【DESCRIPTION】

【Invention Title】

NOVEL LACTOBACILLUS PLANTARUM AND COMPOSITION CONTAINING THE SAME

【Technical Field】

The present invention relates to novel *Lactobacillus plantarum* variant and a composition comprising the same. More particularly, the present invention relates to a novel *Lactobacillus plantarum* variant useful in the prevention and treatment of enteropathy and immune diseases and a composition comprising the same.

【Background Art】

Lactic acid bacteria are abundantly present in fermented foods such as Kimchi and usually dwell in the digestive tract, functioning to degrade fibroid materials and composite proteins into useful metabolites. As such, live microorganisms that confer a health benefit on the host by improving the microbial environment in the gut are generally called probiotics. To function as probiotics after oral uptake, microorganisms must reach the intestine alive and must stay on intestinal surfaces. Therefore, they are fundamentally required to have a tolerance to acid and bile acid and an ability to adhere to intestinal epithelial cells.

Representative among probiotics are *Lactobacillus* sp. microorganisms, which are abundantly present in Korean conventional fermented foods such as Kimchi. *Lactobacillus* sp. microorganisms are homo- or hetero-fermentative lactic acid bacilli which can be readily found in human and animal guts or in the fermentation process of diary products or vegetables. *Lactobacillus* sp. microorganisms are known to have the

beneficial functions of vitamin synthesis, anticancer activity and blood cholesterol reduction in addition to maintaining the intestines at an acidic pH that inhibits the excessive growth of harmful bacteria, such as *E. coli*, or *Clostridium* and improving diarrhea and constipation. Acidophilin, produced by fermenting lactic acid bacilli, can act like an antibiotic and inhibit the growth of dysentery bacilli, salmonella, staphylococci, E. coli, etc. Reportedly, this natural antibiotic can bind the bowel by suppressing the growth of diarrhea-causing bacteria and normalizing the intestinal microfloral environment (Michael and Philippe, Probiotics and prebiotics: Effects on diarrhea, The journal of nutrition, Volume 137, March 2007, pages 803S-811S; Roberfroid, Prebiotics and probiotics: Are they functional foods?, American journal of clinical nutrition, Volume 71, June 2000, pages 1682S-1687S).

Active research has been made to take advantage of Lactobacillus sp. microorganisms in the developmentt of probiotic agents and animal feeds. Bacterial diarrhea causes livestock to lose weight or even die. To increase livestock production by preventing the onset of diseases, antibiotics have been generally added to the feedstock of livestock. However, because the use of antibiotics causes the advent of resistant bacteria and a residuum of antibiotics within livestock products, it is regulated by law and therefore, and organic methods of raising livestock have been recommended (Korean Patent Laid-Open Publication No. 1998-78358)(McEwen and Fedorka-Cray, Antimicrobial use and resistance in animals, Clinical infectious Diseases, Volume 34, June 2002, pages S93-S106).

In addition, lactic acid bacteria such as Lactobacillus sp. microorganisms are known to exhibit immunopotentiating activity. With the aggravation of environmental pollution in the world and the increased uptake of instant food, allergy and atopic diseases associated with abnormal immunomodulation have been increased rapidly over the world including Korea. In Europe, keen attention has been paid to

bacteriotherapy in which pathogenic microorganisms are displaced with beneficial bacteria by the oral administration of, for example, lactic acid bacteria, thereby treating or alleviating diseases.

A report has it that the administration of *Lactobacillus rhamnosus* GG has reduced the onset of atopic diseases in infants to half the level (Kalliomäki *et. al.*, Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial, Lancet, Volume 357, April 2001, pages 1076-1079). Also, children with atopic dermatitis were reported to undergo a decrease in both the area and extent of dermatitis when they were administered with *Lactobacillus rhamnosus* and *L. reuteri* (Rosenfeldt *et. al.*, Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis, Dermatologic and ocular diseases, Volume 111, February 2003, pages 389-395).

The immunopotentiating mechanism of lactic acid bacteria has been steadily researched and has yet to be proven. It is generally inferred that after being introduced via an oral route, lactic acid bacteria settle down and inhabit the gut, positively affecting the intestinal immune system. For example, the uptake of lactic acid bacteria with yogurt is reported to increase the antibacterial activity of Peyer's patch's lymphocytes. Tests with animals and humans showed that lactic acid bacteria potentiate the response of IgA. Further, lactic acid bacteria have an influence on both innate immunity and adaptive immunity. In the intestinal immune system, the cells imparting innate immunity defend the host from infection of pathogens by recognizing and killing them. In adaptive immunity, macrophages, which plays a role in phagocytosing pathogens and presenting antigens, are activated to stimulate the production of various cytokines, inter alia, IL12 and IL-18. In this regard, some of the constituents of the cell wall of lactic acid bacteria are known to activate the NF-κB and STAT signaling pathway in macrophages and thereby stimulate the production of

cytokines. In addition lactic acid bacteria increase the production of IL-12, IL-18, and TNF-α in dendritic cells, which are specialized antigen-presenting cells abundantly found in the lymph nodes and the mucous membranes of the digestive tract, as well as the expression of T-lymphocyte activating surface molecules such as MHC class II and B7-2 (Cross *et. al.*,Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria?, International Immunopharmacology, Volume 1, May 2001, pages 891-901).

T lymphocytes play a central role in adaptive immunity. There is a Th1 response leading to cell-mediated immunity and also a Th2 response leading to humoral immunity in the adaptive immunity. The cytokines produced by antigen-presenting cells differ between the Th1 response to Th2 response. IL-12, IL-18, and interferon (IFN) are predominantly produced in the Th1 response whereas the Th2 response predominantly results in the production of PGE2, IL-4, and IL-10. For immune system homeostasis, there must be an appropriate balance between the Th1 and Th2 responses. The disruption of Th1/Th2 balance gives rise to immune-mediated diseases. Generally, Th1 cells are more effective against infection, while Th2 cells are responsible mainly for allergic and inflammatory responses. When acting normally, Th2 cells protect the body from dust and other undesired substances. When excessively activated, Th2 cells induce the hyperproduction of IgE antibodies, giving rise to allergic reactions to proteins which are not normally antigenic (e.g., pollen, foods). Th1 responses must be in balance with Th2 responses. A surplus or deficiency of either of them causes diseases. Chronic stress induces the continuous release of cortisol, which causes a decrease in Th2 response but an increase in Th1 response, resulting in the induction of cancer, atopy, allergy, and autoimmune diseases (Elenkov and Chrousos, Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease, Trends in Endocrinology and Metabolism,

Volume 10, November 1999, pages 359-368).

