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EVALUATION OF OXALATE-DEGRADING ACTIVITY AND MOLECULAR RECOGNITION OF OXC, FRC GENES IN LACTIC ACID BACTERIUM OF INHABIT IN HUMAN COLON

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Abstract

Hypercalciuria and hyperoxaluria are among the most important pathophysiologic causes of kidney stone formation. An increased intestinal absorption of oxalate is known to lead to hyperoxaluria with a significantly enhanced risk of urinary stone formation. More recent studies have demonstrated the presence of several oxalate-degrading bacteria in human gut and their ability to control oxalate levels by influencing intestinal absorption of dietary oxalate. Lactobacilli are common inhabitants of the human gut and are widely included in fermented dairy products as probiotic adjuncts.

Materials and Methods: This case-control study was conducted on serum, urinary, and fecal samples. The research population included a total of 200 subjects divided in two equal groups. They were selected from the patients with urinary tract stones, visiting urologist, and also normal people. The level of calcium, oxalate, and citrate in the urinary samples, parathyroid and calcium in the serum samples, and degrading activity of fecal lactobacillus strains of all the subjects were evaluated. Then, data analysis was carried out using SPSS-11.5, Fisher's exact test, and analysis of variance.

Results: The results revealed that the patients had higher urinary level of oxalate and calcium, as well as higher serum level of parathyroid hormone than normal people. In contrast, urinary level of citrate was higher in normal people. In addition, there was a significant difference between the oxalate-degrading capacities of lactobacillus isolated from the patients and their normal peers.

Conclusion: Reduction of digestive lactobacillus-related oxalate-degrading capacity and increased serum level of parathyroid hormone can cause elevated urinary level of oxalate and calcium in people with kidney stone.

Key words: Kidney stone, Oxalate-degrading capacity, Lactobacillus.

Introduction: Hyperoxaluria is a major risk factors for kidney stones that the urine load of oxalate and calcium plays an important role in calcium-oxalate stone formation. Oxalic acid is a strong dicarboxylic acid that is found in dietary sources (such as coffee , tea ,…) or produced by intestinal microflora from ascorbic acid. In the intestine, oxalate may combine with, especially Ca^{+2} to form nonsluble salts. But they also make oxalate toxic for most forms of life, especially mammals. In humans, the high concentration of oxalic acid can result in a number of pathologic conditions, including hyperoxaluria, urolithiasis, renal failure, kidney stone formation,…The colon is the major site of oxalate absorption,that though around 10% of dietary oxalate being absorbed under normal cantions. Highly intestinal obsorption of oxalate my lead to hypreoxaluria and increased risk of urinary stone formation. Prevention of recurrence of urinary calculi is one of the biggest challenges facing the modern urologist. With the detection and identification of oxalate –degrading bacteria, which can apromising treatment for decrease of urinary oxalate levels in kidney stone disease. For the first time in 1985 (Allison et al) reported that the presence of oxalate degrading bacteria in the human intestine have been shown to play a key role in reducing absorption of (enteric) intestine oxalate. Oxalobacterformigenes was the first of oxalate degrading bacteria for specifically, which metabolizing oxalate for give to energy. In thismicroorganism, oxalate is decarboxylated to give CO_2 and Formic acid, by of the key enzyme, oxalyl-COA decarboxylase(OXC) and formyl-COA transferase(FRC). Previous studies have suggested a (correlation) relationship between increased of urinary oxalate levels and the absence of oxalobacterformigenes colonization. Further of oxalate –degrading bacteria have been isolated from the human intestine tract, including EubacteriumLentum (9), Enterococcus faecalis (10), and providencia.rettgeri (11). Evidence in dicatesthat this bacterium have oxalate degrading capacity and decreasing oxalic acid levels in plasma and urine. Recently, probiotic bacteria belonging to Bifidobacterium and lactobacillus have been studied for their potential capability to degrade oxalate demonstrated the prevention of nephrolithiasis by lactobacillus in stone-forming rate.Reported the efficacy of the treatment with Lactobacillus in patients with idiopathic calcium-oxalate urolithiasis and enteric hyperoxalutia. Furthermore, the analysis of the genome sequence of lactobacillus strains revealed the presence of an operon involved in oxalate catabolism, which contains genes homologous to formyl-coAtransferase(frc) and oxalyl-coA

decarboxylase(oxc) genes of oxalobacterformigenes. The Aim of the Present Study was evaluation of Decreasing of urinary oxalate levels by probiotic-lactobacillus in kidney stone patients**.**

