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# Beneficial Effect of Viscous Fermented Milk on Blood Glucose and Insulin Responses to Carbohydrates in Mice and Healthy Volunteers: Preventive Geriatrics Approach by "Slow Calorie"

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## 1. Introduction

WHO-coordinated Cardiovascular Disease and Alimentary Comparison (CARDIAC) Study covering over 61 populations of 25 countries in the world revealed the significant inverse association of the biomarkers of fish and soybean intakes in 24-hour urine with age-adjusted mortality rates of coronary heart diseases (CHD) (Yamori, 2006a; Yamori et al., 2006), indicating high fish and soybean consumptions might contribute to the No.1 average life expectancy of the Japanese with the lowest CHD mortality rates among developed countries (Yamori, 2006b). The mechanisms by which these food factors such as isoflavones and magnesium from soybeans as well as n-3 fatty acids and taurine from seafood could work as nutrients good for longevity have been studied experimentally and epidemiologically (Yamori, 2006c; Yamori, 2009; Yamori et al., 2010).

In contrast to soybeans and fish, fermented milk, yogurt is the common daily food consumed by some populations well-known for their longevity as Georgian and Uygur peoples studied by CARDIAC Study (Mori et al., 2006). However, the possible mechanisms for yogurt to contribute to their longevity have not been investigated. "Caspian Sea Yogurt" was introduced to Japan after CARDIAC Study for long lived populations in 1980's and this home-made fermented milk spread all over Japan due to its mild taste with less acidity and its smooth viscous character (Mori et al., 2006). Moreover, the health effects of this yogurt such as an improvement of defecation and the immunopotentiality of influenza vaccination were observed by a randomized placebo-controlled study (Toda et al., 2005; Mori et al., 2006). Therefore, in the present studies we investigated the effect of "Caspian Sea Yogurt" on the glucose absorption from simple and complex carbohydrates because our previous

comparative studies on palatinose and sugar indicated slow absorption of glucose beneficially reduced risks of metabolic syndrome (Yamori et al., 2007; Matsuo et al., 2007; Holub et al., 2010; Okuno et al., 2010).

## 2. Caspian Sea Yogurt, its introduction and development in Japan

After the introduction of Caspian Sea Yogurt to Japan for nutritional analysis in CARDIAC Study, strains from the fermented milk were characterized (Ishida et al., 2005). Strain FC was Gram-positive, facultatively anaerobic cocci, and strain FA was Gram-negative, with aerobic rods. Phylogenetic analysis based on 16S rDNA sequences showed that strain FC formed a cluster with *Lactococcus (L) lactis* strains and was most closely related to *L. lactis* subsp. *cremoris*. Strain FA was included in the genus *Acetobacter* cluster and was most closely related to *A. orientalis*. Biochemical tests and DNA-DNA hybridization clarified that strain FC belongs to *L. lactis* subsp. *cremoris* and strain FA belongs to *A. orientalis*. Since the smooth viscous character of *L. lactis* subsp. *cremoris* is due to the bacterial exopolysaccharide (EPS), EPS non-producing variant (FC-EPS(-)) was isolated in order to clarify the effect of EPS by incubating the *L. lactis* subsp. *cremoris* FC-EPS(+) (stock strain of the Fujicco Co., Ltd. (Kobe, Japan)) in M17G broth at elevated, sublethal temperatures (37°C) for 72 hours. After growth on M17G agar at 25°C for 48 hours, colonies were isolated and maintained in the same medium. Thus, isolated non-ropy variant *L. lactis* subsp. *cremoris* FC-EPS(-) and the original ropy strain of *L. lactis* subsp. *cremoris* FC-EPS(+) were grown on M17G agar or broth (Difco) for 18 hours at 30°C. The existence of EPS forming was confirmed by Indian ink staining under microscopic observation (Figure 1).

