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[TYPE OF PAPER] SPECIFICATION

[TITLE OF INVENTION] LACTIC ACID BACTERIA OF LACTOBACILLUS
HELVETICUS HAVING HIGH TRIPEPTIDE PRODUCTIVITY

[SCOPE OF CLAIMS]

- 5 1. Lactic acid bacteria of *Lactobacillus helveticus*
characterized in that said bacteria have the following
bacteriological properties, that said bacteria, when cultured
in a medium of animal milk containing 9wt% or more of solid of
non-fat milk, produce tripeptides Val-Pro-Pro and/or Ile-
10 Pro-Pro in an amount of not less than 60µg in terms of Val-
Pro-Pro per ml medium, and that said bacteria exhibit
extracellular proteinase activity of not less than 400U/OD₅₉₀:
(Morphological Properties)
- 1) Shape of Cell; rod,
15 2) Motility; none,
3) Spore Formation; none,
4) Gram Stain; positive
(Physiological Properties)
- 1) Catalase Production; negative,
20 2) Indole Production; negative,
3) Nitrate Reduction; negative,
4) Aerobic Growth; facultative anaerobic,
5) Formation of D(-)lactic acid from glucose by homolactic
fermentation without formation of gases
25 6) Carbohydrate Degradation
glucose; +

lactose; +
mannose; +
fructose; +
galactose; +
5 sucrose; -
maltose; -
xylose; -
rhamnose; -
cellobiose; -
10 trehalose; -
melibiose; -
raffinose; -
stachyose; -
mannitol; -
15 sorbitol; -
esculin; -
salicin; -.

2. The lactic acid bacteria of *Lactobacillus helveticus* of claim 1 wherein said lactic acid bacteria is *Lactobacillus*
20 *helveticus* CM4 strain (deposition number at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology; FERM BP-6060).

3. The lactic acid bacteria of *Lactobacillus helveticus* of claim 1 or 2 wherein double-digestion product of a chromosomal
25 DNA of said bacteria by restriction enzymes PstI and EcoRI contains a characteristic DNA fragment of 15 to 17 kb.



4. A fermented milk product obtained by fermenting an animal milk using said lactic acid bacteria of any of claims 1 to 3.

[DETAILED DESCRIPTION OF THE INVENTION]

[Field of Invention]

5 The present invention relates to lactic acid bacteria of *Lactobacillus helveticus* that can produce a particular tripeptide having high hypotensive effect and stress-relieving effect when they are cultured in an animal milk medium and that have high extracellular proteinase activity.

10 [Prior Art]

Lactobacillus helveticus has been employed for producing fermented milk for a long time as a typical lactic acid bacteria starter for dairy milk products. Lactobacillus helveticus has high proteolytic activity, and particularly, its extracellular
15 proteinase having high activity plays an important role in fermentation of animal milk. That is, the extracellular proteinase digests animal milk proteins to produce various peptide fragments. The produced peptides are further subjected to the action of peptidases to become peptides of lower molecular
20 weight. It is known that a part of peptides produced in a medium due to the action of proteinase enzymes is taken into cells of the lactic acid bacteria and utilized as a nitrogen source. It has also been reported that some of the peptides produced in the medium have an inhibitory activity against angiotensin
25 converting enzyme (ACE) which causes hypertension. (J. Dairy Sci. 78:777-783(1995)).



As peptides for inhibiting ACE activity and suppressing rise in blood pressure, various effective peptides have been reported, such as those derived from degradation products of milk proteins, soybean proteins or fish meat proteins. It is confirmed that
5 the ACE inhibitory peptides in the fermented milk of *Lactobacillus helveticus* are Val-Pro-Pro and Ile-Pro-Pro (abbreviated hereinbelow as VPP and IPP, respectively. These peptides are collectively referred to hereinbelow as
lactotripeptides). These lactotripeptides have been confirmed
10 to have a strong hypotensive effect by experiments using spontaneously hypertensive rat (SHR) (J. Dairy Sci. 78:1253-1257(1995)). In addition to the hypotensive effect, the lactotripeptides are suggested to have an stress-relieving effect.

