

**Effect of a Probiotic Supplement Containing *Lactobacillus Acidophilus* and *Bifidobacterium Animalis Lactis* on Urine Oxalate in Calcium Stone Formers with Hyperoxaluria:  
A Randomized, Placebo-controlled, Double-blind and In-vitro Trial**

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**Purpose:** To determine the effect of a probiotic supplement containing native *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium animalis lactis* (*B. lactis*) on 24-hour urine oxalate in recurrent calcium stone formers with hyperoxaluria. Moreover, the in-vitro oxalate degradation capacity and the intestinal colonization of consumed probiotics were evaluated.

**Materials and Methods:** The oxalate degrading activity of *L. acidophilus* and *B. lactis* were evaluated in-vitro. The presence of oxalyl-CoA decarboxylase (*oxc*) gene in the probiotic species was assessed. One hundred patients were randomized to receive the probiotic supplement or placebo for four weeks. The 24-hour urine oxalate and the colonization of consumed probiotics were assessed after weeks four and eight.

**Results:** Although the *oxc* gene was present in both species, only *L. acidophilus* had a good oxalate degrading activity, in-vitro. Thirty-four patients from the probiotic and thirty patients from the placebo group finished the study. The urine oxalate changes were not significantly different between groups ( $57.21 \pm 11.71$  to  $49.44 \pm 18.14$  mg/day for probiotic, and  $56.43 \pm 9.89$  to  $50.47 \pm 18.04$  mg/day for placebo) ( $P = .776$ ). The probiotic consumption had no significant effect on urine oxalate, both in univariable ( $P = .771$ ) and multivariable analyses ( $P = .490$ ). The consumed probiotics were not detected in the stool samples of most participants.

**Conclusion:** Our results showed that the consumption of a probiotic supplement containing *L. acidophilus* and *B. lactis* did not affect urine oxalate. The results may be due to a lack of bacterial colonization in the intestine.

**Keywords:** *lactobacillus acidophilus*; *bifidobacterium animalis subsp. lactis*; hyperoxaluria; probiotics; urolithiasis; calcium oxalate.

## INTRODUCTION

Hyperoxaluria is a common urinary metabolic risk factor in calcium stone formers<sup>(1)</sup>. Although it is more prevalent among Iranians and some other Asian countries, the global prevalence of hyperoxaluria in stone-forming patients has increased over the last two decades<sup>(1)</sup>. Despite its prevalence and significance, the level of evidence for hyperoxaluria management is low in current kidney stone guidelines<sup>(2)</sup>. Moreover, the most common approach for hyperoxaluria management is based on dietary limitation, which may not be applicable or accepted by all patients<sup>(3)</sup>. The oxalate in the human body originates from dietary intake and liver metabolism<sup>(4)</sup>. The liver is the primary source of oxalate generation in the human body, which metabolizes several precursors, such as glycine, glyoxylate, and ascorbic acid, to oxalate<sup>(4,5)</sup>. Since the human body could not degrade oxalate, controlling the intestinal absorption of dietary oxalate could be a treatment modality for hyperoxaluria management<sup>(4,6)</sup>. The intestinal microbiota has a known contribution to

kidney stone pathophysiology<sup>(7,8)</sup>. Studies demonstrated that intestinal microbiota could metabolize oxalate and reduce its absorption from the intestine<sup>(9)</sup>. The well-known oxalate degrading bacterium in the gut microbiota is *Oxalobacter formigenes* (*O. formigenes*)<sup>(7)</sup>. *O. formigenes* exclusively depends on oxalate as its obligatory energy source. Oxalyl-CoA decarboxylase (OXC) is one of the critical bacterial enzymes for oxalate degradation that catalyze Oxalyl-CoA to CO<sub>2</sub> and Formyl-CoA<sup>(7)</sup>.

*Lactobacillus* and *Bifidobacterium* sp. occur in high numbers in the human gut and have been used extensively as probiotics for health improvement<sup>(5)</sup>. Few studies evaluated their effect on hyperoxaluria. However, most of these studies could not find an oxalate-lowering effect of evaluated probiotics<sup>(9)</sup>. Since there is a controversy in the effect of probiotics on hyperoxaluria, the selection of species with the most significant oxalate degrading activity may elicit a more favorable response<sup>(10)</sup>. Some in-vitro studies suggested that the *oxc* gene, encoding OXC, is not present in all the Lacto-

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**Table 1.** The sequence and amplicon size of the primers

Bacteria/gene	Sequence (5'-3')	Amplicon Size (bp)	Reference
oxc gene in <i>Lactobacillus</i> sp.	F: AGCCTCGTCACCGTCTTG R: ACCAAATGCTGAGTACCTTC	125	(21)
oxc gene in <i>Bifidobacterium</i> sp.	F: ACCTTCGTCGTGCTCAAC R: ACCTTCGTCGTGCTCAAC	107	(21)
Probiotic <i>L. acidophilus</i>	F: AACCAACAGATTCACCTCG R: CTCTCAACTCGGCTATGC	250	This study
Probiotic <i>B. lactis</i>	F: AGCGAACAGGATTAGATACC R: GAAGGGAAACCGTGTCTC	254	This study
Universal primer for total bacteria	F: AG(A/C)GTT(T/C)GAT(T/C)(A/C)TGGCTCAG R: GCTGCCTCCCGTAGGAGT	314-373	(25)

**Abbreviations:** oxc: Oxalyl-CoA decarboxylase; *L. acidophilus*: *Lactobacillus acidophilus*; *B. lactis*: *Bifidobacterium animalis lactis*

bacillus and *Bifidobacterium* sp.; therefore, these species showed highly variable oxalate degrading capacity<sup>(11,12)</sup>. *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium animalis lactis* (*B. lactis*) showed the highest oxalate degrading activity in in-vitro<sup>(11-13)</sup> and animal studies<sup>(14,15)</sup>. However, the effect of the simultaneous use of both species on urine oxalate was not assessed in clinical trials.

