Dietary Zinc Alters the Microbiota and Decreases Resistance to *Clostridium difficile* Infection

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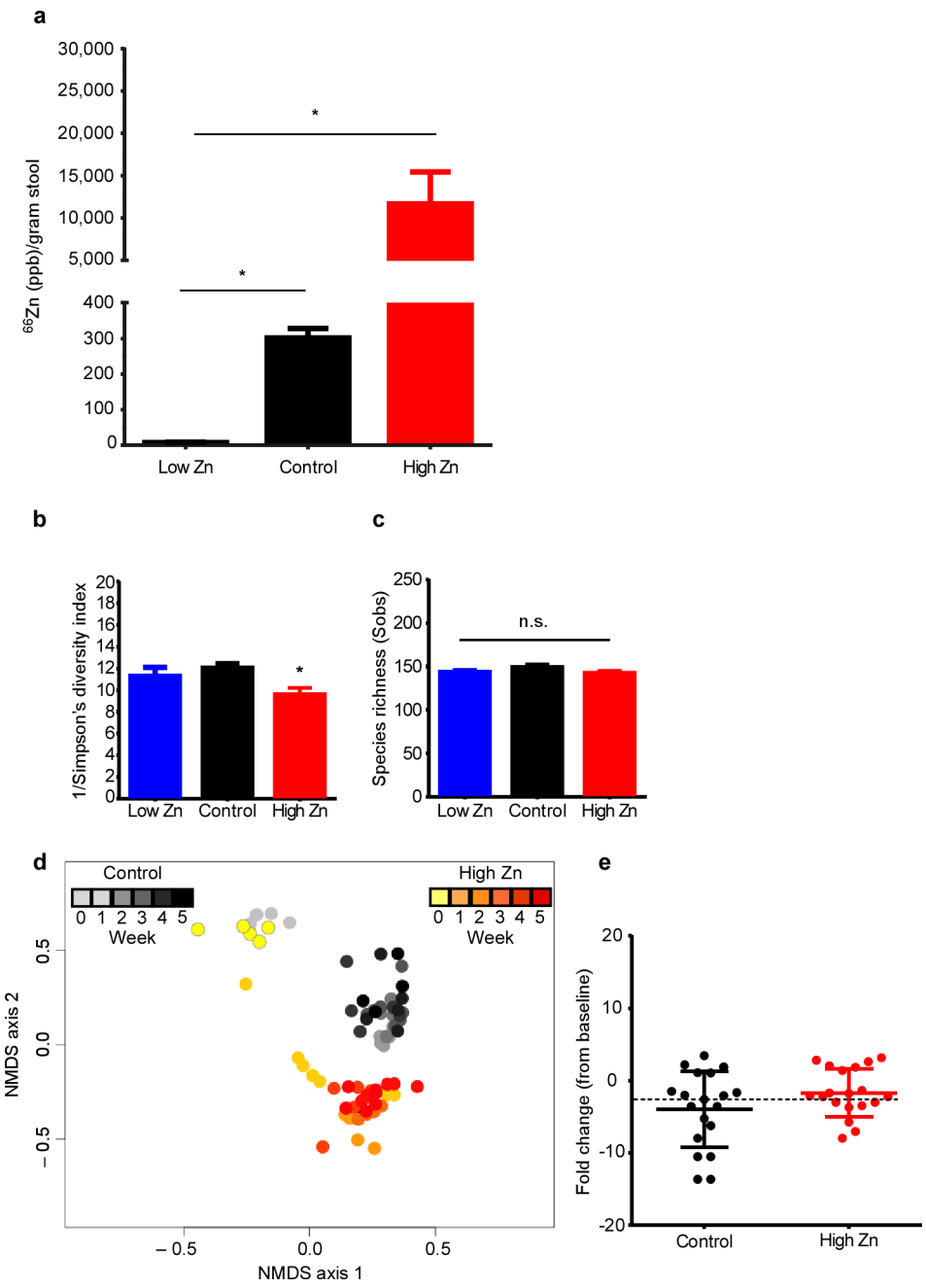
Supplementary Materials:



