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# Effects of a New Compound of Calcium Gluconate and Calcium Lactate on the *in vivo* and *in vitro* Growth of *Bifidobacterium*

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**Effects of calcium gluconate (GACa) and a new compound of calcium gluconate and calcium lactate (Medical Super Ca; MSCa) on the growth of *Bifidobacterium* were investigated *in vivo* and *in vitro*. In *in vitro* experiments, GACa and MSCa stimulated the growth of *B. bifidum* in synthetic VLM medium. However, these substances did not stimulate, rather inhibited in part, the growth of *B. bifidum* in the complex medium (GAM broth). When 2.2 mg per mouse per day of GACa or MSCa was administered to mice, and feces were assessed for anaerobic bacteria on VLM medium and for facultative anaerobic bacteria on BTB-L agar, there was no difference among the 3 groups of mice administered with or without GACa or MSCa. Colony forming units (CFU) of *B. bifidum* in feces of the mice drinking 2.2 mg of MSCa was $3.5 \times 10^5$ organisms per g of feces, a value intermediate between those drinking 2.2 mg GACa ($6.8 \times 10^4$) and controls ($3.3 \times 10^6$). These results suggested that GACa and MSCa stimulated the growth of *B. bifidum* in synthetic media, and that the effects of MSCa on the growth of bifidobacteria *in vivo* and *in vitro* were intermediate between those of GACa and control.**

**Key words:** *Bifidobacterium*; calcium gluconate; calcium lactate; MPN medium; VLM medium

Many substances are well known as so-called "foods for health". These include fructo-oligosaccharides (Gibson and Wang, 1994; Gibson et al., 1995; Sghir et al., 1998; Sharp et al., 2001), galacto-oligosaccharides (Sharp et al., 2001), gluconic acid (Asano et al., 1994), lactic acid, and lactosucrose (Ohkusa et al., 1995). Fructo-oligosaccharide stimulates the growth of *Bifidobacterium* (Gibson and Wang, 1994; Gibson et al., 1995; Sghir et al., 1998; Sharp et al., 2001) and administration of fructo-oligosaccharide increases fecal bifidobacteria in healthy humans (Bouhnik et al., 1999).

Gluconic acid is widely distributed in natural products such as rice, honey, wine, vinegar, etc., and has been shown to stimulate the growth of *Bifidobacterium* in the human intestine and to have beneficial effects on the bacterial flora in the human intestine (Asano et al., 1994). Calcium ions are necessary for the strengthening of bones and teeth. However, the ordinary Japanese diet is lacking in calcium. Calcium gluconate (GACa) is stable at room temperature (Rubio and Moldenhauer, 1995) and has been suggested to be useful as a health-promoting food additive.

Calcium lactate dissolves readily and freely in water and reduces intraoral demineralization of enamel if ingested in the diet (Kashket and Yaskell, 1992, 1997).

In the present study we investigated the effects of a compound of calcium gluconate and calcium lactate (MSCa) on the growth of *Bifidobacterium in vivo* and *in vitro*. MSCa dissolves more rapidly and freely in water than GACa, and is therefore expected to be a better food additive.

Abbreviations: BTB-L agar, bromthymol blue lactose agar; GACa, calcium gluconate; GAM, Gifu anaerobic medium; MSCa, a compound of calcium gluconate and calcium lactate

## Materials and Methods

### Reagents and media

Calcium gluconate (GACa) was purchased from Sigma (St. Louis, MO). A compound of calcium gluconate and calcium lactate (MSCa), which is freely soluble in water, was obtained from Nihon Medical (Tottori, Japan). GAM for anaerobic bacteria and bromthymol blue lactose agar (BTB-L agar) were obtained commercially from Nissui Seiyaku (Tokyo, Japan). VLM medium was used as a nonselective medium for anaerobic bacteria (Tanaka et al., 1997). Resazurin was purchased from Wako Pure Chemicals (Osaka, Japan) and cysteine-HCl was obtained from Sigma. MPN medium was used as a selective medium for *Bifidobacterium* (Tanaka and Mutai, 1980). Biotin, riboflavin, adenine, guanine, uracil and Tween 80 were purchased from Wako Pure Chemicals. Xanthine and pyruvic acid were from Sigma, and pantothenic acid and nalidixic acid were from Nakalai Chemicals (Kyoto, Japan). GACa and MSCa were dissolved in GAM medium and VLM medium, and autoclaved before use. The ingredients in 100 mL of anaerobic dilution fluid were as follows: 3.75 mL of 0.78% $K_2HPO_4$, 3.75 mL of a salt solution consisting of 0.47% $KH_2PO_4$, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% of $CaCl_2$ and 0.25% $MgSO_4 \cdot H_2O$, 0.1 mL of 0.1% resazurin, 0.1 mL of 5% L-cysteine·HCl, 0.2 mL of 25% L-ascorbic acid, 5 mL of 8% $Na_2CO_3$ and distilled water.

