

PATENT SPECIFICATION

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(54) FERMENTED MILK PRODUCT CONTAINING VIABLE BIFIDOBACTERIA AND METHOD FOR PRODUCING THE SAME

(71) We, KABUSHIKI KAISHA YAKULT HONSHA, of 1-19, Higashi Shinbashi 1-chome, Minato-ku Tokyo, Japan, a Japanese Body Corporate, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a fermented milk product containing a viable Bifidobacterium which may be used as a foodstuff or a drink or as an additive thereto, and to a process for producing same.

Bifidobacteria are well known as dominant bacteria found in the intestinal flora of a breast-fed infant. Bifidobacteria are known to have various physiological effects, for example:

- (a) suppression of putrefactive bacteria;
- (b) inhibition of production of toxic amines;
- (c) promotion of digestion of human milk casein by the action of phosphoprotein phosphatase;
- and
- (d) growth inhibition of pathogenic bacteria in conjunction with lowering the pH in the intestines by production of acids for example, lactic acid, acetic acid and formic acid.

However, an infant who is bottle-fed has very few intestinal Bifidobacteria which, as indicated above, help to keep the intestines healthy. This is considered to be the reason why a bottle-fed infant is more susceptible to intestinal diseases than is a breast-fed infant. A modified milk powder has therefore been prepared as a substitute for human milk so that a bottle-fed infant is able to maintain an intestinal flora containing a Bifidobacterium.

The cultivation of a standard strain of a Bifidobacterium by the conventional method in a medium of, for example, milk has not been practically carried out on a

commercial scale, for the following reasons:

(a) the conditions for cultivation must be strictly anaerobic;

(b) the nutritional requirement for the cultivation is complicated and strict, and therefore a bacterium does not propagate in a pure milk medium which does not contain any added growth-promoting substance, for example yeast extract; and

(c) it is difficult to maintain a bacterium viable for a long time at a low pH if the acid-resistance of the bacterium is low.

These difficulties are not encountered with lactic-acid forming bacteria, which are widely used in milk processing.

If a growth-promoting substance for a Bifidobacterium is added to a milk medium, the Bifidobacterium can be cultivated even under aerobic conditions. However, the product from this culture often impairs the taste and flavour of the product. In addition to this disadvantage, growth-promoting substances are generally expensive, and therefore not practically used.

We have discovered new strains of Bifidobacteria, having special properties, for example:

(a) such strains can be actively propagated under aerobic conditions without any added growth-promoting or reducing agents;

(b) a cultivated product free from unfavourable tastes can be produced, and the seasoning of such a product is easy;

(c) since additives (for example growth-promoting substances) are not required for cultivation, a food product suitable as a food for infants or as an additive to a food for infants may be produced; and

(d) such strains are essentially oxygen resistant and the nutrition conditions required for their cultivation are simple.

As a result of these advantages, the bacteria in a cultivated product remain viable during preservation or processing of the product into a foodstuff or a drink.

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Thus the present invention consists in a method of producing a fermented milk product which comprises cultivating in a medium comprising milk *Bifidobacterium bifidum* YIT-4002 or *Bifidobacterium bifidum* YIT-4005. These strains are here classified in accordance with Bergey's Manual of 1974, in view of their taxological properties described below. These new strains are deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology No. 8-1, Inage Higashi-5-chome, Chiba-shi, Chiba-ken, Japan, the deposit number of strain YIT-4002 is FERM-P No. 3371 and the deposit number of strain YIT-4005 is FERM-P No. 3372.

Bifidobacterium bifidum YIT-4002 was discovered in successive transfer cultures of *Bifidobacteria* taken from the faeces of a healthy breast-fed infant, and has the following properties:

1. This strain is a Gram-positive, non-spore forming bacillus of size $0.5 \times 2-3 \mu$. It has intracellular granules having an affinity with methylene blue. Observation under a microscope reveals bacteria of this strain to be polymorphous and to have a branched outline, for example a "Y"-shape or a bent-shape. A colony formed by a double-layer plate method is cylindrical, convex or lens-shaped, colonies formed at the bottom of the plate are variable in shape. When the strain was cultivated in a VL-G liquid medium under anaerobic conditions for 72 hours acetic acid and lactic acid were formed in the ratio of 1.6 ± 0.5 moles of acetic acid to 1 mole of lactic acid. The lactic acid so formed had a specific optical rotation of -0.038° which corresponds to 24% DL and 76% L (+). *Bifidobacterium bifidum* YIT-4002 was tested for various properties, as follows: negative results were obtained for catalase activity, carbon dioxide gas production, hydrolysis of gelatine, nitrate reducing activity, indole production and hydrogen sulphide generation; a positive result was obtained for the coagulation of milk. This pattern of properties is characteristic of the genus *Bifidobacterium*.