Supplementary Fig. 1. **The impact of dietary Zn on the gastrointestinal tract and gut microbiota**. Zn concentration in the stool of mice fed altered Zn diets (**a**). ICP-MS was performed on stool samples from mice fed altered Zn diets for five-weeks (n=5/group). Inverse Simpson's diversity for mice fed low Zn,

control, or high Zn diets (**b**). Species richness measured by Sobs (number of observed OTUs) for each altered Zn diet (**c**). NMDS plot depicting time course of diet mediated microbiota alterations (**d**). Grey scale indicates samples from control diet. Red scale indicates samples from High Zn diet. 16S rRNA gene copy number following dietary Zn alterations (**e**). Fold change is calculated relative to baseline microbiota samples. Data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-Whitney test.

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42 **Supplementary Fig. 2**. **Model of diet manipulation and *C. difficile* infection**. Mice are put on altered
43 Zn diets at four weeks of age. Five weeks of diet manipulation is followed by cefoperazone treatment in
44 the drinking water. Mice are infected via gavage with 10^5 spores of *C. difficile* strain 630 or R20291.
45 Cefoperazone concentration and length of infection following gavage varies as described in text and
46 methods.

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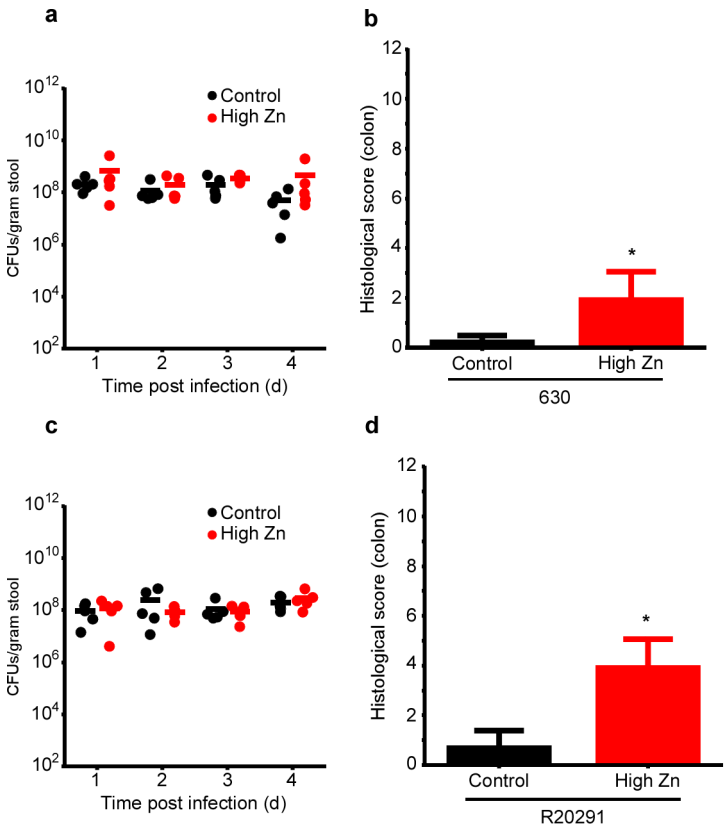
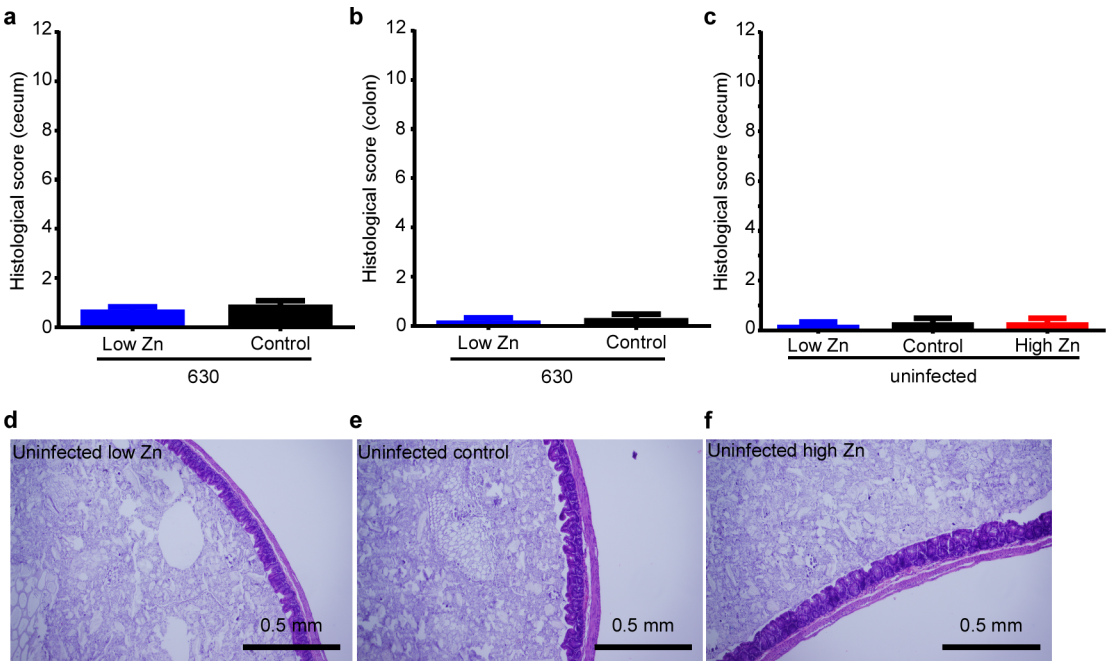


