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Original article

Anti-glycative effect of yogurt: Prevention of advanced glycation end product formation

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Abstract

***Purpose*:** Existence or none-existence of advanced glycation end products (AGEs) inhibiting effect in yogurt was verified.
***Method*:** Specimens were 12 commercially available yogurt products in Japan. Aminoguanidine was used as a positive control for the AGE-inhibiting effect. Yogurt was centrifuged at 20 centigrade degrees (ºC), at a rate of 3,000 rpm (1,210 g) for 15 minutes, and divided into milk serum (supernatant) and milk solid (sediment). After the milk solid was thus obtained and dispersed in purified water by ultrasonication, a water-soluble extract was obtained. The specimens for AGE-inhibiting effect were added to glucose and human serum albumin (HSA) reaction system and reacted with it at the temperature of 60 ºC for 40 hours. The fluorescent AGE amount (excitation wavelength: 370 nm, detection wavelength: 440 nm) was then measured and the AGE production inhibition rate was calculated to determine for any AGE-inhibiting effect. The 50 % inhibitory concentration against AGEs (IC_{50}: mg/mL) was defined as being anti-glycation activity.
***Results*:** Anti-glycation activity (IC_{50} < 50 mg/mL) was confirmed in 9 out of 12 yogurt products (IC_{50} < 18.91 mg/mL on average). The AGE-inhibiting effect of yogurt was recognized in the milk serum, but not the milk solid. As a result of the fractionation of the milk serum, the AGE-inhibiting effect was strongly recognized with a fraction of a molecular weight of less than 10,000. Furthermore, as a result of the fractionation of the milk serum fraction of the molecular weight less than 10,000 using hydrophobic cartridge column, the AGE-inhibiting effect was recognized in hydrophilic fraction.
***Conclusion*:** The AGE-inhibiting effect was recognized in yogurt. The components for the AGE-inhibiting effect exists in the milk serum, and they were possibly hydrophilic components with a molecular weight of less than 10,000.

***KEY WORDS*:** yogurt, lactic acid bacteria, fermentation, advanced glycation end products (AGEs)

Introduction

Glycation *in vivo* is a process where the reducing sugars, including glucose, non-enzymatically react with proteins and advanced glycation end products (AGEs) are generated and accumulated. It is known that the generation and accumulation of AGEs *in vivo* are causes of chronic diseases including diabetic complications and the progression of aging [1].

Meanwhile, in order to obtain a vigorous healthy longevity, it is important to re-evaluate Japanese traditional food, rectify its drawbacks, learn the dietary habits of regions of the world with greater longevity and enjoy a well-balanced diet [2]. The example of the integration of macrobiotic diets in the East and West is to take Japanese traditional ingredients such as soybean, fish, vegetable, seaweed and others as regular food along with utilizing fermented dairy products such as yogurt and cheese, typical macrobiotic food of the West. It is recommended to eat fermented dairy products on a daily basis, for both the prevention of diseases and promotion of health [3-6].

Fermented dairy products are processed using the work of microorganisms within the foodstuff. Among the effects born from the fermentation of foodstuffs by microorganisms, there are the intestinal regulating function and the strengthening of bones [7], cholesterol absorption inhibition [8] and a strengthening of antioxidant effect [9]. It is presumed that these effects lead to various health benefits and the prevention of diseases [10]. In lactic acid bacteria, in particular, there are the ingredients producing polyamine which promotes the growth of suckling babies [3]; gassericin A, a cyclic bacteriocin [11] and the main active component of the NK activity enhancing effect, exocellular polysaccharide [12]. Recently, yogurt with various functionalities has been

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commercialized using the technique of mass-screening for lactic acid bacteria [13-17]. It is possible that there are some functionalities that are not known yet in these yogurts. However, there has been no report that yogurt is involved in glycation inhibition.

In this research, the AGE-inhibiting effect of yogurt, one of many fermented foods, was verified using an *in vitro* glucose and human serum albumin (HSA) reaction model (glucose and HSA model) [18].

