Urol Res (2006) 34: 265–270
DOI 10.1007/s00240-006-0054-4

ORIGINAL PAPER

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Prevention of nephrolithiasis by *Lactobacillus* in stone-forming rats: a preliminary study

Received: 13 December 2005 / Accepted: 7 March 2006 / Published online: 22 April 2006

Abstract Hyperoxaluria is a risk factor for renal stones. It appears to be sustained by increased dietary load or increased intestinal absorption. The aim of this study was to evaluate whether oral administration of lactobacilli could prevent urolithiasis in stone-forming rats. Oxalate-degrading activities of lactobacilli were evaluated by measuring the oxalate level in a culture medium after inoculation with lactobacilli. Only the strains of *Lactobacillus* having oxalate-degrading activity were used. Sprague–Dawley rats were fed a powdered standard diet containing 3% sodium oxalate and/or received 100 mg/kg of celecoxib for the first 8 days by gavage, before or after the beginning of this experiment (groups with previous treatment or with co-treatment). Rats were sacrificed after 4 weeks and kidneys were harvested for the assay of crystal formation under a dissecting microscope. Twenty-four-hour urine collections were performed before kidney harvest. Only two strains, *Lactobacillus casei* HY2743 and *L. casei* HY7201 out of 31 strains of *Lactobacillus* were able to degrade oxalate. In both groups of co-treatment and previous treatment with *L. casei* HY2743 and *L. casei* HY7201, urine oxalate excretion decreased compared to the group without lactobacilli. The dissecting microscope examination of kidneys in the rats in two previous treatment groups and the co-treatment group with *L. casei* HY7201 showed less abundant crystals than control groups. Our results show that lactobacilli may be used as a potential therapeutic strategy in the prevention of urinary stones.

Keywords *Lactobacillus* · Nephrolithiasis · Oxalate · Rat

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Introduction

The prevalence of urinary stone disease is estimated to be 2–3%. The lifetime prevalence rate of urolithiasis in Korea is known to be 6.0% in men and 1.8% in women [1]. The recurrence rate without treatment for calcium oxalate renal stones is about 10% at 1 year, 35% at 5 years and 50% at 10 years [2]. Calcium oxalate stone is the most common cause of urolithiasis and accounts for 81.6% of the total number of urinary tract stones in Korea [3].

Even though around 10% of dietary oxalate is absorbed under normal conditions [4], the increased intestinal absorption of oxalate may lead to hyperoxaluria and a significantly enhanced risk of urinary stone formation [5, 6]. Prevention of recurrence of urinary calculi is one of the biggest challenges facing the modern urologist.

Based on several studies demonstrating the presence of oxalate-degrading bacteria in the human intestine [7–9], the metabolism of oxalate by intestinal bacteria might be a focus of investigation in subjects with recurrent production of urinary stones.

Lactic acid bacteria are a group of bacteria belonging to diverse genera used in the dairy industry and are composed mainly of bacteria, of which the primary metabolic end-product of carbohydrate metabolism is lactic acid. The use of lactic acid bacteria to reduce the accumulation of uremic toxins by modifying the intestinal flora has already been employed in humans [10, 11]. In addition, Campieri et al. [12] demonstrated that the urinary excretion of oxalate was reduced with treatment using freeze-dried lactic acid bacteria. We evaluated

whether oral administration of lactobacilli could prevent urolithiasis in stone-forming rats.

Materials and methods

Strains

Thirty-one strains of *Lactobacillus* were purchased from R&D center of Korea Yakult Co. Ltd (Yongin, Korea). These strains were tested by colonization on medium B which was the selective medium for *Oxalobacter formigenes* (under aerobic conditions, at 37°C) [7].

Media and culture condition

Medium B was used in the selection of lactobacilli capable of degrading oxalate. Medium B was prepared as follows. The mixture was boiled and after it had been cooled at 50°C, 20 ml of trace metal solution ($ZnSO_4 \cdot 7H_2O$ 10.0 mg, $MnCl_2 \cdot 4H_2O$ 3.0 mg, H_3BO_3 30.0 mg, $CoCl_2 \cdot 6H_2O$ 20.0 mg, $CuCl_2 \cdot 2H_2O$ 1.0 mg, $NiCl_2 \cdot 6H_2O$ 2.0 mg, $Na_2MoO_4 \cdot 2H_2O$ 3.0 mg) was added. The pH was adjusted to 7.0, and 180 ml of distilled water was added. The mixture of total 1 l of solution was sterilized using the filtration method. Prior to inoculation, medium B was incubated in an anaerobic chamber (Bactron Anaerobic/Environmental Chamber, USA) for 24 h.

