

## DESCRIPTION

ENDOMETRIOSIS PREVENTION AND/OR IMPROVING AGENT, AND FOOD OR DRINK  
COMPOSITION CONTAINING SAMETechnical Field

The present invention relates to agents for preventing and/or improving endometriosis. In particular, the present invention relates to agents containing specific lactic acid bacteria for preventing and/or improving endometriosis, and food and beverage compositions containing the lactic acid bacteria.

Background Art

Endometriosis is a disease in which the endometrium or a similar tissue shed during menstruation regurgitates through the fallopian tubes and proliferates within the peritoneal cavity outside the uterus. It has been estimated that 6 to 10% of women in reproductive years are affected by the disease, and thus it is an extremely serious gynecological disease. While many reports have shown that the disease arises due to abnormalities in the intraperitoneal immune surveillance system, little is known about the pathogenic mechanism. Normally, immunocompetent cells such as macrophages and NK cells eliminate endometrial tissues that flow into the peritoneal cavity. It is however believed that when the function of immunocompetent cells is impaired due to some problems, endometrial tissues survive and proliferate in the peritoneal cavity. In particular, reduced NK activity has been reported to significantly correlate with the severity (Non-patent Document 1). This finding supports the NK cell hypothesis that the reduction of intraperitoneal NK cell activity to eliminate ectopic endometrial cells is involved in the development and progression of endometriosis.

Meanwhile, endometriosis is often painful and significantly impairs the Quality of Life (QOL). Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to ease pain during menstruation. On the other hand, NSAIDs are known to cause gastrointestinal disorders such as corrosive lesion and ulcer because of their pharmacological nature to inhibit cyclooxygenase, resulting in inhibition of the production of prostaglandin which has mucosal protective function from arachidonic acid. In addition, hormone-related adverse effects are observed in endometriosis therapy using danazol, gonadotropin-releasing hormone agonists, or the like.

Thus, substances that enhance NK cell activation have been searched in various fields. Examples of substances known to date to be effective to some extent include polysaccharides

(lentinan, chitin, chitosan, Maitake D-Fraction, nigero-oligosaccharides, etc.) and bacterial cells. However, none of these has been sufficiently assessed for endometriosis.

On the other hand, IL-12 is a cytokine that has the effect of activating Th1 cells, NK cells, etc., and has been reported to produce an inhibitory effect on the ectopic growth of endometrial cells (Non-patent Document 2).

#### Prior Art Documents

##### Non-patent Documents

Non-patent Document 1: Oosterlynck DJ, Meuleman C, Waer M, Vandeputte M, and Koninckx PR, The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. Fertil.Steril., 58, 290-295(1992)

Non-patent Document 2: Somigliana E, Vigano P, Rossi G, Carinelli S, Vignali M, and Panina-Bordignon P. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. Hum.Reprod., 14, 2944-2950(1999)

#### Summary of the Invention

##### [Problems to be Solved by the Invention]

However, the above-described compounds for enhancing NK cell activation and compounds capable of inducing the IL-12 production to activate NK cells have not been assessed sufficiently in terms of their relevance to the development of endometriosis.

Thus, an objective of the present invention is to provide agents for preventing and/or improving endometriosis by enhancing NK activity. Another objective of the present invention is to provide food and beverage compositions which comprise lactic acid bacteria for preventing and/or improving endometriosis. Still another objective of the present invention is to provide methods for preventing and/or improving endometriosis. In particular, an objective of the present invention is to provide orally ingestible agents for preventing and/or improving endometriosis that can produce a pain-relieving effect and are highly safe without adverse effects.

##### [Means for Solving the Problems]

The present inventors have investigated probiotic lactic acid bacteria in terms of their ability to induce IL-12. Specifically, the present inventors have performed various tests to investigate whether lactic acid bacteria that have the activity of enhancing IL-12 production and potentiating the cytotoxicity of NK cells can exhibit an endometriosis-improving effect.

The present inventors conducted dedicated studies in view of the conventional problems described above, and as a result demonstrated that some lactic acid bacteria, in particular, lactic

acid bacteria of the genus *Lactobacillus* have an excellent inhibitory effect against the growth of endometriosis lesions. The present inventors thereby completed the present invention.

5 Lactic acid bacteria of the genus *Lactobacillus* were prescribed for endometriosis patients, particularly those that complained of pelvic pain which is a major factor impairing QOL. The lactic acid bacteria were revealed to have an effect of improving endometriosis, in particular, menstrual cramping and dysmenorrhea. As described above, the present inventors discovered that lactic acid bacteria are effective for improving the QOL of endometriosis patients. The  
10 present inventors thereby completed the present invention.

In one aspect, there is provided the use of an agent comprising a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.

In another aspect, there is provided the use of a food or beverage composition  
15 comprising a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.

In another aspect, there is provided the use of a composition comprising an effective amount of a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.

20 Specifically, in one embodiment, the present invention includes agents that contain lactic acid bacteria for preventing and/or improving endometriosis.

There is no limitation on the lactic acid bacteria contained in the agents for preventing and/or improving endometriosis, but they are preferably those of the genus *Lactobacillus*, more preferably *Lactobacillus gasseri*, and in particular, most preferably *Lactobacillus gasseri* strain  
25 OLL2809.

In another embodiment, the present invention includes food and beverage compositions that contain an effective amount of lactic acid bacteria for preventing and/or improving endometriosis.

There is also no limitation on the lactic acid bacteria in the food and beverage  
30 compositions for preventing and/or improving endometriosis, but they are preferably those of the genus *Lactobacillus*, more preferably *Lactobacillus gasseri*, and in particular, most preferably *Lactobacillus gasseri* strain OLL2809.

The above-described food and beverage compositions are preferably fermented milk food and beverage, and preferably yogurt foods and beverages such as yogurt and yogurt drinks;  
35 however, they are not limited thereto.

In still another embodiment, the present invention includes methods for preventing and/or improving endometriosis comprising administering a composition containing an effective amount of lactic acid bacteria at a desired dose.