2. *Bifidobacterium bifidum* YIT-4002 will not propagate at a temperature below 25°C or above 45°C or at a pH lower than 5.0. The optimum temperature is from 36 to 38°C and the optimum pH is from 6 to 7.

3. The fermentation pattern of *Bifidobacterium bifidum* YIT-4002 completely corresponds to that of a standard strain of *Bifidobacterium* (for example *Bifidobacterium bifidum* E 319). This pattern is positive fermentation of glucose, fructose, lactose and galactose, and negative fermentation of arabinose, xylose, salicin, mannose, mannitol, melzitose, cellobiose, sorbitol,

inulin, trehalose, rhamnose, maltose, ribose and sorbose.

4. A population of from 10^7 to 10^8 cells/ml of *Bifidobacterium bifidum* YIT-4002 was incubated at 37°C in a medium of reduced skimmed milk (containing no added growth-promoting substances and having a milk solids content of 12%) saturated with oxygen (about 6.5 ppm). The count of bacteria reached more than 10^9 cells/ml in 24 hours. A standard strain of *Bifidobacteria* hardly propagates under these conditions, as is illustrated in Example 2 and Figure 3 herein.

Bifidobacterium bifidum YIT-4005 was isolated from the faeces of a healthy breast-fed infant by successive treatment in a low pH buffer solution. The following properties of *Bifidobacterium bifidum* YIT-4005 are, with the exception of acid resistance, almost identical to those of *Bifidobacterium bifidum* YIT-4002:

1. This strain is a Gram-positive, non-spore forming bacillus with intracellular granules having an affinity with methylene blue. Observation under a microscope reveals this strain to be polymorphous and to have a bifid outline, for example a "Y"-shape or a bent-shape. A colony formed by a double-layer plate method is cylindrical, convex or lens-shaped, colonies formed at the bottom of the plate are variable in shape. When the colony was cultivated in a medium of a non-fat milk (having a milk solids content of 12%) for 72 hours acetic acid and lactic acid were formed in the ratio of 1.7 ± 0.5 moles of acetic acid to 1 mole of lactic acid. *Bifidobacterium bifidum* YIT-4005 was tested for various properties, as follows: negative results were obtained for catalase activity, gelatine hydrolysis, nitrate reducing activity, indole production and hydrogen sulphide generation; a positive result was obtained for milk coagulating activity.

2. *Bifidobacterium bifidum* YIT-4005 will propagate at a temperature from 25 to 45°C and at a pH from 5 to 7. The optimum temperature is from 36 to 38°C and the optimum pH is from 6 to 7.

3. *Bifidobacterium bifidum* YIT-4005 has the following fermentation pattern: positive fermentation of glucose, fructose, lactose and galactose and negative fermentation of arabinose, xylose, salicin, mannose, mannitol, melzitose, cellobiose, sorbitol, inulin, trehalose, rhamnose, maltose, ribose and sorbose.

4. *Bifidobacterium bifidum* YIT-4005 is acid resistant. This strain and *Bifidobacterium bifidum* E 319 (a "standard" strain) were each cultivated in a VL-G liquid medium, under anaerobic conditions for 48 hours. The cultivated bacteria were collected

under aseptic conditions by centrifugation. Washed sulphur suspensions (OD at $660\text{ m}\mu = 1.5$) were then added to buffer solutions of various pH, the resulting solutions were kept at 5°C . For pH 6 a 0.01 M phosphoric acid buffer was used and for pH from 4 to 5 0.01 M acetic acid buffers were used. Table 1 shows the variation in the size of the two populations (expressed as counts of viable

bacteria per ml) with time. Similarly various strains of Bifidobacteria were cultivated in a non-fat milk medium (having a milk solids content of 10%) containing 0.3% by weight of yeast extract until the pH of the culture reached the values 4.61 or 4.21. The cultures were then rapidly cooled and kept at 5°C . Table 2 shows the variation in the counts with time.

