

DESCRIPTION

ANTI-STRESS AGENT

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Technical Field

The present invention relates to stress-reducing agents comprising *Lactobacillus gasseri* as an active ingredient. The present invention also relates to stress-reducing agents additionally comprising α -lactalbumin (α LA) as an active ingredient.

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Background Art

In recent years, chronic (accumulated) stress due to insufficient reduction of short-term stress or light stress loads over a long period of time has become an issue. Although such chronic stress might be low in intensity, the accumulation will lead to mental and physical effects greater than those of highly intense short-term stress, and death from overwork apparently caused by chronic stress has become a social problem.

Stress is defined as a biological reaction against stimuli that impair the homeostasis of a living body. Causes of stress are called stressors, and are classified according to the types of external stimuli into physical stressors (cold, noise, radiation, etc.), chemical stressors (oxygen, drugs, etc.), biological stressors (inflammation and infection), and psychological stressors (anger, anxiety, and such).

There are mainly two types of systems for transmitting orders from the brain to the body to deal with stressors. One of them is the hypothalamic-sympathetic-adrenal-medullary system, (sympathetic-adrenal-medullary axis or SAM axis) known to be an emergency response to stress. The other is hypothalamic-pituitary-adrenocortical system (Hypothalamo-Pituitary-Adrenal axis or HPA axis), known to be a non-specific stress response to various demands from the external environment.

The HPA axis which is a non-specific stress response, first involves secretion of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH causes the pituitary to secrete the adrenocorticotrophic hormone (ACTH). ACTH promotes secretion of glucocorticoids such as cortisol (mainly corticosterone in rats) from the adrenal cortex. These glucocorticoids are generally called stress hormones.

Many of the actions of glucocorticoids are involved with the physical response to stress (activation of energy metabolism and enhancement of cardiac function), and play important roles in maintenance of homeostasis. On the other hand, glucocorticoids are also known to have bad effects on the living body such as lowering the function and proliferative capacity of immune

cells. For example, continuous secretion of glucocorticoids due to chronic stress causes secondary symptoms such as decrease in the ability to protect against infection (decrease in immune function), emotional disorders such as depression and anxiety, and sleep disorders such as insomnia.

5 Recently in focus is the "brain-gut axis". Brain-gut axis refers to transmission of various signals produced in the intestine to the nervous system and to the central nervous system *via* commonly shared receptors and signaling substances, such as hormones and cytokines which influence the course of this information processing (Non-patent Document 1). More specifically, stimulation from the intestine by intestinal bacteria, probiotics, and such may
10 attenuate sensitivity to stress and reduce the stress response.

 The intestine is controlled by the brain through the autonomic nerve, and when the brain recognizes stress, that information is transmitted to the intestine by the autonomic nerves, or by signaling substances such as endocrine hormones and cytokines, and this induces abnormalities in peristaltic movement and such. On the other hand, when symptoms such as constipation or
15 diarrhea take place in the intestine, that is communicated to the brain as stress. This two-way signal transduction mechanism between the brain and the intestine is called the "brain-gut axis" (Non-patent Document 1). Recently, influence of intestinal bacteria on signal transduction from the intestine to the brain is becoming elucidated with regard to this brain-gut axis. Accordingly, stimulation from the intestine by probiotics such as lactic acid bacteria may
20 attenuate sensitivity toward stress and reduce the stress response.

 For example, Non-patent Document 2 discloses that administration of *Lactobacillus johnsonii* La1 to rats enhances the activity of the parasympathetic nerves that govern the function of the stomach and digestive tract. Patent Document 1 discloses that *Lactobacillus paracasei* *subsp. paracasei* lactic acid bacteria FERM-19169 inhibits stress by suppressing excessive
25 production of cytokines such as TNF- α , IFN- γ , and IL-6; effectively defending against infection by pathogens; suppressing excessive inflammatory response; and suppressing deterioration due to diseases and such to a minimum. Furthermore, Patent Document 2 discloses that sour milk produced by fermenting livestock milk using lactic acid bacteria belonging to the genus *Lactobacillus* which includes *Lactobacillus helveticus* ATCC8205, has effects of preventing or
30 reducing mental and physical symptoms caused by stress.

 However, Non-patent Document 2 features an approach targeting acute stress such as gastrointestinal injury and inflammatory response. While Patent Document 1 examines mental and physical symptoms, it only confirms effects on transitory stress, and it is unclear whether there are sufficient effects on chronic stress which is of concern in modern society.

35 So far, the inventors have found that a probiotic lactic acid bacteria, *Lactobacillus gasseri* OLL2809, has the action of stimulating the immune response of a living organism, such

as strongly inducing IL-12 from mouse splenocytes. The inventors have revealed that the lactic acid bacteria act on the host's immune response mechanism and are effective for immediate allergic diseases (Patent Document 3), delayed hypersensitivity (Patent Document 4), and alleviation of endometriosis (Patent Document 5). IL-12 induces the Type-1 helper T cells (Th1) and also has the action of enhancing the cytotoxic activity of NK cells (Patent Document 3). NK cells play important roles in natural immunological mechanisms such as defense against viral infection and elimination of tumor cells.

Furthermore, the inventors have elucidated that α LA has the action of inhibiting cyclooxygenase-2 activity to suppress production of prostaglandins from arachidonic acid and also suppress IL-6 production. Due to these actions, α LA has been found to contribute in improving QOL of healthy women of reproductive age by alleviating pain during the menstrual period caused by inflammation (Patent Document 6). Prostaglandin is one of the inflammatory mediators and brings about various inflammatory symptoms such as pain.

However, there is no description at all regarding whether the above-mentioned *Lactobacillus gasseri* OLL2809 and α LA have effects on reduced immune functions that occur under the influence of glucocorticoids due to stress that has become chronic (chronic stress); effects on psychological stress such as anxiety and fatigue; and effects under conditions of being subjected to chronic stress.