Lactic acid bacteria stimulate the production of the Th1 cytokine IFN-γ, but suppress the release of the Th2 cytokines IL-4 and IL-5 in T lymphocytes, as shown in an *in vivo* experiment (Matsuzaki *et. al.*, The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice, Journal of Dairy Science, Volume 81, January 1998, pages 48-53). Another experiment exhibited that when ovalbumin-primed mice that showed a Th2 bias were administered orally with lactic acid bacteria, the IFN-γ level of the splenocytes increased but the IL-4, IL-5 and IgE levels decreased and that incubating the splenocytes isolated from ovalbumin-primed mice with a Th2 bias, together with lactic acid bacteria, brought about a change in cytokine and IgE levels in agreement with the results of the oral administration experiment. However, because the incubation of only T lymphocytes together with lactic acid bacteria did not lead to a significant increase in IFN-γ level, T lymphocytes are thought to require antigen-presenting cells such as macrophages and dendritic cells for their IFN-γ production (Kato *et. al.*, Lactic acid bacterium potently induces the production of interleukin-12 and interferon-gamma by mouse splenocytes, International Journal of Immunopharmacology, Volume 21, February 1999, pages 121-131). IL-12 and IL-18, which are cytokines playing an important role in differentiating Th0 lymphocytes into Th1 lymphocytes, are produced in macrophages or dendritic cells. When treated with lactic acid bacteria, splenocytes or microphages are known to increase the production of IL-12, IL-18 and IFN-α in dose-dependent manners. As such, lactic acid bacteria increases the production of IL-12, IL-18 and IFN-α in macrophages, thus promoting differentiation into Th1 cells with the concomitant induction of IFN-γ production, so that they can act to drive a Th2-predominant condition toward a Th1/Th2 balance (Cross *et. al.*, Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria?,

International Immunopharmacology?, Volume 1, May 2001, pages 891-901). Therefore, lactic acid bacteria are reported to be useful in the prevention or treatment of immune-mediated diseases such as cancers, atopy, allergy and autoimmune diseases, which are caused by the disruption of the Th1/Th2 balance triggered by an excessive Th2 response.

【Description of Drawings】

FIG. 1 is a graph showing the acid resistance of *Lactobacillus plantarum* CJLP56.

FIG. 2 is a graph showing the bilic acid resistance of *Lactobacillus plantarum* CJLP56.

FIG. 3 is a graph showing an adhesion ability of *Lactobacillus plantarum* CJLP56 to intestinal epithelial cells.

FIG. 4 is a graph showing the concentrations of of the Th1 response-inducing cytokine IL-12 produced in the splenocytes of the ovalumin-primed mice showing Th2 bias after they are treated with *Lactobacillus plantarum* CJLP56 and other comparative lactic acid bacteria.

FIG. 5 is a graph showing the concentrations of of the Th2 response-inducing cytokine IL-4 produced in the splenocytes of the ovalumin-primed mice showing Th2 bias after they are treated with *Lactobacillus plantarum* CJLP56 and other comparative lactic acid bacteria.

FIG. 6 is a graph showing the concentrations of IL-12 and IL-10 produced in the macrophage cell strain RAW264.7 treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by ELISA.

FIG. 7 is a graph showing the the concentrations of IL-12 and IL-10 produced in the dendritic cell line JAWSII treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by ELISA.

FIG. 8 is a graph showing the expression levels of IL-12p40 and IL-18 mRNA in the macrophage cell line RAW264.7 treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by RT-PCR.

FIG. 9 is a graph showing the expression levels of IL-12p40 and IL-18 mRNA in the dendritic cell line JAWSII treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by RT-PCR.

【Disclosure】

【Technical Problem】

Leading to the present invention, intensive and thorough research into probiotics, conducted by the present inventors, resulted in the finding that a novel Lactobacillus sp. strain was isolated from Korean traditional fermented foods and was identified to have an excellent modulatory effect on Th1/Th2 imbalance in favor or Th2 cell mediated responses.

It is therefore an object of the present invention to provide a novel Lactobacillus sp. strain useful as a probiotic which has an excellent immunopotentiating effect, particularly an immunomodulatory effect on the Th1/Th2 imbalance in favor of a Th2 shift as well as showing excellent acid- and bile acid-tolerance and adhesion on intestinal epithelial cells.

It is another object of the present invention to provide a composition for the prevention or treatment of enteropathy, comprising the novel Lactobacillus sp. strain.

It is a further object of the present invention to provide an immunopotentiating composition comprising the novel Lactobacillus sp. strain.

【Technical Solution】

In order to accomplish the above objects, the present invention provides

Lactobacillus plantarum CJLP56 (deposited with the Korean Collection for Type Cultures on Oct. 16, 2008, with accession No. KCTC 11402BP).

In addition, the present invention provides a composition for the prevention or treatment of enteropathy, comprising *Lactobacillus plantarum* CJLP56.

Further, the present invention provides an immunopotentiating composition comprising *Lactobacillus plantarum* CJLP56.

A detailed description will be given of the present invention, below.

Lactobacillus plantarum CJLP56 according to the present invention is a novel strain of *Lactobacillus plantarum* which was isolated and identified from Korean traditional fermented foods. Examples of the traditional fermented foods include, but are not limited to, Kimchi, vegetable ferments, fermented soybean paste, soy source, fast-fermented soybean paste, and pickled seafoods.