Method

Specimen

For the evaluation of the AGE-inhibiting effect, 12 yogurt products obtained from a supermarket in Kyotanabe, Kyoto in May-June, 2013 were used. Aminoguanidine was used as a positive control substance for the AGE-inhibiting effect (***Table 1***). After each yogurt was evenly stirred, 10 mL was taken with a centrifuge tube and then centrifuged at the temperature of 20 ºC, at a rate of 3,000 rpm (1,210 g) for 15 minutes. It was then divided into milk serum (or whey) (supernatant) and milk solid (sediment). The supernatant thus obtained was used as the milk serum specimen. The milk solid was then resuspended in 10 mL of distilled water, centrifuged again at the temperature of 20 ºC, at a rate of 5,000 rpm (3,350g) and the supernatant was removed. This series of operations was repeated three times and the milk serum components were removed. The milk solid was suspended in 10 mL distilled water and destructed three times using an ultrasonic homogenizer (Misonix S-4000, NY, USA) at an amplitude of 40, pulse-on time 1 sec, and pulse-off time 3 sec. Then, the supernatant obtained after centrifugal separation at a temperature of 20 ºC at 5,000 rpm (3,350 g) was used as the liquid specimen extracted from the milk solid. 5 mL of each specimen thus obtained was placed in an aluminum tray, dried in an incubator set at the temperature of 110 ºC for four hours and weighed. The solid content concentration (mg/mL) of the specimen solution was calculated using the weights of aluminum tray before and after drying.

Table 1. Material profile.

ID	Materials	Product name	Seller / Manufacturer / Importer	Characteristics
1	Yogurt A	Koiwau Namanyu 100% Yogurt	Koiwai Dairy Products (Tokyo, Japan)	*Bifidobacterium lactis* BB12
2	Yogurt B	AEON Namanyu 100% Plain Yogurt	AEON (Chiba, Japan)	Lactobacteria (Bifidobacteria, Acidophilus), *Streptococcus thermophiles, Lactobacillus bulgaricus, Bifidobacterium lactis*
3	Yogurt C	StyleONE Probiotics Yogurt LKM512 Low Fat	Kyodo Milk Industry (Tokyo, Japan)	*Bifidobacterium lactis* LKM512
4	Yogurt D	StyleONE Probiotics Yogurt LKM512	Kyodo Milk Industry	*Bifidobacterium lactis* LKM512
5	Yogurt E	Caspian Sea Yogurt Plain	Fujicco (Kobe, Japan)	*Streptococcus cremoris* FC
6	Yogurt F	Morinaga Bifidus Plain Yogurt BB536	Morinaga Milk Industry (Tokyo Japan)	*Bifidobacterium longum* BB536
7	Yogurt G	Meiji Bulgaria Yogurt LB81 Plain	Meiji (Tokyo, Japan)	LB81 (*Streptococcus thermophilus* 1131, *Lactobacillus bulgaricus* 2038)
8	Yogurt H	Nature Megumi Plain Yogurt	Megmilk Snow Brand (Sapporo, Japan)	*Lactobacillus gasseri* SP, *Bifidobacteria* SP
9	Yogurt I	TOPVALU Bifidus Plain Yogurt	AEON	Bifidobacteria
10	Yogurt J	Namanyu 100% Plain Yogurt	Yasuda Yogurt (Niigata, Japan)	*Bifidobacterium brevis*
11	Yogurt K	Probiotics Plain Yogurt	AEON	Bifidobacteria, Casei bacteria, Acidophilus
12	Yogurt L	AEON Plain Yogurt	AEON	*Streptococcus thermophiles, Lactobacillus bulgaricus*
13	Positive control	Aminoguanidine	Wako Pure Chemical Industries (Osaka, Japan)	—

