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DESCRIPTION CN115851529A

Modified culture medium for *Akkermansia muciniphila*

[0001]

Technical Field

[n0001]

This invention belongs to the field of bacterial culture medium technology, specifically relating to a modified culture medium for *Akkermansia muciniphila*.

[0003]

Background Technology

[n0002]

Akkermansia muciniphila is a strictly anaerobic intestinal bacterium belonging to the phylum Verrucomicrobia, G⁺. It is a non-motile, non-spore-forming oval bacterium with a cell length of 0.6-1 µm. It can colonize the mucus layer of the gastrointestinal tract in humans and many animals. It has been widely reported to be associated with diseases such as obesity, inflammatory bowel disease, hypertension, and atherosclerosis, and is expected to become a next-generation probiotic drug.

However, in vitro culture of *Akkermansia muciniphila* is not easy.

In 2004, researchers first successfully cultured *A. muciniphila* in vitro using a culture medium containing porcine gastric mucin.

Subsequently, it was discovered that *Akermansia muciniphila** could grow in a variety of enrichment media, such as Columbia Broth (peptone, corn starch, sodium chloride, defibrinated sheep blood) and Brain Heart Infusion (brain heart extract, D-glucose, peptone,

sodium chloride, disodium hydrogen phosphate). However, because these culture media used for in vitro culture of *Akkermansia muciniphila* contain animal-derived components, which may contain viruses, bacteria, allergens, pathogens, etc., the subsequent application of *Akkermansia muciniphila* as a probiotic will be limited. To this end, some researchers have proposed a non-animal-derived culture medium for the in vitro culture of *Akkermansia muciniphila*, whose main components include soybean peptone, glucose, and amino acids. However, this culture medium can cause changes in the morphology of the bacteria, including uneven shape, elongation of the bacteria, and even the appearance of a "tripod" shape, which may affect the viability of subsequent strains.

[n0003]

Therefore, it is necessary to develop a culture medium that does not contain animal-derived components, is free of allergens, can effectively maintain the excellent in vitro growth of *Akkermansia muciniphila*, and does not alter the normal morphology of the bacteria. This medium would be suitable for large-scale fermentation culture of *Akkermansia muciniphila*, enabling it to be widely developed and used as a next-generation probiotic.

[0006]

Summary of the Invention

[n0004]

To overcome the shortcomings of the prior art, this invention proposes a modified culture medium for *Akkermansia muciniphila*. This culture medium does not contain animal-derived components, which can maintain the excellent in vitro growth of *Akkermansia muciniphila* without changing the normal morphology of the bacteria, and is also suitable for large-volume culture of *Akkermansia muciniphila*.

[n0005]

To achieve the above objectives, the technical solution adopted by the present invention is as follows:

[n0006]

This invention provides a modified culture medium for *Akkermansia muciniphila*, the culture medium comprising yeast extract, EGCG, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, glucose, N-acetyl-glucosamine, N-acetyl-D-galactosamine, urea, threonine, proline, L-cysteine hydrochloride, sodium pyruvate, ferric pyrophosphate, and vitamin K3.

[n0007]

The improved culture medium of this invention contains only non-animal-derived components, ensuring high safety. It contains only 0.5 g/L ferric pyrophosphate (15.9% of the virulence genes in the genome of *Akkermansia muciniphila* are annotated to be related to iron ions) and 5 mg/L vitamin K3, without using any other elements or vitamins.

Among them, the addition of yeast extract to the culture medium at a level of 30 g/L can maintain the excellent in vitro growth of *A. muciniphila*; the use of urea to replace peptone components can maintain the excellent in vitro growth of *A. muciniphila* while also maintaining the normal morphology of the bacteria; the addition of green tea extract EGCG can significantly improve the in vitro growth rate and amount of *A. muciniphila*.

The synergistic effect of yeast extract, N-acetyl-D-galactosamine, urea and EGCG in the modified culture medium can significantly increase the in vitro growth rate and amount of *Akkermansia muciniphila*.