### Bacterial strain

*Bifidobacterium bifidum* strain Hino used in this experiment was kindly supplied by Dr. M. Ueda, Tottori University College of Medical Care Technology and stored in GAM broth at –30˚C. An aliquot of the stock solution of *Bifidobacterium* was added to fresh GAM broth in a test tube with a tight-fitting cap, anaerobically incubated at 37˚C overnight and used for the experiments.

### Growth of Bifidobacterium in culture media

Aliquots of 10 μL of the culture were added under a stream of hydrogen peroxide gas to test tubes with GAM broth containing GACa at a final concentration of 1% (GACa1) or 2% (GACa2) or MSCa at a final concentration of 1% (MSCa1) or 2% (MSCa2). These test tubes were closed tightly with a cap, and anaerobically incubated at 37˚C for 16 h. After serial dilution with anaerobic dilution fluid under a stream of hydrogen peroxide gas, aliquots of 100 μL of the suspension were put onto VLM agar plates, incubated in an anaerobic jar at 37˚C for about 1 week and the viable organisms were enumerated by the colony counting method. Similar experiments were performed using VLM medium containing GACa1 or GACa2 and MSCa1 or MSCa2, and put onto VLM agar plates for colony counting.

### Isolation of bacteria from feces of mice

GACa and MSCa were suspended in water at a concentration of 0.75% and sterilized by autoclaving. Six-week-old BALB/c mice (4 to 6; female) were bred in cages, and were given free access to solid food (CE-2; Clea Japan, Tokyo) and water containing GACa or MSCa. The doses of GACa and MSCa consumed by mouse per day were calculated from the decrease in amount of water in the bottle. In another experiment, granules of 20 mg of MSCa were administered directly into the animal's mouth with a small spoon.

After at least 2 weeks under these conditions, the feces were collected directly in a vinyl bag with 1 mL of anaerobic dilution fluid. Immediately after closing the vinyl bag and weighing, the suspension of feces was serially diluted with anaerobic dilution fluid under a stream of carbon dioxide gas. Aliquots of 100 μL of each suspension were put onto VLM agar plates, MPN agar plates and BTB-L agar plates, and incubated in an anaerobic jar at 37˚C for about 2 weeks, followed by colony counting.

### *Statistical analysis*

Student's *t*-test was used for statistical analysis to test for differences between the groups.

## Results

### *Growth of B. bifidum in GAM broth and VLM medium*

*B. bifidum* grew in GAM broth and reached 2.1 × $10^8$ cells per mL after 16 h in culture at 37˚C. The numbers of bacteria in GAM broth containing GACa1 and GACa2 were 60% and 46%, respectively, of those in control GAM broth with no additive. More bacteria grew in GAM broth containing MSCa1 or MSCa2 than in that containing GACa1 or GACa2 (Fig. 1). Interestingly, the numbers of bacteria grown in all GAM broth containing GACa or MSCa were less than that in control GAM broth. Thus, GACa and MSCa did not have any positive effect on the growth of *Bifidobacterium*. The numbers of viable organisms in GAM broth containing GACa and MSCa decreased in a dose-dependent manner.