Lactobacilli were subcultured in MRS medium (Difco, MI, USA) with 0.5% (v/v) inoculum each and incubated at 37°C for 18 h. For the in vivo experiment, 2% (v/v) of lactobacilli were inoculated into the 10% (w/v) skim milk and cultured at 37°C for 24 h.

Oxalate-degrading activity of *Lactobacillus*

Oxalate-degrading activities of *Lactobacillus* were measured using Sigma Diagnostics Oxalate Kit (Sigma Diagnostics Inc., St Louis, MO, USA). Oxalate level was measured in non-inoculated medium B, as an initial positive control. Then, oxalate levels were measured daily for 7 days after inoculating 50 and 100 μl of lactobacilli into 10 ml medium B.

Oral administration of *Lactobacillus* into stone-forming rats

We previously reported on a stone-forming animal model using selective cyclo-oxygenase 2 inhibitor [13] and the animal model was used in this experiment.

Four-week-old male Sprague–Dawley rats with an average initial body weight of 130–140 g were maintained under specific pathogen-free conditions in the animal facility of the Clinical Research Institute and fed water and pellet feed ad libitum for 1 week. Forty-five rats were then equally divided into seven groups ($n = 5$, each). Group 1 rats were maintained on the powdered regular diet for the whole study with each rat receiving 1 ml of normal saline given by esophageal gavage. Group 2 rats received the powdered regular diet supplemented with 3% (w/v) sodium oxalate. Group 3 rats were maintained on the powdered regular diet supplemented with 3% (w/v) sodium oxalate for the whole study with each rat receiving 1 ml of celecoxib (100 mg/kg) for the first 8 days. In addition to the diet and celecoxib used in group 3 rats, group 4 rats received 1.5 ml of 10% (w/v) skim milk and group 5 (*Lactobacillus casei* HY2743) and group 6 (*L. casei* HY7201) rats received 1.5 ml of lactobacilli (final concentration 5×10^8 cfu/ml diet) daily 1 week prior to initiation of the experiment for the whole study. In addition to the diet and celecoxib used in group 3 rats, group 7 rats received 1.5 ml of 10% (w/v) skim milk and group 8 (*L. casei* HY2743) and group 9 (*L. casei* HY7201) rats received 1 ml of lactobacilli (final concentration 5×10^8 cfu/ml diet) daily starting the experiment for the whole study. At day 28, the rats were transferred to metabolic cages for environmental adaptation for 24 h and at day 29, a 24-h urine collection was performed. The rats were sacrificed at day 30 and morphologic examination involving crystal formation was observed under a microscope.

Observation of crystal formation in kidneys

The animals from the above groups were anesthetized with Nembutal (50 mg/kg body wt.). A midline laparotomy was performed and the abdominal aorta was cannulated using a 20-gauge angiocath. The kidney was flushed retrograde with 10 ml cold (0°C) Krebs Ringer Bicarbonate (KRB) buffer concentrations in mM: NaCl, 118; KCl, 5; $CaCl_2$, 0.25; $MgSO_4$, 1; $NaHCO_3$, 24; KH_2PO_4, 1.2; glucose, 10. Buffer pH was adjusted at 7.4 by bubbling with 95% O_2/5% CO_2 for 1 h before use. The kidneys were quickly excised, longitudinally bisected and placed in a beaker of cold KRB (0°C). The bisected kidneys were examined under a dissecting microscope (under 2.5× power field) for crystal numbers and distribution. Organs including heart, lung, liver, spleen, testis and intestine were dissected. They were fixed with 5% formalin and embedded with paraffin.

Determinations of urinary constituents of urolithiasis

Twenty-four-hour urine was collected at day 29 and its volume and pH were measured. The creatinine clearance was measured for evaluating renal function. Concentrations of urinary calcium, oxalate and uric acid, known as the constituents of urinary stones, and citrate and magnesium, known as inhibitors of urinary stones, were determined using a chemical method.

Statistical methods

Statistical analysis was done using the SPSS 10.0 program for personal computers. A Mann–Whitney test was done for simple comparisons among experimental groups and differences were considered significant if the probability of chance occurrence was < 0.05.