[Prior Art Documents]

[Patent Documents]

[Patent Document 1] Japanese Patent Application Kokai Publication No. (JP-A) 2010-96751 (unexamined, published Japanese patent application)

[Patent Document 2] JP-A (Kokai) H10-045610

[Patent Document 3] International Publication No. WO2006/093022 pamphlet

[Patent Document 4] International Publication No. WO2012/086150 pamphlet

[Patent Document 5] International Publication No. WO2012/073924 pamphlet

[Patent Document 6] International Publication No. WO2008/140041 pamphlet

[Non-patent Documents]

[Non-patent Document 1] Aziz Q, Thompson DG., Gastroenterol, 114: 559-578 (1998).

[Non-patent Document 2] Tanida M, Yamano T, Maeda K, Okumura N, Fukushima Y, Nagai K., Neurosci Lett, 389:109-114 (2005).

Summary of the Invention

[Problems to be Solved by the Invention]

As discussed above, it is known that stress-suppressing agents known to date improve gastrointestinal disorders and stimulation of the immune system, but their actions toward stress,

particularly various symptoms accompanying chronic stress, are not at all known. Therefore, an objective of the present invention is to provide stress-reducing agents that adjust physical and psychological conditions by inhibiting decrease in immune function and excessive response of the HPA axis due to chronic stress, and by reducing psychological stress represented by a feeling of fatigue, anxiety and/or tension, wherein the agents
 5 can be used regularly as a pharmaceutical without side effects, and can be taken in easily as foods, drinks, and the like.

[Means for Solving the Problems]

The present invention demonstrates that *Lactobacillus gasseri* inhibits decrease in immune function
 10 and excessive response of the HPA axis due to chronic stress and has the action of reducing psychological stress such as anxiety and/or tension, which were hitherto unknown properties, and that *L. gasseri* is useful as a stress-reducing agent. Furthermore, use of α LA in combination with *Lactobacillus gasseri* was found to reduce daily fatigue that can be felt as one of the symptoms of chronic stress, and was found to be useful as a stress-reducing agent.

15 The basic characteristics and various embodiments of the present invention are listed below:

- [1] a stress-reducing agent comprising *Lactobacillus gasseri* as active ingredient;
- [2] the agent of [1], wherein the stress is chronic stress;
- [3] the agent of [1] or [2], which further comprises α -lactalbumin (α LA) as active ingredient;
- [4] *Lactobacillus gasseri* which has a stress-reducing action;
- 20 [5] the *Lactobacillus gasseri* of [4], which has the action of suppressing increase in stress hormone in blood;
- [6] the *Lactobacillus gasseri* of [4] or [5], which has the action of suppressing increase in adrenocorticotrophic hormone in blood and/or glucocorticoid in blood;
- [7] a composition comprising 1×10^7 or more of *Lactobacillus gasseri* cells and 40 wt% or more of α LA;
- [8] the agent of any one of [1] to [3], wherein the *Lactobacillus gasseri* is *Lactobacillus gasseri* OLL2809
 25 deposited under the accession number NITE BP-72;
- [9] the *Lactobacillus gasseri* of any one of [4] to [6], which is deposited under the accession number NITE BP-72;
- [10] the composition of [7], wherein the *Lactobacillus gasseri* is *Lactobacillus gasseri* OLL2809 deposited under the accession number NITE BP-72; [10a] In one aspect the present invention refers to a non-therapeutic
 30 method of reducing stress, comprising administering *Lactobacillus gasseri* and α -lactalbumin (α LA) to the subject in need thereof, wherein the *Lactobacillus gasseri* is *Lactobacillus gasseri* OLL2809 deposited under the accession number NITE BP-72. [11] Also disclosed herein is a method of reducing stress, which uses *Lactobacillus gasseri*;
- [12] the method of [11], wherein the stress is chronic stress; [13] the method of [11] or [12] which additionally
 35 uses α LA; and [14] the method of any one of [11] to [13], wherein the *Lactobacillus gasseri* is *Lactobacillus*

gasseri OLL2809 deposited under the accession number NITE BP-72.

[Effects of the Invention]

The present inventors have been examining the effects of *Lactobacillus gasseri* and α LA for some time. As a result, they performed water immersion-induced stress tolerance test on *Lactobacillus gasseri* OLL2809-administered rats and discovered that *Lactobacillus gasseri* OLL2809 administration slows down increase in stress hormones such as corticosterone in blood, and inhibits decrease in NK activity due to the stress load. Furthermore, tests in which the subjects were Athletes performing daily vigorous exercise showed that *Lactobacillus gasseri* OLL2809 suppresses decrease in NK activity due to vigorous exercise and reduces psychological stress such as anxiety and tension.

Furthermore, the present inventors discovered through tests using as subjects Athletes performing daily vigorous exercise that the everyday feeling of fatigue is relieved by combined intake of OLL2809 and α LA. This effect was proven objectively by significant decrease in the levels of Transforming Growth Factor (TGF)- β 1 and derivatives of Reactive Oxygen Metabolites (dROMs) in serum. The above-mentioned results were found to be useful for adjusting physical conditions, for example, for excellent athletic performance by Athletes.

Brief Description of the Drawings

Figure 1 shows graphs indicating the concentrations of stress hormones (ACTH and corticosterone) in the blood of rats after the water immersion-induced stress tolerance test. The results are shown as the mean value + standard deviation. The white, gray, and black bars indicate the normal group, stress group, and *Lactobacillus gasseri* OLL2809 group, respectively. Evaluations were performed on the 14th day of testing. a, b show that there is a significant difference between different signs (One-way ANOVA, Fisher's PLSD test, $P < 0.05$).

Figure 2 is a graph showing the concentration of an oxidative stress marker (8-OHdG) in the blood of rats after the water immersion-induced stress tolerance test. The results are shown as the mean value + standard deviation. The white, gray, and black bars indicate the normal group, stress group, and *Lactobacillus gasseri* OLL2809 group, respectively. Evaluations were performed on the 14th day of testing. a, b show that there is a significant difference between different signs (One-way analysis of variance (One-way ANOVA), Fisher's PLSD test, $P < 0.05$).

