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ORIGINAL PAPER

Synbiotic supplementation and oxalate homeostasis in rats: focus on microbiota oxalate-degrading activity

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Abstract

The present study aimed (i) to evaluate whether ceftriaxone treatment could affect not only intestinal oxalate-degrading bacteria number but also their total activity to degrade oxalate and influence oxalate homeostasis in rats, (ii) and to estimate the ability of commercially available inulin-contained synbiotic to restore fecal oxalate-degrading activity and ceftriaxone-induced disruption of oxalate homeostasis in rats. Twenty-eight female Wistar rats (200–300 g) were randomly divided into four groups ($n=7$). Group 1 was treated with vehicle sterile water (0.1 ml, i.m., 14 days); Group 2 received synbiotic (30 mg/kg, per os, 14 days); Group 3 was treated with ceftriaxone (300 mg/kg, i.m., 7 days); Group 4 was supplemented with ceftriaxone and synbiotic. Oxalate-degrading bacteria number and their total activity, urinary and plasma oxalate concentrations were measured on days 1 and 57 after the treatment withdrawal. The redoximetric titration with $KMnO_4$ was adopted to evaluate the total oxalate-degrading activity in highly selective Oxalate Medium. Ceftriaxone treatment reduced total fecal oxalate-degrading activity independently on oxalate-degrading bacteria number and increased urinary and plasma oxalate concentrations. The synbiotic had higher oxalate-degrading activity vs probiotics and was able to restore fecal oxalate-degrading activity and significantly decrease urinary oxalate excretion in antibiotic-treated rats. Total fecal oxalate-degrading activity but not oxalate-degrading bacteria number should be thoroughly examined in the future to develop predictive diagnostics methods, targeted prevention and personalized treatment in kidney stone disease. Synbiotic supplementation had a beneficial effect on the total oxalate-degrading activity of gut microbiota, which resulted in decreased UOx excretion in rats.

Keywords Ceftriaxone · Oxalate-degrading bacteria · Oxalate-degrading activity · Urine oxalate excretion · Plasma oxalic acid · Synbiotic · Probiotic

Abbreviations

CaOx	Calcium oxalate
CEF	Ceftriaxone
CEF + SYN	Ceftriaxone plus symbiotic
KSD	Kidney stone disease
ODA	Oxalate-degrading activity
ODB	Oxalate-degrading bacteria
Pox	Plasma oxalic acid
UOx	Urinary oxalate

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SYN Symbiotic
VEH Vehicle

Introduction

There has recently been a trend of rising scientific interest in the field of intestinal, extraintestinal and systemic effects of gut microbiota, including the "gut-kidney axis" in urolithiasis [1–4]. The role of gut microbiota in the development and progression of kidney stone diseases (KSD) has historically focused on the presence of *Oxalobacter formigenes (O. formigenes)*. The deficiency of *O. formigenes* in fecal samples was considered a fundamental risk factor for nephrolithiasis [1, 2]. However, current progress in metagenomic approaches has overturned the *O. formigenes*-based hypothesis and demonstrated that reduction of overall microbial biodiversity poses a lithogenic risk [5, 6]. Moreover, with the increasing number of published data, it has become clear that the *O. formigenes* colonization is almost similar in non-calcium oxalate (CaOx) formers and patients with KSD [2, 7, 8] and, thus, may not be the only risk factor for the CaOx lithiasis formation. The emerging data highlight the ability of not only *O. formigenes* but many other intestine oxalate-degrading bacteria (ODB) (e.g. *Enterococcus spp., Lactobacillus spp., Bifidobacterium spp., Bacillus spp*) to degrade oxalate and stimulate its endogenous secretion [1, 2, 4]. These data have found a clinical application, and a strong association between the low abundance of ODB in the gut microbiota community and hyperoxaluria has been demonstrated confirming the potential role of ODB in the maintenance of oxalate homeostasis [9–11]. However, the majority of the existing studies have largely focused on the quantitative determination of ODB in feces [1, 10–12] but not on their total oxalate-degrading activity (ODA).

The term of total ODA in fecal microbiota could be defined as the general ability of different strains of ODB to metabolize oxalate [13]. Several in vitro studies have been devoted to oxalate degradation abilities of *O. formigenes, Bifidobacterium* and *Lactobacillus* strains in which the efficiency degrade oxalate from 15 to 98% has been reported [14–16]. Therefore, the presence of ODB in the intestine might not mean their sufficient functional capabilities, which was confirmed by multi-omics data (> 3000 samples from > 1000 subjects) showing the dichotomy between metagenomic and metatranscriptomic of the human microbiota ODB [17]. It should be noted, that to the best of our knowledge, there is a general lack of experimental or clinical studies on total ODA in fecal microbiota. There is only the early study where the authors have measured total ODA directly in human fecal samples in anaerobic dilution solution with [14C]-oxalate [18].

