Published in final edited form as:

Urolithiasis. 2015 April ; 43(2): 107–117. doi:10.1007/s00240-014-0728-2.

***Bifidobacterium animalis* subsp. *lactis* decreases urinary oxalate excretion in a mouse model of primary hyperoxaluria**

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Abstract

Hyperoxaluria significantly increases the risk of calcium oxalate kidney stone formation. Since several bacteria have been shown to metabolize oxalate in vitro, including probiotic bifidobacteria, we focused on the efficiency and possible mechanisms by which bifidobacteria can influence oxalate handling in vivo, especially in the intestines, and compared these results with the reported effects of *Oxalobacter formigenes*. *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and *B. adolescentis* ATCC 15703 were administered to wild-type (WT) mice and to mice deficient in the hepatic enzyme alanine-glyoxylate aminotransferase (*Agxt*^{-/-}, a mouse model of Primary Hyperoxaluria) that were fed an oxalate-supplemented diet. The administration of *B. animalis* subsp. *lactis* led to a significant decrease in urinary oxalate excretion in WT and *Agxt*^{-/-} mice when compared to treatment with *B. adolescentis*. Detection of *B. animalis* subsp. *lactis* in feces revealed that 3 weeks after oral gavage with the bacteria 64 % of WT mice, but only 37 % of *Agxt*^{-/-} mice were colonized. Examining intestinal oxalate fluxes showed there were no significant changes to net oxalate secretion in colonized animals and were therefore not associated with the changes in urinary oxalate excretion. These results indicate that colonization with *B. animalis* subsp. *lactis* decreased urinary oxalate excretion by degrading dietary oxalate thus limiting its absorption across the intestine but it did not promote enteric oxalate excretion as reported for *O. formigenes*. Preventive or therapeutic administration of *B. animalis* subsp. *lactis* appears to have some potential to beneficially influence dietary hyperoxaluria in mice.

Keywords

Probiotics; *Oxalobacter formigenes*; Hyperoxaluria; Calcium oxalate; Kidney stones; Intestinal transport

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Conflict of interest The authors declare that they have no conflict of interest.

Introduction

Systemic levels of oxalate are derived from two sources, endogenously through metabolic production by the liver and by absorption across the gastrointestinal tract from dietary sources. Since mammals lack the enzymes necessary for its utilization, oxalate is a waste product that must be excreted, occurring primarily in the urine [1]. An imbalance in oxalate production/absorption and its excretion can lead to hyperoxaluria, a major risk factor for calcium oxalate kidney stone formation which affects approximately 1 in 11 of the population in the US [2, 3]. Primary hyperoxaluria type 1 (PH 1) is a rare genetic disease caused by a deficiency of the liver enzyme alanine-glyoxylate aminotransferase (AGT) and is associated with increased endogenous oxalate production and serious complications from oxalate deposition in various organs, particularly the kidney resulting in progressive renal failure. Presently, there is no effective pharmacological treatment for PH 1 and the only available cure is a combined liver–kidney transplant [4].

The gut microbiota has emerged as an important factor influencing oxalate handling in the intestine [5–7] and *Oxalobacter formigenes*, a specialist oxalate degrader [8], is among the most widely studied in this regard [9–14]. In addition to consuming luminal oxalate, thus limiting its absorption across the intestine, rodent studies have shown that *O. formigenes* colonization also stimulates net oxalate secretion by the intestinal epithelium itself, thereby actively reducing the overall systemic oxalate burden [10–12]. Consequently, there is considerable interest in the therapeutic potential of *O. formigenes* and other probiotics for the benefit of patients with PH 1 [13]. Whether the ability to promote intestinal oxalate secretion is a strategy unique to *O. formigenes* or shared by other commensal or probiotic bacteria is not yet known. Interestingly, a study comparing gut microbiota composition between black and white South Africans showed higher diversity of lactobacilli and bifidobacteria in black South Africans with overall lower colonization with *O. formigenes* [5]. Moreover, prior studies showed that although black South Africans diet contained much more oxalate and their urinary oxalate levels were significantly higher, the incidence of calcium oxalate kidney stone disease was significantly lower than in white South Africans [15, 16]. Thus, these studies indirectly suggest that colonization with lactobacilli and bifidobacteria may be also important for intestinal oxalate handling. Indeed, further studies have shown that several strains of bacteria, including lactobacilli and bifidobacteria, harbor genes encoding the enzymes necessary for oxalate utilization [17–19], metabolize oxalate in vitro [17, 19, 20], and reduce urinary oxalate excretion and calcium oxalate stone formation in animal models of hyperoxaluria [20, 21].