TABLE 1

pH	Strain*	Preservation Days				
		0	3	5	7	10
6.0	V	4.7×10^8	3.8×10^8	3.7×10^8	4.0×10^8	3.0×10^8
	S	4.5×10^8	3.1×10^8	6.8×10^7	4.2×10^7	2.1×10^7
5.0	V	5.0×10^8	3.8×10^8	2.4×10^8	3.0×10^8	3.3×10^8
	S	4.9×10^8	3.1×10^8	5.5×10^7	1.9×10^7	2.1×10^6
4.6	V	4.2×10^8	5.1×10^8	4.2×10^8	2.0×10^8	9.3×10^7
	S	4.5×10^8	1.0×10^7	3.6×10^5	4.0×10^3	$< 10^2$
4.3	V	4.6×10^8	4.0×10^8	2.1×10^8	6.9×10^7	3.0×10^7
	S	4.2×10^8	3.9×10^6	2.1×10^4	$< 10^2$	$< 10^2$
4.1	V	4.7×10^8	9.5×10^7	3.4×10^7	5.6×10^6	4.3×10^5
	S	4.5×10^8	4.1×10^4	1.0×10^3	$< 10^2$	$< 10^2$
4.0	V	5.0×10^8	7.0×10^6	8.0×10^5	2.8×10^5	2.9×10^4
	S	4.9×10^8	1.2×10^3	$< 10^2$	$< 10^2$	$< 10^2$

TABLE 2

pH	Strain*	Preservation Days				
		0	3	5	7	10
4.61	V	4.1×10^9	3.1×10^9	2.0×10^9	7.6×10^8	4.3×10^8
	S	6.8×10^8	2.0×10^7	4.3×10^6	5.3×10^5	1.6×10^4
	A	3.3×10^9	5.6×10^8	1.5×10^7	4.3×10^6	2.1×10^5
	B	1.5×10^9	2.7×10^8	5.3×10^7	9.6×10^6	7.7×10^6
	C	8.8×10^8	6.2×10^7	2.5×10^6	5.1×10^5	2.9×10^4
	D	4.3×10^9	7.1×10^8	7.9×10^7	6.3×10^6	4.4×10^5
4.21	V	1.8×10^9	7.6×10^8	9.8×10^8	7.0×10^7	2.4×10^6
	S	2.5×10^8	2.0×10^6	4.5×10^4	$<10^2$	$<10^2$
	A	9.9×10^8	7.5×10^6	8.2×10^5	2.3×10^3	$<10^2$
	B	4.2×10^9	2.5×10^7	4.9×10^5	2.4×10^3	$<10^2$
	C	2.1×10^9	1.5×10^7	8.7×10^4	$<10^2$	$<10^2$
	D	7.7×10^8	2.1×10^7	6.3×10^5	1.5×10^4	$<10^2$

*V: *Bifidobacterium bifidum* YIT-4005

S: *Bifidobacterium bifidum* E319

A: *Bifidobacterium bifidum*

B: *Bifidobacterium bifidum* YIT-4002

C: *Bifidobacterium longum* standard strain

D: *Bifidobacterium longum*

It can be seen that *Bifidobacterium bifidum* YIT-4005, which has a count greater than 10^4 /ml after seven days of preservation at pH 4.0 under the conditions used in preparing the data of Table 1, has a greater acid resistance than the standard strain. In processing or preservation of cultivated foods or drink products it is desirable to maintain a viable population of bacteria. The high acid resistance of *Bifidobacterium bifidum* YIT-4005 is useful in this respect.

5. Various strains of *Bifidobacterium*, including *Bifidobacterium bifidum* YIT-4005 were separately cultivated in a medium of non-fat milk (having a milk solids content of 12%) which had been sterilised by heating, and then cooled to 37°C after which air had been introduced to the medium. The changes in the populations of viable bacteria and in the total acid content of the cultures (measured as mls of 0.1 N sodium hydroxide required to neutralise 10 mls of culture) during cultivation are shown in Figure 1 of the accompanying drawings. It can be seen that the count of *Bifidobacterium bifidum* YIT-4005 reached 10^9 viable

cells/ml from the initial population of 10^7 — 10^8 cells/ml in 24 hours, whereas the standard strains fail to propagate. Substantially the same results were obtained under anaerobic conditions.

Since the two variant strains described above do not require special conditions for successful cultivation, it is possible to carry out a mixed cultivation of either of these strains with a lactic acid bacterium. Although added growth-promoting and reducing agents are not necessary for the cultivation of these strains, such additives may be employed advantageously to fully promote propagation so long as they do not hinder the use of the cultured products.