Results

Oxalate-degrading activity of *Lactobacillus*

To isolate lactobacilli capable of degrading oxalate, 31 strains of *Lactobacillus* were tested by colonization in medium B. The colonizations in medium B were observed in several strains, which were tested by the proliferation and acid-forming rate in 10% skim milk. Finally, two strains were isolated and named as *L. casei* HY2743 and *L. casei* HY7201, respectively. And these strains confirmed the oxalate-degrading activities by using an oxalate kit. Oxalate level was measured in non-inoculated medium B, as an initial positive control. Following inoculation of 50 µl of *L. casei* HY2743 in medium B, oxalate levels decreased after 5 days and following 100 µl, oxalate levels decreased after 4 days. In the case of *L. casei* HY7201, oxalate levels decreased after 3 days following inoculation of 50 and 100 µl (Table 1).

Effect of *Lactobacillus* on urinary excretion of oxalate and citrate

To determine the effects of lactobacilli on renal function, we measured a variety of urinary parameters including volume, urinary excretion of oxalate, citrate, calcium, magnesium, phosphorus, uric acid and creatinine. Table 2 shows the results of urinary excretion of oxalate and citrate in rats in the various treatment groups expressed as µmol excreted daily. There were no significant differences in the mean levels of urinary excretion of calcium, magnesium, phosphorus and uric acid between groups (data not shown). The volume of 24-h urine ranged within 10 ml across the experimental groups. Animals in all groups containing the control and celecoxib-treated groups retained relatively normal renal function as judged by creatinine clearance. In the oxalate-treated and celecoxib plus oxalate-treated groups, the levels of oxalate excretion were significantly higher than those seen in the control group, considered to be physiologic results of the nephrolithiasis model. The levels of oxalate secretion in the groups (4 and 7) which received 10% skim milk with celecoxib plus oxalate were not different from that in the group treated with celecoxib plus oxalate. In comparison with the stone-forming group (group 3), all groups with *Lactobacillus* treatment (groups 5, 6, 8 and 9) showed a lower excretion level of urine oxalate, and especially in group 5 of the pretreatment groups with lactobacilli and group 8 of the co-treatment groups with lactobacilli, the level of oxalate in urine was significantly lower than in group 3 ($P < 0.05$). In addition, compared to the stone-forming control groups (groups 4 and 7), all groups with *Lactobacillus* treatment (groups 5, 6, 8, 9) showed significantly lower excretion levels of urine oxalate. Urine citrate levels between the stone-forming group and *Lactobacillus* treatment groups had no significant differences.

Table 1 Oxalate level in medium B broth inoculated with *Lactobacillus*

Incubation time (days)	Oxalate level (mmol/l)			
	L. casei HY2743		*L. casei* HY7201	
	50 µl	100 µl	50 µl	100 µl
0	2.029		3.546	
1	1.752	1.745	3.720	3.605
2	1.983	1.765	3.546	3.886
3	2.281	1.898	0.501	0.337
4	2.053	0.114	0.495	0.208
5	0.199	0.016	0.376	0.159
6	0.167	0.163	0.168	0.245
7	0.078	0.217	0.298	0.102

Clinical signs and body weights

Mortalities, changes of motor activity, appearances and other clinical signs were checked daily during the study period. Neither mortalities nor abnormal clinical signs were observed during the study period. No statistical differences were found in body weights during the study period (Fig. 1).

Inhibitory effect of *Lactobacillus* against crystal formation

As can be seen in Fig. 2, group 3 receiving both celecoxib and oxalate, group 4 receiving prophylactic skim milk and group 7 receiving skim milk during the study period had larger numbers of crystals than the control (119.6, 107.0 and 135.8 vs. 1.4, $P < 0.01$). All groups with *Lactobacillus* treatment had smaller numbers of crystals than group 3 (stone-forming group). Both pretreatment and co-treatment with *Lactobacillus* showed an inhibitory effect on stone formation, but while all pretreatment groups inhibited stone formation significantly ($P < 0.05$), only group 9 (co-treatment with *L. casei* HY7201) of the co-treatment groups had a significant decrease of crystals. Especially in group 5 (pretreatment with *L. casei* HY2743), the number of crystals was the lowest among *Lactobacillus* treatment groups, even similar to the control (2.7 vs. 1.4).

Compared with the stone-forming control groups (groups 4 and 7), pretreatment with two strains (groups 5 and 6) and co-treatment with *L. casei* HY7201 (group 9) decreased the number of crystals in the kidneys of rats significantly.