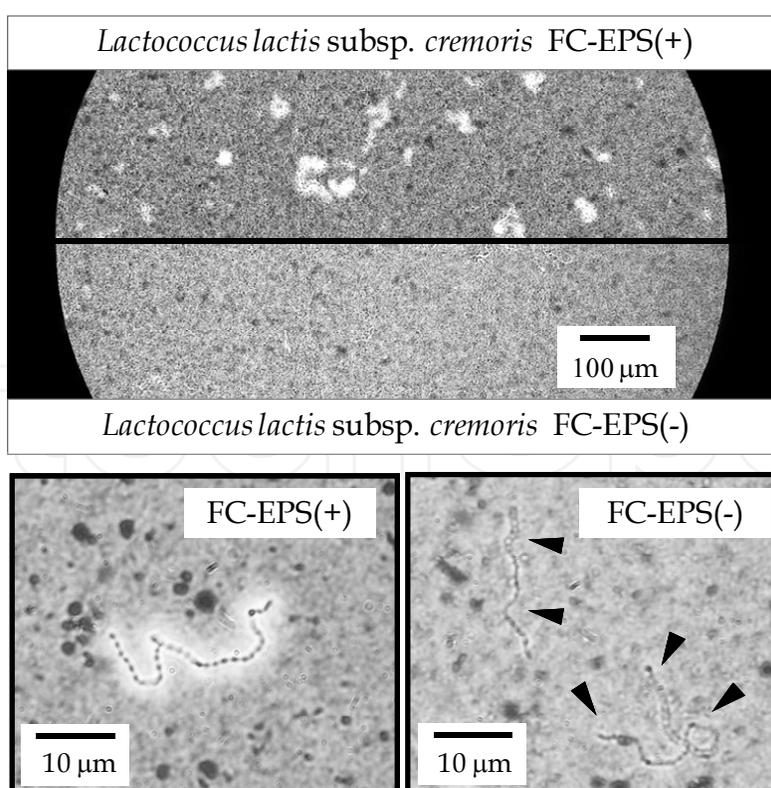


Fig. 1. The image of *Lactococcus lactis* subsp. *cremoris* FC-EPS(+) and FC-EPS(-) under the microscope.

Fig. 1 showed the photomicrograph of *L. lactis* subsp. *cremoris* EPS producing FC-EPS(+) and EPS non-producing variant FC-EPS(-) with Indian ink mixture. EPS was visible transparent white under the microscope. Fermented commercial milk with FC-EPS(+) or FC-EPS(-) was boiled at 85°C for 15 minutes and treated with protease at 37°C for 24 hours. Proteins were precipitated by addition of trichloroacetic acid (final concentration, 4%). After centrifugation and filtration, equivalent of acetone was added to the supernatant. The precipitated EPS from the yogurt fermented by FC-EPS(+) was 21 mg/kg, but was not detected from the yogurt by FC-EPS(-).

### 3. Caspian Sea Yogurt's effect on postprandial glycemia in mice

Since the viscous constituents derived from EPS of Caspian Sea Yogurt are supposed to affect glucose absorption, we compared in our animal studies the effect of non-fermented milk, the milk fermentation with *L. Lactis* subsp. *cremoris* FC (FC-EPS(+)) and the variant of *L. lactis* subsp. *cremoris* FC (EPS non-producing strain, FC-EPS(-)) on postprandial blood glucose levels by glucose tolerance tests.

#### 3.1 Experimental animals and oral glucose tolerance test

Male ICR mice were purchased from Crea Japan, Inc. (Tokyo, Japan) and fed a commercial diet (MF; Oriental Yeast Co., Ltd., (Tokyo, Japan)) for a week. Mice were divided into a control group, milk group and two kinds of FC yogurt groups, and 7 mice with similar body weight in each group were housed in a plastic cage with controlled lighting (a 12-hour light/12-hour dark cycle), temperature ( $25 \pm 1^\circ\text{C}$ ) and humidity ( $60 \pm 5\%$ ) under conventional conditions. Yogurt materials were prepared by fermentation of pasteurized milk with *L. Lactis* subsp. *cremoris* FC-EPS(+) or EPS(-) at 30°C for 6 hours. Each of starter cultures were grown at 30°C for 8 hours and inoculated at 4% (wt/wt) concentration. After fermentation, they were stored at 4°C.

Postprandial blood glucose levels were evaluated using the oral glucose tolerance tests (OGTT). The tests were performed between 09:00 and 12:00h in a day. After fasting for 12 hours, the 30% glucose solution was given mixed with water, milk or fermented milk at the dosage of the glucose solution vs. water, milk or fermented milk for 1.5 g/kg vs. 5.0 g/kg of body weight of mice.