Also in the methods for preventing and/or improving endometriosis, the lactic acid bacteria are preferably those of the genus *Lactobacillus*, more preferably *Lactobacillus gasseri*,  
5 and in particular, most preferably *Lactobacillus gasseri* strain OLL2809.

Furthermore, in the methods for preventing and/or improving endometriosis, the dose is preferably at least 2 mg/kg/day; however, it is not limited thereto.

Alternatively, the present invention relates to:

[1] an agent for either or both prevention and improvement of endometriosis, which comprises a  
10 lactic acid bacterium;

[2] the agent of [1], wherein the lactic acid bacterium is of the genus *Lactobacillus*;

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- [3] the agent of [1], wherein the lactic acid bacterium is *Lactobacillus gasseri*;
- [4] the agent of [1], wherein the lactic acid bacterium is *Lactobacillus gasseri* strain OLL2809;
- [5] a food or beverage composition for either or both prevention and improvement of endometriosis, which comprises an effective amount of a lactic acid bacterium;
- 5 [6] the food or beverage composition of [5], wherein the lactic acid bacterium is of the genus *Lactobacillus*;
- [7] the food or beverage composition of [5], wherein the lactic acid bacterium is *Lactobacillus gasseri*;
- [8] the food or beverage composition of [5], wherein the lactic acid bacterium is *Lactobacillus*
- 10 *gasseri* strain OLL2809;
- [9] the food or beverage composition of [8], which is a fermented milk food or beverage composition;
- [10] the food or beverage composition of [9], wherein the fermented milk food or beverage composition is yogurt;
- 15 [11] a method for either or both prevention and improvement of endometriosis, wherein a composition comprising an effective amount of a lactic acid bacterium is administered at a desired dose;
- [12] the method of [11], wherein the lactic acid bacterium is of the genus *Lactobacillus*;
- [13] the method of [11], wherein the lactic acid bacterium is *Lactobacillus gasseri*;
- 20 [14] the method of [11], wherein the lactic acid bacterium is *Lactobacillus gasseri* strain OLL2809;
- [15] the method of any one of [11] to [14], wherein the dose is at least 2 mg/kg/day;
- [16] a lactic acid bacterium for either or both prevention and improvement of endometriosis;
- [17] the lactic acid bacterium of [16], which is of the genus *Lactobacillus*;
- 25 [18] the lactic acid bacterium of [16], which is *Lactobacillus gasseri*; and
- [19] the lactic acid bacterium of [16], which is *Lactobacillus gasseri* strain OLL2809.

Alternatively, the present invention relates to:

- [101] an agent for preventing and treating endometriosis, which comprises a lactic acid bacterium;
- 30 [102] the agent of [101] for preventing and treating endometriosis, wherein the lactic acid bacterium is of the genus *Lactobacillus*;
- [103] the agent of [101] for preventing and treating endometriosis, wherein the lactic acid bacterium is *Lactobacillus gasseri* strain OLL2809;
- [104] a food or beverage composition for preventing and treating endometriosis, which
- 35 comprises an effective amount of a lactic acid bacterium;

[105] the composition of [104] for preventing and treating endometriosis, wherein the lactic acid bacterium is of the genus *Lactobacillus*;

[106] the food or beverage composition of [105] for preventing and treating endometriosis, wherein the lactic acid bacterium is *Lactobacillus gasseri* strain OLL2809;

5 [107] the food or beverage composition of [106] for preventing and treating endometriosis, which is a fermented milk food or beverage;

[108] the food or beverage composition of [107] for preventing and treating endometriosis, wherein the fermented milk food or beverage is a yogurt food or beverage such as yogurt or yogurt drink;

10 [109] a method for preventing and treating endometriosis comprising administering a desired dose of a composition comprising an effective amount of a lactic acid bacterium;

[110] the method of [109] for preventing and treating endometriosis, wherein the lactic acid bacterium is of the genus *Lactobacillus*;

15 [111] the method of [109] for preventing and treating endometriosis, wherein the lactic acid bacterium is *Lactobacillus gasseri* strain OLL2809; and

[112] the method of any one of [109] to [111] for preventing and treating endometriosis, wherein the dose is at least 2 mg/kg/day.

#### [Effects of the Invention]

20 The present invention provides agents for preventing and/or improving endometriosis, which contain a certain type of lactic acid bacteria, in particular, lactic acid bacteria of the genus *Lactobacillus*. Lactic acid bacteria of the genus *Lactobacillus* of the present invention have been demonstrated to produce an unprecedentedly significant effect in improving endometriosis, menstrual cramping and dysmenorrhea, specifically by increasing one's own immunity through  
25 enhancement of the NK activity. Thus, the lactic acid bacteria of the genus *Lactobacillus* can be used to prevent and/or improve such disorders. Agents of the present invention for preventing and/or improving endometriosis are effective for improving the QOL of endometriosis patients.

Furthermore, the agents of the present invention for preventing and/or improving  
30 endometriosis can be ingested simply by oral ingestion. While prevention and improvement using hormonal agents or such can sometimes cause adverse effects, the present invention does not have such risk. The agents are highly safe enough to be treated as food. Thus, there is little obstacle for patients to ingest the preventive and/or improving agents of the present invention, and prevention and/or improvement of endometriosis becomes possible without  
35 imposing burden on daily life. Moreover, due to the above-mentioned characteristics, the

agents of the present invention can be easily made into food or beverage compositions, and this can facilitate convenient ingestion.

In addition, the agents of the present invention for preventing and/or improving endometriosis have a unique effect in easing pain which is characteristic of the disease, and are particularly effective in improving the QOL of endometriosis patients.

#### Brief Description of the Drawings

Fig. 1 is a graph showing comparisons of the weight (Fig. 1(A)) and area (Fig. 1(B)) of endometriosis lesions after administration of OLL2809 and such to endometriosis model mice.

a-c: there is a significant difference between different alphabetical labels ( $P<0.05$ ).

Fig. 2 is a graph showing enhanced expression levels of the IL-2 gene (Fig. 2(A)) and Ncr1 gene (Fig. 2(B)) in peritoneal cells in peritoneal lavage fluid. a and b: there is a significant difference between different alphabetical labels ( $P<0.05$ ).

