) resveratrol stock solution and dilute to 1L of distilled water. After mixing well, adjust the pH of the culture medium to 6.5 ± 0.2 , autoclave at 121°C for 20min, then take 150mL of the sterilized solution and add 50mL of the mucin solution from step S1. After mixing well, add 1mL of L-cysteine hydrochloride solution from step S3 and 200uL of NaHCO₃-NER9 stock solution from step S4 to obtain the modified BHI medium (if a solid medium is required, add 1-1.5% agar before sterilization).

[n0040]

7. After the above-mentioned mixed modified BHI medium solid medium has cooled to about 40-50°C, add 0.2mL of the stock solution of regulator A, regulator B, and regulator C to the modified BHI medium, shake well, and pour into plates before solidification to obtain the compound regulator modified BHI medium.

[n0041]

Example 2

[n0042]

Methods for specific screening of Akk bacteria using compound regulators

[n0043]

The specific steps are as follows:

[n0044]

1. Source of materials

[n0045]

Three fecal samples were collected from a county in Guangxi Zhuang Autonomous Region on September 25, 2020. The samples were aliquoted into sterile 2mL centrifuge tubes and stored at -80°C for later use. The sample numbers are DL-01, DL-02, and DL-03.

[n0046]

2. Sample gradient dilution and plate coating

[n0047]

Remove the frozen fecal sample from the -80°C freezer and thaw it at room temperature. Prepare sterile phosphate buffer dilution tubes, each containing 9 mL of phosphate buffer containing L-cysteine hydrochloride. Add 1 g of fecal sample to 9 mL of sterile phosphate buffer containing L-cysteine hydrochloride and mix well. This is recorded as the 10_NER10 dilution gradient. Then, transfer 1 mL of the mixture to the next 9 mL dilution tube and mix well. This is recorded as the 10_NER11 dilution gradient. Continue this process down to the 10_NER12 dilution gradient.

[n0048]

Three gradients, 10⁻², 10⁻³, and 10⁻⁴, were selected, and 100 µL of each gradient was taken and diluted and spread on BHI agar plates modified

with composite regulator. Two plates were spread for each gradient and placed in an anaerobic workstation (90% N₂, 5% CO₂, 5% H₂) for constant temperature anaerobic incubation at 37°C for 48-72 h.

[n0049]

3. Microscopic observation and colony PCR identification

[n0050]

Remove the plate containing the colonies and, in a clean bench, select the colonies that are free of surrounding bacteria, teardrop-shaped, and have smooth edges for Gram staining. Observe them under a regular optical microscope and select Gram-negative (red), round or oval strains.

[n0051]

The strains exhibiting this characteristic were subjected to colony PCR using Akk1 (CAGCACGTGAAGGTGGGGAC) primers specific to Akk2 myxophilus. The two primer pairs were upstream primer Akk1 (CCTTGCGGTTGGCTTCAGAT).

PCR was performed in a 50 µL system. The single-clone colonies growing on the plate were used as templates. 2 µL of upstream primer, 2 µL of downstream primer, and 25 µL of Taq enzyme were added, and the volume was brought up to 50 µL with ddH₂O.

After adding the system, use a pipette to repeatedly blow and aspirate the mixture to mix it thoroughly. After mixing, centrifuge it on a small centrifuge.

[n0052]

Table 1 PCR reaction system

[n0054]

After centrifugation, place the sample into a gradient PCR instrument for PCR reaction.

[n0055]

Table 2 PCR reaction procedure

[n0057]

Take 2.0g of agarose powder into 100mL of 1× electrophoresis buffer, stir to dissolve evenly, then heat in a microwave oven until boiling. Remove and shake well, then heat in a microwave oven again until boiling. Repeat the above operation 2-3 times until the agarose is completely dissolved.

Remove the gel from the microwave and place it in a fume hood until it cools to 40-50°C. Add 10 µL of 4SGelRed nucleic acid dye and mix well. Pour the mixture into a horizontal plate with a comb inserted and allow it to cool and solidify to obtain a gel.

Place the gel plate in the electrophoresis buffer and add 6 µL of Marker, PCR reaction products of different colony samples, and negative control from left to right.

Set the voltage to 100 V and start electrophoresis. After 20-30 minutes, remove the gel plate and irradiate it for observation. The target band is the 327 bp fragment. The presence or absence of the target fragment determines whether the strain is *Ackermania gravidarum*.*.