Blood samples were collected from tail vein at 0, 30, 60, 90 and 120 minutes after administration and allowed to stand at room temperature for 30 minutes. Serum was prepared by centrifugation at 800×g for 15 minutes at 4°C and applied to the assay of glucose. Serum glucose concentration was quantified using the glucose CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

All data are expressed as means  $\pm$  S.D. The trapezoidal rule was used to determine the area under the curve (AUC). All areas below baseline were excluded from the calculations. Statistical analyses were performed using the Statcel2 (OMS publishing Inc., Saitama, Japan) and SPSS for windows package version 10.0J (SPSS Inc, Chicago, IL). P value below 0.05 was set as the level of significance. Differences between treatments at each time point were analyzed with a two-way repeated-measures ANOVA followed by *Tukey's-kramer test* or *Student's t-test*.

### 3.2 Postprandial hyperglycemia after fermented milk and milk administration

The blood glucose response 30 minutes after administration of milk fermented with *L. cremoris* FC-EPS(+) was significantly lower than the control. In the non-fermented milk group, the similar tendency was observed (Figure 2). However, after administration of milk fermented with FC-EPS(+), the curve of the blood glucose drew a slow arc and kept high value at 90 and 120 min.

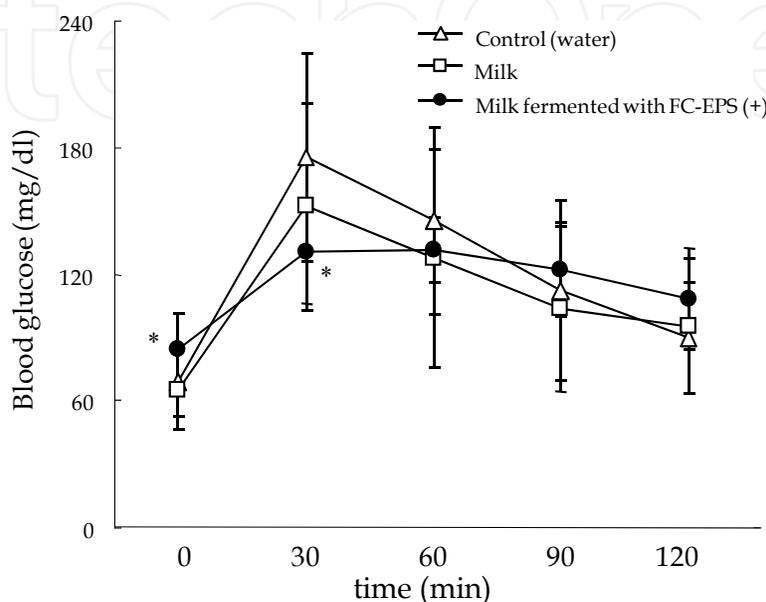


Fig. 2. Effects of fermented milk and milk on glucose tolerance test in ICR mice. Serum glucose concentrations were determined during the oral glucose tolerance test after the 30% glucose solution was administered at 1.5 g/kg body weight with 5.0 g/kg body weight of control (water), milk or fermented milk. \*Significantly different from the response to control,  $P < 0.05$ .

### 3.3 Differential effect on postprandial hyperglycemia by EPS-producing and –non producing fermented milk administration

The glucose levels were compared by glucose tolerance test after FC-EPS(+) and FC-EPS(-) fermented milk administration. FC-EPS(+) fermented milk group showed overall lower curve than FC-EPS(-) fermented milk group, and the levels at 60 and 90 minutes were significantly lower (Figure 3). The AUC of FC-EPS(-) fermented milk was significantly greater, over 1.5 times greater than that of FC-EPS(+) fermented milk (Figure 4). The elevation of blood glucose level was significantly suppressed and the AUC was decreased by the intake of FC-EPS(+) fermented milk, indicating EPS could attenuate postprandial hyperglycemia.

## 4. Caspian Sea Yogurt's effect on postprandial glycemia in humans

Based on the previous data obtained in mice, the effect of the Caspian Sea Yogurt fermented with FC-EPS(+) and of non-fermented milk on glucose tolerance was observed in healthy volunteer humans by a randomized crossover study.

