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(54) **MIXTURE, DISSOLVING SOLUTION AND PHARMACEUTICAL AGENT EACH COMPRISING THERMOPHILIC MICROORGANISM**

MISCHUNG, AUFLÖSUNGSLÖSUNG UND PHARMAZEUTISCHES MITTEL MIT THERMOPHILEN MIKROORGANISMEN

MÉLANGE, SOLUTION DISSOLVANTE ET AGENT PHARMACEUTIQUE CONTENANT CHACUN UN MICROORGANISME THERMOPHILE

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**Description**

## TECHNICAL FIELD

5 **[0001]** The present invention relates to a mixture, a dissolving solution and a pharmaceutical agent each comprising a thermophilic microorganism, which are capable of activating mucous membrane immune systems and regulating metabolisms of animals including humans.

## BACKGROUND ART

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**[0002]** Probiotics using microorganisms have been known to improve the enterobacterial flora of animals, prevent diarrhea, activate immunity, and so on. For example, Patent Document 1 discloses pasteurized ingredients derived from bacterial cells to prevent diarrhea in animals. Also, Patent Document 2 discloses a compound containing one kind of lactic acid bacteria, *Lactobacillus*. In addition, Patent Document 3 discloses an antimicrobial compound derived from *Bacillus subtilis*, which is a species of the genus *Bacillus*. Furthermore, Patent Document 4 discloses microorganisms having ability to colonize on the gastrointestinal tract, which are symbionts including Yeast, *Lactobacillus* and *Bifidobacterium*. Furthermore, Patent Document 5 discloses an immunopotentiator including one kind of lactic acid bacteria, such as *Lactobacillus*. Probiotics disclosed in these patent documents are those using microorganisms proliferative at normal temperatures, but not using any thermophilic microorganism.

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**[0003]** Furthermore, the followings are examples of influences of administering microorganisms proliferative at normal temperatures to animals on immune systems and metabolic regulations and the action mechanisms of such influences.

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**[0004]** In Non-Patent Document 1, it is reported that *Bacillus subtilis* increases CCL21 gene expression by symbiosis with the *Bacteroides* in the appendix of a rabbit. Furthermore, in Non-Patent Document 2, it is reported that *Salmonellas* known as pathogenic bacteria derived from animals inhibit the expression of chemokines CXCL13 and CCL21, which are chemostatic factors for B cells in the immune system, through sensors, Toll-like receptor 4, in the mucous membrane immune system. Furthermore, Non-Patent Document 3 discloses that the above chemokine CXCL13 and the like play a role in development of lymph nodes in the living body, and Non-Patent Document 4 discloses that they relate to the formation of immune functions in the respiratory system.

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**[0005]** In Non-Patent Document 5, furthermore, segmented filamentous bacteria are disclosed as bacteria that regulate the functions of the Peyer's patches, the regulatory site of the immune system in the intestinal tract. Furthermore, in Non-Patent Document 6, an attempt to introduce human's flora is also carried out by introduction of the special bacteria such as those described above into germ-free animals (axenic animals).

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**[0006]** On the other hand, Patent Documents 6 to 8 disclose techniques using thermophilic microorganisms. The techniques using thermophilic microorganisms have a great advantage in that, for example, they allow organic waste materials to be recycled and thus formulated for respective applications. Each of the above patent documents discloses promotion of making compost from feces and urine, reduction of smells, and the like when administering *Bacillus* having chitin degradation ability to farm animals. However, the mechanisms of action of these techniques are not described in detail. In other words, these patent documents do not disclose direct effects of administration of thermophilic microorganism to an animal on a living body, particularly an influence thereof on the immune or endocrine system.

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In Chie Niisawa et al.: "Microbial analysis of a composted product of marine animal resources and isolation of bacteria antagonistic to a plant pathogen from the compost.", *The Journal of general and applied Microbiology*, Vol. 54, No. 3, 1 June 2008 (2008-06-01), Pages 149-158, a study result is disclosed that performed a microbial analysis of compost having high bacteria antagonistic activity. In Miyamoto et al., 2008, *Journal of Japanese Biochemical Society*, 1P-1278, it is described that the feed with microorganisms as disclosed in Chie Niisawa et al. affect the intestinal flora and the blood component of rats administered the said feed. Further, from EP 2 135 614 A1 an intestinal dismicrobism compound is known having anti-diarrhoea, antiinflammatory and lactose and lactose derivative intolerance lenitive properties.

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**[0007]** As described above, any of the conventional techniques for regulating the immune system is just something to use only a microorganism proliferative at normal temperatures. In particular, the conventional techniques cannot simultaneously attain advantageous effects of enhancing muscle-building effects, regulating gas metabolism and fat metabolism to reduce in greenhouse gases generated from intestinal contents, and regulating a fat accumulation in the body. Alternatively, the conventional techniques using thermophilic microorganisms declare effects on manure and feed, and environmental improvement effects.

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## CITATION LIST

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## PATENT DOCUMENTS

**[0008]**

Patent Document 1: Japanese Patent 2621588  
Patent Document 2: Japanese Patent 3338446  
Patent Document 3: JP 2006-514019 A  
Patent Document 4: JP 2009-137962 A  
Patent Document 5: JP 2006-76961 A  
Patent Document 6: Japanese Patent 3146305  
Patent Document 7: Japanese Patent 3314302  
Patent Document 8: JP 2003-219864 A

NON-PATENT DOCUMENTS

[0009]

Non-Patent Document 1: Nicholas B et al., Microbial induction of B and T cell areas in rabbit appendix. Dev Comp Immunol. 2008; 32(8): 980-981  
Non-Patent Document 2: Asheley L st John et al., Salmonella disrupts lymph node architecture by TLR-4mediated suppression of homeostatic chemokines. Nature Medicine 2009; 15(11): 1259-1266  
Non-Patent Document 3: Serge A van de Pavert et al., ChemokineCXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. Nature Immunology 2009; 10(11): 1193-1200  
Non-Patent Document 4: Juan E Moyron-Quiroz, et al. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nature Medicine. 2004; 10(9): 927-934  
Non-Patent Document 5: Klaasen HLBM et al., Infection and Immunity61: 303-306, 1993 etc.  
Non-Patent Document 6: Journal of Intestinal Microbiology 22: 109-114,2008

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0010] However, the techniques disclosed in the respective patent and non-patent documents are insufficient in data for interactive verification between the mechanisms of action about influence on animals and the influence to be affected on general health conditions. In particular, the conventional techniques using thermophilic microorganisms do not relate to the results of researches with experimental animals, and are insufficient in findings about fundamental researches or the like that intend to apply the techniques to animals other than farm animals, specifically to humans.

[0011] The present invention has been made in consideration of the above situation, and intends to provide a mixture, a dissolving solution and a pharmaceutical agent using a thermophilic microorganism, which allow regulation of a mucous membrane immune system gene cluster and metabolism-related gene clusters of the intestines and liver based on the data of researches using rats and mice which are experimental animals with accumulated universal data.

SOLUTIONS TO THE PROBLEMS

[0012] The invention pertains to a mixture or dissolving solution according to claim 1 and a pharmaceutical agent according to claim 2. A mixture or a dissolving solution according to the present disclosure is a mixture or a dissolving solution for regulating at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines and a metabolism-related gene cluster in the liver of an animal by being administered to the animal, prepared by fermentation of an organic material containing a thermophilic microorganism at a temperature of 50°C or more and 90°C or less, wherein the thermophilic microorganism includes at least one species of the genus Bacillus, Oceanobacillus, Paenibacillus, Anoxybacillus, Lysinibacillus, Methanopyrus, Geogemma, Pyrolobus, Pyrodictium, Hyperthermus, Pyrococcus, Pyrobaculum, Thermococcus, Aeropyrum, Aquifex, Thermotoga, Thermodesulfobacterium, Thermus, Geobacillus, Thermomyces, and Clostridium.

[0013] Herein, the above thermophilic microorganism refers to those that meet the criteria for thermostability described in Schlegel, "General Microbiology" (Thieme Verlag Stuttgart, fifth edition, the column of "173 Highly thermophilic bacteria and extremely thermophilic bacteria") (optimum growth temperature of 40°C or more).

[0014] The mixture or the dissolving solution according to the present invention includes thermophilic mixed bacteria BP-1051 as a thermophilic microorganism.

[0015] The mixture or the dissolving solution according to the present invention includes, as a thermophilic microorganism, BP-863 having an ability to degrade persistent sugar, which is a related species of Bacillus thermoamylovorans.

[0016] The mixture or the dissolving solution according to the present disclosure includes thermophilic seed bacteria PTA-1733.

[0017] The pharmaceutical agent includes either the above mixture or the dissolving solution as an active component.

#### EFFECTS OF THE INVENTION

5 [0018] The mixture or dissolving solution of the present invention includes, as the thermophilic microorganism, at least one of thermophilic microorganisms of the genus *Bacillus*, *Oceanobacillus*, *Paenibacillus*, *Anoxybacillus*, *Lysinibacillus*, *Methanopyrus*, *Geogemma*, *Pyrolobus*, *Pyrodictium*, *Hyperthermus*, *Pyrococcus*, *Pyrobaculum*, *Thermococcus*, *Aeropyrum*, *Aquifex*, *Thermotoga*, *Thermodesulfobacterium*, *Thermus*, *Geobacillus*, *Thermomyces*, and *Clostridium*. Thus, when administered to animals including humans, the mixture or the dissolving solution is expected to regulate expression of at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines, a metabolism-related gene cluster in the liver while coexisting with the host intestinal flora. Also, the mixture or the dissolving solution is expected to regulate expression of a mucous membrane immune system gene cluster or the like by administration to animals including humans, under aseptic environment.