The current study aimed to determine the effect of a probiotic supplement containing native *L. acidophilus* and *B. lactis* on 24-hour urine oxalate in recurrent calcium stone formers with hyperoxaluria in a randomized, placebo-controlled, double-blind trial. These species were selected according to the results of previous studies<sup>(11-15)</sup>. Besides the clinical trial, the in-vitro oxalate degradation capacity of native *L. acidophilus* and *B. lactis*, and the presence of oxc gene in these species were evaluated. We also assessed the colonization of consumed probiotics in the intestine by real-time PCR of the mentioned species in the stool<sup>(16,17)</sup>.

## MATERIALS AND METHODS

### In-vitro study

Bacterial species, chemicals, and media

Two probiotic species, *L. acidophilus* (PTCC No: 1643) and *B. lactis* (PTCC No: 1736), were provided by Tak Gen Zist Pharmaceutical Company, Tehran, Iran. Both strains were isolated from Iranian native foods and used for the production of the probiotic supplements. Both bacteria were verified for genus and species by 16S rRNA gene sequence typing. *O. formigenes* (DSM 4420) was purchased from the DSMZ-German collection of microorganisms and cell cultures (Braunschweig, Germany).

Proteose peptone, yeast extract, TWEEN® 80, potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), sodium acetate, di-ammonium hydrogen citrate, magnesium sulfate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O), manganese sulfate monohydrate (MnSO<sub>4</sub>·H<sub>2</sub>O), di-ammonium oxalate monohydrate, D (+)-glucose anhydrous, L-Cysteine, and Anaerocult® A gas pack were purchased from Merck (Darmstadt, Germany). The De Man-Rogosa and Sharpe (MRS) broth was purchased from Liofilchem® (Roseto Degli Abruzzi (TE), Italy). D (+)-sucrose and sodium oxalate were purchased from Carlo Erba Reagents (Val de Reuil, France).

### Pre-adaptation to high oxalate contents

The in-vitro study was conducted in the microbiology laboratory of the National Nutrition and Food Technology Research Institute, Shahid Beheshti University of

Medical Sciences, Tehran, Iran. All the bacteria were pre-adapted to non-inhibitory concentrations of oxalate before the oxalate degradation assay<sup>(12)</sup>. Anaerobic conditions were achieved in all experiments in anaerobic jars supplemented with a pad of Anaerocult® A.

After anaerobic growth of the bacteria in MRS broth for 16 hours, *L. acidophilus* was pre-adapted to high oxalate contents: First, *L. acidophilus* was grown in MRS broth containing 0.35 mmol/L sodium oxalate (pH 5.5) and incubated at 37°C for 16 hours. At the next step, growing microorganisms were transferred to MRS broth containing 35 mmol/L of sodium oxalate (pH 5.5) and incubated at 37 °C for 16 hours.

As the method mentioned above, the *B. lactis* was pre-adapted to high oxalate contents after anaerobic growth of the bacteria in MRS broth + L-cysteine (0.5 g/L) for 16 hours. The pre-adaptation steps were growth within two incubation cycles at 37 °C for 16 hours, first in MRS broth + L-cysteine containing 0.35 mmol/L sodium oxalate (pH 5.5), and then MRS broth + L-cysteine, containing 35 mmol/L of sodium oxalate (pH 5.5).

*O. formigenes* was pre-adapted to high oxalate contents, as we previously published, using 0.35 mmol/L and 35 mmol/L ammonium oxalate<sup>(18)</sup>.

### Oxalate degradation estimation

The base culture medium used for oxalate degradation assay was prepared as the method used by Campieri et al.<sup>(19)</sup>. This enriched media contained proteose peptone (10 g), yeast extract (5 g), TWEEN® 80 (1 mL), KH<sub>2</sub>PO<sub>4</sub> (2 g), sodium acetate (5 g), di-ammonium hydrogen citrate (2 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.05 g), and MnSO<sub>4</sub>·H<sub>2</sub>O (0.05 g). All these materials were dissolved in distilled water, with a final volume of 500 mL. After sterilization, sodium oxalate (25 mmol/L), D (+)-glucose anhydrous (10 mL), and D (+)-sucrose (10 mL) (all sterilized using 0.46-µ filters) were added to the medium. The final pH of the medium was 5.5. The media were inoculated with study species (2\*10<sup>8</sup> CFU/mL) and incubated for four days. *O. formigenes* has been employed as a positive reference to validate oxalate degradations assay, and an un-inoculated medium was used as a negative control. All the cultivations were performed in a Bioscreen C system (Growth Curves Ltd, Finland), which also measured the population of bacteria by a turbidometric method.

The oxalate contents of culture broth samples, positive control, and negative control were measured at the beginning and every day until the fourth day of the study. After pasteurization of media at 90 °C for 15 min, the

**Table 2.** Baseline Demographic and clinical characteristics of the studied participants

	Probiotic (n = 34)	Placebo (n = 30)	P-value
Age, years, Mean (SD)	46.1 (12.7)	50.4 (9.3)	.130 <sup>a</sup>
Gender, Number (percentage)			.325 <sup>b</sup>
Female	13 (38.2%)	8 (26.7%)	
Male	21 (61.8%)	22 (73.3%)	
BMI, Kg/m <sup>2</sup> , Mean (SD)	28.85 (4.75)	30.45 (4.66)	.281 <sup>a</sup>
Positive family history, Number (percentage)	17 (50%)	16 (53.3%)	.790 <sup>b</sup>
Disease duration, years, Median (IQR)	7.00 (4.00-15.00)	19.00 (5.00-29.00)	.055 <sup>c</sup>
Cigarette smoking, number (percentage)			.314 <sup>d</sup>
Yes	1 (2.9%)	2 (6.7%)	
No	33 (97.1%)	26 (86.7%)	
Past smoking	0 (0.0%)	2 (6.7%)	

**Abbreviations:** SD: Standard deviation; BMI: Body mass index; IQR: Interquartile range.

<sup>a</sup>Independent T test

<sup>b</sup>Chi square Test

<sup>c</sup>Mann-Whitney test.

