

DESCRIPTION

NUTRITIONAL COMPOSITION FOR IMPROVING INTESTINAL FLORA

5

Technical Field

The present invention relates to nutritional compositions for improving intestinal flora.

Background Art

10 The intestinal environment is thought to be involved in various diseases and aging. It is said that in the elderly, the bacterial count of *Bifidobacterium* is decreased and the intestinal flora is unbalanced. It has been pointed out that such deterioration of the intestinal flora possibly promotes aging. Bad bacteria in the intestinal tract produce harmful putrefaction products (such as ammonia, amine, phenol, and indole). Such putrefaction products directly
15 damage the intestinal tract and are also partially absorbed into the body, not only promoting aging of the host throughout life, but also causing onset of diseases such as cancer, myocardial infarction, and hypertension. On the other hand, good bacteria such as *Bifidobacterium* in the intestinal tract inhibit proliferation of bad bacteria. Therefore, presence of bacteria belonging to *Bifidobacterium* in the intestinal tract is instrumental in maintaining health, and these bacteria
20 are suggested to be important throughout the host life as useful intestinal bacteria. That is, decrease in the bacterial count or disappearance of *Bifidobacterium* in the intestinal tract indicates an unhealthy state (Non-Patent Document 1).

 Enterally-fed patients are thought to have lower bacterial count of *Bifidobacterium* in the intestinal tract compared to healthy individuals (Non-Patent Documents 2 and 3), and have
25 deteriorated intestinal flora compared to healthy individuals. Therefore, the intestinal flora imbalance of enterally-fed patients must be normalized.

 In addition to probiotics such as *Bifidobacterium* and *Lactobacillus*, culture supernatants of propionic acid bacteria and lactic acid bacteria (*Enterococcus* and *Lactococcus*) (Patent Document 1), and fructo-oligosaccharides (Non-Patent Document 3) and such are known as
30 foods having effects of improving the intestinal flora. However, they are literally nutritional compositions for supplementing nutrition (liquid diet) and those for promoting proliferation of good bacteria of the genera *Bifidobacterium* and/or *Lactobacillus* are not known.

[Prior Art Documents]

[Patent Document]

35 [Patent Document 1] Japanese Patent Application Kokai Publication No. (JP-A) H05-041995 (unexamined, published Japanese patent application).

[Non-Patent Documents]

[Non-Patent Document 1] Mitsuoka T, Intestinal Flora and Aging, Nutrition Reviews, 1992; 50(12):438-46.

[Non-Patent Document 2] Del Piano M, Clinical Experience With Probiotics in the Elderly on Total Enteral Nutrition, Journal of Clinical Gastroenterology, 2004; 38(6 Suppl): S111-4.

[Non-Patent Document 3] Bouhnik Y *et al.*, The capacity of short-chain fructo-oligosaccharides to stimulate faecal bifidobacteria: a dose-response relationship study in healthy humans, Nutrition Journal, 2006; 5:8.

10 Summary of the Invention

[Problems to be Solved by the Invention]

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide nutritional compositions that concurrent with the supplying of nutrients, also promote proliferation of bacteria of the genera *Bifidobacterium* and/or

15 *Lactobacillus* in the intestine (in the intestinal tract).

[Means for Solving the Problems]

As a result of dedicated research to solve the above-mentioned problems, the present inventors discovered that a nutritional composition containing milk protein hydrolysates, fermented milk proteins, phospholipids, oleic acid-containing oils and fats, and isomaltulose has

20 the effect of promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, and thereby completed the present invention.

Specifically, in an *in vivo* experiment using rats, the above-mentioned nutritional composition was found to increase the percentage and bacterial count of *Bifidobacterium* and *Lactobacillus* without changing the total bacterial count in the intestine. The results revealed

25 that the above-mentioned nutritional composition promotes proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*. Furthermore, decrease in pH in the cecum, increase in the weight of cecal tissue and cecal contents, and increase in the amount of organic acids in the cecal contents were confirmed. Therefore, the composition of the present invention is useful for improving intestinal flora.

30 Specifically, the present invention consists of the following:

[1] a nutritional composition for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising a milk protein hydrolysate and a fermented milk protein as proteins, oleic acid-containing oil and fat as well as milk phospholipid and/or soybean lecithin as lipids, and isomaltulose as a carbohydrate;

35 [2] the nutritional composition of [1], wherein the milk protein is selected from the group consisting of casein, a milk protein concentrate (MPC), a whey protein concentrate (WPC),

a whey protein isolate (WPI), α -lactoalbumin, β -lactoglobulin, and lactoferrin;

[3] the nutritional composition of [1] or [2], wherein the milk protein hydrolysate is contained at 0.9 g to 5.0 g per 100 mL of the nutritional composition;

[4] the nutritional composition of any one of [1] to [3], wherein the milk protein hydrolysate is obtained by hydrolyzing a whey protein concentrate (WPC) and/or whey protein isolate (WPI) with *Bacillus licheniformis*-derived alcalase or porcine pancreas-derived trypsin;

[5] the nutritional composition of any one of [1] to [4], wherein the milk protein hydrolysate is a permeate fraction obtained by treatment with an ultrafiltration membrane having a cutoff molecular weight of 10,000;

[6] the nutritional composition of any one of [1] to [5], wherein the fermented milk protein is derived from cheese;

[7] the nutritional composition of [6], wherein the cheese is quark;

[8] the nutritional composition of any one of [1] to [7], wherein the fermented milk protein is contained at 0.5 g to 6 g per 100 mL of the nutritional composition;

[9] the nutritional composition of any one of [1] to [8], wherein isomaltulose is contained at 4 g to 15 g per 100 mL of the nutritional composition; and

[10] the nutritional composition of any one of [1] to [9], wherein the lipids contain oleic acid at a proportion of 30% or more of the total fatty acid composition.

[Effects of the Invention]

The nutritional compositions of the present invention can supply nutrients, and concurrently improve intestinal flora. For example, when a nutritional composition of the present invention is used for a liquid diet, or oral / enteral / tube feeding, and such, the intestinal flora of an individual who takes it can be improved, in addition to supplying nutrients.

Furthermore, since the compositions of the present invention can be taken safely, they are suitable for nutritional management during long-term care.

Brief Description of the Drawings

Fig. 1 depicts graphs showing the weight of the cecum, etc., and pH in the cecum determined in Example 1. The graphs show mean $\pm$ SD. *: $p < 0.05$.

Fig. 2 depicts a graph showing the amount of short-chain fatty acids in cecal contents determined in Example 1. The graph shows mean $\pm$ SD. *: $p < 0.05$.

Fig. 3 depicts graphs showing the analysis results of *Bifidobacterium* and *Lactobacillus* in Example 1. The graphs show mean $\pm$ SD. *: $p < 0.05$.

Mode for Carrying Out the Invention

The present invention will be described in detail below. However, the present invention is not limited to the following preferred embodiments, and can be altered freely within the scope of the present invention.