For identification and classification thereof, the novel strain according to the present invention was subjected to 16S rRNA base sequencing. As a result, it was found to have the highest molecular phylogenetic relationship with the reference strain *Lactobacillus plantarum* (*Lactobacillus plantarum* NBRC15891^T, GenBank accession number AB326351) due to the highest homology (99.9%) therebetween. Therefore, the novel strain was identified as a strain of *Lactobacillus plantarum*, named *Lactobacillus plantarum* CJLP56, and deposited with the Korean Collection for Type Cultures on Oct. 16, 2008 (accession number KCTC 11402BP). The nucleotide sequence of 16S rRNA of *Lactobacillus plantarum* CJLP56 is represented by SEQ ID NO. 1 as given in the following sequence list text.

Lactobacillus plantarum CJLP56 is Gram positive and facultative anaerobic so that it can grow both aerobically and anaerobically. The novel bacteria does not form a spore nor move, and has a rod shape. More concrete morphological and physiological properties of *Lactobacillus plantarum* CJLP56 were analyzed using well-

known methods and the results are summarized in Table 1, below.

TABLE 1

Morphological, Physiological and Biochemical Properties	**Results**
Morphology	Bacillus (Rod)
Motility	-
Spore	-
Catalase	-
Yeast- heterofermentation	Facultative heterofermentation
Proliferation at 15℃	+
Proliferation at 45℃	-
Proliferation at 3% NaCl	+
Anaerobic growth	+
Formation of CO_2 using glucose	-
Sugar fermentation	
Glycerol	-
Erythritol	-
D-arabinose	-
L-arabinose	+
Ribose	+
D-xylose	-
L-xylose	-
Adonitol	-
Xyloside	-
Galactose	+

D-glucose	+
D-fructose	+
D-mannose	+
L-sorbose	-
Rhamnose	+
Dulcitol	-
Inositol	-
Mannitol	+
Sorbitol	+
D-mannoside	+
D-glucoside	-
Glucosamine	+
Amygdalin	+
Albutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Saccharose	+
Trehalose	+
Inulin	+
Melizitose	+
D-raffinose	+
Amidon	-
Glycogen	-

Xylitol	-
Gentiobiose	+
D-turanose	+
D-lyxose	-
D-tagatose	-
D-fucose	-
L-fucose	-
D-arabitol	-
L-arabitol	-
Gluconate	-
2-gluconate	-
5-gluconate	-

+ : positive

- : negative

For long-term storage, preferably, the inventive novel strain *Lactobacillus plantarum* CJLP56 may be cryopreserved with a storage solution prepared from a mixture of water and glycerol at -70℃ or may be suspended in sterile 10% skim milk before lyophilization.

Also, the inventive novel strain *Lactobacillus plantarum* CJLP56 serves as probiotics that show the activities of gastrointestinal protection and immunopotentiation.

As used herein, the term "probiotic" is understood to be a live microorganism that benefits the health on the host by improving the microbial environment in the gastrointestinal tract. Probiotics, that is, live microorranisms with probiotic activity, may be single or composite strains and may beneficially affect intestinal flora in the host after uptake thereof in the form of dried cells or fermented products. To serve as

probiotics, first, microorganisms must pass through the stomach into the intestine in a living state and have a tolerance to gastric and bile juice. Also, they must settle down and inhabit the intestine and have a beneficial influence on intestinal microflora. Therefore, they should be resistant to gastric juice and bile acid and also be able to adhere to intestinal epithelial cells. Next, the microorganisms must be safe to the body. In this regard, a gelatin liquefaction test, a phenylalanine deaminase test, an ammonia formation test, and a hemolysis test are conducted. *Lactobacillus plantarum* CJLP56 according to the present invention was observed to be negative to the gelatin liquefaction test, the phenylalanine deaminase test, and the ammonia formation test, as well as to show excellent tolerance to acid and bile acid and ability to adhere to intestinal epithelial cells. Also, α-hemolysis was observed, indicating that *Lactobacillus plantarum* CJLP56 is safe to the body.

Thanks to its excellent ability to be tolerant to acid and bile acid and adhere to intestinal epithelial cells, *Lactobacillus plantarum* CJLP56 according to the present invention is anticipated to have excellent gastrointestinal protecting effects. Therefore, in accordance with another aspect thereof, the present invention provides a composition for preventing or treating intestinal diseases, comprising *Lactobacillus plantarum* CJLP56.

The composition for treatment of intestinal diseases comprising the inventive microorganism may be useful for the prevention or treatment of intestinal diseases of mammals including humans, such as livestock, e.g., cattle, horses, and pigs. The term "intestinal diseases," as used herein, is intended to refer to bacterial infectious or inflammatory diseases in the intestine. Examples of the intestinal diseases include, but are not limited to, infectious diarrhea caused by pathogenic microorganisms (E. coli, salmonella, and clostridium), gastroenteritis, inflammatory bowel diseases, psychogenic enteritis syndrome, overgrowth of microorganisms in the small intestine,

diarrhea, and the like. *Lactobacillus plantarum* CJLP56 contained in the composition for treating intestinal diseases may be alive or dead, and preferably alive. In general, live bacteria have an ability to treat or improve general symptoms caused by the abnormal fermentation of intestinal flora, to inhabit the intestinal tracts with the concomitant prevention of allowing harmful bacteria to adhere to the intestinal tracts in humans and animals, and to produce lactic acid to lower the intestinal pH, thereby suppressing proliferation of harmful bacteria. In addition, the administered live bacteria produce bacteriocin and peroxides to suppress the proliferation of pathogens and promote the activity of intestinal villi to absorb nutrients. Further, live bacteria may produce materials helpful for the absorption and utilization of nutrients, improve livestock feed requirements, and generate materials neutralizing the toxic materials of pathogens.

The composition for the prevention or treatment of intestinal diseases in accordance with the present invention may be preferably orally administered, but the method of administering the composition is not limited thereto. The dose may vary depending on various factors including the type and severity of intestinal diseases, the patient's age, gender and ethnicity, and the purpose of prevention. Typically, the bacteria may be administered in an amount of from 10 million to 100 billion cells a day to an adult.