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15 [0019] The mixture or the dissolving solution of the present invention includes thermophilic mixed bacteria BP-1051 as the thermophilic microorganism. Thus, the natural immune system that promptly responds to bacterial and viral infections can be activated by administration of the mixture or the dissolving solution to animals (including humans) under any of aseptic conditions without the presence of micro flora in the intestines and ordinary environment conditions with the presence of micro flora. Therefore, the mixture or the dissolving solution is expected to regulate expression of a mucous membrane immune system gene cluster, and also regulate expression of gene clusters related to intestine and liver metabolisms.

20 [0020] The mixture or the dissolving solution of the present disclosure can exert an effect similar to one described above by inclusion of any of BP-863 having an ability to degrade persistent sugar and thermophilic seed bacteria PTA-1773.

25 [0021] In addition, it is assumed that the above BP-863 activates development of the intestinal Peyer's patches and in vitro IL-18 production. Generally the Peyer's patch takes production regulation of immunoglobulin, and IL-18 is known to induce production of gamma interferon. Therefore, the presence of BP-863 contributes to activation of the natural immune system that promptly responds to bacterial and viral infections under aseptic conditions or ordinary environment conditions.

30 [0022] The pharmaceutical agent of the present invention can exert effects similar to those described above by inclusion of either the mixture or the dissolving solution as an active component. In addition, the pharmaceutical agent of the present invention can be administered orally or trans-bronchially to animals including humans.

#### BRIEF DESCRIPTION OF THE DRAWINGS

35 [0023]

FIG. 1 is a conceptual diagram illustrating a work mechanism of a mixture or a dissolving solution of the present invention in the intestinal tract.

40 FIG. 2 is a conceptual diagram illustrating an influence of the mixture or the dissolving solution of the present invention on the intestinal functions of a germ-free mouse.

FIG. 3 is a photograph of a culture of the related species of *Bacillus thermoamylovorans*, strain N-11 (NITE BP-863).

FIG. 4 is an electron micrographic image of the culture of the related species of *Bacillus thermoamylovorans*, strain N-11 (NITE BP-863).

FIG. 5 is a CT-scan image of the trunk of a mouse fed with high fat food (drinking water: tap water).

45 FIG. 6 is a CT-scan image of the trunk of a mouse fed with high fat food (drinking water: tap water with dissolving solution 1-B in a concentration of 1.0%).

FIG. 7 is a diagram depicting the content of IL-18 in liver of a germ-free mouse receiving a thermophilic microorganism.

50 FIG. 8 is a diagram depicting the concentration of secretory IgA in feces of a germ-free mouse receiving a thermophilic microorganism.

#### EMBODIMENTS OF THE INVENTION

55 [0024] Hereinafter, embodiments of the present invention will be described with reference to drawings. First, a mixture or a dissolving solution of the present invention will be described. The mixture or the dissolving solution of the present invention is obtained by high-temperature fermentation of an organic material containing a thermophilic microorganism. It is administered to animals including humans to regulate expression of at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines, and a metabolism-related gene cluster in the liver.

**[0025]** The thermophilic microorganism is a microorganism having an optimum growth temperature of 40°C or more as described in the above description. Specifically, such a microorganism includes thermophilic microorganisms of the genus *Bacillus*, *Oceanobacillus*, *Paenibacillus*, *Anoxybacillus*, and *Lysinibacillus*. The microorganism further includes thermophilic microorganisms of the genus *Methanopyrus*, *Geogemma*, *Pyrolobus*, *Pyrodictium*, *Hyperthermus*, *Pyrococcus*, *Pyrobaculum*, *Thermococcus*, *Aeropyrum*, *Aquifex*, *Thermotoga*, *Thermodesulfobacterium*, *Thermus*, *Geobacillus*, *Thermomyces*, and *Clostridium*. More specifically, the microorganism includes thermophilic seed bacteria PTA-1773, thermophilic mixed bacteria BP-1051, the related species (N-11) BP-863 of *Bacillus thermoamylovorans*, *Bacillus thermocloacae* related species belonging to the genus *Firmicutes* as a phylum of bacteria (registered under No. AB298562 in GeneBank database), and *Bacillus thermoamylovorans* related species (registered under No. AB298559 in the same database).

**[0026]** In addition, the above thermophilic seed bacteria PTA-1773 are internationally deposited to ATCC (American Type Culture Collection, 10801 University Boulevard Manassas, Virginia 20110-2209 U.S.A.) (Accession No: PTA-1773). The thermophilic seed bacteria PTA-1773 include a group of microorganisms with high chitin degradation ability and thermophilic lactic acid bacteria. Specifically, the bacteria PTA-1773 include microorganisms of *Actinomycetales* bacterium, *Alicyclobacillus*, *Amphibacillus*, *Anoxybacillus*, *Atopostipes*, *Brachybacterium*, *Brevibacterium*, *Cerasibacillus*, *Clostridium*, *Corynebacterium*, *Curtobacterium*, *Georgenia*, *Gracilibacillus*, *Jeotgalicoccus*, *Salinibacillus*, *Tissierella*, *Ureibacillus*, *Vagococcus*, *Virgibacillus*, and *Weissella*. Furthermore, thermophilic mixed bacteria BP-1051 are internationally deposited on January 18, 2011 to an independent administrative institution, the Patent Microorganisms Depository of the National Institute of Technology and Evaluation (NPMD) (2-5-8 Kazusakamatar, Kisarazu-shi, Chiba Prefecture, 292-0818, Japan) (accession No: NITE BP-1051). In addition, *Bacillus thermoamylovorans* related species (N-11), BP-863, is internationally deposited on January 15, 2010 to the independent administrative institution, the Patent Microorganisms Depository of the National Institute of Technology and Evaluation (NPMD) 2-5-8 Kazusakamatar, Kisarazu-shi, Chiba Prefecture, 292-0818, Japan) (accession No: NITE BP-863).

**[0027]** The organic material includes the thermophilic microorganisms as described above and can be subjected to high-temperature fermentation. Specifically, the organic material includes marine products, farm products, and their residues such as organic wastes and wood chips, which contain the above thermophilic microorganism. Here, the above farm products include raw materials such as corn husk, corn core (corn cob), soybean meal, strawberry, and mushroom, which contain persistent sugar alcohols such as arabinose, xylitol, and xylan.

**[0028]** Here, to prepare the mixture or the dissolving of the present invention, the organic material is fermented at a temperature of 50°C or more and 90°C or less. Here, if the fermentation temperature of the organic material is lower than 50°C, it is not adequate because the growth of the above thermophilic microorganism may hardly progress and the growth of a microorganism proliferative at normal temperatures may increase. In addition, if the fermentation temperature of the organic material is higher than 90°C, it is not adequate because the thermophilic microorganism may die out.

**[0029]** The mixture or the dissolving solution of the present invention can be prepared from a fermentation product obtained by the above fermentation. For example, the mixture of the present invention can be prepared directly from the fermentation product or from a mixture thereof with feed or the like. Alternatively, the dissolving solution of the present invention can be prepared by dilution of the fermentation product with water. Furthermore, the mixture or the dissolving solution of the present invention can be made by any method with the proviso that the above thermophilic microorganism does not die out.

**[0030]** The mixture or the dissolving solution of the present invention prepared as described above can be administered to animals (including humans) orally or trans-bronchially to regulate expression of at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines, and a metabolism-related gene cluster in the liver.

**[0031]** It is assumed that the above functions of the mixture or the dissolving solution of the present invention may result from the mechanism as described, for example, in FIG. 1. In other words, the thermophilic microorganism included in the mixture or the dissolving solution of the present invention acts in the mucous membrane immune system and metabolic system when it coexists with the intestinal flora of the host. First, as an action on the mucous membrane immune system, it activates natural killer cells (NK cells) in the intestinal tract and promotes expression of chemokine and the related gene cluster to enhance the formation of lymphoid tissues and migration of B cells, thereby causing an increase in antibody production. Furthermore, as an action on the metabolic system, it regulates the level of expression of gene cluster for fat metabolism to reduce neutral fats or regulates the level of expression of gene cluster for gas metabolism to prevent methane gas production.

**[0032]** Also, it is assumed that the thermophilic microorganism included in the mixture of the present invention or the metabolic system may act on the mucous membrane immune system directly. This is based on the fact that, as illustrated in FIG. 2, in germ free mice (axenic mice) receiving the mixture or the dissolving solution of the present invention, development of the Peyer's patches, normalization of the fecal metabolism, and mild activation of the mucous membrane immune system are performed. Therefore, even if intestinal environment is aseptic, the mixture or the dissolving solution of the present invention may be expressed as probiotics that regulate intestinal metabolism in a manner close to the

properties thereof. For example, it is expected to be applied to postoperative therapy requiring nothing by mouth in medical field.

**[0033]** Furthermore, the mixture or the dissolving solution of the present invention can be utilized while being prevented from contamination with various germs because the containing microorganism is thermophilic and sterilization almost at 60°C is possible before use. Furthermore, the more simple culture technique allows probiotics and pre-probiotics with new functions to be produced in large amount.

**[0034]** Next, the pharmaceutical agent of the present invention will be described. Since the pharmaceutical agent of the present invention includes the mixture or the dissolving solution of the present invention as an active component, it exerts an effect by regulating at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines and a metabolism-related gene cluster in the liver of an animal receiving the mixture or the dissolving solution of the present. In addition, the present pharmaceutical agent can be administered orally or trans-bronchially to animals (including humans).

**[0035]** If desired, the pharmaceutical agent of the invention can be realized by being mixed with a pharmaceutically acceptable carrier or additive suitably selected from excipients, extending agents, binders, wetting agents, disintegrants, surfactants, lubricants, dispersants, buffers, preservatives, solubilizers, flavoring agents, soothing agents and stabilizers, and formulated in solution, capsule, tablet, granule, or the like.

**[0036]** For example, the excipients include sugars such as milk sugar and saccharide, and starch. The disintegrants include cellulose derivatives and starch. The binders include macromolecules such as gelatin and Arabian gum. The lubricants include waxes and stearic acid.