15 The lactotripeptides are thus being studied as a practical hypotensive agent or stress-relieving agent that are natural and having low toxicity and high safety, and that can be taken orally.

However, the lactotripeptide-containing fermented milk
20 produced by fermenting animal milk with conventional *Lactobacillus helveticus* strains can hardly be taken as it is, because it exhibits high acidity due to a large quantity of lactic acid generated as the fermentation progresses.

Given that fermented milk with higher content of the
25 lactotripeptides with respect to the content of the lactic acid generated in the fermented milk can be produced, it will be

possible to easily produce products having function and in an agreeable form to take for consumer's use, by addition of a small amount of such fermented milk to various foods and beverages. Therefore, it is desired a strain of lactic acid bacteria that
5 can produce higher amount of the lactotripeptides.

[Problems Solved by the Invention]

It is an object of the present invention to provide a lactic acid bacteria strain which can produce a large amount of lactotripeptide.

10 It is another object of the present invention to provide a fermented milk product which contains a large amount of an ingredient having hypotensive activity and stress-relieving effect and which can be taken pleasantly as foods or beverages.

[Means for Solving the Problem]

15 According to the present invention, there is provided lactic acid bacteria of *Lactobacillus helveticus* characterized in that said bacteria have the following bacteriological properties, that said bacteria, when cultured in a medium of animal milk containing not less than 9wt% or more of solid of non-fat milk,
20 produce tripeptides Val-Pro-Pro and/or Ile-Pro-Pro in an amount of not less than 60µg in terms of Val-Pro-Pro per ml medium, and that said bacteria exhibit extracellular proteinase activity of not lower than 400U/OD₅₉₀:

(Morphological Properties)

- 25 1) Shape of Cell; rod,
2) Motility; none,

3) Spore Formation; none,

4) Gram Stain; positive

(Physiological Properties)

1) Catalase Production; negative,

5 2) Indole Production; negative,

3) Nitrate Reduction; negative,

4) Aerobic Growth; facultative anaerobic,

5) Formation of D(-)lactic acid from glucose by homolactic
fermentation without formation of gases

10 6) Carbohydrate Degradation

glucose; +

lactose; +

mannose; +

fructose; +

15 galactose; +

sucrose; -

maltose; -

xylose; -

rhamnose; -

20 cellobiose; -

trehalose; -

melibiose; -

raffinose; -

stachyose; -

25 mannitol; -

sorbitol; -

esculin; -

salicin; -.

According to the present invention, there is also provided the lactic acid bacteria of *Lactobacillus helveticus* wherein
5 said lactic acid bacteria is *Lactobacillus helveticus* CM4 strain (deposition number at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology; FERM BP-6060).

According to the present invention, there is further
10 provided the lactic acid bacteria of *Lactobacillus helveticus* wherein double-digestion products of a chromosomal DNA of said bacteria by restriction enzymes PstI and EcoRI contains a characteristic DNA fragment of 15 to 17 kb.

According to the present invention, there is also provided
15 a fermented milk product obtained by fermenting an animal milk with the lactic acid bacteria.

[Embodiment of the Present Invention]

The lactic acid bacteria of the present invention belong to *Lactobacillus helveticus*, and produce tripeptides Val-
20 Pro-Pro and/or Ile-Pro-Pro in an amount of not less than 60 μ g, and preferably not less than 70 μ g in terms of Val-Pro-Pro per ml medium when cultured in a medium of animal milk containing not less than 9wt% solid of non-fat milk, and exhibit extracellular proteinase activity of not lower than 400U/OD₅₉₀,
25 and preferably not lower than 430U/OD₅₉₀. The maximum productivity of VPP and/or IPP is not particularly limited, but

can be achieved when all of Val-Pro-Pro and Ile-Pro-Pro included as the sequences in the protein of the medium are taken out as the tripeptides by digestion. The upper limit of the extracellular proteinase activity is not limited either, but
5 is usually 800U/OD₅₉₀.