The present invention relates to nutritional compositions for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising the followings:

1. Proteins;
2. Lipids; and
3. Carbohydrates.

The nutritional compositions of the present invention contain milk protein hydrolysates and fermented milk protein as proteins. They contain oleic acid-containing oils and fats and phospholipids as lipids. They contain isomaltulose as a carbohydrate. Herein below, the components of the compositions of the present invention will be described specifically.

1. Proteins

1-1 Milk protein hydrolysates

At least one milk protein selected from casein, whey protein (whey protein concentrate (WPC), whey protein isolate (WPI), α -lactalbumin (α -La), and β -lactoglobulin (β -Lg)), milk protein concentrate (MPC, also referred to as total milk protein (TMP)), and a combination thereof can be presented as examples of the milk protein serving as the source material.

Milk protein hydrolysates will be described using hydrolysis of whey protein as an example. Enzymes generally used for hydrolysis of whey proteins include, pepsin, trypsin, and chymotrypsin. However, there are also reports of studies that use plant-derived papain, and proteases derived from bacteria and fungi (Food Technol., 48: 68-71, 1994; Trends Food Sci. Technol., 7: 120-125, 1996; Food Proteins and Their Applications, pp. 443-472, 1997).

Therefore, these enzymes may be used in the present invention. Accordingly, the compositions of the present invention include those that can be obtained by hydrolyzing the above-mentioned milk protein using at least one enzyme selected from the group consisting of these enzymes.

Activities of enzymes that hydrolyze whey protein vary greatly depending on their types.

Those skilled in the art can select the optimum enzyme based on the activity of each enzyme.

Pepsin degrades α -La and denatured α -La, but not native β -Lg (Neth. Milk dairy J., 47: 15-22,

1993). Trypsin slowly degrades α -La, but hardly degrades β -Lg (Neth. Milk dairy J., 45:

225-240, 1991). Chymotrypsin rapidly degrades α -La, but slowly degrades β -Lg. Papain

degrades bovine serum albumin (BSA) and β -Lg, but does not easily degrades α -La and α -La is tolerant to papain (Int. Dairy Journal 6: 13-31, 1996a). However, under acidic pH, papain

degrades α -La not bound to Ca completely (J. Dairy Sci., 76: 311-320, 1993).

By controlling the degree of hydrolysis of a milk protein and by modifying the milk protein, the functional characteristics of the milk protein can be altered over a wide range of pH

and processing conditions (Enzyme and Chemical Modification of proteins in Food proteins and their Applications pp. 393-423, 1997, Marcel Dekker, Inc., New York, 1997; Food Technol., 48: 68-71, 1994).

Hydrolysis of peptide bonds increases the number of charged groups and hydrophobicity, decreases molecular weight, and modifies molecular configuration (J. Dairy Sci., 76: 311-320, 1993). Changes in functional properties of the milk protein depend greatly on the degree of hydrolysis. The greatest changes commonly observed in whey protein functionality are, for example, increased solubility and decreased viscosity. When the degree of hydrolysis of whey protein is high, the resulting hydrolysates often will not precipitate, even upon heating, and the solubility increases at pH 3.5 to 4.0. The hydrolysates also have far lower viscosity than intact proteins. These differences are especially prominent when protein concentration is high. Other effects include altered gelation properties, enhanced thermostability, decreased emulsifying ability, and decreased foam stability (Int. Dairy journal, 6: 13-31, 1996a; Dairy Chemistry 4, pp. 347-376, 1989; J. Dairy Sci., 79: 782-790, 1996).

Various bioactive peptides derived from milk proteins are known. For example, it has been pointed out that α s2-casein-derived peptides disrupt microorganisms (*Rhodotorula rubra*, *Escherichia coli*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Staphylococcus carnosus*, and *Bacillus subtilis*) that are not naturally present in ordinary intestinal flora (Japanese Patent Kohyo Publication No. (JP-A) 2000-507941 (unexamined Japanese national phase publication corresponding to a non-Japanese international publication)). It has also been pointed out that with a nutritional composition using whey protein hydrolysate alone as the protein source, proliferation of lactobacilli and enterococci of the jejunum/ileum is suppressed compared to a nutritional composition using intact whey protein alone as the protein source. However, bifidobacteria were not detected in both test groups (JP-A(Kohyo) 2004-536143). Furthermore, it has been pointed out that κ -casein-derived peptides and lactoferrin-derived peptides obtained from products generated by pepsin treatment of milk proteins promote more growth (preferential growth) of *Bifidobacterium bifidum in vitro* relative to *E. coli* (JP-A (Kohyo) 2001-516570). However, specific experimental data has not been disclosed.

In addition to the scientific literature exemplified above, many patent documents (published patent applications and granted patents) that disclose preparation methods exist with regard to milk protein hydrolysates. Those skilled in the art can prepare milk protein hydrolysates according to such documents. Examples include:

- the method of separate hydrolysis of casein and whey protein, followed by adsorption and removal of the hydrophobic portion, and then mixing the two in a designated ratio (Japanese Patent No. 2,986,764);
- the method of hydrolyzing whey protein with a *Bacillus*-derived protease and an

mycobacteria-derived protease, and then removing the enzymes and insoluble hydrolysates (Japanese Patent No. 3,222,638);

- the method of enzymatically degrading β -Lg to obtain a peptide mixture in which the mole ratio of branched-chain amino acids / aromatic amino acids is 10 wt% or more, in which aromatic amino acids are less than 2.0 wt%, and in which the average molecular weight is

several hundreds to several thousands (Japanese Patent No. 3,183,945);

- the method of selective enzymatic degradation of β -Lg in whey protein (Japanese Patent No. 2,794,305); and

- the method of hydrolyzing whey proteins with proteases derived from *B. licheniformis* and/or *B. subtilis* using the non-pH-stat technique to hydrolysis degree (DE) of 15% to 30%, and then obtaining the permeate from an ultrafiltration membrane with a cutoff value exceeding 10,000 (Japanese Patent No. 3,167,723),

but are not limited thereto. The milk protein hydrolysates of the present invention also include those prepared by methods and techniques other than those described in these patent documents.

In a preferred embodiment of the present invention, whey protein hydrolysates can be prepared, for example, by the method including the following steps:

(1) obtaining an aqueous protein solution by dissolving a whey protein isolate (WPI, Davisco) with protein content of approximately 90% (w/w) calculated as dry material in distilled water to produce a protein concentration of 8% (w/v);

(2) denaturing the protein by heat treatment of this aqueous solution at 85°C for two minutes. The pH of the aqueous solution after this heat treatment can be, for example, approximately 7.5;

(3) then, adding Alcalase 2.4L (enzyme, Novozymes) at 2.0% (w/w) relative to the concentration of the protein (substrate), and maintaining the aqueous solution at 55°C for three hours for hydrolysis;

(4) then, adding PTN 6.0S (enzyme, Novozymes Japan), which is pig-derived trypsin, at 3.0% (w/w) relative to the concentration of the protein (substrate), and maintaining the aqueous solution at 55°C for three hours for hydrolysis. That is, time for hydrolysis may be a total of six hours. The pH of the aqueous solution at completion of the hydrolysis reaction can be, for example, approximately 7.0.

