

SUPPORTING INFORMATION EXPERIMENTAL SECTION

TiO₂NPs, SiO₂NPs and ZnONPs Dose Calculations

Briefly, the daily intake of TiO₂NPs was estimated to be 10¹¹-10¹³ particles per meal¹⁸. Considering that the total intestinal surface area is approximately 2×10⁶ cm, ingesting 10¹³ particles the small intestine will expose the intestine to 10⁶ particles/cm²⁴⁶. In the case of SiO₂NPs, it was found that adults may ingest around 35 mg of fine (0.1-1 μm) or ultrafine (<100 nm) silicate per day¹⁹, which means that the human small intestine would be exposed to 4.67×10⁸ particles/cm² approximately⁴⁵. In the case of exposure doses of ZnONPs, these were extrapolated from the content of Zn released from cans into the food matrix (10 mg) assuming that a person consumes 1000 calories from canned food. Then, the number of NPs per meal were estimated to be approximately 1×10⁹ particles/cm²¹⁵.

Ion Release

ZnO- and Fe₂O₃NP are amphoteric, and this means that they can behave differently depending on the pH of the solution that they are suspended in. The ZnO- and Fe₂O₃NP can readily change between electron donor and acceptor and are more prone to release ions when they are placed in acidic solutions^{91,92}. In order to evaluate the differences between the ZnO- and Fe₂O₃NP that have been subjected to an *in vitro* digestion compared to those that are suspended in 18 MΩ DI water, samples of the digested supernatant of ultracentrifuged solutions were analyzed. The NP suspensions were prepared as described in the *in vitro* digestion and NP exposure sections, then they were diluted to the low, medium and high doses (Table 1) in water. Next, they were centrifuged at 10,600 x g, for 10 minutes (Eppendorf Centrifuge 5417 R, with a rotor F-45-30-11, Brinkmann Instruments, Inc, Westbury, NY). The supernatant was analyzed with ICP-MS.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

After the centrifugation time described in the ion release section, 100 μL of the tested samples was pre-digested in boro-silicate glass tubes with 3 mL of a concentrated ultra-pure nitric acid and perchloric acid mixture (60:40 v/v) for 16 h at room temperature. Samples were then placed in a digestion block (Martin Machine, Ivesdale, IL, USA) and heated incrementally over 4 h to a temperature of 120°C with refluxing. After incubation at 120°C for 2 h, 5 mL of concentrated ultra-pure nitric acid was subsequently added to each sample before raising the digestion block temperature to 145°C for an additional 2 h. The temperature of the digestion block was then raised to 190°C and maintained for at least ten minutes before samples were allowed to cool at room temperature. Digested samples were re-suspended in 20 mL of ultrapure water prior to analysis using ICP-MS (inductively coupled plasma-mass spectroscopy; Agilent ICP-MS 7500 Series, Agilent Technologies, Santa Clara, Ca, USA) with quality control standards (High Purity Standards, Charleston, SC, USA) following every 10 samples. Yttrium purchased from High Purity Standards (10M67-1) was used as an internal standard. To ensure batch-to-batch accuracy and to correct for matrix inference, all samples were digested and measured with 0.5 $\mu\text{g}/\text{mL}$ of Yttrium (final concentration). The concentration of iron is expressed as the number of micrograms per gram of samples. Limit of detection for iron and zinc analysis is 5×10^{-6} to 1×10^{-5} micrograms per gram of sample.

Ionic Controls

Because of the amphoteric nature of ZnO- and Fe₂O₃NP, ionic controls for these NP were used for all experiments. This makes it possible to discern if any toxicological effects seen in the results are due to the nature of NP (reactivity, high surface area/volume ratio) or due to ions being released

from the NP. ZnCl_2 and FeCl_3 were selected because these salts contain Zn and Fe at the same oxidation state as the ZnO- and Fe_2O_3 NP respectively, and because Cl is present in the *in vitro* digestion solutions due to salts. The number of moles of Zn and Fe in ZnO- and Fe_2O_3 NP respectively were calculated, and an equivalent number of moles was calculated from the FeCl_3 and ZnCl_2 salts. The medium and high doses were selected because these are the doses where significant changes are expected in the enzymatic activity experiments (Table S1). The calculations are shown in the supplementary information (Equation S1 and S2).