Examples of the animal milk containing not less than 9wt% solid of non-fat milk may include a usual animal milk containing a protein including the sequence Val-Pro-Pro and/or Ile-Pro-Pro.

10 The fermentation may be performed by inoculating the animal milk with the lactic acid bacteria and fermenting at 37° C for 24 hours.

As an example of the present lactic acid bacteria, Lactobacillus helveticus CM4 strain is deposited as FERM BP-6060
15 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology. Lactobacillus helveticus CM4 strain has the following bacteriological properties:

1. Morphological Properties

- 20 1) Shape of Cell; rod,
2) Motility; none,
3) Spore Formation; none,
4) Gram Stain; positive

2. Physiological Properties

- 25 1) Catalase Production; negative,
2) Indole Production; negative,

- 3) Nitrate Reduction; negative,
4) Aerobic Growth; facultative anaerobic,
5) Formation of D(-)lactic acid from glucose by homolactic
fermentation without formation of gases
- 5 6) Carbohydrate Degradation
- glucose; +
lactose; +
mannose; +
fructose; +
10 galactose; +
sucrose; -
maltose; -
xylose; -
rhamnose; -
15 cellobiose; -
trehalose; -
melibiose; -
raffinose; -
stachyose; -
20 mannitol; -
sorbitol; -
esculin; -
salicin; -.

The aforementioned bacteriological properties are
25 identical with publicly known *Lactobacillus helveticus*
NCDO-099 strain when examined by the method of Mitsuoka et al.

(Rinshoukensa 18, 1163(1974)). However, as to the following properties, which are not described in Mitsuoka et al., CM4 is clearly distinguished from NCDO-099.

7) Fermentability; the acidity of the lactic acid of not less
5 than 2.0wt% when cultured at 37° C for 24 hours in 9wt% skim milk having been sterilized at 95° C for 5 minutes.

8) Extracellular proteinase activity; not less than 400U/OD₅₉₀

9) Lactotripeptide productivity; production of two sorts of
tripeptides (VPP and/or IPP) in an amount of 60µg or more in
10 terms of VPP per ml fermented liquid when cultured in a medium containing 9wt% skim milk at 37° C for 24 hours

The extracellular proteinase activity is measured in accordance with the method of Yamamoto et al. (Yamamoto, N. et al. J.Biochem. (1993) 114, 740) based on the method of Twining
15 et al. (Twining, S. Anal. Biochem. 143 3410 (1984)), and expressed by defining the amount of enzyme exhibiting 1% fluorescent intensity to be 1U/OD₅₉₀.

The productivity of the lactotripeptides is measured by culturing the strain in 9wt% skim milk at 37° C for 24 hours,
20 centrifuging 1ml of the fermented milk at 15,000rpm for 10 minutes, subjecting the supernatant to measurement for the amounts of VPP and IPP, and converting the amounts into the VPP amount. Converted amount of the lactotripeptide in terms of VPP is calculated by the following equation since ACE inhibitory
25 activity of IPP per unit weight is 1.7 times that of VPP;

Converted amount of lactotripeptide (μg in terms of VPP per ml)
= amount of IPP ($\mu\text{g}/\text{ml}$) $\times$ 1.7 + amount of VPP ($\mu\text{g}/\text{ml}$)

The lactic acid bacteria strain of the present invention
5 may be obtained by, e.g., the following screening.