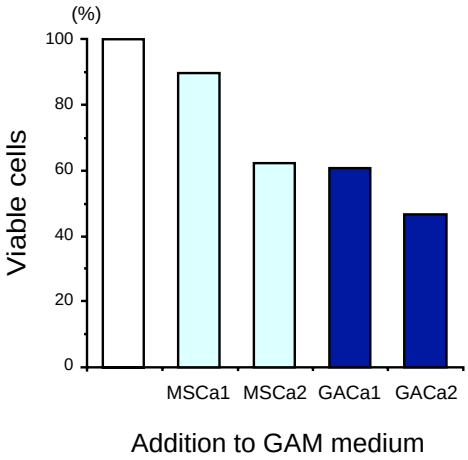
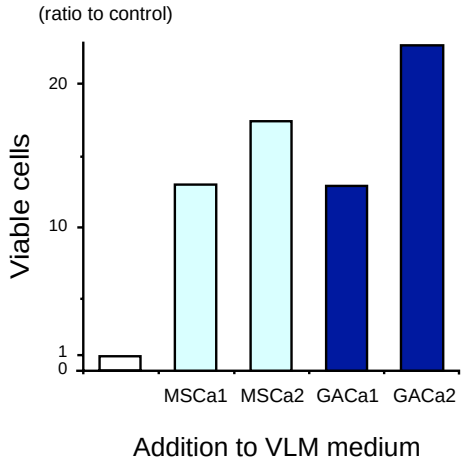



**Fig. 1.** Growth of *Bifidobacterium bifidum* in GAM broth supplemented with MSCa and GACa. The growth of *B. bifidum* in GAM broth was approximately 2.1 × $10^8$ organisms per mL of medium, and this is shown as 100% viable organisms. Other data are shown as percentages relative to this value. MSCa1 (□) and MSCa2 (□) indicate GAM broth containing MSCa at final concentrations of 1% and 2%, respectively. GACa1 (■) and GACa2 (■) indicate GAM broth containing GACa at final concentrations of 1% and 2%, respectively. (–) indicates GAM broth with no additive (□). Typical data from 1 of 3 separate experiments are shown.




*B. bifidum* grew in VLM medium and reached 2.0 × $10^7$ organisms per mL. In contrast to GAM broth, *B. bifidum* grew well in VLM medium containing GACa1 and GACa2, and reached 2.6 × $10^8$ and 4.5 × $10^8$ organisms per mL, respectively, which were 13-fold and 23-fold, respectively, greater than the control (Fig. 2). The bacteria also grew in VLM medium containing MSCa1 and MSCa2, as shown in Fig. 2. The viable cell number in VLM medium containing GACa and MSCa increased in a dose-dependent manner.

**Fig. 2.** Growth of *B. bifidum* in VLM medium supplemented with MSCa and GACa. The growth of *B. bifidum* in VLM medium was approximately 2.0 × $10^7$ organisms per mL of medium. *B. bifidum* growth in VLM medium containing MSCa and GACa is shown as the relative ratio of the number of viable organisms to that in controls. Symbols are the same as in Fig. 1.

### *Isolation of bacteria from feces of mice*

The number of bacteria isolated on VLM agar plates from the feces of mice fed approximately 2.2 mg of GACa or MSCa per mouse per day was about $1.3 \times 10^9$ organisms per g of feces (Fig. 3). There was no difference among the 3 groups of mice examined. However, feces of mice drinking 2.2 mg of GACa yielded only $6.8 \times 10^4$ organisms per g of feces on MPN agar plates. That value was significantly less than that ($3.3 \times 10^6$ organisms) in the control group. The number of viable bacteria in feces of mice drinking 2.2 mg of MSCa, was $3.5 \times 10^5$ organisms per g of feces, a value intermediate between the group drinking 2.2 mg of GACa and controls.

To search for facultative anaerobic bacteria and anaerobic bacteria in feces, we put the samples from feces onto BTB-L agar plates and VLM agar plates. BALB/c mice were orally administered 20 mg of MSCa every day and their feces were collected for isolation of bacteria. As shown in Fig. 4, there were no differences in the number of viable organisms associated with use of BTB-L agar plates and VLM agar plates.