It is little wonder that antibiotics would affect gut microbiota and reduce the number of ODB [19, 20]. The evidence of antibiotics' effect on ODB was generally based on quantification before and after antibacterial treatment [19, 20]. It has been demonstrated that the administration of different antibiotic classes reduces ODB in the gut for a long-time period after antibiotic exposure [19–21]. However, the effect of antibiotics on total ODA in fecal microbiota has never been evaluated before. Consequently, it is still unclear whether the use of antibiotics could affect not only the number of ODB but their ODA and thus influence the blood and urine oxalate concentrations.

The overwhelming majority of the published experimental and in vitro studies have demonstrated a promising effect of probiotics on increasing ODB number and reducing urinary oxalate (UOx) excretion [17, 22, 23]. However, systematic reviews of open-label and randomized placebo studies suggested that currently available *O. formigenes* formulation, *Lactobacillus* or other probiotic products and/or regimens of their administration have limitations related to insignificant or temporal efficiency to reduce UOx excretion [23, 24]. In addition, little is known about the synbiotic effect on ODB and their ability to degrade oxalate. To the best of our knowledge, only two recent studies have been focused on the new avenue for reduction of the UOx excretion by using the combination of probiotic and prebiotic (synbiotic) in which the authors found synbiotic more efficient than probiotics and prebiotics alone [25, 26]. By in vitro study, the authors confirmed that the addition of inulin-based prebiotic increased ODA of *Lactobacillus spp* probiotic [25]. In this context, we hypothesized that the use of probiotic bacteria (e.g. *Lactobacillus spp*.) in prebiotic inulin-contained media might be effective to overcome antibiotic-induced disturbance of oxalate homeostasis.

Therefore, the present study aimed (i) to evaluate whether ceftriaxone treatment could affect not only intestinal ODB number but also their total ODA and influence oxalate homeostasis in rats, (ii) and to estimate the ability of commercially available inulin-contained synbiotic to restore ODA and ceftriaxone-induced disruption of oxalate homeostasis in rats.

Materials and methods

Animals

Twenty-eight female Wistar rats (200–300 g) were bred and housed in the conventional animal facility of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Kyiv, Ukraine) under standard environmental conditions (12-h light/dark cycle at a constant temperature of 22 °C). All the rats were kept in a standard

cage and had free access to a standard stock diet and tap water provided ad libitum. To ensure standardized gut microbiota, rats from all groups were kept in the same room and maintained by the same person. The "Guide for the Care and Use of Laboratory Animals" (National Research Council 2011) was followed. The procedures used and the care of animals were approved by the animal committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Protocol # 18/01/2020).

Sample size

Sample size consideration was based on previous reports on a similar topic [26, 27] and calculated using G*Power Software, version 3.1.9.4. The effect sizes for means obtained in these studies varied from 0.93 to 2.56 with sample sizes ranging between 6 and 8 animals in each group. Therefore, we expected that a sample size of 7 animals in each group would be sufficient to achieve a power of 0.80 and an alpha of 0.05 detecting differences between the groups using the same data variability in non-parametric tests. Similarly, we would need a minimum sample size of 25 rats to achieve a power of 0.80 and an alpha of 0.05 in the correlation analysis.

Study design

Animals were numbered and randomly divided into four groups of seven rats each: Group 1 received sterile water (Vehicle group), Group 2 was supplied with synbiotic (Synbiotic group), Group 3 was intramuscularly injected with ceftriaxone (Ceftriaxone group) and Group 4 was given ceftriaxone and synbiotic simultaneously (Ceftriaxone + Synbiotic group) (Table 1). Bodyweight and lethargy were controlled throughout the experimental period.

On day 1 after treatment withdrawal, the amount of ODB and their total ODA in fecal microbiota, as well as urinary oxalate (UOx) and creatinine excretion were measured in each group of rats. Then, we repeated the same tests in 8 weeks (day 57) after treatment withdrawal.

On day 57 of the experimental period, all rats were anesthetized with urethane (1.1 g/kg, i.p. Sigma-Aldrich, Germany). Blood samples were collected by cardiac puncture immediately after death, and in addition to the aforementioned markers in the rats' feces and urine, plasma oxalic acid (POx) was determined. The experiment design and outline are depicted in Fig. 1.

Experimental substances

Antibiotic

The rat equivalent dose of ceftriaxone (Ind. Stock Company Darnytsya, Ukraine) was calculated based on body surface area by multiplying the human dose (50 mg/kg) by the K_m value (6 for rats) [28].