In addition to the gastrointestinal protecting effect, *Lactobacillus plantarum* CJLP56 of the present invention has an excellent immunopotentiating effect compared to conventional lactic acid bacteria. *Lactobacillus plantarum* CJLP56 is found to promote the production of IL-12 leading to a Th1 response, but suppress the production of IL-4 leading to Th2 responses in splenocytes. Further, the inventive novel strain stimulates immunomodulatory cells, such as macrophages and dendritic cells, which are antigen-presenting cells capable of modulating the immune responses of T cells, to produce cytokines that induce Th0 lymphocytes to differentiate into Th1 lymphocytes, thus

driving the Th2-shifted Th1/Th2 imbalance toward Th1 responses. A detailed description is now given of the immunopotentiating effect of *Lactobacillus plantarum* CJLP56, below.

In the murine splenocytes which were allowed to shift toward Th2 responses by the administration of ovalbumin (OVA), *Lactobacillus plantarum* CJLP56 induced the production of IL-12, a cytokine leading to Th1 response, at a level 5.8 - 8.4 times as high that of a negative control and suppressed the production of IL-4, a cytokine leading to Th2 response, at a level of 10.7 - 12.9 % as high as that of the negative control. The inventive novel strain was thus found to have significant advantages in terms of the immunomodulative activity over other typical lactic acid bacteria *Lactobacillus rhamnosus*GG (KCTC 5033), *Lactobacillus casei* (KCTC 3109), and *Lactobacillus sakei* CJLS118 (KCTC13416). Therefore, *Lactobacillus plantarum* CJLP56 is highly immunomodulative such that it promotes Th1 responses with the concomitant suppression of Th2 responses, to modulate the Th1/Th2 imbalance in favor of a Th2 shift.

The immunopotentiating activity of *Lactobacillus plantarum* CJLP56 was also proven in the microphage cell line RAW264.7 and the dendritic cell line JAWSII which were cultured together with the inventive novel strain. When treated with *Lactobacillus plantarum* CJLP56, the microphage cell line RAW264.7 and the dendritic cell line JAWSII were induced to produce IL-12 and IL-18, cytokines dictating differentiation into Th1, at high levels, and suppress the production of IL-10, a cytokine inhibiting differentiation into Th1, to a level lower than that of IL-12, thus promoting differentiation into Th1. It was also understood from these results that *Lactobacillus plantarum* CJLP56 has an immunomodulatory activity of modulating a Th2-shifted Th1/Th2 imbalance by promoting Th1 responses and suppressing Th2 responses.

IL-4 is produced by Th2 cells and plays a central role in Th2-specific cell-

mediated immunity. It also functions as an anti-inflammatory cytokine, that is, it inhibits the production of IL-12, a cytokine of Th1 cells. Recently, it has been reported that the peripheral blood and skin lesions of atopic dermatitis patients are relatively increased in Th2 cells, which are responsible mainly for the production of IL-4 and IL-5 (Miraglia *et. al*, Immune dysregulation in atopic dermatitis, Allergy and Asthma Proceedings, Volume 27, November-December 2006, pages 451-455). Thus, a Th1/Th2 imbalance in favor of Th2-mediated immune responses induces diseases such as atopic dermatitis. In addition, as described above, a surplus or deficiency of one of Th1 or Th2 over the other causes the outbreak of diseases. For example, a relative decrease in Th1 response or a relative increase in Th2 response is known to induce the onset of immune cell-mediated diseases such as cancers, atopic diseases, allergies, and autoimmune diseases (Elenkov and Chrousos, Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease, Trends in Endocrinology and Metabolism, Volume 10, November 1999, pages 359-368). Thus, it is expected that *Lactobacillus plantarum* CJLP56 may be applied to the prevention or treatment of atopic diseases and allergies as well as cancers and autoimmune diseases because *Lactobacillus plantarum* CJLP56 can modulate the production of cytokines from immunmodulatory cells such as Th1, Th2, macrophages and dendritic cells to drive a Th2-shifted Th1/Th2 imbalance toward Th1 responses.

In accordance with another aspect thereof, the present invention provides an immunopotentiating composition comprising *Lactobacillus plantarum* CJLP56. The immunopotentiating composition of the present invention is effective at enhancing immune response because *Lactobacillus plantarum* CJLP56 is a lactic acid bacterium that is effective for enhancing immune response as described above. Particularly, as will be proven in the following Example Section, the immunopotentiating composition of the present invention is effective at preventing or treating diseases caused by a Th1/Th2

imbalance in favor of a Th2 shift, because *Lactobacillus plantarum* CJLP56 can promote Th1 responses. Thus, the immunopotentiating composition of the present invention may be effectively used to prevent or treat atopic disease, allergies, cancer and autoimmune disease. The autoimmune diseases include asthma and hay fever, but are not limited thereto.

The composition for enhancing an immune response may be orally administered, but the method of administering the composition is not limited thereto. The dose may vary depending on various factors including the type of the disease which needs immunopotentiation for its treatment, the severity of the disease, the patient's age, gender, and ethnicity, and the purpose of treatment or prevention. In general, the bacteria is administered in an amount of 10 million to 100 billion cells a day to an adult.

Comprising *Lactobacillus plantarum* CJLP56, the safety of which has been proven, the composition for the prevention or treatment of intestinal diseases, and the immunopotentiating composition in accordance with the present invention can be applied to pharmaceuticals, functional food, cosmetics, livestock feeds, or feed additives to livestock, without any concern about side effects.

When used as pharmaceuticals, the composition may be formulated into pharmaceutical preparations that are commonly used in the art. The pharmaceuticals may be formulations for oral dosage forms such as liquids, suspensions, powder, granules, tablets, capsules, pills, or extracts.

Pharmaceutically acceptable excipients or additives suitable for formulations may be used. For example, formulations suitable for oral administration may include at least one carrier selected from the group consisting of a diluent, a lubricant, a binder, a disintegrant, a sweetener, a stabilizer, and a preservative, and at least one additive selected from the group consisting of a flavoring agent, a vitamin, and an antioxidant.