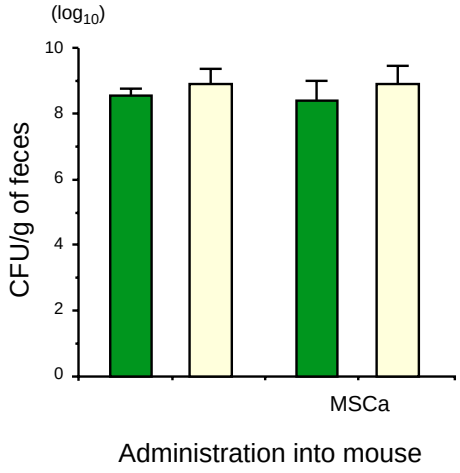



**Fig. 4.** Numbers of colony forming units (CFU) of mouse intestinal bacteria. Bacterial suspensions were prepared from the feces of mice, fed 20 mg of MSCa every day. A fixed amount of the suspension was serially diluted with anaerobic dilution fluid, and viable organisms were enumerated on BTB-lactose agar plates (■) and VLM agar plates (□). (–) indicates CFU from feces of mice without MSCa. Each column shows the average ± SD of 5 to 8 separate experiments.

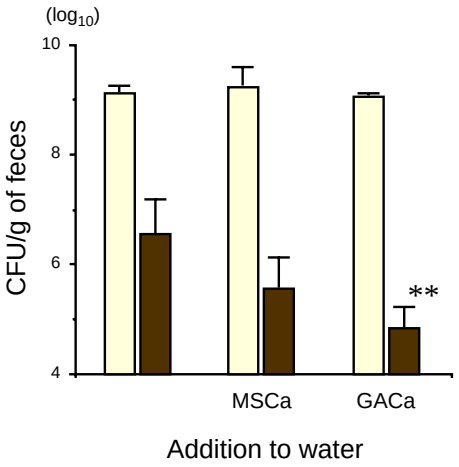



**Fig. 3.** Isolation of anaerobic bacteria from feces of mice given water containing MSCa or GACa. Numbers of colony forming units (CFU) of viable organisms in feces were assessed on VLM agar plates (□) and MPN agar plates (■). Each column shows an average ± SD of 3 to 5 experiments. $^{**}P < 0.05$ relative to control.

## Discussion

*Bifidobacterium* is thought to exert health-promoting effects for humans (Sghir et al., 1998). Its effects include inhibition of abnormal fermentation, production of vitamin B complexes, acceleration of peristaltic movement, stimulation of the immune system and inhibition of the production of carcinogenic substances. The *in vitro* experiments indicated that the growth of *Bifidobacterium* was stimulated, while that of *Clostridium perfringens* was not stimulated, rather inhibited, by gluconic acid or oligofructose (Asano et al., 1994; Catala et al., 1999).

The growth of *Bifidobacterium* was inhibited by adding GACa and MSCa to GAM broth and stimulated by adding GACa and MSCa to

VLM medium (Figs. 1 and 2). As GAM broth contains peptone, soy peptone, proteose peptone, decomposed serum, yeast extract and liver extract, it is a nutrient-rich medium. Thus, GAM broth may contain sufficient amounts of gluconic acid and calcium ions for bacterial growth. However, a large excess of calcium ions might inhibit the growth of *Bifidobacterium*. VLM medium is less nutrient-rich, and therefore addition of gluconic acid and calcium ions to this medium might improve the growth of *Bifidobacterium*.

There are large numbers of anaerobic bacteria and facultative anaerobic bacteria in the mouse intestine. The total numbers of these anaerobic bacteria and facultative aerobic bacteria in the feces of the mice drinking water supplemented with 0.75% MSCa were similar to those in the feces of controls (Fig. 4). However, there were fewer *Bifidobacterium* organisms in mouse feces as compared with the human feces described by Asano *et al*. (1994). When 2.2 mg of GACa was given to mice in the drinking water, the number of *Bifidobacterium* was significantly decreased. Administration of 2.2 mg of MSCa reduced the number of *Bifidobacterium* but this effect was not significant. As the solid food given to mice is nutrient-rich and may contain sufficient amounts of substances similar to gluconic acid and calcium ions, it might be unnecessary to add MSCa to the feed. The excessive administration of calcium ions might inhibit the growth of *Bifidobacterium* in the mouse intestine.

In these experiments, we found that the growth of *B. bifidum* in GAM broth supplemented with MSCa was inhibited, but not to a greater extent than that in GAM broth with GACa. Addition of MSCa to VLM medium stimulated the growth of *B. bifidum*. Furthermore, by administration of MSCa in the drinking water, the number of *Bifidobacterium* organisms isolated from the feces of the mice was reduced relative to the controls, although this effect was not significant. For application of MSCa for human use, further studies on the isolation of *Bifidobacterium* from the feces of subjects taking MSCa are required.

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