**[0037]** Hereinafter, the present invention will be described in more detail with reference to examples. However, these examples do not limit the present invention at all. In addition, the contents of the description in each document cited in the examples shall be incorporated herein by reference.

#### Example 1

##### (1-1) Preparation of dissolving solutions 1-A and 1-B

**[0038]** A dissolving solution 1-A was prepared using a high-temperature fermentation product reported by Niisawa et al. (Niisawa C, Oka S, Kodama H, Hirai M, Kumagai Y, Mori K, Matsumoto J, Miyamoto H, Miyamoto H (2008) Microbial analysis of composted product of marine animal resources and isolation of antagonistic bacteria to plant pathogen from the compost. J Gen Appl Microbiol 54: 149-158) such that the product was diluted 200 times by weight and then subjected to aeration by diffused air at 60 to 70°C for 6 hours or more. Furthermore, a dissolving solution 1-B was prepared by co-cultivating thermophilic microorganisms included in the dissolving solution 1-A with PTA-1773.

##### (1-2) Analysis of microorganisms in dissolving solution 1-A

**[0039]** The dissolving solution 1-A includes various kinds of thermophilic microorganisms, and thermophilic mixed bacteria BP-1051 as the dominant bacterial species. Their base sequences (16SrDNA sequences) were analyzed. The analysis was performed such that the microorganisms included in the dissolving solution 1-A were inoculated into standard culture media, nutrient agar culture media, heart infusion culture medium, or the like, and DNAs were then extracted from growing bacterial strains. Furthermore, this analysis employed a known method (Lane, D.J. (1991) 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematics. Stackebrandt, E. and Goodfellow, M. eds., John Wiley & Sons Ltd., Chichester, England, pp. 115-175) to conduct PCR reaction in which 27F and 1525R were used as universal primers. A reaction solution was prepared by mixing 25 µL of 2 x GoTaq Hot Start Colorless Master Mix (Promega Co., WI, USA) and 2 pmole of the primer, and dissolving a sample with 50 µL of sterilized water. A PCR reaction was performed by 94°C for 15 minutes and then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds, followed by a reaction at 72°C for 7 minutes. Furthermore, A PCR fragment of 1.5 kbp in length was purified using QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and the base sequence thereof was then determined by a full-automatic DNA analyzer system (Applied Biosystems Inc., CA, USA) using BigDye Terminator Cycle Sequencing Kit. Furthermore, matching retrieval was performed using database of the GenBank (<http://www.ncbi.nlm.nih.gov/>) or the like. The base sequences of the respective microorganisms analyzed by this analysis are represented by SEQ ID NOs. 1 to 8.

**[0040]** Here, *Bacillus badius* related species (IP-2) having a base sequence represented by SEQ ID NO. 1 is 97.3% homologous with the type strain (B NBRC15713<sup>T</sup>) of *Bacillus badius*. The characteristics of *Bacillus badius* related species (IP-2) include Gram positive, 2 µm in width, 2 µm in length, sporulation, no ability for glycolysis of sugars such as glucose and lactose, catalase positive, oxidase positive, and so on. *Bacillus badius* is known to have genes for nitrogen metabolism.

**[0041]** The related species (IP-3) of *Anoxybacillus kamchatkensis* representing a base sequence in SEQ ID NO. 2 is

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99.5% homogenous to the type strain (IAM11061<sup>T</sup>) of *Anoxybacillus kamchatkensis*. Also, the characteristics of the related species (IP-3) of *Anoxybacillus kamchatkensis* include Gram positive, 0.4 μm in width, 3 to 4 μm in length, sporulation, presence of ability for glycolysis of starch and glucose, catalase positive, oxidase positive, reduction of nitrate into nitrite, and so on. It is assumed that *Anoxybacillus kamchatkensis* may have lipase activity, and a high ability to degrade fat.

**[0042]** The related species (IP-9) of *Virgibacillus pantothenicus* representing a base sequence in SEQ ID NO. 3 is 99.5% homologous to the type strain (DSM14988<sup>T</sup>) of *Virgibacillus pantothenicus*. Also, the characteristics of the related species (IP-9) of *Virgibacillus pantothenicus* include Gram negative, 0.5 μm in width, 5 to 6 μm in length, large sporulation, presence of ability for glycolysis of starch, glucose, and tagatose, catalase positive, oxidase positive, reduction of nitrate to nitrite, and so on. *Virgibacillus pantothenicus* has ectoine which is a salt-resistant component and known as a moisture-retention component.

**[0043]** The related species (IP-14) of *Bacillus fortis* representing a sequence in SEQ ID NO. 4 is 99.7% homologous to the type strain (LMG22079<sup>T</sup>) of *Bacillus fortis*. Also, the characteristics of the related species (IP-14) of *Bacillus fortis* include Gram positive, 0.5 μm in width, 1 μm in length, sporulation, no ability for glycolysis of starch and glucose, presence of ability to degrade trehalose, catalase positive, oxidase positive, no reduction of nitrate into nitrite, and so on.

**[0044]** The related species (IP-23) of *Lysinibacillus xylanilyticus* representing a sequence in SEQ ID NO. 5 is 95.0% homologous to the type strain (YC6957<sup>T</sup>) of *Lysinibacillus xylanilyticus*. Also, the characteristics of the related species (IP-23) of *Lysinibacillus xylanilyticus* include Gram positive, 0.5 μm in width, 3 to 5 μm in length, sporulation, no ability for glycolysis, presence of ability for peptone degradation, catalase positive, oxidase positive, no reduction of nitrate into nitrite, and so on. *Lysinibacillus xylanilyticus* is known to have a degradation characteristic of persistent xylan. However, the related species (IP-23) of *Lysinibacillus xylanilyticus* may be a new bacterial species because it shows no ability for glycolysis of sugars at all but shows high usage of peptone only.

**[0045]** The related species (IP-60) of *Paenibacillus timonensis* representing a base sequence in SEQ ID NO. 6 is 96.9% homologous to the type strain (CIP108005<sup>T</sup>) of *Paenibacillus timonensis*. Also, the characteristics of the related species (IP-60) of *Paenibacillus timonensis* include Gram positive, 0.5 μm in width, 3 to 5 μm in length, sporulation, presence of ability for glycolysis of starch, xylitol and xylan, catalase negative, oxidase negative, reduction of nitrate into nitrite, and so on. *Paenibacillus timonensis* is unknown to have ability for glycolysis of xylan. However, the related species (IP-60) of *Paenibacillus timonensis* is assumed as a new bacterial species because of its high xylan degradation ability as described above. Besides, its ability to degrade persistent sugar alcohol is as high as that of BP-863.

**[0046]** The related species (IP-75) of *Paenibacillus curdlanolyticus* representing a base sequence in SEQ ID NO. 7 and is 94.6% homologous to the type strain (IFO15724<sup>T</sup>) of *Paenibacillus curdlanolyticus*. Also, the characteristics of the related species (IP-75) of *Paenibacillus curdlanolyticus* include Gram positive, 0.5 μm in width, 3 to 5 μm in length, sporulation, presence of ability for glycolysis of lactose, catalase negative, oxidase negative, no reduction of nitrate into nitrite, and so on. *Paenibacillus curdlanolyticus* is known to have a degradation characteristic of persistent xylan. Also, the related species (IP-75) of *Paenibacillus curdlanolyticus* is assumed as a new bacterial species because the ability to degrade persistent sugar alcohol is as high as that of BP-863.

**[0047]** Furthermore, the related species (IP-95) of *Bacillus ruris* representing a sequence in SEQ ID NO. 8 is 99.9% homogenous to the type strain (LMG22866<sup>T</sup>) of *Bacillus ruris*. Also, the characteristics of the related species (IP-95) of *Bacillus ruris* include Gram positive, 1 μm in width, 2 μm in length, sporulation, presence of ability for glycolysis of starch, glucose and trehalose, catalase positive, oxidase positive, reduction of nitrate into nitrite, and so on.

### Example 2

#### (2-1) Preparation of dissolving solution 2

**[0048]** A dissolving solution 2 was prepared by fermenting an organic material containing marine residues with microorganisms included in the dissolving solution 1-B in an air permeable three-staged fermenter installed in Miroku Co., Ltd at 70°C or more and 90°C or less, diluting the final fermentation product 100 times with water, and dissolving it therein at 60°C or less for 10 hours or more under aeration conditions.

#### (2-2) Analysis of microorganism dominant in cecal feces of germ-free mouse with administration of dissolving solution 2

**[0049]** A dissolving solution 2 at a concentration of 0.5% was administered to aseptically breeding Balb/c mice (male, 10 weeks of age) for three weeks, and the base sequences (16SrDNA sequences) of microorganisms isolated from cecal feces of the mice were then analyzed. Here, the above Balb/c mice were bred in isolators (manufactured by ICM Co., Ltd.) in a breeding room controlled at a room temperature of 24 ± 1°C and a humidity of 55 ± 5%, and the feed used was one sterilized by radiation (product name CMF, manufactured by Oriental Yeast Co., Ltd.). Also, the analysis of the base sequences was carried out by the same method as one described in (1-2) of Example 1. The base sequences



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of the respective microorganisms in the dissolving solution 2 analyzed by this analysis are represented by SEQ ID NOs. 9 and 10.

**[0050]** The related species (N-11) of *Bacillus thermoamylovorans* representing a base sequence in SEQ ID NO. 9 is the above BP-863 and 99.9% homogenous to the type strain (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans*. The biochemical properties of the related species (N-11) of this *Bacillus thermoamylovorans* are listed in Table 1, and photographs of the culture of the related species (N-11) of *Bacillus thermoamylovorans* are shown in FIG. 3 and FIG. 4.