The administration of lactobacilli and bifidobacteria in dairy products or as nutritional supplements has revealed various beneficial effects on human health. Although these bacteria are mainly studied from an immunological perspective, they can also effectively modulate intestinal solute transport, e.g., reducing chloride secretion and enhancing electrolyte absorption in epithelial cells treated with *Bifidobacterium breve* C50 [22] and *Lactobacillus acidophilus* ATCC 4357 [23], respectively. In addition to *O. formigenes*, some lactobacilli and bifido-bacteria represent potential candidates for use in the prevention and treatment of hyperoxaluria. However, modern therapeutic approaches do not currently emphasize administration of oxalate-metabolizing bacteria either as a common part of

supportive therapy or in the treatment of hyperoxaluria [24]. This may be due to inconsistent results from recent clinical trials [25–32]. One of the first studies provided very promising results where urinary and fecal oxalate levels were reduced in patients with idiopathic hyperoxaluria after treatment with a mixture of three *Lactobacillus* strains (*L. acidophilus*, *L. plantarum* and *L. brevis*), together with *B. infantis*, and *Streptococcus thermophilus* [25]. Despite this, a subsequent study reported no effect on urinary oxalate levels in healthy volunteers treated with a similar probiotic preparation [26]. However, variability in the probiotic strains or mixtures of strains administered and differences in the diets and treatment schedules of patients and volunteers make comparison between clinical trials difficult. These studies also highlight that, unlike *O. formigenes*, not all lactobacilli and bifidobacteria metabolize oxalate and, although some strains may efficiently degrade oxalate in vitro, this does not necessarily translate in vivo. Furthermore, we do not know whether the ability to directly promote oxalate secretion by the intestine is a trait unique to *O. formigenes* or shared by other commensal bacteria. Therefore, a thorough evaluation of the efficacy and underlying mechanisms of the effects of these oxalate-degrading probiotic strains is necessary.

In the present study, we focused on two strains of bifidobacteria, *B. animalis* subsp. *lactis* DSM 10140, known to degrade oxalate in vitro [17] and, as a negative control, *B. adolescentis* ATCC 15703, which does not metabolize oxalate [18]. Despite the demonstrated oxalate-metabolizing capability of *B. animalis* subsp. *lactis*, its effect on urinary oxalate excretion and on oxalate transport across different intestinal segments has not been determined in vivo. We therefore studied the effects of colonization with either *B. animalis* subsp. *lactis* or *B. adolescentis* on urinary oxalate excretion and intestinal oxalate handling in wild-type mice and *Agxt*^{-/-} mice, a mouse model of PH 1 [33].

Materials and methods

Cultivation of bacteria

Bifidobacterium animalis subsp. *lactis* DSM 10140 and *B. adolescentis* ATCC 15703 were obtained from BCCM™/LMG Bacteria Collection at Gent University, Gent, Belgium. The bacteria were grown anaerobically at 37 °C in Medium 58 (Bifidobacterium medium, Deutsche Sammlung von Mikroorganismen und Zellkulturen) containing (in gram per liter) casein peptone—tryptic digest, 10; meat extract, 5; soytone, 5; yeast extract, 5; glucose, 10; NaCl, 5.08; K₂HPO₄, 2.04; KH₂PO₄, 0.04; NaHCO₃, 0.4; MgSO₄·7H₂O, 0.22; MnSO₄·H₂O, 0.05; CaCl₂·2H₂O, 0.01; cysteine HCl·H₂O, 0.5; resazurin, 0.25; and Tween 80, 1 ml. To determine the oxalate-degrading capacity of the bacteria, the media for pure culture growth were supplemented with sodium oxalate to final concentrations of 5, 10 and 20 mM. One week following inoculation, residual oxalate levels in the media were measured by colorimetric assay (Trinity Biotech, St. Louis, MO). Bacteria for the gavage procedure were cultivated overnight in media containing 10 mM sodium oxalate.

Animals and colonization study

All animal studies were approved by University of Florida Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health

Guide for the Care and Use of Laboratory Animals. Wild-type (WT) C57BL/6 male mice (25–30 g) were obtained from Charles River Laboratories (Wilmington, MA) or similar mice reared locally were used. A colony of *Agxt*^{-/-} mice on a C57BL/6 background bred in the local animal facility for several generations was used in experiments addressing PH 1 [12]. All animals had free access to water and standard mouse chow (1 % calcium; diet 2018S, Harlan Teklad).