Under ordinary conditions the population of a viable *Bifidobacterium* reaches a maximum in from 18 to 24 hours and as the cultivation proceeds, acid is generated and the pH falls. Acetic acid production, in the case of *Bifidobacterium bifidum* YIT-4005, begins at the initial stages of cultivation and the mole ratio of acetic acid to lactic acid remains substantially constant during the cultivation at 1.7 ± 0.5 moles of acetic acid

to 1 mole of lactic acid. If cultivation of a variant strain is continued further the population begins to decrease but the generation of acid continues for a while until the culture becomes saturated with the acid. The cultivation is therefore generally stopped at a suitable stage depending on the intended use of the final product. It is desirable that a cultured product should contain at least 10^7 viable bacteria cells/ml, and a population of at least 10^9 cells/ml can be obtained by the process of the present invention.

Convenient milk media for use in the process of the present invention include whole milk or a lactose-containing component of milk, for example reconstituted milk, milk whey or skimmed milk or a preparation made from any of these ingredients. A cultured product prepared by the method of the present invention, containing a large population of a viable *Bifidobacterium* and its milk metabolite (particularly an organic acid mixture predominantly of acetic acid and lactic acid), may be used as a drink or foodstuff or as an additive thereto. Alternatively the cultured product may be diluted with water or mixed with a seasoning, for example a sweetener, a fruit juice or other flavourings to produce a novel fermented milk beverage. The cultured product may be freeze-dried or spray-dried to produce a powdered product. Such a powdered product, which may contain more than 10^9 viable *Bifidobacteria* cells per gram dry weight, is easy to handle and can therefore be more widely used. For example, a cultured product or its direct equivalent, produced using an acid resistant strain of *Bifidobacterium bifidum*, can be used in combination with a food or drink so long as it is not necessary to heat the material to a temperature lethal to the bacteria. Such foods and drinks include: milk, condensed milk, powdered milk, raw cream, vegetable juice, fruit juice, soybean milk, fermented soybean milk, mayonnaise dressing, ketchup, paste, jam, fish flour, baby food and diet food. A cultivated product containing a viable population of a species of *Bifidobacterium* may either be mixed with any of these foods and drinks during their preparation or be added to them up to 12 hours before serving. In this way foods and drinks containing a viable *Bifidobacterium* and having a pleasant flavour may be produced. The dried powdered products may be mixed, for example, with yeast powder, Chlorella powder, sugar or drugs such as digestives. A mixture containing a powdered product may be made into a tablet.

The present invention is further illustrated by the following Examples. Examples 1 to 5 illustrate the production of a fermented culture containing a viable

species of *Bifidobacterium* and Examples 6 to 18 illustrate the preparation of fermented milk-food and drinks.

Example 1.

Bifidobacterium bifidum YIT-4002, referred to in this Example as the variant, and three standard strains of *Bifidobacteria* were separately cultivated under anaerobic conditions. Seed cultures were prepared by cultivating each strain in a modified medium of Rogosa (1 ml of pyruvic acid and 0.2 g of reduced glutathione were added per litre of liquid medium free of agar), the cultivated bacteria were collected and washed under sterile conditions and then suspended in a 0.01 M phosphate buffer solution (pH = 6.8). The resulting suspension had an optical density of 1.5 at 660 m μ . A reduced non-fat milk medium (having a milk solids content of 12%) was used for the main propagation. The medium was sterilised and placed in a jar, having an anaerobic atmosphere of 95% nitrogen and 5% carbon dioxide. The medium had a dissolved oxygen content of 0.1 ppm at the beginning of the cultivation.

Figure 2 of the accompanying drawings shows the variation in the counts of viable *Bifidobacteria* and the change in the total acid content and pH with cultivation time. The population of the variant strain reached 6.5×10^9 cells/ml in 24 hours and with this increase in population a considerable amount of acid was generated resulting in a fall in pH. In contrast, only a small degree of propagation of the standard strain was noticed. This demonstrates that these standard strains do not propagate substantially even under aerobic conditions.

Example 2.

Bifidobacterium bifidum YIT-4002, referred to in this Example as the variant, and three standard strains of a *Bifidobacteria* were separately cultivated under aerobic conditions using the same seed culture and medium as used in Example 1 herein. After sterilising the medium, it was saturated with oxygen by passing oxygen gas through it; the amount of dissolved oxygen was 6.5 ppm.

Figure 3 of the accompanying drawings shows the change in the population of viable *Bifidobacteria* and the change in the total acid content and pH with cultivation time. The results are substantially the same as those obtained in Example 1 above; the population of the variant reached 5.0×10^9 viable cells/ml after a period of 24 hours. The standard strains failed to propagate substantially and in one case the population of viable cells decreased immediately on adding the seed culture to the medium.

Example 3.