[Table 1]

Characteristics	Related species (N-11) of <i>Bacillus thermoamylovorans</i>	Type bacterial strain (LMG 18084 <sup>T</sup> ) of <i>Bacillus thermoamylovorans</i>
Colony and microscopy		
Color of colony	Cream	Cream
Shape of bacteria	Bacillus	Bacillus
Dimension of bacteria	0.5 × 2-5 μm	0.45-0.5 × 3-4 μm
Gram stain	+	+
Spore stain	+	+
Sporular position	End (subterminal)	End (subterminal)
Mobility	V	V (variable among the strain)
Other biochemical characteristics		
Indole production	-	-
IPA production	-	-
H <sub>2</sub> S production	-	-
Ureolysis	-	-
Nitrate reduction	+	+
Catalase	+	+
Oxidase	+	+
Acid-producing ability test		
Glucose	+	+
Lactose	+	+
Maltose	+	+
Galactose	+	+
Trehalose	+	+
Mannose	+	+
Sucrose	+	+
Fructose	+	+
Cellobiose	+	+
Ribose	+	+
Xylose	+	V (variable among the strain)
Rhamnose	+	-
D-arabinose	+	-

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(continued)

Characteristics	Related species (N-11) of <i>Bacillus thermoamylovorans</i>	Type bacterial strain (LMG 18084 <sup>T</sup> ) of <i>Bacillus thermoamylovorans</i>
Colony and microscopy		
Turanose	+	ND
Sodium gluconate	+	-
Inositol	+	-
Xylitol	+	-
Dulcitol	+	-
Erythritol	+	-
Sorbitol	+	-
Mannitol	+	-
Lactic acid	+ (weak)	ND
Xylan	+	ND

**[0051]** Here, in Table 1, the biochemical properties of the related species (N-11) of *Bacillus thermoamylovorans* are listed in comparison with the type strain (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans*. In addition, the biochemical properties of the type strain (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans* are based on the contents of the following documents:

Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B.K.C., Dwivedi, P.P., Pot, B., Prensier, G., Garcia, J.L. (1995) *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *Int. J. Syst. Bacteriol.* 45: 9-16; and

Coorevits, A., Logan, N., Dinsdale, A., Halket, G., Scheldeman, P., Heyndrickx, M., Schumann, P., VanLandschoot, A., De Vos, P. (2010) *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. *Int. J. Syst. Microbiol.* 56: 781-786.

**[0052]** As shown in Table 1, the related species (N-11) of *Bacillus thermoamylovorans* has high ability to degrade persistent sugar alcohols such as arabinose and xylitol, compared with the type strain (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans*. Therefore, the related species (N-11) of *Bacillus thermoamylovorans* is expected, when used in fermentation feed or the like, to have effective ability to degrade corn husk, wheat meal, soybean meal, mushroom, vegetable meal, and so on, which contain persistent sugar alcohols or the like and have conventionally little values as feed. In addition, it is also confirmed that the related species (N-11) of *Bacillus thermoamylovorans* has ability to degrade xylan which is one of persistent polysaccharides. Furthermore, as represented in an electron microgram in FIG. 4, the related species (N-11) of *Bacillus thermoamylovorans* in spore form coexists with bacillus under normal culture conditions.

**[0053]** The related species (N-16) of *Bacillus coagulans* representing a base sequence in the above SEQ ID NO. 10 is 99.9% homogenous to the type strain (ATCC7050<sup>T</sup>) of *Bacillus coagulans*. The characteristics of the related species (N-16) of *Bacillus coagulans* include Gram positive, 0.7 μm in width, 3 to 5 μm in length, sporulation, no ability to degrade starch, presence of ability for glycolysis of glucose, trehalose and tagatose, catalase positive, oxidase negative, no reduction of nitrate to nitrite, and so on.

(2-3) Preparation of dissolving solutions 3, 4 and 5

**[0054]** A dissolving solution 3 was prepared as a dissolving solution containing only the related species (N-11) (BP-863) of *Bacillus thermoamylovorans*. A dissolving solution 4 was prepared as a dissolving solution containing only the related species (N-16) (isolated bacteria containing in BP-1051) of *Bacillus coagulans*. A dissolving solution 5 was prepared as a dissolving solution containing only a type bacterial strain (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans*.

## Example 3

## (3-1) Verification experiment [1]

5 for dissolving solution 1-A

[0055] An experiment for verifying a regulatory effect of the dissolving solution 1-A on expression of gene cluster by administration of the dissolving solution 1-A to Wistar rats (male, 3 weeks of age) (obtained from Kyudo Co., Ltd.). In this experiment, the following three groups were prepared and compared with one another.

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Group (1-A): A normal feeding group (controls)

Group (1-B): A group in which the dissolving solution 1-A was added to drinking water.

Group (1-C): A group in which the dissolving solution 1-A (but sterilized with 0.02  $\mu$ m) was added to drinking water.

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[0056] Here, the Wistar rats used in the experiment were preliminarily bred in groups (1-A) to (1-C) for five days. Furthermore, the Wistar rats were divided into five animals per group and each was bred in its own gauge (manufactured by Natsume Seisakusyo Co., Ltd.). Furthermore, the feed used was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.). Each Wister rat was fed ad libitum within the intake limits of 25 g per day. In addition, drinking water was taken ad libitum using tap water for the group (1-A), tap water with addition of the dissolving solution 1-A for the group (1-B), and tap water with addition of 1.0% of the dissolving solution 1-A sterilized by 0.02  $\mu$ m filter for the group (1-C).

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[0057] The Wistar rats of groups (1-A) to (1-C) were bred for three months, respectively. Subsequently, their intestines, livers, spleens, bloods, and so on were collected and quickly frozen with liquid nitrogen, followed by being stored in a refrigerator at -80°C.

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[0058] A change in expression of gene cluster in each group was analyzed using the collected small intestines. Specifically, RNA extraction was performed on parts of the collected small intestines, except the Peyer's patches. The RNA extraction was performed by adding 1000  $\mu$ l of Isogen (manufactured by Nippon Gene Co., Ltd.) to a tissue of 100 mg or less, crushing the resultant with a mortar while being frozen by liquid nitrogen, and using RNeasy mini kit (manufactured by Quiagen). Subsequently, the expression level of each gene cluster was digitized by calculation and calibration of each spot fluorescence level using a array scanner (manufactured by Agilent Co., Ltd.) after hybridization of the extracted RNA on a microarray (manufactured by Agilent Co., Ltd.) on which all genes were mounted and washing thereof. The results are listed in Tables 2 and 3.

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[Table 2]

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Gene	Gene Symbol	Regulation
immunoglobulin related gene	XM_213585, LOC500183, RGD1359539, Z93370, XM_345745, X60291, A2m	up
vitamin D-binding protein precursor	TC641315	up
chemokine (C-C motif) ligand,	Ccl21b, Scya11, Scya28, Sdf1, LOC498335	up
chemokine (C-C motif) receptor	Cxcr4, Ccr5, Ccr6, Ccr7	up
nuclear receptor coactivator 7	XM_571285	up
granzyme B /natural killer cell protease precursor	M_224224, RGD1562700_predicted Gzmb, RGD1562700_predicted	up up

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[Table 3]

55

Gene	Gene Symbol	Regulation
HBV pX associated protein 8 large isoform	Hbxap_predicted	drown
carbonic anhydrase	ENSRNOT00000051309	down
apolipoprotein A-V	Apoa5	down
endothelin	Edn3	down

(continued)

Gene	Gene Symbol	Regulation
heat shock protein 4	Hspa4	down

**[0059]** Table 2 shows upper six gene clusters among those in group (1-B) each having an expression level of 2.0 or more times higher than that of group (1-A). Among these gene clusters, immunoglobulin related gene, chemokine (C-C motif) ligand, chemokine (C-C motif) receptor, and granzyme B/natural killer cell protease precursor are mucous membrane immune system gene clusters, and vitamin D-binding protein precursor is a metabolism-related gene cluster. Furthermore, but not listed in the tables, "tumor necrosis factor receptor" provided as a mucous membrane immune system gene cluster in group (1-B) had an expression level of 1.8 times higher than that in group (1-A).

**[0060]** Furthermore, as the above immunoglobulin related gene, anti-idiotypic immunoglobulin M light chain, immunoglobulin gamma2a constant region, NGF-binding Ig light chain, Ig gamma-1, chain C region, gamma-2a immunoglobulin heavy chain, and immunoglobulin kappa chain variable region were confirmed. Furthermore, for the above chemokine (C-C motif) ligand, small inducible cytokine B13 precursor (CXCL13) (B lymphocyte chemoattractant) was significantly expressed. The expression levels were quantified in Real time PCR, showing that group (1-B) had an increasing tendency of 3.6 times (n = 3) higher than that of group (1-A).

**[0061]** Table 3 shows upper five gene clusters among those in group (1-B) having an expression level of one half or less times compared with that of group (1-A). Among these gene clusters, HBV pX associated protein is responsible for control of virus infection, carbonic anhydrase for gas metabolism, apolipoprotein A-V for fat metabolism, endothelin for blood pressure regulation, heat shock protein 4 for gene expression, protein function regulation, and intracellular signaling, and so on.

**[0062]** Here, group (1-C) is different from group (1-B) in that changes in expression levels of gene clusters were small with respect to those in group (1-A).

(3-2) Verification experiment [1]

for dissolving solution 1-B

**[0063]** The dissolving solution 1-B was administered to germ-free mice to carry out an experiment for verifying a regulatory effect of the dissolving solution 1-B on the expression level of gene cluster. In this experiment, the following two groups were prepared and compared with each other.

Group (2-A): A normal feeding group (controls)

Group (2-B): A group in which the dissolving solution 1-B was added to drinking water.

**[0064]** Here, five germ-free mice (obtained from Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, and bred in this laboratory at our request) were used for each of groups (2-A) and (1-B), and bred in the same isolator (manufactured by ICM Co., Ltd.) provided for each group. Furthermore, the feed used was one sterilized by radiation (product name CMF, manufactured by Oriental Yeast Co., Ltd.). The mice were allowed to take the feed ad libitum. Furthermore, drinking water for group (2-A) was water sterilized by UV and autoclave. Drinking water for group (2-B) was water sterilized by UV and autoclave, to which 0.5% of the dissolving solution 1-B was added. The mice were allowed to drink the drinking water ad libitum.