[n0008]

Preferably, the culture medium comprises 25-35 g/L yeast extract, 0.4-0.6 g/L EGCG, 0.4-0.6 g/L potassium dihydrogen phosphate, 1-2 g/L disodium hydrogen phosphate, 0.5-1.5 g/L sodium chloride, 4-5 g/L glucose, 5-6 g/L N-acetyl-glucosamine, 20-30 g/L N-acetyl-D-galactosamine, 7-15 g/L urea, 3-5 g/L threonine, 3-5 g/L proline, 4-6 mg/L L-cysteine hydrochloride, 0.5-1.5 g/L sodium pyruvate, 0.4-0.6 g/L ferric pyrophosphate, and 4-6 mg/L vitamin K3.

[n0009]

More preferably, the culture medium comprises 30 g/L yeast extract, 0.5 g/L EGCG, 0.5 g/L potassium dihydrogen phosphate, 1.5 g/L disodium hydrogen phosphate, 1.0 g/L sodium chloride, 4.5 g/L glucose, 5.53 g/L N-acetyl-glucosamine, 25 g/L N-acetyl-D-galactosamine, 10 g/L urea, 4 g/L threonine, 4 g/L proline, 5 mg/L L-cysteine hydrochloride, 1 g/L sodium pyruvate, 0.5 g/L ferric pyrophosphate, and 5 mg/L vitamin K3.

[n0010]

Preferably, the pH of the culture medium is 6-7.

More preferably, the pH of the culture medium is 6.5.

[n0011]

The present invention also provides another modified culture medium for *Akkermansia muciniphila*, the culture medium comprising yeast extract, EGCG, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, glucose, N-acetyl-glucosamine, threonine, proline, L-cysteine hydrochloride, sodium pyruvate, ferric pyrophosphate, and vitamin K3.

[n0012]

Based on the first improved culture medium mentioned above, although the absence of N-acetyl-D-galactosamine and urea leads to a decrease in the in vitro growth of *A. muciniphila*, the combination of yeast extract and EGCG can still promote the growth of *A. muciniphila* in large-volume in vitro culture, maintaining its excellent growth rate and quantity, which are still superior to those of existing culture media.

Moreover, omitting N-acetyl-D-galactosamine and urea can significantly reduce the cost of the culture medium, making it more suitable for industrial applications.

[n0013]

Preferably, the culture medium comprises 25-35 g/L yeast extract, 0.4-0.6 g/L EGCG, 0.4-0.6 g/L potassium dihydrogen phosphate, 1-2 g/L disodium hydrogen phosphate, 0.5-1.5 g/L sodium chloride, 4-5 g/L glucose, 5-6 g/L N-acetyl-glucosamine, 3-5 g/L threonine, 3-5 g/L

proline, 4-6 mg/L L-cysteine hydrochloride, 0.5-1.5 g/L sodium pyruvate, 0.4-0.6 g/L ferric pyrophosphate, and 4-6 mg/L vitamin K3.

[n0014]

More preferably, the culture medium comprises 30 g/L yeast extract, 0.5 g/L EGCG, 0.5 g/L potassium dihydrogen phosphate, 1.5 g/L disodium hydrogen phosphate, 1.0 g/L sodium chloride, 4.5 g/L glucose, 5.53 g/L N-acetyl-glucosamine, 4 g/L threonine, 4 g/L proline, 5 mg/L L-cysteine hydrochloride, 1 g/L sodium pyruvate, 0.5 g/L ferric pyrophosphate, and 5 mg/L vitamin K3.

[n0015]

Preferably, the pH of the culture medium is 6-7.

More preferably, the pH of the culture medium is 6.5.