Figure 3 is a graph showing the splenic NK activity after the water immersion-induced stress tolerance test on rats. The results are shown as the mean value + standard deviation. The white, gray, and black bars indicate the normal group, stress group, and *Lactobacillus gasseri* OLL2809 group, respectively. Evaluations were performed on the 14th day of testing.

a, b show that there is a significant difference between different signs (One-way ANOVA, Fisher's PLSD test, $P < 0.05$).

Figure 4 shows graphs indicating the effects of *Lactobacillus gasseri* OLL2809 and α LA upon evaluation of conditions of mood and feelings using the Profile of Mood States (POMS). In the graph, the black dots indicate the results from each subject, and the bars indicate the average values. Evaluations were performed before intake of the test substance, and after four weeks of intake. #, *: $P < 0.1, 0.05$. Comparison between before intake of the test substance and after four weeks of intake shows significant difference (two-way repeated measures analysis of variance (Two-way rm ANOVA), Student's paired t test (Bonferroni corrected)).

Figure 5 is a graph indicating the effects of *Lactobacillus gasseri* OLL2809 and α LA on the feeling of fatigue before and after exercise load examined using the visual analogue scale (VAS). The results are shown as the mean value $\pm$ standard deviation. The white, gray, and black bars indicate placebo, OLL2809 alone, and OLL2809 + α LA, respectively. Evaluations were performed before and after exercise prior to intake of the test substance, and before and after exercise after four weeks of intake (Student's paired t-test).

Figure 6 is a graph showing the effects of *Lactobacillus gasseri* OLL2809 and α LA on decrease in NK cytotoxic activity caused by a one-hour exercise on a bicycle ergometer. The results are shown as the mean value $\pm$ standard deviation. The white and black bars indicate before and after exercise, respectively. *: $P < 0.05$, comparison between before and after exercise shows significant difference (Two-way rm ANOVA, Student's paired t test (Bonferroni corrected)). n.s.: no significant difference.

Mode for Carrying Out the Invention

In the present invention, stress refers to phenomena of biologically tense states caused by stressors such as physical stressors (cold, noise, radiation, etc.), chemical stressors (oxygen, drugs, etc.), biological stressors (inflammation and infection), and psychological stressors (anger, anxiety, and such).

Stress-reducing agents of the present invention can be used to alleviate stress. The stress-reducing agents of the present invention have no side-effects as pharmaceuticals do, so can be used regularly, and may also be taken easily as foods, drinks, and such.

For example, stress-reducing agents of the present invention can suppress the decrease in immune function and such caused by stress. In particular, the stress-reducing agents of the present invention have the function of decreasing acute stress and chronic stress due to the above-mentioned stressors, and can be used favorably in the case of chronic stress where there is continuous stress. For example, use of the stress-reducing agents of the present invention for

chronic stress enables inhibition of decrease in NK activity caused by glucocorticoids such as cortisol, corticosterone, and cortisone continuously secreted in response to chronic stress, and also enables reduction of the psychological feeling of fatigue.

5 The stress-reducing agents of the present invention can improve or prevent secondary symptoms during chronic stress, such as decrease in immune function, increase in psychological stress, delayed wound healing, or metabolic disorders.

10 The stress-reducing agents of the present invention can alleviate indisposition caused by stress. The stress-reducing agents of the present invention can reduce, for example, mental stress (psychological stress) such as anxiety, tension, and feeling of fatigue; everyday feeling of fatigue (chronic fatigue); or chronic fatigue syndrome. Furthermore, the stress-reducing agents of the present invention adjust physical conditions by inhibiting increase in stress hormones caused by stress stimuli such as vigorous exercise, reducing increase in oxidative stress markers, and inhibiting decrease in immune function due to stress load. Therefore, the stress-reducing agents of the present invention can be used with the objective of maintaining homeostasis, 15 conditioning, and adjusting physical conditions. In one example, the stress-reducing agents of the present invention can be used as a sports conditioning agent that reduces physical fatigue and increases immune response so that athletes can demonstrate excellent athletic performance or for preventing or reducing overtraining.

20 The stress-reducing agents of the present invention comprise *Lactobacillus gasseri*, or *Lactobacillus gasseri* and α LA as the active ingredient. The stress-reducing agents of the present invention comprise *Lactobacillus gasseri* as the lactic acid bacteria. As long as there are effects of reducing stress and effects of reducing chronic fatigue, and as long as they are *Lactobacillus gasseri*, any *Lactobacillus gasseri* may be used.

25 In the present invention, *Lactobacillus gasseri* which is used particularly favorably is *Lactobacillus gasseri* OLL2809 which was isolated independently by the present inventors from adult human feces. This bacterial strain has been deposited, with the Incorporated Administrative Agency, National Institute of Technology and Evaluation, Patent Microorganisms Depositary (2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) under the accession number NITE P-72 on February 1, 2005 (day of the original deposit), which was 30 transferred from the original deposit to a deposit under the Budapest Treaty on January 18, 2006, and deposited under the accession number NITE BP-72.

Media suitable for lactobacilli in general may also be used as a medium for culturing *Lactobacillus gasseri* which is the active ingredient of the stress-reducing agents of the present invention. Thus, any medium containing, other than the main carbon source, a nitrogen source, 35 inorganic substances, and other nutrients to a sufficient extent can be used. Depending on the assimilating ability of the bacteria used, 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 protein 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 of *Lactobacillus gasseri* which is the active ingredient of the stress-reducing agents of the present invention 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 nitrogen 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 10 40°C; however, other temperature conditions may be used, so long as they allow the *Lactobacillus gasseri* to grow. The pH of the medium is preferably maintained between 4.0 and 7.0 during culture; however, other pH conditions may be used, so long as the pH allows growth of *Lactobacillus gasseri*. Moreover, culture can also be carried out under batch culture 15 conditions. In general, the duration of culture preferably ranges between 10 and 24 hours; however, other culture periods may be used, so long as they permit growth of *Lactobacillus gasseri*.

Lactobacillus gasseri which is the active ingredient of the stress-reducing agents of the present invention may be live bacteria or dead bacteria, and wet or dry bacteria. Herein, 20 "*Lactobacillus gasseri*" refers to both the bacterial strain and bacterial cells of *Lactobacillus gasseri*.