Cell viability

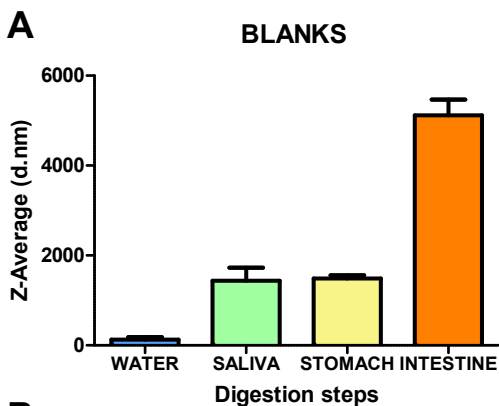
To determine the cytotoxic effects of all four NPs onto the *in vitro* monolayer and to use the selected concentrations for further experiments, the LIVE/DEAD[®] Viability/Cytotoxicity kit *for mammalian cells* (Invitrogen, Ltd.) was used. Here, the polyanionic dye calcein AM is used to stain live cells (ex/em ~ 495 nm/~515 nm), while the ethidium homodimer-1 enters cells the damaged membranes and undergoes fluorescence upon binding to nucleic acids, thereby staining dead cells (ex/em ~ 495 nm/~635 nm) (Invitrogen Molecular Probes, 2005). Briefly, Caco-2/HT29-MTX cell lines were seeded in 96 well-plates at the ratio of 75:25, cell density of 50,000 cells/cm² and was allowed to differentiate for a period of up to 15 days. At day 15, *in vitro* monolayers were exposed to low, medium and high doses of digested TiO_2 NPs, SiO_2 NPs, ZnONPs and Fe_2O_3 NPs for 4 h. After the exposure, the supernatant was discarded and 100 μL of LIVE/DEAD[®] working solution (0.6 μM calcein AM/ 0.9 μM EthD-1 in 1x PBS) was added and the plate was incubated at RT for 30-45 min. A plate reader (Infinite M200PRO, TECAN Trading AG, Switzerland) was used to excite the fluorophores and read its fluorescence emissions. As a qualitative assessment, fluorescent images of all treatments were done using a fluorescent

microscope (OLYMPUS BX43, OLYMPUS CORPORATION) and the Olympus cellSens platform. Image J was used to further edit the images.

Bacterial Viability

Using 24-well plates, both *E. coli* and *L. rhamnosus* at a cell density of 10^3 CFU/mL were exposed to low, medium and high concentrations of digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs diluted in DMEM +10% Hi-FBS. Bacterial cells were left in the incubator for a period of 4 h at 37 °C in 5% CO₂. After the exposure time, the drop plate method was carried out to determine the number of viable bacteria in the well. To harvest the bacterial cells, the cell suspension was homogenized and transferred with a pipette tip into a microfuge tube, serially diluted (using a 0.85% NaCl solution) and drop plated onto the agar plates. Colonies were allowed to develop for a period of 24 h for *E. coli* and 48 h for *L. rhamnosus* at 37 °C in 5% CO₂. *E. coli* viability was determined using Nutrient agar (Becton, Dickinson and Company, Franklin Lakes, NJ) while *L. rhamnosus* viability was determined using Lactobacillus MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ). Three independent experiments were performed using triplicates per treatment (n=9).