Measurement of AGE inhibiting effect using glucose and HSA model

The specimen was added to the reaction solution inclu The specimen was added to the reaction solution including 0.05 mol/L NaH_2PO_4-$Na_2H_2PO_4$ phosphate buffer solution (pH 7.4), 8 mg/mL human serum albumin (HSA) (Sigma-Aldrich, MO, USA) and 0.2 mol/L glucose, at a proportion of 1 : 10 (glucose / HSA model), and then incubated at a temperature of 60 ºC for 40 hours. The specimens were diluted with purified water to three concentrations of one time (undiluted), 10 times and 100 times. For the control of the glycation reaction, the reaction solution added with purified water in place of the specimen was used. After the completion of the glycation reaction, 200 µL of the reaction solution was dispensed into each well of a black 96-well micro-plate and the AGE-derived fluorescence of the reaction solution (excitation wavelength: 370 nm, detection wavelength: 440 nm) was measured using a microplate reader (SpectraMax Paradigm Multi-Mode Microplate Reader, Molecular Devise, Sunnyvale, California, USA). The measured fluorescence value was converted to the relative value of the time when the fluorescence value of the water solution of 5 µL/mL quinine sulfate and 0.1 N sulfuric acid is defined as 1,000. In the case of glucose and HSA model reaction solution, relative fluorescence values of (A) reaction solution added with specimen and glucose water solution, (B) reaction solution not added with glucose water solution, but added with distilled water and specimen, (C) reaction solution not added with specimen, but added with glucose water solution and (D) reaction solution not added with both specimen and glucose water solution were substituted to the following equation and AGE inhibition rate (%) was calculated.

$$\text{AGE Inhibition rate (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

From the AGE inhibition rates of the three specimen concentrations (concentrations of milk solid), IC_{50} (50% inhibitory concentration) was calculated and defined as anti-glycation activity. The smaller the value of IC_{50} is, the stronger anti-glycation activity is. When IC_{50} is less than 50 mg/mL, anti-glycation activity is evaluated as existing.

Fractionation of specimen using ultrafiltration membrane

Each specimen was fractionated into a molecular weight of 10,000 or more (10 K ≧) and that of less than 10,000 (10 K <). Each 450 µL specimen was put in a cup on the upper part of the membrane of the Amicon Ultra – 0.5 mL 10 K (Merck Millipoe, Darmstadt, German) and centrifuged at a temperature of 20 ºC at 10,000 rpm (13,420 g) for 30 minutes. The residue remaining in the cup on the upper part of membrane was diluted with 450 µL of purified water and used as a fractionated specimen of 10 K ≧. The specimen liquid that passed through the ultrafiltration membrane was used as a fractionated specimen of 10 K <.

Fractionation by hydrophobic cartridge column

The fractionated specimen of 10K< that was fractionated using hydrophobic cartridge column (Oasis HLB Plus Light Cartridge, 30 mg Sorbent per Cartridge, 30 µm Particle Size, Nihon Waters K.K., Shinagawa, Tokyo). The hydrophobic cartridge column was conditioned with 3 mL acetonitrile (ACN) and 3 mL distilled water being flowed before use; 1.0 mL specimen was poured and specimen was dissolved l.0 mL, one time, with purified water including 0 ~ 50% of ACN, in stages.

Statistical analysis

The measured values were expressed as mean ± standard deviation (n = 3). Tukey's test was used for the differences among the specimens for AGE inhibition rate and anti-glycation activity. As a result of the analysis using a two tailed test, a value of $p < 0.05$ was defined as significant. IMB SPSS Statics 24 (IBM Japan, Minato-ku, Tokyo) was used for this statistical analysis.

Results

AGE-inhibiting effect

Twelve yogurt products, and the AGE inhibition rates and anti-glycation activity (IC_{50}) by the glucose and HSA model using aminoguanidine are shown in ***Fig. 1***. Anti-glycation activity was recognized in 9 out of 12 products excluding D, G and I. The average IC_{50} value of these nine products was 18.9 mg/mL and it was 160 times greater than that of the aminoguanidine. The maximum difference of anti-glycation activities (IC_{50}) of the nine specimens in which anti-glycation activity was recognized was 8.2 times (minimum: 4.2 mg/mL ~ maximum: 34.5 mg/mL).

Fractionation using ultrafiltration membrane

The milk serum and milk solid water diluted specimens of Yogurt A (*Bifidobacterium lactis* BB12-derived) were fractionated into a molecular weight of 10,000 or more (10 K ≧) and that of less than 10,000 (10 K <) using an ultrafiltration membrane. The AGE inhibition rate (%) at that time was measured (***Table 2***). At the same time, the milk serum specimens of Yogurt C (*Bifidobacterium lactis* LKM512-derived), Yogurt B (*Lactobacteria (Bifidobacteria, Acidophilus)*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus, Bifidobacterium lactis*-derived) and Yogurt J (*Bifidobacterium brevis*-derived), which have different origin or different strain, were fractionated into 10 K ≧ and 10 K < using ultrafiltration membrane, and the AGE inhibition rates (%) of these fractionated liquids were measured. (***Table 2***).