Material and methods

Selection of Patients: Idiopathic calcium oxalateurolithiasis patients registered in the urology Department of Motahari Hospital Jahrom-Iran. Complete Clinical evaluation such as screening of urinary tract calculi by plain x-ray and ultrasound of abdomen (KUB) region was done by our urologists . A total of 100 patients (62 males and 38 females) and 100 normal , healthy volunteers (64 males and 36 females).

Collection Of Human Stool Samples: Samples were collected from each participant with consent during their routine hospital admission or visit to the urology OPD. Approximately 20-30 mg stool was collected as fecal swab using the fecal collection and transport to laboratory of university (jahrom-iran).

Detection of Lactobacillus Of Stool Culture : All of the stool samples after three-day incubation in Demann,Rogosa and sharpe (MRS) Broth (merck) and medium B Broth (culture medium 419 modified oxalate).Than , to medium B and M.R.S agar with 0/01%(w/v) ammonium oxalate transferred for 48-72h. Using the method of Allison et al. Medium B comprised:K2HPO4 0.25 g, KH2PO4 0.25 g (NH4)2SO4 0.5 g,MgSO4 .7H2O 0.025 g, sodium acetate 0.82 g, yeastextract 1.0 g, trypticase 1.0 g, sodium oxalate 5.0 g, resazurin 0.001 g, Na2CO3 4.0 g, and cysteine HCl .H2O 0.5 g in 800 mL of distilled water.

The mixture was boiled and after it had been cooled to 50C0, 20 mL of trace metal solution (ZnSO4 . 7H2O 10.0 mg, MnCl2.4H2O 3.0 mg, H3BO3 30.0 mg, CoCl26H2O 20.0 mg, CuCl2 . 2H2O 1.0 mg, NiCl2 . 6H2O 2.0 mg, Na2MoO4 . 2H2O 3.0 mg) was added. The pH was adjusted to 6.5, 180 mL of distilled water added andThan lactobacillus were subcultured in selective –medium ofRogosa agar (merck) and Tomato juice agar (merck) with 0/01%(w/v) ammonium oxalate transferred for 48-72h.(Condition cultures were incubated statically at 37^0C In an anaerobic environment for two-three day,obtain pH5.5 to 6.5).Following purification, single strain cultures were identified on the basis of Colony Morphology , Gram Reaction ,Catalase Activity assay.Gram-positive, Catalase Negative Rode were genetically Characterized using primers specific for the 16s rRNA.

Identification and presentation of lactobacillus by using of molecular methods

Isolation of Genomic DNA : DNA extracts of each sample were prepared using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the protocol provided. The resulting solutions of chromosomal DNA of the fecal bacteria were used as templates for PCR .

Oligonucleotides: Bacterial DNA was isolated from colony by DNP$^{T.M}$ kit.The genus specific oligonucleotides for oxalate-degrading lactobacillus and Bac primers used for PCR were purchased from CinnaGen Co.Tehran-Iran. The oligonucleotide sequences used are as follows: The primer sets oxc-L (5-CTT GAA ATG CAA GAT GAA AGCA-3) , oxc-R (5-CTT CAG TCA TTA TTT ATT CTC C-3) and frc-L (5-GGA GAA TAA ATA ATG ACT GAA GA-3) , frc-R (5-CGG TAA AAA TTA ATT ATT CAC C-3) , The primer pairs used were oxcL / oxc-R and frc-L / frc-R for oxalate-degrading lactobacillus (kummar 2002, Altermman 2005,Turroni 2007), and Bac-1/Bac-2 were used to amplify the genomic DNAs.