<sup>d</sup>Fisher exact test

media were centrifuged at  $5000 \times g$  for 10 min, and the supernatants were used to assess concentrations of residual oxalate. The oxalate content was assessed using a colorimetric enzymatic method (Darman Faraz Kave kit, Tehran, Iran), as we previously reported<sup>(20)</sup>. The oxalate degradation was reported as the percentage of oxalate utilized versus the initial values. All experiments were performed in triplicates.

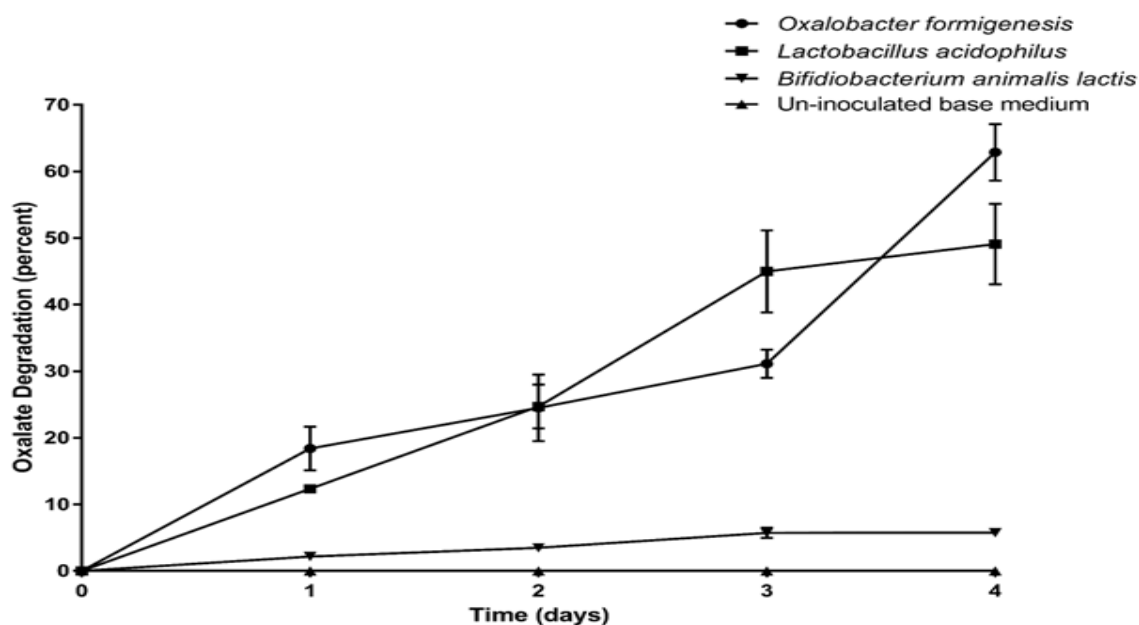
Assessment of *oxc* gene in the probiotic species  
The presence of *oxc* gene in the genomic DNA of probiotic species, i.e., *L. acidophilus* and *B. lactis*, was assessed by polymerase chain reaction (PCR). The genomic DNA was extracted by boiling lysis and quantified using a WPA spectrophotometer (Biochrom, Cambridge, UK)<sup>(21)</sup>. The used primers (Table 1) and positive controls were adapted from our previous study<sup>(21)</sup>. Each PCR reaction was composed of 5 ng of genomic DNA, 20 pmol of each forward and reverse primers, 10 mmol

of each dNTP, 1.5 mmol/L of MgCl<sub>2</sub>, 0.5 units of Taq polymerase, and double distilled water to reach a final volume of 25  $\mu$ L. The reactions were performed in a thermocycler (Mastercycler, Eppendorf). PCR products were electrophoresed on agarose gel (3%) and stained with Ethidium bromide to visualize the amplicons. A gel documentation system (Syngene, Cambridge, UK) was used to capture the gel images.

### Randomized clinical trial

#### Study design and participants

The second step of the study was a randomized, placebo-controlled, double-blind clinical trial. The study had a parallel-group design, with an allocation ratio of 1:1. The clinical trial aimed to evaluate the effect of a probiotic supplement containing the same *L. acidophilus* and *B. lactis* species as In-vitro study, on the urine oxalate of recurrent calcium stone formers with hyperoxaluria.



**Figure 1.** The oxalate depredeating behavior of study *L. acidophilus* and *B. lactis*, compared with *O. formigenes*. Each point represents mean and error bars represent the standard deviation values.

**Table 3.** 24-hour urine metabolites of the study groups at baseline, at the end of the intervention (post-treatment) and 4 weeks after the end of the intervention (post-follow-up). All values stand for mean (standard deviation)