**[0065]** The germ-free mice of groups (2-A) and (2-B) were bred for three weeks, respectively. Subsequently, their intestines, livers, spleens, bloods, and so on were collected and quickly frozen with liquid nitrogen, followed by being stored in a refrigerator at -80°C. A change in expression level of gene cluster in each group was analyzed in the same manner as in the example (3-1).

**[0066]** From the results of the analysis, the following facts were found: in the germ-free mice, administration of the dissolving 1-B led to a small change in number of gene clusters similar to the change in the case of Wistar rats represented in Tables 2 and 3, however, immunoglobulin related gene, chemokine (C-C motif) ligand, and tumor necrosis factor receptor, which are similar mucous membrane immune system gene clusters represented in Table 4, are expressed high, and the expression of metabolism-related gene cluster is also regulated. Here, it is assumed that such results may be due to the effect of short-term administration because a time period of administration to the germ-free mice is as short as three weeks.

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[Table 4]

Gene	Mouse Gene Symbol	Regulation
immunoglobulin related gene	Igsf9, Igsf3, Semas3b	up
chemokine (C-C motif) ligand	Ccl25	up
tumor necrosis factor receptor	Tnfrsf1b	up

(3-3) Verification experiment [2]

for dissolving solution 1-A

**[0067]** The liver of Wistar rat collected in the example (3-1) was subjected to an analysis of a change in expression level of gene cluster in each of groups (1-A) and (1-B). This analysis was performed in the same manner as in the example (3-1). The analytical results are listed in Tables 5 and 6.

[Table 5]

Gene	Gene Symbol	Regulation
olfactory receptor 1148 (predicted)	Olr1148	up
immunoglobulin related gene	RGD1562855 predicted, IgK	up
UDP glycosyl transferase 2 family, polypeptide B	Olr1330	up
TRAF2 binding protein	LOC310877; Ab2-389	up
alcohol dehydrogenase 6 (class V)	Adh6	up

[Table 6]

Gene	Gene Symbol	Regulation
mesothelin	Msln	down
prolactin receptor	RATPRLR; MGC105486	down
Nocturnin (CCR4 protein homolog)	LOC310395	down
hydroxysteroid (17-beta) dehydrogenase 2	Iisd17b2	down
apelin, AGTRL1 ligand	Apel	down
ring finger protein 187 (predicated)	RGD1308636	down
SNF1-like kinase	Sik	down
stearoyl-Coenzyme A desaturase	Scd1, Scd2	down

**[0068]** Table 5 shows upper five main gene clusters among those in group (1-B) each having an extremely high expression level compared with that of group (1-A). Among these gene clusters, immunoglobulin related gene is of the mucous membrane immune system, and alcohol dehydrogenase 6 (class V) is of the metabolism-related system. Furthermore, olfactory receptor 1148 (predicted), UDP glycosyltransferase 2 family polypeptide B, TRAF2 binding protein are responsible for other physiological reactions. Furthermore, but not listed in the tables, glucokinase and so on, which were genes of the metabolism-related gene cluster, were also expressed highly.

**[0069]** Here, examples of the immunoglobulin related gene include those of Ig kappa chain, Ig germline kappa-chain C-region gene, 3' end, anti-NG F30 antibody light chain mRNA, variable and constant regions, and immunoglobulin alpha heavy chain.

**[0070]** Table 6 shows upper eight gene clusters among those in group (1-B) being expressed in significantly low level in the liver, compared with that of group (1-A). In these gene clusters, hydroxysteroid (17-beta) dehydrogenase 2 influences an increase or decrease in testosterone, and stearoyl-coenzyme A desaturase influences the entire fat metabolism, such as a decrease in triglyceride. Furthermore, apelin increases in the case of chronic liver disease or obesity.

(3-4) Verification experiment [2]

for dissolving solution 1-B

5 **[0071]** The liver of the germ-free mouse collected in the example (3-2) was subjected to an analysis of a change in expression level of gene cluster in each of groups (2-A) and (2-B). This analysis was performed in the same manner as in the example (3-1). The analysis results are listed in Tables 2 and 3.

[Table 7]

Gene	Mouse Gene Symbol	Regulation
major facilitator superfamily domain containing 2	Mfsd2	up
procollagen, type IV, alpha 2	Col4a2	up
purinergic receptor P2Y, G-protein coupled 2	P2ry2	up
ERBB receptor feedback inhibitor 1	Errfil 1	up
Klypican 1	Gpcl	up

[Table 8]

Gene	Mouse Gene Symbol	Regulation
gene mode 837, (NCB1), transcript variant 1 (Gm837)	Thsd7a	down
phospholipase C, beta 1	Plcb1	down
mesothelin	Msln	down
solute carrier family 17	Slc17a8	down
disrupted in renal carcinoma 2	Dirc2	down

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30  
35 **[0072]** Table 7 shows upper five main gene clusters among those in group (2-B) each having an extremely high expression level compared with that of group (2-A). Table 8 shows upper five gene clusters among those in group (2-B) being expressed in significantly low level in the liver compared with that of group (2-A). Here, it is assumed that such results may be due to the effect of short-term administration because a time period of administration to the germ-free mice is as short as three weeks. As is evident from Tables 7 and 8, when comparing with the liver of Wistar rat in the example (3-3), the liver of the germ-free mouse coincides therewith in terms of a significantly decrease in expression level of mesothelin, but does not coincide therewith in terms of other gene clusters.

40 (3-5) Verification experiment [3]

for dissolving solution 1-B

45 **[0073]** An experiment for verifying a regulatory effect of the dissolving solution 1-B on lipid energy metabolism was performed by administration of the dissolving solution 1-B to Wistar rats (male, 3 weeks of age) (obtained from Kyudo Co., Ltd.). In this experiment, the following four groups were prepared and compared with one another.

Group (3-A): A normal feeding group (controls)

Group (3-B): A group in which regular diet was fed and the dissolving solution 1-B was added to drinking water

Group (3-C): A group bred with high-fat diet

Group (3-D): A group in which high-fat diet was fed and the dissolving solution 1-B was added to drinking water.

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55 **[0074]** Here, the Wistar rats used in the experiment were preliminarily bred in groups (3-A) to (3-D) for five days. Furthermore, the Wistar rats were divided into five animals per group and each was bred in its own gauge (manufactured by Natsume Seisakusyo Co., Ltd.). Furthermore, the feed used for groups (3-A) and (3-B) was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.). The feed used for groups (3-C) and (3-D) was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.) with addition of lard so as to be

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20% in content (prepared in KBT Oriental Co., Ltd.). Each Wistar rat was fed ad libitum within the intake limits of 25 g per day. For drinking water, groups (3-A) and (3-C) were allowed to take tap water ad libitum, and groups (3-B) and (3-D) were allowed to take tap water with 1.0% addition of the dissolving solution 1-B ad libitum.

[0075] Wistar rats of the above groups (3-A) to (3-D) were bred for three months, respectively. Then, each of them was subjected to collection of blood and so on and weighed. Here, the statistical work handling of weight is carried out by an ANOVA (analysis of variance), and the results are listed in Table 9. Here, NS in the table represents that there is no significant difference.

[Table 9]

Normal diet	Mean value	Standard deviation	Mean value	Standard deviation	Significant difference (LF-T vs. LF-C)	Significant difference (C vs. HF-C)
Drinking days	Group (3-A) (LF-C)		Group (3-B) (LF-T)			
Before experiment	88.6	0.5	89.4	4.9	NS	NS
Three months after experiment	485.2	35.3	486.6	49.7	NS	NS
High-fat diet	Mean value	Standard deviation	Mean value	Standard deviation	Significant difference (HF-T vs. HF-C)	Significant difference (LF-T vs. HF-T)
Drinking days	Group (3-C) (HF-C)		Group (3-D) (LF-T)			
Before experiment	88.6	3.4	88.0	2.9	NS	NS
Three months after experiment	456.8	35.7	516.4	31.7	p<0.05	NS

[0076] As is evident from Table 9, there was no significant difference between group (3-A) and group (3-B) with respect to the weights of Wistar rats. However, there was a significant difference between group (3-C) and group (3-D), which were fed with regular diet, with respect to the weights of Wistar rats. In other words, the Wistar rats of group (3-D), which was a group in which high-fat diet was fed and the dissolving solution 1-B was added to drinking water, increased in weight in comparison with Wistar rats of group (3-C), which was a group bred with high-fat diet.

[0077] Here, between group (3-C) and group (3-D), a significant difference was not found in the results of blood analysis of the Wistar rats. Furthermore, significant fat deposition was not found in the Wistar rats in group (3-D) in anatomical findings. In addition, a decrease in number of lipid droplets was found in immunohistological staining of the liver.

(3-6) Verification experiment [4]

for dissolving solution 1-B

[0078] The dissolving solution 1-B was administered to Balb/c mice (male, 3 weeks of age) (obtained from Kyudo Co., Ltd.) to carry out an experiment for verifying a regulatory effect of the dissolving solution 1-B on fat energy metabolism. In this experiment, the following four groups were prepared and compared with one another.

Group (4-A): A normal feeding group (controls)

Group (4-B): A group in which regular diet was fed and the dissolving solution 1-B was added to drinking water

Group (4-C): A group bred with high-fat diet

Group (4-D): A group in which high-fat diet was fed and the dissolving solution 1-B was added to drinking water.