Polymerase Chain Reaction (PCR) : PCR amplification was performed in 50 µl reaction mixtures containing 150–200 ng DNA, 200 µM dNTPs, 1 µM of each primer, 2.5 µl PCR buffer and 1.5 U Taq polymerase (Cina Gene). Hot start (94 °C for 1 min), The optimal reaction profile was found to be 94 for 5 minutes followed by 35 cycles of denaturation for 1 minute at 94 °C , annealing temperature of 55 °C to 60°C (reducing gradient $\frac{^{\circ}C}{s}$ ./1) for 1 minute , and extention for 1 minute at 72 in Thermocycler (Techne) .The amplified PCR products were electrophorized in 1.5% agarose gels , visualized with UV light and documented. Each set of PCR performed was controlled negatively using a reaction containing all the PCR components except for template DNA and positively using a reaction containing lactobacillus acidophilus PTCC1643 and lactobacillus cassei PTCC1608 DNA as the template.The primer pairs used were oxc/frc for oxalate-degrading lactobacillus and Bac-1/Bac-2 for bacterial DNA.

Oxalate-degrading activity in Lactobacillus strains: To evaluate the oxalate-degrading property, Lactobacillus cultures were transferred two times in MRS with 4 mmol/l ammonium oxalate (Merck, Germmany) at 37oC (incubated in an anaerobic condition) . Cultures were then inoculated at 1% into MRS with 0. 5%, 0.1%, 0.15%,0.2% ammonium oxalate concentreations (4,8,12,16,24 mmol/l ammonium oxalate, respectively). Cultures were incubated statically at 37oC and samples were taken at 24 and 48 hours, centrifuged, neutralized to obtain pH values between 5 and 6 (according to the manufacturer's instructions) with 1 N sodium hydroxide , and stored at –20°.

Cultures were pasteurized at 90 C for 15 minutes and then centrifuged at 5000 rpm for 10 minutes, and finally, the supernatant was filtered with a 0.45 micron filter. Oxalic acid in the cultural media was determined with the “Oxalic acid” Kit (Darman Kave Kit, Isfahan, Iran., 2005) based on the oxidation of oxalate by oxalate oxidase. The analysis was performed by spectrophotometer (Appel,Co, Japan) at 578 nm.Lactobacillus Acidophilus PTCC1643 and Lactobacillus Cassei PTCC1608 were used as a positive control , while E. Coli PTCC1399 and Salmonella Para

Typhoid BRTCC1624 were used as the negative control . The obtained results were analyzed using SPSS-11.5), χ^2 test, Fisher's exact test, and ANOVA. The significant threshold of $p<0.05$ was set.

Hour Urine Analysis: 24-hour urine sample was collected from the 100 patients and 100 controls. The urine was collected in containers containing Thymolasa preservative and was subsequently analyzed for urine volume, pHand excretion of ionic components such as oxalate, calcium, citrate, sodium, potassium, phosphate, uric acid and creatinine. Oxalate was determined by the oxalate oxidase enzymatic method using kit (Darman Keve Res,Lab ,Isfahan,Iran,2005); Other biochemical parameters such as calcium, creatinine, sodium, potassium, phosphorus and uric acid were assayed spectrophotometrically (Appel. Japan) and using method using kit (Darman Keve Res, Lab, Isfahan, Iran, 2005). Metabolic abnormality was assessed in terms of five categories: low urinary volume (<1500 ml/24 h), hyperoxaluria (>.45 mmol/24 h), hypercalciuria (>7.5 mmol/24 h),hypocitraturia (<1.56 mmol/24 h) and hyperuricosuria (>4.46mmol/24 h).

Results

1. Patient profile:

In the present study were 100 patients with calcium oxalate stone forming selection of people was by x-ray and ultrasound. All of them,diviton to 5 group of age .then, using of statistical analysis of ANOVA determind. Significant differences were observed, between age and gender in each group. Results showed which, stone formation in men higher than that in women lower ages.

2. Nutrient intakes:

According to the questionnaire, 46% case of patients and 34% of control group consuming high-oxalate diet also,67% of patients and 33% of case control using calcium-rich diet daily(table1). The difference in calcium and oxalate intake between both groups were significant.