Table 2 Results of urinary excretion of metabolic constituents after experiment

Group no.	Volume (ml ± SE daily)	Oxalate (μmol ± SE daily)	Citrate (μmol ± SE daily)	Creatinine clearance (ml/min)
1	8.2 ± 0.5 (6.7–9.7) [a]	12.13 ± 1.84 (7.03–17.23)[b,c]	14.68 ± 1.75 (9.81–19.54)[d]	1.52 ± 0.09 (1.26–1.78)
2	14.7 ± 0.6 (12.9–16.5)	20.88 ± 1.78 (15.93–25.82)[e,f]	25.36 ± 0.96 (22.69–28.03)	1.40 ± 0.19 (0.81–2.00)
3	14.8 ± 1.9 (9.4–20.2)	36.86 ± 7.78 (15.26–58.47)[g]	19.93 ± 2.07 (14.20–25.67)	1.46 ± 0.08 (1.25–1.68)
4	11.9 ± 3.2 (8.0–16.5)	31.42 ± 4.42 (15.74–35.96)[h,i]	21.88 ± 4.82 (18.26–30.13)	32 ± 0.25 (1.00–1.50)
5	11.4 ± 1.7 (6.8–16.0)	15.36 ± 2.08 (9.58–21.14)[g]	20.45 ± 1.58 (16.07–24.82)[f]	1.38 ± 0.14 (0.92–1.83)
6	13.1 ± 0.8 (10.9–15.3)	21.51 ± 2.88 (13.53–29.50)	20.19 ± 2.64 (12.86–27.51)	1.46 ± 0.07 (1.26–1.66)
7	15.4 ± 2.3 (12.0–18.0)	30.94 ± 3.21 (15.18–35.93)[j,k]	22.00 ± 2.79 (18.72–26.03)	1.61 ± 0.23 (1.30–2.00)
8	16.7 ± 2.9 (8.8–24.6)	16.49 ± 2.89 (8.46–24.52)[e]	23.24 ± 8.12 (0.69–45.78)	1.50 ± 0.36 (1.39–1.61)
9	14.2 ± 0.9 (11.8–16.6)	19.58 ± 2.98 (11.30–27.85)	24.04 ± 1.30 (20.43–27.65)	1.66 ± 0.11 (1.35–1.97)

Group 1, regular chow and saline; group 2, sodium oxalate; group 3, sodium oxalate and celecoxib;group 4, prophylactic skim milk; group 5, prophylactic *Lactobacillus casei* strain HY2743; group 6, prophylactic *L. casei* strain HY7201; group 7, co-treatment with skim milk, group 8: treatment with *L. casei* strain HY2743; group 9, treatment with *L. casei* strain HY7201

[a]Compared to other groups, $P < 0.05$ using Mann–Whitney test

[b]Compared to group 2, $P < 0.05$ using Mann–Whitney test

[c]Compared to groups 3, 4, and 7, $P < 0.01$ using Mann–Whitney test

[d]Compared to other groups except groups 6 and 8, $P < 0.05$ using Mann–Whitney test