Table 1 Experimental groups and interventions

Groups ($n = 28$)	Interventions
Vehicle (VEH, $n = 7$)	Sterile water 0.1 ml, intramuscularly for 14 days
Synbiotic (SYN, $n = 7$)	Synbiotic 30 mg/kg, *per os* for 14 days
Ceftriaxone (CEF, $n = 7$)	Ceftriaxone 300 mg/kg, intramuscularly for 7 days
Ceftriaxone + Synbiotic (CEF + SYN, $n = 7$)	Ceftriaxone (300 mg/kg, intramuscularly for 7 days) and synbiotic (30 mg/kg, *per os* for 14 days) simultaneously

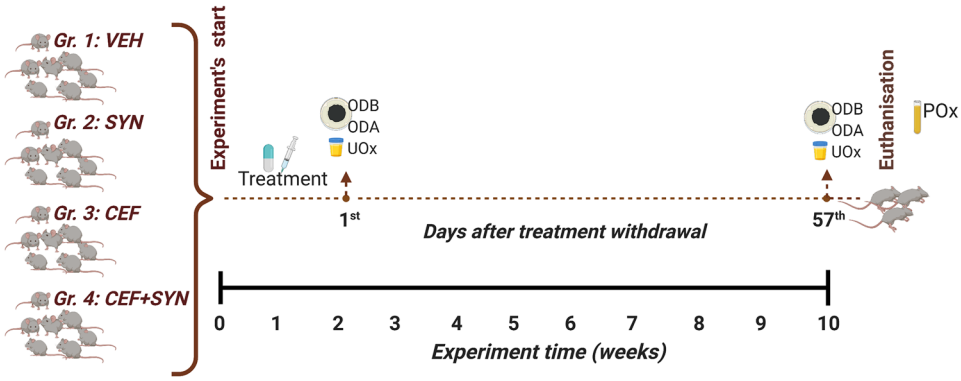

Fig. 1 Experiment design and outline (created with BioRender.com). *CEF* ceftriaxone, *CEF + SYN* ceftriaxone plus symbiotic, *Gr* group, *ODA* oxalate-degrading activity, *ODB* oxalate-degrading bacteria, *POx* plasma oxalic acid, *SYN* symbiotic, *UOx* urinary oxalate, *VEH* vehicle

Synbiotic

The synbiotic was manufactured by LLC "Element of Health", Ukraine, and consisted of 4 strains of *Lactobacillus spp. (L. acidophilus* 5×10^8 CFU/g*, L. rhamnosus* 9×10^8 CFU/g*, L. plantarum* 2×10^7 CFU/g*, L. casei* 4×10^8 CFU/g*),* 2 strains of *Bifidobacterium spp. (B. bifidum* 5×10^8 CFU/g and *B. longum* 8×10^8 CFU/g*), Saccharomyces boulardii* 3×10^7 CFU/g*,* selenium 0.05 mg, oligofructose 40 mg and inulin 450 mg.

Processing of fecal specimens for ODB and total ODA analysis

Rats' fecal samples (1 g) were collected and immediately dispersed in 9 ml highly selective media Oxalate Medium (g/L): K_2HPO_4 – 0.25, KH_2PO_4 – 0.25, $(NH_4)_2SO_4$ – 0.5, $MgSO_4{\cdot}7H_2O$ – 0.025, CH_3COON – 0.82, yest extract – 1.0, rezazurin – 0,001, Na_2CO_3 – 4, L-cystein-HCl – 0.5, $Na_2C_2O_4$ – 5.0, Trace element solution SL-10 – 1 ml (mix/L: HCl (25%; 7.7 M) – 10.00 ml, $FeCl_2\times4H_2O$ – 1.50 g, $ZnCl_2$ – 70.0 mg, $MnCl_2\times4H_2O$ – 100.0 mg, H_3BO_3 – 6.0 mg, $CoCl_2\times6H_2O$ – 190.0 mg, $CuCl_2\times2H_2O$ – 2.0 mg, $NiCl_2\times6H_2O$ – 24.0 mg, $Na_2MoO_4\times2H_2O$ – 36.0 mg) [29]. Oxalate Medium has 5.0 g/L $Na_2C_2O_4$ as a single energy source and favors the growth of oxalate-degrading bacteria.

For determination of ODB number, serial dilutions (10^{-3}, 10^{-5}, 10^{-7}) of each dispersed sample were prepared. One aliquot of 0.1 ml of each dilution was seeded by a pure plate technique and cultured anaerobically (BD BBL™ $C0_2$ gas generators, BioMerieux, France) at 37 °C for 5 days on a solid sterile Oxalate Medium. Finally, we determined the quantitative composition of microorganisms, which grew as single colonies. The number of fecal ODB was calculated as lg of colony-forming unit per 1 g of feces (lg CFU/g).

For determination of the ODA in feces, we transferred 5 ml of dispersed feces samples to 45 ml Oxalate Medium and anaerobically incubated for 48 h at 37 °C in tightly closed 50 ml bickers (test solution).

For determination of the synbiotic ODA rate, the one dose of synbiotic was diluted in 20 ml of Oxalate Medium and anaerobically incubated for 48 h at 37 °C in tightly closed 50 ml bickers (test solution).