In Yogurt A, the AGE-inhibiting effect was recognized only in the milk serum and not in the milk solid water diluted extract. The AGE inhibiting effect of the milk serum was recognized in B, C, and J as well as A. The AGE inhibition rates of these four yogurt products showed that the fractionated specimens of 10 K < were 4.4-13.5 times larger when compare with that of 10 K ≧ ($p < 0.01$).

Fractionation using hydrophobic cartridge column

The milk serums of Yogurt A, B, C and J were fractionated into 10 K < as shown above. These solutions were fractionated using a hydrophobic cartridge column and the AGE inhibition rate was measured for each eluted fraction (***Fig. 2***). In each of the four specimens, the AGE-inhibiting effect was recognized in the fraction eluted with purified water (fractions 1 and 2). In Yogurt B, the AGE-inhibiting effect was recognized in the fractions eluted with ACN 5% ~ 10% (fractions 3-7).

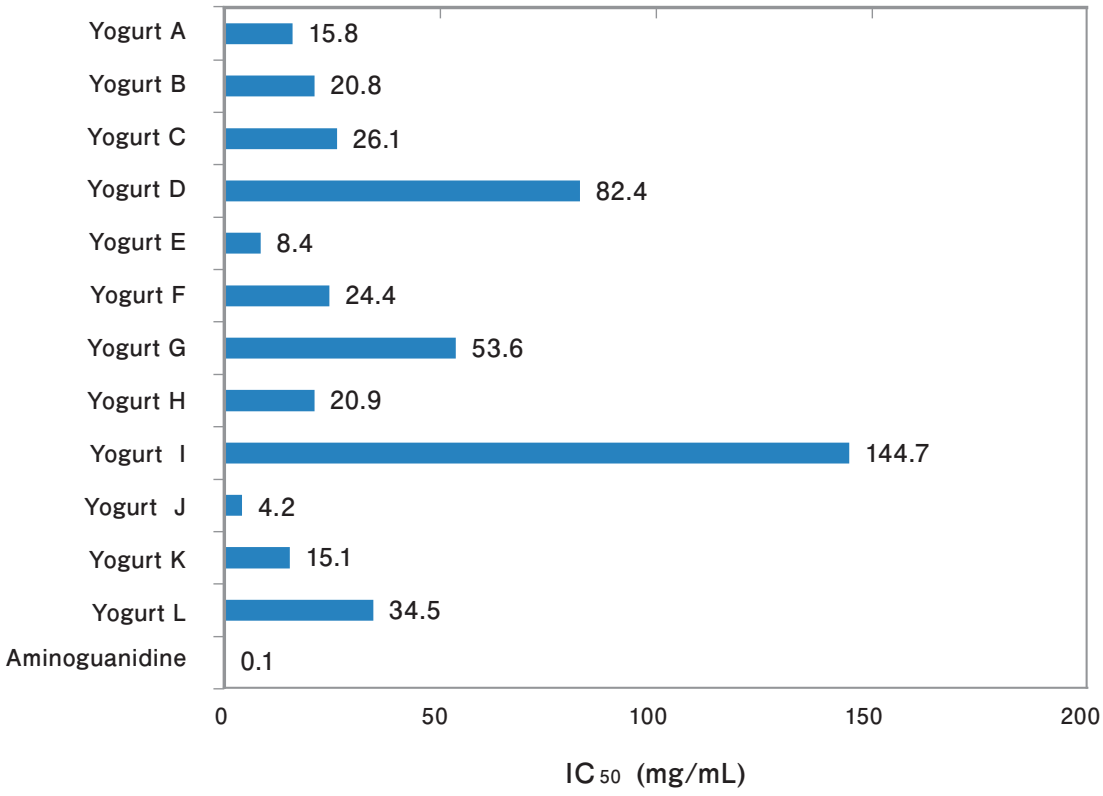

Fig. 1. **Anti-glycative activity of yogurt and starter lactobacillus.**
Inhibitory activity against fluorescent AGE formation (370 nm/440 nm) in a glucose/HSA model is measured. IC_{50} values are expressed in mg/mL. Results are expressed as mean ± standard deviation (n = 3). AGE, advanced glycation end product; HSA, human serum albumin; IC_{50}, 50% inhibitory concentration.