The colonization study schedule is described in Table 1 and is based on an experimental design that was previously adopted by Hatch et al. [12]. Since oxalate is an essential growth substrate for *O. formigenes*, and regular mouse chow contains little or no oxalate, mice were primed by administration of the diet supplemented with oxalate prior to oral gavage to optimize the colonization procedure. Our present study employed the same dietary regimen, so we could fully determine the efficacy whereby intestinal colonization with bifidobacteria was able to impact urinary oxalate excretion and compare it with reported effects of *O. formigenes*. Two baseline urine collections were carried out, the First (BL1) after feeding regular chow containing 1 % calcium, and the second (BL2) following an oxalate challenge with a specialized oxalate-supplemented chow (0.5 % calcium; diet 89222, Harlan Teklad) containing 1.5 % potassium oxalate. Mice were gavaged with 0.5 ml media containing about 45 mg wet weight of the overnight bacterial culture on day 15 and 17. Subsequently, two postgavage urine collections (PG) were made, the First (PG1) on oxalate-supplemented diet and the second (PG2) after the mice were switched back to standard chow. Mice were euthanized by 100 % CO₂ inhalation 22–35 days from the beginning of the study. Whole blood was collected by cardiac puncture and plasma creatinine was determined by colorimetric assay (modification of Jaffé reaction [34]). Tissues from the terminal ileum, caecum and distal colon were used for studies of intestinal oxalate fluxes described below.

Urine collection and analyses

Mice were housed individually in metabolic cages and urine was collected over 24 h into containers prefilled with 75 µl of hydrated mineral oil and 20 µl 2 % sodium azide. The total volume of urine was measured and all samples were subsequently acidified with 3 N HCl to pH 1. Colorimetric assays were used for determination of urinary oxalate (Trinity Biotech), calcium (Pointe Scientific, Canton, MI), and creatinine (modification of Jaffé reaction).

DNA extraction and quantitative polymerase chain reaction (qPCR)

The colonization status of mice with bifidobacteria was monitored by PCR on weekly basis (i.e., BL1, BL2, PG1, PG2, and PG3 collections). Fresh fecal pellets (10–220 mg) were collected and stored at –80 °C. Total DNA was extracted using QIAamp DNA stool mini kit (Qiagen, Valencia, CA) with minor modifications to ensure lysis of the difficult-to-lyse bacteria. To achieve this, samples were preincubated in lysis buffer (20 mM Tris–Cl; 2 mM sodium EDTA, pH 8; 1.2 % Triton® X-100) containing lysozyme (20 mg/ml; Fisher Scientific) for 60 min at 37 °C. Subsequent purification was carried out according to manufacturer's protocol. The concentration and purity of extracted DNA was assessed by optical density measurement at 260 and 280 nm using Synergy HT-1 spectrophotometer (BioTek Instruments, Winooski, VT).

Specific bacterial standards for qPCR were prepared and the number of gene copies was calculated as described by Junick et al. [35]. Aliquots of standards were stored at -80°C until analysis and fresh serial 20-fold dilutions were used to generate standard curves for qPCR.

The qPCR reaction mixture contained SYBR Green PCR Master Mix 2 $\times$ (Life Technologies, Carlsbad, CA), forward and reverse primers (Table 2), and 50 ng of sample DNA. The initial cycle consisted of 10 min of DNA polymerase activation/sample denaturation at 94°C , 1 min of annealing (see the specific temperatures in Table 2), and 1 min of elongation at 72°C . After this step, samples were subjected to 45 cycles at 94°C for 30 s, strain-specific annealing temperature for 30 s, and 72°C for 30 s, and the final extension step at 72°C for 10 min. Product specificity and size were determined by melting curve analysis and electrophoresis in 2 % agarose gel, respectively. All qPCR reactions were performed with a DNA Engine Opticon® 2 System (Bio-Rad, Hercules, CA).

Intestinal oxalate transport

Oxalate flux measurements have been described in detail previously [36]. Briefly, immediately following exsanguination, the entire large intestine and distal portion of the ileum were removed and rinsed in ice-cold buffered saline. Tissue pairs from each segment were mounted as a fat sheet into a cassette covering 0.3 cm^2 aperture, which was then placed in a modified Ussing chamber (Physiologic Instruments, San Diego, CA). Unidirectional mucosa-to-serosa ($J_{\text{ms}}^{\text{Ox}}$, absorptive) and serosa-to-mucosa ($J_{\text{sm}}^{\text{Ox}}$, secretory) fluxes of oxalate were measured using $0.2\text{ }\mu\text{Ci }^{14}\text{C}$ -oxalate (Amersham, Piscataway, NJ) to determine the magnitude and direction of net oxalate flux ($J_{\text{net}}^{\text{Ox}} = J_{\text{ms}}^{\text{Ox}} - J_{\text{sm}}^{\text{Ox}}$). Open-circuit potential (mV) and short-circuit current ($\mu\text{A}/\text{cm}^2$) were recorded at 15-min intervals during flux experiments and used to calculate transepithelial conductance (mS/cm^2) based on Ohm's Law.