The relation between the degree of

propagation of the *Bifidobacterium bifidum* YIT-4002 and the amount of starter used was investigated. A starter culture was prepared by cultivating the bacterium in non-fat milk (having a milk solids content of 8%) for 20 hours. The starter culture was then preserved at a low temperature for 3 days. This starter culture was then aerobically cultivated in a reduced non-fat milk medium in amounts ranging from 1 to 10% by weight based on the weight of the reduced non-fat milk medium. When the starter was present in amounts of up to 3%, the amount of acid generated increased in proportion to the amount of starter used. When the starter was added in amounts of more than 3% the amount of acid generated was substantially the same. A pilot test indicated that from 1 to 2% of the starter culture is sufficient. Conventionally, the starter has generally been used in amounts of from 5 to 10%. In order to prepare such a large amount of starter, the starter itself has to be prepared by the starter method.

Example 4.

The relations between the degree of propagation of a culture of *Bifidobacterium bifidum* YIT-4002 and the temperature of the culture was investigated. A medium of

reduced non-fat milk, having a milk solids content of 16%, was prepared. The medium was sterilised and then cooled to 37°C during which time the sterilised air was passed through it. A starter culture was added to the medium and five separation cultivations were conducted, each at a different temperature. The starter used was prepared as described in Example 3 herein, the amount used was 2% by weight based on the weight of the main milk medium. The bacterium propagated most successfully at 37°C., proagation at 34°C and at 40°C was good but propagation at 30°C and at 43°C was considerably reduced.

Example 5.

The relation between the viability of *Bifidobacterium bifidum* YIT-4002 and the pH of its culture was investigated. The bacterium was innoculated into four media of non-fat milk, each having a milk solids content of 12%. The media were cultivated until the pH reached 5.0, 4.6, 4.3, and 4.1 respectively. The four media were then preserved by rapid cooling to 5°C. The populations of viable bacterium, measured as cells/ml, recorded over a period of 10 days is shown in Table 3.

TABLE 3

pH	Total Acid Content	Preservation Days				
		0	3	5	7	10
5.0	18.0	4.4×10^9	3.2×10^9	9.6×10^8	4.2×10^8	6.8×10^7
4.6	19.6	5.4×10^9	7.2×10^8	2.3×10^8	8.0×10^7	1.2×10^8
4.3	23.0	3.0×10^9	1.7×10^7	5.7×10^5	2.6×10^5	1.5×10^4
4.1	26.8	6.0×10^8	8.5×10^5	4.7×10^3	$<10^1$	$<10^1$

It can be seen from Table 3 that the decrease in the population of the bacterium with time depends on the pH of the medium. The population of viable cells rapidly decreases with time in a medium having a pH of 4.3 or less. The population decreased far less rapidly when the pH of the medium was at least 4.6.

Substantially the same results were obtained when the cultivation was conducted in whole milk, non-fat milk or in a mixture of whole milk and non-fat milk.

Example 6.

A fermented milk product was prepared in the following way. Non-fat powdered milk (160 g) was dissolved in water (sufficient to produce a 1 litre solution), and the resulting

solution was then sterilised by heating it at 100°C for 30 minutes. The sterilised solution was then cooled, 2% of a starter culture was added and the cultivation was allowed to continue for 16 hours at 37°C (the starter culture had been previously prepared by cultivating *Bifidobacterium bifidum* YIT-4002 under pure conditions). The cultivated solution (525 ml) was mixed with a syrup (475 ml) containing sugar (35 g) and sorbitol (35 g). Spice was then added to the mixture and the mixture was homogenised to produce a fermented milk product. The product thus obtained had a total acid content of 7.0 (the number of mls of 0.1 N sodium hydroxide required to neutralise 10 mls of culture) and a pH of 4.7. The population of viable *Bifidobacteria* was 2.9×10^9 cells/ml.

Example 7.

A mixture having a non-fat milk solids content of 16% was prepared by adding non-fat powdered milk to whole milk. A *Lactobacillus acidophilus* starter (5%) by weight, based on the weight of the mixture and a *Bifidobacterium bifidum* YIT-4002 starter (1% by weight, based on the weight of the mixture) were added to the mixture, and were cultivated for 20 hours at 37°C. The cultivated solution (525 mls) were mixed with a syrup (472 mls) containing glucose (20 g) and fructose (35 g). After adding spice, the mixture was homogenised to produce a fermented milk product. The product thus obtained had a total acid content of 3.3 (the number of mls of 0.1 N sodium hydroxide required to neutralise 10 mls of the culture) and a pH of 4.8. The population of viable *Bifidobacterium bifidum* YIT-4002 was 1.9×10^9 cells/ml and that of *Lactobacillus acidophilus* was 5.0×10^8 cells/ml.

