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Modulation of Urinary Siderophores by the Diet, Gut Microbiota and Inflammation in Mice

Xia Xiao¹, Beng San Yeoh¹, Piu Saha¹, Yuan Tian², Vishal Singh¹, Andrew D. Patterson², and Matam Vijay-Kumar^{1,3,*}

¹Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802

²Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802

³Department of Medicine, The Pennsylvania State University Medical Center, Hershey, PA 17033

Abstract

Mammalian siderophores are believed to play a critical role in maintaining iron homeostasis. However, the properties and functions of mammalian siderophores have not been fully clarified. In this study, we have employed Chrome Azurol S (CAS) assay which is a well-established method for bacterial siderophores study, to detect and quantify mammalian siderophores in urine samples. Our study demonstrates that siderophores in urine can be altered by diet, gut microbiota and inflammation. C57BL/6 mice, fed on plant-based chow diets which contain numerous phytochemicals, have more siderophores in the urine compared to those fed on purified diets. Urinary siderophores were upregulated in iron overload conditions, but not altered by other tested nutrients status. Further, germ-free mice displayed 50% reduced urinary siderophores, in comparison to conventional mice, indicating microbiota biotransformation is critical in generating or stimulating host metabolism to create more siderophores. Altered urinary siderophores levels during inflammation suggest that host health conditions influence systemic siderophores level. This is the first report to measure urinary siderophores as a whole, describing how siderophores levels are modulated under different physiological conditions. We believe that our study opens up a new field in mammalian siderophores research and the technique we used in a novel manner has the potential to be applied to clinical purpose.

* **Corresponding Author:** Matam Vijay-Kumar (Vijay), PhD, Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802, Ph: 814-867-3537, FAX: 814-863-6103, mvk13@psu.edu.

Author contribution:

X.X. designed and performed the experiments, analyzed the data and co-wrote the paper. B.S.Y. designed and performed experiments, and involved in data interpretation and discussion. P.S. and V.S. performed animal experiments and analyzed the data. Y.T. performed ¹H NMR analysis. A.P. contributed to the experimental design and analysis. M.V-K. original idea, directed the project, analyzed the data and co-wrote the paper.

Conflict of Interests

The authors have declared that no conflict of interest exists.

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Keywords

Purified diets; Iron Chelation; Iron toxicity; Hypoferremia; Antibiotics; Gut Bacteria

1. Introduction

Iron, a redox metal ion, is vital to almost all living aerobic organisms except *Lactobacillus* and *Borrelia* species [1, 2]. Although ferric iron is the most abundant transition metal on the biosphere, its bioavailability to microbes is very limited because of its insolubility. To acquire this essential nutrient, microorganisms including bacteria and fungi secrete high affinity siderophores to capture ferric iron from the environment. Many bacterial pathogens are also known to employ siderophores to steal iron from host ferroproteins such as transferrin and ferritin. For instance, enterobactin is one such prototypical bacterial siderophores that chelates iron with extremely high affinity (10^{-49} M) [3].

Siderophores (Greek; *iron carriers*) are small, secreted and low molecular weight (<1 kDa) iron chelators that have high affinity towards ferric, but not ferrous iron [4]. In the past, studies were mainly focused on siderophores of microbial origin and it was not until much recently that research began to elucidate mammalian siderophores. Mammalian siderophores are structurally distinct from microbial siderophores, yet they perform similar functions in chelating and solubilizing ferric iron for transport and systemic utilization. The chelation of ferric iron by siderophores also facilitates their binding to host innate immune protein, lipocalin 2 (*aka* siderocalin) which chelates and stabilizes the iron in its non-reactive ferric state [4]. Previous studies by Bao *et al*/identified catechol as an endogenous siderophore, which is derived from the metabolism of dietary compounds such as polyphenol/quinic acid-rich foods [5, 6]. Currently known mammalian siderophores are citrate, pyrogallol, gentistic acid, pyrocatechol and 3-methylcatechol, which are either derived from the diet, host and/or gut microbiotal activity [4, 7]. In addition, plant-derived phytochelators such as epigallocatechin-3-gallate (EGCG; a major catechin in green tea) have also been characterized to display strong siderophore-like properties [8].

The vast majority of microbes residing in the large intestine are collectively known as the gut microbiota, whose metabolic activity is comparable to that of the liver. Gut microbiota can metabolize many dietary compounds and generate ‘microbial signature compounds’ (*e.g.* short chain fatty acids, indole, secondary bile acids), which are absent or substantially reduced in germ-free (GF) mice. In addition to generating their own siderophores, the gut microbiota can convert diet-derived components into iron-binding molecules [9]. Conversely, the gut microbiota can also abrogate iron-binding properties of plant-derived compounds such as ellagic acid by metabolizing it into urolithin A, which lacks iron-binding property [10].