Lactobacillus gasseri which is the active ingredient of the stress-reducing agents of the present invention may also be a "processed product". In the present invention, "processed products" of *Lactobacillus gasseri* are not particularly limited, and examples include a 25 suspension of *Lactobacillus gasseri* after culture termination (suspension solution), a culture which is the culturing solution as it is (containing the bacterial cells, culture supernatant, and medium components); fermentation product (product obtained by fermenting raw milk, powdered skim milk, or soymilk); product subjected to heat treatment; disinfected or sterilized product produced by disinfection or sterilization by heating, radiation, or such; homogenate 30 produced by ultrasonication and such; concentrate; product in the form of a paste; dried product (spray-dried product, freeze-dried product, vacuum-dried product, drum-dried product, etc.); liquid product; and diluted product. The bacterial cell concentration of *Lactobacillus gasseri* which is the active ingredient of the stress-reducing agents of the present invention is not particularly limited, but it is preferably 1×10^6 cells/g or more as a concentrated solution, and 1×10^7 cells/g or more as a dried product. 35

The stress-reducing agents of the present invention further comprise α LA in addition to

Lactobacillus gasseri as the active ingredient. α LA of the present invention can be of any origin, but is preferably derived from humans and/or non-human mammals (bovine, sheep, goat, horse, etc.), and is more preferably human or bovine α LA. The amino acid sequences and nucleotide sequences of such α LA are already known, and are registered in databases such as EMBL, DDBJ, NCBI, and such. For example, bovine α LA is registered in DDBJ under
 5 ACCESSION No. J05147.

α LA can be produced from milk and such of mammals including humans, bovine, sheep, and goat, by known techniques such as ammonium sulfate precipitation ("Saishin Kaiko Nyugyo Gijyutsu Binran (Latest Revised Handbook of Dairy Technology)" Rakuno Gijyutsu Fukyu
 10 Gakkai (Society for spreading dairy technology), (1975) pp. 122-125), ultrafiltration (JP-A (Kokai) H05-268879), and ion exchange (Japanese Patent No. 2916047). The α LA content in cow's milk differs somewhat depending on the place of production, feed, and such, but Japanese cow's milk usually contains 1.2 g/kg, and if cow's milk is used as the raw material for production, α LA can be prepared in large amounts. Furthermore, the production can be carried out by
 15 chemical synthesis based on the amino acid sequence and nucleotide sequence, or by genetic engineering techniques well known to those skilled in the art. More simply, commercially available α LA (for example, that manufactured by Davisco) may be used.

The stress-reducing agents of the present invention may be pharmaceuticals or foods for humans or animals, or they may be raw materials or formulations to be mixed with the
 20 pharmaceuticals or foods. Here, the foods include functional food, food for patients, and food for specified health uses aimed at alleviation of chronic stress, recovery from physical and/or psychological feeling of fatigue and maintenance of homeostasis, and with the concepts of conditioning and adjustment of physical conditions, where the aims and concepts are indicated when necessary.

When the stress-reducing agents of the present invention are used as pharmaceuticals, dosage forms can be selected depending on the purpose and administration route, and examples include tablets, coated tablets, pills, capsules, granules, powders, liquids, suspensions, emulsions, syrups, injections, suppositories, infusions, decoctions, and tinctures, and the pharmaceuticals are preferably used as oral agents or as parenteral agents such as nutritional agents for tube
 30 feeding.

These various formulations can be formulated using, in addition to the main agent, known auxiliary agents that can generally be used in the technical field of pharmaceutical formulation, such as fillers, expanders, excipients, binding agents, moisturizing agents, disintegrants, surfactants, lubricants, coloring agents, corrigents, solubilizing agents, suspending
 35 agents, and coating agents according to standard methods when necessary. Coloring agents, preservatives, perfumes, flavoring agents, sweeteners, and such as well as other pharmaceuticals

may also be included in the pharmaceutical formulations.

The stress-reducing agents of the present invention are effective for lowering physical fatigue and enhancing immune response in humans and animals through oral administration by itself or as a mixture with other components that may be used ordinarily in pharmaceuticals and foods, or by combined use with other microorganisms, compounds, or such having effects of lowering physical fatigue and enhancing immune response. Examples include administration in combination with antianxiety drugs (for example, chlordiazepoxide, oxazolam, or diazepam), hypnotics (for example, nitrazepam, estazolam, or haloxazolam), or vitamin preparations (for example, vitamin A, vitamin B1, vitamin B2, vitamin B6, niacinamide, pantothenic acid, vitamin B12, vitamin E, biotin, or vitamin C).

Since the stress-reducing agents of the present invention do not have side-effects and can be used regularly without problems, they may be used not only as pharmaceuticals but also as foods and drinks. Specifically, they may be in any food or drink form such as milk, soft drinks, milk beverages, fermented milk, yogurt, cheese, ice creams, bread, biscuits, crackers, confectionary, tablet candy, pizza crusts, prepared powdered milk, fluid diet, dietary supplements, beauty food, health food, food for the sick, food such as powdered milk for infants, food such as powdered milk for nursing women, nutritive foods, food for sport. As to the properties of the foods and drinks, the stress-reducing agents of the present invention can be provided in any of the forms ordinarily used for foods and drinks such as solid forms (powders, granules, etc.), paste forms, gel forms, jelly forms, liquid forms, and suspended forms.

The foods can be produced according to procedures common for ordinary food and drink compositions such as using *Lactobacillus gasseri* of the present invention and α LA as they are, or by mixing with other foods or food components.

Other food components are not particularly limited, and examples include water, proteins, sugars, lipids, vitamins, minerals, organic acids, organic bases, fruit juices, flavors, and such.

Examples of proteins include animal or plant proteins, such as whole powdered milk, powdered skim milk, partially-skimmed milk, casein, whey powder, whey proteins, whey protein concentrates, whey protein isolates, α -casein, β -casein, κ -casein, β -lactoglobulin, lactoferrin, soy bean proteins, chicken egg proteins, and meat proteins as well as their hydrolysates.

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 oils, 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.

5 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.

Two or more of these components can be used in combination, and synthetic products and/or foods containing many of them may also be used.