Determination of ODA

The redoximetric titration with $KMnO_4$ was adopted to evaluate the total ODA of bacteria in culture media, as described previously [13, 29]. In brief, an aliquot of 10 ml test solution and 10 ml Oxalate Medium (control solution) was centrifuged at 3000*g* for 15 min at room temperature. Supernatant 10 ml was precipitated with 10 ml of 0.4 M $Ca(NO_3)_2$. The filtered precipitated calcium oxalate was acidified with H_2SO_4 (1:4), mixed with 20 ml of deionized water and heated to 80 °C. Immediately, 10 ml of H_2SO_4 (1:4) solution was added before titration. The solution was titrated with $KMnO_4$ (0.02 N) until a pink color persisted for 30 s. The results were expressed in % degradation of sodium oxalate per dose of synbiotic; and % degradation of sodium oxalate per 0.01 g of feces for rat fecal specimens.

Plasma and urine oxalate measurements

After sample collection, the blood tubes were delivered to the Laboratory and centrifugated at 2000×*g* for 15 min at room temperature. After centrifugation, the plasma was separated into 1.5 ml Eppendorf tubes labeled with the animals' number and analyzed immediately. POx concentration was measured spectrophotometrically using a commercially available kit (MAK315, Sigma, Barcelona, Spain) according to the manufacturer's protocols. Rats' 24-h urine samples were collected in individual metabolic cages and delivered to the Laboratory immediately. Daily UOx excretion was determined using an oxalate oxidase/peroxidase reagent (BioSystems, Barcelona, Spain). Urinary creatinine level was additionally measured by kinetic Jaffe reaction on Flexor Junior Analyzer to calculate urinary oxalate to creatinine(Ox:Cr) ratio and validate UOx excretion results.

Statistical analysis

Data analysis and all graphs were performed using the MedCalc Statistica Software version 20.011 (Ostend, Belgium). Since most of the data were not normally distributed, the median (Me) and interquartile ranges (Q25; Q75) were calculated. The normality of the data distribution was tested using the Shapiro–Wilk test. Differences between the groups were evaluated using the non-parametric Kruskal–Wallis test with the Conover test for post hoc comparisons. The Spearman's test was used for the correlation analysis. Two-factor analysis with repeated measures ANOVA was performed followed by Turkey post hoc test for multiple comparisons (main treatment effect).

Results

Seven days of ceftriaxone treatment induced a shift in oxalate homeostasis in rats

Contrarily to our expectation, on day 1 after treatment withdrawal, rats in ceftriaxone-treated groups (CEF) demonstrated a significantly higher ODB amount compared to the vehicle group ($p<0.001$) (Supplementary Table S1, Fig. 2A). However, ODA rate did not increase proportionally to the ODB level and tended to decrease compared to

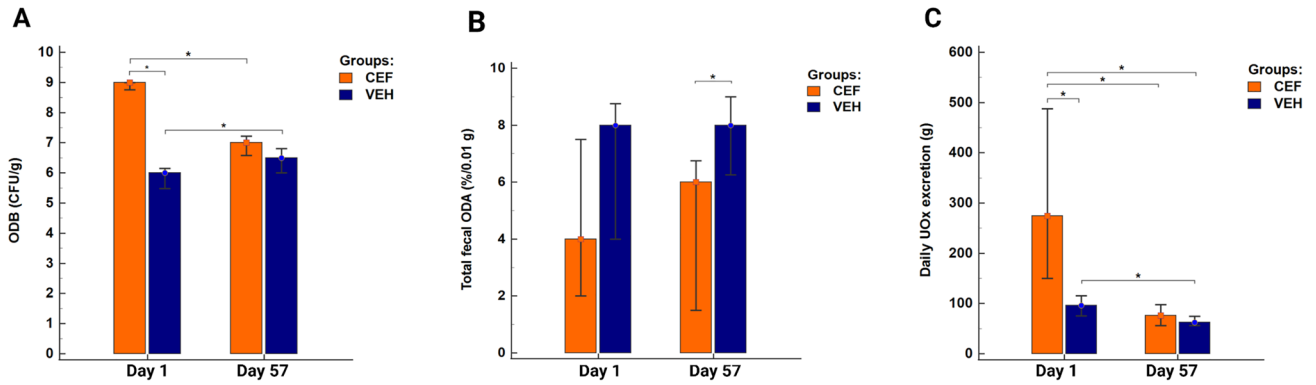

Fig. 2 Ceftriaxone administration (300 mg/kg, i.m., for 7 days) induced changes in the ODB number and their activity in rats' feces and UOx excretion. The data are presented as Me (Q25–Q75) and compared using the two-factor repeated ANOVA analysis. **A** Changes in ODB number in the rats' fecal microbiota during the experimental period; (*): $p < 0.001$. **B** Changes in total fecal ODA in rats during the experimental period. **C** Changes in UOx excretion during the experimental period; (*): $p < 0.001$

the vehicle group (Fig. 2B). These changes were associated with a significant increase in the UOx excretion in the ceftriaxone-treated group *vs* vehicle group ($p < 0.001$) (Fig. 2C). On day 57 after ceftriaxone withdrawal, the number of ODB and UOx excretion did not differ between groups while ODA was statistically lower in the ceftriaxone-treated group compared to the vehicle-treated group ($p = 0.03$) (Fig. 2A–C). Moreover, POx concentration in the ceftriaxone-treated rats was significantly high compared to the vehicle-treated group ($p = 0.02$) (Supplementary Table S1).