(5) Treating the whey protein hydrolysate with an ultrafiltration (UF) membrane with a cutoff molecular weight of 10,000 (Millipore, Ultrafree-MC), following centrifugation (20,000 x g, 10 min).

Preliminary heating, enzyme-to-substrate ratio (E/S), pH, hydrolyzing temperature, time for hydrolyses, and such are five parameters to be optimized in the method for preparing protein hydrolysates, and the following conditions can be presented as examples. Specifically, they are: preliminary heating: 65 to 90°C; E/S: 0.01 to 0.2; pH: 2 to 10; hydrolyzing temperature: 30

to 65°C; and time for hydrolyses: 3 to 20 hours.

Examples of the enzymes used for protein hydrolysis include the following enzymes which are available from suppliers such as Novo Nordisk:

1) Endo-type proteases

- 5 *Bacillus licheniformis*-derived: Alcalase
- B. lentus*-derived: Esperase
- Bacillus subtilis*-derived: Neutrase
- Bacteria-derived: Protamex
- Porcine pancreas-derived: PTN (trypsin)

10 2) Exo-type proteases

- Aspergillus oryzae*-derived: Flavorzyme
- Porcine or bovine viscera-derived: Carboxypeptidase

15 Examples other than the above-mentioned enzymes include animal-derived pancreatin, pepsin, plant-derived papain, bromeline, microorganism (such as, lactobacilli, yeasts, molds, and mycobacteria)-derived endoprotease and exoprotease, and their crudely purified products and bacterial homogenates. Furthermore, combinations of *B. licheniformis*-derived alcalase and porcine pancreas-derived PTN (trypsin) are often used as an enzyme mixture.

20 The milk protein hydrolysates of the present invention include: protein hydrolysates themselves, retained solutions (retentates) or permeates after ultrafiltration treatment, and commercial milk protein hydrolysates which show similar activity required for the present invention. For example, the retentates subjected to ultrafiltration treatment through a membrane with a cutoff molecular weight of any one of 5000, 6000, 7000, 8000, 9000, and 10000 for the lower limit ("or more" or "higher than"), and any one of 15000, 20000, 25000, and 30000 for the upper limit ("or less" or "lower than"), preferably a membrane with a cutoff

25 molecular weight of 10000, can be used as the milk protein hydrolysates of the present invention.

30 The blended quantity of milk protein hydrolysate can be adjusted appropriately according to: the blended quantity of other components (fermented milk protein, oleic acid-containing oils and fats, milk phospholipid, soybean lecithin, isomaltulose, and such), the pathological condition, symptoms, age, and body weight of the enterally-fed patient, uses and such. Specifically, the blended quantity of milk protein hydrolysate may be 0.9 to 5.0 g, preferably 0.9 to 3.0 g, more preferably 1.0 to 2.5 g, even more preferably 1.2 to 2.0 g per 100 mL of the nutritional composition, but are not limited to these ranges.

1-2. Fermented milk proteins

35 Next, fermented milk proteins will be described. So-called fermented milk (liquid milk prepared by using one or a combination of two or more types of milk sources such as

livestock milk including cow milk, buffalo milk, goat milk, sheep milk, and horse milk and/or partially skimmed milk, skim milk, reduced whole milk, reduced skim milk, reduced partially-skimmed milk, butter, cream, and such thereof, fermented by using a starter such as lactic acid bacteria) can be used as a source of the fermented milk proteins of the present invention. For example, fresh cheeses, natural cheeses, yogurt, and whey cheeses are included in the fermented milk proteins of the present invention. Cheeses in the present invention refer to those produced by removing whey from curd formed by fermenting milk, butter milk, or cream with lactic acid bacteria or by adding enzymes to milk, butter milk, or cream. It may or may not be solidified or ripened. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* may be generally used as the starters for producing fermented milk, but starters are not limited to these, and for example, lactobacilli and bifidobacteria such as *Streptococcus lactis*, *Streptococcus cremoris*, *Streptococcus diacetylactis*, *Enterococcus faecium*, *Enterococcus fecalis*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus murinus*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus gasseri*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, or *Bifidobacterium breve* may be used. In addition, microorganisms used when producing fermented milk such as *Propionibacterium* bacteria may be used in combination. The nutritional compositions of the present invention may be prepared using any fermented milk but preferably they are prepared using fresh cheeses or yogurt, or more preferably quark or yogurt.

There are many types of fresh cheeses, including cottage, quark, string, neuchatel, cream cheese, mozzarella, ricotta, and mascarpone. Quark is a type of non-ripened (fresh) cheese and has a low fat content and a refreshing flavor and acidic taste. A common method for producing quark is described below.

First, skim milk is pasteurized, and then fermented by seeding lactic acid bacteria starter (generally, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at 0.5% to 5% (w/w). When the pH of the solution reaches 4.6, curd is formed. Then, whey is separated by centrifugation using a quark separator, and the obtained curd is cooled. An example of a composition of the quark of the present invention is 17% to 19% (w/w) total solid, 11% to 13% (w/w) protein, less than 1% (w/w) fat, 2% to 8% (w/w) carbohydrates, and less than 2% (w/w) lactose. Those produced by solidification using rennet are also included in the quark of the present invention. In addition, those obtained by adding a mixed culture of *Lactococcus lactis*, *Lactococcus cremoris*, and bacterial species of the genus *Leuconostoc* to skim milk, culturing it, and then removing whey, is also included in the quark of the present invention. Furthermore, those obtained by cutting curd obtained in a manner similar to the above-mentioned method using a cutter and then separating whey while heating the solution is also included in the fresh cheeses of the present invention.

It is widely known that lactic acid bacteria have probiotic effects. Furthermore, whey of fresh cheeses obtained by lactobacilli fermentation is known to have antimicrobial effects against *E. coli*, *Vibrio parahaemolyticus*, bifidobacteria, *Bacteroides*, and such (JP-A (Kokai) H07-155103).

Blended quantity of fermented milk protein can be adjusted appropriately according to the blended quantity of other components (milk protein hydrolysate, oleic acid-containing oils and fats, milk phospholipid, soybean lecithin, isomaltulose, and such), pathological condition, symptoms, age, and body weight of the subject who takes it, uses and such. Specifically, the blended quantity of fermented milk protein may be 0.5 to 6 g, preferably 2 to 6 g, or more preferably 2.5 to 4.5 g, when calculated in terms of protein, per 100 mL of the nutritional composition, but are not limited to these ranges.

The Japanese Ministerial Ordinance Concerning Compositional Standard, etc., for Milk and Milk Products (Ministerial Ordinance No. 52 from Ministry of Health and Welfare in December 27, 1951) defines that "cows, goats, or sheep must not be milked five days within calf delivery" as a basis for compositional standard and methods for producing milk and such, in general. That is, this ordinance restricts the use of colostrum in milk products.