Fig. 3 is a graph showing a time course of menstrual pain assessed with a visual analogue scale.

\* and \*\*:  $P<0.05$  and  $0.01$ , respectively.

Fig. 4 is a graph showing changes in menstrual pain before and after intake assessed with a visual analogue scale. \*\*:  $P<0.01$ .

Fig. 5 is a graph showing a time course of dysmenorrhea score during menstruation. \*:  $P<0.05$ .

Fig. 6 is a graph showing changes in the dysmenorrhea score during menstruation before and after intake. (\*):  $P<0.1$ .

#### Mode for Carrying Out the Invention

Hereinbelow, the present invention will be described more specifically, but is not to be construed as being limited to any embodiment described below.

The present invention provides agents for preventing and/or improving endometriosis, which contain lactic acid bacteria (hereinafter referred to as "preventive/improving agents of the present invention"). The preventive/improving agents of the present invention can also be referred to as:

- pharmaceutical compositions that contain lactic acid bacteria for preventing and/or improving endometriosis;
- agents that contain lactic acid bacteria for inhibiting the growth of endometriosis lesions; or
- pharmaceutical compositions that contain lactic acid bacteria for inhibiting the growth of endometriosis lesions.

Herein, "prevention and/or improvement" can also be referred to as "either or both of prevention and improvement". Thus, in the present invention, "prevention and/or improvement" includes:

- prevention and improvement;
- prevention; and
- improvement.

Herein, "improvement" can also be referred to as "treatment".

5 Preventive/improving agents of the present invention contain lactic acid bacteria as an active ingredient. The lactic acid bacteria may be of any type, as long as they have a high capacity of inducing IL-12 production. To date, lactic acid bacteria have been grouped into more than 30 genera, including the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Wissella*, *Tetragenococcus*, *Oenococcus*, *Enterococcus*,  
 10 *Vagococcus*, and *Carnobacterium*. Such lactic acid bacteria can be used for the preventive/improving agents of the present invention. The lactic acid bacteria are preferably those of the genus *Lactobacillus*, more preferably *Lactobacillus gasseri*, and particularly preferably *Lactobacillus gasseri* strain OLL2809 (Accession Number: NITE BP-72); however, they are not limited thereto. Alternatively, two or more different types of lactic acid bacteria  
 15 may be used in combination.

The present inventors deposited *Lactobacillus gasseri* OLL2809 with the Incorporated Administrative Agency, National Institute of Technology and Evaluation, Patent Microorganisms Depository. The specific content of the deposition is detailed below:

- (1) Name of depositary institution: The Incorporated Administrative Agency, National Institute  
 20 of Technology and Evaluation, Patent Microorganisms Depository.
- (2) Contact: 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818.  
 Phone: 0438-20-5580
- (3) Accession number: NITE BP-72
- (4) Identification label: *Lactobacillus gasseri* OLL2809
- 25 (5) Date of original deposition: February 1, 2005
- (6) Date of transfer to the depositary under the Budapest Treaty: January 18, 2006.

*Lactobacillus gasseri* OLL2809 (Accession Number: NITE BP-72) is a Gram-positive bacillus. The morphology of colonies on the Lactobacilli MRS Agar, Difco0, is round, light yellow, and flat. It possesses physiological characteristics such as homolactic fermentation,  
 30 growth at 45°C, and fermentability for glucose, mannose, fructose, galactose, sucrose, cellobiose, lactose, and trehalose.

Media suitable for culturing lactobacilli in general may also be used to culture the *Lactobacillus gasseri* OLL2809 strain (Accession Number: NITE BP-72). Thus, any medium containing, other than the main carbon source, a good extent of a nitrogen source, inorganic  
 35 substances, and other nutrients can be used. Depending on the assimilating ability of the bacteria selected, lactose, glucose, sucrose, fructose, starch hydrolysate, molasses, and such can

be used as the carbon source. Organic nitrogen-containing materials, such as casein hydrolysate, whey protein hydrolysate, and soybean hydrolysate, can be used as the nitrogen source. In addition, meat extracts, fish meat extracts, yeast extracts, and such can be used as growth-promoting agents.

5 Culture is preferably performed under anaerobic conditions; however, it may also be performed under microaerobic conditions, for example in the context of the commonly used stationary liquid culture. Known methods, such as the method of culturing under a carbon gas layer, can be applied to the anaerobic culturing, though other methods are contemplated as well. In general, the culture temperature preferably ranges between 30 and 40°C; however, other  
10 temperature conditions may be used, so long as they allow the bacteria to grow. The pH of the medium is preferably maintained between 6.0 and 7.0 during culture; however, other pH conditions may be used, so long as the temperature allows bacterial growth. Moreover, culture can also be carried out under batch culture conditions. In general, the duration of culture preferably ranges between 10 and 24 hours; however, other culture periods may be used, as long  
15 as they permit bacterial growth.

Other strains of *Lactobacillus gasseri* that can be used in the context of the present invention should possess characteristics similar to those of *Lactobacillus gasseri* OLL2809, such as Gram positivity and other physiological characteristics. Those skilled in the art can isolate such *Lactobacillus gasseri* from human feces and such based on known physiological  
20 characteristics. Culture can also be performed using methods similar to those described above for *Lactobacillus gasseri* OLL2809.

The preventive/improving agents of the present invention may contain lactic acid bacteria as they are or as a processed product obtained by certain processing. For example, lactic acid bacterial suspensions, lactic acid bacterial culture (bacterial cells and culture  
25 supernatants (including medium components)), lactic acid bacterial fermentation products (lactic fermented milk beverage, sour milk, yogurt, etc.), concentrates, paste-processed products, spray-dried products, freeze-dried products (at least one selected from spray-dried products, freeze-dried products, vacuum-dried products, and drum-dried products), vacuum-dried products, drum-dried products, liquid products obtained by dispersing the bacteria in a medium, dilutes  
30 prepared by diluting the bacteria with a diluent, and crushed products obtained by grinding the dried bacteria with a mill or the like may be used as processed products. The lactic acid bacteria can be appropriately used in the form of live bacteria, wet bacteria, dried bacteria, or such. Lactic acid bacteria may be dead bacterial cells prepared by sterilization via heating, irradiation, or crushing. Such processing steps may be used alone or in combination.