Oxalate-degrading activity of the commercially available synbiotic

To assess the ability of commercially available synbiotic to degrade oxalate, we tested in vitro its ODA rate. We found, that the synbiotic consisted of *Lactobacillus spp. (L. acidophilus,, L. rhamnosus, L. plantarum, L. casei), Bifidobacterium spp. (B. bifidum, B. longum), Saccharomyces boulardii,* selenium, oligofructose, and inulin, in 48 h was able to degrade 69% of oxalate in highly selective Oxalate Medium (the only source of energy is sodium oxalate) and was chosen for further investigation in vivo to restore ODA rate and oxalate homeostasis in ceftriaxone-treated rats.

Synbiotic supplementation restored ODA and decreased the level of UOx excretion in rats following ceftriaxone treatment

The two-factor repeated ANOVA demonstrated that both antibiotic and synbiotic administration as well as time after treatment withdrawal influence oxalate homeostasis in the rats. In particular, the ceftriaxone use in both CEF and CEF + SYN groups resulted in a compensatory increase of ODB number on day 1 following the treatment compared to the other groups while the use of the synbiotic did not increase the number of ODB compared to Vehicle Group immediately after the treatment (Fig. 3A). However, synbiotic supplementation led to statistically increased ODB number on day 57 after treatment withdrawal while the ODB number in ceftriaxone-treated groups (CEF and CEF + SYN) decreased to those level in the Vehicle Group by this time (group interaction $F = 42.6$, $p < 0.0001$; group and time interaction $F = 63.3$, $p < 0.0001$).

That is, by the end of the experimental period (in 8 weeks following the treatment), the number of ODB in rats' fecal microbiota did not differ between the groups, regardless of the treatment performed. However, despite the identical ODB number in all experimental groups, the total ODA in fecal microbiota was significantly higher in the synbiotic-treated groups (CEF + SYN and SYN), compared with Vehicle and Ceftriaxone Groups (group interaction $F = 4.91$, $p = 0.008$; group and time interaction $F = 7.51$, $p = 0.001$). It should be noted, that despite ceftriaxone treatment, total fecal ODA in rats of the CEF + SYN Group was significantly higher compared with those in the Ceftriaxone Group (Supplementary Table 1, Fig. 3B).

UOx excretion in the rats of the Synbiotic group was significantly lower compared to all other groups at both times of point (Fig. 3C). A similar result was obtained when we compared urinary Ox:Cr ratios between the groups. On day 1 after treatment withdrawal, the urinary Ox:Cr ratio of the Synbiotic group was significantly lower compared to all other groups, while the Ox:Cr ratio of the CEF + SYN group was practically equal to that in the Vehicle Group (Supplementary Fig. S1). In 8 weeks following the treatment urinary Ox:Cr ratio was significantly decreased compared with the previous one in all groups but the lowest level was observed in synbiotic-treated groups (see Supplementary Fig. S1).