[0079] Here, the Balb/c mice were four or six in one group. In the case of four mice, these mice were bred in one gauge (manufactured by Natsume Seisakusho Co., Ltd.). In the case of six mice, these mice were divided and bred in two gauges (manufactured by Natsume Seisakusho Co., Ltd.). Furthermore, the feed used for groups (4-A) and (4-B) was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.). The feed used for groups (4-C) and (4-D) was one sterilized by radiation (product name MF, manufactured by Oriental Co., Ltd.) with addition of lard so as to be 20% in content (prepared in KBT Oriental Co., Ltd.). The mice were allowed to take the feed ad libitum. For drinking water, groups (4-A) and (4-C) were allowed to take tap water ad libitum, and groups (4-B) and (4-D) were allowed to take tap water with 1.0% addition of the dissolving solution 1-B ad libitum. Furthermore, the Balb/c mice of

groups (4-A) to (4-D) were bred for three months, respectively, and then subjected to measurement of percent of body fat by CT scanning and also weighed.

[0080] CT-scanning images of the trunks of the respective Balb/c mice in groups (4-C) and (4-D) were shown in FIGS. 5 and 6, respectively. In the bodies of the Balb/c mice, dark and gray portions on the portion near the peripheries of FIGs. 5 and 6 and the upper center portion shown in FIG. 6. Therefore, even though the Balb/c mice in group (4-D) tends to increase in weight compared with the Balb/c mice in group (4-C), each of them has a little body fat. Actually, the percent of body fat was about 20% lower. In addition, a similar tendency is represented in the femoral regions of the Balb/c mice. The tendency suggests that accumulation of body fat may decrease and the muscle may tend to be built.

(3-7) Verification experiment [5]

for dissolving solution 1-B

[0081] The dissolving solution 1-B was administered to Wistar rats (male, 3 weeks of age) (obtained from Kyudo Co., Ltd.) and then subjected to an experiment for measuring changes in Clostridium clusters IV and Clostridium subcluster XIVa, which were Clostridium as residential flora in the intestines. In this experiment, the following two groups were prepared and compared with each other.

Group (5-A): A normal feeding group (controls)

Group (5-B): A group in which regular diet was fed and the dissolving solution 1-B was added to drinking water

[0082] Here, the Wistar rats used in the experiment were preliminarily bred in both groups (5-A) and (5-B) for five days. Furthermore, the Wistar rats were divided into five animals per group and each was bred in its own gauge (manufactured by Natsume Seisakusyo Co., Ltd.). Furthermore, the feed used was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.). Each Wister rat was fed ad libitum within the intake limits of 25 g per day. For drinking water, group (5-A) was allowed to take tap water ad libitum, and group (5-B) was allowed to take tap water with 1.0% addition of the dissolving solution 1-B ad libitum.

[0083] The Wistar rats in the above groups (5-A) and (5-B) were bred for three months, respectively. Subsequently, changes in Clostridium clusters IV and Clostridium subcluster XIVa in their feces were confirmed using a T-RFLP (Terminal Restriction Fragment Length Polymorphism Analysis). The results are listed in Table 10.

[Table 10]

	Group (5-A)	Group (5-B)
Clostridium cluster IV	3.38	7.17
Clostridium subcluster XIVa	9.50	14.04

[0084] Therefore, it was found that both Clostridium cluster IV and Clostridium subcluster XIVa were increased in group (5-B) in comparison with group (5-A).

(3-8) Findings obtained from verification experiment for dissolving solutions 1-A and 1-B

[0085] The following findings were obtained by examining the results of the respective experiments described in the examples (3-1) to (3-7).

[0086] From the experimental results of the examples (3-1) and (3-3), there is a tendency of activation of the immune system and normalization of functions of the intestines by microorganisms included in the dissolving solution 1-A. For example, anti-IgM antibodies are known to contribute to activation of naive B cells instead of antigen (Mora et al. Generation of Gut-Homing Ig A- secreting B cells by intestinal dendritic cells. Science 2006; 314: 1157-1160). Likewise, furthermore, production of anti-NGF antibodies can be presumed. The anti-NGF antibodies are known to suppress an abnormal increase in Parietal cell permeability in the intestines (Barreau, et al. Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. J Physiol. 2007; 580(1): 347-356).

[0087] Next, from the experimental results of the example (3-1), the microorganisms included in the dissolving solution 1-A caused an increase in expression level of vitamin D-binding protein precursor. The vitamin D-binding protein precursor is known to contribute to the activation of macrophages, and suggested to have anti-cancer functions (Kisker et al., Vitamin D binding protein-Macrophage activating factor (DBP-maf) inhibits angiogenesis and tumor growth in mice. Neoplasia 2003; 5(1): 32-40). Furthermore, the expression level of HBV pX associated protein was decreased. However,



the HBV pX associated protein (HBV pX gene) is known to promote p53-induced type cell death.

**[0088]** Also, expression level of carbonic anhydrase was decreased. However, it is known that the carbonic anhydrase is included in the metabolism-regulating system and regulates the amount of carbonate ion. Carbonate ions in feces are a source of methane gas generated from the intestinal flora. Thus, it is expected that a decrease in expression level of carbonic anhydrase may lead to a decrease in methane gas production in the intestines. In a separate experiment, this does not contradict a fact that smells may be decreased at the time of fermentation of feces when high-temperature fermentation feed (feed including a fermentation product containing the same microorganisms as those in the dissolving solution 1-A) is administered. In general, animal feces tend to be anaerobically fermented when subjected to composting, so that methyl mercaptan may be generated from methane gas and hydrogen sulfide in the feces. Therefore, if the feces originally contain methane in small amount, the amount of the methyl mercaptan generated may become small. Furthermore, methane gas has 20 times larger warming coefficient than carbon dioxide. Thus, it is significant if methane gas can be regulated from the inside of the intestines.

**[0089]** Furthermore, the expression level of apolipoprotein A-V is decreased. It is known that a decrease in expression level of apolipoprotein A-V may contribute to a reduction in level of neutral fat as a result of a decrease in its expression level. Furthermore, as a result of analyzing the liver or the like collected in the example (3-1), there is a tendency of a decrease in triglyceride in the serum. A decrease in deposition of triglycerides in the liver is also confirmed by immunohistochemical staining.

**[0090]** Furthermore, in group (1-C) subjected to a sterile treatment, it is suggested that the presence of thermophilic microorganisms is important for exerting a regulatory effect of the expression level of gene cluster based on the fact that no change in expression level of gene cluster for group (1-A) is observed in contrast to group (1-B).

**[0091]** Next, from the experimental results of the example (3-3), an increase in expression level of alcohol dehydrogenase 6 (class V) occurs due to the microorganism clusters in the dissolving solution 1-A. The alcohol dehydrogenase 6 (class V) may be expected to have relevance with fat metabolism. Furthermore, there is a decrease in expression level of mesothelin. This mesothelin is a cancer-related gene cluster and a decrease in expression is considered preferable.

**[0092]** Furthermore, the expression level of Nocturnin is decreased. It is known that the decrease in expression level of Nocturnin tends to cause a decrease in fat level. When the liver collected in the example (3-1) is analyzed, the deposition of neutral fat is decreased and the results do not contradict the decrease in expression level of Nocturnin. In addition, there is a decrease in hydroxysteroid (17-beta) dehydrogenase 2. It is expected that the decrease in hydroxysteroid (17-beta) dehydrogenase 2 may influence on the steroid metabolic system.

**[0093]** Next, the experimental results of the examples (3-2) and (3-4) for germ-free mice suggest that the microorganisms included in the dissolving solution 1-B may directly exert an effect. From the experimental results from the examples (3-1) and (3-3) for Wistar rats, regulatory effects on expression of gene cluster for the mucous membrane immune system and gene cluster for the metabolism system may be obtained by corporation with the flora in the host. However, it is speculated that the direct effects may be different at all.

**[0094]** Next, the experimental results of the examples (3-5) and (3-6) show that the microorganisms included in the dissolving solution 1-B may regulate metabolism of lipid energy in the intestines. Actually, a separate experiment also reveals that the composition of organic acid, which is an enteral energy source, can be changed when high-temperature fermentation feed (containing the same microorganisms as those in the dissolving solution 1-B) is administered.

**[0095]** Next, the experimental results for the example (3-7) show that the microorganisms included in the dissolving solution 1-B can cause an increase in clostridium clusters IV and XIVa (Clostridium leptum and coccoides groups), which are the resident flora, harmless Clostridium. Therefore, it was found that the microorganism included in the dissolving solution 1-B can induce a change in enterobacterial flora of animals receiving the dissolving solution 1-B. It is also considered in combination with the experimental results of the examples (3-5) and (3-6), an increase in Clostridium stimulates Toll-like receptor 5 (TLR5) so on to regulate the fat metabolism. Thus, as represented by a CT-scan image shown in FIG. 6, any mechanism may be present to prevent visceral fat from being accumulated even under high-fat diet conditions.

**[0096]** The TLR5, a receptor of the intestinal immune system, acts as a receptor for Clostridium or the like having flagella and is considered to regulate the metabolic syndrome as well as regulate the natural immune system. Furthermore, it is reported that a mouse with defected TLR-5 gene becomes metabolic syndrome, and when the enterobacterial flora derived from the mouse is orally administered to a germ-free mouse, the germ-free mouse also becomes metabolic syndrome (Matam Vijay-Kumar, et al. Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like Receptor 5. Science 2010; 328: 228-231). This fact means that metabolic syndrome may be caused when the stimulation with Clostridium is not applied through TLR5. Thus, the presence of Clostridium as residential flora in the intestines will come under question.

**[0097]** It is also reported that the above Clostridium induces control of the intestinal immune system, particularly expression of CD4-positive regulatory T cells (Treg cells), where CD4 expresses transcription factor forkhead box P3 (Foxp3), to cause less incidence of inflamed enterocolitis or allergic reaction (Koji Atarashi, et al. Induction of Colonic

Regulatory T Cells by Indigenous Clostridium Species. Journal Science, electronic edition (published on December 24, 2010), Science 2011, 311 337-341). Furthermore, there is the report that these cells are decreased in the patient with human ulcerative colitis. In addition, the above regulatory T cells are considered to carry an important role about the regulation of the autoimmune disease with a kind of T cells controlling the overactivity of the immune system.

