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Fortsættes ...

US-A1- 2015 306 152

MARIA CARLOTA DAO ET AL: "Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology", GUT MICROBIOTA, vol. 65, no. 3, 22 June 2015 (2015-06-22), pages 426-436, XP055698426, UK ISSN: 0017-5749, DOI: 10.1136/gutjnl-2014-308778

Zhao Fan , Li Chun-bao: "Characteristics of intestinal bacterium Akkermansia muciniphila and the association with host health", Microbiology China, vol. 44, no. 6, 20 June 2017 (2017-06-20), pages 1458-1462, XP055790692, ISSN: 0253-2654, DOI: 10.13344/j.microbiol.china.160740

Maria Carlota Dao, Amandine Everard, Judith Aron-Wisnewsky, Nataliya Sokolovska, Edi Prifti, Eric O Verger, Brandon D Kayser, Flor: "Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology", Gut microbiota, vol. 65, no. 3, 22 June 2015 (2015-06-22), pages 426-436, XP055698426, ISSN: 0017-5749, DOI: 10.1136/gutjnl-2014-308778

MARIA CARLOTA DAO ET AL: "Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology", GUT MICROBIOTA, vol. 65, no. 3, 22 June 2015 (2015-06-22), UK, pages 426 - 436, XP055698426, ISSN: 0017-5749, DOI: 10.1136/gutjnl-2014-308778

ZHAO FAN , LI CHUN-BAO: "Characteristics of intestinal bacterium Akkermansia muciniphila and the association with host health", MICROBIOLOGY CHINA , vol. 44, no. 6, 20 June 2017 (2017-06-20), pages 1458 - 1462, XP055790692, ISSN: 0253-2654, DOI: 10.13344/j.microbiol.china.160740

MARIA CARLOTA DAO, AMANDINE EVERARD, JUDITH ARON-WISNEWSKY, NATALIYA SOKOLOVSKA, EDI PRIFTI, ERIC O VERGER, BRANDON D KAYSER, FLOR: "Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology", GUT MICROBIOTA, vol. 65, no. 3, 22 June 2015 (2015-06-22), pages 426 - 436, XP055698426, ISSN: 0017-5749, DOI: 10.1136/gutjnl-2014-308778

DESCRIPTION

[0001] The present invention claims the priority of CN2018111328821, filed on September 27, 2018.

Technical Field

[0002] The present invention relates to the field of microorganisms in the field of biotechnology, and specifically relates to a strain for preventing and treating metabolic diseases and the strain for use as a preparation for preventing and treating metabolic diseases.

Background Arts

[0003] Obesity is a worldwide health problem. According to the research of the Global Burden of Disease, the global epidemiology of obesity is gradually deteriorating, which has a negative impact on physical hygiene and health economics. Internationally, the body mass index (BMI) is usually used as the standard to judge the degree of obesity. The healthy range for BMI is between 18.5 and 24.9, overweight is defined as a body mass index of 25 or higher, and obese is defined as a body mass index of 30 or higher. Data of a study of 195 countries worldwide showed that the prevalence of obesity has doubled since 1980. At present, 5% of children and 12% of adults are obese. The prevalence of obesity is similar to that of type 2 diabetes, and with this trend, 1/5 of the population worldwide will be obese. From 1975 to 2014, the global average BMI increased from 21.7 to 24.2 kg/m² for men, and from 22.1 to 24.4 kg/m² for women. As of 2014, the prevalence of obesity among Chinese adults was 12.9%, the prevalence of overweight was 41.2%, and the prevalence of central obesity was 20.9%, which was increasing year by year. Obesity destroys the metabolic homeostasis of body, which in turn causes metabolic diseases. Obesity is a critical risk factor for the development of type 2 diabetes. Current treatments are mainly to reduce calorie intake and prevent calorie absorption. Gastric volume reduction surgery can reduce energy intake, basic metabolism and exercise can increase calorie consumption and loss, and the increase in brown and beige adipose tissue can increase heat production. In addition, intestinal cells and flora are involved in feeding behavior and can be used as potential targets for treating obesity.

[0004] Obesity and type 2 diabetes are affected by environmental and genetic factors. The genes of the human flora also play an important role in both diseases; the flora may affect obesity by influencing, e.g., the absorption of calories, and obesity may in turn affect the composition of intestinal flora. There are more than 1,000 different types of bacteria in the human intestine, with a total of about 10¹⁴. Dysbiosis of the intestinal flora is associated with many diseases, including obesity, with a decrease in the anti-inflammatory *Akkermansia*

muciniphila (hereinafter referred to as 'Akkermansia') in obese patient, and an increase in Proteobacteria, Bacteroides, Campylobacter and Shigella spp. The ratio of Firmicutes to Bacteroidetes not only affects the metabolism of carbohydrates, but also alters the yield of short-chain fatty acids. Increased blood acetic acid levels can lead to increased insulin resistance and ghrelin production, of which the flora may be the most important variable factor. Intestinal flora can change a variety of metabolic pathways centered on energy metabolism balance, thereby affecting the occurrence and development of obesity. Probiotics and prebiotics can play a role in combating obesity by reducing intestinal bacterial lipopolysaccharide, changing the composition of the flora, and reducing fat storage.