Hypoferremia (anemia) of inflammation is a well-known phenomenon in response to acute and chronic inflammation [11]. This host response is a primitive protective mechanism, which reduces the systemic iron availability for invading pathogen(s), and also limits the participation of iron in Fenton's reaction. For instance, administration of lipopolysaccharide (LPS) results in more than 50% reduction in the systemic iron in mice [12]. To thrive in such

condition of iron deficiency, certain members of the gut microbiota i.e. *Escherichia coli* can adapt by increasing enterobactin secretion. However, the extent to which the pool of microbial-, dietary- and mammalian-derived siderophores is modulated during hypoferremia of inflammation remains to be elucidated.

In this study, we have investigated how the diet, microbiota and inflammation can influence urinary siderophore levels, which in turn reflects the iron homeostasis of the organism. We employed the Chrome Azurol S (CAS) assay, a methodology initially developed for microbial siderophore research, as an innovative approach to measure urinary siderophores. When compared to mice on lab chow, purified diet-fed mice excrete substantially reduced urinary siderophores. Conditions of iron overload due to either the diet or genetics significantly increased the levels of urinary siderophores. Interestingly, in GF mice urinary siderophore levels are substantially reduced when compared to conventional mice. Further, gut/systemic acute and chronic inflammation also alters urinary siderophores. Altogether, our findings provide new insights on endogenous siderophores homeostasis, which may be useful for rapid screening for iron deficiency or overload.

2. Materials and methods

2.1 Reagents

Ferrous sulfate heptahydrate and sodium dihydrogen citrate were obtained from Alfa Aesar (Ward Hill, MA). Dextran sodium sulfate (DSS) was obtained from MP Biomedicals, LLC (Solon, OH). Epigallocatechin-3-gallate, lipopolysaccharide (LPS; *E. coli* O128:B12), ferric chloride hexahydrate, pyrocatechol, deferoxamine mesylate salt, enterobactin (*Escherichia coli*), pyoverdines (*Pseudomonas fluorescens*), ferrichrome (*Ustilago sphaerogena*) were purchased from Sigma-Aldrich (St. Louis, MO). *Salmonella typhimurium* (SL3201) was a generous gift from Dr. Andrew Gewirtz (Georgia State University). All other fine chemicals used were reagent grade and procured from Sigma-Aldrich (St. Louis, MO).

2.2 Mice

C57BL/6 mice used in this study were bred and maintained in cages (n=5 mice/cage) with sawdust as bedding material and paper nestlets. The cages were housed at 23 °C and maintained a 12-h light and 12-h dark cycle. Lipocalin 2 (Lcn2) knockout mice in C57BL/6 background generated by Dr. Shizuo Akira (Japan) were obtained via Dr. Kelly Smith (University of Washington) and bred with wild type (WT) mice. The resulting offspring were crossed to generate homozygous Lcn2KO mice and their WT littermates. All mice were maintained on chow (Lab Diet 5001) or indicated diets *ad libitum* and have unlimited access to water throughout the study. Urine collected from hemochromatosis protein (Hfe)-deficient mice and their WT littermates were provided by Dr. James Connor (The Pennsylvania State University Hershey Medical Center, PA). Germ-free (GF) urine samples were obtained from Gnotobiotic Facility at Penn State University. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at The Pennsylvania State University.

2.3 Dietary studies

Four weeks old male WT mice (n=4-5) were fed on diets which were modified AIN-93G (Research Diets Inc., New Brunswick, NJ) containing adequate iron (37 ppm iron: ferrous citrate) or excess iron 10,000 ppm (carbonyl iron) or iron deficient diets (no added iron). Mice were monitored for body weight and food intake weekly. For high-fat diet (HFD) study, six weeks old WT male mice (n=4) were maintained on HFD (60% fat; D12492) and the control diet (10% fat; D12450J) from Research Diets for three weeks. For autoclave diet feeding study, 8 weeks old male WT mice (n=5) were maintained on autoclaved Lab Diet 5010 which has the exact diet composition as Lab Diet 5001 for one week. The urine was collected before and after autoclaved diet treatment for siderophores analysis. Urine samples from vitamin D (1, 25-(OH)₃ V_D)-supplemented mice or vitamin A-deficient diet fed mice (for 3 generations) and their control diets mice were collected from Dr. Margherita Cantorna laboratory (The Pennsylvania State University, PA). Urine from altered dietary selenium [adequate (178 ppm), deficient (0 ppm), excess (890 ppm)] fed mice was obtained from Dr. Sandeep Prabhu laboratory (The Pennsylvania State University, PA).