Supplementary Fig. 3. The impact of dietary Zn on *C. difficile* colonization and colitis. CFU analysis (**a**) and blinded histology scoring of colons (**b**) from mice fed a high Zn or control diet and infected with *C. difficile* strain 630 (n=5/group). CFU analysis (**c**) and blinded histology scoring of colons (**d**) from mice fed a high Zn or control diet and infected with R20291 (n=5/group). All infections were performed on susceptible mice pre-treated with 0.5 mg/ml cefoperazone. Blinded histological scores were performed four days post-infection. CFU data are represented as mean and histological data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-Whitney test.



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60 **Supplementary Fig. 4. Dietary Zn alterations do not impact pathogenesis in mice in the absence of**
61 ***C. difficile***. Blinded histological scoring from ceca (n=10/group) (**a**) or colons (n=5/group) (**b**) of mice
62 infected with *C. difficile* strain 630 following 5-weeks of low Zn or control diets (n=10/group). Blinded
63 histology scoring of ceca from mice fed a low Zn, control, or high Zn diet prior to infection (**c**)
64 (n=5/group). Representative H&E stained images from mice fed the low Zn (**d**), control (**e**), or high Zn
65 diet (**f**). Scale, 0.5 mm. All data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-
66 Whitney test. Images are representative of 5 replicate ceca per group.