[e]Compared to group 4, $P < 0.05$ using Mann–Whitney test

[f]Compared to group 7, $P < 0.01$ using Mann–Whitney test

[g]Compared to groups 5 and 8, $P < 0.05$ using Mann–Whitney test

[h]Compared to groups 5 and 8, $P < 0.01$ using Mann–Whitney test

[i]Compared to groups 6 and 9, $P < 0.05$ using Mann–Whitney test

[j]Compared to groups 5 and 8, $P < 0.01$ using Mann–Whitney test

[k]Compared to groups 6 and 9, $P < 0.05$ using Mann–Whitney test

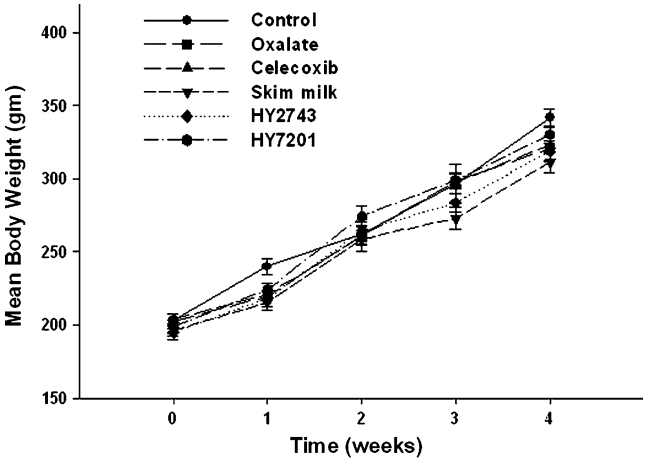

Fig. 1 Mean body weights of rats in each experimental group

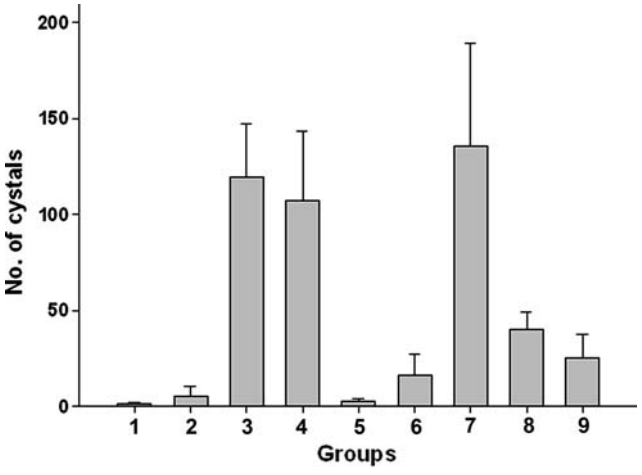

Fig. 2 Numbers of crystals in the kidney of rats after the experiment. Significant differences in crystal numbers were observed between groups. *P*-values by Mann–Whitney tests were as follows: (1) group 3 vs. group 1 ($P = 0.003$), group 2 ($P = 0.001$), group 5 ($P < 0.001$), group 6 ($P = 0.009$) and group 9 ($P = 0.035$); (2) group 4 vs. group 1 ($P = 0.001$), group 2 ($P = 0.001$), group 5 ($P < 0.001$), group 6 ($P = 0.006$) and group 9 ($P = 0.019$); (3) group 7 vs. group 1 ($P < 0.001$), group 2 ($P = 0.001$), group 5 ($P < 0.001$), group 6 ($P = 0.001$) and group 9 ($P = 0.035$); (4) group 8 vs. group 1 ($P < 0.001$), group 2 ($P = 0.002$) and group 5 ($P < 0.001$). Group 1, regular chow and saline; group 2, sodium oxalate; group 3, sodium oxalate and celecoxib; group 4, prophylactic skim milk; group 5, prophylactic *Lactobacillus casei* strain HY2743; group 6, prophylactic *L. casei* strain HY7201; group 7, co-treatment with skim milk; group 8, treatment with *L. casei* strain HY2743; group 9, treatment with *L. casei* strain HY7201

Crystal formation in kidneys

The morphological examination of bisected kidneys under the dissecting microscope showed that crystals were found profoundly in the corticomedullary junction of kidneys in group 3 (no treatment group), but few crystals in the co-treatment group with HY (Fig. 3).

Discussion

Several studies have demonstrated the presence of oxalate-degrading bacteria in the human intestine [7, 9, 14, 15], suggesting that the maintenance of a normal ecology among the bacterial species that constitute the endogenous digestive microflora is a natural defense mechanism against urolithiasis. Based on these data, the metabolism

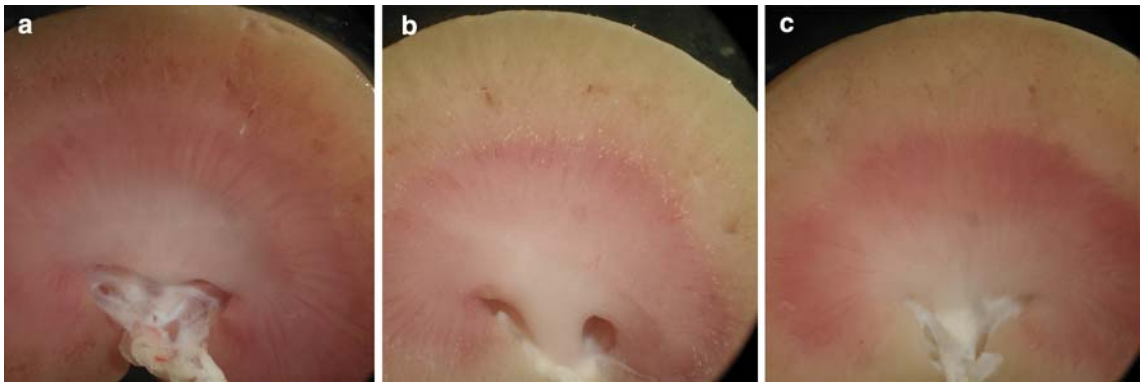

Fig. 3 The figures of bisected kidney. **a** Group 1 with normal diet showed no crystals. **b** Group 3 receiving celecoxib and oxalate showed many crystals in the corticomedullary junction. **c** Group 5 receiving co-treatment with HY2743 had few crystals, similar to group 1

of oxalate by intestinal bacteria might be a focus of investigation for urinary stones.