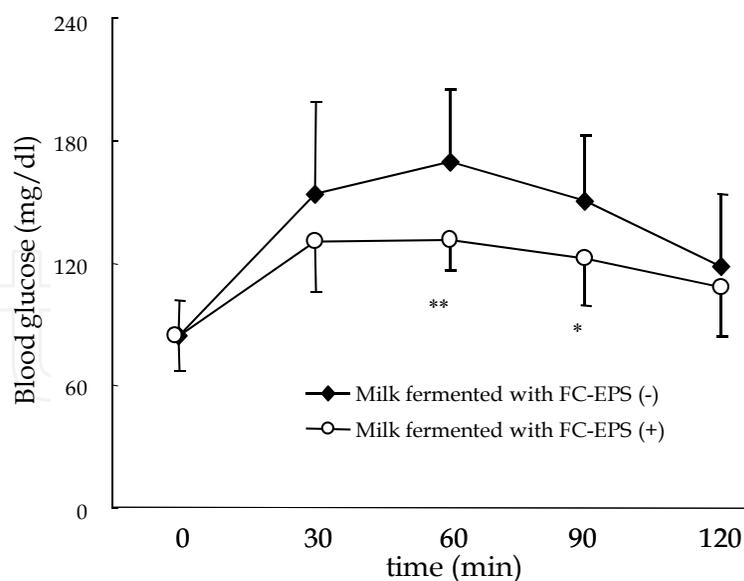


Fig. 3. Comparison of the effect of EPS producing (FC-EPS(+)) and non-producing (FC-EPS(-)) fermented milk on glucose tolerance test in ICR mice. Glucose responses were determined during the oral glucose tolerance test after the administration of the 30% glucose solution containing 1.5 g/kg body weight of glucose with 5.0 g/kg body weight of FC-EPS(+) or (-) fermented milk. Results are means  $\pm$  S.D. (n=12-14). \*Significantly different from milk fermented with FC-EPS(-) group,  $P < 0.05$  and \*\*  $P < 0.01$ .

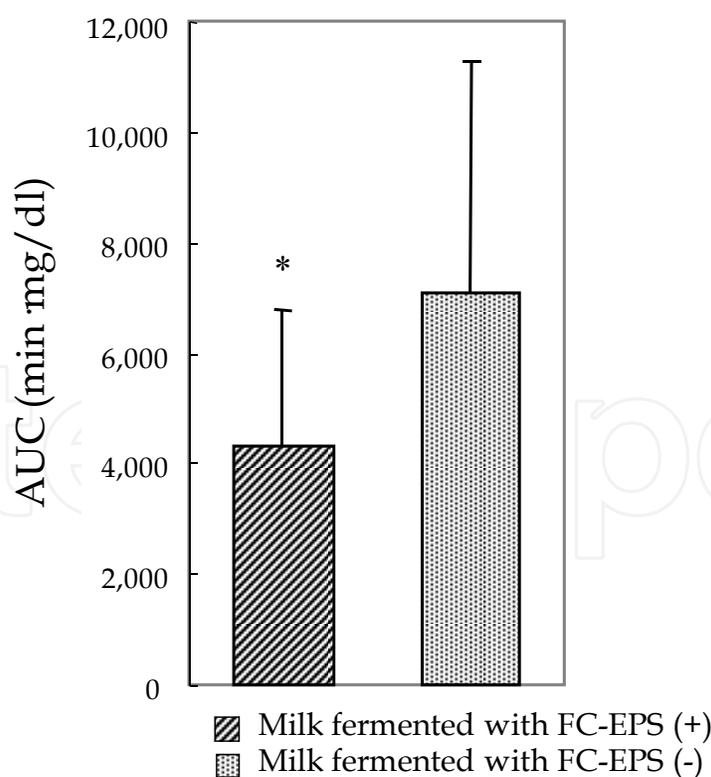


Fig. 4. Area under the curve for glucose (AUC), calculated using the trapezoidal rule by oral glucose tolerance test after FC-EPS(+) or (-) fermented milk administration. \*Significantly different from milk fermented with FC-EPS(-) group,  $P < 0.05$ .