35 The preventive/improving agents of the present invention can be used alone to prevent or improve human and animal endometriosis. The agents of the present invention may also be

combined with ingredients that are commonly used for foods or pharmaceutical agents, and administered orally. Furthermore, the agents of the present invention can be used in combination with other compounds, microorganisms, and such, which are known to be effective in preventing or improving endometriosis.

5           The effects of preventing and treating endometriosis can also be assessed, for example, with subjective evaluation using visual analogue scale (VAS) or dysmenorrhea score, as described in the Examples.

          An appropriate dose of a preventive/improving agent of the present invention can be appropriately determined by considering various factors such as administration route as well as  
10   age, weight, and symptoms of subject animals including humans. The present invention is preferably administered as an active ingredient at least 2 mg/kg/day, but it is not limited thereto. On the other hand, when ingested for the purpose of long-term prevention and/or improvement, the dose may be lower than the range described above. Alternatively, since there is no problem with the safety of active ingredients of the preventive/improving agents of the present invention  
15   as food products, they may be used at a dose greater than the range described above.

          The preventive/improving agents of the present invention can be administered either orally or parenterally (intramuscularly, subcutaneously, intravenously, as a suppository, transdermally, via a gastric tube, etc.).

          The dosage form of a preventive/improving agent of the present invention can be  
20   selected depending on the purpose of prevention or improvement, administration route, and so on. The dosage form includes, for example, tablets, coated tablets, pills, capsules, granules, powders, solutions, suspensions, emulsions, syrups, injections, suppositories, infusions, decoctions, and tinctures. These various preparations can be formulated by conventional methods using known adjuvants that are commonly used in the technical field of pharmaceutical formulation, such as  
25   fillers, expanders, excipients, binders, humectants, disintegrators, surfactants, lubricants, colorants, corrigents, solubilizing agents, suspending agents, and coating agents for the main agent according to needs. The pharmaceutical preparations may also contain other pharmaceutical agents as well as colorants, preservatives, perfumes, flavors, sweeteners, and such.

30           In the present invention, lactic acid bacteria can be used in the form of a pharmaceutical agent or food/drink. The bacteria are expected to prevent and/or improve endometriosis, for example, when they are directly administered as a pharmaceutical agent or directly ingested as a food for special uses such as a food for specified health uses, or a food with nutrient function claims. Thus, the present invention provides food and beverage compositions for preventing  
35   and/or improving endometriosis which contain an effective amount of lactic acid bacteria that constitute the preventive/improving agents of the present invention. The form of a food or

beverage composition includes prepared powdered milk and fermented milk food and beverage. The fermented milk food and beverage include yogurt and cheese; however, they are not limited thereto.

The food and beverage compositions of the present invention can also be applied to a food with health claims or a food for diseased people, including beverages and fermented milk such as yogurt. The system of food with health claims was created by taking into consideration domestic and international trends as well as compliance with the conventional system of "Food for Specified Health Use". It was created not only for ordinary foods but also foods in a tablet shape, capsule shape, etc., and consists of two types: food for specified health uses (product-based approval type) and food with nutrient function claims (regulatory standard type). Endometriosis can be prevented and/or improved via direct ingestion of food for special uses such as food for specified health uses, or food with nutrient function claims, which contain lactic acid bacteria that constitute the preventive/improving agents of the present invention.

Specifically, it is possible to ingest the food and beverage compositions of the present invention obtained by adding the lactic acid bacteria that constitute the preventive/improving agents of the present invention to various foods/drinks (milk, soft drinks, fermented milk, yogurt, cheese, bread, biscuits, crackers, pizza crusts, prepared powdered milk, fluid diet, food for sick people, food such as powdered milk for infants, food such as powdered milk for nursing women, nutritive food, etc.). The active ingredient can be used as it is or after combining with other foods or food ingredients according to conventional methods for common food and beverage compositions, and may be in a form usually used in food/beverage, for example, in the form of solid (powder, granule, etc.), paste, liquid, or suspension.

Other ingredients are also not particularly limited; however, water, proteins, sugars, lipids, vitamins, minerals, organic acids, organic bases, fruit juice, flavors, and such can be used as the main ingredients for food and beverage compositions containing lactic acid bacteria that constitute the preventive/improving agents of the present invention. Proteins include, for example, plant and animal proteins, such as whole milk powder, powdered skim milk, partially-skimmed milk, casein, whey powder, whey protein, whey protein concentrates, whey protein isolates,  $\alpha$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, lactoferrin, soy bean protein, chicken egg protein, and meat protein; hydrolysates thereof; and various milk-derived components such as butter, whey minerals, cream, whey, non-protein nitrogen, sialic acid, phospholipids, and lactose. Examples of sugars include saccharides, processed starch (dextrin, soluble starch, British starch, oxidized starch, starch ester, starch ether, etc.), and dietary fibers. Examples of lipids include animal fat and oil such as lard and fish oils, as well as their separated oils, hydrogenated oils, and interesterified and/or transesterified oils; and vegetable oils such as palm oil, safflower oil, corn oil, rapeseed oil, coconut oil, as well as their

separated oils, hydrogenated oils, and interesterified and/or transesterified oils. Examples of vitamins include vitamin A, carotenes, vitamin B group, vitamin C, vitamin D group, vitamin E, vitamin K group, vitamin P, vitamin Q, niacin, nicotinic acid, pantothenic acid, biotin, inositol, choline, and folic acid. Examples of minerals include calcium, potassium, magnesium, sodium, copper, iron, manganese, zinc, selenium, and whey minerals. Examples of organic acids include malic acid, citric acid, lactic acid, and tartaric acid. Of these components, two or more may be used in combination. It is also possible to use synthetic products and/or foods which are rich in these components.