Example 8.

Non-fat powdered milk (100 g) and whole powdered milk (60 g) were dissolved in sufficient water to produce 1 litre of solution, the solution was then sterilised. A *Bifidobacterium bifidum* YIT-4002 starter (3% by weight based on the weight of the solution) was added to the solution, and then cultivated for 12 hours at 37°C. The cultivated solution (525 mls) was mixed with a syrup containing 100 g of fruit juice (tangerine and apple), sugar (40 g) and sorbitol (35 g). Spice was then added to the mixture and the mixture was homogenised to produce a fermented milk product. The product thus obtained had a total acid content of 4.0 (number of mls 0.1 N sodium hydroxide required to neutralise 10 mls of the culture) and a pH of 4.6. The population of viable *Bifidobacteria* was 1.5×10^9 cells/ml.

Example 9.

A *Bifidobacterium bifidum* YIT-4005 starter culture was added to 10 litres of heat-sterilised skimmed milk and cultivated for 3 hours at 37°C. The amount of starter used was 10% by weight, based on the weight of the heat-sterilised skimmed milk. Curd was formed by adding rennet powder (0.05%, 30,000 units) to the cultivated product. The curd was then sliced, and washed with water to remove the whey. The fat content of the curd was adjusted to 30% by weight by adding 12% by weight of cream (based on the weight of the curd). Flavouring and a stabiliser were then mixed with the curd to produce a final cheese product containing *Bifidobacterium bifidum*. Immediately after its preparation the cheese thus prepared contained 7.6×10^7 viable cells/g. The count of viable bacteria was 2.6×10^7 cells/g on the tenth day of preservation.

Example 10.

A *Bifidobacterium bifidum* YIT-4005 starter was added to heat-sterilised reconstituted skimmed milk (having a milk solids content of 16%) which had been cooled by introducing sterile air and stirring. After incubation for 48 hours at 37°C, the culture (525 mls) was mixed with a syrup (475 mls) containing sugar (80 g). Flavours were then added to the mixture. The resulting mixture was then homogenised to prepare a fermented milk product containing viable *Bifidobacterium bifidum*. The fermented milk product thus prepared had a pH value of 4.30 and the count of the viable bacterium was 3.6×10^9 cells/ml. The count of viable *Bifidobacteria* had decreased to 4.9×10^7 cells/ml on the seventh day of preservation.

Example 11.

A milk medium having a milk solids content of 16% was produced by adding skimmed dried milk to whole milk, the medium was then sterilised. A starter culture of *Lactobacillus acidophilus* (5% by weight, based on the weight of the milk medium) and a starter culture of *Bifidobacterium bifidum* YIT-4005 (1% by weight, based on the weight of the milk medium) were added to the medium and cultivated for 20 hours at 37°C. 200 mls of fruit juice (consisting of 3 parts of banana juice to 1 part of tangerine juice) and 475 mls of syrup containing sugar (60 g) and flavourings were added to 525 mls of the culture. The resulting mixture was then homogenised. The fermented milk product thus prepared had a pH of 4.40 and contained 2.6×10^9 viable *Bifidobacterium bifidum* cells/ml and 4.8×10^8 viable *Lactobacillus acidophilus* cells/ml. The count of viable *Bifidobacterium bifidum* and viable *Lactobacillus acidophilus* had decreased to 6.8×10^7 cells/ml and 2.0×10^6 cells/ml respectively, on the seventh day of preservation.

Example 12.

Defatted soybean powder (70 g), milk whey powder (30 g) and lactose (10 g) were added to sufficient water to produce a 1 litre medium, the medium was then sterilised. A *Bifidobacterium bifidum* YIT-4005 starter culture (5% by weight, based on the weight of the medium) was then added to the medium and was incubated at 37°C for 72 hours. A syrup (500 mls) containing sugar (60 g), sorbitol (50 g) and flavouring was then added to the culture (500 mls). The resultant mixture was homogenised. The fermented soybean milk product thus prepared, had a pH value of 4.3 and contained 1.6×10^9 viable *Bifidobacterium* cells/ml. The count of viable *Bifidobacteria* decreased to 3.8×10^7 cells/ml on the sixth day of preservation.

Example 13.