In the present invention, milk (normal milk, or also referred to as mature milk) is preferably used for preparation of milk protein hydrolysates and fermented milk proteins. The milk may be derived from any animal including cows, buffalos, goats, sheep, horses, and humans.

2. Lipids

2-1. Phospholipids

In the present invention, combinations of milk phospholipids and soybean-derived lecithin or egg yolk lecithin may be used as phospholipids. Alternatively, milk phospholipids, soybean-derived lecithin, or egg yolk lecithin may be used alone as the phospholipid. Examples of the preferred phospholipids of the present invention include milk phospholipid and/or soybean-derived lecithin, or milk phospholipid and/or egg yolk lecithin, but are not limited thereto. In fields such as biochemistry, medicine and pharmacology, the term "lecithin" is used as a term that refers only to phosphatidylcholine. However, in commercial or industrial fields, it is used as a general term for phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, and a mixture of other phospholipids. In "Japan's Specifications and Standards for Food Additives", 7th edition (1999), lecithin is defined as "a substance obtained from oilseed or animal sources, whose main component is phospholipids". In the present invention, lecithin includes these phospholipids used in the commercial or industrial definition.

Milk-phospholipids

Milk phospholipids consist of sphingomyelin (SM) , phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and lysophosphatidylcholine (LPC), and is localized in milk fat globule membranes (MFGM). As shown in Table 1, milk phospholipid characteristically includes a large amount of SM which is not included in soybean lecithin.

[Table 1]

PHOSPHOLIPID COMPONENTS	WT%
SPHINGOMYELIN	22
PHOSPHATIDYLCHOLINE	36
PHOSPHATIDYLETHANOLAMINE	27
PHOSPHATIDYLINOSITOL	11
PHOSPHATIDYLSERINE	4
LYSOPHOSPHATIDYLCHOLINE	2

Soybean lecithin

While soybean lecithin is widely used as a natural food additive in the field of foods, polyenephosphatidylcholine is also used as a pharmaceutical (applications: improvement of liver function in chronic liver disease, fatty liver, and hyperlipidemia).

A series of the so-called "natural" phospholipid products are usually ranked according to their PC content. Various types of phospholipids upgraded according to their use have been produced. As Table 2 indicates, soybean lecithin products are conveniently categorized according to the main difference in PC content, based on purification and fractionation of soybean lecithin (Fujikawa, T., Oil Chemistry, Vol. 40(10): pp. 951-p58, 1991).

[Table 2]

TYPE	PC CONTENT (%)
PASTE LECITHIN	15 ~ 20
PURIFIED LECITHIN	20 ~ 25
EXTRACTED LECITHIN	30 ~ 40
PC CONCENTRATED LECITHIN	45 ~ 60
PC HIGHLY PURIFIED LECITHIN	75 ~ 95
PHOSPHOLIPIDS (PC, PE, PS, PG, etc.)	EACH 98% OR MORE

In the present invention, milk phospholipid and soybean lecithin may be used alone, or in combination. The blended quantity of milk phospholipid and/or soybean lecithin can be adjusted appropriately according to the blended quantity of other components (milk protein hydrolysate, fermented milk protein, oleic acid-containing oils and fats, isomaltulose, and such), the pathological condition, symptoms, age, and body weight of the subject who takes it, and uses and such. Specifically, the blended quantity of milk phospholipid and/or soybean lecithin may be 0.01 to 0.5 g, 0.05 to 0.5 g, 0.1 to 0.5 g, or 0.2 to 0.3 g in total per 100 mL of the nutritional composition, but are not limited to these ranges.

2-2. Other lipids

Compositions of the present invention include oleic acid-containing oils and fats as lipids. The Ministry of Health, Labour and Welfare prescribes the desirable intake ratio of saturated fatty acids (SFA: palmitic acid, stearic acid, etc.) : monovalent unsaturated fatty acids (MUFA: oleic acid, etc.) : polyvalent unsaturated fatty acids (PUFA: linolic acid, linolenic acid, etc.) as conventional 1:1.5:1 to 3:4:3, and the ratio of n-6 fatty acid : n-3 fatty acid as 4:1. One of the reasons for this recommendation is that in Japan it is difficult to practice a diet in which the intake ratio of MUFA is elevated to 1.5 times that of SFA and PUFA. Therefore, in order to realize a desirable fatty acid intake ratio (SMP ratio), monovalent unsaturated fatty acids (MUFA) content in the fatty acid composition of lipids is attempted to be increased. To this end, oleic acid, which is a monovalent unsaturated fatty acid, can be mixed into the nutritional composition of the present invention. Lipid sources containing a large amount of oleic acid include high oleic-sunflower oil containing high oleic acid, rapeseed oil, olive oil, high oleic acid safflower oil, soybean oil, corn oil, and palm oil. Furthermore, nutritionally adjusted oils and fats (NOF Corporation) are also examples of a lipid source containing oleic acid. Sunflower oil, rapeseed oil, olive oil, and a mixture with olive oil may be used.

The blended quantity of oleic acid can be adjusted appropriately according to the blended quantity of other components (milk protein hydrolysate, fermented milk protein, milk phospholipid, soybean lecithin, isomaltulose, and such), pathological condition, symptoms, age, and body weight of the subject who takes it, and uses and such. Specifically, the blended quantity of oleic acid is, for example, 25% or more, preferably 30% or more, and more preferably 30% to 50% in the fatty acid component of the nutritional composition of the present invention, but is not limited to these ranges. Furthermore, polyvalent unsaturated fatty acids such as DHA, EPA, and arachidonic acid, and medium-chain fatty acids such as caprylic acid, capric acid, and lauric acid are added to adjust the saturated fatty acids : monovalent unsaturated fatty acids : polyvalent unsaturated fatty acids ratio to a value close to 3:4:3.

3. Carbohydrates and dietary fibers

In the present invention, isomaltulose may be generally used as the carbohydrate. Examples of other carbohydrates that may be used include sugar alcohols (sorbitol, xylitol, maltitol, etc.), honey, granulated sugar, glucose, fructose, and invert sugar.

Isomaltulose is a disaccharide in which one molecule each of glucose and fructose are joined by an α -1,6 bond. It is a structural isomer of sucrose and is also called 6-O-(α -D-Glucopyranosyl)-D-fructose or isomaltulose, or palatinose. Isomaltulose is a substance having a molecular weight of 342.297, registered as Cas. No. 13718-94-0, and is used as a sweetener or such. It is included in very small quantities in honey and sugar cane.