10 The amount of intake of the stress-reducing agents of the present invention cannot be generalized since it differs depending on the gender, age, health condition, and such of the person taking the agent, but in the case of oral agents, the daily amount of the active ingredient *Lactobacillus gasseri* OLL2809 is, for example, 1×10^7 cells or more, preferably 1×10^8 cells or more, more preferably 1×10^9 cells or more, particularly preferably 1×10^{10} cells or more, and even more preferably 1×10^{11} cells or more. The daily amount of the active ingredient α LA is, 15 for example, 100 mg or more, preferably 250 mg or more, more preferably 500 mg or more, and particularly preferably 1000 mg or more. There is no particular upper limit for the amount of intake of these stress-reducing agents since *Lactobacillus gasseri* and α LA which are the active ingredients are present as ordinary food materials. However, if upper limits are to be given, they are for example, 1×10^{15} cells per day for *Lactobacillus gasseri* OLL2809, and 2000 mg/kg 20 per day for α LA. Furthermore, the number of intake per day may be once or multiple times. Herein, the term "intake" includes "administer".

The stress-reducing agents of the present invention can be administered at once or in portions to patients in need of treatment by a composition of the present invention, before meals, after meals, between meals, and/or before bedtime. The dose can be set individually depending 25 on the age and weight of the patient receiving the administration, and the purpose of administration.

The present invention also provides methods for regulating physical conditions, methods for reducing or alleviating chronic stress, as well as methods for suppressing increase of stress hormones in blood due to chronic stress, and methods for alleviating or reducing 30 psychological stress due to chronic stress, which comprise eating and drinking, or administering or feeding to mammals an effective amount of the *Lactobacillus gasseri* of the present invention, or *Lactobacillus gasseri* and α LA.

The effective amount of *Lactobacillus gasseri*, or *Lactobacillus gasseri* and α LA mentioned above means the same amount as the amount of intake of *Lactobacillus gasseri*, or 35 *Lactobacillus gasseri* and α LA in the above-mentioned stress-reducing agents.

Stress against which the methods of the present invention can be applied include all

phenomena of biologically tense states which are caused by stressors physiologically harmful to animals. Stress-reducing agents of the present invention are particularly useful against stress response mediated by the HPA axis.

Symptoms caused by stress include decrease in the ability to protect against infection, delay in wound healing, decrease in immune function, and metabolic disorders, and also circulatory impairments (for example, tightness in the chest, palpitations, arrhythmia), gastrointestinal disorders (for example, decreased appetite, stomach pain, nausea, gastric ulcers, constipation, diarrhea), stiff shoulders, headache, dizziness, anacatesthesia, eyestrain, general malaise, feeling of fatigue, psychological stress such as tension and anxiety, and depression.

Stress-reducing agents of the present invention are particularly useful against decrease in immune function, decrease in infection-protective ability, general malaise, feeling of fatigue, and psychological stress such as tension and anxiety.

The alleviation or reduction methods of the present invention lead to relief from the aforementioned stress, suppress increase in glucocorticoids, inhibit decrease in immune function accompanying stress, and alleviate the feeling of fatigue and psychological stress such as anxiety and tension, and thus, homeostasis in a living organism can be maintained and daily physical conditions can be regulated.

All prior art documents cited herein are incorporated herein by reference.

20 Examples

Herein below, the present invention will be explained more specifically based on the Examples, but it should not be construed as being limited thereto.

25 [Example 1] Examination of stress-reducing effects and immunosuppression-improving effects of *Lactobacillus gasseri* OLL2809

[Experiment Method]

Six-week old male Sprague Dawley rats (SPF) were habituated to individual housing, and then divided into three groups (Day 1): "normal group" not subjected to stress load; "stress group" subjected to stress load; and "OLL2809 group" subjected to stress load while administering *Lactobacillus gasseri* OLL2809. All groups were reared normally as during the habituation period, and then the stress group and OLL2809 group were reared for five days from Day 9 (Day 9 to Day 13) in a cage filled with water to a depth of 1.5 cm from the bottom. From Day 1 to Day 14, 40 mg / kg body weight / day of OLL2809 strain was administered to the OLL2809 group by forced intragastric administration using a gastric tube. Only water, which is the solvent, was similarly administered to the normal group and the stress group. The rats were autopsied on Day 14, and NK activity of splenocytes and stress hormones in the blood were

measured.

For NK activity of splenocytes, YAC-1 cells were used as the target cells and a method that uses flow cytometry was employed. 1×10^6 cells of rat splenic effector cells and 2.5×10^4 cells of Dio (3,3'-diiodo-4,4'-dimethoxy-5,5'-diphenylquinone perchlorate (Dio; Sigma-Aldrich, St. Louis, MO))-labeled YAC-1 target cells were mixed in a 96-well round bottom plate, and then cultured in a CO₂ incubator at 37°C for four hours. Fifteen minutes before end of culturing, 20 µL of 0.5 mg/mL propidium iodide (Sigma-Aldrich) was added to stain the dead cells. Death of YAC-1-target cells was analyzed by FACSCalibur flow cytometry and by using the CellQuest software (BD Biosciences, Franklin Lakes, NJ), and the death rate was regarded as the NK activity.

Regarding stress hormones in blood, three were measured: adrenocorticotrophic hormone (ACTH); corticosterone; and 8-OHdG. They were measured using kits: ACTH (Rat) EIA KIT (Phoenix Pharmaceuticals, Burlingame, CA) for ACTH; Corticosterone EIA Kit (Enzo Life Science, Farmingdale, NY) for corticosterone; and high sensitive 8-OHdG Check (Nikken Seil, Shizuoka) for 8-OHdG.

[Experiment Results]

Measurement of corticosterone and ACTH which are substances that transmit stress response through the HPA axis showed that they are increased significantly in the stress group than in the normal group (Fig. 1). On the other hand, while these values increased due to stress load in the OLL2809 group than in the normal group, the increases were not significant. Furthermore, similar changes were also indicated by 8-OHdG which is an oxidative stress marker (Fig. 2).