2.4 Systemic iron administration

Eight weeks old WT female mice (n=5) were administered ferrous sulfate (FeSO₄·7H₂O i.p.; 25 mg/kg body weight). Urine samples were collected before and after (4, 24 h) injection.

2.5 Oral epigallocatechin-3-gallate (EGCG) administration

Six weeks old female WT mice (n=5) were treated with one dose EGCG (50 mg/kg BW) by oral gavage. Urine samples were collected before EGCG treatment and 4, 8, 12, 24 h post-treatment for siderophore quantification.

2.6 Antibiotics administration

Six weeks old female WT mice (n=4) were maintained on 1.0 g/L ampicillin and 0.5 g/L neomycin containing drinking water for 4 weeks. Urine and feces were collected weekly. Serum was collected at the end of experiment. This antibiotic regimen ablates 90% of gut bacterial load as measured by qRT-PCR using universal primers for 16S rRNA [13].

2.7 DSS-induced colitis in mice

Six weeks old female WT mice (n=8) were administered 1.5% DSS (MP Biomedicals) in drinking water over a period of 7 days [14]. Induction of colonic inflammation was confirmed by fecal occult blood, diarrhea and loss in body weight. Urine samples were collected for urinalysis.

2.8 Lipopolysaccharides (LPS)-induced acute inflammation

Eight weeks old male WT mice (n=5) were challenged with 10 µg of LPS from *E. coli* O128:B12 intraperitoneally. Before and after 24 h treatment, urine samples were collected.

2.9 *Salmonella* infection

Salmonella enterica serovars *typhimurium* (SL3201) was grown under microaerophilic conditions overnight as previously described [15]. Briefly, 8 weeks old male WT mice were

orally inoculated with (10^8 CFU/mouse) *Salmonella* to mimic natural infection and induce septic-like systemic inflammatory response. Mice were monitored for body weight and urine was collected every other day until day 10.

2.10 Urine samples processing

Urine samples were centrifuged at 5,000 rpm for 5 min and particulate-free urine were stored at -80°C until analysis. To study the heat stability of siderophores, urine samples were subjected to boiling for 5 min.

2.11 Quantification of urinary creatinine

Urinary creatinine levels were measured using a kit from Randox (Crumlin, UK) according to the manufacturer's instructions.

2.12 Chrome Azurol S (CAS) liquid assay

Liquid CAS solution was prepared according to the procedure outlined in [16]. CAS gives a distinctive blue color when it is in complex with iron. When iron is instead chelated by siderophores, the complex results in the orange colored chromogen. The intensity of orange color is directly proportional to the concentration of siderophores. Urine samples were diluted 100x in double-distilled water and 100 μL of diluted samples were mixed with 100 μL of CAS solution in 96-well plate. After 20 min incubation at room temperature, optical density was read at 630 nm. Pyrocatechol was used as a standard curve (range 10-0.625 mM) against the blank (double distilled water). EGCG, enterobactin (Ent), deferoxamine (DFO) pyoverdines (PYO), ferrichrome (FCM) and citrate were also employed in this assay and results were presented as percentage of iron chelation. To confirm that urinary siderophores chelate specifically ferric iron, assay was performed in the presence of exogenously added ferric iron to the urine samples. Briefly, samples were spiked with or without $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma) at indicated concentrations and then CAS liquid assay was performed as above. We also employed zinc chloride, manganese chloride, and calcium chloride to study the specificity of the urinary siderophores. Urinary siderophores levels were normalized to creatinine and expressed as mol/g creatinine.

2.13 CAS plate assay

CAS agar plates were prepared according to the protocol described previously [17]. The mechanism of CAS plate assay is the same as the above CAS liquid assay. However, when compared to CAS liquid assay, CAS plate assay is less sensitive. For all tested samples, 10 μL was applied on CAS plate at indicated concentrations and monitored for orange-color halo formation at indicated time points.

2.14 Serum total iron, total iron binding capacity (TIBC), transferrin saturation, catalytic iron measurement

Serum total iron was measured as described previously [18]. Briefly, to precipitate serum proteins and liberate protein-bound iron, serum samples were mixed with an equal volume of hydrochloric acid (0.1 mg/mL), trichloroacetic acid (1.0 M) and thioglycolic acid (30 mL/L). After centrifugation (6,200 g for 10 min), the supernatants were collected and mixed

with equal volume of chromogen solution containing ferrozine (1.5 M) and saturated sodium acetate (1.5 M). The optical density of the chromogen was read at 562 nm. Total iron levels were estimated using a standard curve generated with the iron AA standard (Ricca Chemical Company). Serum TIBC and catalytic iron was analyzed as described in detail elsewhere [19, 20]. Transferrin saturation was calculated as serum total iron/TIBC $\times$ 100 [21].