Isomaltulose may also be produced using *Protaminobacter rubrum*-derived α -glucosyl transferase and such to sucrose to convert the α -1,2 bond to an α -1,6 bond. The sweetness of isomaltulose is similar to that of sucrose, but the degree of sweetness is approximately half that of sucrose. Orally ingested isomaltulose is degraded by isomaltase in the digestive tract, and in a similar manner to sucrose, it is digested to glucose and fructose and then absorbed (Goda, T. *et al.*, Journal of Japanese Society of Nutrition and Food Science, Vol. 36(3): 169-173, 1983). Since other carbohydrates that are digested by isomaltase, such as isomaltose, panose, and isomaltotriose, compete with digestion of isomaltulose, intake of isomaltulose is said to suppress digestion and absorption (Journal of Japanese Society of Nutrition and Food Science, Vol. 36(3): 169-173 (1983)). Isomaltulose has an energy value of 4 kcal/g. In the present invention, isomaltulose includes palatinose syrup, reduced palatinose, or palatinose starch syrup. Palatinose starch syrup is a liquid substance in the form of starch syrup containing as the main ingredient an oligosaccharide such as tetrasaccharide, hexasaccharide, and octasaccharide produced when isomaltulose is condensed by dehydration.

It is known that isomaltulose is assimilated highly selectively by butyric acid bacteria *Clostridium butyricum*, and promotes its growth (T. Mizutani, "Characteristics and use of

palatinose oligosaccharide", New Food Industry, Food Material Study Group, 1991, Vol. 33, No. 2, p. 9-16). It is also known that an aqueous solution produced by dissolving a combination of a crystalloid osmotic pressure regulator such as isomaltulose and a colloid osmotic pressure regulator such as dextrin/indigestible dextrin in water and adjusting the osmotic pressure to 200 to 440 mOsm/L removes harmful bacteria in the intestine and prepares a growth environment for useful bacteria (WO2004/067037).

The blended quantity of isomaltulose can be adjusted appropriately according to the blended quantity of other components (milk protein hydrolysate, fermented milk protein, oleic acid-containing oils and fats, milk phospholipid, soybean lecithin, and such), pathological condition, symptoms, age, and body weight of, and uses by the subject who takes it, and such. Specifically, the blended quantity of isomaltulose may be, for example, 4 to 15 g. or preferably 5 to 7 g per 100 mL of the nutritional composition, but are not limited to these ranges.

Energy of the nutritional compositions of the present invention can be adjusted suitably by adding proteins, lipids, and carbohydrates. The energy of the nutritional composition of the present invention may be, for example, 50 to 150 kcal, or preferably 80 to 120 kcal per 100 mL of the nutritional composition, but are not limited to these ranges.

Furthermore, the energy ratio for proteins, lipids, and carbohydrates with respect to the nutritional composition as a whole in the nutritional composition of the present invention is nearly the same as that in the "Recommended Dietary Allowance for the Japanese (6th revision)". Specifically, examples include 15-25% for proteins, 20-30% for lipids, and 45-65% for carbohydrates, but are not limited to these ranges.

The nutritional composition of the present invention may also include dietary fibers. Dietary fibers refer to substances in food that cannot be hydrolyzed by human digestive enzymes, and are classified into water-soluble dietary fibers and insoluble dietary fibers, based on affinity to water. Indigestible oligosaccharides, such as lactulose, lactitol, raffinose can be used as water-soluble dietary fibers. The known biological function of indigestible oligosaccharides is to improve the intestinal environment, i.e., to regulate intestinal function, by reaching the large intestine undigested and contributing to the activation and growth of intestinal bifidobacteria. Pectin (protopectin, pectinic acid, and pectic acid), enzymatic decomposition product of guar gum, tamarind seed gum, and such can be used as other candidates for water-soluble dietary fibers.

Furthermore, candidates for water-soluble dietary fibers include, as high molecular weight water-soluble dietary fibers: soybean polysaccharide thickener, konjac glucomannan, alginic acid, low molecular weight alginic acid, psyllium, gum arabic, seaweed polysaccharides (cellulose, lignin-like substances, agar, carrageenan, alginic acid, fucodine, and laminarin), gums produced by microorganisms (welan gum, curdlan, xanthan gum, gellan gum, dextran, pullulan,

and rhamosan gum), other gums (seed-derived locust bean gum, tamarind gum, tara gum, sap-derived karaya gum, and tragacanth gum); and as low molecular weight water-soluble dietary fibers: polydextrose, indigestible dextrin, pine fiber, and maltitol.

Insoluble dietary fibers increases the bulk of undigested material in the large intestine and shortens its transit time. This increases the frequency of defecation and increases the quantity of stool. Examples of candidates for insoluble dietary fibers include cellulose, hemicellulose, lignin, chitin, chitosan, soybean bran, wheat bran, barley bran, pine fiber, corn fiber, beet fiber, oat bran, rye bran, adlay bran, rice bran, bran of miscellaneous grains such as millet, foxtail millet, barnyard millet, sorghum, bran of bean grains (legume family), bran of pseudo cereals such as buckwheat, sesame bran, and tofu refuse.

Water, proteins, carbohydrates, lipids, vitamins, minerals, organic acids, organic bases, fruit juice, flavors, emulsions, thickening agents, stabilizers, and such may be used in the nutritional compositions of the present invention, besides the aforementioned proteins, lipids, carbohydrates, and dietary fibers. Examples of proteins include animal and plant proteins such as whole milk powder, powdered skim milk, powdered partially skimmed milk, casein, whey powder, whey protein, whey protein concentrate, whey protein isolate, whey protein hydrolysate, α -casein, β -casein, κ -casein, β -lactoglobulin, α -lactoalbumin, lactoferrin, soybean protein, hen egg protein, meat protein, and degradation products thereof; and various types of milk-derived components such as butter, whey mineral, cream, whey, non-protein nitrogen, sialic acid, phospholipid, and lactose. Peptides and amino acids such as casein phosphopeptides and lysine may also be included. Examples of carbohydrates include saccharides, processed starch (in addition to dextrin, soluble starch, British starch, oxidized starch, starch ester, starch ether, etc.), and dietary fibers. Examples of lipids include animal oils and fats, such as lard and fish oils, as well as their separated oils, hydrogenated oils, and 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 transesterified oils. Examples of vitamins include vitamin A, carotenes, vitamin B group, vitamin C, vitamin D group, vitamin E, vitamin K group, vitamin P, vitamin Q, niacin, nicotinic acid, pantothenic acid, biotin, inositol, choline, and folic acid. Examples of minerals include calcium, potassium, magnesium, sodium, copper, iron, manganese, zinc, and selenium. Examples of organic acids include malic acid, citric acid, lactic acid, tartaric acid, and erythorbic acid. Furthermore, materials having fecal odor-reducing effects (for example, 5 mg to 500 mg (0.005% to 0.5%) of champignon extract), a carotenoid preparation (for example, 10 μ g to 200 μ g (0.00001% to 0.0002%) of a preparation containing α -carotene, β -carotene, lycopene, lutein, and such), or an antioxidant (catechin, polyphenol, etc.) can be added. Two or more types of these components can be used in combination, and furthermore, synthesized components and/or food containing these components in abundance may also be used. The

form of the food may be any of a solid form, liquid form, or gel form.

Production of the nutritional compositions of the present invention can be carried out by methods well known in the art. A portion or all of the aforementioned raw materials are blended, and followed by homogenization when necessary. Homogenization refers to sufficiently mixing each of the blended components to homogeneity, and mechanically miniaturizing fat globules and crude large particles of other components to prevent floating and aggregation of fats and such, as well as producing a homogeneous emulsified nutritional composition.