Variable	Probiotic group	Placebo group	P-value <sup>a</sup>	
			Time effect	Group effect
24-hour urine oxalate, mg/day (n = 64)			.017*	.776
Baseline	57.21 (11.71)	56.43 (9.89)		
Post-treatment	53.82 (16.13)	51.17 (18.32)		
Post-follow up	49.44 (18.14)	50.47 (18.04)		
Calcium oxalate supersaturation (n = 64)			.618	.804
Baseline	7.44 (2.83)	6.96 (3.41)		
Post-treatment	6.91 (3.88)	6.78 (4.31)		
Post-follow up	6.61 (3.30)	6.74 (3.80)		
24-hour urine volume, mL/day (n = 64)			.557	.615
Baseline	2398.5 (747.0)	2233.3 (624.8)		
Post-treatment	2359.9 (658.7)	2311.7 (696.8)		
Post-follow up	2257.4 (717.4)	2240.0 (740.5)		
24-hour urine Calcium, mg/day (n = 64)			.596	.241
Baseline	237.76 (113.00)	183.60 (77.04)		
Post-treatment	230.88 (125.89)	222.47 (125.26)		
Post-follow up	229.44 (120.48)	212.97 (107.30)		
24-hour urine Phosphor, gr/day (n = 62)			.979	.640
Baseline	0.81 (0.27)	0.76 (0.22)		
Post-treatment	0.73 (0.25)	0.83 (0.26)		
Post-follow up	0.78 (0.27)	0.80 (0.28)		
24-hour urine magnesium, mg/day (n = 64)			.524	.824
Baseline	99.68 (39.67)	103.63 (50.98)		
Post-treatment	97.82 (38.06)	98.93 (40.56)		
Post-follow up	102.85 (46.83)	104.40 (52.68)		
24-hour urine sodium, mEq/day (n = 64)			.362	.305
Baseline	181.29 (64.60)	149.93 (50.84)		
Post-treatment	181.06 (57.62)	178.47 (80.83)		
Post-follow up	174.03 (69.50)	172.60 (69.52)		
24-hour urine potassium, mEq/day (n = 64)			.445	.315
Baseline	87.48 (134.81)	58.83 (21.59)		
Post-treatment	61.42 (23.23)	64.57 (29.30)		
Post-follow up	65.73 (22.68)	62.10 (25.01)		
24-hour urine citrate, mg/day (n = 64)			.448	.317
Baseline	823.24 (343.95)	640.47 (338.34)		
Post-treatment	736.38 (340.89)	719.67 (401.90)		
Post-follow up	713.79 (320.76)	664.30 (398.75)		
24-hour urine uric acid, mg/day (n = 64)			.246	.982
Baseline	545.88 (196.80)	476.83 (152.03)		
Post-treatment	507.32 (162.32)	468.00 (160.41)		
Post-follow up	409.59 (161.76)	520.00 (217.89)		
24-hour urine urea, gr/day (n=61)			.134	.704
Baseline	37.48 (10.34)	34.04 (8.72)		
Post-treatment	32.41 (9.66)	34.5 (9.97)		
Post-follow up	34.15 (9.49)	34.90 (8.85)		
24-hour urine creatinine, gr/day (n = 64)			.751	.759
Baseline	1.18 (0.41)	1.19 (0.38)		
Post-treatment	1.23 (0.38)	1.21 (0.39)		
Post-follow up	1.21 (0.45)	1.26 (0.39)		

<sup>a</sup>repeated measures ANOVA analyses.

\*  $P < .05$ . Bold values emphasize statistical significance.

Patients with a history of at least two radiopaque stone episodes<sup>(22)</sup> and hyperoxaluria (24-hour urine oxalate  $\geq$  40 mg/24h) were recruited from the stone prevention clinic of Shahid Labbafinejad medical hospital, Tehran, Iran. All patients aged 18-70 who were capable of giving informed consent and had no history of surgical interventions or stone expulsion in the last twenty days<sup>(2)</sup>. Patients were not included if they had a history of primary or enteric hyperoxaluria (urine oxalate  $>$  80 mg/24h), chronic kidney disease, current urinary tract infection, chronic diarrhea, thyroid or parathyroid diseases, diabetes mellitus, hepatic failure, cancers or immunologic diseases and in case of pregnancy or lactation. Patients were also excluded from the study if they used antibiotics, any medication influencing urine oxalate homeostasis, including calcium and magnesium supplements and pyridoxine, or if they had a new prescription or changed the dose of thiazides and potassium citrate during the study. Patients using antibiotics

two weeks before the start and during the study were also excluded.

All patients gave written informed consent, and the study was performed in accordance with the 1964 Declaration of Helsinki. The study was approved by the National Institute for Medical Research Development (NIMAD) (grant number: 940329) and Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The ethics committee of NIMAD approved the study (reference number: IR.NIMAD.REC.1394.014). The trial was registered on the Iranian Registry of Clinical Trials (IRCT) (IRCT registration number: IRCT2016020626406N1). Interventions

The used probiotic supplement was a commercial product, containing assessed *L. acidophilus* and *B. lactis* species, produced by Tak Gen Zist Pharmaceutical Company, Tehran, Iran. The supplement was approved by the Food and Drug Organization of Iran. Each cap-

**Table 4.** The effect of probiotic consumption on urinary oxalate using multi-variable General Linear Model (GLM) with Generalized Estimating Equation (GEE) approach.

Variables		Univariable analysis B (CI)	P-value	Multivariable analysis B (CI)	P-value
Group	Probiotic	0.80 (-4.58, 6.19)	.771	-2.13 (-8.18, 3.91)	.490
	Placebo	Reference		Reference	
Time	Post-follow-up	-6.92 (-11.66, -2.18)	.004**	-6.31 (-12.48, -0.14)	.045*
	Post-treatment	-4.26 (-9.00, 0.47)	.078	-3.07 (-9.05, 2.91)	.314
	Baseline	Reference		Reference	
Gender	Male	6.88 (1.63, 12.13)	.010**	6.12 (0.20, 12.03)	.043*
	Female	Reference		Reference	
24-hour urine magnesium		-0.05 (-0.10, -0.007)	.025*	-0.04 (-0.11, 0.02)	.182
24-hour urine urea		0.05 (-0.18, 0.29)	.649	0.07 (-0.19, 0.34)	.602
24-hour urine sodium		0.03 (0.00, 0.07)	.045*	0.06 (0.01, 0.10)	.009**
Age		0.13 (-0.07, 0.34)	.207	0.27 (-0.005, 0.54)	.054
BMI		0.50 (-0.28, 1.28)	.214	0.17 (-0.36, 0.71)	.518
Duration of disease		0.01 (-0.007, 0.02)	.245	-0.02 (-0.04, 0.002)	.075