3. Relationship between urinary /serum parameters and calcium oxalate stone forming

Differences of estimated diet composition between both groups corresponded to differences in urinary parameters.Evaluation of 24-h urine showed that hyperoxaluria was the most common abnormality found in patient group (56% in patients, 0% in control group), followed by hypocitraturia (68% and 8%, respectively). Hypercalciuria was markedly higher in patients (72%) than in normal individual (8%). Also hyperuricosuria (54% in patients, 38 % in control group). The mean oxalate and calcium level in urinary were significantly higher in stone formers than in controls. Mean oxalate, calcium and uric acid levels increased in patients. Difference in serum

calcium and uric acid concentration was found in patient group. Also the serum creatinine concentration was higher in calcium oxalate stone formation with hyperoxaluria.($p>0.05$).The results showed that appearly, there were statistial relationship between increasing level of urinary oxalate and calcium and using of diet rich in oxalate and calcium. In additoin the chance formation of calcium oxalate stone in patients was higher compared to safe group.

4. Relationship between stone formation and oxalate-degrading bacteria detection by PCR:

Detection rate of oxalate-degrading bacteria in stool by PCR method andbiochemical teste tended to be reduced as the number of stone formation increased ($p>0.05$). The bacteria isolated were 128 (33%) lactobacillus strain;44% of patients and 46% of normal individual , positive for colonozation lactobacillus intestine . The detection rate oxalate-degradinglactobacillus in patients with urolithiasis was significantly lower than that in healthy volunteers ($p>0.05$). The result by PCR showed that significant difference between high –level of urinary oxalate and lack of oxalate-degrading lactobacillus colonization human digestive tract.the average urinary oxalate level was 0.64 mmol/day in patients tested positive($p>0.05$).Table 1,2

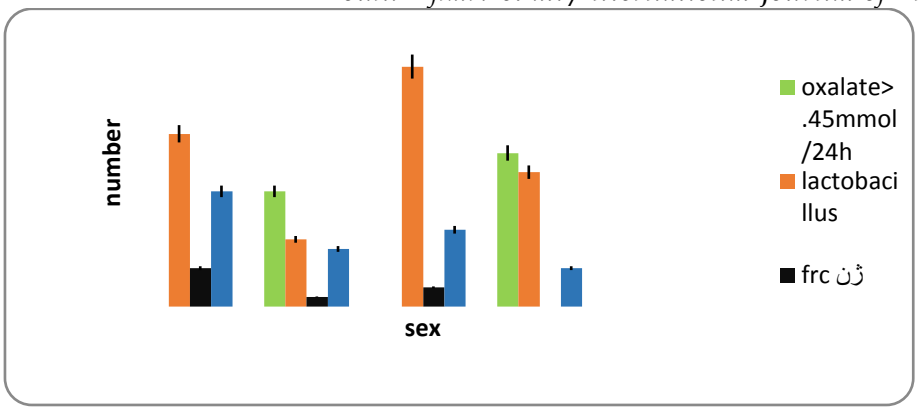
5. Degrad of Oxalate by Oxalate-Degrading Lactobacillus:

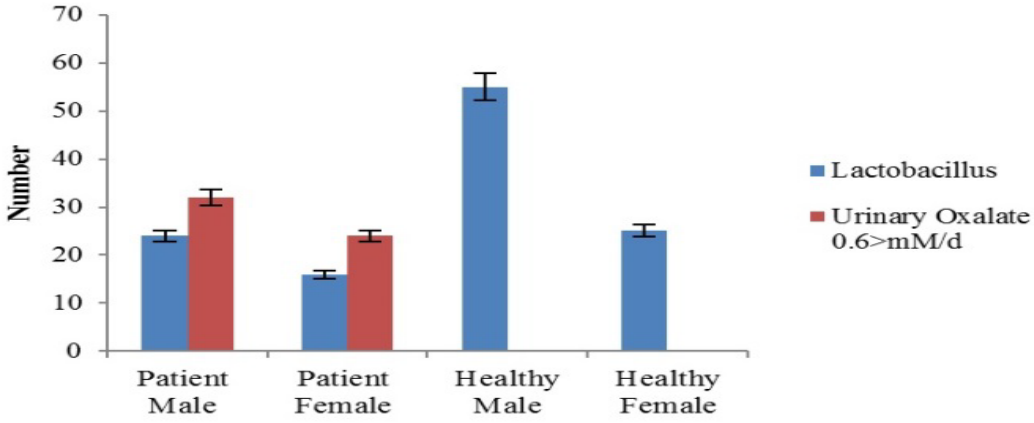
The isolated lactobacillus were cultured in MRS medium with different concentration .5 ,.1,.15 and .2 percentage of ammunium oxalate, then, observed that in concentration of %.5 and .1% , the resulted was 100% and 90% degradation of oxalate, respectively. Although, only in 56.5% of lactobacillus selected could grow and degrad of oxalate in 15% of ammunium oxalate concentration .none no bacteria grow in .2% concentration.