[0005] Chinese invention patent application with application number CN201380070847.0 and publication number CN104918626A discloses "USE OF AKKERMANSIA FOR TREATING METABOLIC DISORDERS", and the *Akkermansia muciniphila* strain ATCC BAA835 screened by this patent application can be used to promote weight loss for subjects in need. Also, US 2015/306152 A1 (GANI PATRICE [BE] ET AL) discloses the strain *Akkermansia muciniphila* Muc (ATCC BAA-835) for similar use. However, the inventors had shown through their research that although *Akkermansia muciniphila* strain ATCC BAA835 was generally accepted as the most classic and effective Akkermansia strain, the strain was not ideal in respect of fermentation performance, storage stability, acid and bile resistance, and the effect on treating metabolic diseases.

[0006] MARIA CARLOTA DAO ET AL (*Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology, DOI: 10.1136/gutjnl-2014-308778) reports on the abundance of *Akkermansia muciniphila*, the mucin-degrading bacterium, which has been found inversely associated with body fat mass and glucose intolerance in mice. However, the provision of an alternative as no improved effect is disclosed.

Content of the present invention

[0007] The technical problem to be solved by the present invention is to overcome the defects of the existing *Akkermansia muciniphila* strain ATCC BAA835 in the prior art, which is not ideal in respect of fermentation performance, storage stability, acid and bile resistance, and the effect on treating metabolic diseases.

[0008] The present invention is as defined in the appended claims and provides a strain for preventing and treating metabolic diseases. The strain is *Akkermansia muciniphila* strain, which is named *Akkermansia muciniphila* SSYD-3 with an accession number of CGMCC No. 14764 for the deposit.

[0009] The present invention provides a use of the *Akkermansia muciniphila* strain in preparing preparations for preventing and treating metabolic diseases.

[0010] In the present disclosure, metabolic diseases have conventional meanings in the art, comprising diabetes, obesity, insulin resistance, alcoholic and non-alcoholic fatty liver, hypertension, hyperlipidemia, hypercholesterolemia, heart disease and other diseases. The preventive and therapeutic effects refer to the prevention of the occurrence and development of metabolic diseases, and the treatment and auxiliary treatment of metabolic diseases, respectively.

[0011] In particular, the use of the *Akkermansia muciniphila* strain for treating and preventing metabolic diseases in the present invention comprises one or more of the following uses: for treating and preventing obesity, type 2 diabetes and metabolic syndrome, and other diseases that combine weight gain and glucose metabolism disorders. More particularly, the strain is used for one or more of the following uses: delaying weight gain, reducing visceral adipose tissue, reducing blood-glucose after 15-30 minutes of glucose injection, and reducing fasting blood-glucose and blood-glucose within 60 minutes after insulin injection.

[0012] The form of the preparation is a conventional form in the art, comprising medicines, health foods, functional foods, foods, beverages, feed products and dietary supplements containing the *Akkermansia muciniphila*.

[0013] The *Akkermansia muciniphila* refers to a living organism or a pasteurized inactivated organism.

[0014] The preparation is prepared by using an effective dose of the *Akkermansia muciniphila* as the active ingredient, and adding pharmaceutical excipients such as conventional excipients, flavoring agents, disintegrants, binders, adjuvants, thickeners, solubilizers, preservatives, lubricants according to certain preparation techniques. Particularly, the dosage form is a lyophilized bacterial agent. Particularly, the count of viable bacteria is 10^{10} CFU/g.

[0015] Wherein, the effective dose means that *Akkermansia muciniphila*, as the main active ingredient in a solid viable bacterial preparation, has a viable count of 10^6 - 10^{14} CFU/g. Wherein, the effective dose means that *Akkermansia muciniphila*, as the main active ingredient in a liquid viable bacterial preparation, has a viable count of 10^6 - 10^{14} CFU/g.

[0016] On the basis of the common sense in the art, the above-mentioned preferred conditions can be combined arbitrarily to obtain preferred examples of the present invention.

[0017] The reagents and raw materials used in the present invention are all commercially available.

[0018] The positive and progressive effects of the present invention are as follows: studies in the present invention shows that *Akkermansia muciniphila* SSYD-3 is significantly better than the standard strain of *Akkermansia muciniphila* (ATCC BAA835) in respects of fermentation performance, storage stability, acid and bile resistance, and is significantly better than the

standard strain (ATCC BAA835) in respects of delaying weight gain, reducing visceral adipose tissue, reducing blood-glucose after 15-30 minutes of glucose injection, and reducing fasting blood-glucose and blood-glucose within 60 minutes after insulin injection.

Information of deposit of microorganism material

[0019] The *Akkermansia muciniphila* SSYD-3 (strain W03 for short) of the present invention has been deposited in China General Microbiological Culture Collection Center (CGMCC) on September 29, 2017. The deposit address: Building 3, Lane 1, NO.1 West Beichen Road, Chaoyang District, Beijing 100101, the accession number is CGMCC No. 14764, the culture name is *Akkermansia muciniphila* SSYD-3, and the proposed taxonomic name is *Akkermansia muciniphila*.

Brief description of the drawings

[0020]

Fig. 1 shows the growth curve of *Akkermansia muciniphila* in Example 3.

Fig. 2 shows the variation curve of the bacterial content of *Akkermansia muciniphila* in Example 5 under the storage condition of 4°C.