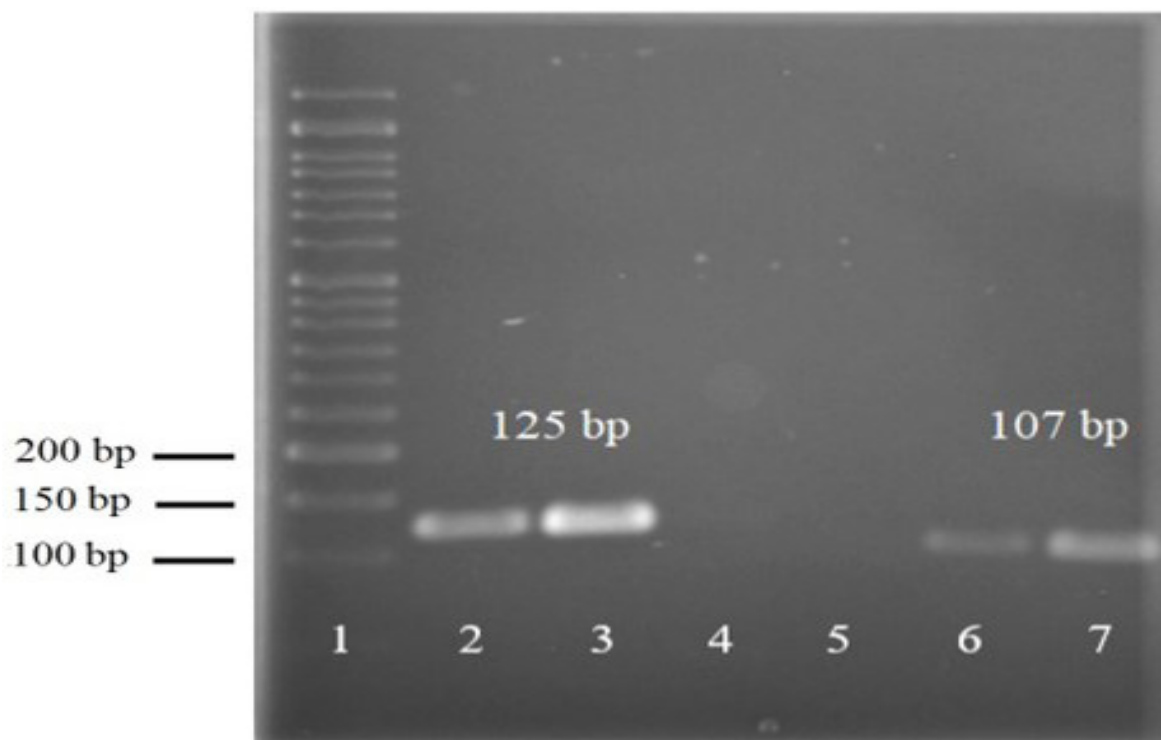
**Abbreviations:** CI: Confidence Interval; BMI: Body mass index.  
\*  $P < .05$ . \*\*  $P < .01$ . Bold values emphasize statistical significance.

sule of the supplement contained  $1.8 \times 10^9$  CFU of the following species with the ratio of 1:1:1:1 *L. acidophilus*, *B. lactis*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*. The placebo capsules had the same color, shape, size, and package.

All study patients received two capsules of either probiotic or placebo every day for four weeks. Both groups had the usual nutritional consult and suggestions of the stone prevention clinic according to the European Association of Urology (EAU) guidelines, including normal calcium and restricted oxalate intake<sup>(2)</sup>. Drug compliance was defined as the ratio of consumed to total pills<sup>(23)</sup>.

### Sample size, randomization, and blinding

The sample size was calculated to have the power to detect five units decrease in urine oxalate with a sensitivity of 80%. Considering a 40% loss to follow up in nephrolithiasis prevention clinic (unpublished data), the study sample size was 50 patients in each group. We used permuted-block randomization, with the allocation ratio of 1:1, to divide patients into blocks with the size of four. In each block two patients were allocated to probiotic group and other two were allocated to placebo group. A random sequence was generated before the patient allocation by one of the study investigators. The same investigator sequentially coded the drug and pla-



**Figure 2.** Agarose gel electrophoresis of PCR amplification products in *L. acidophilus* (lane 2), and *B. lactis* (lane 6). Lane 3 and 4: positive control for *oxc* gene in *Lactobacillus* sp. and corresponding negative control, respectively. Lane 7 and 5: positive control for *oxc* gene in *Bifidobacterium* sp. and corresponding negative control, respectively. Positive controls were adapted from<sup>(21)</sup>.