(1) Primary screening

(Selection of strain having high ACE inhibitory activity in the
fermented milk)

The strains to be screened are cultured in a 9wt% skim milk
10 medium at 37°C for 24 hours. After finishing the culturing,
the number of the lactic acid bacteria, acidity of the lactic
acid, and ACE inhibitory activity are measured. Strains
producing 1×10^8 cells/ml or more of lactic acid bacteria, and
exhibiting acidity of the lactic acid of 1.6wt% or more and ACE
15 inhibitory activity of 40 unit/ml or more are selected. ACE
inhibitory activity is measured by Cushman and Cheung's method
(Cushman, D.W. and Cheung, H.S. Pharmacol., 20 1637(1971)).

(2) Secondary Screening

(Selection of strain having high lactotripeptide productivity)

20 The cultured liquids of the strains selected by the primary
screening are centrifuged at 15,000rpm for 10 minutes, and the
supernatants thereof are subjected to HPLC for quantifying the
lactotripeptide. Strains which produced not less than 30 μg in
terms of VPP per ml are selected.

25 (3) Tertiary Screening

(Selection of strain having high extracellular proteinase

activity)

Each of the strains selected by the secondary screening is cultured in a 9wt% skim milk medium while pH thereof is maintained at 6. Sample is taken in the middle of logarithmic growth phase, and admixed with 1 wt% of sodium citrate, and centrifuged at 5,000rpm for 10 minutes to harvest cells. The harvested cells were washed with 50mM β -glycerophosphoric acid, and suspended in 50mM Tris-HCl buffer (pH7.8) to adjust turbidity (OD_{590}) to 1. Proteinase activity on the cell surface is then measured. It will be confirmed that the result is correlative with lactotripeptide productivity of strains measured in the secondary screening.

The lactic acid bacteria strain of *Lactobacillus helveticus* selected by the above method can be distinguished from other lactic acid bacteria strains by, e.g., the aforementioned lactotripeptide productivity and extracellular proteinase activity.

The selected lactic acid bacteria of *Lactobacillus helveticus* can more clearly be distinguished from other strains of the same species on the basis of the specific molecular weight distribution of DNA fragments obtained by digestion of chromosomal DNA. Specifically, if double-digestion product of the chromosomal DNA with restriction enzymes PstI and EcoRI contains a characteristic DNA fragment of 15 to 17kb, the lactic acid bacteria can be identified as that of the present invention.

The existence of the characteristic DNA fragment of 15 to

17 kb may be confirmed by extracting the chromosomal DNA of the lactic acid bacteria in accordance with the method of Leenhouts et al. (Leenhouts, K. (1990) Appl. Environ. Microbiol. 56:2726), digesting the chromosomal DNA with EcoRI and PstI, performing
5 0.8% agarose gel electrophoresis of the digested fragments, and analyzing the resulting electrophoresis pattern. Upon electrophoresis, the existence of the DNA fragment is clearly confirmed by subjecting λ phage DNA digested with a restriction enzyme Hind III to parallel electrophoresis as a size marker.

10 The fermented milk product of the present invention is obtained by fermentation of a medium with the lactic acid bacteria.

The medium for obtaining the present fermented milk product may be milk mediums such as cow's milk, goat's milk, horse's
15 milk, or skim milks of these.

The fermentation may be performed at 25 to 50° C and preferably 30 to 45° C, for 6 to 30 hours and preferably 10 to
24 hours. The fermentation is preferably performed such that the amount of the lactotripeptide is not less than 60 μ g in terms
20 of VPP per ml of the resulting fermented milk. Specifically, for example, when cow's milk is employed as a medium, fermentation at 25 to 40° C for 12 to 48 hours results in a fermented milk product containing the lactotripeptide in an amount of not less than 70 μ g in terms of VPP per ml.

25 The present fermented milk product obtained by the above fermentation as it is may be provided as a product. If necessary,

the fermented milk may be cooled and stored at 5°C, and then admixed with other components to prepare a product such as a chilled product. Alternatively, the fermented milk may be subjected to heat sterilization treatment, and, if necessary,
5 powdered by spray drying to prepare a product for distributing at an ordinary temperature.