So long as they are pharmaceutically acceptable, any excipient or additive may be used. For example, a diluent may be lactic acid, corn starch, soybean oil, microcrystalline cellulose, or mannitol. Examples of the lubricant include magnesium stearate and talc. Polyvinyl pyrrolidone or hydroxypropylcellulose may be suitable as a binder. In addition, a disintegrant may be preferably selected from amongst calcium carboxymethylcellulose, sodium starch glycolate, polacrilin potassium, and crospovidone. The sweetener may be white sugar, fructose, sorbitol, or aspartame, the stabilizer may be sodium carboxymethylcellulose, β-cyclodextrin, white wax, or xanthan gum, and the preservative may be methyl paraoxybenzoate, propyl paraoxybenzoate, or potassium sorbate.

In addition to the above substances, a natural flavor such as plum flavor, lemon flavor, pineapple flavor, or herb flavor, a natural fruit juice, a natural colorant such as chlorophylin or flavonoid, a sweetener agent such as fructose, honey, sugar alcohol, or sugar, or an acidifier such as citric acid or sodium citrate, or combinations thereof may be added to the formulation in order to improve the taste.

Formulation techniques, and excipients and additives necessary for formulation are described in detail in Remington's Pharmaceutical Sciences (19th ed., 1995).

The composition of the present invention may also be used as a food. Among them are functional foods and everyday foods. For use as a function food, the composition may be formulated into a variety of formulations that are commonly used in the art with sitologically acceptable excipients or additives. Examples of the functional food include powder, granules, tablets, capsules, suspensions, emulsions, syrups, liquids, extracts, tea, jelly, drinks, or the like. So long as it is well known in the art, any sitologically acceptable excipient or additive may be used.

Thanks to its preventive or therapeutic effect on atopic diseases, the

composition of the present invention may be used in cosmetics. The composition used in cosmetics may be formulated into various cosmetic preparations that are commonly used in the art. When the composition is formulated, excipients or additives acceptable for cosmetics may be added thereto.

The composition may be used as a livestock feed or a feed additive.

For use as a feed additive, the composition may be formulated into a liquid with a high concentration of from 20 to 90% or may be prepared as a powder or granules. The feed additive may include at least one selected from the group consisting of an organic acid such as citric acid, fumaric acid, adipic acid, lactic acid, or malic acid, a phosphate salt such as sodium phosphate, potassium phosphate, acidic pyrrophosphate, or polyphosphate (polymerized phosphate), and a natural antioxidant such as polyphenol, catechin, α-tocopherol, rosemary extract, vitamin C, green tea extract, licorice extract, chitosan, tannic acid, or phytic acid. The composition used as livestock feed may be formulated into various forms that are commonly used in the art with ingredients commonly used in livestock feed.

The feed additive and livestock feed may include grains such as powdered or pulverized wheat, oats, barley, corn, or rice; plant protein livestock feed containing rape, bean, or sunflower as a main ingredient; animal protein livestock feed such as blood powder, meat powder, bone powder, or fish powder; sugar; and dairy products such as powdered milk and whey powder. The feed additive and livestock feed may further include nutrient supplements, digestion- and absorption-assisting agents, growth promoting substances, or the like.

The livestock feed additive may be administered, alone or in combination with another edible excipient, to animals. In addition, the livestock feed additive may be administered as a top dressing to the livestock feed or as a mixture with the livestock feed, or in separate oral form. If the feed additive is administered separately from the

livestock feed, it is combined with a pharmaceutically acceptable vehicle to prepare an immediate release or sustained release formulation. The edible vehicle may be solid or liquid, such as corn starch, lactose, sucrose, bean flake, peanut oil, olive oil, sesame oil, or propylene glycol. When a solid vehicle is used, the feed additive may be in the form of tablets, capsules, powder, troches or lozenges, or a non-dispersed top dressing. As for a liquid vehicle, the feed additive may be in the form of gelatin soft capsules, a syrup suspension, an emulsion, or a solution.

The livestock feed may include protein-containing organic cereal flour that is commonly used to satisfy the dietary demand of animals. The protein-containing cereal flour may comprise corn, bean flour, or a corn/bean flour mix.

In addition, the feed additive and livestock feed may include a supplement such as a preservative, a stabilizer, a wetting agent, an emulsifier, and a solubilizer. The feed additive may be added to the livestock feed in an infiltration, spraying, or mixing manner.

The livestock feed or feed additive may be applied to meals for various animals such as mammals, poultry, and fish. The mammals include pigs, cows, sheep, goats, rodents for experimentation, and pets (e.g., dogs and cats). Examples of the poultry include chicken, turkeys, ducks, geese, pheasants, and quails, and the fish includes trout, but are not limited thereto.

【Advantageous Effects】

Having excellent resistance to acid and bile acid and adhering strongly to intestinal epithelial cells, as described above, *Lactobacillus plantarum* CJLP56 according to the present invention can be used as a probiotic that provides effective gastrointestinal protection. Further, *Lactobacillus plantarum* CJLP56 promotes a Th1 response so that it is used to modulate a Th1/Th2 imbalance in favor of a Th2 shift. Therefore,

Lactobacillus plantarum CJLP56 according to the present invention may be applied to a composition for treating intestinal diseases and an immunopotentiating composition. Particularly, *Lactobacillus plantarum* CJLP56 is effective at treating diseases induced by a Th1/Th2 imbalance in favor of a Th2 shift.

【Best Mode】

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as limiting the present invention.

EXAMPLE 1: Isolation and identification of *Lactobacillus plantarum* CJLP56 strains

Lactobacillus plantarum CJLP56 strains isolated from kimchi were smeared onto 1.5% agar MRS plates (Difco, USA), and incubated at 37°C for 24 hours. Colonies that were proven to be pure were collected using a loop and incubated at 37°C for 18 to 24 hours in a liquid MRS medium (Difco, USA).

Then, morphological and physiological properties of *Lactobacillus plantarum* CJLP56 strains were determined using a method reported by Kim *et. al.* (Kim *et. al.*, *Leuconostoc inhae* sp. nov., a lactic acid bacterium isolated from kimchi, International Journal of Systematic and Evolutional Microbiology, Volume 53, July 2003, pages 1123-1126), and API50CH and API50CHL kits (Biomerio). The identified morphological and physiological properties of *Lactobacillus plantarum* CJLP56 are summarized in Table 1 above.