Fig. 3 shows the variation curve of the bacterial content of *Akkermansia muciniphila* in Example 5 under the storage condition of 25°C.

Fig. 4 shows the weight gain results of the mice that was administrated with W03 and ATCC BAA835 in Example 8.

Fig. 5 shows the visceral adipose tissue of the mice that was administrated with W03 and ATCC BAA835 in Example 8.

Fig. 6 shows the glucose tolerance of the mice that was administrated with W03 and ATCC BAA835 after intraperitoneal injection of gluc in Example 8.

Fig. 7 shows the blood-glucose of the mice that was administrated with W03 and ATCC BAA835 after intraperitoneal injection of insulin in Example 8.

Fig. 8 shows the change in body fat of the mice that was administrated with W03 and ATCC BAA835.

Detailed description of the preferred embodiment

[0021] The following examples further illustrate the present disclosure. In the following examples, unless specified otherwise, the experimental methods are selected according to conventional methods and conditions, or according to the product specification.

[0022] The experimental data of each group were expressed as mean $\pm$ standard deviation, and then collated and imported into SPSS18.0 statistical software for data analysis. The difference between the means of each group was tested by analysis of variance. In animal experiments, the data distribution was checked using Shapiro-Wilk, and the homogeneity of variance was further tested using Levene's test. The importance of each two specified groups was assessed using corresponding t-test. $P < 0.05$ means the difference is a statistically significant difference.

Example 1 Screening of bacteria

[0023]

1. 1. Culture medium

Isolation medium: 0.4g KH_2PO_4 ; 0.53g Na_2HPO_4 ; 0.3g NH_4Cl ; 0.3g NaCl ; 0.1g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 0.11g CaCl_2 ; 0.5mg resazurin; 4g NaHCO_3 ; 0.25g $\text{Na}_2\text{S} \cdot 7-9\text{H}_2\text{O}$; 0.25% mucin.

Fermentation medium 1: brain heart infusion broth (BHI)

Fermentation medium 2: 5g tryptone, 5g soybean peptone, 5g beef powder, 5g yeast powder, 4g glucose, 3g NaCl , 1.5g MgSO_4 , Na_2HPO_4 , KH_2PO_4 , 1L water, $\text{pH} = 7.2$.

The isolation medium and the fermentation medium were autoclaved at 121°C for 15 minutes for later use.

2. 2. Isolation and identification of strains

① The prepared mucin isolation medium was placed in a serum bottle (liquid volume 10/30 mL).

② 0.5g stool of healthy adult was placed in sterile PBS (phosphate saline buffer) and dispersed evenly. The stool was 10-fold gradiently diluted in PBS solution, and 1mL of the dilution was inoculated into the serum bottle containing mucin.

③ The serum bottle containing mucin was placed at 37°C and incubated for 48h in an anaerobic environment.

④ The mucin fermentation broth with the highest dilution gradient that was visible to the naked eye was taken and diluted to 10^{-6} - 10^{-9} with PBS buffer.

⑤ The diluent was spread on the brain heart infusion agar medium and incubated for 24-48 hours, and colonies of 1mm in size was picked, purified and cultured, then the strain identification was performed using bacterial 16S rDNA.

[0024] Results: The morphological, physiological and biochemical characteristics of the colonies were combined with the amplification of bacterial 16S rDNA to identify the bacterial species. W03 bacterial 16S rDNA sequence of is shown in SEQ ID No. 1 in the Sequence Listing. Then the sequence obtained by sequencing was compared with the 16S rDNA gene sequence registered in GenBank for homology. Finally, the isolated bacterium was identified to be *Akkermansia muciniphila* and named *Akkermansia muciniphila* SSYD-3, with a maximum sequence similarity of 99% to the existing sequences.

Example 2: Akkermansia fermentation

[0025]

- ① *Akkermansia muciniphila* was inoculated on brain heart infusion agar using three sector streak, and incubated anaerobically at 37°C for 48 hours.
- ② Single colonies of *Akkermansia muciniphila* were picked from the agar plate and inoculated in 15mL of brain heart infusion broth, and incubated anaerobically at 37°C for 24h.
- ③ 10mL fermentation broth was transferred from 15mL anaerobic tube to 200mL brain heart infusion broth, and incubated anaerobically at 37°C for 24h.
- ④ 200mL fermentation broth of *Akkermansia muciniphila* was inoculated into brain heart infusion broth, 4L of mixed fermentation broth was loaded into a 5L fermentor. The anaerobic gas was a binary gas mixed by nitrogen and carbon dioxide (N₂:CO₂=9:1). The mixed fermentation broth was incubated anaerobically at 37°C for 24h with stirring speed of 100r/min.

Example 3: Fermentation of *Akkermansia muciniphila*

[0026]

- ① Single colonies of W03 and standard strain ATCC BAA835 (purchased from the American Type Culture Collection) were respectively inoculated into 15mL fermentation medium 2, and incubated anaerobically at 37° C for 24h.
- ② 10mL fermentation broth was transferred from 15mL anaerobic tube to 200mL fermentation medium 2, and incubated anaerobically at 37°C for 24h.