The fermented milk product of the present invention may be provided as a product in the form of yogurt, milk-containing acidified beverages, cheese, processed foods containing
10 fermented sour milk, healthy foods, powders, granule foods and tablets.

Since the fermented milk product of the present invention contains high content of the lactotripeptide, it is capable of exhibiting hypotensive effect and anti-stress effect when taken
15 by human being. For example, for a human with hypertension, intake of about 100ml of the present fermented milk product in daily food consumption results in improvement of health. For a human having much stress, intake of the same amount of the present fermented milk product will also exhibit the effect.

20 [Effect of the Invention]

Since the lactic acid bacteria of the present invention can produce a large amount of the lactotripeptide by culturing them in the animal milk medium, the bacteria can produce a fermented milk product containing a large amount of ingredients having
25 hypotensive effect and stress-relieving effect in agreeable form to take.

Since the fermented milk product of the present invention contains a large amount of the lactotripeptides, it can be used as foods having hypotensive effect and anti-stress effect, such as functional foods, healthy foods, foods for specified health
5 use, foods for specified use for elder people, or the materials thereof.

[Examples of the Invention]

The present invention will be explained more in detail hereinbelow referring to the Examples, but the present invention
10 is not limited thereto.

Among the *Lactobacillus helveticus* strains used in the Examples, CM4 strain is deposited at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology and has been accorded accession number FERM
15 BP-6060. ATCC15009, NCDO-099, JCM1006, ATCC10797, JCM1062, JCM1103, JCM1120 and JCM1004 are publicly available strains. Strains other than the above strains used in the Examples are selected from strain collection of the applicant.

[Example 1]

20 (Selection of strains giving fermented milk having high ACE inhibitory activity)

36 strains of *Lactobacillus helveticus* isolated from various dairy products were screened. ACE inhibitory activity of milk fermented with each of the strains was measured by the
25 following procedure. Each of the *Lactobacillus helveticus* strains was cultured in a 9wt% solid of non-fat milk medium at

37° C for 24 hours. The cultured medium was added to a fresh medium of the same type in such an amount that the new medium contains 3wt% of the cultured medium. Fermentation was further performed at 37° C for 24 hours. After finishing the
5 fermentation, acidity of lactic acid (wt%), the amount of the peptide in the whey (mg/ml), the number of cells and ACE inhibitory activity (U/ml) were measured. The results are shown in Table 1. 7 strains out of 36 strains had very weak fermentability. 15 strains produced fermented milk with the
10 acidity of the lactic acid generated of not less than 1.6 wt%. Out of the 15 strains, 8 strains having ACE inhibitory activity of not less than 40U/ml whey in its fermented milk were selected. (Measurement of ACE inhibitory activity of fermented milk)

ACE inhibitory activity was measured in accordance with
15 Cushman and Cheung's method (Cushman, D.W. and Cheung, H.S. Pharmacol., 20 1637 (1971)). That is, each of the fermented milk was centrifuged at 15,000rpm for 5 minutes to obtain the supernatant (whey). The whey was suitably diluted for
measurement. 80μl of the diluted whey was put in a tube,
20 admixed with 0.2 ml of 0.1M boric acid buffer (containing 0.3M NaCl, pH7.3) containing 5mM hippuryl histidine leucine (Hip-His-Leu, manufactured by SIGMA CHEMICALS CO.) as a substrate, and further admixed with 20μl of enzyme solution (0.1U/ml, manufactured by SIGMA CHEMICALS CO.). The resulting
25 mixture was reacted at 37°C for 30 minutes, and then admixed with 250μl of 1N hydrochloric acid for terminating the reaction.