Table 2. Percent inhibition of purified fraction by ultrafiltration membrane against fluorescent AGE formation.

Sample name	Whey (except solid content)	MW ≧ 10,000	MW < 10,000	p value (vs MW ≧ 10,000)
Yogurt A (whey)	37.0 ± 2.8	3.1 ± 2.0	41.8 ± 2.7 *	< 0.01
(Solid content extract)	-7.8 ± 3.0	-4.2 ± 1.4	-2.9 ± 2.6	0.795
Yogurt B	42.5 ± 5.2	9.5 ± 3.7	49.7 ± 3.5 *	< 0.01
Yogurt C	41.7 ± 5.3	9.6 ± 5.1	48.4 ± 3.8 *	< 0.01
Yogurt J	52.3 ± 3.5	13.7 ± 2.7	59.8 ± 1.2 *	< 0.01

Fluorescent AGE formation (370 nm/440 nm) in a glucose/HSA model is measured. Results are expressed as maen ± standard deviation (n = 3). *p < 0.05 vs fractions with MW≧10,000 by Tukey's test. AGE, advanced glycation end product; HSA, human serum albumin; MW, molecular weight.

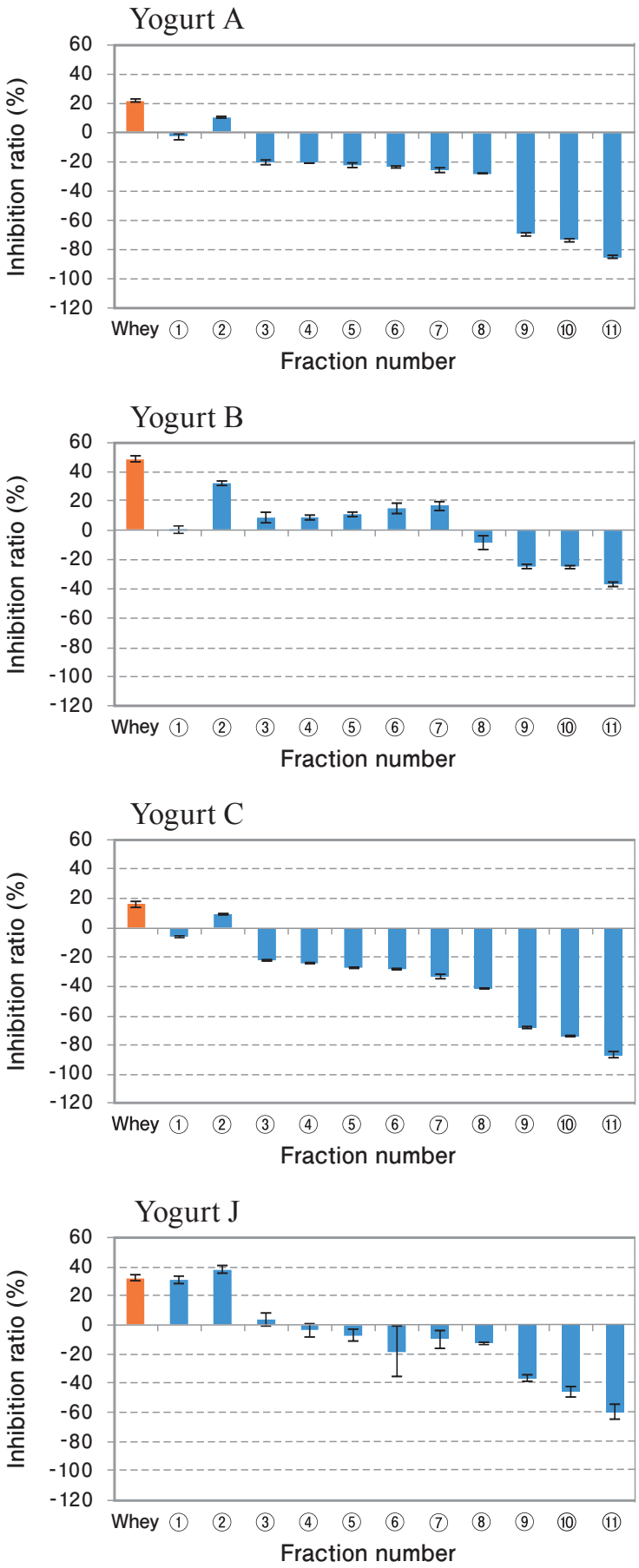

Fig. 2. **Difference of inhibition ratio between separated fractions by hydrophobic cartridge column.**

Fraction: ① - ③ Purified water; ④ - ⑤ ACN 5%; ⑥ - ⑦ ACN 10%; ⑧ - ⑨ ACN 30%; ⑩ - ⑪ A CN 50%. Fluorescent AGE formation (370 nm / 440 nm) in a glucose / HSA model is measured. Results are expressed as mean ± standard deviation (n = 3). AGEs, advanced glycation end products; ACN, acetonitrile.

Discussion

The AGE-inhibiting effect was recognized in 9 out of 12 commercially available yogurt products used. There were various kinds and strains in the fermentation bacteria used in these yogurt products. However, no relationship between the kinds of bacteria and AGE-inhibiting effect was recognized. Meanwhile, although the same fermentation bacteria (*Bifidobacterium lactis* LKM512) was used in Yogurt C and D, anti-glycation activity was observed only in C (C: IC_{50} = 26.1 mg/mL, D: IC_{50} > 50 mg/mL). Yogurt C is the product type marketed as being low fat with a milkfat content of 1.0%. On the other hand, Yogurt D was a normal type with a milkfat content of 3%. Therefore, it was possible that the difference in the AGE-inhibiting effect was involved in the constituents and their percentages in the yogurt. It was also presumed that differences in the constituents were caused by the variations in the raw milk as well as different fermentation conditions at the time of manufacturing.