SUPPORTING INFORMATION FIGURES



B

POLYDISPERSITY INDEX

NPs	WATER		SALIVA		STOMACH		INTESTINE	
	PdI	SEM	PdI	SEM	PdI	SEM	PdI	SEM
TiO ₂ NPs	0.521	0.035	0.35	0.077	0.83	0.170	0.585	0.207
SiO ₂ NPs	0.72	0.021	0.876	0.062	0.55	0.017	0.601	0.092
ZnONPs	0.584	0.054	0.591	0.241	0.821	0.073	0.683	0.075
Fe ₂ O ₃ NPs	0.154	0.011	0.329	0.018	0.274	0.006	0.389	0.023

Figure S1. Further nanoparticles (NPs) characterization. (A) Hydrodynamic size of the blanks of each digestion step. (B) Polydispersity index (PdI) of TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs in each step of the digestion process was measured by dynamic light scattering (DLS). Results are represented as mean ± SEM.

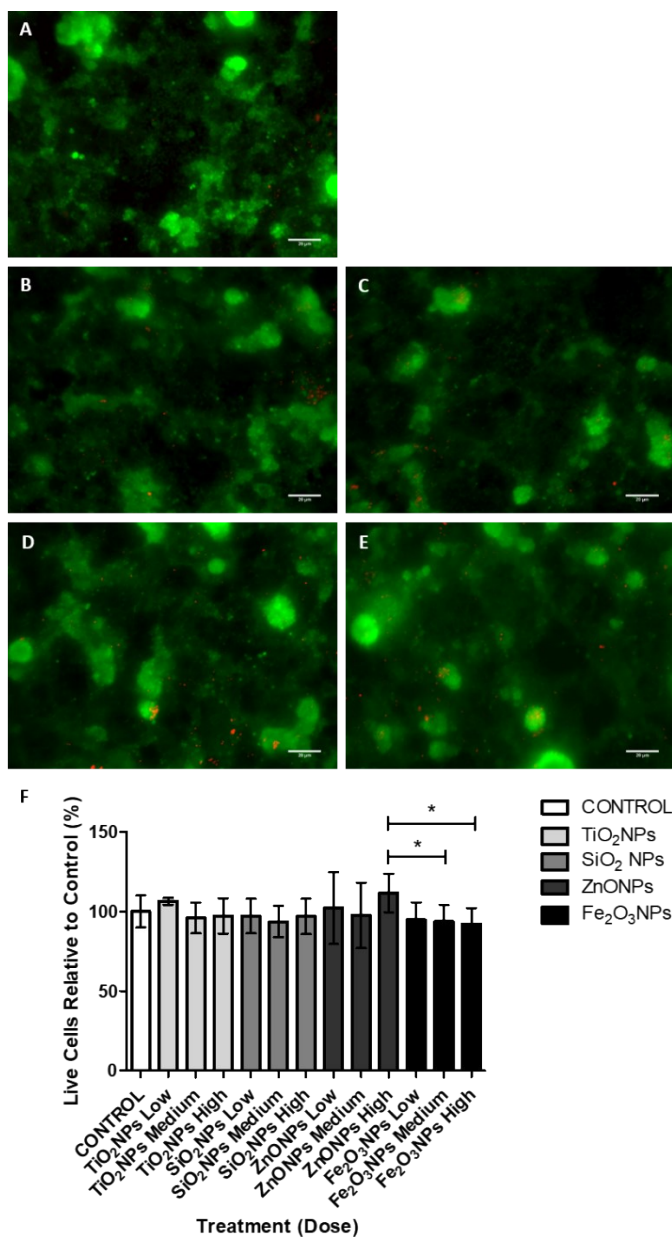


Figure S2. Mammalian cell viability. Fluorescent microscopy images of Caco-2/HT29-MTX-E12 barrier after 4 h of exposure to *in vitro* digested and physiologically relevant doses of negative control (A), TiO₂NPs (B), SiO₂NPs (C), ZnONPs (D) and Fe₂O₃NPs (E), respectively (Scale bars = 20 μm). Calcein AM/Propidium Iodide assay Kit was used, and green/ red represents live and dead cells, respectively. (F) Percentage of live cells relative to control

measured with a fluorescent plate reader. Data represented as mean \pm coefficient of variability (CV%) and analyzed according to a one-way ANOVA with Tukey's post-test. (*) $P < 0.05$.