Heat treatment or heat disinfection is carried out in the production of the nutritional compositions of the present invention. Common food disinfection conditions can be used for heat disinfection conditions, and heat disinfection can be performed using commonly used equipment. Disinfection at 62 to 65°C x 30 minutes, 72°C or higher x 15 seconds or longer, 72°C or higher x 15 minutes or longer, or 120 to 150°C x 1 to 5 seconds, or sterilization at 121 to 124°C x 5 to 20 minutes and 105 to 140°C, retort (pressurized heat) disinfection, high pressure steam sterilization, and such may be used, but the heat sterilization is not limited thereto. Heat disinfection can be performed preferably under pressure.

For example, the following methods may be employed without limitation: the method of heat-sterilizing a liquid nutritional composition first and then filling it aseptically into a container (for example, a method that uses UHT sterilization and aseptic packaging in combination), or the method of filling a liquid nutritional composition into a container, and then heat-sterilizing the contents with the container (for example, using an autoclave method), the method of filling a can or various containers used for liquid diet or oral or tube feeding (the so-called soft bags and nutrient bag) followed by retort disinfection (for example, 115 to 145°C for 5 to 10 seconds), or the method of filling a can or various containers used for liquid diet or oral or tube feeding (the so-called soft bags and nutrient bags) followed by retort disinfection, subjecting it to further heat disinfection at approximately 140 to 145°C for approximately 5 to 8 seconds, cooling, and then filling it aseptically.

Heat treatment or heat disinfection kills the starter (lactobacilli, bifidobacteria, or *Propionibacterium* bacteria) derived from the fermented milk which is the source material.

The compositions of the present invention can be used for normalization and maintenance of intestinal flora. The nutritional composition of the present invention can be used, for example, as a liquid diet, oral tube feeding, beverage, gelatinous food (particularly, the so-called functional food), and for nutritional management of patients with oral and enteral feeding and the elderly, infants, and such.

The nutritional composition of the present invention have an osmotic pressure of approximately 500-1000 mOsm/L, for example, approximately 550-750 mOsm/L. When

measured at room temperature, the nutritional composition have a viscosity of 20-100 cp (1 cp = 0.001 Pa·s), preferably 25-60 cp, or more preferably 30-50 cp, but it is not limited to these ranges.

The pH of the nutritional composition of the present invention can be adjusted to 4.6 or less, preferably 3.0 to 4.3, or more preferably 3.8 to 4.2, but it is not limited to these ranges.

The intestinal environment is thought to be involved in various diseases and aging. It is said that in the elderly, the bacterial count of *Bifidobacterium* is decreased and the intestinal flora is unbalanced. It has been pointed out that such deterioration of the intestinal flora possibly promotes aging. Bad bacteria in the intestinal tract produce harmful putrefaction products (ammonia, amines, phenols, indoles, etc.). Such putrefaction products directly damage the intestine and are also partially absorbed into the body, not only promoting aging of the host throughout life, but also causing onset of diseases such as cancer, myocardial infarction, and hypertension. On the other hand, good bacteria such as *Bifidobacterium* in the intestinal tract inhibit proliferation of bad bacteria. Therefore, presence of lactic acid bacteria of the genus *Bifidobacterium* in the intestinal tract is instrumental in maintaining health, and these bacteria are suggested to be important throughout the host life as useful intestinal bacteria. That is, decrease or disappearance of lactic acid bacteria of *Bifidobacterium* in the intestinal tract indicates an unhealthy state.

Enterally-fed patients are thought to have lower bacterial count of *Bifidobacterium* in the intestinal tract compared to healthy individuals and have deteriorated intestinal flora compared to healthy individuals. Therefore, the intestinal flora imbalance of enterally-fed patients must be normalized.

Many reports indicate that administration of probiotics such as bifidobacteria and lactobacilli is related to increase in the amount of organic acids and decrease in putrefaction products in the intestine. Furthermore, there are reports that administration of probiotics causes bifidobacteria and lactobacilli in the intestine to increase, and the accompanying decrease in pH in the intestine inhibits increase of bacteria that produce harmful substances (for example, decrease in *Clostridium*).

Liquid diet and enteral feeding agents are used for the elderly and various pathological conditions. Since the intestinal function of the subject ingesting them is not sufficiently operating, the liquid diet and enteral feeding agents are often supplemented with a small amount of dietary fibers, and currently, there are few liquid diets and enteral feeding agents that take into account cecal fermentation. Common liquid diets and enteral feeding agents do not contain or contain insufficient amount of dietary fibers, and therefore, cecal fermentation becomes insufficient in the individuals taking them. By including fermented milk proteins and dietary fibers, the nutritional compositions of the present invention maintains cecal fermentation in a

normal range, and thereby brings about increase in cecal tissue weight and increase in cecal contents. Increase in cecal contents means increase in intestinal flora and increase in the amount of total short chain fatty acids. Short chain fatty acids are absorbed in the cecum and large intestine, and has been known to be involved in promotion of peristaltic movement in the intestine and proliferation in cecum and large intestine. Therefore, the present invention provides compositions for promoting increase of cecal tissue weight and compositions for promoting increase in cecal contents, comprising milk protein hydrolysate and fermented milk protein as proteins, oleic acid-containing oil and fat as well as milk phospholipid and/or soybean lecithin as lipids, and isomaltulose as carbohydrate.

It was confirmed in an *in vivo* experiment using rats that the nutritional composition of the present invention increased the percentage and bacterial count of *Bifidobacterium* and *Lactobacillus* in cecal contents and stool without changing the total bacterial count in the intestine. Furthermore, decrease in pH in the cecum, and increase in weight of cecal tissue / cecal contents and increase in amount of organic acids in the cecal contents were also confirmed. Therefore, the compositions of the present invention are useful for improving intestinal flora. Furthermore, the present invention provides compositions for promoting decrease in pH in the intestine and compositions for promoting increase in the amount of organic acids in the intestine, comprising milk protein hydrolysates and fermented milk proteins as proteins, oleic acid-containing oils and fats as well as milk phospholipids and/or soybean lecithin as lipids, and isomaltulose as carbohydrate. Examples of the intestine include small intestine, large intestine, and cecum.

The nutritional compositions of the present invention are effective against various bacteria of the genera *Bifidobacterium* and *Lactobacillus*. Examples of bacteria of the genus *Bifidobacterium* include *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium animalis*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, and *Bifidobacterium catenulatum*. Examples of bacteria of the genus *Lactobacillus* include *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus murinus*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus gasseri*, and *Lactobacillus paracasei*.

Whether or not promotion of proliferation of bacteria of the genera *Bifidobacterium* and *Lactobacillus* took place can be determined by methods known to those skilled in the art, such as real time PCR and observation of bacterial colonies.