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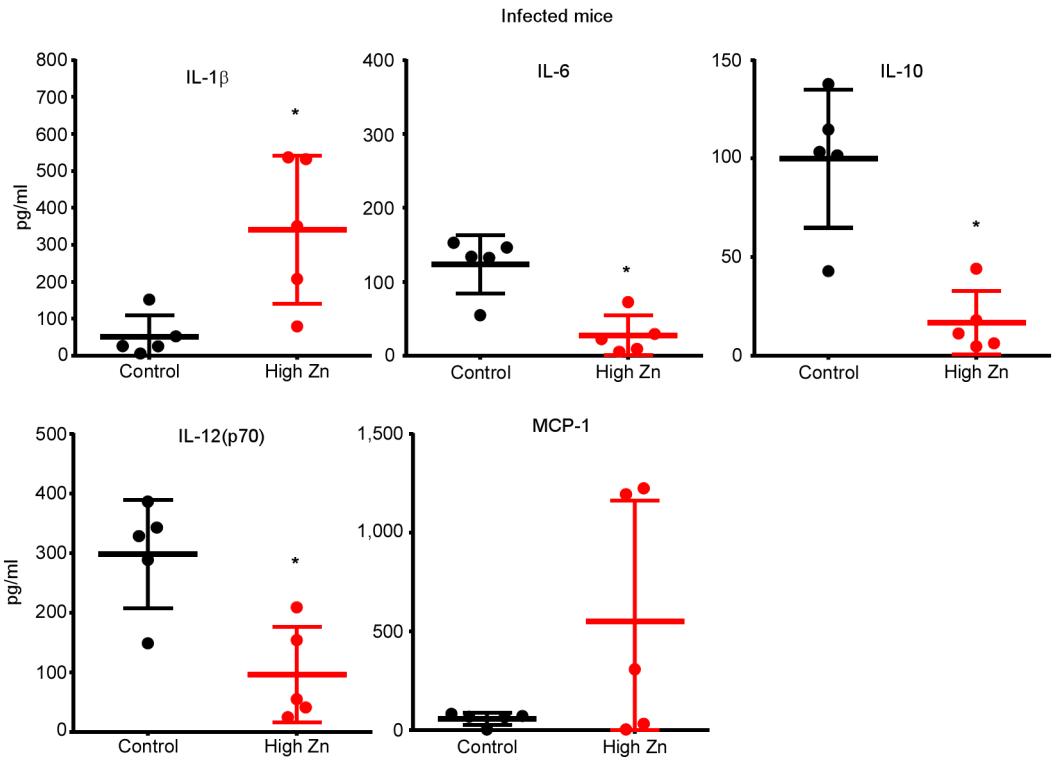
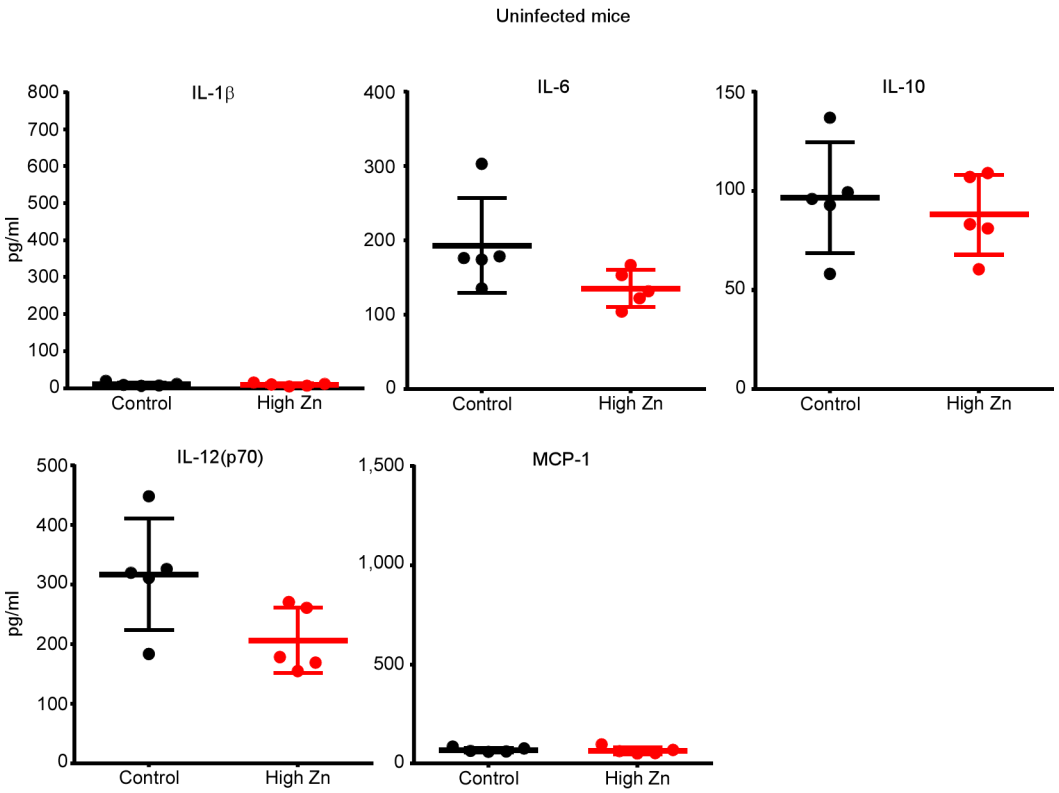
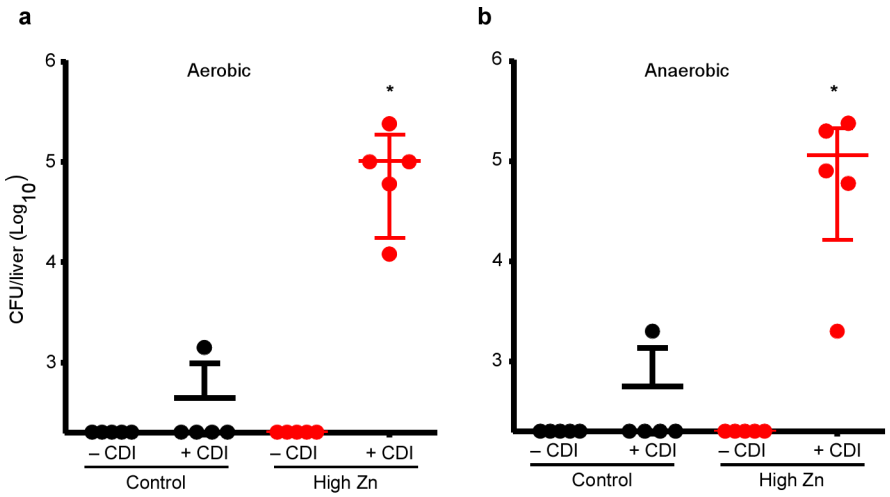