Statistical analyses

One-way analysis of variance followed by Tukey's multiple comparison tests was used to compare multiple experimental groups. Differences between two individual groups were evaluated by an unpaired t test. Data normality was verified by D'Agostino and Pearson omnibus normality test, and for data failing to meet the assumptions of approximate normality and equality of variance, the equivalent non-parametric tests were performed. Data are presented as the mean $\pm$ standard error (SEM) or as percentages of difference between measured parameters. The data were considered statistically significant at $p < 0.05$. The GraphPad Prism statistical software (version 5.03, GraphPad Software, Inc., La Jolla, CA) was used for the analyses.

Results

Bifidobacterium animalis subs. lactis degrades oxalate in vitro

Of the two strains of bifidobacteria chosen for this study, only *B. animalis* subsp. *lactis* possesses the genes encoding enzymes permitting oxalate utilization, whereas *B. adolescentis* does not. After 1 week of cultivation at 37°C , *B. animalis* subsp. *lactis* had

degraded virtually all of the oxalate present in the test media compared to *B. adolescentis* (Fig. 1). These robust differences in the in vitro oxalate-degrading capabilities confirmed previous studies [17, 18] and validated use of the bifidobacteria in the subsequent in vivo studies.

Colonization of WT and *Agxt*^{-/-} mice with *B. animalis* subsp. *lactis* decreased urinary oxalate levels

Since colonization of mice with *O. formigenes* has shown to significantly decrease urinary oxalate excretion [10, 12], we tested the ability of bifidobacteria to alter urinary oxalate levels in WT and hyperoxaluric *Agxt*^{-/-} mice. Baseline urinary oxalate excretion was significantly increased when the mice were switched to an oxalate-supplemented diet (Table 3). Five days after the second gavage (PG1) with *B. animalis* subsp. *lactis*, there was significant 44 and 33 % reduction in urinary oxalate excretion for WT and *Agxt*^{-/-} mice, respectively, resulting in levels close to BL1. In contrast, administration of *B. adolescentis* had no significant effect on urinary oxalate excretion. Unlike WT mice, urinary oxalate excretion in *B. animalis* subsp. *lactis*-colonized *Agxt*^{-/-} mice at PG2 was not significantly different from BL2 ($p = 0.7$), but was comparable in magnitude to BL1. This was in contrast to *B. adolescentis*-colonized *Agxt*^{-/-} mice at PG2 where urinary oxalate excretion remained higher in the absence of an oxalate-supplemented diet. Neither bifidobacteria influenced 24 h urinary creatinine excretion in WT and *Agxt*^{-/-} mice as there were no significant differences before or after gavage as well as between bacterial strains (Table 3), except for *Agxt*^{-/-} mice in which urinary creatinine excretion after gavage with *B. adolescentis* was ~40 % higher, despite no change in urine volume. Nevertheless, by normalizing urinary oxalate excretion to creatinine, generating the oxalate-to-creatinine (Ox:Cr) ratios, we found changes comparable to those observed in urinary oxalate excretion in WT and *Agxt*^{-/-} mice following dietary oxalate supplementation and gavage with bifidobacteria (Figs. 2, 3). Moreover, compared to corresponding values in WT mice, both BL1 and BL2 Ox:Cr ratios were significantly higher in *Agxt*^{-/-} mice ($p = 0.002$ and $p = 0.009$, respectively), which is in accordance with previously published data [12].

Urinary calcium excretion decreased significantly by ~50 % in all animals when fed with an oxalate-supplemented diet with 0.5 % calcium content compared to 1 % calcium in the regular chow. No changes in plasma creatinine among control, *B. animalis* subsp. *lactis*, and *B. adolescentis* groups were observed in WT or *Agxt*^{-/-} mice at the time of euthanasia (Table 3). Experimental data were collected from two to three independent experiments with at least 6 mice per group.

Colonization with bifidobacteria was transient

Variability in the persistence of colonization with *O. formigenes* in WT and *Agxt*^{-/-} mice has been reported previously [12]. Prior to gavage, no animal was found to be colonized with either *B. animalis* subsp. *lactis* or *B. adolescentis*, as determined by PCR. Both WT and *Agxt*^{-/-} mice were successfully colonized by two doses of bifidobacteria cultures and PCR confirmed the presence of administered bacteria in fecal samples collected in the weeks following oral gavage (Fig. 4a). All WT mice gavaged with *B. adolescentis* remained colonized for at least 2 weeks but this fell dramatically by 75 % during the third week.