2.15 Quantitative RT-PCR

Mouse liver was collected in *RNA Later* (Sigma) and stored in -80°C until process. mRNA was extracted using Trizol reagent (Sigma) according to the manufacturer's protocol. mRNA (0.8 μg) was used to synthesize cDNA for qRT-PCR using SYBR green (Quanta) as described in manufacturer's protocol. The following primers were used to assess gene expression: *Hamp* (hepcidin) 5'-AGAAAGCAGGGCAGACATTG-3' and 5'-CACTGGGAATTGTTACAGCATT-3' [22]; *36B4* 5'-TCCAGGCTTTGGGCATCA-3' and 5'-CTTTATTTCAGCTGCACATCACTCAGA-3' [23]. *36B4* was used to normalize relative mRNA expression using Ct ($2^{-\text{Ct}}$) method. Fold change was determined by comparison to the untreated control group.

2.16 Nuclear magnetic resonance spectroscopy (NMR) analysis

Urine samples were processed as previously described [24] for NMR analysis. All ^1H NMR spectra of sample were recorded at 298K using a Bruker Avance III 600 MHz NMR spectrometer. Multivariable data analysis was carried out with the SIMCAP+ software (version 13.0, Umetrics, Sweden).

2.17 Statistical Analysis

All values in the results were expressed as mean $\pm$ SEM. The significance of difference between different groups was determined by one way ANOVA followed by post hoc Tukey's multiple comparison test in case of 3 or more groups and unpaired student 't' test in case of 2 groups. $P < 0.05$ (*) was considered as significant. GraphPad Prism 6.0 software was used to calculate statistical significance.

3. Results

3.1 Standardization of CAS liquid assay for siderophores detection

CAS assay is widely used to screen for siderophore-producing bacteria in microbiology research; however, its utility in estimating mammalian siderophores is yet to be elucidated. Hence, we first examined the sensitivity of CAS liquid assay to detect the activity of microbial enterobactin (Ent), deferoxamine (DFO) pyoverdines (PYO), ferrichrome (FCM), plant EGCG and chemical (pyrocatechol, citrate) siderophores. We observed that CAS liquid assay has high sensitivity for EGCG ($\sim 3 \mu\text{M}$; 10% chelation) and Ent ($\sim 10 \mu\text{M}$; 10% chelation) when compared to citrate ($\sim 50 \mu\text{M}$; 10% chelation), pyrocatechol and DFO ($\sim 300 \mu\text{M}$; 10% chelation) (Fig 1A). The activity of most siderophores peaked approximately 20 min at room temperature, with the exception of DFO, PYO and FCM. To further distinguish the iron binding properties DFO, PYO and FCM, we next performed time-dependent CAS liquid and plate assays. DFO continued to chelate iron up to 48 h (Fig 1B and Fig S1). Similar trends were also observed with PYO (Fig 1C) and FCM (Fig 1D) by CAS liquid

assay, indicating that DFO, PYO and FCM are slow, but strong ferric iron chelators. Among these three siderophores, DFO is the most potent since the lowest concentration (~1.5 μ M) of DFO continued to chelate iron up to 48 h, but not the PYO and FCM.

Urine is enriched with a spectrum of small chemical molecules, many of which may be metabolic byproducts of host and gut microbiota. Hence, we next examined whether urine contains siderophores, which can be detected by CAS assay under healthy conditions. Indeed, we observed that mice urine display siderophoric activity. Specifically, in CAS liquid assay, we found that urine samples need to be diluted at least 100 times for optimal reading within the detection range using pyrocatechol as standard (0-10 mM) (Fig 1A). We observed that gender differences did not affect the levels of urinary siderophores (data not shown) neither did genotype difference (Lcn2KO). Similar levels of urinary siderophores between Lcn2KO and their WT littermates may indicate that these urinary siderophores are Lcn2 resistant or that chelated urinary siderophores by Lcn2 may still have iron binding capacity (Fig S2A). To verify that our assay detects urinary siderophores but not other non-specific reactivity between urinary metabolites or pH with CAS constituents, we spiked the urine samples with increasing concentration of ferric iron and then analyzed on CAS liquid assay. As expected, the addition of competing ferric iron dose-dependently blunted the readout in the CAS assay (Fig S2B). In contrast, the addition of other metal ions such as zinc, manganese or calcium into the urine samples did not alter the assay readout (Fig S2C-E), thus confirming that the assay is detecting specifically only iron-free siderophores. Intriguingly, the urine samples retained their siderophoric activity even after boiling at 100 °C for 5 min, implicating that these siderophores are heat-stable (Fig. S2F). Similarly, we noted that both microbial and chemical siderophores retained their substantial siderophoric activity on CAS plate assay even after boiling (Fig. S2G).