[n0016]

Compared with the prior art, the beneficial effects of the present invention are:

[n0017]

This invention discloses a modified culture medium for *Akkermansia muciniphila*, comprising yeast extract, EGCG, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, glucose, N-acetyl-glucosamine, N-acetyl-D-galactosamine, urea, threonine, proline, L-cysteine hydrochloride, sodium pyruvate, ferric pyrophosphate, and vitamin K3 (N-acetyl-D-galactosamine and urea may be omitted).

This improved culture medium is composed of plant-derived components and contains no animal-derived components, making it highly safe. It can maintain the excellent growth of *Akkermansia muciniphila* in vitro without altering the normal morphology of the bacteria, and is also suitable for large-volume in vitro culture of *Akkermansia muciniphila*.

[0021]

Attached Figure Description

[n0018]

Figure 1 shows the growth curves of *A. muciniphila* JF3 in vitro on culture media 1, 2, 7, 8 and 9.

[n0019]

Figure 2 shows the cell morphology of *A. muciniphila* JF3 in media 1, 2 and 6 during the logarithmic and stationary phases;

[n0020]

In Figure 2, A and D: cell morphology of *A. muciniphila* JF3 in medium 1 during the logarithmic and stationary phases; B and E: cell morphology of *A. muciniphila* JF3 in medium 2 during the logarithmic and stationary phases; C and F: cell morphology of *A. muciniphila* JF3 in medium 6 during the logarithmic and stationary phases; G: statistical graph of cell length of *A. muciniphila* JF3 in media 1, 2, and 6 during the logarithmic and stationary phases (10 fields of view were randomly selected, and cell length was randomly

measured); H: cell morphology of *A. muciniphila* JF3 in medium 2 under transmission electron microscopy.

[n0021]

Figure 3 shows the in vitro growth curves of A. muciniphila JF3 in media 1, 2, 6 and 9.

[n0022]

Figure 4 shows the in vitro growth curves of A. muciniphila JF3 in culture media 1, 2, 5, 6, 10, 11 and 12.

[n0023]

Figure 5 shows the cell morphology of *A. muciniphila* JF3 in media 10 and 11 during the logarithmic and stationary phases;

[n0024]

In Figure 5, A and C: cell morphology of *A. muciniphila* JF3 in medium 10 during the logarithmic and stationary phases; B and D: cell morphology of *A. muciniphila* JF3 in medium 11 during the logarithmic and stationary phases; E: statistical graph of cell length of *A. muciniphila* JF3 in media 1, 2, 6, 10, and 11 during the logarithmic and stationary phases (10 fields of view were randomly selected, and cell length was randomly measured).

[0029]

Detailed Implementation

[n0025]

The specific embodiments of the present invention will be further described below.

It should be noted that the description of these embodiments is for the purpose of helping to understand the present invention, but does not constitute a limitation of the present 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.

[n0026]

Unless otherwise specified, the experimental methods used in the following embodiments are conventional methods, and the experimental materials used in the following embodiments are all available through conventional commercial channels.

[n0027]

The pH of the culture media used in the following examples is 6.5.

[n0028]

The preservation information of **Akermansia japonicus** JF3 used in the following examples is as follows:

[n0029]

Deposit date: September 27, 2022;

[n0030]

Name of depositary institution: China Center for Type Culture Collection (CCTCC);

[n0031]

Accession number: CCTCC No: M 20221499;

[n0032]

Address of the depositary institution: China.

Wuhan.

Wuhan University;

[n0033]

Category name: *Akkermansia muciniphila*.

[n0034]

Example 1: Exploration of non-animal-derived culture media for *Akkermansia muciniphila*

[n0035]

According to existing reports, *Akkermansia muciniphila* can grow on media containing monosaccharides, nitrogenous monosaccharide derivatives, and amino acid sources, which can replace mucin or other animal-derived components. This suggests that *Akkermansia muciniphila* can be fermented using media with safer non-animal-derived components.

Therefore, this embodiment further improves the in vitro growth medium for *Akkermansia muciniphila* based on the existing culture media (No. 1 and No. 2) (Table 1) to more effectively increase the in vitro growth rate and amount of *Akkermansia muciniphila*. The basic components of the improved culture medium are shown in Table 2.