5 A milk medium was prepared by dissolving skimmed milk powder (100 g) in water (900 mls). The medium was then sterilised. A *Bifidobacterium bifidum* YIT-4005 starter culture (2% by weight, based on the weight of the milk medium) was added to the milk medium and cultivated for 24 hours at 37°C. The cultivated milk product medium contained 2.8×10^9 viable *Bifidobacteria* cells/ml and had a total acid content of 10.5 (number of mls of 0.1 N sodium hydroxide required to neutralise 10 mls of the medium). Acetic acid and lactic acid were produced in the ratio of 1.7 moles of acetic acid to 1 mole of lactic acid.

10 The culture was then frozen and dried for about 7 hours under a maximum pressure of 2 mm Hg. 105 g of a dried material containing 5.5×10^9 viable *Bifidobacteria* cells/g were obtained.

Example 14.

Reconstituted milk media (having a milk solids content of 16%) containing; no added growth-promoting agents, added peptones, added corn steep liquor (CSL) and added yeast extract, were prepared. A *Bifidobacterium bifidum* YIT-4005 starter (2% by weight, based on the weight of the milk medium) was added to each milk medium. The cultivation was carried out 37°C for 15 hours. Fermented milk products were prepared from these media by the method described in Example 10. Table 4 shows the total acid content, the pH and the counts of viable *Bifidobacteria* cells/ml of these cultures. Table 5 shows a taste evaluation of the four cultivated products by a panel of 36 people and a statistical treatment of these results by Scheffe's paired comparative method for the 12 combinations of pair samples.

TABLE 4

Growth-promoting substance		Acidity	pH	Counts of Viable <i>Bifidobacteria</i> /ml
None		7.0	4.75	1.5×10^9
Peptone	0.5%	8.0	4.55	2.0×10^9
CSL	1.0%	7.2	4.60	1.6×10^9
Yeast extract	0.3%	7.0	4.75	2.5×10^9

TABLE 5

Growth-promoting Substance		Taste Evaluation*			
None		+0.3 ~ 1.3			
Peptone		-1 ~ 0			
CSL		-0.5 ~ +0.4			
Yeast extract		-1 ~ +0.1			
Significance of Difference					
Factorial effect	Sum of squares	Degree of freedom	Unbiased variance	F ₀	Level of significance
Main effect	46.042	3	15.347	24.444	up to 1%
Combination effect	0.291	3	0.097	0.155	—
Ordinal effect	32.000	6	5.333	8.494	up to 1%
Error	37.667	60	0.628	—	—

* -1: poor 0: average
+1: good +2: better

Example 15.

A medium was prepared having a pH of 6.8 and containing 3% of yeast extract powder, 5% of corn steep liquor, 0.5% of citric acid, 0.5% of ammonium sulphate, 1% of lactose and 0.03% of cysteine hydrochloride. These percentages are percentages by weight, based on the weight of the medium plus additives. 3% by weight of a starter culture of *Bifidobacterium bifidum* YIT-4005 was then added to this medium, the cultivation was conducted at 37°C for 72 hours. The culture was centrifuged and the collected bacteria were suspended in water containing 5% by weight of skimmed milk which had been hydrolysed by protease, 5% by weight of sugar and 1% by weight of vitamin C. The suspension was then freeze-dried.

The dried bacterial powder thus obtained was added to sterilised milk in an amount of 2% by weight, based on the weight of the sterilised milk. The mixture was then homogenised, producing a milk product containing 4.6×10^8 viable *Bifidobacteria* cells/ml. The count of viable cells decreased to 2.2×10^6 cells/ml on the sixth day of preservation.

Example 16.

A starting material of ice cream (150 litres) was produced by dissolving raw cream (7 kg) having a fat content of 40%, sweetened condensed whole milk (20 kg), milk (64 litres), skimmed milk powder (2 kg), sugar (6 kg) and a stabiliser (0.3 kg) in water. The mixture was then heated, sterilised, homogenised and cooled (to a temperature not exceeding 40°C). 1% of a dry bacterial powder as prepared in Example 15 was then added to the mixture. The mixture was then dispensed into cups, and solidified by cooling to -20°C. The resulting ice cream contained 3.0×10^8 viable *Bifidobacteria* cells/ml. The count of viable cells decreased to 2.2×10^6 cells/ml in the third month of preservation.

Example 17.

Sliced vegetables (130 g of carrot, 100 g of tomato, 3 g of celery and 100 g of cabbage) were boiled in 500 mls of water. The boiled vegetables were then mashed in a mixer and filtered through a cloth to obtain the juice of the vegetables. Chlorella powder (10 g), table salt (0.5 g), spices (0.1 g) and dry bacterial powder (1 g) as prepared in Example 15 were added to the vegetable juice (100 mls). The resulting vegetable juice product contained 2.8×10^8 viable *Bifidobacteria* cells/ml. The count of viable cells

decreased to 6.5×10^7 cells/ml on the tenth day of preservation.