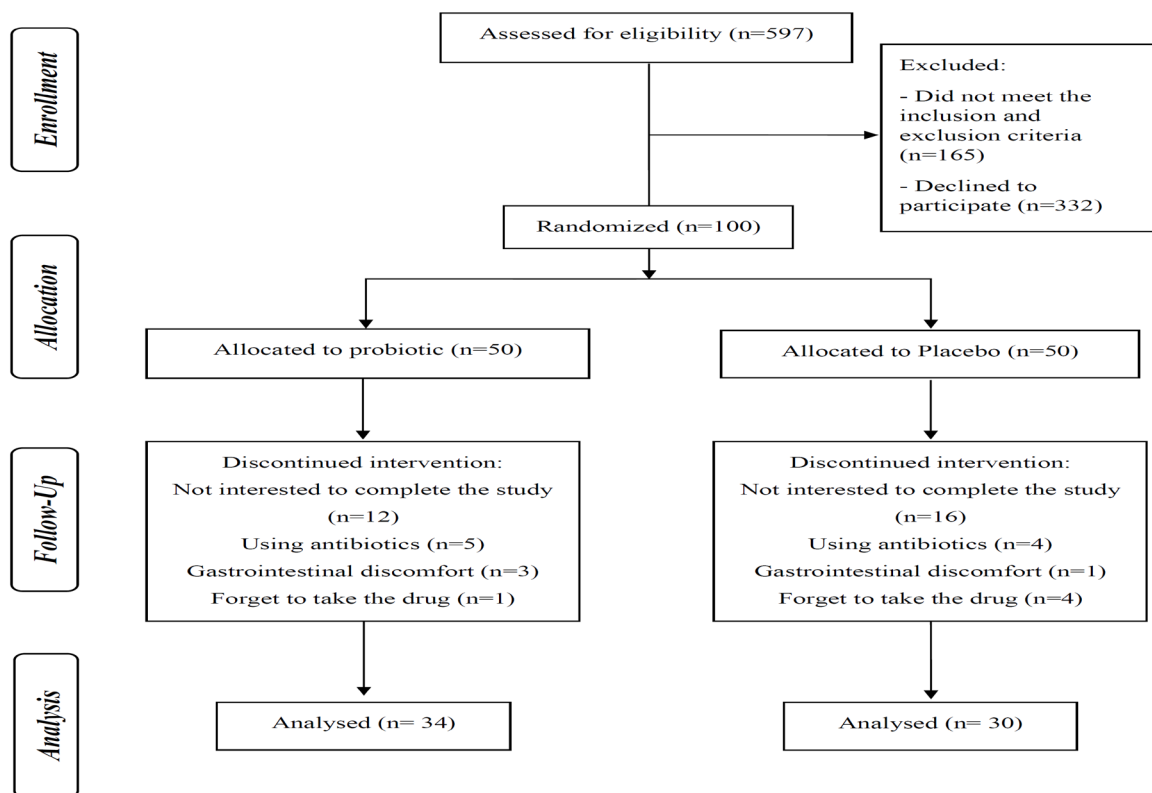


Figure 3. Flow diagram for participants included in the study.

cebo containers and kept the code secret until the end of data analysis. Other researchers and study participants were unaware of study allocation.

**Para-clinic assessments**

All study participants were assessed in three time-points: before the interventions (baseline), at the end of the interventions (post-treatment), and four weeks after the end of the interventions (post-follow up). The col-

lected samples in assessment visits were one 24-hour urine sample (to evaluate the urine oxalate and other metabolites and calcium oxalate relative supersaturation (CaOXSS) values) and one fresh stool sample (to evaluate the colonization of the study probiotic bacteria). The 24-hour urine sample collection and urine metabolite analyses were performed as previously published<sup>(24)</sup>. The CaOXSS values were calculated using LithoRisk software (Biohealth, Italy).

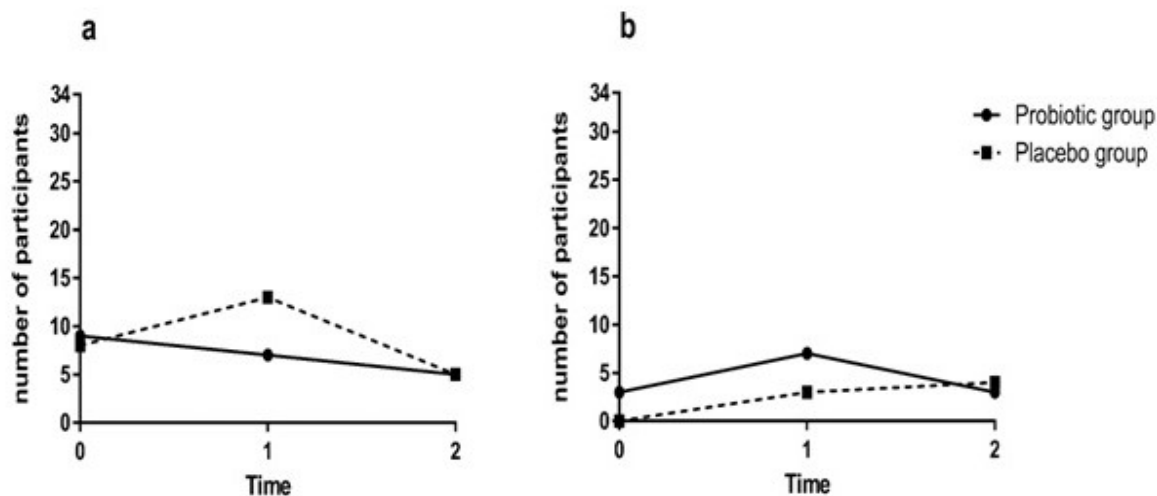


Figure 4. Number of positive stool samples for probiotic species at each group and time point, investigated by real-time PCR. A: L. acidophilus, B: B. lactis. Time 0: Baseline, Time 1: Post-treatment, Time 2: Post-follow-up

### Microbial assessment of stool samples

The colonization of consumed probiotics in the intestine was assessed by fecal microbial analyses<sup>(16,17)</sup> at three time-points: baseline stool samples (S1), post-treatment stool sample (S2), and post-follow up stool samples (S3). The stool sample bacterial DNA was extracted by the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Dusseldorf, Germany) per the manufacturer's guidelines. The extracted DNA was quantified using a WPA spectrophotometer (Biochrom, Cambridge, UK)<sup>(21)</sup>. We used quantitative real-time PCR to assess the relative amount of study species, i.e., *L. acidophilus* and *B. lactis*. The primers for the detection of *L. acidophilus* and *B. lactis* were designed by AlleleID 6 software (Table 1). These primers were designed to detect specific conserved sequences of 16S rRNA of bacterial species. Besides, a universal primer for bacterial 16S rRNA gene (Table 1) was used to quantify the total number of the Eubacteria<sup>(25)</sup>, as we reported previously<sup>(21)</sup>. The presence of *L. acidophilus* and *B. lactis* were normalized to the total Eubacteria to calculate the relative abundance of each specie.

Quantitative real-time PCR reactions were composed of RealQ Plus 2x Master Mix Green (Ampliqon, Denmark), specific primers (0.4 micromoles of each primer), and extracted bacterial DNA (50 ng). Using the Rotor-Gene instrument (Qiagen), PCR reactions were conducted by the following parameters: 95°C for 15 min to activate the enzyme, 40 cycles of 95°C for 20 seconds, followed by 60 °C for 60 seconds. All reactions were performed in duplicates. Amplification of specific sequences was monitored by melt curve analysis and electrophoresis of PCR products. The evaluated probiotic *L. acidophilus* and *B. lactis* genomic DNAs were used as positive controls.

### Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp.). The final oxalate degrading activity of the bacteria on the fourth day of the study were compared with the Kruskal-Wallis test. Post-hoc Bonferroni correction was performed to compare the activity of *O. formigenes* with *L. acidophilus* and *B. lactis*. The Chi-Square and Fisher exact tests were used to compare the groups in case of categorical data. The normality of numeric variables was checked using the Shapiro-Wilk test. The differences in continuous data between study groups were assessed using the Independent t-test tests or Mann-Whitney U test in the case of non-normal variables. The effect of probiotic consumption on 24-hour urine metabolites during the study at baseline, post-treatment, and post-follow up were explored using Repeated measures ANOVA. General Linear Model (GLM) with Generalized Estimating Equation (GEE) approach was applied to investigate the univariable and multivariable effect of the consumed probiotic on the 24-hour urine oxalate changes over time. The significance level was considered as  $p < 0.05$ .

## RESULTS

### In-vitro study

#### Oxalate degrading activity

Figure 1 shows the degradation of oxalate salt by study species. As shown in Figure 1, *L. acidophilus* degraded  $49.08 \pm 6.05$  percent, *B. lactis* degraded  $5.75 \pm 0.50$  per-

cent, and *O. formigenes* degraded  $62.88 \pm 4.26$  percent of the media oxalate at the end (fourth day) of the study, which was significantly different ( $P = .007$ ). Pairwise comparisons showed that there was no significant difference between *L. acidophilus* and *O. formigenes* ( $P = .226$ ). However, *B. lactis* showed a low oxalate degrading activity that was significantly lower than *O. formigenes* ( $P = .004$ ). The population of bacteria in all cultures were 106-108 CFU/mL in all measurements.

Assessment of *oxc* gene in the probiotic species  
The presence of *oxc* gene in the genomic DNA of probiotic species, i.e., *L. acidophilus* and *B. lactis*, was assessed by PCR. The results are presented in Figure 2. As shown in the figure, the *oxc* gene was present in the genomic DNA of both species.

### Randomized clinical trial

#### Effect of probiotic consumption on 24-hour urine oxalate

Five-hundred and ninety-seven patients with hyperoxaluria were screened, and from them, 100 patients were randomized to the probiotic ( $n = 50$ ) and placebo ( $n = 50$ ) groups, from September 2017 to March 2019. Thirty-four patients from the probiotic group and thirty patients from the placebo group finished the study. All the participants consumed more than 80% of the probiotic or placebo and reported no severe side effects. The CONSORT participant flow diagram is presented in Figure 3.

The baseline characteristics of the probiotic and placebo groups are presented in Table 2. The study groups were not different in the case of baseline characteristics. Table 3 shows the 24-hour urine metabolites and CaOXSS of the probiotic and placebo groups at baseline, post-treatment, and post-follow-up time points. The results of repeated measures ANOVA analyses showed that although there was a significant decrease in the mean of 24-h urine oxalate in both groups ( $P = .017$ ), the changes were not significantly different between groups ( $P = .776$ ). The consumption of the probiotic supplement did not significantly affect other urinary metabolites and CaOXSS. Moreover, none of these variables changed significantly over time (Table 3).

The effect of probiotic consumption on urinary oxalate was analyzed using both univariable and multivariable GLM with GEE approach (Table 4). In agreement with the previous results, the univariable analysis revealed that the urinary oxalate decreased significantly at the end of the study in both groups ( $P = .004$ ). However, the decrease was not different between the probiotic and placebo groups ( $P = .771$ ). These results were confirmed in the multivariable analysis, which showed that probiotic consumption had no significant effect on urinary oxalate after adjusting for confounders ( $P = .490$ ) (Table 4).

#### Colonization of consumed probiotics in the stool samples

The extracted DNA from stool samples were analyzed for the presence of studied *L. acidophilus* and *B. lactis* species (Figure 4). *L. acidophilus* was detected in 9 (26.5%) S1 samples of the probiotic group and in 8 (26.7%) S1 samples of the placebo group. Regarding S2 samples, *L. acidophilus* was detected in 7 (20.6%) and 13 (43.3%) cases from the probiotic and the place-

bo groups, respectively. Five (14.7%) cases in probiotic group and 5 (16.7%) in placebo group showed positive signals (proper amplification) for the presence of *L. acidophilus* in S3 samples (Figure 4). Regarding *B. lactis* probiotic, a positive signal was observed in 3 (8.8%) patients of the probiotic group and 0 (0%) patients of the placebo group in S1 samples. In S2 samples, 7 (20.6%) in the probiotic group and 3 (10%) in the placebo group were positive for *B. lactis*. Finally, *B. lactis* was detected in 3 (8.8%) probiotic and 4 (13.3%) placebo S3 samples (Figure 4). Since the studied bacteria were not detected in most participants, we could not compare the relative abundance of bacteria between groups.

## DISCUSSION

Hyperoxaluria is a known urinary metabolic risk factor in calcium stone formation. Alteration in intestinal microflora is suggested as an important cause of secondary hyperoxaluria; therefore, modification of intestinal microbiome with oxalate degrading bacteria could be a treatment modality in this situation<sup>(26,27)</sup>. Since *Lactobacillus* and *Bifidobacterium* sp. are safe for human consumption as probiotics, they may be a good option for managing hyperoxaluria. However, most of previous studies could not show the oxalate-lowering effect of *Lactobacillus* and *Bifidobacterium* sp.<sup>(3,28-30)</sup>. The main reason for such these findings may be that *Lactobacillus* and *Bifidobacterium* sp. have various oxalate degrading activity in-vitro<sup>(11,12,19,31,32)</sup> and most of the previous studies did not select species that efficiently degrade oxalate in-vitro<sup>(3)</sup>.