Colonization with *B. animalis* subsp. *lactis* decreased from 100 % in the first week to 64 and 43 % in the second and third week, respectively. Colonization of *Agxt*^{-/-} mice showed similar pattern but loss of colonization of both bacteria was much quicker. Although all *Agxt*^{-/-} mice were colonized in the first week after gavage, 1 week later, only 50 and 38 % of mice were colonized with *B. adolescentis* and *B. animalis* subsp. *lactis*, respectively. Three weeks after gavage, the number of colonized *Agxt*^{-/-} mice dropped further to just 25 % for both strains of bacteria.

To investigate the extent of colonization in more detail, we used qPCR to compare the *B. animalis* subsp. *lactis* counts in fecal samples from WT and *Agxt*^{-/-} mice (Fig. 4b). Although both strains of mice were colonized with comparable amounts of bacteria 5 days after gavage (PG1), 1 week later (PG2) only WT mice remained colonized with numbers that were detectable by qPCR, whereas counts of *B. animalis* subsp. *lactis* in *Agxt*^{-/-} mice were below the detection limit for quantification.

Influence of bifidobacteria on transepithelial oxalate fluxes across the intestine

Earlier studies with *O. formigenes* had shown that this bacterium promoted oxalate secretion into gut lumen by intestinal epithelial cells and thus greatly influenced urinary oxalate excretion in both WT and *Agxt*^{-/-} mice [10, 12]. To test whether this capability is unique to *O. formigenes* or perhaps a characteristic shared by other bacteria, we measured transepithelial fluxes of ¹⁴C-oxalate across paired tissues from distal ileum, caecum and distal colon of WT and *Agxt*^{-/-} mice colonized with bifidobacteria (Figs. 5, 6). All flux studies were carried out 1–2 weeks after gavage, when the bacteria were still detectable in the intestinal contents. Colonization with *B. animalis* subsp. *lactis* or *B. adolescentis* did not lead to significant changes in the net transepithelial flux of oxalate. While there were some changes observed in the unidirectional fluxes for some tissues that reached statistical significance, these coordinated changes did not result in any significant effect on $J_{\text{net}}^{\text{Ox}}$ in any of the groups. Transepithelial conductance and short-circuit current from all three intestinal segments in WT and *Agxt*^{-/-} mice are summarized in Table 4. In general, no significant differences in net electrogenic transport (I_{sc}) or tissue permeability (G_t) were observed, with the exception of significantly larger short-circuit current in *Agxt*^{-/-} mice colonized with *B. animalis* subsp. *lactis* and lower transepithelial conductance in *Agxt*^{-/-} mice colonized with *B. adolescentis* compared to control mice.

In vivo studies on oxalate handling by probiotic bacteria can help define features important for their potential use in the prevention and treatment of hyperoxaluria, like their efficiency and effect on transepithelial transport within the complex environment of the gut. Based on recent in vitro studies [17, 18], we used two strains of bifidobacteria: *B. animalis* subsp. *lactis*, an oxalate degrader, and the oxalate non-metabolizing *B. adolescentis*, as a negative control. We found that WT as well as *Agxt*^{-/-} mice fed an oxalate-supplemented diet significantly decreased urinary oxalate excretion after gavage with *B. animalis* subsp. *lactis*, whereas after gavage with *B. adolescentis* did not. In addition to degrading luminal oxalate and limiting its absorption across the gut, another mechanism for reducing urinary oxalate levels is promotion of net secretion of oxalate into the intestinal lumen, which has been previously demonstrated in *O. formigenes*-colonized rodents [10–12]. Here, we found that