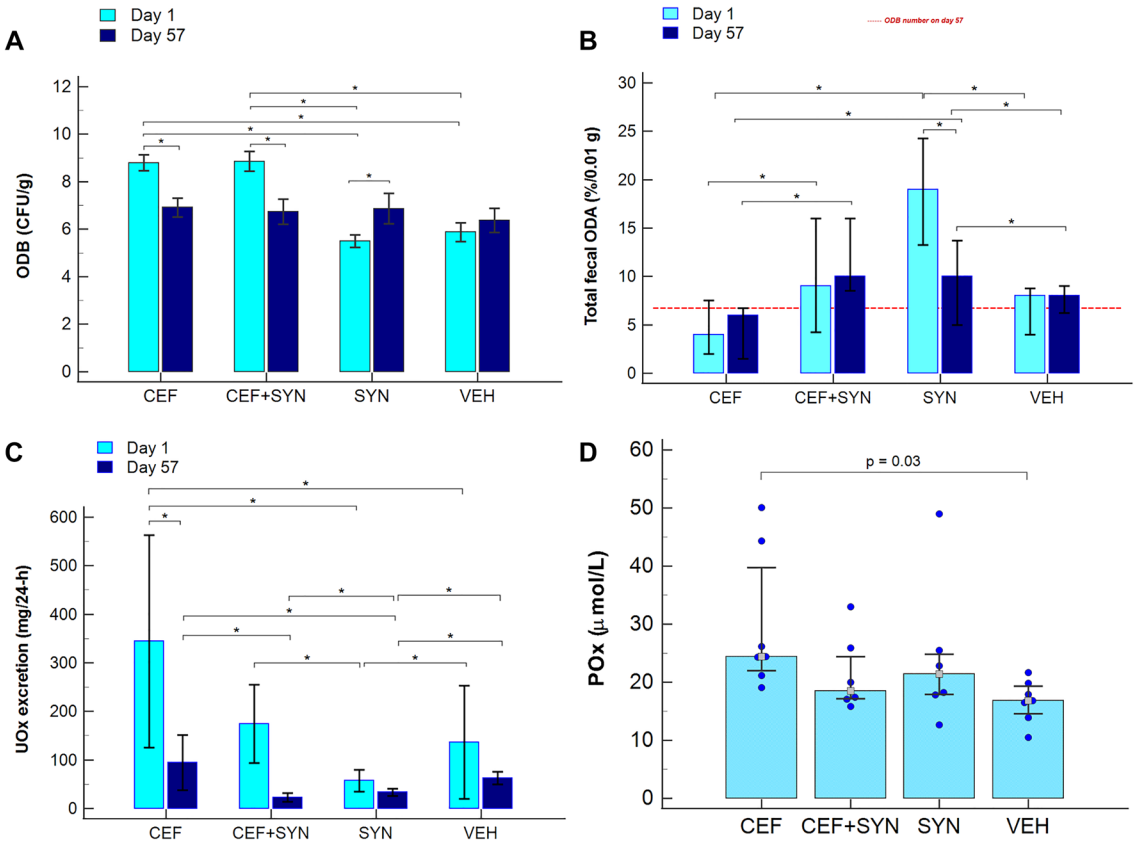

Fig. 3 Synbiotic treatment (30 mg/kg, *per os* for 14 days) restored total fecal ODA and decreased UOx excretion in rats on days 1 and 57 following ceftriaxone treatment (300 mg/kg, i.m., 7 days). The data are presented as Me (Q25–Q75) and compared using the two-factor repeated ANOVA analysis or the Kruskal–Wallis test with Conover post hoc. **A** Changes in ODB number in the rats' fecal microbiota during the experimental period; (*): $p < 0.0001$. **B** Changes in total fecal ODA in the rats during the experimental period; (*): $p < 0.001$. The reference line demonstrates the average ODB number (CFU/g) on day 57 following ceftriaxone treatment. **C** Changes in UOx excretion during the experimental period; (*): $p < 0.001$. **D** Changes in POx concentration on day 57 of the experimental period

There was no difference in POx concentration between the Vehicle and synbiotic-treated groups on day 1 after treatment withdrawal. Nonetheless, as was mentioned above, the POx concentration of the Ceftriaxone Group was significantly higher compared to the Vehicle Group (Fig. 3D).

Total ODA in rats' feces was inversely associated with plasma and urine oxalate concentrations

To further confirm the significance of total ODA *vs.* ODB number in supporting overall oxalate homeostasis, we next performed correlation analysis through all studied groups independently of the treatment protocol (see Table 1). Interestingly, the ODB number was not associated with their total ODA in rats' fecal microbiota on the 1st and the 57th experimental days ($r = -0.13$, $p = 0.36$ and $r = 0.07$, $p = 0.72$, respectively). However, both the ODB number and total ODA in fecal microbiota were associated with UOx excretion on day 1 after the treatment withdrawal ($r = 0.54$, $p = 0.003$ and $r = -0.44$, $p = 0.002$, respectively). On day 57, the rats' UOx excretion was not associated with the ODB number ($r = -00.3$, $p = 0.88$) but had a strong inverse correlation with total ODA in fecal microbiota (Fig. 4A). A similar result was observed in correlation analysis between ODB, total fecal ODA and POx concentration on day 57 after the treatment withdrawal. POx concentration was not associated with ODB number in rats' fecal microbiota ($r = 0.02$, $p = 0.89$), but showed a significant correlation with total fecal ODA (Fig. 4B).

The correlation analysis within each group also showed no significant association between ODB number and their ODA in rats' fecal microbiota as well as UOx excretion either on the 1st or the 57th experimental days (Supplementary Table S2). However, in contrast to the result of the whole rats' cohort, ODB number measured on the 1st day after antibiotic withdrawal was significantly associated with POx concentration in the ceftriaxone-treated ($r = -0.78$, $p = 0.03$) and the synbiotic ($r = -0.77$, $p = 0.04$) groups.

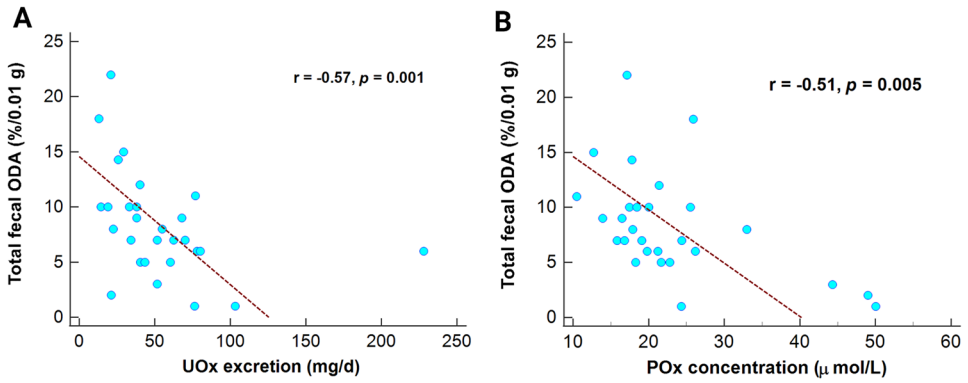

Fig. 4 The association between total fecal ODA and (**A**) UOx excretion and (**B**) POx concentration in all experimental rats in 8 weeks after the treatment withdrawal. *ODA* oxalate-degrading activity, *POx* plasma oxalic acid, *UOx* urinary oxalate