Frequency (%) of urinary abnormalities in controls and patients with kidney stone disease

Urinary abnormality	**Control**	**patients**
	n=100	**n=100**
low urinary volume(<1500ml/24h)	**40**	**60**
hyperoxaluria (>.45 mmol/24 h)	**0**	**56**
hypercalciuria (>7.5 mmol/24 h)	**8**	**72**
hypocitraturia (<1.56 mmol/24 h)	**8**	**68**
hyperuricosuria (>4.46mmol/24 h)	20	**76**

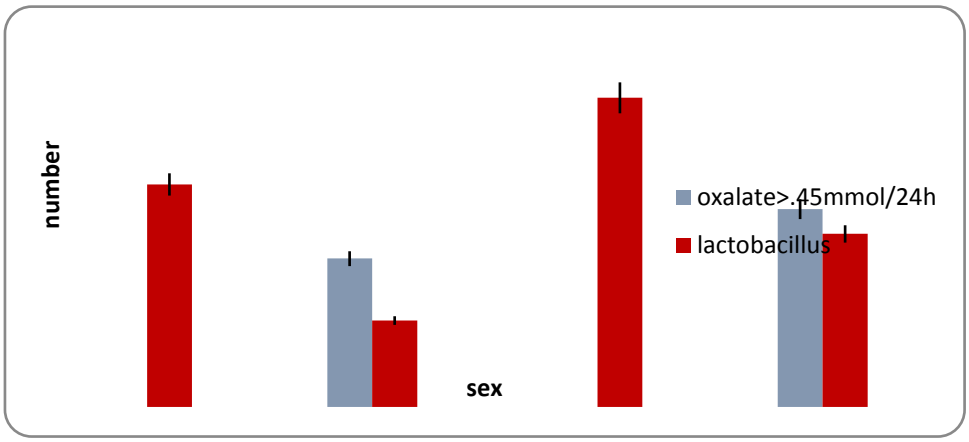
Frequency of lactobacillus and oxalate-degrading genes among people this study