When splenic NK activities were measured, significant decrease was observed in the stress group compared to the normal group (Fig. 3). On the other hand, NK activity was significantly enhanced in the OLL2809 group compared to the stress group, and the activity had recovered to the same level as that of the normal group. This change was opposite to that of corticosterone. The results revealed that intake of the OLL2809 strain maintains NK activity which decreases due to stress at a high level.

Accordingly, these results revealed that administration of the OLL2809 strain reduces the stress response mediated by the HPA axis.

[Example 2] Examination of condition-improving effects of *Lactobacillus gasseri* OLL2809 and αLA on athletes

(1) Subjects

Healthy university students satisfying the following criteria were registered as subjects

(n = 44) of the clinical studies: (i) healthy Japanese male whose age is 30 or younger when obtaining consent; and (ii) performing exercise that requires endurance on a daily basis (five days a week or more).

Furthermore, those that do not conflict with the following exclusion criteria were chosen as the subjects. Exclusion (suspension and/or omission) criteria: (i) subjects who declined to continue the study by free will; (ii) those who developed allergic diseases such as pollinosis during the study; (iii) those who had difficulties in continuing the study due to development of some disease; (iv) those found to be not keeping compliance with or not following the instructions of the physician directing the study or the person in charge of performing and managing the study; and (v) those whose number of times of non-intake reached 10% of the scheduled number of times of intake during the period for test substance intake.

When performing this study, details of the study were explained by the physician in charge of the clinical trial to all members of the subject group, and a written consent was obtained from everyone. When the subject was under the age of 20, consent was obtained from the subject himself and his guardian. This study was conducted in compliance with the Helsinki Declaration, and considering the safety of the subjects as the first priority. This study was performed following approval by the Meiji Institutional Review Board.

(2) Study procedure

A double-blind, randomized, placebo-controlled trial (three-group, parallel, comparative trial) was performed. After one week of preliminary observation period, the subjects were divided into three groups: placebo group; the group with intake of *Lactobacillus gasseri* OLL2809 alone (hereinafter, referred to as the OLL2809 group), and the group with intake of α LA in addition to *Lactobacillus gasseri* OLL2809 (hereinafter, referred to as the OLL2809 + α LA group). Each subject was made to take placebo tablets or test substance-containing tablets for four weeks. Examinations were performed before and after intake of the test substance, and exercise load, questionnaire, and blood tests were performed as follows. Post-exercise blood test was performed approximately one hour after completion of an hour-long exercise.

[Exercise Load]

Using a bicycle ergometer with a magnetic breaking system, subjects were made to perform one hour of bicycle ergometer exercise at an intensity of 70% of the predicted maximum heart rate reserve (70% HR reserve) maintained throughout the exercise. 70% HR reserve was calculated by Karvonen formula shown below:

$$70\% \text{ HR reserve} = [(220 - \text{age}) - \text{resting heart rate}] \times 0.7 + \text{resting heart rate}$$

The heart rate was monitored every five minutes throughout the exercise test.

[Evaluated Points]

[i] POMS

5 The POMS questionnaire was filled out for a total of two times: during the preliminary observation period, and before exercise following the four weeks of intake. Each score was summed up, and the following six subscales were scored: T-A (tension-anxiety), D (depression-dejection), A-H (anger-hostility), V (vigor), F (fatigue), and C (confusion). The sums of raw scores were converted to T scores according to the POMS manual to perform
10 parametric statistical analysis (Yokoyama K, Araki S. POMS Japanese manual. Kaneko Syobo, Tokyo, 1994 (In Japanese)).

[ii] VAS for fatigue

15 VAS for fatigue was used to measure fatigue of the subjects. A total of four evaluations were performed: before and after exercise load prior to intake, and before and after exercise load after four weeks of intake. In the VAS, the right end was "feeling no fatigue at all" and the left end was "strongest fatigue ever experienced", and the degree of fatigue was evaluated on a 100-mm straight line. Subjects were asked to place a mark on the line at a position that best describes what the subject himself recognizes as his current state. Next, VAS
20 fatigue score was determined by measuring the distance (mm) from the left end of the line to the mark.

[iii] NK activity

25 In each of the two observation periods before and after intake, blood samples were collected twice, that is before and after the exercise load, for a total of four times, and measurements of NK activities were performed at BML Co.

[iv] Blood test and biochemical examination of blood

30 Blood test (red blood cell count, white blood cell count, platelet count, and hemoglobin) and biochemical examination of blood (blood glucose level, total protein, alanine aminotransferase (ALT), asparagine aminotransferase (AST), lactic acid dehydrogenase (LDH), total bilirubin, γ -transpeptidase, alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, uric acid, total cholesterol, triglyceride, and high-density lipoprotein (HDL)) were performed before exercise prior to intake and after four weeks of intake. These were evaluated
35 mainly as safety indicators.

Furthermore, TGF- β 1 and dROMs were evaluated by performing a total of four blood

collections, corresponding to the two times before and after the exercise load in each of the two observation periods before and after intake.

(3) Production of test substances

5 Test substances were produced at Meiji Co. Ltd.

Freeze-dried *Lactobacillus gasseri* OLL2809 powder was produced by inoculating a medium whose major ingredients are whey and lactose (whey degradation medium) with a preculture solution of *Lactobacillus gasseri* OLL2809 at 1%, and culturing the cells for 18 hours under a stream of nitrogen on the surface at 37°C while setting the lower limit of pH to 4.5
10 (automatic adjustment using 6 mol/L NaOH). Thereafter, the culture solution was concentrated 20-fold, and this was subjected to heat treatment at 85°C for 60 minutes. After cooling, this was freeze-dried and the dried substance was crushed to produce the freeze-dried *Lactobacillus gasseri* OLL2809 powder.

The OLL2809 tablet taken by the OLL2809 group was produced by mixing 16.7 mg of
15 the freeze-dried *Lactobacillus gasseri* OLL2809 powder with excipients and such, so that each tablet was 500 mg. The OLL2809 + α LA tablet taken by the OLL2809 + α LA group was produced by mixing 16.7 mg of the freeze-dried *Lactobacillus gasseri* OLL2809 powder and 150 mg of α LA with excipients and such, so that each tablet was 500 mg. The placebo tablet taken by the placebo group was produced by mixing dextrin, instead of the freeze-dried *Lactobacillus*
20 *gasseri* OLL2809 powder and α LA, with excipients and such so that each tablet was 500 mg. They were produced so that all of the tablets were not distinguishable from their appearance, color, or taste.