③200mL *Akkermansia* fermentation broth was inoculated into fermentation medium 2, 4L of mixed fermentation broth was loaded into a 5L fermentation tank. The anaerobic gas was a binary gas mixed by nitrogen and carbon dioxide ($N_2:CO_2=9:1$). The mixed fermentation broth was incubated anaerobically at 37°C for 24h while controlling pH of 6.5 in the process, until there was no change in OD.

[0027] Results: As shown in Figure 1, the maximum OD of W03 strain was 2.26 (2.13×10^9 CFU/mL) after 13 hours of fermentation while the growth of bacteria reached stationary phase, and the maximum OD of ATCC BAA835 was 1.87 (9.67×10^8 CFU/mL) after 18 hours of fermentation while the growth of bacteria reached stationary phase. The fermentation time of W03 strain is shorter, which is more conducive to industrial production. The OD during fermentation of W03 strain was significantly higher than that of ATCC BAA835 with a higher fermentation unit.

Example 4: Lyophilized inoculum of *Akkermansia muciniphila* CGMCC No. 14764

[0028]

- ① Lyophilized protectant: trehalose, sucrose and milk powder.
- ② The fermentation broth obtained in Example 3 was centrifuged at 8000R for 20 minutes to collect the bacteria.
- ③ The bacteria obtained by centrifugation of *Akkermansia muciniphila* was mixed with lyophilized protectant and water to make sure the mass percentages of trehalose, sucrose and milk powder in the solution were 5%, 5% and 10% respectively before lyophilization. Then the solution was lyophilized to obtain a lyophilized bacterial powder, and the number of viable bacteria of the *Akkermansia muciniphila* strain with the accession number of CGMCC No. 14764 for the deposit in the bacterial powder was 10^{10} CFU/g.

Comparative Example 1: Lyophilized inoculum of *Akkermansia muciniphila* strain ATCC BAA835

[0029] The preparation method and parameter conditions of the lyophilized bacterial inoculum in Comparative Example 1 are the same as those in Example 4, except that the strain is *Akkermansia muciniphila* strain ATCC BAA835.

Example 5: Storage stability of *Akkermansia muciniphila* powder

[0030] W03 bacterial powder obtained in Example 4 and ATCC BAA835 bacterial powder obtained in Comparative Example 1 were stored at 4°C for 24 months and at 25°C for 6 months to compare their storage stability.

[0031] Results: It can be seen from Figure 2 that after 2 years of storage at 4°C, the number of viable bacteria of W03 bacterial powder is 4.85×10^8 CFU/g, which was one order of magnitude higher than that of ATCC BAA835 bacterial powder (6.69×10^7 CFU/g). This means that W03 strain is more stable and has a longer shelf life. It can be seen from Figure 3 that after 6 months of storage at 25°C, the number of viable bacteria of W03 bacterial powder was 8.23×10^8 CFU/g, which was one order of magnitude higher than that of ATCC BAA835 bacterial powder (7.79×10^7 CFU/g). This means that W03 strain is more stable in storage.

Example 6: Viability of *Akkermansia muciniphila* in simulated gastric juice

[0032] 2.0 sodium chloride and 3.2g pepsin were dissolved in 7.0ml hydrochloric acid and water and the solution was replenished to 1000mL. The artificial gastric juice with pH 1.2 was filtered through 0.22µm filter to get sterilized. 20 ml sterilized artificial gastric juice was filled into 30 mL sterile vials with a total of 3 vials, then sealing the vials with aluminum caps, and the headspace gas was replaced with nitrogen for protection. The lyophilized bacterial powder of W03 and ATCC BAA835 was each added to 1 mL sterile PBS, and 1 mL of the bacterial suspensions was each inoculated into the above vials. The vials were placed at 37°C, and the solutions were sampled at different times to calculate the survival rate.

[0033] Results: The viability of two strains in a simulated gastric juice environment was investigated in this experiment. It can be seen from Table 1 that the survival rates of the two strains showed a significant downward trend with time. The survival rate of W03 was higher than that of ATCC BAA835 at different times, and W03 had stronger acid tolerance.

Table 1. Viability of *Akkermansia muciniphila* in simulated gastric juice

Strain	0 h	1 h	2 h	3 h	4 h	2h viability
W03 (10^9 CFU/mL)	6.35	5.68	4.32	1.32	1.04	68.03 %
ATCC BAA835 (10^9 CFU/mL)	6.52	4.52	3.18	1.19	0.67	48.77%

Example 7: Viability of *Akkermansia muciniphila* in simulated intestinal juice

[0034] 6.8 g of potassium dihydrogen phosphate and 3 g of bile salt No. 3 were added into 500 mL of water, followed by adjusting the pH to 6.8 with 0.4% sodium hydroxide solution; then 10

g of pancreatin was dissolved in appropriate amount of water. After mixing the two liquids, the mixed solution was replenished to 1000 mL with water, then filtered with 0.22µm filter membrane for sterilization. 20 ml sterilized artificial gastric juice was filled into 30 mL sterile vials with a total of 3 vials, then sealing the vials with aluminum caps, and the headspace gas replaced with nitrogen for protection. The lyophilized bacterial powder of W03 and ATCC BAA835 was each added to 1 mL sterile PBS, and 1 mL of the bacterial suspensions was each inoculated into the vials described above. The vials were placed at 37°C, and the solutions were sampled at different times to calculate the survival rate.