The amount of lactic acid bacteria that constitute the preventive/improving agents or the food and beverage compositions of the present invention can be determined arbitrarily depending the purpose and use (pharmaceutical agent, or food and beverage composition). The content is typically 0.001 to 100% (w/w), and particularly preferably 0.01 to 100% (w/w) of the total weight; however, the present invention is not limited thereto.

When lactic acid bacteria are used to produce food and beverage compositions, or pharmaceutical agents, they can be produced using methods known to those skilled in the art. Those skilled in the art can manufacture desired foods and pharmaceutical agents by appropriately combining a step of mixing lactic acid bacteria or processed products thereof with other components, molding step, sterilization step, fermentation step, baking step, drying step, cooling step, granulating step, packing step, etc.

When lactic acid bacteria are used to produce various milk products, desired milk products can also be produced using methods known to those skilled in the art. For example, in the case of yogurt, yogurt can be produced via steps of preparing a starter using lactic acid bacteria, adding the starter to pre-treated milk and culturing it, cooling, flavoring, filling, etc. Cheese can be produced, for example, via steps of adding the lactic acid bacteria of the present invention as a starter to milk pretreated by sterilization or such and performing lactic acid fermentation; producing cheese curd by adding rennet; cutting the curd; discarding whey; adding salt; and maturation. Alternatively, in the above-described production of various types of milk products, other lactobacilli may be used as the starter in place of a specific lactic acid bacterium (for example, *Lactobacillus gasseri*), and the specific lactic acid bacteria (for example, *Lactobacillus gasseri*) or a processed product thereof can be added thereto during the manufacturing process.

The present invention also relates to methods for preventing and/or improving endometriosis, which comprise the step of orally administering lactic acid bacteria to animals. Subjects to be administered with lactic acid bacteria include mammals. Mammals include humans and nonhuman mammals, preferably humans and monkeys, and more preferably humans.

Furthermore, the present invention relates to lactic acid bacteria that are used for preventing and/or improving endometriosis. The present invention also relates to the use of lactic acid bacteria in the production of agents for preventing and/or improving endometriosis. The present invention also relates to the use of lactic acid bacteria for preventing and/or improving endometriosis. The present invention also relates to methods for producing agents for preventing and/or improving endometriosis, which comprise the step of combining lactic acid bacteria with pharmaceutically acceptable carriers.

Lactic acid bacteria preferably include those of the genus *Lactobacillus*, more preferably *Lactobacillus gasseri*, and particularly preferably *Lactobacillus gasseri* strain OLL2809; however, they are not limited to the above examples.

The present invention also provides agents that contain lactic acid bacteria for inhibiting the growth of endometriosis lesions. The present invention also provides methods for inhibiting the growth of endometriosis lesions, which comprise the step of administering lactic acid bacteria to animals. The present invention also relates to lactic acid bacteria used for inhibiting the growth of endometriosis lesions. The present invention also relates to the use of lactic acid bacteria in the production of agents for inhibiting the growth of endometriosis lesions. The present invention also relates to the use of lactic acid bacteria for inhibiting the growth of endometriosis lesions. The present invention also relates to methods for producing agents for inhibiting the growth of endometriosis lesions, which comprise the step of combining lactic acid bacteria with pharmaceutically acceptable carriers.

The growth inhibitors of the present invention can be formulated with carries suitable for oral administration. The growth inhibitors of the present invention may be administered as a food to inhibit the growth of endometriosis lesions.

Endometriosis develops mainly on the surface of organs such as the peritoneum, ovary, uterus, Douglas' pouch, intestines and rectum; inside the ovary; and among cells of uterine muscles and such. Whether the growth inhibitors of the present invention inhibit the growth of lesions can be determined, for example, by measuring the area, volume, or weight of the lesions using methods known to those skilled in the art. All prior art documents cited herein are incorporated by reference herein.

### Examples

Hereinbelow, the present invention will be specifically described with reference to the Examples, but the present invention should not be construed as being limited thereto. Herein, % sign refers to weight %, unless otherwise specified.

[Example 1]

Endometriosis model mice were prepared using the method described below, and the preventive/improving agents of the present invention were assessed for their preventive and improving effect.

5 [Experimental animals]

Six weeks old female BALB/c mice (Japan SLC, Inc.) which were ovariectomized at five weeks of age were used. The mice were fed with the CRF-1 diet (Oriental Yeast Co., Ltd.), grouped and housed in cages in a room controlled at room temperature  $21 \pm 2^\circ\text{C}$  and humidity  $55 \pm 15\%$  under a 12/12 hour light-dark cycle. The mice were allowed to freely feed and consume  
10 water.

Endometriosis model mice were prepared using a modified method based on the method of Somigliana *et al.* (Non-patent Document 2).

Specifically, pairs of uterine horns from donor mice described below were cut into fine pieces and suspended in 0.6 ml of phosphate buffered saline (PBS; pH 7.2). Using an 18G  
15 needle, the endometrial suspension was injected at 0.3 ml/head into the peritoneal cavities of recipient mice (defined as day 0). The recipient mice had been ovariectomized at five weeks of age (seven days before transplantation). Estrogen (Wako Pure Chemical Industries, Ltd.; 100  $\mu\text{g/kg}$ ) dissolved in castor oil was administered into the thigh muscle for a total of four times: seven days before transplantation, on day 0, day 7, and day 14. As with the recipient mice, the  
20 donor mice also underwent ovariectomy and were administered with estrogen seven days before transplantation. Ovariectomy and estrogen administration were performed to synchronize the sexual cycle in the mice so that they all come into estrus.

Recombinant mouse IL-12 (Wako Pure Chemical Industries, Ltd.) was dissolved in PBS and administered at 0.15  $\mu\text{g}/0.4\text{ ml}$  into the peritoneal cavities for a total of five consecutive days  
25 from two days before to two days after transplantation. To the other groups, the medium alone was administered under the same conditions.