(4) Amount of intake of the test substance

25 In all groups, each individual was made to continuously intake the tablets to be taken at two tablets per intake, three times a day (morning, noon, night) for four weeks. That is, the amount of daily intake by the OLL2809 group was 100 mg of the freeze-dried *L. gasseri* OLL2809 powder (5×10^{10} cells), and the amount of intake by the OLL2809 + α LA group was 100 mg of the freeze-dried *Lactobacillus gasseri* OLL2809 powder (5×10^{10} cells) and 900 mg
30 of α LA.

(5) Statistical Analysis

Blinded study results were reviewed before unblinding, and how dropped cases should be handled was determined based on the blinded study data. The evaluations were carried out
35 on all subjects who completed the study.

The results are shown as the mean value $\pm$ standard deviation. Background factors of

the subjects were analyzed by one-way ANOVA. Investigation of the feeling of fatigue by the POMS and VAS methods, and investigation of NK activity were performed by two-way repeated-measures (rm) ANOVA using two parameters, a test substance parameter and an observation period parameter before or after intake (for investigation of the feeling of fatigue and NK activity, the difference before and after exercise in each of the observation periods were used). When found to be significant, post hoc analysis was performed, and comparison before and after intake within the group was performed by Student's paired t-test (Bonferroni corrected). Statistical significance was accepted at $P < 0.05$.

10 (6) Results

[Background factor of the subjects]

There were 44 participants in the study (14 individuals in the placebo group, 15 individuals in the OLL2809 group, and 15 individuals in the OLL2809 + α LA group), and all completed the study period. There were no cases of suspension and omission due to violation of compliance or adverse events. Ultimately all subjects were selected for analyses. The background factors of the subjects recorded prior to intake are shown in Table 1. In all of the points, no significant differences were observed among the three groups.

Table 1

20 Background factors of the subjects

	Placebo	OLL2809	OLL2809 $\pm$ α LA	Difference between groups
Number of subjects	14	15	15	n.s.
Age (years old)	20.2 $\pm$ 1.1	19.8 $\pm$ 0.9	19.9 $\pm$ 0.9	n.s.
Height (cm)	171.1 $\pm$ 4.8	171.9 $\pm$ 5.0	171.3 $\pm$ 4.6	n.s.
Body weight (kg)	65.0 $\pm$ 5.2	64.4 $\pm$ 5.3	64.1 $\pm$ 5.9	n.s.

The results are shown as the mean value $\pm$ standard deviation.

n.s.: no significant difference (one-way ANOVA)

[POMS]

Results of POMS are shown in Table 2 and Fig. 4. Upon comparison of values prior to intake and the values four weeks later within each group, with regard to "tension-anxiety", no significant difference was observed in the placebo group where the values prior to intake and four weeks later were 44.9 ± 9.9 and 43.0 ± 6.0 , respectively. However, significant decrease was observed in the OLL2809 group where the values prior to intake and four weeks later were 43.6 ± 8.2 and 40.6 ± 6.9 , respectively ($P = 0.006$). Furthermore, a decreasing trend was observed in the OLL2809 + α LA group where the values prior to intake and four weeks later

were 42.6 ± 6.1 and 39.7 ± 6.3 , respectively ($P = 0.095$).

With regard to "fatigue", no significant difference between before and after intake was observed in both the placebo group and the OLL2809 group, however a decreasing trend was observed in the OLL2809 + α LA group alone where the values prior to intake and four weeks later were 43.8 ± 6.1 and 40.3 ± 5.2 , respectively.

From the above-mentioned results, OLL2809 was found to reduce psychological stress such as tension and anxiety, and OLL2809 with α LA was found to have effects of reducing daily fatigue felt during chronic stress.

Table 2

Effects of OLL2809 and α LA on Profile of Mood States (POMS) scores
POMS before and after intake of test substance

	Placebo		OLL2809		OLL2809 + α LA	
	Before intake	4 weeks later	Before intake	4 weeks later	Before intake	4 weeks later
Tension - anxiety	44.9 ± 9.9	43.0 ± 6.0	43.6 ± 8.2	$40.6 \pm 6.9^*$	42.6 ± 6.1	$39.7 \pm 6.3^{\#}$
Depression - dejection	47.0 ± 7.6	47.1 ± 6.9	44.8 ± 6.4	45.1 ± 5.1	44.9 ± 6.2	43.9 ± 7.2
Anger - hostility	46.5 ± 7.3	43.9 ± 4.9	45.3 ± 6.4	44.8 ± 7.3	42.6 ± 6.3	40.9 ± 5.2
Vigor	50.6 ± 10.9	$47.0 \pm 10.6^{\#}$	51.3 ± 8.1	$47.5 \pm 6.7^{\#}$	48.3 ± 6.1	$45.1 \pm 11.5^{\#}$
Fatigue	45.5 ± 8.3	43.6 ± 5.5	45.4 ± 4.9	42.9 ± 8.2	43.8 ± 6.1	$40.3 \pm 5.2^{\#}$
Confusion	46.3 ± 8.7	47.7 ± 6.2	44.8 ± 5.8	45.9 ± 4.7	46.7 ± 6.7	45.7 ± 4.5

The results are shown as the mean value $\pm$ standard deviation.

*, *: $P < 0.1$, 0.05 (comparison to before intake within the group by Student's paired t test)

[Evaluation of the feeling of fatigue caused by exercise load using the VAS method]

The results of evaluating the feeling of fatigue caused by exercise load using the VAS method are shown in Figure 5. Upon comparison of the feeling of fatigue before exercise before and after intake within each group, the OLL2809 + α LA group showed significant decrease in the VAS scores four weeks later ($P = 0.026$). This result matched the POMS result. Accordingly, when correlation analysis was performed on the values obtained by examination before exercise, significant positive correlation was found for the feeling of fatigue between the POMS and VAS methods ($r = 0.293$, $n = 88$, $P = 0.0053$). These results suggested that four weeks of test substance intake had reduced chronic feeling of fatigue in the OLL2809 + α LA group.