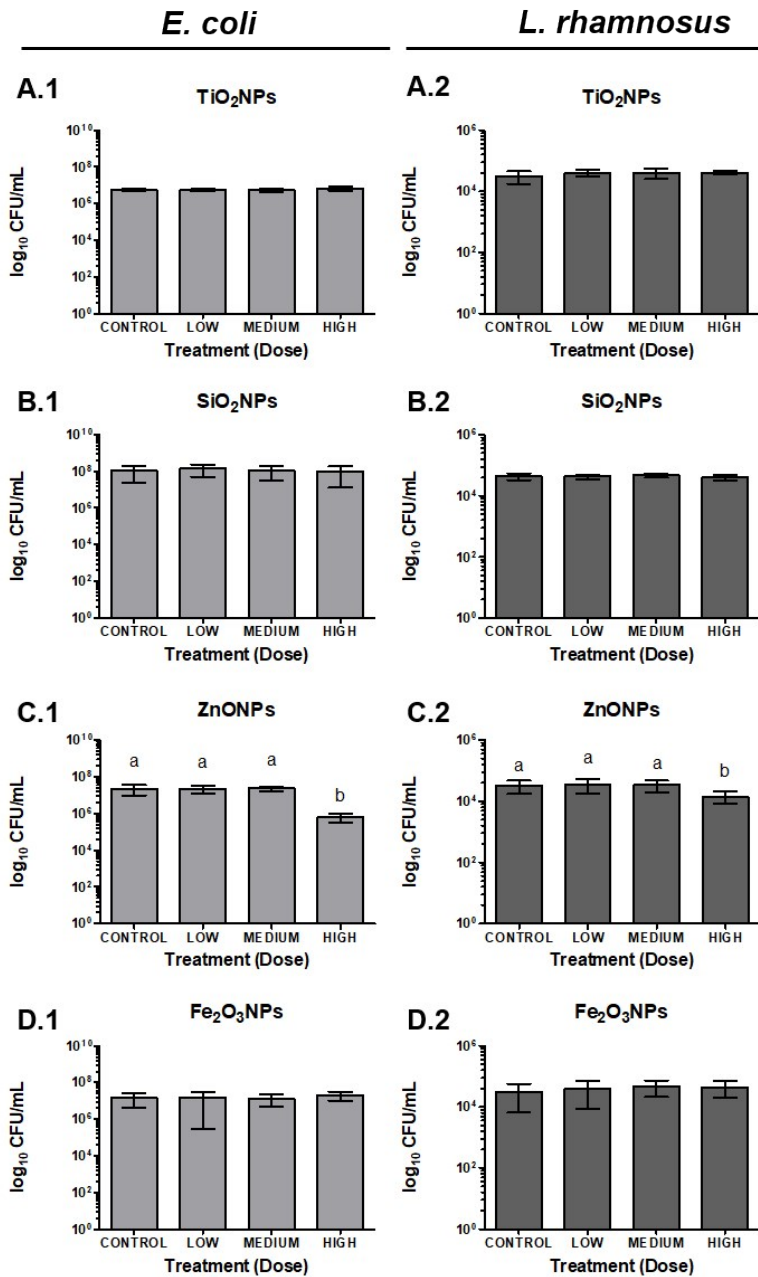


Figure S3. Bacteria viability. Viable counts (CFU/mL) plotted in Log base 10 for *E. coli* (A.1, B.1, C.1 and D.1) and *L. rhamnosus* (A.2, B.2, C.2 and D.2) were performed after 4 h of exposure to *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs, respectively. Results

are represented as mean \pm SEM. Bars that do not share any letter are significantly different according to the one-way ANOVA with Tukey's post-test ($P < 0.05$).