3.2 Plant-based lab chow increases urinary siderophores compared to purified diet

Plant-derived polyphenols/hydroxybenzenes such as caffeic acid, catechin and chlorogenic acid, can undergo conversion into metabolites that contribute to the pool of mammalian siderophores [25]. To test whether dietary components can modulate urinary siderophores, we orally administered single dose of EGCG (50 mg/kg body weight) to 6 weeks old female mice and collected urine samples at indicated time points. We found that urinary siderophoric activity increased after 4 h and peaked at 8 h post-treatment (Fig 2A), but became attenuated at later time points. We confirmed that the halo formed was due to iron chelation by performing iron saturation assay at selected time points (Fig 2B). The upregulated siderophores after EGCG treatment can be due to either other siderophores excretion stimulated by EGCG, or EGCG itself that was excreted through urine or by exerting its influence on systemic iron levels.

After establishing that the administration of a single dietary component can modulate urinary siderophore levels, we next inquired whether the diet, as a whole, could also mediate similar effects. The chow diet (rich in plant materials) and compositionally defined purified diet (almost no plant derived material) are commonly used as maintenance or control diet in most animal experiments. Intriguingly, chow diet-fed mice excreted about 10-fold more siderophores in urine when compared to purified diet-fed mice (Fig 3A). Iron saturation

assay confirmed that halo formation is due to ferric iron chelation by siderophores in a dose-dependent manner (Fig 3B).

We next speculate that the 7 fold more iron in chow diet (~250 ppm) than purified diets (37 ppm) may affect urinary siderophores by changing serum iron level. However, the serum iron levels were comparable between these 2 groups (Fig 3C). Therefore, the higher levels of urinary siderophores in chow diet-fed mice could potentially be explained by the higher content of plant-derived components in chow diet than purified diet. To identify these urinary siderophores, we next performed metabolomic profiling of urine by ¹H NMR. As shown in Fig 3D and 3E, chow diet-fed mice displayed increased citrate levels, suggesting that citrate may be the major siderophore in the urine. We also detected high levels of hippuric acid, which is the glycine conjugate of aromatic phenolic acids excreted from the liver [26]. Since glycine is widely used as glycine-conjugated iron to increase the bioavailability of iron supplement [27], we thus hypothesized that hippuric acid, which contains glycine in its structure, may also have iron chelation property. However, we did not detect any siderophoric activity for hippuric acid by both CAS assays (data not shown). Although ¹H NMR provided insights on the urinary siderophore pool, we note that it is not sensitive enough to detect catechol which has been demonstrated to be secreted in the urine [5, 28]. We envision that the use of high-resolution chromatography-based techniques (GC-MS, LC-MS) in future studies may be required to screen for other potential urinary siderophore candidates.

Besides purified diet, we further explored the effect of purified high-fat diet (HFD; 60%) on urinary siderophores excretion. There is a 38% decrease of siderophores in HFD-fed mice urine compared to the control group (10% of fat) (Fig 3F). HFD or HFD-induced obesity has been demonstrated to induce hypoferremia/iron deficiency via low-grade inflammation or decreased iron absorption [29, 30]. Thus, we next confirmed that serum total iron levels were decreased after 3 weeks of HFD treatment (Fig 3G). This observation suggests urinary siderophores excretion may be influenced by host iron status or inflammation.