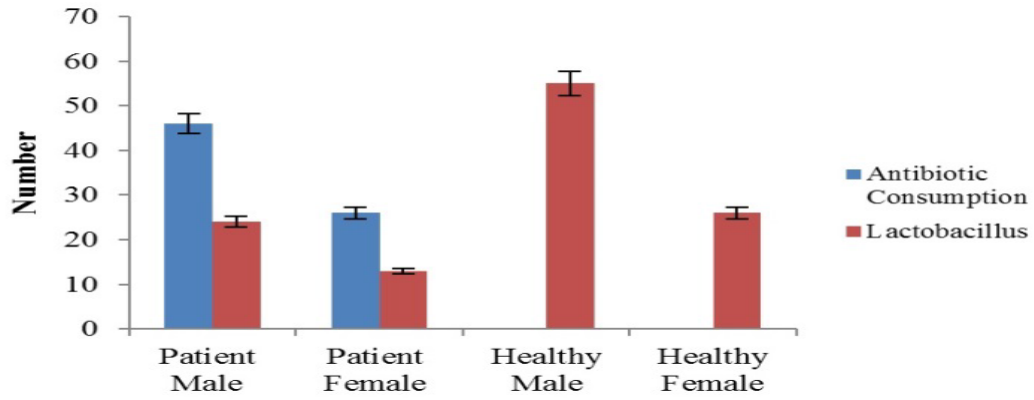




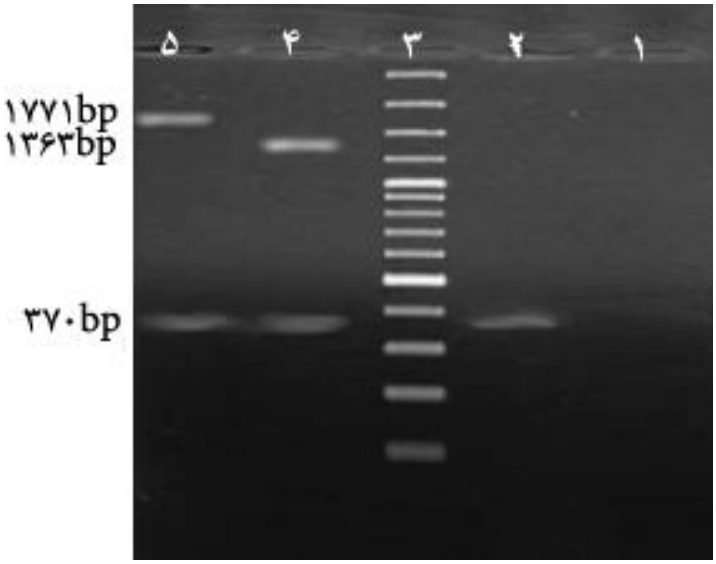
Absolute frequency and the ratio of the studied subjects based on the existence of lactobacillus and increase concentration of urinary oxalate.

Frequency of urinary abnormalities and lactobacillus among individual in this stu.





Absolute frequency and the ratio of the studied subjects based on history of taking antibiotic and lactobacillus isolation



PCR amplification of oxalate-degrading genes. 370 bp related to 16SrRNA, 1363bp and 1771bp related to frc (Line4) , oxc (Line5) respectively.line 1 is negative control and line 2 is positive control, line 3 is 100bp marker

Discussion

Urolithiasis is a common urinary tract problem in Iran where approximately 1 to7 patients have calcium oxalate stones. Hyperoxaluria, the major risk factor in these patients can be due to a high absorption of oxalate from the GI tract [12]. In some countries such as Korea, the prevalence of the disease is 60%, but in Europe the prevalence is between 1 to 5 out of every 100,000 children .Oxalate has been shown to be absorbed all along the GI tract resulting in 10–50% contribution of dietary oxalate to the urinary pool [13]. Hesse et al. [14] have demonstrated a significant increase in the intestinal absorption of oxalate in recurrent calcium oxalate stone formers using a ^{13}C-oxalate absorption test. They also showed a significant correlation between intestinal oxalate absorption and oxalate excretion in 24h urine among the stone formers. Oxalyl-CoA decarboxylase and formyl-CoA transferase are key enzymes involved in the bacterial detoxification ofoxalate within the human intestinal tract. More recent studies have demonstrated the presence of several oxalate-degrading bacteria in human gut and their ability to control oxalate levels by influencing intestinal absorption of dietary oxalate (13). Lactobacilli are common inhabitants of the human gut and are widely included in fermented dairy products as probiotic adjuncts. Recently, Azcarate-Peril et al. (14-17), by using a whole-genome microarray of Lact. acidophilus NCFM, showed the presence of two adjacent genes encoding a formyl-CoA transferase and an oxalyl-CoA decarboxylase that are highly similar to O. formigenes frc and