[0035] Results: It can be seen from Table 2 that under the same conditions, W03 strain has better bile tolerance than the control strain ATCC BAA 835, and W03 strain is more conducive to resist high bile salt environment to colonize the intestine.

Table 2. Viability of *Akkermansia muciniphila* in simulated intestinal juice

Strain	0h	1h	2h	3h	4h	2h viability
W03 (10 ⁹ CFU/mL)	6.16	1.70	1.32	1.04	0.88	21.43%
ATCC BAA835 (10 ⁹ CFU/mL)	6.29	1.23	0.87	0.81	0.75	13.83%

Example 8: Application of *Akkermansia muciniphila* for treating metabolic diseases

[0036] Eight-week-old male C57 BL/6 mice (no specific pathogens, Shanghai Lab Animal Research Center) were fed food *ad libitum* in groups of 3 mice per cage (filter cage) during a strict 12-hour photoperiod. After adapting to the new environment, they were fed a standard diet or a high-fat diet with 60 kcal% fat (Research Diet, D12492i). The animals were randomly allocated into 2 groups and administrated with 200 microliters of sterile PBS and *Akkermansia muciniphila* bacterial solution (10⁸ CFU/100 microliters), respectively, once a day for 8 weeks.

[0037] Body weight was measured before intragastric administration and at the end of the experiment. The food intake of 7 consecutive days was recorded by cage (3 mice per cage) to calculate the average food intake per mouse per day. Stool samples were collected before intragastric administration and at the end of the experiment, and immediately stored in a refrigerator at -80°C for further analysis. Body fat content was assessed with a body composition analyzer (EchoMRI). Whole blood was collected from the tail vein of mice, and the blood glucose level and insulin level were measured with a glucometer (LifeScan). To ensure that the starting points were the same, this study did not use random allocation, but grouped mice that had adapted to the environment according to their body weight. All operations were approved by the Animal Ethics Committee of Shanghai Jiaotong University School of Medicine.

[0038] The experimental results are as follows:

1. W03 delays the weight gain of obese mice induced by high-fat diet with better effect

than PBS and positive control ATCC BAA835. The weight gain percentage of mice administrated with W03 is lower than that of ATCC BAA835 mice with a significant difference, ** $p < 0.01$ (Figure 4: $n=9-10$ per group, indicating by mean $\pm$ standard error, * means the difference is statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

2. 2. The application of W03 reduces the visceral adipose tissue of obese mice fed with high-fat diet, the effect is superior to the positive control ATCC BAA835 with a significant difference, *** $p < 0.001$; there is no significant difference between ATCC BAA835 and PBS control group, i.e., $P > 0.05$, indicating that ATCC BAA835 strain does not improve the visceral adipose tissue of high-fat obese mice (Figure 5: $n=9-10$ per group, indicating by mean $\pm$ standard error, * means the difference is statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Visceral adipose deposition is the main feature of central obesity, while visceral adipose is considered to be significantly associated with obesity-related metabolic complications such as insulin resistance and cardiovascular risk events. Therefore, reducing the visceral adipose tissue has a good preventive effect on the occurrence of metabolic-related complications and cardiovascular risk events in obese patients.
3. 3. The application of W03 improves the glucose tolerance of obese mice fed with high-fat diet, whose blood-glucose is significantly lower than the positive control ATCC BAA835 group after 15min and 30min of glucose injection, with significant differences at 15min and 30min (Figure 6: $n=9-10$ per group, indicating by mean $\pm$ standard error, * means the difference is statistically significant compared with PBS, # means the difference is statistically significant compared with ATCC BAA835* # $p < 0.05$, ** ## $p < 0.01$, *** ### $p < 0.001$). High-fat diet can cause impaired glucose regulation, while W03 treatment can alleviate the degree of impairment, which manifested as that the tolerance of mice with intraperitoneal glucose injection is improved compared with the PBS group. The improvement of glucose tolerance after the application of W03 is more significant than that of ATCC BAA835, and the effect is better than the positive control ATCC BAA835. It is suggested that W03 can improve the impaired glucose regulation in pre-diabetes, which has a certain contribution to preventing the occurrence and development of diabetes.
4. 4. The application of W03 improves the insulin tolerance of obese mice fed with high-fat diet, which manifested as the fasting blood-glucose and blood-glucose at 15min, 30min, 60min and 120min after insulin injection are all lower than the control group PBS, wherein the fasting blood-glucose and blood-glucose at 15min, 30min, 60min and 120min after insulin injection are even better than the positive control ATCC BAA835 (Figure 7: $n=9-10$ per group, indicating by mean $\pm$ standard error, * means the difference is statistically significant compared with PBS, # means the difference is statistically significant compared with ATCC BAA835 * # $p < 0.05$, ** ## $p < 0.01$, *** ### $p < 0.001$). High-fat diet leads to decreased insulin sensitivity and deterioration of insulin tolerance, while the application of W03 can alleviate the deterioration of insulin tolerance induced by high-fat diet, with better effect than the positive control ATCC BAA83 5. It is suggested that the application of W03 can benefit patients with reduced insulin sensitivity, thereby preventing the occurrence and development of diabetes.
5. 5. The body fat composition of the model animals in this study is relatively complicated.