Example 4

(4-1) Verification experiment [6]

for dissolving solution 1-B

**[0098]** The dissolving solution 1-B was administered to aseptically breeding Balb/c mice and then subjected to an experiment for verifying the development of the Peyer's patches or the like in the intestinal tract. In this experiment, the following two groups were prepared and compared with each other: a normal feeding group; and a group in which the dissolving solution 1-B was added to drinking water.

**[0099]** In addition, five aseptically breeding Balb/c mice (obtained from Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, and bred in this laboratory at our request) were used for each of groups, and bred in the same isolator (manufactured by ICM Co., Ltd.) provided for each group. Furthermore, the feed used was one sterilized by radiation (product name CMF, manufactured by Oriental Yeast Co., Ltd.). The mice were allowed to take the feed ad libitum. Furthermore, drinking water for a control group was water sterilized by UV and autoclave. Drinking water for group (1-B) was water sterilized by UV and autoclave, to which 0.5% of the dissolving solution 1-B was added. The mice were allowed to drink the drinking water ad libitum.

**[0100]** The aseptically breeding Balb/c mice in both groups were bred for three weeks, respectively. Then, after the breeding, the aseptically breeding Balb/c mice in both groups were compared with the control group and showed a tendency to normalize feces of the group in which the dissolving solution 1-B was added to drinking water. Also, comparing with the control group, it is confirmed that the Peyer's patches of the intestinal tract of the group in which the dissolving solution 1-B was added to drinking water can be developed. Furthermore, it is confirmed that the group in which the dissolving solution 1-B was added to drinking water may tend to cause about 1.5 times higher level of the secretary IgA in the colon feces. In addition, the intestinal intensity increased more than the control group and the feces were nearly normal.

(4-2) Verification experiment [7]

for dissolving solution 1-B

**[0101]** The dissolving solution 1-B was administered to germ-free mice to carry out an experiment for verifying a change in IL-18 content in the liver. In this experiment, the following two groups were prepared and compared with each other: a normal feeding group (controls); and a group in which the dissolving solution 1-B was added to drinking water.

**[0102]** In addition, five germ-free mice (obtained from Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, and bred in this laboratory at our request) were used for each of groups, and bred in the same isolator (manufactured by ICM Co., Ltd.) provided for each group. Furthermore, the feed used was one sterilized by radiation (product name CMF, manufactured by Oriental Yeast Co., Ltd.). The mice were allowed to take the feed ad libitum. Furthermore, drinking water for the control group was water sterilized by UV and autoclave. Drinking water for the group in which the dissolving solution 1-B was added to drinking water was water sterilized by UV and autoclave, to which 0.5% of the dissolving solution 1-B was added. The mice were allowed to drink the drinking water ad libitum.

**[0103]** The Germ free mice of both groups were bred for three weeks, respectively. Then, after the feeding, the livers of the germ-free mice of both groups were analyzed. As a result, comparing with the control group, the content of IL-18 in the liver of the group in which the dissolving solution 1-B was added to drinking water was increased as shown in FIG. 7.

(4-3) Verification experiment for dissolving solutions 2, 3, 4, and 5

**[0104]** Dissolving solutions 2, 3, 4, and 5 were added to Balb/ c mouse (male, 3 weeks of age) (obtained from Kyudo Co., Ltd.) and then subjected to an experiment for verifying a change in level of secretary IgA in each of the intestines and spleen, respectively. In this experiment, the following five groups were prepared and compared with one another.

Group (6-A): A normal feeding group (controls)

Group (6-B): A group in which the dissolving solution 2 was added to drinking water.

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Group (6-C): A group in which the dissolving solution 3 was added to drinking water.

Group (6-D) A group in which the dissolving solution 4 was added to drinking water.

Group (6-E): A group in which the dissolving solution 5 was added to drinking water.

5 **[0105]** Furthermore, the Balb/c mice were four or six in each group. In the case of four mice, these mice were bred in one gauge (manufactured by Natsume Seisakusho Co., Ltd.). In the case of six mice, these mice were divided and bred in two gauges (manufactured by Natsume Seisakusho Co., Ltd.). Furthermore, the feed used was one sterilized by radiation (product name MF, manufactured by Oriental Yeast Co., Ltd.). The mice were allowed to take the feed ad libitum. Furthermore, drinking water was tap water for group (6-A), tap water with 1.0% addition of the dissolving solution 2 for group (6-B), tap water with 1.0% addition of the dissolving solution 3 for group (6-C), tap water with 1.0% addition of the dissolving solution 4 for group (6-D), and tap water with 1.0% addition of the dissolving solution 5 for group (6-E). The mice were allowed to take the feed ad libitum.

10 **[0106]** The Balb/c mice of the above groups (6-A) to (6-E) were bred for three months, respectively, and then subjected to a measurement for the level of secretory IgA in the feces to estimate the level of secretory IgA in each of the intestines and the spleen. Small intestinal data is shown in FIG. 8. As a result of the analysis, the groups (6-B), (6-C), and (6-D) respectively receiving the dissolving solution 2, 3, and 4 showed significant increases in level of secretory IgA, compared with the control group, group (6-A). In addition, it was found that the increment of the secretory IgA level of group (6-E) receiving the dissolving solution 5 with respect to group (6-A) was small, compared with groups (6-B), (6-C), and (6-D). Such tendencies were also confirmed in the spleen.

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(4-4) Findings obtained from verification experiment for dissolving solutions 1-B, 2, 3, 4, and 5

**[0107]** The following findings were obtained by examining the results of the respective experiments described in the examples (4-1) to (4-3).

25 **[0108]** The experimental results of the examples (4-1) and (4-2) for aseptically breeding Balb/c mice and germ-free mice suggest that the microorganisms included in the dissolving solution 1-B may have a regulatory effect directly on expression of gene cluster of the mucous membrane immune system. This is because, while in general the Peyer's patches are known to induce the production regulation of immunoglobulin and so on and IL-18 is known to induce production of gamma interferon, from the results of the experiment, it is assumed that the microorganism, such as the related strain (N-11) of *Bacillus thermoamylovorans*, included in the dissolving solution 1-B may activate the development of the Peyer's patches in the intestinal tract and the production of IL-18 in the living body.

30 **[0109]** Next, the experimental results of the example (4-3) for Balb/c mice suggest that the microorganisms included in the dissolving solution 2 coordinate with the established intestinal flora in the host to exert a regulatory effect on expression of gene cluster of the mucous membrane immune system. Then, the expression level of Foxp3 in the large intestinal tissue, which could be expressed in Treg cells, regulatory cells of the immune system, was investigated using real time PCR. As a result, group (6-C), a single BP-863-administration group, has an expression level of about 1.4 times higher than group (6-A), a normal feeding group (control group), and group (6-E), a type-strain administration group. From the results, it is speculated that the administration of thermophilic BP-863 may accumulate treg cells, which are regulatory cells in the immune system, and an immunoregulation mechanism such as allergic prophylaxis may work.

35 **[0110]** Furthermore, the related species (N-11) (BP-863) of *Bacillus thermoamylovorans* and the related species (N-16) of *Bacillus coagulans*, which are the microorganisms in the dissolving solution 2, are different from the type bacterial species (LMG18084<sup>T</sup>) of *Bacillus thermoamylovorans* in that, even in the case of administration as isolated bacteria, it can exert a regulatory effect of expression of gene cluster of the mucous membrane immune system as illustrated in FIG. 8. Furthermore, from the results of the verification examinations for (3-5) and (3-6) of Example 3, and so on, the group receiving the related species (N-11) of *Bacillus thermoamylovorans* is provided with an improved feed efficiency and simultaneously gains a weight equal to or more than the weight of the mice bred with high fat diet 10% or higher calories, resulting an improvement in weight-increasing rate. This may be caused by that the related species (N-11) of *Bacillus thermoamylovorans* breaks down persistent sugar in the feed to increase use efficiency.

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### INDUSTRIAL APPLICABILITY

**[0111]** The mixture, dissolving solution, and pharmaceutical agent of the present invention can be used as those capable of regulating the mucous membrane immune system gene clusters and the metabolism-related gene clusters in the intestines and liver of an animal by being administered to the animal.

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### SEQUENCE LISTING

**[0112]**

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Kanazawa University Independent Administrative Institution National Fisheries University Miroku Co., Ltd.

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Claims

1. A mixture or a dissolving solution for regulating at least one of a mucous membrane immune system gene cluster, a metabolism-related gene cluster in the intestines and a metabolism-related gene cluster in the liver of an animal by being administered to the animal, prepared by fermentation of an organic material containing a thermophilic microorganism at a temperature of 50°C or more and 90°C or less, wherein the thermophilic microorganism includes thermophilic mixed bacteria BP-1051, deposited as NITE-BP-1051, or a related species of *Bacillus thermoamylo-*

rans, BP-863, deposited as NITE-BP-863, where the BP-863 has an ability to degrade persistent sugar and wherein said organisms are included in said mixture or dissolving solution.

2. A pharmaceutical agent, comprising:

a mixture or dissolving product as described in claim 1.

### Patentansprüche

1. Mischung oder Lösung zum Regulieren von mindestens einem aus einem Gencluster des Schleimhaut-Immunsystems, einem Stoffwechsel-bezogenen Gencluster in den Eingeweiden und einem Stoffwechsel-bezogenen Gencluster in der Leber eines Tieres, indem sie dem Tier verabreicht wird, hergestellt durch Fermentation eines einen wärmeliebenden Mikroorganismus enthaltenden organischen Materials bei einer Temperatur von 50°C oder mehr und 90°C oder weniger, wobei der wärmeliebende Mikroorganismus wärmeliebende gemischte Bakterien BP-1051, hinterlegt als NITE-BP-1051, oder eine verwandte Spezies von *Bacillus thermoamylovorans*, BP-863, hinterlegt als NITE-BP-863 beinhaltet, wobei BP-863 die Fähigkeit hat, persistenten Zucker abzubauen und wobei die Organismen in der Mischung oder Lösung beinhaltet sind.