#### 4.1 Volunteers for glycemic response test and study design

Ten males aged 21-24 years were recruited in this randomized, crossover study. All subjects with normal body mass indexes ( $21.0 \pm 0.83 \text{ kg/m}^2$ ) were not allergic to milk products and not taking either medicines or nutritional supplements. The study protocol was ethically approved by Japan Medical Laboratory Co., Ltd. (Osaka, Japan) and Mukogawa Women's University. Written informed consent was obtained from all subjects.

As for the test meals two types of milk products, normal fat milk enriched 5% skim milk (M) and fermented milk (F), were prepared by Fujicco Co., Ltd. Compositional information of normal fat milk (per 200 ml) was obtained from the suppliers (total carbohydrates 9.5 g, protein 6.6 g, fat 7.8 g). Skim milk contained no less than 95% milk-solids-nonfat, between 0-0.1% fat and between 0-0.5% water. F was manufactured from 90% milk, 5% skim milk and 5% *L. lactis* subsp. *cremoris* FC-EPS(+) and yogurt starter cultures. Both test meals, M and F, were served 180 g at a test. A rice ball consisting of 75 g available carbohydrates (R) was served as a complex carbohydrate product. White rice was boiled in a pan individually and made to be a rice ball. Trelan-G75 (T: 75 g/225 ml of glucose; Ajinomoto Pharma Co.,Ltd., Tokyo, Japan) was used for a simple carbohydrate source as a reference. For three tests, the combinations of meals were milk and Trelan (M/T), milk and a rice ball (M/R) and fermented milk and a rice ball (F/R).

Each participant was assigned to three treatments M/T, M/R and F/R on separate days at 7-day intervals. On each trial, after an overnight fast, blood samples were taken before and after a meal (0, 30, 60, 90, and 120 minutes) for analysis of glucose and insulin. The subjects were asked to eat up a milk product, M or F first and after finishing eating it, a carbohydrate product, T or R was followed. Subjects were instructed to finish eating within 10 minutes and asked to consume test meals in nearly the same length of time.

Plasma glucose and insulin levels were analyzed by Japan Medical Laboratory Co., Ltd. Glucose was determined by hexokinase method using the JCA-BM9020 analyzer (JEOL Ltd., Tokyo, Japan) and insulin was analyzed with the ADVIA Centaur immunoassay system (both Siemens Healthcare Diagnostics, Fernwald, Germany).

All statistical analyses were performed using the SPSS for windows package version 15 (SPSS Inc, Chicago, IL). Results were presented as means  $\pm$  S.E. A *P* value of 0.05 was set as the level of significance. Differences between treatments at each time point were analyzed with the general linear model (analysis of variance) followed by *Tukey's post-hoc test*.

#### 4.2 "Slow calorie" as a characteristic slow glycemic response of Caspian Sea Yogurt

Three subjects were excluded from all analysis because two did not meet the homeostasis model assessment-insulin resistance requirements in fasting ( $<2.5$ ) and one did not complete the study for a personal reason.

The plasma glucose response at 30 minutes after the M/T meal ( $140.7 \pm 20.7 \text{ mg/dl}$ ) was significantly higher than those of the M/R and Y/R meal (M/R;  $108.6 \pm 18.5$ , Y/R;  $100.3 \pm 8.2 \text{ mg/dl}$ , respectively) (Figure 5-A). Peak glycemic responses appeared at 30 minutes after the consumption of M/T and M/R, whereas the peak delayed at 60 minutes after the consumption of Y/R ( $111.7 \pm 17.5 \text{ mg/dl}$ ). Insulin response showed a similar pattern to

glucose response; the peak occurred 30 minutes after the consumption of M/T and M/R ( $61.8 \pm 23.6$  and  $46.6 \pm 9.2$   $\mu$ IU/ml, respectively) (Figure 5-B), and 60 minutes after the consumption of Y/R ( $40.5 \pm 18.2$   $\mu$ IU/ml).