Campieri et al. [12] revealed that feeding a mixture of freeze-dried lactic acid bacteria led to a significant reduction of the urinary excretion of oxalate in a group of patients with idiopathic calcium oxalate urolithiasis and mild hyperoxaluria although the sample size was small. They suggested that lactic acid bacteria may have oxalate-degrading activity.

We hypothesized that the colonization of the gut of stone-forming rats using lactic acid bacteria could be an alternative approach to reduce the intestinal absorption of oxalate and the resulting urinary excretion and crystal formation in the kidney.

If injury of the renal tubule is induced, as well as adding the constituents of urinary stone, the formation of urinary stone could be induced in the animal model. Kumar et al. [16] developed an animal model of nephrolithiasis by inducing moderate crystalluria in animals with altered proximal tubular functions, administering gentamicin subcutaneously and supplementing 3% sodium oxalate. Their model featured papillary calcium oxalate plaque formation and normal or near-normal renal function without histological evidence of renal damage as well as a higher frequency of stones in a shorter period of time. We confirmed the reproducibility of their model of nephrolithiasis. However, this model could have a limitation to use in the present study because the administration of antibiotics, gentamicin, could prevent the colonization of bacteria in the gut of rats. Celecoxib, a COX-2 inhibitor, has a low GI toxicity whereas most nonsteroidal anti-inflammatory agents have significant toxicities. Therefore, we used celecoxib instead of gentamicin as a source for renal tubular injury [13].

Our study shows that oral administration of celecoxib and oxalate led to a significant elevation of the urinary excretion of oxalate, elevated activities of urinary *N*-acetyl glucosaminase and gamma glutamyl transpeptidase, and a significant increase in crystal formation in the rat kidney. Although the mechanism of nephrotoxicity by a selective COX-2 inhibitor, celecoxib, is obscure, this model shows the formation of more abundant crystals than in rats with only oxalate administration. In addition, there were no gross and pathologic abnormalities in rats after oral administration of celecoxib and oxalate.

We isolated *L. casei* HY2743 and *L. casei* HY7201 out of numerous lactic acid bacteria since these lactobacilli had survived in medium B. After being cultured in medium B, the levels of oxalate in medium B colonized with these lactobacilli was decreased. These lactobacilli were presumed to use yeast extracts as an energy source initially and oxalate after depletion of yeast extracts in medium B.

To our knowledge, our in vivo study is the first report about the inhibitory role of *Lactobacillus* in urolithiasis associated with hyperoxaluria. Prophylactic oral administration of *L. casei* HY2743 led to a significant reduction of the urinary excretion of oxalate and a significant reduction of crystal formation in the rat kidney compared to the stone-forming rats in the present study. These results suggest that the prophylactic colonization of *Lactobacillus* in the GI tract inhibits the intestinal absorption of oxalate.

Since oxalate-degrading bacteria are present in the endogenous microflora of human intestine, the colonization of the gut of subjects with hyperoxaluria using oxalate-degrading bacteria could be an alternative approach to reducing the intestinal absorption of oxalate, the urinary excretion and crystal or plaque formation in the kidney. A few candidate bacteria could be used for this purpose. While an oxalate-degrading strain of *Enterococcus faecalis* has been isolated from human stools under anaerobic conditions [9], it is a potentially pathogenic organism that cannot be safely used for oral bacteriotherapy.

Another candidate bacterium has been *O. formigenes*. *O. formigenes* apparently regulates the absorption of oxalic acid through the gut when present at a concentration ranging from 10^6 to 10^8 cfu/g of feces [17]. The observation that not all patients who tested negative for *O. formigenes* were also hyperoxaluric suggested that several other bacteria might degrade oxalate [18, 19]. In

addition, we failed to isolate and culture *O. formigenes* from human stools [20].

The advantages of *Lactobacillus* could be that it is not pathogenic for humans, easily obtainable, and able to colonize the gut. Based on these advantages and our results, *Lactobacillus* may be used as a potential therapeutic strategy in the prevention of urinary stone.

Acknowledgment This work was supported by the Clinical Research Institute, Seoul National University Hospital (06-2002-028).

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