2. Pharmazeutisches Mittel, umfassend:

eine Mischung oder Lösung gemäß Anspruch 1.

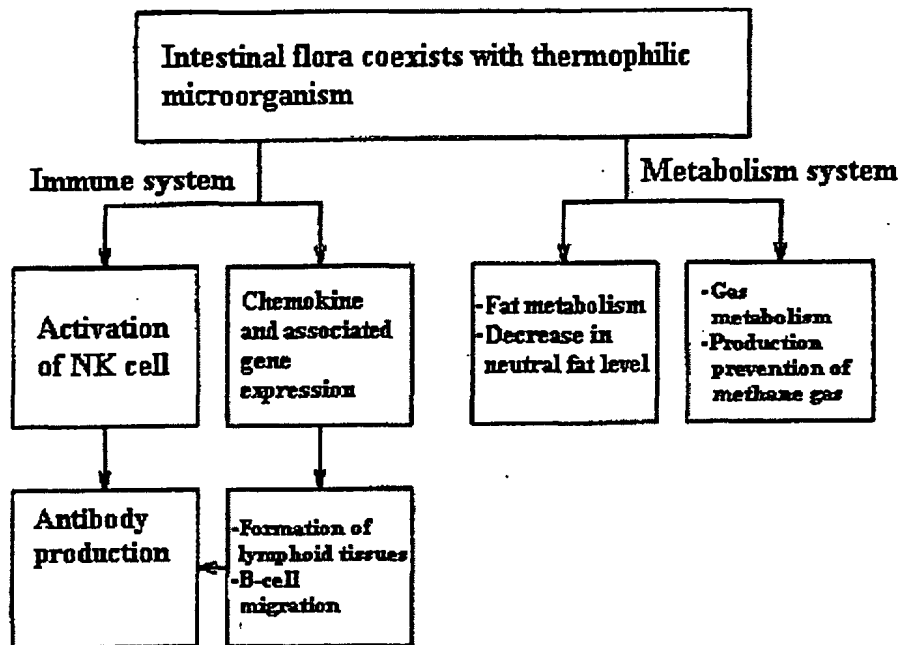
### Revendications

1. Mélange ou solution pour contrôler au moins un d'un amas de gènes du système immunitaire des muqueuses, un amas de gènes concernant le métabolisme dans les intestins et un amas de gènes concernant le métabolisme dans le foie d'un animal, en étant administré à l'animal, préparé par fermentation d'un matériau organique qui contient un micro-organisme thermophile chez une température de 50°C ou plus et 90°C ou moins, le micro-organisme thermophile incluant des bactéries thermophiles mixtes BP-1051, déposées comme NITE-BP-1051, ou un espèce apparenté de *Bacillus thermoamylovorans* BP-1051, déposé comme NITE-BP-863, ou BP-863 a l'aptitude de dégrader du sucre persistant, et lesdits organismes sont inclus dans ledit mélange ou ladite solution.

2. Agent pharmaceutique, comportant :

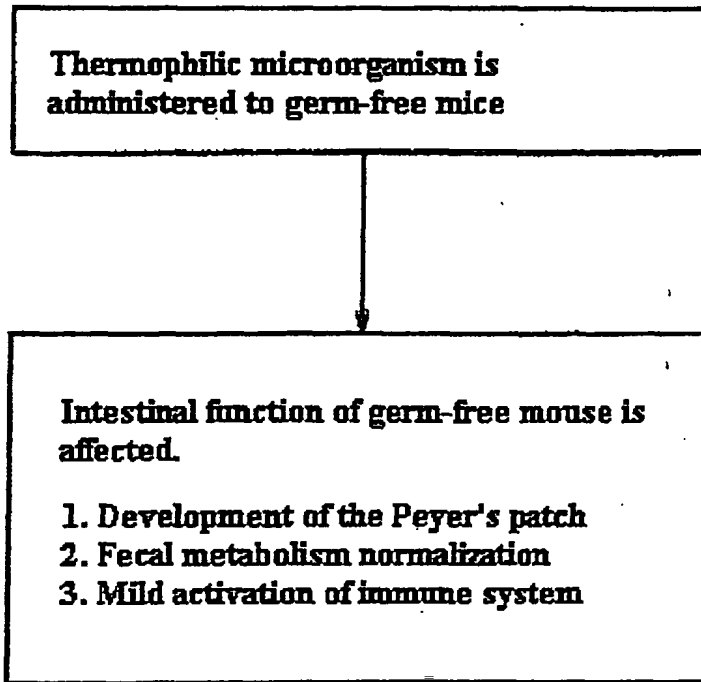
un mélange ou une solution comme décrite dans la revendication 1.

【 F i g . 1 】

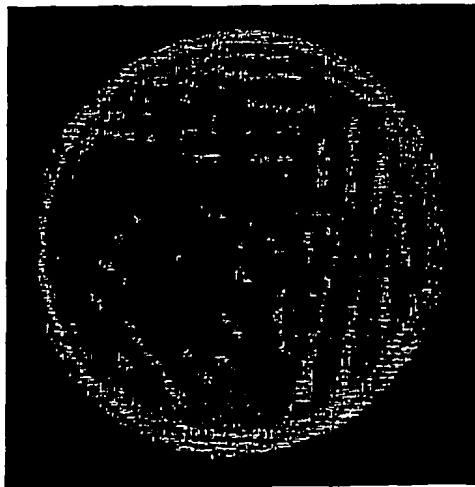




**[ F i g . 2 ]**



**[ F i g . 3 ]**



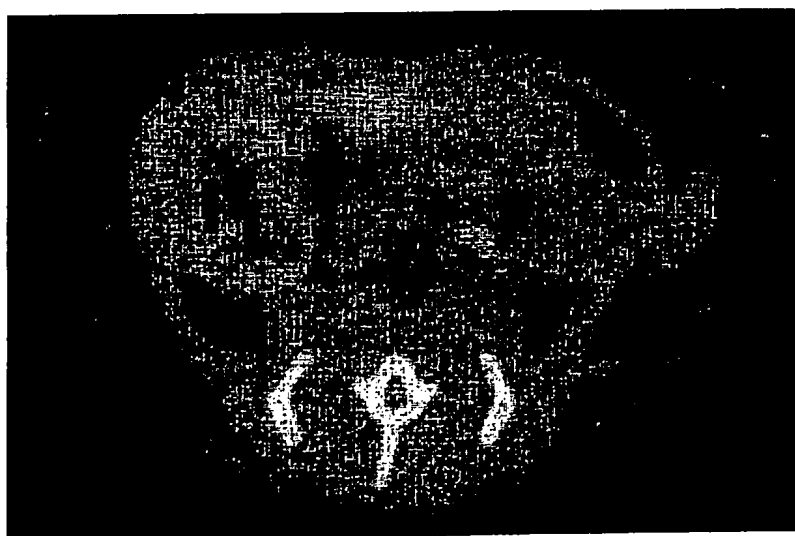
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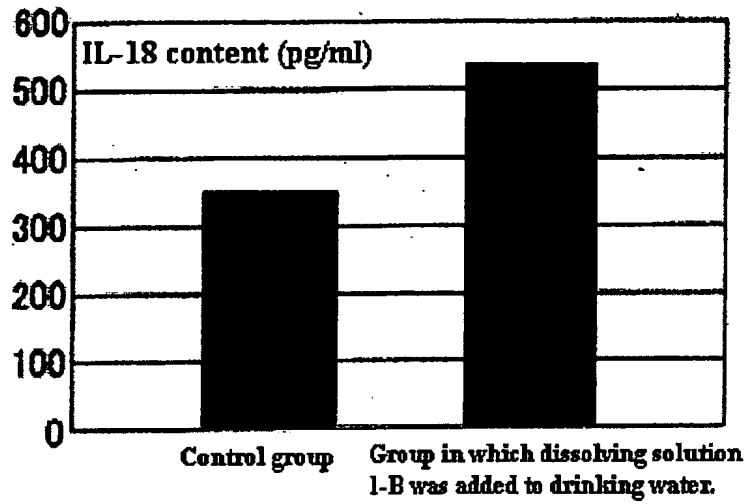
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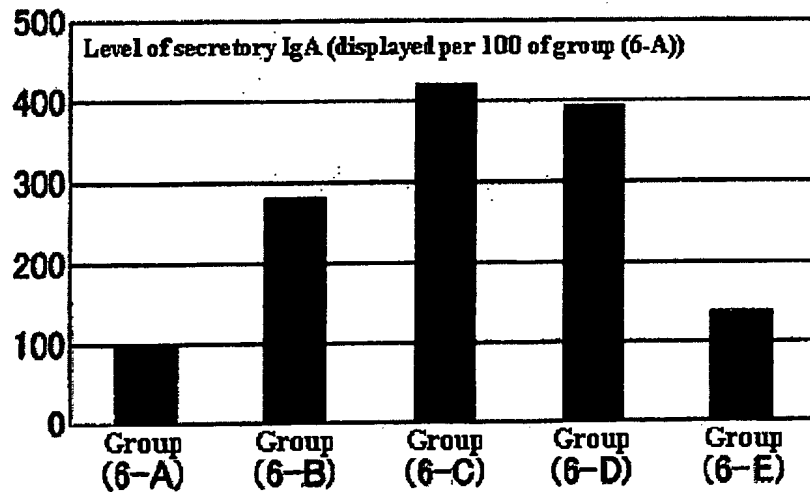
【F i g . 6】



[ Fig. 7 ]



[ Fig. 8 ]



## REFERENCES CITED IN THE DESCRIPTION

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