*Lactobacillus gasseri* OLL2809 was cultured twice for activation in Lactobacilli MRS broth (Difco) ( $37^\circ\text{C}$ , 18 hours). The activated bacterial cells were inoculated at 1% to the same medium and cultured at  $37^\circ\text{C}$  for 18 hours. After harvesting, the bacterial cells were washed  
30 twice with physiological saline and then once with sterile distilled water. The cells were sterilized by heating at  $75^\circ\text{C}$  for 60 minutes and then freeze-dried. The freeze-dried bacterial cells were suspended at a concentration of 2 mg/0.5 ml in water, and administered at a dose of 2 mg/0.5 ml/head via a gastric tube for a period from the day of transplantation to the day before dissection (day 20). To the other groups, the medium alone was administered under the same  
35 conditions.

On day 21 after endometrial transplantation, the mice were euthanized by cervical dislocation, and laparotomized after collecting peritoneal lavage fluid. The size of endometrial lesions (the major and minor axes) was measured with a vernier caliper, and then the lesions were excised to measure their weight. When cyst formation was seen on the lesions, the measurement was carried out after aspiration of cyst fluid.

Furthermore, before laparotomy, 1 ml of PBS containing 1% FCS was injected into the peritoneal cavities, and 0.5 ml of peritoneal lavage fluid was collected to determine the concentrations of cytokines in the ascites. Cytokine assay was carried out using the Bio-plex Cytokine Assay Kit (Bio-Rad; Hercules, CA).

After 0.5 ml of peritoneal lavage fluid was collected, 4 ml of PBS containing 1% FCS was additionally injected into the peritoneal cavities, and 3 ml of lavage fluid was collected for peritoneal cells. Cells were isolated by centrifugation from 0.5 ml of the peritoneal lavage fluid collected for cytokine assay, and they were used as peritoneal cells.

Total RNAs were extracted from peritoneal cells using a Trizol reagent (Invitrogen; Calsbad, CA). cDNAs were prepared by reverse transcription with the PrimeScript RT Reagent Kit (TaKaRa Bio Inc.) using 1 µg of extracted total RNAs as template. Real-time PCR assay was carried out with ABI Prism 7300 (Applied Biosystems; Foster City, CA) using the FastStart Universal SYBR Green Master Reagent (Roche; Indianapolis, IN). Oligonucleotide primers for the IL-2 gene were prepared with reference to Overberg *et al.* (Overberg L, Giulietti A, Valckx D, Decallonne B, Bouillon R, and Mathieu C. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. J. Biomol. Tech., 14, 33-43(2003)). The TaqMan probe (Applied Biosystems) was used in NCR1 gene assay. The expression levels of respective genes were normalized with the level of glyceraldehyde-3-phosphate dehydrogenase expression.

Results are shown as mean ± standard error. Multiple testing of parametric data in statistical analysis was carried out using one-way analysis of variance. When significant differences were observed, Fisher's PLSD test was used to analyze the significant differences between groups. Multiple comparison of non-parametric data was carried out according to the method of Steel-Dwass.  $P < 0.05$  was considered significant.

#### Effect of *Lactobacillus gasseri* OLL2809 administration on endometriosis lesions

No endometriosis lesion was seen in the normal (non-treated) group. In the control group, on the other hand, two to seven lesions were observed in each animal, and values of both area and weight were significantly greater as compared to those of the normal group (Fig. 1). When *Lactobacillus gasseri* OLL2809 was administered, values of both area and weight were significantly lower as compared to those of the control group. Thus, it was demonstrated to

have an inhibitory effect on lesion growth. The effect was comparable to that observed in the IL-12 administration group as a positive control. As seen from the above, the administration of preventive/improving agents of the present invention was demonstrated to be effective in preventing and improving endometriosis.

#### Gene expression analysis of peritoneal cells

In peritoneal lavage fluid, the levels of most cytokines tested were below the detection limit. The cell type ratio in the peritoneal cells also did not suggest any significant effect by administration of *Lactobacillus gasseri* OLL2809. Thus, the gene expression levels in the peritoneal cells were measured. The result showed that when compared to the normal group, IL-2 was significantly lower in the control group (Fig. 2(A)). By contrast, in both OLL2809 and IL-12 administration groups, the expression level was restored to be comparable to that in the normal group (Fig. 2(B)). The IL-2 gene is a cytokine produced mainly by Th1 cells, and enhances the growth and differentiation of B, T, NK cells and such. The IL-2 gene has been demonstrated to inhibit endometriosis in rats (Velasco I, Quereda F, Bermejo R, Campos A, and Acien P. Intraperitoneal recombinant interleukin-2 activates leukocytes in rat endometriosis. J. Reprod. Immunol., 74, 124-132 (2006), etc.). The expression level of the IL-2 gene was enhanced by administration of *Lactobacillus gasseri* OLL2809. This confirms the effectiveness of the preventive/improving agents of the present invention.

Furthermore, significant enhancement was observed in the expression level of natural cytotoxicity triggering receptor 1 (NCR1) gene which is one of the NK cell activation markers. Human NCR1 is also referred to as Ly94 or NKp46, and is known to be expressed on the surface of activated NK cells. Measurement of gene expression levels was performed using cDNAs prepared from the same concentration of total RNAs derived from the same number of peritoneal cells collected from each animal. Meanwhile, a FACS-based population analysis of peritoneal cells did not reveal any difference in the NK cell count between groups. In conclusion, although the NK activity itself was not significantly enhanced upon administration of *Lactobacillus gasseri* OLL2809, the gene-level analysis suggested enhanced activity of individual cells.

Accordingly, the preventive/improving agents of the present invention, which comprise *Lactobacillus gasseri* OLL2809, were demonstrated to have an inhibitory effect on the growth of endometrial lesions.

[Example 2]

In Example 2 below, the effect of the preventive/improving agents of the present invention in improving menstrual cramping and dysmenorrhea in endometriosis patients was examined.

Subjects were selected from patients who were diagnosed with endometriosis, and met the selection criteria but did not fit the exclusion criteria shown below. The diagnostic criteria for endometriosis were the same as described in "6<sup>th</sup> Obstetrics and Gynecology, 2003 (published by People's Medical Publishing House)". Specifically, a patient was determined to be affected when she had one of symptoms (1) to (5), and either physical sign (6) or (7).