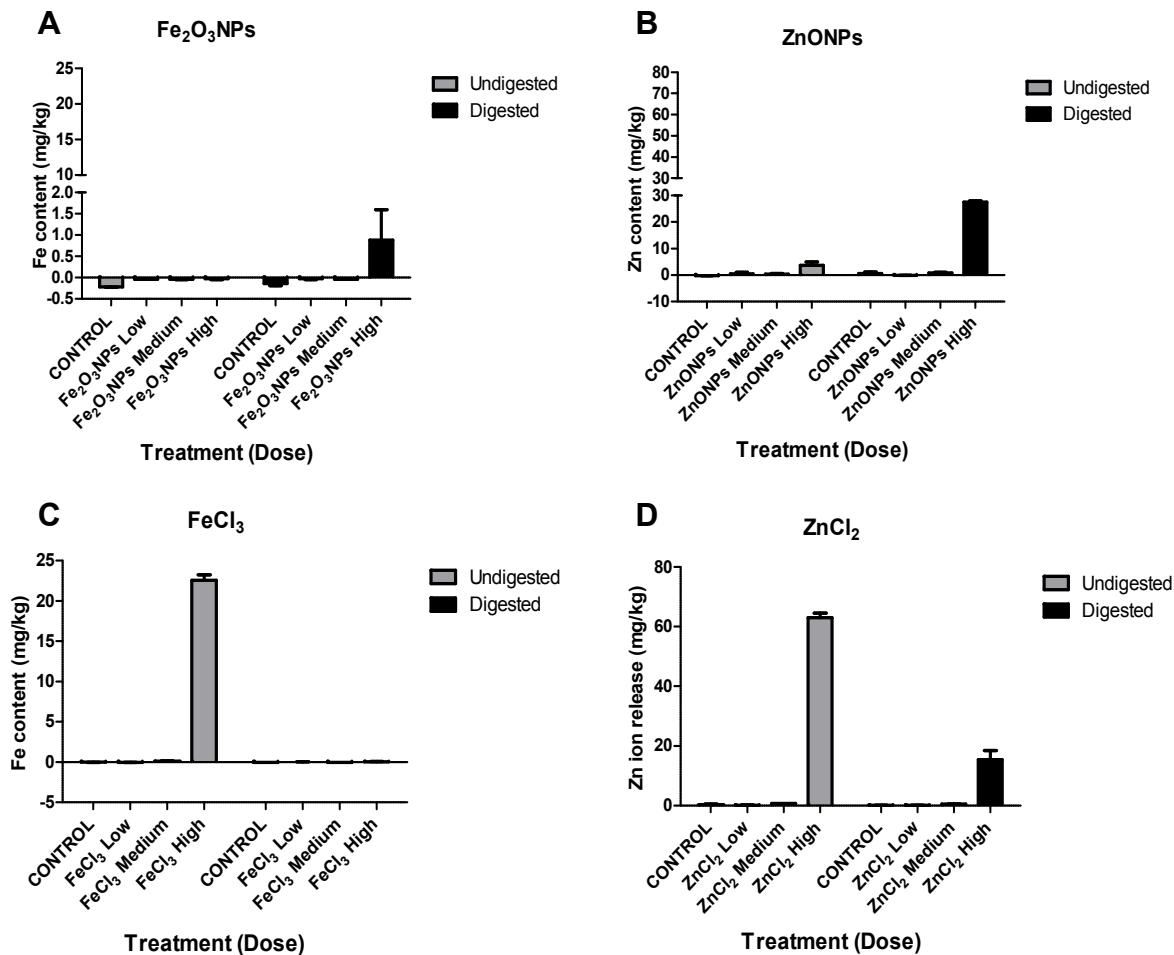


Figure S4. Measurement of the ZnONPs and Fe₂O₃NPs ionic release by ICP-MS.

Undigested or *in vitro* digested (A) Fe₂O₃NPs, (B) ZnONP, (C) FeCl₃ and (D) ZnCl₂ were ultra-centrifuged (10,600 \times g) for 10 min. The supernatant was analyzed with ICP-MS for total ionic Zn or Fe content. Results represent mean \pm SEM.