In addition, the base sequence of a 16S rRNA gene was analyzed in order to identify and classify lactic acid bacteria. The base sequence of 16S rRNA gene was determined and analyzed using the method of Kim *et. al.* (Kim *et. al.*, *Leuconostoc kimchii*

sp. nov., a new species from kimchi. International Journal of Systematic and Evolutional Microbiology, Volume 50, September 2000, pages 1915-1919). The determined base sequence of 16S rRNA gene of *Lactobacillus plantarum* CJLP56 is given in the sequence list text attached hereto (SEQ ID NO: 1).

As a result of 16S rRNA base sequencing, the strain was found to have the highest homology (99.9%) with a *Lactobacillus plantarum* standard strain (*Lactobacillus plantarum* NBRC15891^T, GenBank accession number AB326351), and was identified to be a *Lactobacillus plantarum* strain and named *Lactobacillus plantarum* CJLP56, and deposited with the Korean Collection for Type Cultures on Oct. 16, 2008 (accession No.: KCTC11402BP).

EXAMPLE 2: Assay of *Lactobacillus plantarum* CJLP56 for Resistance to Acid of Artificial Gastric Juice and to Bile Acid of Artificial Bile Juice

An acid resistance test was conducted with an artificial gastric juice prepared by a modified method of Kobayashi *et. al.*, (Kobayashi *et. al.*, Studies on biological characteristics of *Lactobacillus*: II. Tolerance of the multiple antibiotic resistance strain, *L. casei* PSR3002, to artificial digestive fluids. Japan Journal of Microbiology. Volume 29, July 1974, pages 691-697). In detail, the artificial gastric juice was prepared by adjusting the pH of a liquid MRS medium to 2.5 with 1N HCl, adding pepsin in a concentration of 1000 unit/mL, and sterilizing the medium.

Lactobacillus plantarum CJLP56, isolated and identified in Example 1, was incubated in an MRS medium at 37°C for 18 hours and centrifuged to precipitate a cell pellet. This was washed twice with sterilized saline (0.85% NaCl) and the cell suspension was inoculated onto a control medium and the artificial gastric juice to a concentration of about 10^7 cfu/mL. During incubation at 37°C, viable cells were

counted 0 and 3 hours after inoculation. The total count of the cells was measured by diluting the cells 10 times in a phosphate-buffered solution (pH 6.8) containing KH_2PO4, Na_2HPO, L-cysteine, HCl, and Tween 80.

A bile resistance test in artificial bile juice was performed using the method of Casey *et. al.* (Casey *et. al.*, Isolation and characterization of anti-Salmonella lactic acid bacteria from the porcine gastrointestinal tract, Letters in Applied Microbiology. Volume 39, 2004, pages 431-438). In this regard, *Lactobacillus plantarum* CJLP56 was incubated in a medium which was prepared by adding 0.3% bull bile to the liquid MRS medium used in the acid resistance test above. The cells were inoculated in the same manner as in the acid resistance test above, and viable cells were counted 0, 12 and 24 hours after inoculation.

Separately, the typical lactic acid bacteria strains *Lactobacillus casei* (KCTC 3109), *Lactobacillus sakei* CJLS118 (KCTC13416) , and *Lactobacillus rhamnosus* GG (KCTC 5033) were subjected to the same acid and bile acid resistance tests as described above.

The results are shown in FIGS. 1 and 2. FIG. 1 is a graph illustrating the acid resistance of *Lactobacillus plantarum* CJLP56. FIG. 2 is a graph illustrating the bile resistance of *Lactobacillus plantarum* CJLP56.

With reference to FIGS. 1 and 2, *Lactobacillus plantarum* CJLP56 had equal or greater acid resistance and bile resistance compared to the comparative lactic acid bacteria strains. This result indicates that *Lactobacillus plantarum* CJLP56 of the present invention may be alive as it proceeds through gastric juice to the intestine and survives the bile within the intestine.

EXAMPLE 3: Assay of *Lactobacillus plantarum* CJLP56 for Ability to Adhere to intestinal Epithelial Cells

For use in a test for adhesion to intestinal epithelial cells, HT-29 was obtained from the Korean Cell Line Bank (KCLB), and the test was conducted using the methods of Kim *et. al.* (Kim *et. al.*, Probiotic properties of *Lactobacillus* and *Bifidobacterium* strains isolated from porcine gastrointestinal tract, Applied Microbiology and Biotechnology, Volume 74, April 2007, pages 1103-1111) and of Hirano et al. (Hirano *et. al.*, The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells *in vitro*, Microbiology and Immunology, Volume 47, 2003, pages 405-109)

HT-29 cells were cultured in a RPMI 1640 (Gibco, USA) medium containing heat-inactivated 10% fetal bovine serum (FBS), 1% L-glutamine, penicillin G (100 IU/mL), and streptomycin (100 mg/mL) at 37°C under a 5% CO_2 atmosphere. For assaying adhesion ability and adhesion inhibitory ability, first, HT-29 cells were seeded at a density of 1.0×10^5 cells/mL per well in 24-well plates, and cultured to the extent of forming a complete monolayer, with the replacement of the medium with a fresh one on alternate days. The complete monolayer of HT-29 cells was washed five times with PBS buffer solution at 25°C, followed by the addition of 0.5 mL of an antibiotic-free RPMI 1640 medium thereto.

Lactobacillus plantarum CJLP56 was suspended in an RPMI medium to a concentration of about 1.0×10^9 cfu/mL, and the suspension was inoculated into the 24-well plates and incubated at 37°C for 2 hours under a 5% CO_2 atmosphere. After the completion of incubation, the 24-well plates were washed three times with PBS buffer while stirring at 200 rpm for 3 min in order to remove the cells which remained unattached and to determine the adhesion ability over the washing. After washing, 0.2% trypsin-EDTA was added into the wells to detach the attached cells. The cells thus separated were diluted in peptone water in a serial dilution manner and smeared on MRS-agar plates, followed by incubation at 37°C for 24 hours. Thereafter, the cells were counted.