25

[NK cytotoxic activity]

Results of determining NK cytotoxic activity in peripheral blood before and after exercise performed prior to intake of the test substance, and peripheral blood NK activity before

and after exercise performed after four weeks of intake are shown in Figure 6.

Prior to the intake, NK activities decreased after one hour of exercise, and were $-12.4 \pm 11.0\%$, $-12.2 \pm 12.3\%$, and $-14.5 \pm 11.4\%$ in the placebo group, OLL2809 group, and OLL2809 + α LA group, respectively. On the other hand, four weeks later, the differences between before and after exercise were $-8.8 \pm 12.0\%$, $-2.7 \pm 14.2\%$, and $-4.3 \pm 10.2\%$ in the placebo group, OLL2809 group, and OLL2809 + α LA group, respectively.

Comparison before and after exercise load in each group showed that NK activity decreases significantly due to exercise load in all groups prior to intake of the test substance. On the other hand, four weeks later, the placebo group ($P = 0.016$) showed significant decrease due to exercise load similar to that prior to intake, whereas in the OLL2809 group ($P = 0.476$) and the OLL2809 + α LA group ($P = 0.125$), significant decrease in NK activity was not observed. More specifically, intake of the OLL2809 strain was found to relieve decrease in NK activity due to chronic stress which refers to exercise load.

15 [dROMs and TGF- β 1 in blood]

The results of evaluating dROMs and TGF- β 1 in peripheral blood are shown in Table 3. dROMs is an indicator that shows the degree of oxidative damage caused by reactive oxygen species and/or free radicals, or in other words, oxidative stress. When only the values of dROMs prior to exercise are compared, the values in the placebo group prior to intake and four weeks later were 303 ± 56 U.CARR and 298 ± 80 U.CARR, respectively, and did not show significant difference; however, the values in the OLL2809 group prior to intake and four weeks later were 298 ± 50 U.CARR and 283 ± 27 U.CARR, respectively, and showed a decreasing trend ($P = 0.089$). Furthermore, the values in the OLL2809 + α LA group prior to intake and four weeks later were 317 ± 58 U.CARR and 284 ± 53 U.CARR, respectively, and showed a significant decrease ($P = 0.033$). Effects of exercise load were not observed in these values.

TGF- β 1 is a cytokine suggested to be a substance that causes central fatigue. When only the values of TGF- β 1 prior to exercise were compared, the values for the placebo group, OLL2809 group, and OLL2809 + α LA group prior to intake were 1.29 ± 0.35 , 1.33 ± 0.27 , and 1.35 ± 0.32 ng/ml, respectively, and after four weeks of intake were 1.23 ± 0.18 , 1.20 ± 0.15 , and 1.19 ± 0.19 ng/ml, respectively, and no significant differences among the groups were observed. When compared to the values prior to intake in each group, the placebo group did not show a significant change, whereas the OLL2809 group showed a decreasing trend ($P = 0.075$). Furthermore, the OLL2809 + α LA group showed significant decrease ($P = 0.035$).

35 Table 3

Effects of test substance intake on TGF- β 1 and dROMs in peripheral blood

	Exercise	Placebo		OLL2809		OLL2809 + α LA	
		Before intake	4 weeks later	Before intake	4 weeks later	Before intake	4 weeks later
dROMs	Before	303 $\pm$ 56	298 $\pm$ 80	298 $\pm$ 50	283 $\pm$ 27 [#]	317 $\pm$ 58	284 $\pm$ 53 *
(U.CARR)	After	308 $\pm$ 54	297 $\pm$ 52	308 $\pm$ 40	288 $\pm$ 46	326 $\pm$ 53	302 $\pm$ 56
TGF- β 1	Before	1.29 $\pm$ 0.35	1.23 $\pm$ 0.18	1.33 $\pm$ 0.27	1.20 $\pm$ 0.15 [#]	1.35 $\pm$ 0.32	1.19 $\pm$ 0.19*
(ng/mL)	After	1.27 $\pm$ 0.28	1.30 $\pm$ 0.26 ^{\$}	1.49 $\pm$ 0.44 ^{\$}	1.43 $\pm$ 0.32 ^{\$}	1.37 $\pm$ 0.31 ^{\$}	1.29 $\pm$ 0.20 ^{\$}

The results are shown as the mean value $\pm$ standard deviation.

*, *: P<0.1, 0.05 (comparison to before intake within the group by Student's paired t test)

^{\$}, ^{\$}: P<0.1, 0.05 (comparison to before exercise by Student's paired t test)

- Assessment of effects of *Lactobacillus gasseri* OLL2809 and α LA on improving the condition of athletes showed that intake of *Lactobacillus gasseri* OLL2809 reduces mental stress, and is effective for maintaining immune strength that decreases after vigorous exercise. Furthermore, taking α LA in addition to *Lactobacillus gasseri* OLL2809 was found to have an effect of alleviating the daily feeling of fatigue. The above-mentioned results elucidated that intake of *Lactobacillus gasseri* OLL2809 alone or intake of α LA in addition to *Lactobacillus gasseri* OLL2809 is useful for regulating the physical condition (improving the condition) of Athletes, since it exhibits effects of reducing decrease in immune function as well as alleviating psychological responses such as the feeling of fatigue (psychological stress) against Athletes' chronic stress represented by their daily training..

Industrial Applicability

- Oral intake of stress-reducing agents provided based on the present invention, which contains *Lactobacillus gasseri* or *Lactobacillus gasseri* with α LA as the active ingredient, alleviates poor physical conditions caused by chronic stress.

CLAIMS

1. A non-therapeutic method of reducing stress, comprising administering *Lactobacillus gasseri* and α -lactalbumin (α LA) to the subject in need thereof, wherein the *Lactobacillus gasseri* is
5 *Lactobacillus gasseri* OLL2809 deposited under the accession number NITE BP-72.
2. The non-therapeutic method of claim 1, wherein the stress is chronic stress.