In addition to the measurable inguinal fat (subcutaneous fat) and paradidymal fat (visceral adipose), there is lots of fat that is hard to collect and measure like omental fat. Therefore, the body composition of the model animal was analyzed with a small animal body fat imager, and the results are shown in Figure 8. The results show that compared with the original strain ATCC BAA835, the visceral adipose tissue of model animals applied with W03 is reduced, and the increase in non-body fat content (muscles, bones, etc.) is obvious.

[0039] The positive clinical significance in pharmacodynamic experiments is as follows:

Nearly 50% of obese patients have 'Triple-H' symptoms, i.e. hyperglycemia, hyperlipidemia, and hypertension. Visceral adipose deposition is the main feature of central obesity. Meanwhile, visceral adipose is considered to be significantly associated with insulin resistance, cardiovascular and cerebrovascular risk events and other obesity-associated metabolic complications. Therefore, reducing the visceral adipose tissue has a good preventive effect on the occurrence of the dangerous events described above. In respect of improving oral glucose tolerance, mice administrating with W03 showed a 3-4mmol/L reduction in intravenous blood-glucose at 30min and 60min postprandial, thus allowing for 1.5-2U less insulin or one less second-line hypoglycemic drug for non-severe diabetic patients clinically. The use of W03 can also improve the intravenous insulin tolerance of high-fat-induced obese mice. Its practical significance is that a large part of insulin resistance is caused by fat accumulation, therefore improving insulin resistance can also slow down the progression of pre-diabetes to diabetes and reduce insulin dosage in patients that were injected with insulin. Because long-term clinical use of insulin has the risk of weight gain, reducing the amount of insulin used is of great practical significance for obese patients with diabetes. Meanwhile, many drugs need to be metabolized and detoxified by the liver, and the livers of obese patients have low inflammation and a slightly abnormal liver function. Reducing the use of drugs is of great significance to reduce the body burden and cost for treatment of the patients with chronic diseases such as obesity or obesity with diabetes.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- CN2018111328821 [0001]
- CN201380070847 [0005]
- CN104918626A [0005]
- US2015306152A1 [0005]

Non-patent literature cited in the description

- **MARIA CARLOTA DAO et al.** Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity relationship with gut microbiome richness and ecology, [0006]

**STAMME TIL FOREBYGGELSE OG BEHANDLING AF STOFSKIFTESYGDOMME,
OG BRUGEN HERAF**

Patentkrav

1. Stamme til anvendelse i en fremgangsmåde til forebyggelse og behandling af stofskiftesygdomme, hvor stammen er *Akkermansia muciniphila*-stammen, som benævnes *Akkermansia muciniphila* SSYD-3 med stammesamlingsnummer CGMCC No. 14764 for deponeringen.
2. Stamme ifølge anvendelsen i krav 1, hvor stammen er omfattet i et præparat.
3. Præparat til anvendelsen i krav 2, hvor præparatet formuleres ved hjælp af en effektiv dosis af *Akkermansia muciniphila*-stammen som en aktiv ingrediens og tilsætning med farmaceutiske hjælpestoffer.
4. Præparat til anvendelsen i krav 2 eller 3, hvor præparatet er et frysetørret bakterielt inokulum, med et kimtal på 10^{10} CFU/g levedygtige bakterier af *Akkermansia muciniphila*-stammen.
5. Præparat til anvendelsen i krav 3, hvor den effektive dosis betyder at *Akkermansia muciniphila*, som den vigtigste aktive ingrediens i et fast præparat med levedygtige bakterier, har et levedygtigt kimtal på 10^6 - 10^{14} CFU/g.
6. Præparat til anvendelsen i krav 3, hvor den effektive dosis betyder at *Akkermansia muciniphila*, som den vigtigste aktive ingrediens i et flydende præparat med levedygtige bakterier, har et levedygtigt kimtal på 10^6 - 10^{14} CFU/g.
7. Præparat til anvendelsen i krav 2, hvor stammen anvendes med en effektiv dosis.
8. Præparat til anvendelsen i krav 7, hvor den effektive dosis betyder at stammen har et levedygtigt kimtal på 10^6 - 10^{14} CFU/g.
9. Præparat til anvendelsen i mindst et af kravene 2-8, hvor stofskiftesygdommen er en eller flere af de følgende sygdomme:

overvægt, type 2 diabetes og metabolisk syndrom, og andre sygdomme der kombinerer vægtstigning og forstyrrelser i glukosemetabolismen.

10. Præparat til anvendelsen i mindst et af kravene 2-9, hvor præparatet anvendes til en eller flere af de følgende anvendelser: forsinkelse af vægtstigning, reduktion af visceralt adipøst væv, reduktion af blodglukose 15min~30min efter glukoseinjektion, og reduktion af fastebloodglukose og blod-blodglukose inden for 60 minutter efter insulininjektion.