Subsequently, the mixture was admixed with 1.7ml of ethyl acetate, stirred for 20 seconds, and then centrifuged at 3,000rpm for 10 minutes to recover 1.4ml of ethyl acetate layer (upper layer). The upper layer was heated at 120°C for 40
5 minutes to remove the solvent, admixed with 1ml of distilled water, and stirred for about 20 seconds. The absorbance of hippurylic acid extracted was measured at 228nm. The enzyme activity in unit was calculated by the following equation with the amount which gives 50% inhibition of the ACE activity being
10 defined as one unit.

$$\text{Amount of the enzyme (unit)} = ((A-B) / (A-C)) \times 100 \times 1/50$$

A: Absorbance at 228nm without sample

B: Absorbance at 228nm with sample

15 C: Absorbance at 228 without enzyme nor sample

(Quantitative analysis of amount of peptides in the fermented milk)

Quantitative analysis of the peptides was performed in
20 accordance with OPA method (Charch, F.C. et al. J.Dairy Sci. 66
1219(1983). As a standard substance for generating a calibration curve, casein digested with trypsin was employed.

Table 1

Strains	Acidity (wt%)	Amount of Peptides (mg/ml)	Number of Cells ($\times 10^8$ cells/ ml)	ACE Inhibitory activity(U /ml)
strain 1	-	-	-	-
strain 2	-	-	-	-
strain 3	-	-	-	-
strain 4	-	-	-	-
strain 5	-	-	-	-
strain 6	-	-	-	-
strain 7	-	-	-	-
strain 8	0.498	1.59	0.29	26.4
strain 9	2.022	1.99	9.53	34.5
strain 10	1.709	2.10	8.53	24.5
strain 11	0.615	1.76	1.28	29.1
strain 12	0.411	1.35	0.38	17.6
strain 13	0.917	1.57	3.63	19.9
strain 14	1.026	1.71	5.78	9.4
strain 15	0.517	1.59	0.56	26.9
strain 16	1.532	4.69	5.97	102.5
strain 17	2.101	2.01	6.09	98.9
strain 18	1.783	1.94	5.38	21.9
strain 19	1.955	1.69	5.31	100.6
strain 20	2.095	1.74	7.16	61.4
strain 21	1.963	2.03	6.05	125.3
strain 22	1.798	2.85	6.19	54.2
strain 23	1.604	2.32	6.81	36.6
strain 24	1.932	1.77	7.97	47.7
strain 25	1.885	1.51	4.91	18.3
strain 26	1.862	1.46	5.69	26.2
strain 27	1.063	3.01	2.78	76.9
strain 28	0.457	1.98	0.50	52.4
strain 29	0.516	2.55	1.13	92.6
JCM1006	1.872	2.35	6.97	48.5
JCM1062	1.109	2.60	8.50	78.4
JCM1103	1.244	1.36	3.69	31.0
ATCC10797	1.359	2.11	8.56	13.8
ATCC15009	1.454	1.81	5.16	16.6
NCDO-099	1.769	2.76	6.59	25.5
CM4	1.635	3.12	4.44	130.0

(Selection of strains having high lactotripeptide productivity)

Subsequently, the 8 strains which gave fermented milk having high ACE inhibitory activity as selected above were measured for VPP and IPP in their fermented milk.

1ml of the fermented milk was centrifuged at 15,000rpm for 10 minutes. The supernatant thereof, i.e. whey, was collected. 0.3ml of the whey was subjected to Sep-Pak Cartridge (manufactured by WATERS INC.) adsorption, washed with distilled water, and then eluted with 5ml of methanol. The eluate was dried under centrifugation and reduced pressure. The dried product was dissolved in 0.3ml of a 0.05% aqueous solution of trifluoroacetic acid, and subjected to HPLC (high performance liquid chromatography) analysis under the following conditions.

Apparatus Employed:

Hitachi L4000UV detector (at 215nm)
L6200 intelligent pump
L5030 column oven (35°C)

Condition of Elution: Flow rate 0.5ml/min.

Eluent: Aqueous solution containing 0.3M NaCl and 0.05% trifluoroacetic acid

Column: Asahipak GS320 (Φ 3.9x600mm)

The results are shown in Table 2.