3.3 Urinary siderophores excretion is altered by host iron status

To investigate the hypothesis that dietary iron and host iron status can also modulate urinary siderophore levels, we placed mice on iron-adequate (Fe-A; 37 ppm), iron-deficient (Fe-D; 2.9 ppm) or iron-excess (Fe-E; 10,000 ppm) diet. After 8 weeks of feeding, we confirmed the mice iron status by analyzing their serum total iron levels (Fig 4A). As expected, Fe-E diet increased serum total iron by one-fold, whereas Fe-D diet decreased serum total iron by 70% when compared to control diet. Accordingly, serum TIBC, transferrin saturation, catalytic iron and liver hepcidin expression (Fig S3A-D) are all reflecting the mice are in iron deficient/overload status. Remarkably, the urinary siderophores excretion in the Fe-E group significantly increased by 4-fold (Fig 4B). The Fe-D group also slightly increased their urinary siderophore levels, albeit did not reach significance, when compared to the Fe-A group (Fig 4B). Iron saturation assay was used to confirm that the orange chromogen formation is due to ferric iron chelation by siderophores in a dose-dependent manner (Fig 4C). Along with urinary siderophores, similar trend was observed in citrate levels analyzed by ¹H NMR (Fig 4D). To further confirm whether iron status can influence urinary

siderophore levels, we next examined the hemochromatosis gene-deficient (Hfe-KO) mice that are widely studied for their genetic iron overload phenotype. Similar to Fe-E diet-fed mice, Hfe-KO mice also excreted more iron-free siderophores than WT littermates (Fig 4E). Iron saturation assay was also performed (Fig 4F).

To verify whether other nutrients can affect urinary siderophores excretion, we analyzed urine samples from mice fed on diets with altered levels of vitamin A, vitamin D and selenium. However, none of these diet alternations affected urinary siderophores excretion (Fig S3A-C). These findings suggest that iron is probably the major nutritional factor which affects urinary siderophores excretion.

When mice were administered with a single dose of iron (25 mg/kg BW of i.p. iron treatment), the urinary siderophores levels were surprisingly reduced at 4 and 24 h post-iron treatment (Fig 4G). Since CAS assay could only detect iron-free siderophores, it is likely that the acute reduction of urinary siderophore activity may be due to the loss of their iron-free state upon binding to iron for urinary excretion. Although these observations seemingly implicate a potential role of urinary siderophores in facilitating iron excretion, we note that further in-depth studies are required to examine this possibility.

3.4 Germ-free (GF) mice excrete less urinary siderophores

Among factors that influence the metabolism of dietary components, the gut microbiota has emerged as a key player in metabolizing and generating various nutrients required by the host. Indeed, mice with sterilized gut were previously reported to display a two-fold reduction in urinary catechol levels [9]. Since GF mice are typically used to study the influence of the gut microbiota on host metabolism [31], we therefore analyzed the siderophores levels in the urine collected from age and gender matched GF and conventionally-housed mice. Interestingly, GF mice urine contained only half of WT urinary siderophores levels (Fig 5A), which implies that microbiota contributes about half of the urinary siderophores. The halo formed was confirmed to be due to iron chelation by performing iron saturation assay (Fig 5B). We considered the possibility that the reduced siderophore excretion in chow diet-fed GF mice could be due to the use of sterilized (autoclaved) diet, which may destroy potential siderophore precursors in the diet. However, the level of urinary siderophores was comparable between conventional and autoclaved diet-fed mice (Fig 5C), thus reaffirming the involvement of microbiota in mediating generation of urinary siderophores. We also suspect that gut microbiota may alter iron status, since recent study has demonstrated that iron absorption could be altered by gut microbiota [32]. Yet, we did not observe significant difference for serum iron levels (data not shown).

Antibiotics (Abx) treated mouse model is a good alternative to GF mouse model. Thus, we asked to what extent Abx treatment could recapitulate the observation made with GF mice. We measured urinary siderophore levels in mice administered broad-spectrum antibiotics in drinking water. Surprisingly, the result is opposite to what we found with GF mice. As Abx treatment continued, siderophores excretion increased (Fig 5D) and we performed iron saturation assay to confirm that the chromogen formed was due to ferric iron chelation (Fig 5E). Considering that this antibiotic regimen can kill 90% of bacteria in the gut [13], it is possible that the 10% survived microbiota have more potential to affect siderophores

metabolism. In addition, antibiotics treatment can promote inflammation via translocating native commensal colonic bacteria [33] and gut microbiota dysbiosis associated with lipid metabolism dysregulation [34]. Accordingly we observed low-grade inflammation in Abx treated mice as their fecal, serum and urinary Lcn2 levels were increased (data not shown) with 25% decrease in serum iron levels (Fig 5F).

3.5 Inflammation modulates urinary siderophores excretion

Inflammation generally associates with reduced systemic iron levels *aka* hypoferremia. Next, we investigated to what extent inflammation can influence urinary siderophores excretion by using well-established models of inflammation [12, 23, 35]. First, we studied acute sepsis model by giving LPS (10 µg/mouse, i.p.) to mice and found urinary siderophores increased (60%) significantly at 24 h post treatment (Fig 6A). Mice with acute colitis, induced by a chemical colitogen dextran sodium sulfate (DSS), also displayed elevated urinary siderophores by more than 2 folds (Fig 6B). However, substantial reduction in urinary siderophores by 50% (Fig 6C) was observed in mice with chronic systemic inflammation induced by oral inoculation of an enteropathogen, *Salmonella*, which is known to cause sepsis. These results suggest that acute and chronic inflammation differentially influence urinary siderophores excretion.