DRAWINGS

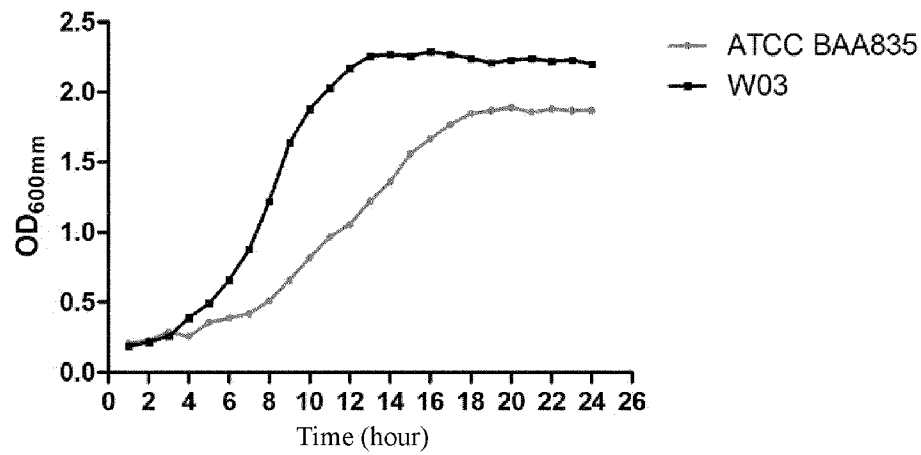


Fig. 1

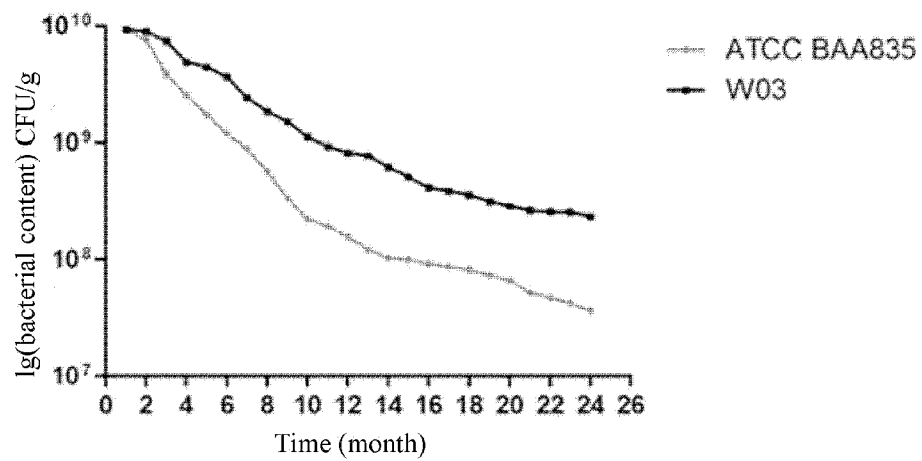


Fig. 2

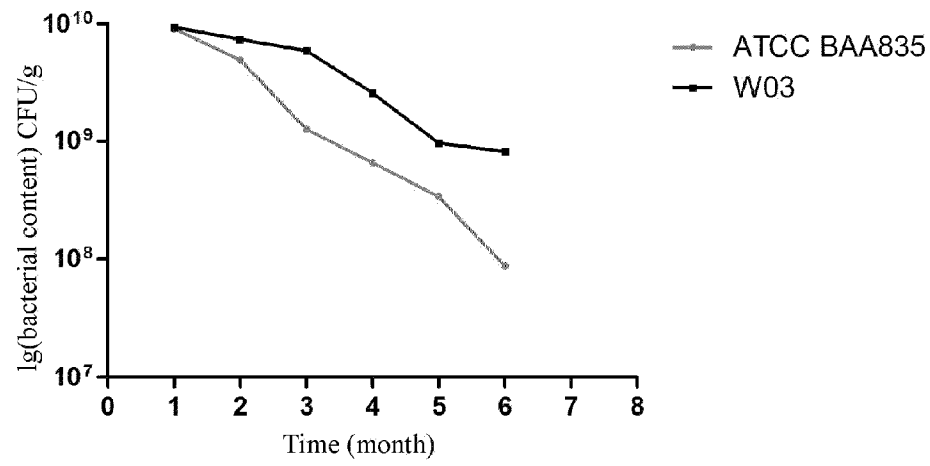


Fig. 3

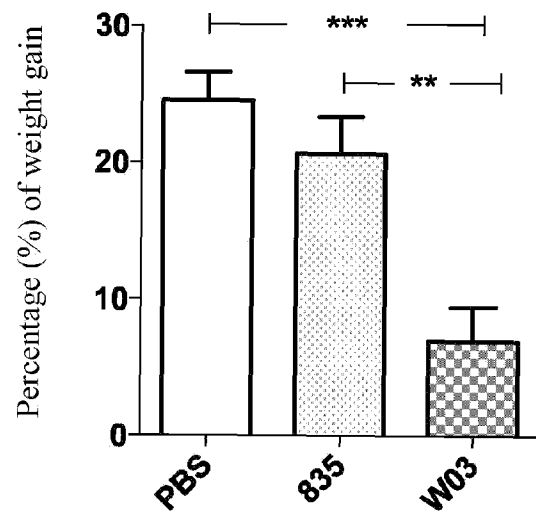


Fig. 4

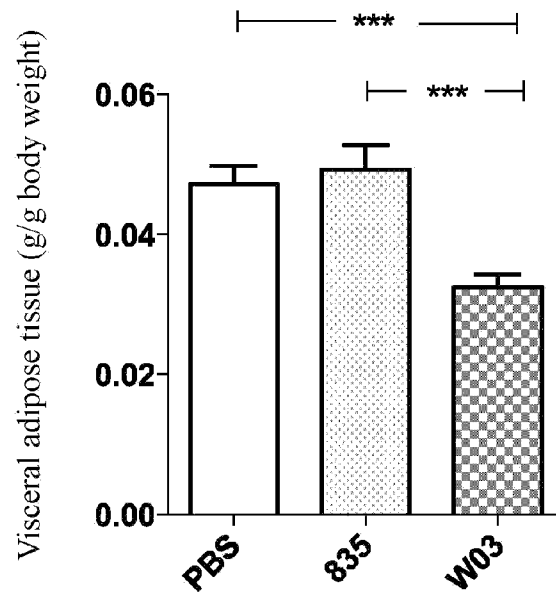


Fig. 5