[n0058]

4. Streak purification and preservation

[n0059]

Stranding the observed target bands onto modified BHI solid medium for purification, and obtaining purified monoclonal strains after 2-3 generations of streak purification, inoculating them into 10 mL of modified BHI liquid medium for enrichment culture, and after the medium becomes turbid, taking 1 mL of bacterial solution and mixing it with 1 mL of 40% glycerol, and storing it in a -80°C freezer.

[n0060]

Example 3

[n0061]

Screening effects of adding compound regulators to different culture media

[n0062]

1. Preparation of different culture media

[n0063]

Three Akk bacteria screening media were used to test the screening effect of adding a

compound regulator. The three screening media are as follows: ① Modified BHI medium: 38.5 g brain heart extract broth, 2.5g mucin, 5g yeast extract powder, 2.5g L-cysteine, 1mg resazurin, 0.294g sodium bicarbonate, and distilled water to a final volume of 1L. If a solid medium is used, 1.5% agar is added.

② Ordinary mucin medium: 38.5g brain and heart infusion broth, 4g mucin, 0.5g L-cysteine, distilled water to a final volume of 1L. If using solid medium, add 1.5% agar.

③ Synthetic culture medium: 38.5g brain and heart infusion broth, 16g soybean peptone, 11.3 g glucose, 5.5g N-acetylglucosamine, 4g L-threonine, 0.5g L-cysteine, and distilled water to a final volume of 1L. If using solid culture medium, add 1.5% agar.

Except for the regulator, which was filtered through a 0.22µm sterile filter membrane, all other components were sterilized at 121°C for 20 minutes after being stirred evenly, cooled to about 50°C, and then the culture medium was poured into petri dishes in a clean bench and left to stand for 1 hour to allow the culture medium to completely solidify and dry out before use.

[n0064]

2. Screening effect under the action of compound regulators

[n0065]

(1) Experimental controls with and without the compound regulator were set up by controlling whether the compound regulator was added. After the plating plates were prepared, the Akk bacteria screening experiment in the fecal samples was carried out according to Example 2.

After the culture dishes have grown bacteria, observe the growth effect of the same fecal sample at the same dilution on screening plates containing and without the regulator.

[n0066]

(2) The positive rate of Akk bacteria on different screening plates was calculated by morphological observation, Gram staining and PCR identification.

Taking culture medium ① as an example, comparative experiments showed that the composite regulator screening plate can help eliminate the interference of miscellaneous bacteria and improve the screening success rate, with a positive screening rate of 50% for Akk bacteria.

The positive rate of Akk bacteria screening on screening plates without regulators was only 20%.

The method of this invention significantly improves the isolation and screening effect of Akk bacteria.

[n0067]

Example 4

[n0068]

Growth curves of Akk bacteria with and without compound regulators

[n0069]

1. Determination of the growth curve of Akk strain: Using brain and heart broth as the basal culture medium and adding a compound regulator as the experimental control, the growth curves of Akk strain under the action of ordinary culture medium and regulator were determined.

Add 2 mL of seed culture to 200 mL of liquid culture medium and anaerobic culture at 37 °C for 36 h.

The absorbance at OD_{600nm} was measured every 3 hours, and the growth curve of Akk bacteria was plotted based on the OD value.

[n0070]

2. Compare the growth curves of Akk bacteria with and without growth regulators, and analyze the significant differences.

[n0071]

Example 5

[n0072]

Antibiotic susceptibility testing of Akk strain

[n0073]

To investigate the drug susceptibility of Akk bacteria to common antibiotics, the resistance of Akk bacteria to 12 common antibiotics in 9 classes was determined using modified BHI medium as a substrate and the Kirby-Bauer disk diffusion method (K-B method).

The 12 antibiotics used are: tetracycline, rifampin, chloramphenicol, penicillin, erythromycin, kanamycin, vancomycin, gentamicin, norfloxacin, nystatin, doxorubicin hydrochloride, and metronidazole.

Take 100 µL of bacterial suspension with a concentration of 10⁸ CFU/mL and spread it evenly on the modified BHI solid medium. After the bacterial suspension is completely

absorbed, place 3 antibiotic tablets evenly on top of it. Place the medium in an anaerobic workstation at 37.0°C and incubate upside down for 48 h. Measure and record the diameter of the inhibition zone. Perform three replicates for each antibiotic.

[n0074]

According to the implementation standards of the Clinical Laboratory Standards Institute (CLSI) (see Table 1), the strains were determined to be sensitive (S), intermediate (I), or resistant (R) to antibiotics, and the results are shown in Table 2.

[n0075]

Table 1 CLSI Antimicrobial Susceptibility Testing Standards

[n0077]

Table 2 Antibiotic resistance of Akk bacteria