Separately, to identify partial adhesion, a cover glass completely sterilized by immersion in 70% alcohol for one day was placed on the bottom of a petri-dish before HT-29 cells were incubated therein, along with the same amount of lactic acid bacteria as described above. Lactic acid bacteria that were not washed and remained adhering to the HT-29 cells were dried, Gram stained, observed under an optical microscope and counted. *Lactobacillus sakei* CJLS118, and *Lactobacillus rhamnosus*GG (KCTC 5033) were used for comparison in this experiment.

The results are shown in FIG. 3. FIG. 3 is a graph illustrating the ability of *Lactobacillus plantarum* CJLP56 to adhere to intestinal epithelial cells.

Referring to FIG. 3, *Lactobacillus plantarum* CJLP56 was observed to have excellent ability to adhere to intestinal epithelial cells after 24 hours, compared to *Lactobacillus rhamnosus*GG (KCTC 5033) and *Lactobacillus sakei*CJLS118, both commercially well known as a probiotics, and particularly to the former. These results imply that *Lactobacillus plantarum* CJLP56 according to the present invention is capable of adhering to intestinal epithelial cells and thus of improving the intestinal environment.

EXAMPLE 4: Safety Test of *Lactobacillus plantarum* CJLP56

In order to evaluate the safety of the strains isolated in Example 1, a hemolysis test, a gelatin liquefaction test, a harmful metabolite (ammonia) formation test, and a phenylalanine deaminase test were preformed using the safety test methods according to a collective standard of the Korean Bio Venture Association.

The results are shown in Table 2 below.

TABLE 2

Results of Safety Test of *Lactobacillus plantarum* CJLP56

Strain	Test			
	Gelatin Liquefaction Test	Phenylalanine Deamination	α-Hemolysis result	Ammonia formation
CJLP56	negative	Negative	α-Hemolysis, safe	Negative

According to the results, *Lactobacillus plantarum* CJLP56 was negative for the gelatin liquefaction test, the harmful metabolite (ammonia) formation test, and the phenylalanine deaminase test, and showed α-hemolysis which is regarded as not being a pathogen. Thus, *Lactobacillus plantarum* CJLP56 was proven as being safely administered to the body.

EXAMPLE 5: Assay for IL-12 Production In Treated Mouse Splenocyte

To assay the ability of *Lactobacillus plantarum* CJLP56 to promote the production of the Th1 response-inducing cytokine IL-12 in the splenocytes of ovalbumin-primed mice that showed a Th2 bias, the following experiment was conducted with reference to Fujiwara et al. (Fujiwara *et. al.* A double-blind trial of *Lactobacillus paracasei* strain KW3110 administration for immunomodulation in patients with pollen allergy, Allergology International, 2005, volume 54, pages 143-149) and Fujiwara et al. (Fujiwara *et. al.*, The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance, International Archives of Allergy and Immunology, 2004, Volume135, pages 205-215) as follows.

After being incubated at room temperature, a mixture of 1.538 mL of 13

mg/mL alum hydroxide(Sigma), 10 mg of ovalbumin and 0.4615 mL of PBS was intraperitoneally injected into 5 6-week-old female Balb/c mice at a dose of 0.2 mL (1 mg OVA + 2 mg alum) per mouse, followed by intraperitoneal injection at the same dose on day 6 for boosting. The mice were sacrificed on day 13 and the spleens were excised. The splenocytes thus obtained were plated in an amount of 100 μL ($4x10^6$ cells/mL), along with 50 μL of dead cells of test and 50 μL of ovalbumin (4 mg/mL) into cell culture well plates and incubated for 7 days in DMEM-10 in a 10% CO_2 incubated. Thereafter, the supernatant was assayed for IL-12 level using an IL-12 ELISA kit (Biosource).

The dead bacteria of the test were obtained as follows.

Bacteria of the test was inoculated into MRS broth (Difco) and cultured at 37°C for 24 hours, followed by centrifugation at 13,000 rpm for 1 min to obtain cells as a pellet. The cells were then washed twice with physiological saline and harvested. For an animal cell inoculation test, the bacterial cells were heated at 100°C for 10 min in the same volume of sterilized distilled water as that of the original culture medium and harvested by centrifugation at 13,000 rpm for 1 min. The cells were diluted in DMEM to form a concentration of 50 μg/mL and 5 μg/mL of the cell culture medium. *Lactobacillus plantarum* CJLP56 was used as test bacteria. The same experiment was performed with *Lactobacillus rhamnosus* GG (KCTC 5033), *Lactobacillus casei* (KCTC 3109), and *Lactobacillus sakei* CJLS118 (KCTC 13416) for comparison.

The IL-12 assay was performed according to the instructions supplied for the IL-12 ELISA kit. The O.D. values measured in an ELISA reader were used to calculate the level of IL-12 by normalization to the IL-12 sample provided for the kit. The measurements are shown in FIG. 4.

FIG. 4 is a graph showing the concentrations of the Th1 response-inducing cytokine IL-12 produced in the splenocytes of the ovalbumin-primed mice which had a

Th2 bias after they were treated with *Lactobacillus plantarum* CJLP56 and other comparative lactic acid bacteria.

As is apparent from the data of FIG. 4, *Lactobacillus plantarum* CJLP56 was found to remarkably promote the production of the Th1 response-inducing cytokine IL-12, compared to the other lactic acid bacteria. Therefore, *Lactobacillus plantarum* CJLP56 according to the present invention was identified to significantly induce Th1 responses in the mice with a Th2 bias.

EXAMPLE 6: Assay for Inhibitory Activity against IL-4 Production In Treated Mouse Splenocytes

To assay the inhibitory activity of *Lactobacillus plantarum* CJLP56 against the production of the Th2 response-inducing cytokine IL-4 in the splenocytes of ovalbumin-primed mice that showed a Th2 bias, the same procedure as in Example 5 was repeated, with the exception that an IL-4 kit (Biosource) was used instead of the IL-12 kit. The results are shown in FIG. 5.