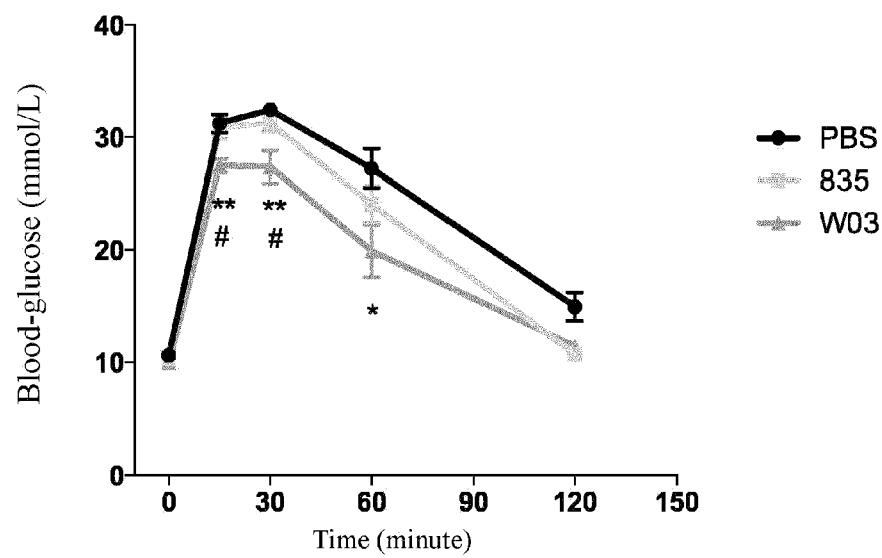


Fig. 6

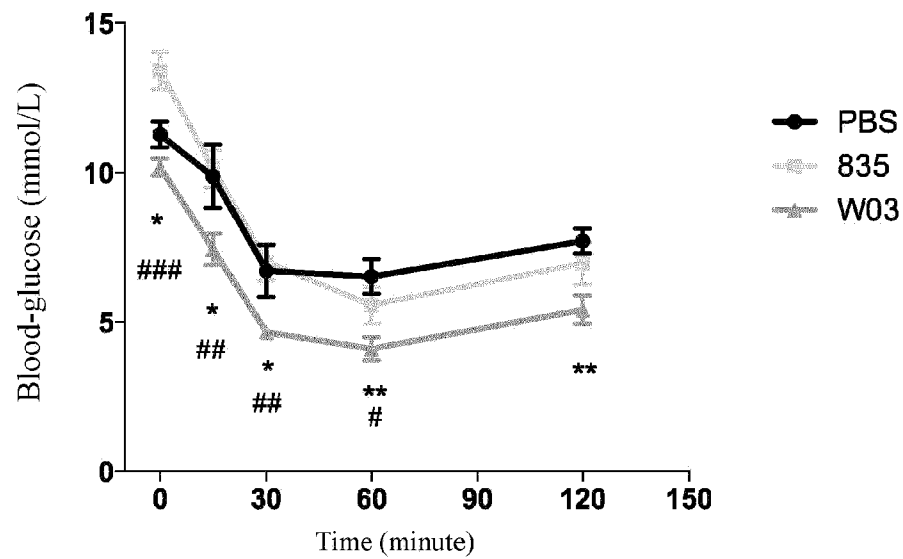


Fig. 7

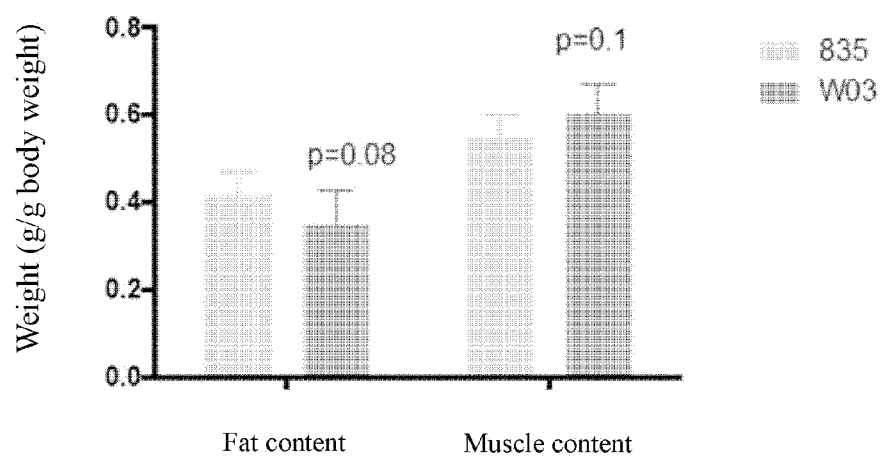


Fig. 8

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

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