Our results showed that the *oxc* gene was detected in both *L. acidophilus* and *B. lactis*. However, only *L. acidophilus* showed an efficient oxalate degrading activity in culture media, and *B. lactis* did not efficiently degrade oxalate. Most previous studies showed that both species efficiently degrade oxalate in-vitro<sup>(11,12,19,31,32)</sup>. However, Mogna et al. reported that *L. acidophilus* is more efficient than *B. lactis* in oxalate degradation<sup>(13)</sup>. The differences in the methods and conditions used to treat the bacteria in these studies are the reason for these controversies<sup>(5,13)</sup>.

The growth and oxalate degrading activity of *L. acidophilus* and *B. lactis* depend on various variables. One of these variables is the oxalate concentration in the culture media. Some studies found that high oxalate levels might inhibit bacterial growth and reduce oxalate degrading activity, consequently<sup>(19)</sup>. Although we used a high oxalate concentration in the culture media to resemble the condition after eating a high oxalate diet, the study species were pre-adapted to high oxalate, and we did not have growth inhibition.

Another variable that affects oxalate degrading activity both in-vitro and in vivo is pH. As reported by Turroni et al.<sup>(12)</sup>, pH is a fundamental variable for expressing genes involved in oxalate catabolism. The best pH for the *oxc* gene expression is 5.5 for *L. acidophilus*<sup>(31)</sup> and 4.5 for *B. lactis*<sup>(12)</sup>. We used a pH of 5.5 in our experiments to simulate a condition near the gut area. However, it is not an optimum pH for the *oxc* gene expression in *B. lactis* and may cause low oxalate degrading activity of the bacteria in our in-vitro study.

Our randomized clinical trial's results showed that probiotic consumption could not decrease urine oxalate. This finding may also be due to the effect of pH on gene expression. The normal colon pH, where *Lactobacillus*

and *Bifidobacterium* sp. are colonized, is 5.5-7.5<sup>(33)</sup>, not the optimum pH for the oxalate degrading activity of *L. acidophilus* and *B. lactis*. We suggest that although previous studies reported that both *L. acidophilus* and *B. lactis* have a good oxalate degrading activity in-vitro<sup>(11,12,31,32)</sup>, these probiotic bacteria are not necessarily efficient in vivo. This hypothesis needs further investigation.

Another reason for our finding may be the lack of bacterial colonization in the intestine. Stool microbial assessment is suggested for the detection of probiotic colonization in the gastrointestinal tract<sup>(16)</sup>. We assessed the stool microbiome, and the results showed that the consumed probiotic bacteria were not detected in stool samples. Our study participants consumed  $1.8 \times 10^9$  CFU of probiotic bacteria. Using higher concentrations of bacteria (as high as  $10^{11}$  CFU) may improve bacterial colonization in the gut<sup>(19)</sup>. However, it should be mentioned that investigating stool samples for bacterial colonization patterns have some potential limitations. The microbial analysis of stool samples does not necessarily demonstrate the gastrointestinal microbial composition and may underestimate the colonization of probiotic species. Assessment of colonic biopsy samples may be more sensitive than stool samples for detecting bacterial colonization<sup>(34)</sup>.

Other points that deserve attention in this context are the recent findings of studies using next-generation-sequencing methods to investigate the association between the gut microbiome composition and kidney stone formation<sup>(8,35-37)</sup>. Some of these studies showed that the abundance of *O. formigenes* was not different between stone formers and healthy people<sup>(8,36)</sup>, suggesting that this bacterium is not necessarily the link between the gut microbiome and urinary stone formation. Although their results had some controversies, these studies demonstrated that kidney stone formers might have a different gut microbiota profile compared to healthy controls at the phylum, genera, and specie levels<sup>(8,35,36,38)</sup>. The study by Liu et al showed that the presence of *O. formigenes* in the colon might be an indicator for the presence of a network of other bacteria<sup>(39)</sup>. Miller et al. showed that whole-community microbial transplants significantly increased oxalate degradation and decreased urine oxalate in a rat model, which persisted nine months after the transplants<sup>(40)</sup>. According to these findings and vast differences between the microbiome of stone formers and healthy controls, Ticinesi et al suggested that a complex microbial network is responsible for the oxalate-degradation. Therefore, consuming a probiotic containing limited oxalate-degrading species may not be sufficient to influence oxalate catabolism<sup>(8)</sup>. It seems that any treatment strategies (such as fecal transplant<sup>(41)</sup>) that could preserve the microbial network needed to maintain *O. formigenes* and other oxalate degrading bacteria would be a more successful treatment strategy for hyperoxaluria. Our current knowledge in this field is limited, and future studies are needed to confirm the efficacy of these treatments.

This study is one of the few randomized clinical trials that evaluated the effect of an oxalate degrading probiotic on urine oxalate. Another strength of our study was that we only recruited the calcium stone formers with hyperoxaluria, and patients with other types of stones and normal urine oxalate were not included. The main limitation that should be considered for this study is



the lack of patients' dietary intake data. However, both groups were asked to follow the same dietary guidelines for urolithiasis prevention, including low oxalate and normal calcium intake. Another major limitation of the study was that we could not produce a supplement that contained only the species with the highest oxalate degrading activity. The daily dose of probiotics may be one more limitation of our study. Higher concentrations of bacteria may be needed for colonization in the intestine.

## CONCLUSIONS

Our results showed that the consumption of a probiotic supplement containing *L. acidophilus* and *B. lactis* did not affect 24-hour urine oxalate. These negative results may be due to a lack of bacterial colonization in the intestine or the effect of colon pH on gene expression of oxalate degrading enzymes. Similar to previous studies' results, our findings could not confirm probiotics' efficacy as a treatment strategy for hyperoxaluria. Further studies in this field are warranted.

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## CONFLICT ON INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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