FIG. 5 is a graph showing the concentrations of the Th2 response-inducing cytokine IL-4 produced in the splenocytes of the ovalbumin-primed mice that had a Th2 bias after they were treated with *Lactobacillus plantarum* CJLP56 and other comparative lactic acid bacteria.

As shown in FIG. 5, *Lactobacillus plantarum* CJLP56 was found to inhibit the production of the Th2 response-inducing cytokine IL-4 to suppress Th2-biased mouse splenocytes from mediating Th2 responses.

EXAMPLE 7: Assay for Expression of the Th1 Differentiation Inducing Cytokines IL-12p40 and IL-18 and the Th1 Differentiation Suppressing Cytokine IL-

10 in Macrophage and Dendritic Cells

Antigen-presenting cells (APCs) such as macrophages and dendritic cells produce IL-12 and IL-18 to induce Th0 to differentiate into Th1 while producing IL-10 to suppress differentiation into Th1. To evaluate the effect of the lactic acid bacteria of the present invention on the production of IL-12, IL-10, and IL-18 in macrophages and dendritic cells, an experiment was performed as follows.

Test bacteria cells were applied at a density of 5×10^7 cells/mL to the macrophage cell line RAW264.7 which was then cultured at 37°C for 48 hours in a 10% CO_2 incubator. The IL-12p40 and IL-10 levels of the supernatant were analyzed using ELISA.

The test bacteria was *Lactobacillus plantarum* CJLP56, with lipopolysaccharide serving as a positive control. The same test was performed on *Lactobacillus rhamnosus* GG (KCTC 5033), *Lactobacillus casei* (KCTC 3109) and *Lactobacillus sakei* CJLS118 (KCTC 13416) for comparison.

The concentrations were measured using an IL-12p40 kit (BD Biosciences, USA) for IL-12 and an IL-10 kit (BD Biosciences, USA) for IL-10 according to the instructions of the manufacturers. The results are shown in FIGS. 6 and 7, respectively.

FIG. 6 is a graph showing the concentrations of IL-12 and IL-10 produced in the macrophage cell strain RAW264.7 treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by ELISA.

FIG. 7 is a graph showing the concentrations of IL-12 and IL-10 produced in the dendritic cell line JAWSII treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by ELISA.

As can be seen in FIGS. 6 and 7, *Lactobacillus plantarum* CJLP56 promotes the

production of the Th1 differentiation-inducing cytokine IL-12, but shows the production of the Th1 differentiation-suppressing cytokine to a significant less extent than that of IL-12, and has a higher potential of producing IL-12 compared to the other lactic acid bacteria.

To ascertain the production of IL-12 and IL-18 at the gene level, first, test bacteria was applied at a density of 5×10^7 cells/mL to the macrophage cell line RAW264.7 which was then cultured at 37°C for 6 hours in a 10% CO_2 incubator. Total RNA was isolated and used to determine the levels of IL-12 and IL-18 mRNA by RT-PCR. The dendritic cell line JAWSII was inoculated with the test bacteria, cultured and subjected to RNA isolation, followed by the determination of IL-12 and IL-18 mRNA levels by RT-PCR in the same manner as in the macrophages.

The results are shown in FIGS. 8 and 9, respectively.

FIG. 8 is a graph showing the expression levels of IL-12p40 and IL-18 mRNA in the macrophage cell line RAW264.7 treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by RT-PCR.

FIG. 9 is a graph showing the expression levels of IL-12p40 and IL-18 mRNA in the dendritic cell line JAWSII treated with *Lactobacillus plantarum* CJLP56 and other references, as measured by RT-PCR.

As can be seen in FIGS. 8 and 9, *Lactobacillus plantarum* CJLP56 promotes the transcription of the mRNA dictating the production of the Th1 differentiation-inducing cytokines IL-12 and IL-18, and shows great superiority in promoting IL-12 mRNA expression to the other lactic acid bacteria.

EXAMPLE 8: Preparation of Probiotic Agent Comprising *Lactobacillus plantarum* CJLP56

The probiotic *Lactobacillus plantarum* CJLP56 identified in Example 1 was produced on a mass scale and lyophilized to make probiotics suitable for use as a raw material of pharmaceuticals, food, livestock feed, feed additives, or cosmetics.

The bacteria were incubated in MRS broth (Difco) at 37°C for 18 hours while its pH was adjusted to 6.0 with a 25% NaOH solution, followed by harvesting the cells by centrifugation. The cells were frozen at -40°C with 5% dextrin and 10% skim milk serving as cryoprotectants, and dried at 37°C. The cells thus lyophilized were powdered using a mixer. The powdered live bacteria were mixed with a suitable amount of an excipient, such as glucose, lactic acid, and skim milk, to adjust the number of bacteria to a desired level, and stored in a sealed aluminum pouch.

To be used in pharmaceuticals, food, livestock feed, cosmetics and so on, the prepared probiotic agent may be mixed with grain powder used as a raw material of the livestock feed, with an excipient or additive for pharmaceuticals, such as tablets and capsules, or with raw materials of cosmetics.

【Sequence List Text】

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【CLAIMS】

【Claim 1】

Lactobacillus plantarum CJLP56 KCTC 11402BP.

【Claim 2】

A composition for prevention or treatment of enteropathy, comprising *Lactobacillus plantarum* CJLP56 KCTC 11402BP.

【Claim 3】

An immunopotentiating composition comprising *Lactobacillus plantarum* CJLP56 KCTC 11402BP.

【Claim 4】

The composition of claim 3, being preventive or therapeutic of an immune disease caused by a Th1/Th2 imbalance in favor of a Th2 shift.

【Claim 5】

The composition of claim 4, wherein the immune disease is selected from the group consisting of allergic diseases, atopy, cancer and autoimmune diseases.

【Claim 6】

The composition of any one of claims 2 to 5, being in a form of a medicine.

【Claim 7】

The composition of any one of claims 2 to 5, being in a form of a food.

【Claim 8】

The composition of any one of claims 2 to 5, being in a form of livestock feed or a feed additive.

【Claim 9】

The composition of any one of claims 2 to 5, being in a form of a cosmetic.