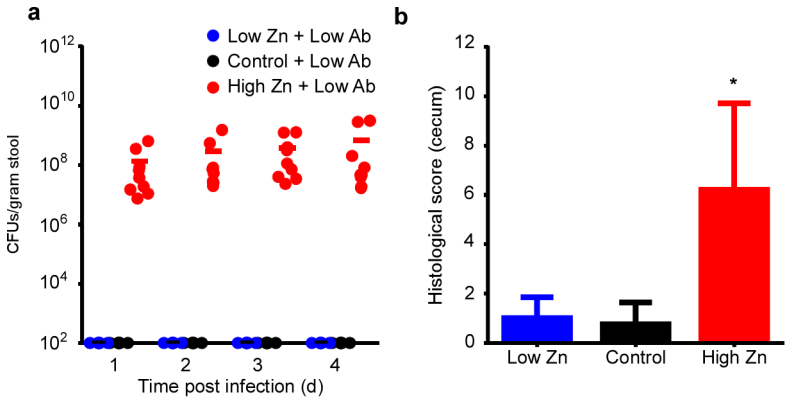
Supplementary Fig. 5. Cytokine production following dietary Zn alteration and infection with *C. difficile*. Cytokine levels were measured from ceca of mice fed either the high Zn or control diet and infected with *C. difficile* strain R20291 (n=5/group). Whole ceca were frozen, homogenized, and normalized to total protein content. Cytokine levels were measured using the Luminex Flexmap 3D platform. All data are represented as mean ± standard deviation. * $P < 0.05$; by Mann-Whitney test.



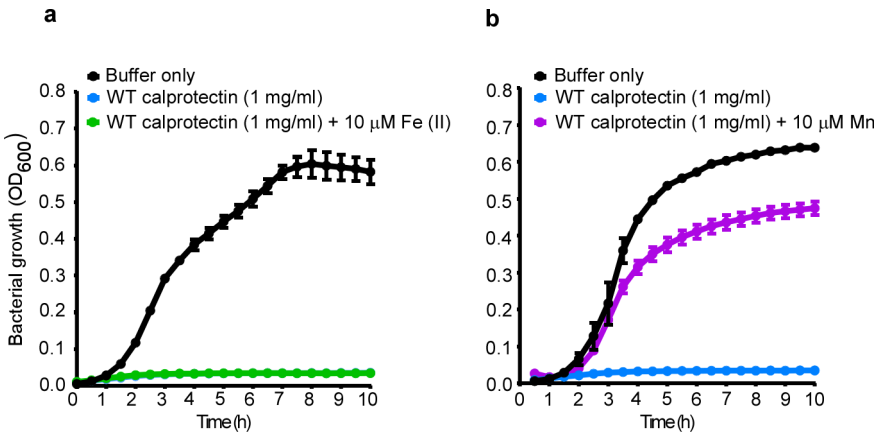
Supplementary Fig. 6. Cytokine production following dietary Zn alteration in the absence of CDI. Cytokine levels were measured from ceca of uninfected mice fed either the high Zn or control diet (n=5/group). Whole ceca were frozen, homogenized, and normalized to total protein content. Cytokine levels were measured using the Luminex Flexmap 3D platform. All data are represented as mean ± standard deviation. * $P < 0.05$; by Mann-Whitney test.