Equation S1

The highest concentration of ZnONPs is 9.7×10^{-2} mg/mL or 9.7×10^{-5} g/mL;

$$\frac{9.7 \times 10^{-5} \text{ g ZnO}}{1} \times \frac{1 \text{ mol ZnO}}{81.38 \text{ g ZnO}} \times \frac{1 \text{ mol Zn}}{1 \text{ mol ZnO}} = 1.19 \times 10^{-6} \text{ mole Zn}$$

$$\frac{136.286 \text{ g ZnCl}_2}{1 \text{ mol ZnCl}_2} \times \frac{1.19 \times 10^{-6} \text{ mol Zn}}{1} \times \frac{1 \text{ mol ZnCl}_2}{1 \text{ mol Zn}} = 1.6 \times 10^{-4} \text{ g ZnCl}_2$$

Equation S2

The highest concentration of Fe₂O₃ NPs 9.7×10^{-2} mg/mL or 9.7×10^{-5} g/mL;

$$\frac{3.8 \times 10^{-5} \text{ g Fe}_2\text{O}_3}{1} \times \frac{1 \text{ mol Fe}_2\text{O}_3}{159.69 \text{ g Fe}_2\text{O}_3} \times \frac{2 \text{ mol Fe}}{1 \text{ mol Fe}_2\text{O}_3} = 4.76 \times 10^{-7} \text{ mol Fe}$$

$$\frac{162.204 \text{ g FeCl}_3}{1 \text{ mole FeCl}_3} \times \frac{4.76 \times 10^{-7} \text{ mol Fe}}{1} \times \frac{1 \text{ mole FeCl}_3}{1 \text{ mol Fe}} = 7.72 \times 10^{-5} \text{ g FeCl}_3$$

Table S1. Ionic control concentrations derived from medium and high doses of ZnONPs and Fe₂O₃NPs.

Concentration	Zinc			Iron		
	ZnO NP (mg/mL)	ZnO NP moles of Zn	ZnCl ₂ NP (mg/mL)	Fe ₂ O ₃ NP (mg/mL)	Fe ₂ O ₃ NP moles of Fe	FeCl ₃ NP (mg/mL)
High	9.7×10^{-2}	1.19×10^{-6}	0.16	3.8×10^{-2}	4.77×10^{-7}	0.077
Medium	9.7×10^{-4}	1.19×10^{-8}	1.6×10^{-3}	3.8×10^{-4}	4.77×10^{-9}	7.7×10^{-4}

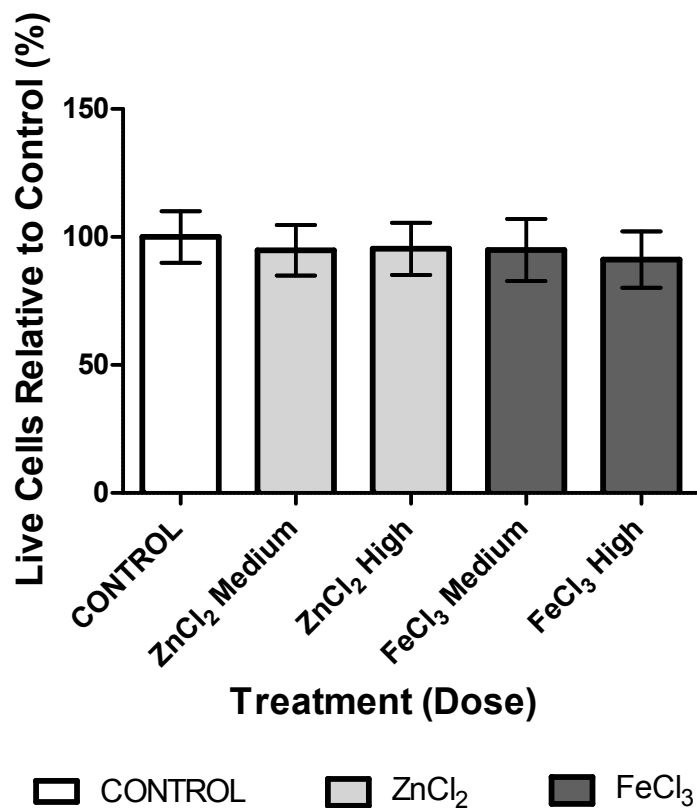


Figure S5. Ionic controls on mammalian cell viability (percentage of live cells relative to control). Calcein AM/Propidium Iodide assay Kit was used. Percentage of live cells relative to control measured with a fluorescent plate reader. Data represented as mean \pm SEM and analyzed according to a one-way ANOVA with Tukey's post-test ($P < 0.05$).