there was no significant increase in net oxalate secretion into the intestinal lumen in any of three studied segments after colonization with *B. animalis* subsp. *lactis*. On the contrary, colonization with *B. adolescentis* significantly increased the unidirectional secretory component of oxalate flux (J_{sm}^{Ox}) in the caecum and distal colon of WT and in the caecum of *Agxt*^{-/-} mice but this did not translate to a significant increase in net secretion because of a coordinated change in the opposing absorptive flux (J_{ms}^{Ox}) and therefore did not confer any additional benefit in reducing urinary oxalate excretion. Moreover, the difference in urinary oxalate excretion after colonization with *B. animalis* subsp. *lactis* was comparable between WT and *Agxt*^{-/-} mice (BL2-PG1; ~0.71 and ~0.84 $\mu\text{mol}/24\text{ h}$ in WT and *Agxt*^{-/-} mice, respectively) indicating that the in vivo benefit of *B. animalis* subsp. *lactis* was similar for WT and *Agxt*^{-/-} mice. Furthermore, the reduction in urinary oxalate excretion was very similar in magnitude to the increase in urinary oxalate after dietary oxalate supplementation (BL2-BL1; ~0.67 and ~1.00 $\mu\text{mol}/24\text{ h}$ in WT and *Agxt*^{-/-} mice, respectively). Therefore, *B. animalis* subsp. *lactis* appears capable of degrading oxalate from the dietary sources and thus limiting its intestinal absorption but it did not reveal any tendency for modifying intestinal oxalate secretion in WT and *Agxt*^{-/-} mice, in contrast to *O. formigenes* [12]. Thus, these results indicate that *O. formigenes* remains unique in the way that it can influence oxalate secretion by the intestinal mucosa. One possible explanation could be that *O. formigenes* requires oxalate as carbon and energy source, whereas lactobacilli and bifidobacteria are not so limited for substrates using oxalate degradation only as detoxification pathway to survive in hostile environment [37]. Further investigations of *O. formigenes*-mucosa interactions may therefore lead to an understanding of mechanisms underlying the stimulation of the host cells to secrete oxalate.

Probiotic strains of bacteria are often isolated from dairy or fermented foods, or from the intestinal contents of different species, hence they may not necessarily colonize the gastrointestinal tract of mice permanently [38]. The strains we used, *B. adolescentis* and *B. animalis* subsp. *lactis*, were originally isolated from human gut and yogurt, respectively. Indeed, we found that the colonization with both bifidobacteria was transient and that the mice lost the bacteria within 4 weeks after gavage. One reason for this may be ecological competition between the bifidobacteria strains and the resident gut micro-biota. Interestingly, we observed that in the mouse model of PH 1 the loss of colonization with bifidobacteria was much quicker. Similar results were also found previously in *O. formigenes*-colonized *Agxt*^{-/-} mice where *O. formigenes* persisted in the gastrointestinal tract of artificially colonized adult mice and naturally colonized pups only 3 and 4 weeks, respectively, compared to WT mice, which remained colonized up to 10 weeks [12]. Similar to the mouse model, PH 1 patients artificially colonized with *O. formigenes* also displayed transient colonization lasting approximately 2 weeks following administration [13], whereas for healthy individuals colonization was longer term—at least 9 months [9]. The transient colonization in hyperoxaluric individuals may be the result of antibiotic treatment [39, 40], although further studies are warranted to determine what other factors are important for sustaining colonization.

To summarize, decrease or loss of oxalate-degrading capability of the gut microbiota is associated with increased oxalate absorption, the subsequent development of hyperoxaluria

and risk of calcium oxalate kidney stone formation. Our study showed that even transient colonization of the gastrointestinal tract of both WT and *Agxt*^{-/-} mice with *B. animalis* subsp. *lactis* was able to reduce urinary oxalate excretion. This bacterium did not influence the intestinal secretion of oxalate, and the mechanism reducing urinary oxalate was solely degradation of dietary oxalate within the gut lumen thus limiting its absorption. Preventive or therapeutic administration of *B. animalis* subsp. *lactis* may have some potential to beneficially influence dietary hyperoxaluria.

Acknowledgments

The authors thank Tisha Van Pelt and Heran Getachew for excellent technical assistance and Dr. Robert W. Freel for comments on the manuscript. This study was supported by National Institute of Health grant DK 088892 and by The Oxalosis and Hyperoxaluria Foundation.

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Fig. 1. Representative results of the in vitro oxalate-degrading capacity of the bifidobacteria determined by culturing in media containing 20 mM oxalate. *a* The supernatant from *B. animalis* subsp. *lactis* cultures showed significantly lower residual oxalate levels than control (no bacteria) and *B. adolescentis* cultures ($p = 0.0001$)

Fig. 2.

Changes in oxalate-to-creatinine ratio for *B. animalis* subsp. *lactis* and *B. adolescentis* groups of WT mice before (combined results; BL1, $n = 11$ and BL2, $n = 19$) and after gavage with *B. animalis* subsp. *lactis* (PG1, $n = 18$ and PG2, $n = 14$) or *B. adolescentis* (PG1, $n = 14$ and PG2, $n = 8$). *a* Significantly different from BL1 ($p < 0.05$); *b* significantly different from BL2 ($p < 0.05$). *BL* baseline, *Cr* creatinine, *Ox* oxalate, *PG* post-gavage

Fig. 3.