The amount of intake of the nutritional composition of the present invention in a pharmaceutical or food and beverage per day is not particularly limited since it differs depending on the pathological condition, age, symptoms, and body weight of the subject who takes it, and uses, and depending on whether the nutritional composition of the present invention is the only

source of nutrition. Specifically, the amount of intake per day for an adult is 400 to 1600 mL, preferably 600 to 1600 mL, and more preferably 800 to 1200 mL. In the case of a healthy adult, for example, up to 3000 mL may be taken per day. Furthermore, the amount of intake may be determined by the physician in charge of the subject who takes it.

Furthermore, the nutritional composition of the present invention may be used in combination with a conventionally known food or pharmaceutical having effects of improving the intestinal flora. Specific examples include fermentation products of propionic acid bacteria, DHNA (2,4-dihydroxy-2-naphthoic acid), ACNQ (2-amino-3-carboxy-1,4-naphthoquinone), and fructo-oligosaccharides, but are not limited thereto.

The nutritional compositions of the present invention can be used in any form of pharmaceuticals, or food and beverages. For example, improvement of intestinal flora can be expected from directly administering the nutritional composition of the present invention as a pharmaceutical, or by directly ingesting it as a food for special dietary use such as food for specified health use, food with nutrient function claims, nutritional supplementary food, liquid diet, and supplement. Their form may be any of a liquid form, paste form, solid form, or powder, and they may be taken by adding to various foods (for example, milk, soft drinks, fermented milk, yogurt, cheese, bread, biscuits, crackers, pizza crust, formulated milk powder, liquid food, food for special dietary use, food for sick people, nutritional food, frozen food, processed food, and other commercially available food). Furthermore, when the nutritional composition is used in the form of a powder, it can be produced using means such as spray drying and freeze drying.

When the nutritional compositions of the present invention are used as pharmaceuticals or supplements, they may be administered in various forms. Examples of such forms include oral administration through tablets, capsules, granules, powders, syrups, and such. These various formulations may be routinely formulated using a known adjuvant for formulation which can be used conventionally in the technical field of pharmaceutical formulation, such as for the main agent and excipients, binders, disintegrants, lubricants, flavoring agents, solubilizing agents, suspending agents, and coating agents. Furthermore, a suitable amount of calcium may be included. In addition, suitable amounts of vitamins, minerals, organic acids, sugars, amino acids, peptides, and such may be added.

The nutritional compositions of the present invention may be described as pharmaceutical agents for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising milk protein hydrolysates and fermented milk proteins as proteins, oleic acid-containing oils and fats as well as milk phospholipids and/or soybean lecithin as lipids, and isomaltulose as carbohydrate.

The present invention also relates to methods for promoting proliferation of bacteria of

the genera *Bifidobacterium* and/or *Lactobacillus*, comprising orally administering the nutritional composition of the present invention to an animal. Subjects to which the nutritional composition of the present invention is administered include mammals. Examples of the mammals are humans and non-human mammals, preferably humans and monkeys, and more preferably humans.

The present invention also relates to nutritional compositions for use in promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising milk protein hydrolysates and fermented milk proteins as proteins, oleic acid-containing oils and fats as well as milk phospholipids and/or soybean lecithin as lipids, and isomaltulose as carbohydrate.

Alternatively, the present invention relates to use of proteins, lipids, and carbohydrates in the manufacture of a nutritional composition for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*. The present invention also relates to use of proteins, lipids, and carbohydrates for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*. The present invention also relates to methods for producing nutritional compositions for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising blending proteins, lipids, and carbohydrates with a pharmaceutically acceptable carrier. Specific examples of the proteins, lipids, and carbohydrates have been described herein.

All prior art references cited herein are incorporated by reference into the specification.

Examples

Herein below, the present invention will be described with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1: Examination of effectiveness using animals]

(Test Samples)

General liquid diet and a nutritional composition of the present invention having the composition shown in Tables 3 and 4 were prepared and subjected to retort (heating and pressurization) disinfection (135 to 145°C x 5 to 10 seconds), then freeze dried, and used as test samples. The oleic acid content in the nutritional composition described in the Tables was 39% of the fatty acid composition.

Control group: intake of a freeze-dried product of a general liquid diet.

Test group: intake of a freeze-dried product of a nutritional composition of the present invention.

[Table 3]

		/100ml	GENERAL LIQUID DIET	NUTRITIONAL COMPOSITION
GENERAL COMPOSITION	PROTEIN	g	4.0	5.0
	LIPID	g	2.8	2.8
	CARBOHYDRATE	g	15.5	14.5
	SUGAR	g	14.5	13.3
	DIETARY FIBER	g	1.0	1.2
	ASH	g	0.7	0.6
	MOISTURE	g	84.5	84.4
	CALORIES	kcal	100.0	100.0
VITAMINS	VITAMIN A	µgRE	60	150
	RETINOL	µgRE		60
	β-CAROTENE	µgRE		90
	VITAMIN D	µg	0.50	0.75
	VITAMIN E	mgα-TE	3.0	5.0
	VITAMIN K	µg	5	3.4
	VITAMIN B1	mg	0.15	0.25
	VITAMIN B2	mg	0.20	0.30
	NIACIN	mgNE	2.4	4.0
	VITAMIN B6	mg	0.30	0.30
	FOLIC ACID	µg	50	50
	VITAMIN B12	µg	0.60	0.60
	BIOTIN	µg	15.0	7.5
	PANTOTHENIC ACID	mg	0.60	1.2
	VITAMIN C	mg	16	50
	CHOLINE	mg	1.7	9.2
MINERALS	SODIUM	mg	110	70
	CALCIUM	mg	60	80
	IRON	mg	1.0	1.0
	PHOSPHORUS	mg	60	70
	MAGNESIUM	mg	20	20
	POTASSIUM	mg	100	80
	COPPER	mg	0.080	0.050
	IODINE	µg	15	9.7
	MANGANESE	mg	0.23	0.175
	SELENIUM	µg	3.5	5.0
	ZINC	mg	0.80	1.0
	CHROMIUM	µg	3.0	2.96
	MOLYBDENUM	µg	2.5	2.5
	CHLORINE	mg	140	80
ENERGY BALANCE	PROTEIN	%	16	20
	LIPID	%	25	25
	CARBOHYDRATE	%	59	55
OSMOTIC PRESSURE		mOsm/L	380	600
pH(20°C)			6.7	4.0

[Table 4]

NUTRIENT COMPOSITION	GENERAL LIQUID DIET		NUTRITIONAL COMPOSITION	
COMPONENTS	SOURCE MATERIAL	g/100ml	SOURCE MATERIAL	g/100ml
PROTEIN	SODIUM CASEINATE	4.0	WHEY PROTEIN HYDROLYSATE	2.0
	MILK PROTEIN		FERMENTED MILK PROTEIN	3.0
LIPID	EDIBLE FAT AND OIL	2.8	EDIBLE FAT AND OIL	1.9
			MILK PHOSPHOLIPID	0.1
			MIDDLE CHAIN FATTY ACID OILS	0.6
			PURIFIED FISH OIL	0.2
SUGAR	DEXTRIN	14.5	DEXTRIN	7.2
	SUCROSE		PALATINOSE	6.2
	DIETARY FIBER	1	DIETARY FIBER	1.5

5 (Animal experiment)

Six-week old male SD rats (Japan SLC) were divided according to body weight into two groups (n=6 for each group), the control group and the test group. From the same day (day 0), they were allowed to freely ingest the test sample for 18 days or 22 days. During this period they also had free access to drinking water. Autopsies were performed on the 18th day (day 18) or 22nd day (day 22) from the start of intake of the test sample. Feces were collected in the morning on the day of autopsy. Autopsy was carried out under ether anesthesia. After blood collection from the heart, the abdominal cavity was opened to remove the cecum, the ileum side and the colon side were ligated with thread, then the cecal weight was measured, and this was frozen and stored at -80°C until measurement.