[n0036]

As a heterotrophic microorganism, *Akkermansia muciniphila* requires organic carbon and nitrogen sources for in vitro growth. To meet the requirements of non-animal-derived ingredients, yeast extract and soybean peptone were selected as organic carbon and nitrogen sources.

In this embodiment, *A. muciniphila* JF3 (accession number CCTCC No: M20221499) was used to determine the in vitro growth curve.

Based on the basic culture medium (Table 2), other components were added to further optimize the culture medium.

Yeast extract (Figure 1, Table 3: media 7 and 9) and soybean peptone (Figure 1, Table 3: media 7 and 8) both promoted the in vitro growth rate and amount of *Ackermania muciniphila** JF3, with yeast extract showing better results.

When the amount of yeast extract was 30 g/L (Figure 1, Table 3: Medium No. 9;), the growth rate and growth amount of *A. muciniphila* JF3 were higher than those after cultivation in the existing medium (Figure 1: Medium No. 1, No. 2 and No. 9; Table 3).

[n0037]

Although it is possible to culture *Akkermansia muciniphila* in vitro, the in vitro culture environment differs from the actual conditions of the strain in the intestinal environment.

Therefore, the whole genome of *A. muciniphila** JF3 in this embodiment was sequenced by Beijing Biomarker Biotechnology Co., Ltd. Analysis of its genomic information predicted that 15.9% of the virulence genes in *A. muciniphila** JF3 were related to iron ions, suggesting that iron ions are related to the growth of *A. muciniphila** JF3 (Table 4).

Although not an essential component, in order to more closely resemble the actual intestinal environment of *A. muciniphila* JF3, 0.5 g/L of ferric pyrophosphate was added to the modified culture medium in this embodiment.

In addition, threonine, proline, L-cysteine hydrochloride and vitamin K3 were added to this embodiment (Table 2).

[n0038]

Table 1. Composition of Culture Media 1 and 2 (Existing Culture Media)

[n0040]

Table 2. Basic Components of the Modified Culture Medium

[n0043]

Table 3. Comparison of New Components in Culture Media Nos. 7, 8, and 9 (Added based on the basic components of the improved culture media)

[n0045]

Table 4. Iron-related virulence factors of **Akkermansia muciniphila** JF3

[n0048]

Example 2: Exploring the replacement of peptone components in culture medium

[n0049]

Although peptones are a relatively ideal organic nitrogen source, they can cause changes in the in vitro morphology of *Akkermansia muciniphila*, mainly manifested as uneven strain shape and elongated cells, but there is still no solution.

This phenomenon was also observed in *A. muciniphila* JF3 of this embodiment. The cell length of *A. muciniphila* JF3 cultured on peptone medium (mediums 1 and 2) was significantly longer than that cultured on medium without peptone and mucin (Figure 2: A, B, D, E, G, H).

Changes in the morphology of *Akkermansia muciniphila* may alter the bacteria's metabolic capacity and its ability to colonize the gut.

Therefore, in this embodiment, urea is preferred as the nitrogen source for replacing peptone components.

When urea replaced peptone at a rate of 10 g/L (Figure 3, Table 5: Medium No. 6), the growth rate and growth volume of *Akkermansia muciniphila* JF3 were lower than those of strains

cultured on media containing peptone (Figure 3, Table 5: Medium No. 9), but still higher than those cultured on the existing media (Figure 3, Table 1: Medium No. 1 and No. 2).

Meanwhile, the urea component does not cause a significant increase in the cell length of *A. muciniphila* JF3, and can maintain the normal cell morphology of *A. muciniphila* JF3 (Figure 2: A-H).

[n0050]

Furthermore, the growth-stimulating effect of epigallocatechin-3-gallate (EGCG) on *A. muciniphila* in vivo has been well demonstrated.