Symptoms:

- (1) slowly-evolving secondary dysmenorrhea;
- (2) nonmenstrual lower-abdominal pain or pelvic pain;
- (3) discomfort or pain during intercourse;
- (4) intermittent diarrhea, constipation, anal tenesmus; and
- (5) infertility.

Physical signs:

- (6) nodules on rectouterine pouch or uterosacral ligament in the lower posterior wall of uterus; and
- (7) cystic masses attached around uterine adnexa.

Selection criteria:

- (1) patients aged 18 to 45 years; and
- (2) patients with regular menstrual cycles of  $28 \pm 3$  days.

Exclusion criteria:

- (1) patients who received hormone therapy within the past three months;
- (2) patients with a history of severe liver disorder, kidney disorder, cardiac infarction, or blood disorder;
- (3) patients with or suspected of having malignant tumor;
- (4) patients whose CA-125 level is 100 IU/ml or higher;
- (5) patients with endometrial lesions at peritoneal dissection sites;
- (6) patients who develop diarrhea after ingesting milk products;
- (7) patients being treated to improve an allergic disease;
- (8) patients taking pharmaceutical agents that may affect the test result (constipation-alleviating agents, gastrointestinal agents, etc.); and

(9) patients ingesting foods that may affect the test result (fermented milk and drinks containing lactic acid bacteria, etc.) four or more days a week.

Discontinuation and withdrawal criteria:

- 5 (1) subjects who voluntarily declined participation of the test;
- (2) subjects who were unable to follow the observation;
- (3) subjects who did not follow compliance norms;
- (4) subjects who developed severe complications or experienced adverse effects; and
- (5) subjects who have taken the tablets for clinical trial at a dose that is 80% or less, or 120% or
- 10 more of the predetermined dose.

Sixty-six persons were diagnosed with endometriosis and selected as subjects according to the above criteria. Of these, 62 persons completed the test. Finally, subjects to be analyzed were 33 persons in the placebo group and 29 persons in the active group.

- 15 Subjects who met the selection criteria and did not fit the exclusion criteria were randomly divided into placebo and active groups by a controller who does not directly participate in the test.

- In the active group, each subject was allowed to take two active tablets/day of a preventive/improving agent of the present invention containing *Lactobacillus gasseri* OLL2809,
- 20 while placebo tablets were given for 12 weeks in the placebo group (three sexual cycles). Before initiation of the test, and one, two, and three months after the start of ingestion, the subjects were monitored monthly through interview and such, and the assessment items described below were recorded.

Assessment was carried out by setting scores for each assessment item shown below.

- 25 The first assessment item: subjective pain level determined with visual analogue scale (VAS) and dysmenorrhea score during a menstrual period.

The second assessment item: sum of VAS outside the menstrual period and pelvic pain score.

The respective scores are shown in Table 1.

[Table 1]

Total dysmenorrhea scores (total scores for the level of dysmenorrhea and use of painkiller)

|                    | Level    | Condition                                                                                             | Score |
|--------------------|----------|-------------------------------------------------------------------------------------------------------|-------|
| Dysmenorrhea level | None     | None                                                                                                  | 0     |
|                    | Mild     | Slight interference with work (schoolwork or domestic work)                                           | 1     |
|                    | Moderate | Interference with work (schoolwork or domestic work) at the level where one wants to lie down to rest | 2     |
|                    | Severe   | So sick in bed that one cannot go to school or do housework for one or more days                      | 3     |
| Use of painkiller  | None     | None                                                                                                  | 0     |
|                    | Mild     | Painkiller was used for one day during the last or current menstrual period                           | 1     |
|                    | Moderate | Painkiller was used for two days during the last or current menstrual period                          | 2     |
|                    | Severe   | Painkiller was used for three or more days during the last or current menstrual period                | 3     |

Total pelvic pain scores outside the menstrual period (total scores for the level of pelvic pain and use of painkiller)

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|                    | Level    | Condition                                                                                                                   | Score |
|--------------------|----------|-----------------------------------------------------------------------------------------------------------------------------|-------|
| Dysmenorrhea level | None     | None                                                                                                                        | 0     |
|                    | Mild     | Slight interference with work (schoolwork or domestic work)                                                                 | 1     |
|                    | Moderate | Interference with work (schoolwork or domestic work) at the level where one wants to lie down to rest                       | 2     |
|                    | Severe   | So sick in bed that one cannot go to school or do housework for one or more days                                            | 3     |
| Use of painkiller  | None     | None                                                                                                                        | 0     |
|                    | Mild     | Painkiller was used one month ago for one day outside the menstrual period after menstruation due to pelvic pain            | 1     |
|                    | Moderate | Painkiller was used one month ago for two to six days outside the menstrual period after menstruation due to pelvic pain    | 2     |
|                    | Severe   | Painkiller was used one month ago for seven or more days outside the menstrual period after menstruation due to pelvic pain | 3     |

The level of pain caused by endometriosis was assessed based on VAS during and outside a menstrual period, with the left end on a 10-cm linear line defined as no pain and the right end defined as the worst pain experienced in the past.

As an objective assessment item for endometriosis, plasma CA-125 was measured before and three months after the start of ingestion.

As safety assessment items, blood test (erythrocyte count, leukocyte count, platelet count, and hemoglobin) and blood biochemistry test (blood glucose level, total protein, alanine aminotransferase (ALT), asparagine aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin,  $\gamma$ -transpeptidase, alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, uric acid, total cholesterol, triglyceride, high-density lipoprotein (HDL)) were carried out before and three months after the start of ingestion.

Materials to be tested were active tablets (Meiji Dairies Corporation) as a preventive/improving agent of the present invention containing *Lactobacillus gasseri* OLL2809 and placebo tablets. The active tablets were prepared by formulating 250-mg tablets with 50 mg of *Lactobacillus gasseri* OLL2809 powder which was obtained by heating a concentrated culture of *Lactobacillus gasseri* OLL2809 at 75°C for 60 minutes followed by lyophilization. The placebo tablets were formulated with dextrin instead of lyophilized bacterial powder.

Results of the blind test were reviewed before key opening, and withdrawal cases were determined based on the blind test data. This assessment was conducted in all subjects who accomplished the test.