Discussion

In the present study, we tested the hypothesis that oxalate homeostasis in rats might be influenced not so much by the quantity of ODB in the intestine microbiota as by the total ability of ODB to degrade oxalate (ODA rate). We assumed that due to its synergistic effect (probiotic and prebiotic), the synbiotic can restore antibiotic-induced disturbance of oxalate homeostasis in rats. To this end, we separately evaluated the changes in the ODB number and their total ODA in fecal microbiota at two-time points after ceftriaxone and synbiotic exposure. In addition, we assessed the interaction between ODB, total fecal ODA, and plasma and urine oxalate concentrations in rats.

There are several new and unexpected findings in the study. First, treatment with ceftriaxone resulted in significant growth in the ODB number on day 1 after the treatment withdrawal, and despite the increase in the ODB quantity, the ceftriaxone exposure substantially reduced total fecal ODA compared to synbiotic-treated and vehicle-treated groups. Moreover, total fecal ODA in ceftriaxone-treated rats remained the lowest in 8 weeks (on day 57) following the treatment, although the ODB number was similar in all the experimental groups. Second, the use of synbiotic did not increase the ODB number as much as enhance their ability to degrade oxalates even when used simultaneously with ceftriaxone, which led to a significant decrease in UOx excretion. Third, ODB number was associated neither with their total ODA in fecal microbiota nor UOx excretion and POx concentration in rats. According to our results, only total fecal ODA was associated with urine and plasma oxalate levels.

The direct link between antibiotics exposure and KSD formation has been previously postulated, mainly in the context of the loss of *O. formigenes* in the gut microbial community [1, 9, 19, 20], the sensitivity *of O. formigenes* strains to commonly prescribed antibiotics [30], or the effect of probiotic interventions [31]. However, in addition to *O. formigenes,* to date, ODA has been identified in many other representatives of the intestinal microbiota (*Enterococcus spp., Lactobacillus spp., Bifidobacterium spp., Bacillus spp*) [10–13, 32]. Nevertheless, the majority of published studies have been focused not on the ODB profile but on differences in the general gut microbial composition between KSD patients and healthy control [2, 33].

To the best of our knowledge, this report is the first to evaluate the total ODB number and their ODA in rats' fecal microbiota in response to ceftriaxone and synbiotic exposure. Surprisingly, according to our findings, the total ODB number was significantly increased after ceftriaxone exposure compared to vehicle- and synbiotic-treated groups. This result is in line with the recent work conducted by R. Chakraborty et al., in which the authors demonstrated a ceftriaxone-induced transient increase in the abundance and extraintestinal dissemination of *Enterococcus spp* and *Lactobacillus spp*. in a mouse model [34]. In another recent study, the authors have observed a substantially increased relative abundance of *Enterococcus spp., Lactobacillus spp*. and *Bifidobacterium spp*. after the use of a combination of four antibiotics (bacitracin, meropenem, neomycin and vancomycin) in mice [35]. From our point of view, these observations might be a consequence of the resistance of certain species of ODB to ceftriaxone, or it could be associated with the growth of some commensals due to ceftriaxone-induced loss of others. In this context, it is logical to assume a compensatory increase in ODB with a lesser ability to degrade oxalate, which could explain a significant decrease in total fecal ODA simultaneously with a transient increase in the ODB number in the ceftriaxone-treated rats. However, simultaneous studies on the ODB number and their total functional ability to degrade oxalate have never been conducted before, hence the obtained results cannot be directly compared with the results of previous reports. Accordingly, the phenomena of ceftriaxone-induced increasing ODB number and a simultaneous decrease in their total ODA in fecal microbiota raises many questions that require further investigations.

Numerous in vitro studies have addressed the beneficial effect of probiotics on the oxalate-degrading capacity of gut microbiota and reducing hyperoxaluria [9, 15]. Clinical results are not as encouraging and need further large-scale

studies [24, 33]. It should be noted that only a few studies were conducted to investigate the synbiotics effect on human health [31, 33] and the only one addressed the effects of prebiotic and synbiotic on oxalate degradation in vitro [25]. Moreover, there are limited data concerning the interaction between antibiotics and synbiotics [35]. Thus, it is not well understood how synbiotics alter antibiotic-induced oxalate homeostasis imbalance and whether the synbiotic supplementation changes the short-term or long-term effects of antibiotics.