EGCG is a component extracted from Chinese green tea. It is the main active and water-soluble component of green tea and has very strong antioxidant activity, playing an important role in anti-cancer and anti-inflammatory effects.

Furthermore, EGCG is derived from green tea, is not animal-derived, is edible, and has no toxic side effects.

By adding EGCG to the culture medium of JF3 (Figure 3: Medium 6; Table 5), the results showed that EGCG could significantly promote the growth rate and growth amount of the JF3 strain.

[n0051]

Table 5. Comparison of New Components in Culture Media No. 6 and No. 9 (Added based on the basic components of the improved culture media)

[n0054]

Example 3: Reducing the cost of growth medium

[n0055]

When developing and utilizing *Akkermansia muciniphila* as a potential probiotic, in addition to considering the safety of the in vitro culture medium and the comprehensiveness of the strain's physiological functions, the development cost also needs to be taken into account.

In this embodiment, N-acetyl-D-galactosamine (D-GalNAc) is relatively expensive, which greatly increases the cost of the culture medium.

Based on the above considerations, this embodiment explores the necessity of D-GalNAc component for the in vitro growth of *A. muciniphila* JF3.

When D-GalNAc was added to the culture medium at a concentration of 25 g/L, the growth rate and quantity of *A. muciniphila* JF3 were higher than those of strains cultured in media without D-GalNAc (Figure 4, Table 6: media 11 and 12). However, after the addition of EGCG, even in media without D-GalNAc, the growth rate and quantity of *A. muciniphila* JF3 were not lower than those of the existing media (Figure 4, Table 1 and Table 6: media 1, 2 and 11).

[n0056]

In addition, urea is not necessary except for D-GalNAc components.

When the culture medium in this embodiment contains D-GalNAc, the addition of urea can effectively increase the growth rate and amount of *Ackermania muciniphila** JF3 (Figure 4, Table 6: Culture media 6 and 10); however, when the culture medium in this embodiment does not contain D-GalNAc, the addition of urea will actually decrease the growth rate and amount of *Ackermania muciniphila** JF3 (Figure 4, Table 6: Culture media 10 and 11).

Meanwhile, regardless of the addition of D-GalNAc and/or urea, EGCG did not affect the cell morphology of *A. muciniphila* JF3 (Figure 5: A-E).

[n0057]

Table 6. Comparison of New Components in Culture Media Nos. 5, 6, 10, 11, and 12 (Added to the basic components of the improved culture media)

[n0059]

Example 4: Large-volume culture of *Akkermansia muciniphila*

[n0060]

To demonstrate that the modified Culture Medium No. 11 has the ability to culture *Akkermansia muciniphila* on a large scale.

In this embodiment, *Ackermania muciniphila** JF3 with an initial concentration of $OD_{600} = 0.025$ was inoculated into 500 mL of medium 11 at pH 6.5. Medium 11

contained 30 g/L yeast extract, 0.5 g/L EGCG, 0.5 g/L potassium dihydrogen phosphate, 1.5 g/L disodium hydrogen phosphate, 1.0 g/L sodium chloride, 4.5 g/L glucose, 5.53 g/L N-acetylglucosamine, 4 g/L threonine, 4 g/L proline, 5 mg/L L-cysteine hydrochloride, 1 g/L sodium pyruvate, 0.5 g/L ferric pyrophosphate, and 5 mg/L vitamin K3.

After 24 hours of culture, the OD_{NER3} value of *Akkermansia muciniphila* JF3 reached 1.5, while maintaining the normal morphology of the bacteria.

This indicates that medium 11 can be an ideal culture medium for the in vitro growth of *A. muciniphila*, which can both promote the high-quality growth of JF3 strain and maintain the normal morphology of the bacteria.

[n0061]

The embodiments of the present invention have been described in detail above, but the present invention is not limited to the described embodiments.

For those skilled in the art, various changes, modifications, substitutions, and variations can

be made to these embodiments without departing from the principles and spirit of the present invention, and these variations still fall within the protection scope of the present invention.