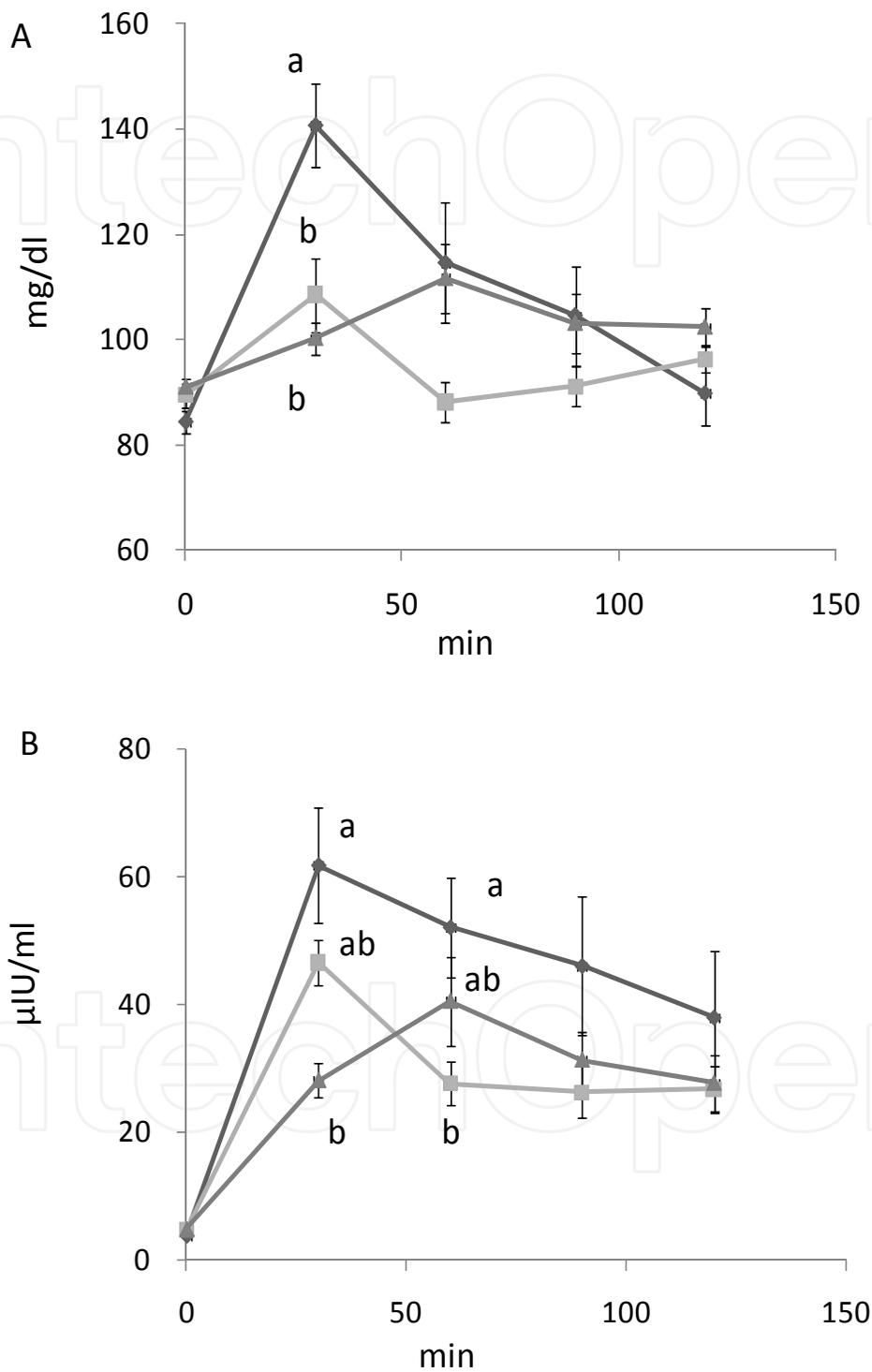


Fig. 5. Mean ( $\pm$  S.E.) plasma glucose (A) and insulin (B) concentrations after M/T ( $\blacklozenge$ ), M/R ( $\blacksquare$ ) and F/R ( $\blacktriangle$ ) meal. Values with different superscript letters are significantly different,  $P < 0.05$ .