Changes in oxalate-to-creatinine ratio for *Agxt*^{-/-} mice before (combined results; BL1, *n* = 21 and BL2, *n* = 21) and after gavage with *B. adolescentis* (PG1, *n* = 14 and PG2, *n* = 8) and *B. animalis* subsp. *lactis* (PG1, *n* = 13 and PG2, *n* = 7). *a* Significantly different from BL1 (*p* < 0.0001); *b* significantly different from BL2 (*p* < 0.05). *BL* baseline, *Cr* creatinine, *Ox* oxalate, *PG* post-gavage

Fig. 4.

a Samples of feces were collected up to 3 weeks post-gavage (PG) and the presence of bifidobacteria determined by PCR. *Agxt*^{-/-} mice colonized with *B. animalis* subsp. lactis (open squares; *n* = 7) and *B. adolescentis* (open circles; *n* = 8) show more rapid loss of both strains than WT mice colonized with either *B. animalis* subsp. lactis (black squares) or *B. adolescentis* (black circles). **b** Quantitative comparison of *B. animalis* subsp. lactis in the feces collected before (BL, *n* = 7) and after gavage (PG, *n* = 7) from WT and *Agxt*^{-/-} mice. Dashed line determines the reaction limit for quantification

Fig. 5.

Unidirectional serosa-to-mucosa (J_{sm}) and mucosa-to-serosa (J_{ms}) oxalate fluxes across short-circuited distal ileum, caecum and distal colon in control ($n = 13, 15, 19$), *B. animalis* subsp. lactis colonized ($n = 8, 11, 11$) and *B. adolescentis*-colonized ($n = 8, 9, 10$) WT mice. *a* Significantly different from control mice ($p < 0.05$); *b* significantly different from mice colonized with *B. animalis* subsp. lactis ($p < 0.05$). J_{net} , net oxalate flux

Fig. 6.

Transepithelial fluxes of oxalate across distal ileum, caecum and distal colon in control ($n = 9, 11, 12$), *B. animalis* subsp. *lactis*-colonized ($n = 9, 9, 12$) and *B. adolescentis*-colonized ($n = 10, 11, 12$) *Agxt*^{-/-} mice. ^a Significantly different from control mice ($p < 0.05$). J_{ms} , mucosa-to-serosa oxalate flux; J_{sm} , serosa-to-mucosa oxalate flux; J_{net} , net oxalate flux

Schedule of dietary oxalate supplementation, bifidobacteria gavage and urine and/or feces collections during the colonization study

Table 1

Day of the study	1	2	8	15 and 17	22	27	29	36
Diet	No Ox, 1 % Ca	1.5 % Ox, 0.5 % Ca	No Ox, 1 % Ca					
Bifidobacteria gavage	–			2 × 45 mg/mouse	–			
Collection	BL1	–	BL2	–	PG1	–	PG2	PG3

BL baseline, Ca calcium, Ox oxalate, PG post-gavage

Table 2

Specific primers and PCR conditions used in the study

Primer	Sequence (5'-3')	Primer conc. (nM)	Annealing temp. (°C)	Product size (bp)	References
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> DSM 10140					
B_ani-F	CACCAATGCGGAAGACCAG	250	62	184	[35]
B_ani-R	GTTGTTGAGAAATCAGCGTGG				
B_ani-std-F	GTCACCGTCGAAAGACAACAACC	200	66	299	[35]
B_ani-std-R	CAGGACTTGAAGGTGCCACGG				
<i>Bifidobacterium adolescentis</i> ATCC 15703					
B_ado-F	CTCCGCCGCTGATCCGGGAAGTCG	300	70	268	[35]
B_ado-R	AACCAACTCGGCGATGTGGACGACA				
B_ado-std-F	GATCAGATCGCTGCCACCCGAAC	200	70	320	[35]
B_ado-std-R	ATCAGCAGCGGCTTGCCGGTCTT				

Table 3

Urine volume, 24 h urinary creatinine, oxalate, and calcium excretions, and final plasma creatinine and body weights for WT and *Agxt*^{-/-} mice before (combined results) and after colonization with bifidobacteria