oxc genes.In the present study, 80% of healthy individuals were observed to be colonized with these lactobacillus whereas in the first and second time stone formers the colonization was in the range of 48%. when compared to normal individuals. So, it is presumed that the absence of oxalate - degrading lactobacillus might have led to the formation of stones. The effect of regular consumption of foods increase oxalate in the urine and the formation of calcium oxalate in calcium oxalate deposits proved. (18)Our results in this study show that calcium oxalate foods, especially spinach, tea, tomatoes significant difference between the patients compared to healthy individuals. A second issue relates to how oxalate-degrading activity may be lost by a significant proportion of adults. The favored explanation is that oxalate-degrading bacteria are susceptible to antibiotics and other therapeutic drugs (15-18). Here, clarithromycin and doxycycline were particularly inhibitory; the effective inhibitory concentration of ca. 1 µg ml-1 would presumably be exceeded in the gut of individuals receiving standard therapeutic doses of ≥300 mg. Doxycycline is used to combat respiratory tract and urinary infections. Moreover, some studies demonstrate that increased consumption of calcium rich foods or excitement of parathyroid hormone secretion can also lead to 35 to 65% increased excretion of urinary calcium and formation of renal calcium oxalate stones [17-19]. Our results from this study show that consumption of oxalate and calcium rich food and increased level of parathyroid hormone secretion are of the major causes of increased excretion of urinary oxalate and calcium and formation of calcium-oxalate stones.

Allison et al., for the first time suggested the role of oxalate-degrading bacteria in ruminant digestive system [20-22]. Studies by Hoppe et al. showed that digestive system had the greatest role in oxalate-degradation. In addition, they demonstrate that the role of gastrointestinal oxalate-degrading bacteria in reducing the risk of kidney stone was greater than that of diet [23]. Studies conducted by Campiri et al., Lieske et al., and Azcarate-Peril et al., on the role of gastrointestinal bacteria and their impact on urinary oxalate-degradation reveal that probiotic bacteria, especially lactobacillus ones, had high oxalate-degrading potential in the environment containing 5-10 mM ammonium oxalate [18, 19, 24]. The underlying reason was the existence of oxalyl-CoA decarboxylase and formyl-CoA transferase as the main causes of oxalate degradation in the mentioned bacteria [17, 25].

We, for the first time in Iran, evaluated the role of oxalate-degrading lactobacillus in reduction of kidney stone disease. The study surveyed the subject by researching one group of healthy people and one group of people with kidney stone. Similar to the mentioned studies, our findings indicate the effective role of lactobacillus bacteria in reducing the incidence of kidney stone problem. In addition, our findings demonstrate that most of the lactobacillus

strains could degrade 4-8 mM ammonium oxalate. This is in consistent with Azcarte-Peril study [26-24]. Lefaucheur et al. in study of the patients, undergoing heart and liver transplant, observed that most of them would encounter with acute renal failures accompanied by acute oxalonephropathy, after a while [27].They showed that long-term use of antibiotics such as cephalosporins, cotrimoxazole, and quinolones by the mentioned patients would cause decreased settlement of intestinal lactobacillus, leading to acute renal failures. Our results also demonstrated a direct correlation between the history of consuming antibiotics, lack of oxalate-degrading lactobacillus settlement, increased level of urinary oxalate, and frequency of stone formation in the patients under investigation. Therefore, it was determined that lack of gastrointestinal lactobacillus was one of the main causes of increased level of urinary oxalate and formation of renal stones in the population under investigation. Since gastrointestinal lactobacillus bacteria are of the most important gastrointestinal probiotic bacteria, further investigation on them can positively influence people health [28].

Regarding the significant correlation between oxalate-degrading bacteria and decreased incidence of kidney stone in the people under investigation, conducting broader studies on different ethnic groups in other parts of the country is recommended. In addition, informing people who are at more risk can have an important role in correcting their diet and kidney stone prevention. However, the main question is that "whether other gastrointestinal bacteria can degrade oxalate and decrease renal complications caused by it?" For a detailed answer to this question, researching broader populations and investigating the role of other bacteria, especially oxalobacter and other probiotic bacteria, are needed.

The results showed that diet, serum parameters especially parathyroid hormone, urinary factors, gastrointestinal oxalate-degrading bacteria, and antibiotic consumption history are the most important causes of kidney stone formation in the population under investigation. Regarding the probiotic role of lactobacillus, molecular monitoring of the strains that produce genes affecting oxalate degradation and evaluation of their probiotic adequacy in future studies are recommended.

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