Results are shown as mean  $\pm$  standard error (SE). The two groups were statistically analyzed with Student's t test or Mann-Whitney U test.  $P < 0.05$  was considered significant.

Background factors of the subjects, which were recorded before ingestion, are shown in Table 2. There is no significant difference between the two groups in any items.

[Table 2]

Background factors in each group before ingestion

|                                          | Placebo group  | Active group   | Significant difference |
|------------------------------------------|----------------|----------------|------------------------|
| Number of subjects                       | 33             | 29             | NS                     |
| Age (year)                               | $32.5 \pm 5.6$ | $31.6 \pm 4.9$ | NS                     |
| Married / Unmarried                      | 31 / 2         | 29 / 0         | NS                     |
| Age of menarche (year)                   | $12.9 \pm 1.2$ | $12.9 \pm 1.3$ | NS                     |
| Average length of menstrual period (day) | $5.3 \pm 0.9$  | $5.2 \pm 0.7$  | NS                     |
| Menstrual cycle (day)                    | $29.0 \pm 1.3$ | $28.9 \pm 1.7$ | NS                     |
| Menstrual cramping (yes/no)              | 33 / 0         | 29 / 0         | NS                     |
| Chronic pelvic pain (yes/no)             | 30 / 3         | 27 / 2         | NS                     |
| Pain during intercourse (yes/no)         | 27 / 6         | 25 / 4         | NS                     |

NS: No significant difference

5 The assessment items are specifically described below with reference to drawings. In the graphs shown in Figs. 3 and 5, closed circle represents the placebo group ( $n = 33$ ) and closed square represents the active group ( $n = 29$ ).

The time course of VAS is shown in Fig. 3. Before the start of ingestion, VAS was  $7.73 \pm 0.26$  in the placebo group and  $7.51 \pm 0.22$  in the active group; thus, there was no significant difference between the two groups. Meanwhile, two and three months after the start of ingestion of the test materials, significant differences in pain during menstruation were observed between the placebo and active groups ( $P < 0.05$  and  $P < 0.01$ , respectively). Specifically, the change in VAS up to three months after the start of ingestion was  $-3.28 \pm 0.36$  in the active group, and it was demonstrated to be significantly improved as compared to  $-2.00 \pm 0.29$  in the placebo group ( $P < 0.01$ , Fig. 4). The result described above revealed that the preventive/improving agent of the present invention (the active tablets described in this Example) is effective for easing menstrual pain.

The time course of dysmenorrhea score for the menstrual period is shown in Fig. 5. The score before the start of ingestion was  $3.45 \pm 0.20$  in the placebo group and  $3.14 \pm 0.17$  in the active group; thus, there was no significant difference between the two groups. The score of the active group after three months was significantly lower than that of the placebo ( $P < 0.05$ ). Furthermore, the change in dysmenorrhea score up to three months after the start of ingestion was  $-1.03 \pm 0.16$  in the placebo group and  $-1.44 \pm 0.17$  in the active group, showing an improving trend ( $P < 0.1$ , Fig. 6). Specifically, the preventive/improving agent of the present

invention (the active tablets described in this Example) was demonstrated to be effective in improving the dysmenorrhea score during menstruation.

There were no significant differences between the groups in blood erythrocyte count, leukocyte count, platelet count, and hemoglobin after ingestion. Furthermore, in each group, there was no difference between before and after ingestion in terms of blood glucose level, total protein, ALT, AST, LDH, total bilirubin,  $\gamma$ -transpeptidase, ALP, BUN, creatinine, uric acid, total cholesterol, triglyceride, and HDL. Thus, the preventive/improving agent of the present invention was demonstrated to be safe without any side effects.

As described above, it was demonstrated that *Lactobacillus gasseri* OLL2809 improves menstrual pain and dysmenorrhea caused by endometriosis and is effective for improving the QOL of patients. Furthermore, since no adverse effect was observed, *Lactobacillus gasseri* OLL2809 is expected to serve as a highly safe preventive/improving agent effective for improving the QOL of endometriosis patients

#### Industrial Applicability

As described above, agents of the present invention for preventing and/or improving endometriosis, which contain a certain type of lactic acid bacteria, in particular, lactic acid bacteria of the genus *Lactobacillus*, produce a unprecedentedly significant effect of improving endometriosis, menstrual cramping and dysmenorrhea specifically by improving one's own immunity through enhancement of the NK activity. Thus, the preventive/improving agents of the present invention are effective in preventing and improving such diseases as well as for improving the QOL of endometriosis patients.

Furthermore, the preventive/improving agents of the present invention can be orally ingested, and thus used simply to prevent or improve endometriosis. Moreover, since the preventive and/or improving agents of the present invention produce no side effects and are highly safe, they are convenient for patients to take and enable prevention and improvement of endometriosis without imposing burden on patients' life. In addition, since the preventive/improving agents of the present invention have the characteristics described above, they can be easily made into food and beverage compositions for further convenient ingestion. Furthermore, the preventive/improving agents of the present invention have an extraordinary effect in relieving pain which is characteristic of endometriosis, and in particular they are effective for improving the QOL of endometriosis patients.

## CLAIMS

1. Use of an agent comprising a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.  
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2. The use of claim 1, wherein the *Lactobacillus gasseri* bacterium is *Lactobacillus gasseri* strain OLL2809.
- 10 3. Use of a food or beverage composition comprising a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.
- 15 4. The use of claim 3, wherein the *Lactobacillus gasseri* bacterium is *Lactobacillus gasseri* strain OLL2809.
5. The use of claim 4, wherein the food or beverage composition is a fermented milk food or beverage composition.
- 20 6. The use of claim 5, wherein the fermented milk food or beverage composition is yogurt.
- 25 7. Use of a composition comprising an effective amount of a *Lactobacillus gasseri* bacterium in the manufacture of a medicament for either or both prevention and improvement of endometriosis.

8. The use of claim 7, wherein the *Lactobacillus gasseri* bacterium is *Lactobacillus gasseri* strain OLL2809.

9. The use of claims 7 or 8, wherein the medicament is administered at least 2  
5 mg/kg/day.