Gavage	WT mice			<i>Agxt</i> ^{-/-} mice					
				<i>B. adolescentis</i>			<i>B. animalis</i>		
	BL1	BL2	PG1	PG2	PG1	PG2	PG1	PG2	PG2
Collection									
U volume, mL/24 h	1.13 ± 0.19 (n=11)	1.50 ± 0.15 (n=20)	0.96 ± 0.10 (n=18)	1.57 ± 0.17 (n=8)	1.60 ± 0.21 (n=14)	1.57 ± 0.17 (n=8)	1.15 ± 0.18 (n=13) ^b	1.27 ± 0.18 (n=7)	1.66 ± 0.18 (n=7)
U-Cr, µmol/24 h	3.17 ± 0.35 (n=10)	4.29 ± 0.36 (n=14)	3.16 ± 0.27 (n=14)	4.23 ± 0.19 (n=13)	3.63 ± 0.29 (n=13)	4.35 ± 0.49 (n=8)	3.97 ± 0.43 (n=12)	4.48 ± 0.44 (n=7)	5.48 ± 0.66 (n=8) ^d
U-Ox, µmol/24 h	0.94 ± 0.18 (n=11)	1.61 ± 0.16 (n=19) ^a	0.90 ± 0.10 (n=18) ^b	1.03 ± 0.10 (n=14) ^b	1.35 ± 0.18 (n=14)	1.08 ± 0.16 (n=8)	1.71 ± 0.20 (n=13) ^b	1.87 ± 0.27 (n=7)	2.72 ± 0.51 (n=8) ^d
U-Ca, µmol/24 h	2.13 ± 0.23 (n=11)	2.38 ± 0.25 (n=14)	0.96 ± 0.07 (n=18) ^{a,b}	2.53 ± 0.28 (n=14)	1.80 ± 0.23 (n=14)	2.65 ± 0.26 (n=7)	0.99 ± 0.12 (n=13) ^{a,b}	1.89 ± 0.16 (n=7)	2.63 ± 0.36 (n=8)
P-Cr (µM)	–	–	31.62 ± 2.78 (n=19)	–	28.61 ± 1.65 (n=18)	–	24.91 ± 2.85 (n=14)	29.85 ± 2.77 (n=14)	–
Body weight (g)	–	–	32.71 ± 1.17 (n=14)	–	35.5 ± 0.89 (n=8)	–	36.0 ± 1.89 (n=8)	36.63 ± 2.62 (n=8)	–

Combined results are presented for BL1 and BL2. All values are mean ± SE

BL baseline, Ca calcium, Cr creatinine, Ox oxalate, P plasma, PG post-gavage, U urine

^a Significantly different from BL1 (*p* 0.05)

^b Significantly different from BL2 (*p* 0.05)

Table 4
Trans epithelial conductances and short-circuit currents recorded from intestinal tissues of WT and Agxt^{-/-} control mice and mice colonized with bifidobacteria

Segment	Distal ileum		Caecum		Distal colon	
	I _{sc}	G _t	I _{sc}	G _t	I _{sc}	G _t
WT mice						
Control non-colonized mice	-7.1 ± 0.6 (n = 31)	39.3 ± 1.8 (n = 31)	-0.7 ± 0.2 (n = 32)	19.7 ± 0.8 (n = 31)	-1.5 ± 0.1 (n = 32)	18.5 ± 1.3 (n = 32)
<i>B. animalis</i> colonized mice	-7.9 ± 0.8 (n = 23)	44.9 ± 3.1 (n = 19)	-0.6 ± 0.1 (n = 23)	23.1 ± 2.1 (n = 24)	-1.6 ± 0.1 (n = 24)	21.2 ± 2.1 (n = 24)
<i>B. adolescentis</i> -colonized mice	-9.3 ± 0.8 (n = 20)	44.0 ± 1.5 (n = 19)	-0.3 ± 0.2 (n = 19)	21.8 ± 1.2 (n = 19)	-1.6 ± 0.2 (n = 18)	21.5 ± 1.8 (n = 19)
Agxt ^{-/-} mice						
Control non-colonized mice	-5.9 ± 0.7 (n = 25)	37.5 ± 2.0 (n = 24)	0.3 ± 0.1 (n = 26)	18.5 ± 0.9 (n = 25)	-1.2 ± 0.1 (n = 25)	23.4 ± 1.4 (n = 26)
<i>B. animalis</i> colonized mice	-8.2 ± 0.8 (n = 28)	42.6 ± 2.7 (n = 26)	-0.2 ± 0.1 (n = 27) ^a	22.3 ± 1.5 (n = 26)	-1.8 ± 0.1 (n = 27) ^a	20.4 ± 1.7 (n = 28)
<i>B. adolescentis</i> -colonized mice	-6.0 ± 0.7 (n = 27)	38.5 ± 2.2 (n = 28)	-0.4 ± 0.1 (n = 27) ^a	19.4 ± 1.2 (n = 27)	-1.6 ± 0.2 (n = 27)	18.0 ± 1.4 (n = 28) ^a

Values are mean ± SE

G_t transepithelial conductance (mS/cm²), I_{sc} short-circuit current (μeq/cm² h)

^aData significantly different from non-colonized control (p 0.05)