4. Discussion

Iron is an essential trace element required for virtually almost all aerobic organisms. Accordingly, various bacteria and fungi have evolved to produce siderophores to acquire this vital nutrient. Recent findings reveal mammals also generate their own pool of endogenous siderophores [4], which have led researchers to begin identifying and characterizing their physiological functions. However, advances in this area of research have been significantly hindered by the lack of tools/methodology to study mammalian siderophores *in vivo*. Herein, we employed the CAS assay, a colorimetric method that detects chelation of iron, as an innovative approach to assess the levels urinary siderophores. In this endeavor, we found that the diet, gut microbiota, and inflammatory state of the host significantly modulate siderophore excretion in the urine.

The reduced urinary siderophores in mice fed on compositionally-defined purified diet could be, in part, due to the lack of plant-derived phytochemicals that potentially serve as metabolic precursors for siderophores excreted in the urine. Previous studies have reported that ingestion of plant quinic and shikimic acid increases urinary excretion of catechol [6], whereas the restriction of dietary plant foods reduces urinary catechol by two-fold [36]. Yet surprisingly, our metabolomics analysis via ¹H NMR reveals that citrate, rather than catechol, is the major siderophore in the urine from mice fed on plant-based chow diet. Although we predict the use of GC-MS or LC-MS may allow better detection of catechol and other siderophore candidates, this consideration does not detract the findings which suggest citrate is relatively more abundant and thus was easily detected by the less sensitive NMR-based methodology. Despite so, it remains to be elucidated whether urinary citrate is derived from the diet or also from host/microbiotal metabolic processes (i.e. TCA cycle that

converts acetyl-CoA and oxaloacetate into citrate) and whether citrate may have anaplerotic functions in modulating iron homeostasis.

The gut microbiota may also constitute a major source of urinary siderophores, given their roles in mediating the biotransformation of various nutrients and other compounds in the gut. Indeed, we observed that GF mice displayed 50% reduction in urinary siderophore activity when compared to conventional mice. Our observation parallels a previous study in which urinary citrate levels were reported to be significantly reduced in GF rats [37]. It is possible that microbial siderophores could leak into systemic circulation and thus excreted into urine. However, we posit that the alteration in the urinary siderophores is more likely to be due to the interplay between host and microbiota metabolic activities (e.g. citrate, catechol), rather than directly from gut microbiota (e.g. Ent). Quite unexpectedly, we found that antibiotics treatment somehow increases urinary siderophore activity. It is intriguing to note a similar counter-intuitive result was also observed in regards to urinary citrate levels, which was reported to be increased in antibiotics-treated rats [38]. The disparity between GF and antibiotics-treated mice could be explained, at least in part, by the physiological effects of antibiotics themselves. Antibiotics exposure could potentially disrupt the gut homeostasis and induce host inflammatory responses [34, 38], which offset factors that modulate the pool of urinary siderophores. Indeed, we confirmed that the antibiotics-treated mice in this study displayed elevated systemic and fecal Lcn2, a sensitive biomarker of inflammation.

Given the extent to which iron homeostasis is tightly regulated during hypoferremia of inflammation, we postulated that inflammation may also impact siderophore homeostasis. Intriguingly, we observed significant upregulation in urinary siderophore excretion (24 h) during conditions of LPS-induced acute systemic inflammation and DSS-induced acute colitis. Yet on the contrary, mice with chronic inflammation (i.e. *Salmonella* infection) instead displayed reduced levels of urinary siderophores. The acute and chronic states of inflammation appear to differentially modulate the levels of urinary siderophores, although further studies are required to elucidate the underlying mechanism and physiological relevance, including their potential role in facilitating iron excretion via urine.