Since the ACE inhibitory activity of IPP per unit weight

is 1.7 times that of VPP, the amount of the lactotripeptides in terms of VPP was calculated from the measured amounts of IPP and VPP in accordance with the following equation. The results are shown in Table 2.

5

Converted amount of lactotripeptide (μg in terms of VPP per ml)=Amount of IPP ($\mu\text{g}/\text{ml}$) $\times$ 1.7 + Amount of VPP ($\mu\text{g}/\text{ml}$)

Table 2

Strains	Amount of Peptide ($\mu\text{g}/\text{ml}$ whey)			Acidity (wt%)
	VPP	IPP	Amount of lactotripeptide in terms of VPP	
Strain 17	15.2	11.1	34.0	1.5
Strain 19	11.2	7.3	23.7	1.4
Strain 20	13.0	8.1	26.8	1.6
Strain 21	16.6	11.4	36.0	1.6
Strain 22	15.8	12.1	36.3	1.5
Strain 24	12.6	8.7	27.4	1.6
JCM1006	12.9	9.3	28.6	1.3
CM4	38.5	23.5	78.5	1.9

10

CM4 fermented milk had the largest amount of the lactotripeptide in terms of VPP, that is, 78.5 $\mu\text{g}/\text{ml}$ whey. Other seven strains gave the average amount of 34.2 $\mu\text{g}/\text{ml}$ whey. (Comparison of growth in milk)

15

Fermentability (growth) of CM4 strain was evaluated using 10 strains, namely Strain 18, Strain 20, Strain 23, Strain 24, Strain 25, Strain 26, JCM 1006 and CM4 strain among the above, in addition to publicly known strains JCM 1120 and JCM 1004. Each strain was cultured in 9wt% skim milk medium at 37° C for

24 hours. The cultured medium was added to a fresh medium of the same type which has been sterilized either at 115° C for 15 minutes or at 95° C for 5 minutes, in such an amount that the new medium contains 3wt% of the cultured medium.

5 Fermentation was further performed at 37° C for 24 hours. After finishing the fermentation, acidity of lactic acid was titrated to evaluate the fermentability. As a result, Strain 25, Strain 26 and CM4 strain exhibited higher fermentability in 95° C sterilized milk than in 115° C sterilized milk. Only CM4 strain
10 exhibited the final acidity of not less than 2.0wt% in 95° C sterilized milk.

(Measurement of extracellular proteinase)

Extracellular proteinase activity was measured of 16 strains which gave relatively good results in fermentability
15 shown in Table 1. Measurement was performed in accordance with the method of Yamamoto et al. (Yamamoto, N. et al. J.Biochem.(1993) 114, 740) based on the method of Twining et al. (Twining, S. Anal.Biochem. 143 3410 (1984)). That is, each strain was cultured in 9wt% skim milk medium while pH thereof
20 was maintained at 6.0. Sample was taken in the middle of logarithmic growth phase, and admixed with sodium citrate so that the final concentration was 1wt%, for clarifying the milk medium. The mixture was centrifuged at 5,000rpm for 10 minutes to collect cells. The cells were washed with 50mM β -
25 glycerophosphoric acid, and suspended in 50mM Tris-HCl buffer (pH7.8) to adjust turbidity (OD_{590} , i.e. measured by absorbance

at 590nm) to 1. 30 μ l of the suspension was admixed with 20 μ l of 0.4% fluorescein-casein (manufactured by SIGMA CHEMICALS CO.), and incubated at 42°C for 1 hour. The mixture was further admixed with 120 μ l of 5% trichloroacetic acid, allowed to stand for 20 minutes, and centrifuged at 15,000rpm for 10 minutes. 60 μ l of the supernatant was added to 3ml of 500mM tris-HCl buffer (pH 8.3), and the fluorescent intensity thereof was measured by detecting the fluorescence of 525nm produced at an excitation wavelength of 490nm. Extracellular proteinase activity in unit was calculated with the amount of the enzyme which exhibits 1% fluorescent intensity under the above conditions being defined as one unit. The results are shown in Table 3.