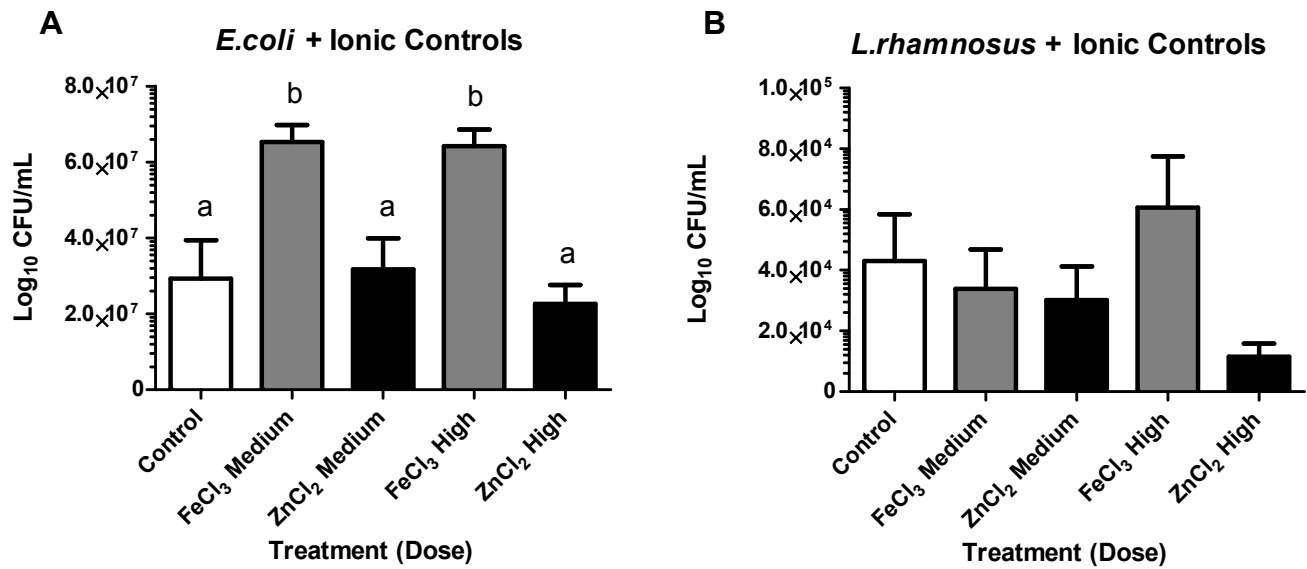


Figure S6. Bacteria viability. Viable counts (CFU/mL) plotted in Log base 10 for *E. coli* (A) and *L. rhamnosus* (B) were performed after 4 h of exposure to both *in vitro* digested FeCl₃ and ZnCl₂. Results are represented as mean ± SEM. Bars that do not share any letter are significantly different according to the one-way ANOVA with Tukey's post-test ($P < 0.05$).