The commercially available synbiotic selected for our study has never been studied before for restoration of oxalate homeostasis. But, the composition of it (*Lactobacillus spp.: L. acidophilus, L. rhamnosus, L. plantarum, L. casei;Bifidobacterium spp.; B. bifidum, B. longum; Saccharomyces boulardii*, selenium, oligofructose and inulin) is very promising. According to the literature [15, 17, 36] and our previous study [32], all the synbiotic-contained *Lactobacillus spp*. and *Bifidobacterium spp.* possess oxalate degradation potential. Moreover, *L. acidophilus* has the highest ODA about 55% [15]. *Saccharomyces boulardii* is known for its clinical and experimental effects to prevent antibiotic-associated diarrhea [37] and its anti-inflammatory effect in the intestine [38]. Therefore, *S. boulardii* might provide an additive beneficial effect for oxalate homeostasis via the restoration of intestinal barrier. However, in addition to seven strains of live probiotic microorganisms, synbiotic consisted of selenium, oligofructose and inulin as a prebiotic supplement. In vitro study showed that the addition of inulin to bacterial incubation medium significantly increased ODA of *Lactobacillus spp* and effect was increased by co-incubation of several *Lactobacillus strains* [25]. In the present study, we tested for the first time the synbiotic total ODA in vitro before in vivo experiment. Synbiotic was able to degrade 69% oxalate after 48 h incubation in highly selective Oxalate Medium. It is somewhat surprising that the synbiotic exposure did not affect the ODB number but more than doubled the total fecal ODA in 2 weeks of its administration. Even with antibiotic therapy, the use of the synbiotic led to an increase in total fecal ODA, which was reflected in a significant decrease in oxaluria on day 1 after treatment withdrawal. At the end of the experiment, the number of ODB increased compared to the first measurement only in the synbiotic group. Interestingly, the synbiotic administration preserved total ODA in the fecal microbiota of ceftriaxone-treated rats during the all post-antibiotic period independently of the ODB number. In fact, on the 57th day following the treatment, the beneficial effect of enhancing total fecal ODA in the group treated simultaneously with ceftriaxone and synbiotic was almost the same as in the isolated synbiotic-treated group. In parallel with our results, A. Jačan et al. have demonstrated that the synbiotic per se did not influence the gut microbiota but was able to modulate the antibiotic-induced dysbiosis in a time-dependent manner [35]. Our data are also consistent with the findings obtained by Ö. Darilmaz et al. in their in vitro study. The authors have shown that the probiotic in a combination with inulin enhanced the degradation of oxalates, thereby highlighting inulin's key role [25]. Moreover, in line with our results, they have not found an association between oxalate degradation rate and bacterial growth after synbiotic exposure [25]. In our opinion, the growth of ODB in our synbiotic-treated group reflects intestinal colonization of the microorganisms present in the synbiotic. In the case of simultaneous use of ceftriaxone, the changes in the ODB number resulted from the growth of enterobacteria and other pathogens, while lacto- and bifidobacteria, stimulated by prebiotic additives, modulated the activity and viability of other intestinal microbiota. These results could explain why not the abundance of ODB but rather their total ODA in fecal microbiota was associated with UOx excretion and POx concentration in the rats.

We clearly assume the limitations of the present study: *(i)* the synbiotic administration should not be considered as substitution therapy in KSD patients but as a way of providing conditions for the restoration of the intestine biocenosis and stimulation of ODB activity; *(ii)* to further confirm the clinical efficacy of synbiotic vs probiotic in the restoration of antibiotic-induced gut dysbiosis and prevention of urolithiasis, its effect should be compared with the same strains of probiotics and *S. boulardii*.

Despite the mentioned limitations, the present study guides several important issues for further possible translation of the study findings to human medicine. First, we believe that total fecal ODA but not the ODB number plays a pivotal role in oxalate homeostasis in rats which should be explored in future clinical studies. Second, we hope our data could be the base of a person-tailored approach to reduce hyperoxaluria and prevent KSD. Finally, our findings are complementary to the published data on the dichotomy between metagenomic and metatranscriptomic of the human microbiota ODB.

Conclusions

Taken together, this study for the first time demonstrated that ceftriaxone treatment decreased total fecal ODA independently of the ODB number and increased urine and plasma oxalate concentrations in the experimental rats. Total fecal ODA but not the ODB number should be thoroughly examined in the future to develop predictive diagnostics methods, targeted prevention and personalized treatment in KSD. Synbiotic supplementation had a beneficial effect on the total ODA of gut microbiota, which resulted in significantly decreased UOx excretion in antibiotic-treated rats. However,

it should be noted that the experimental design of the present study precludes an unambiguous conclusion concerning the use of synbiotics for the treatment of hyperoxaluria or prevention of KSD recurrences in humans.

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Declarations

Conflict of interest The authors have no conflicts of interest to disclose.

Ethical approval The study was reviewed and approved by the Animal Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Protocol #18/01/2020).

Consent to participate All authors have agreed to this submission.

Consent for publication This publication has been approved by all co-authors and by the responsible authorities at the ESC "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv and State Institution «Institute of Nephrology of the National Academy of Medical Sciences», where the work has been carried out.

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