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(Measurements)

- pH in the cecum: The frozen cecum was thawed, a Lacombe tester pH meter (As One Corp.) was inserted into the cecum containing the cecal contents, and pH was measured.
- Cecal tissue weight and cecal content weight: Immediately after measuring the pH in the cecum, the cecal tissue and cecal contents were separated and weighed.
- Amount of short chain fatty acids in cecal contents: Cecal contents were collected and mixed to homogeneity. 400 μ L of MilliQ water was added to 200 mg of cecal contents, evenly homogenized using an ultrasonicator, and then centrifuged at 10,000 rpm, 4°C for ten minutes.

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The supernatant was transferred to a separate microtube, 2.5 μ L each of Carrez I (53.5 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ /100 mL) and Carrez II (17.2 g of $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ /100 mL) were added for every 200 μ L of supernatant, and stirred. This was centrifuged at 10,000 rpm, 4°C for ten minutes, the supernatant was transferred to a microtube equipped with a filter (0.22 μ m), and

centrifuged again under the condition of 10,000 rpm, 4°C for ten minutes, and the supernatant was used as the measurement sample. Organic acids in the measurement sample were

quantified by the post-column pH buffered electric conductivity detection method using an electric conductivity detector (Niwa T., Nakao M., Hoshi S. *et al.* Effect of Dietary Fiber on Morphine-induced Constipation in Rats, Biosci. Biotechnol. Biochem., 66(6), 1233-1240, 2002).

Detector: electric conductivity detector (Shimadzu CDD-10A)

Column: polymer column for organic acid analysis, ICSep ICE-PRE-ORH-801, 6.5 mm I.D. x 300 mm (Tokyo Chemical Industry Co., Ltd.)

Guard column cartridge for organic acid analysis ICSep ICE-ORH-801, 4.0 mm I.D. x 20 mm (Tokyo Chemical Industry Co., Ltd.)

Column temperature: 55°C

Mobile phase: 5 mM aqueous solution of *p*-toluene sulfonic acid, 0.5 mL/min

Reaction solution: 20 mM aqueous Bis-Tris solution containing 5 mM *p*-toluene sulfonic acid and 100 μ M EDTA (2Na), 0.5 mL/min

- Analysis of intestinal flora:

Cecal contents were subjected to freeze-drying treatment.

Bacterial DNAs were extracted from the dried cecal contents and stools, bacterial counts (number of live bacteria + number of dead bacteria) were determined for the genera *Bifidobacterium* and *Lactobacillus* by real time PCR, and the logarithmic value ($\log_{10}$ (bacterial count/g)) of the bacterial count was determined. Furthermore, the proportion of bacterial count for each bacteria with respect to the total bacterial count was calculated as the percentage (%).

Statistical analyses of the results were carried out by Student's t-test (equal variance) or Welch test (unequal variance).

(Results)

Fig. 1 shows the weight of the cecum, etc., and pH in the cecum, for each group. The cecum weight significantly ($p < 0.05$) increased in the test group compared to the control group. This increase was due to increase in the cecal tissue weight and cecal contents. Furthermore, pH in the cecum showed a significantly ($p < 0.05$) low value in the test group compared to the control group.

Fig. 2 shows the amounts of short-chain fatty acids in cecal contents. A significant difference was not observed in the test group compared to the control, but an increasing trend

was observed for short-chain fatty acids.

Fig. 3 depicts the analysis results of intestinal flora of each group. The bacterial count and percentage of *Bifidobacterium* was significantly ($p < 0.05$) increased in both stools and cecal contents for the test group compared to the control group. The bacterial count and percentage of *Lactobacillus* in the cecal contents were also significantly ($p < 0.05$) increased for the test group compared to the control group. Almost no change was observed in the total bacterial count in stools and cecal contents between the two groups (data not shown).

Significant differences were also not observed for body weight and the amount of feed intake during the testing period for both groups. From the above-mentioned results, the nutritional compositions of the present invention were found to supply nutrients and at the same time promote proliferation of *Bifidobacterium* and/or *Lactobacillus*.

Industrial Applicability

The nutritional compositions of the present invention can improve intestinal flora.

Specifically, the compositions of the present invention are useful for promoting proliferation of lactic acid bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, promotion of increase in intestinal weight, promotion of increase in the amount of organic acids in intestinal content, promotion of decrease in pH in the intestine, and promotion of increase in short-chain fatty acids.

CLAIMS

1. A nutritional composition for promoting proliferation of bacteria of the genera *Bifidobacterium* and/or *Lactobacillus*, comprising a milk protein hydrolysate and a fermented milk protein as proteins, oleic acid-containing oil and fat as well as milk phospholipid and/or soybean lecithin as lipids, and isomaltulose as a carbohydrate.
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2. The nutritional composition of claim 1, wherein the milk protein is selected from the group consisting of casein, a milk protein concentrate (MPC), a whey protein concentrate (WPC), a whey protein isolate (WPI), α -lactoalbumin, β -lactoglobulin, and lactoferrin.
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3. The nutritional composition of claim 1 or 2, wherein the milk protein hydrolysate is contained at 0.9 g to 5.0 g per 100 mL of the nutritional composition.
- 15 4. The nutritional composition of any one of claims 1 to 3, wherein the milk protein hydrolysate is obtained by hydrolyzing a whey protein concentrate (WPC) and/or whey protein isolate (WPI) with *Bacillus licheniformis*-derived alcalase or porcine pancreas-derived trypsin.
- 20 5. The nutritional composition of any one of claims 1 to 4, wherein the milk protein hydrolysate is a permeate fraction obtained by treatment with an ultrafiltration membrane having a cutoff molecular weight of 10,000.
6. The nutritional composition of any one of claims 1 to 5, wherein the fermented milk protein is derived from cheese.
- 25 7. The nutritional composition of claim 6, wherein the cheese is quark.
8. The nutritional composition of any one of claims 1 to 7, wherein the fermented milk protein is contained at 0.5 g to 6 g per 100 mL of the nutritional composition.
- 30 9. The nutritional composition of any one of claims 1 to 8, wherein isomaltulose is contained at 4 g to 15 g per 100 mL of the nutritional composition.
- 35 10. The nutritional composition of any one of claims 1 to 9, wherein the lipids contain oleic acid at a proportion of 30% or more of the total fatty acid composition.