The area under the curve (AUC) for glucose (AG) and insulin (AI) responses were calculated using the trapezoidal rule from Figure 5. Although the AG and AI of M/T ( $13405.7 \pm 887.8$  min X mg/ml and  $5424.6 \pm 794.7$  min X  $\mu$ IU/ml, respectively) were significantly different from those of M/R ( $11421.4 \pm 312.5$  min X mg/ml and  $3486.6 \pm 232.3$  min X  $\mu$ IU/ml, respectively) or F/R ( $12355.7 \pm 351.5$  min X mg/ml and  $3487.16 \pm 297.9$  min X  $\mu$ IU/ml, respectively), there was no significant difference between M/R and F/R in both AG and AI.

### 5. Potential benefit of Caspian Sea Yogurt

The animal experiment revealed that Caspian Sea Yogurt produced by *L. lactis* subsp. *cremoris* FC-EPS(+) attenuated postprandial hyperglycemia. The human study confirmed firstly lower glycemic and insulinemic responses after the rice intake with milk than after the ingestion of glucose solution used in oral glucose tolerance test with milk despite the equivalent carbohydrate content, and secondly that plasma glucose level as well as insulin level increased more slowly from fasting to peak following rice consumption after fermented milk intake than after the non-fermented milk, indicating much EPS-producing Caspian Sea Yogurt would slow carbohydrates digestion and glucose absorption to attenuate postprandial hyperglycemia.

The nutritional component of F/R was equal to that of M/R, which resulted in similar AUC for glucose and insulin of them. We further calculated the ratios of AI and AG (AI/AG) at 30, 60, 90 and 120-minute periods of the M/R and F/R meals (Figure 6). The ratios were significantly different between M/R and F/R at 30 ( $P < 0.01$ ) and 60-minute ( $P < 0.05$ ) periods. The results indicated insulin response to glucose rise in F/R was attenuated in comparison with M/R. The lower AI/AG as well as the slower increase of plasma glucose level after F/R meal than that after M/R meal implied that the Caspian Sea Yogurt would increase insulin efficiency. Remarkable characteristics of the Caspian Sea Yogurt consumption before complex carbohydrate intake such as lower AI/AG and peak delay in postprandial glucose level would play an important role for prevention of vascular aging caused by high glucose induced oxidative stress (Labinsky et al., 2009). Furthermore, slow rise of postprandial glucose level may be beneficial on vascular endothelial cells, since intermittent high glucose rather than constant high glucose was revealed to enhance reactive oxygen species-induced apoptosis in human umbilical vein endothelial cells (Risso et al., 2001, Quagliaro et al., 2003).

By the previous studies, palatinose with the same constituents of glucose and fructose as sucrose was proven to be absorbed slower than sucrose and the insulin levels at 30, 60 and 90 minutes after palatinose intake were lower than those after sucrose intake (Kawai et al., 1985, 1989). It was named as "slow calorie sugar". We further demonstrated by a double blind placebo controlled randomized trial that the long-term administration of palatinose in comparison with of sugar resulted in the reduction of visceral fat and blood pressure in high-risk Japanese immigrants in Brazil (Moriguchi et al., 2006; Yamori et al., 2007). Therefore, it is speculated the daily consumption of Caspian Sea Yogurt with meals may attenuate postprandial hyperglycemia and be useful to manage blood glucose level for preventing from the insulin resistance, diabetes and cardiovascular diseases as previously

proven in lower GI diets by epidemiologically (McMillan-Price et al., 2006; Chiu C-J. et al., 2011) and experimentally (Ludwig, D. S. 2002, van Schothorst et al., 2009).

Sedentary lifestyle generally associated with urbanization and aging weaken intestinal motility in the elderly suffering often from constipation. Since Caspian Sea Yogurt was so far proven for its beneficial effect on intestinal motility to improve constipation (Toda et al., 2005) and also for immunopotentiating effect on antibody titer elevation after influenza vaccination in the sedentary elderly (Mori et al., 2006), fermented milk popular in long-lived populations may be recommended for the prevention of communicable and non-communicable diseases from the scope of “preventive geriatrics”.