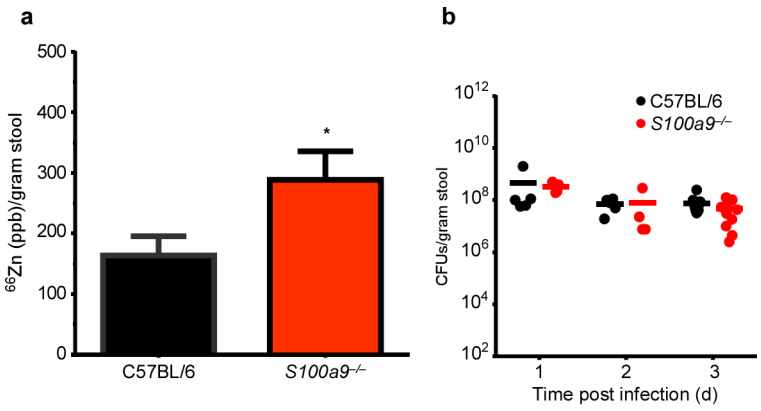
Supplementary Fig 7. Quantification of gut microbiota translocation during CDI. Livers were harvested prior to infection (– CDI) or five-days post-infection (+ CDI) and bacterial burden was quantified under aerobic (**a**) and anaerobic (**b**) conditions (n=5/group). All data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-Whitney test.



Supplementary Fig 8. The impact of dietary Zn alterations on susceptibility to CDI. CFUs were quantified following dietary Zn alterations and low level antibiotic treatment (0.01 mg/ml cefoperazone) (n=9/group) (**a**). Blinded histological scores quantified four-days post low-level cefoperazone infection with R20291 (**b**) (n=9/group). Data shown for control and high Zn treatment groups are the same as Figure 3. CFU data are represented as mean and histological data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-Whitney test.



Supplementary Fig 9. Rescue of calprotectin mediated growth inhibition with metal supplementation. Bacterial growth is shown for *C. difficile* strain R20291 grown in the presence of 1 mg/ml recombinant calprotectin. Medium was supplemented with 10 μM FeSO4 (**a**) or $MnCl_2$ (**b**). Treatment with WT calprotectin (1 mg/ml) resulted in no growth.



Supplementary Fig 10. Calprotectin-deficient mice have increased fecal Zn levels. Zn levels were quantified using ICP-MS from eight-week old wild type C57BL/6 or calprotectin-deficient (*S100a9*$^{-/-}$) mice fed standard chow diets (n=5/group) (**a**). CFUs were quantified following infection of wildtype or calprotectin-deficient (*S100a9*$^{-/-}$) mice with *C. difficile* strain R20291 (day 1-2, n=5/group; day 3, n=10/group). Mice were fed standard chow diets (**b**). CFU data are represented as mean and ICP-MS data are represented as mean ± standard deviation. * $P < 0.01$; by Mann-Whitney test.

Supplementary Tables

	Calprotectin (median, IQR)	*P* value
Fever Yes (n=14) No (n=11)	 5.45 (2.84-22.27) 10.75 (3.48-172.00)	0.55
Blood in stools Yes (n=5) No (n=20)	 171.95 (62.01-647.16) 5.10 (2.85-18.03)	0.023*
Elevated WBC (>15,000) Yes= 5 No= 19 (1 not done)	 66.94 (13.8-736.26) 3.82 (2.84-22.26)	0.0116*
Low albumin (<2.5) Yes= 2 No= 13 (10 not done)	 1006.9 (13.79-2000.00) 6.14 (3.82-62.01)	0.1619

Supplementary Table 1. Relationship between fecal calprotectin and signs of severe CDI in pediatric patients. Fecal calprotectin was measured in twenty-five pediatric patients with CDI and levels were compared between patients with signs of mild and severe CDI. * $P < 0.05$; by Mann-Whitney test.