Taken together, our study herein demonstrated the utility of CAS assay in measuring urinary siderophores in response to the diet, gut microbiota and inflammation. One of the major limitations of CAS assay, however, is that only iron-free siderophores can be detected, but not the iron-bound and total siderophore fractions. Furthermore, CAS assay only detects the cumulative effects of a group of siderophores, and thus requires the use of supplementary methodology (i.e. NMR, LC-MS or GC-MS) in order to identify individual siderophores. Despite our observation that dietary iron but not other vital nutrients (e.g. vitamin A, D and selenium) can modulate urinary siderophore levels, the limitation with CAS assay preclude us from distinguishing whether the changes are due to: (1) decreasing ratio of 'iron-free' to 'iron-bound' siderophore fractions, or (2) reduction in the overall total pool of urinary siderophores. Regardless of these limitations, we note that CAS assay has the potential to be further developed as an innovative methodology that could advance the field of mammalian siderophore research. Furthermore, the CAS assay is fast, inexpensive and non-invasive method which can be exploited for rapid prescreening to diagnose iron dysregulation and additionally microbiota alternation and inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Iron chelation properties of diverse siderophores

Indicated concentrations of EGCG, Ent, citrate, pyrocatechol, DFO, PYO and FCM were assayed for iron chelating capability in CAS liquid assay. (A) Line graph displays the % iron chelation of different siderophores after 20 min of incubation at room temperature. (B-D) Line graphs represent a time-dependent % iron chelation for slow iron chelators DFO (B), PYO (C) and FCM (D). Orange colored chromogen was read at 630 nm at indicated time points.

Fig. 2. EGCG increases urinary siderophores

Six weeks old female WT mice (n=5) were given EGCG orally (50 mg/kg BW). (A) Bar graph and image represent urinary siderophores activity performed by CAS liquid assay (mol/g creatinine) and CAS plate assay at indicated time points. To examine whether the change in CAS reagent color/O.D. is specifically due to chelation of iron, urine was saturated with known concentration of Fe³⁺ in a dose dependent manner. (B) Bar graph displays iron saturation assay for urine collected at indicated time points. Results are represented as mean ± SEM. *Tukey's* post hoc test **p* < 0.05.

Fig. 3. Purified diet decreases urinary siderophores

Four weeks old male WT mice (n=4-5) were maintained on either purified or chow diet for 8 weeks. Urine and serum were collected for analysis. (A) Bar graph and image represent urinary siderophores activity by CAS liquid (mol/g creatinine) and plate assays respectively, and (B) Iron saturation assay (mol/g creatinine). (C) Serum total iron levels (D) Urinary ¹H NMR analysis (E) Citrate levels were indicated as relative values analyzed via NMR. For HFD study, six weeks old male WT mice (n=4) were maintained on HFD for 3 weeks. (F) Urinary siderophores and (G) Serum total iron were measured. Results are represented as mean ± SEM. Unpaired *t*-test **p* < 0.05.

Fig. 4. Iron overload increases urinary siderophores

Four weeks old WT male mice ($n=4-5$) were fed on iron adequate diet (Fe-A), iron deficient diet (Fe-D), or iron excess diet (Fe-E) for 8 weeks. Urine and serum were collected for analysis. (A) Serum total iron (B) Bar graph and image represents urinary siderophores quantification by CAS liquid and plate assays, respectively. (C) Iron saturation assay. (D) Relative citrate levels measured by ^1H NMR analysis. To study the genetic iron overload model, urine was collected from 8 weeks old male Hfe-KO mice and their WT littermates ($n=5-6$). (E) Bar graph and image display the urinary siderophores activity by CAS liquid and plate assays, and (F) Iron saturation assay. For systemic iron administration study, mice ($n=5$) were treated with Fe^{2+} (25 mg/kg BW) intraperitoneally. (G) Urinary siderophores was collected at 4 and 24 h post iron treatment. Results are represented as mean $\pm$ SEM. Unpaired t -test (E) and Tukey's post hoc test (A, B, D, G) $*p < 0.05$

Fig. 5. Gut microbiota affects urinary siderophores

Urine from eight weeks old male germ-free (GF) mice and conventional mice was analyzed for siderophores (A) CAS liquid and plate assays, (B) Iron saturation assay. Eight weeks old male WT mice ($n=5$) were fed with either autoclaved or regular lab chow diet for one week. (C) Urinary siderophore levels by CAS liquid assay. (D) Urinary siderophores of mice treated with broad spectrum antibiotics and, (E) Iron saturation assay (F) Serum total iron was estimated in serum. Results are represented as mean $\pm$ SEM. Unpaired t -test (A, C, F) and *Tukey's* post hoc test (D) * $p < 0.05$

Fig. 6. Inflammation modulates urinary siderophores

Urinary siderophore after analyzed by CAS liquid assay (A) LPS treatment (B) DSS colitis (C) *Salmonella* infection. Results are represented as mean $\pm$ SEM. Unpaired *t*-test (A, B) and *Tukey's* post hoc test (C) * $p < 0.05$