In the comparison between the centrifuged supernatant (milk serum) and milk solid, a strong inhibition effect of AGE formation was found in the milk serum. The AGE-inhibiting effect in the fraction of yogurt purified using ultrafiltration membrane was found in the fraction of 10K <, regardless of which kinds of bacteria used. From the results that the milk serum was fractionated using hydrophobic cartridge column, it was presumed that the active components had hydrophobia. The milk serum of yogurt includes, among others, the proteins lactoferrin, alpha-lactalbumin, beta-tactoglobumin, bovine serum albumin, immunoglobulin among others along with amino acids, calcium, vitamins, minerals and lactic acid bacteria[19]. Because the AGE-inhibiting effect of milk serum was recognized in the fractions of 10K <, it is presumed that amino acids, calcium, vitamins, minerals and others are involved.

It is reported that the daily intake of yogurt supplements minerals including potassium, calcium, magnesium, zinc and others, and vitamins B2 and B12 leads to the improvement of triglyceride, blood sugar, diastolic blood pressure and insulin resistance[20]. It is reported that in the experiment where type 2 diabetic patients took milk products including *Lactobacillus acidophilus* La-5 and *Bifidobacterium animals* subsp lactis BB-12 for six weeks, fructosamine, a glycation marker in blood, decreased[21]. According to the meta-analysis of the results of seven clinical experiments conducted before August 2014, if type 2 diabetic patients take probiotics for more than eight weeks, it leads to a decrease in blood glucose level and HbA1c[22]. The results of these clinical experiments are possibly related to the AGE-inhibiting effect of yogurt newly confirmed in this research.

Conclusion

The AGE-inhibiting effect of commercially available yogurt has been confirmed. It was also suggested that the active components of the AGE-inhibiting effect in yogurt are possibly hydrophilic components of molecular weight 10,000 or less in the milk serum.

Declaration of conflict of interest

The authors claim no conflicts of interest.

Acknowledgements

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