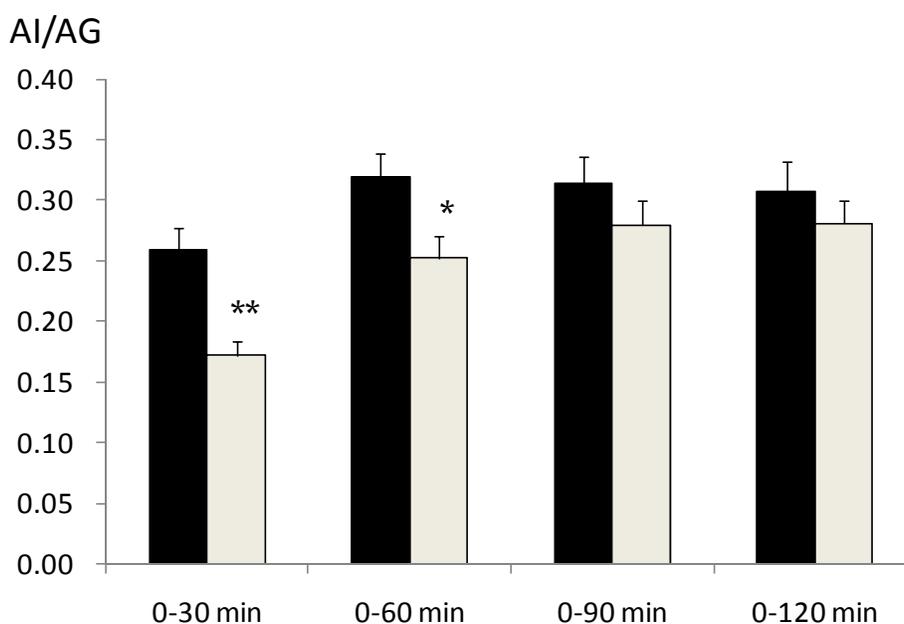


Fig. 6. Mean ( $\pm$  S.E.) ratios of areas under the curve for insulin (AI) and glucose (AG) calculated over 30-, 60-, 90- and 120-min periods after M/R (black column) and F/R (gray column) meal. \*Significantly different from M/R group,  $P < 0.05$  and \*\*  $P < 0.01$ .

## 6. Conclusion

After “Caspian Sea Yogurt” was introduced to Japan from the longevous population living in Georgia, the bacterial strains of this fermented milk was analyzed for the isolation into exopolysaccharide (EPS) producing and non-producing strains. Intestinal glucose absorption after the fermented milk ingestion, particularly the one from EPS producing strain was proven to be attenuated experimentally in mice as well as clinically in humans. Although it is needed to be tested if fermented milk may contribute to cardiovascular risk reduction as indicated in paratinose, the customary intake of fermented milk would contribute to health promotion in the elderly.

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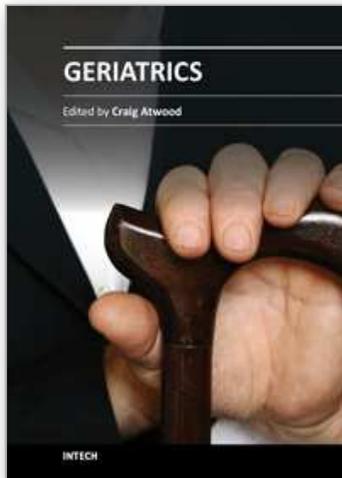
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## **Geriatrics**

Edited by Prof. Craig Atwood

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With the baby boomer generation reaching 65 years of age, attention in the medical field is turning to how best to meet the needs of this rapidly approaching, large population of geriatric individuals. Geriatric healthcare by nature is multi-dimensional, involving medical, educational, social, cultural, religious and economic factors. The chapters in this book illustrate the complex interplay of these factors in the development, management and treatment of geriatric patients, and begin by examining sarcopenia, cognitive decline and dysphagia as important factors involved in frailty syndrome. This is followed by strategies to increase healthspan and lifespan, such as exercise, nutrition and immunization, as well as how physical, psychological and socio-cultural changes impact learning in the elderly. The final chapters of the book examine end of life issues for geriatric patients, including effective advocacy by patients and families for responsive care, attitudes toward autonomy and legal instruments, and the cost effectiveness of new health care technologies and services.

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