15

Table 3

Strains	U/OD ₅₉₀
strain 17	136.7
strain 18	102.8
strain 19	103.2
strain 20	89.9
strain 21	80.1
strain 22	243.3
strain 23	116.6
strain 24	116.6
strain 25	192.6
strain 26	108.4
JCM1006	185.7
JCM1062	96.5
JCM1103	176.3
ATCC15009	168.1
ATCC10797	106.5
NCDO-099	229.7
CM4	452.6

The activity of *Lactobacillus helveticus* CM4 strain was the highest, that is, 450U/OD₅₉₀. Average activity for other 16 strains was 141U/OD₅₉₀, which is about one third of that of CM4 strain.

5 [Example 2]

From 11 strains out of 36 *Lactobacillus helveticus* strains selected in Example 1, chromosomal DNA was extracted in accordance with the method of Leenhouts et al. (Leenhouts, K.(1990) Appl.Environ.Microbiol. 56:2726), digested with
10 several restriction enzymes, and subjected to 0.8% agarose gel electrophoresis to analyze the electrophoresis pattern.

As a result, a characteristic DNA fragment was observed among fragments of chromosome of CM4 strain double-digested with EcoRI and PstI (shown by arrow 1 in Fig.1). Such a fragment
15 was not observed in the fragments of chromosomes from other strains than CM4, and shorter fragments than the characteristic fragment of CM4 were observed in most of other strains (shown by arrow 2 in Fig.1). The molecular weight of the characteristic fragment was measured by comparative electrophoresis of the Hind
20 III digestion products of λ phage DNA as size markers (23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb and 2.0kb, in the order of increasing mobility), and was found to be about 16kb. Thus, it was confirmed that CM4 strain has a chromosomal DNA which gives the characteristic fragment having molecular weight of about 16kb,
25 by double-digestion with EcoRI and PstI. The molecular weight of the fragments in other strains shorter than the

characteristic fragment of CM4 was about 13kb.

[Example 3]

A fermented milk was produced with *Lactobacillus helveticus* CM4 strain selected in Example 1. CM4 strain was cultured in 100g of 9wt% skim milk at 37°C for 12 hours. Subsequently, 3kg of fresh medium was inoculated with the cultured skim milk, and cultured at 37°C for 12 hours. After finishing the fermentation, all of the fermented milk was used as a starter for fermentation of 100kg of 9wt% skim milk at 32°C for 20 hours. After finishing the fermentation, 74.8µg/ml of the lactotripeptide was contained in the fermented milk.

43kg of the obtained fermented milk was admixed with 4kg of granulated sugar, 3kg of water and 0.15kg of high methoxypectin, and homogenized to obtain 50kg of yogurt drink. The yogurt beverage had a preferable mild taste, pH of 3.6 and 4.6×10^8 cells/g of live CM4 cells.

[Example 4]

26.5kg of the fermented milk obtained in Example 3 was admixed with 45.0kg of granulated sugar, 4.7kg of high maltose syrup and 13.8kg of water. 10kg of 3wt% high methoxypectin solution was added to the mixture under stirring. The resulting mixture was homogenized using a laboratory homogenizer (manufactured by ATV GAULIN, INC., Model 15M-8BA) under a treatment pressure of 150kg/cm² and at a treatment flow rate of 2500ml/min. The homogenized liquid was admixed with a vanilla flavor and sterilized by heating up to 85°C. The

fermented milk thus sterilized was charged in a 200ml glass bottle while hot. The content of the lactotripeptide in the sterilized fermented milk product was measured. It was found out that the content of the lactotripeptide corresponded to that
5 in the fermented milk before sterilization.

[Brief Description of Drawing]

Fig.1 is a photograph showing the agarose gel electrophoresis pattern of chromosomal DNA fragments of various *Lactobacillus helveticus* strains in Example 2.