Example 18.

Dry bacterial powder as prepared in Example 15 was mixed with whole milk powder in the ratio of 5 g of bacterial powder to every 100 g of whole milk powder. Nitrogen gas was then added to the mixture to produce a milk powder suitable for infants. The final product contained 1.0×10^9 viable *Bifidobacteria* cells/g. The count of viable cells decreased to 5.1×10^7 cells/g after three months of preservation.

WHAT WE CLAIM IS:—

1. A method of producing a fermented milk product, which comprises cultivating in a medium comprising milk *Bifidobacterium bifidum* YIT-4002 or *Bifidobacterium bifidum* YIT-4005 (deposit numbers FERM—P 3371 and FERM—P 3372 respectively at the Fermentation Research Institute referred to herein).
2. A method according to claim 1, substantially as herein described with reference to any one of the foregoing examples.
3. A fermented milk product when produced by a method according to claim 1 or claim 2, said fermented milk product containing viable *Bifidobacterium bifidum* YIT-4005 and a mixture of organic acids.
4. A fermented milk product according to claim 3, in which said mixture of organic acids is a mixture of acetic acid and lactic acid in the ratio of 1.7 ± 0.5 moles of acetic acid to 1 mole of lactic acid.
5. A fermented milk product when produced by a method according to claim 1 or claim 2, said fermented milk product containing viable *Bifidobacterium bifidum* YIT-4005 and a mixture of organic acids.
6. A fermented milk product according to claim 5, in which said mixture of organic acids is a mixture of acetic acid and lactic acid in the ratio of 1.6 ± 0.5 moles of acetic acid to 1 mole of lactic acid.
7. A fermented milk product according to claim 3 or claim 5 substantially as herein described with reference to any one of the foregoing examples.
8. A food stuff or a drink containing a fermented milk product according to any one of claims 3 to 7.
9. A dry fermented milk product produced by drying a fermented milk product according to any one of claims 3 to 7.
10. A food stuff or drink containing a dried fermented milk product according to claim 9.

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A medium was prepared having a pH of 6.8 and containing 3% of yeast extract powder, 5% of corn steep liquor, 0.5% of citric acid, 0.5% of ammonium sulphate, 1% of lactose and 0.03% of cysteine hydrochloride. These percentages are percentages by weight, based on the weight of the medium plus additives. 3% by weight of a starter culture of *Bifidobacterium bifidum* YIT-4005 was then added to this medium, the cultivation was conducted at 37°C for 72 hours. The culture was centrifuged and the collected bacteria were suspended in water containing 5% by weight of skimmed milk which had been hydrolysed by protease, 5% by weight of sugar and 1% by weight of vitamin C. The suspension was then freeze-dried.

The dried bacterial powder thus obtained was added to sterilised milk in an amount of 2% by weight, based on the weight of the sterilised milk. The mixture was then homogenised, producing a milk product containing 4.6×10^8 viable *Bifidobacteria* cells/ml. The count of viable cells decreased to 2.2×10^6 cells/ml on the sixth day of preservation.

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2. A method according to claim 1, substantially as herein described with reference to any one of the foregoing examples.

3. A fermented milk product when produced by a method according to claim 1 or claim 2, said fermented milk product containing viable *Bifidobacterium bifidum* YIT-4005 and a mixture of organic acids.

4. A fermented milk product according to claim 3, in which said mixture of organic acids is a mixture of acetic acid and lactic acid in the ratio of 1.7 ± 0.5 moles of acetic acid to 1 mole of lactic acid.

5. A fermented milk product when produced by a method according to claim 1 or claim 2, said fermented milk product containing viable *Bifidobacterium bifidum* YIT-4005 and a mixture of organic acids.

6. A fermented milk product according to claim 5, in which said mixture of organic acids is a mixture of acetic acid and lactic acid in the ratio of 1.6 ± 0.5 moles of acetic acid to 1 mole of lactic acid.

7. A fermented milk product according to claim 3 or claim 5 substantially as herein described with reference to any one of the foregoing examples.

8. A food stuff or a drink containing a fermented milk product according to any one of claims 3 to 7.

9. A dry fermented milk product produced by drying a fermented milk product according to any one of claims 3 to 7.

10. A food stuff or drink containing a dried fermented milk product according to claim 9.

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