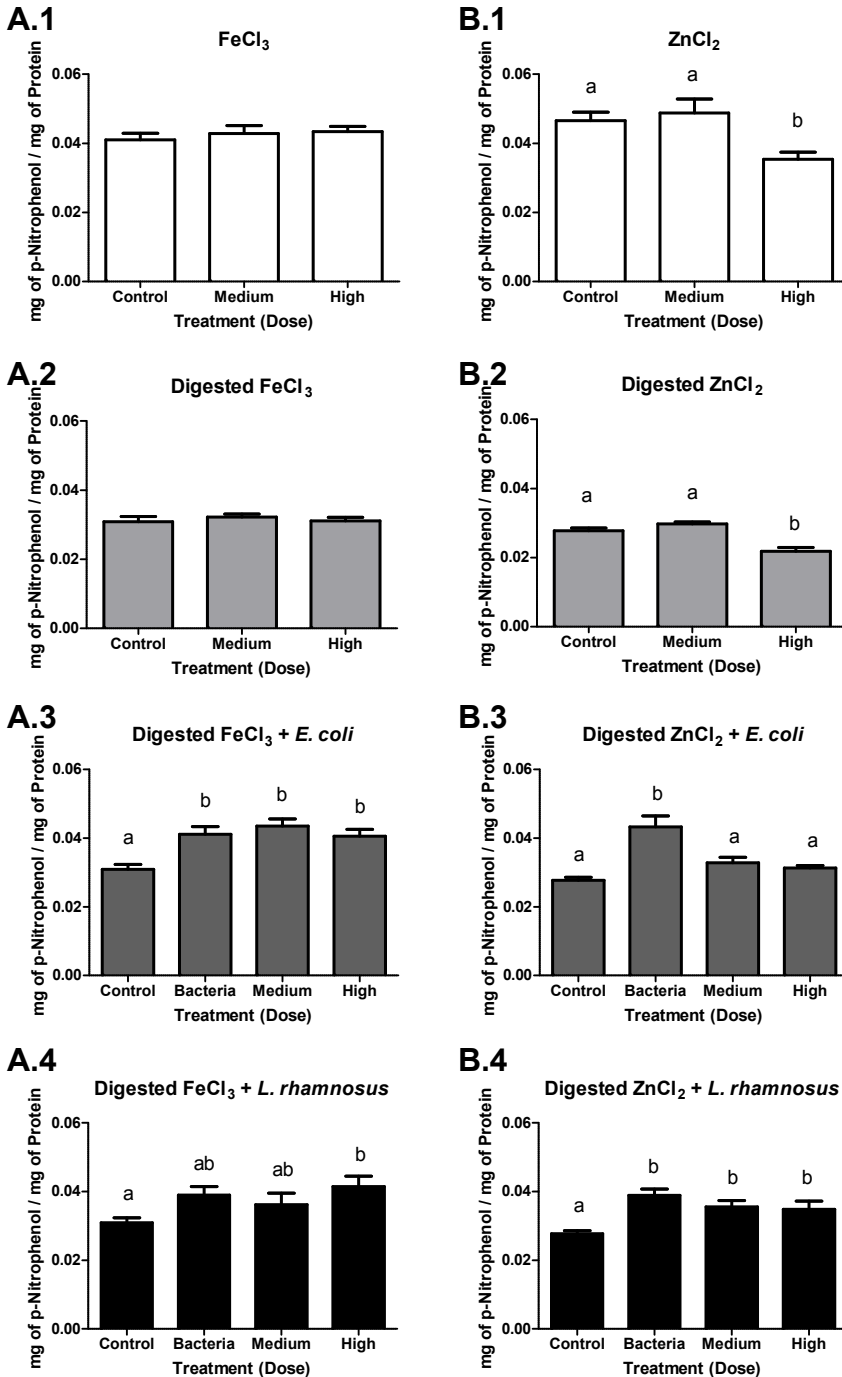


Figure S7. Intestinal alkaline phosphatase (IAP) activity. The activity (mg of p-Nitrophenol/mg of Protein) of IAP was measured after exposing (4h) the Caco-2/HT29-MTX-E12 barrier to undigested FeCl_3 and ZnCl_2 (**A.1** and **B.1**), *in vitro* digested FeCl_3 and ZnCl_2 (**A.2** and **B.2**), and *in vitro* digested FeCl_3 and ZnCl_2 co-exposed with either *E. coli* (**A.3** and **B.3**) or *L. rhamnosus* (**A.4** and **B.4**). “Control” is the untreated group, and “Bacteria” is the group exposed to bacteria only. Data is represented as mean \pm SEM. Bars with no letters in common are significantly different according to a one-way ANOVA with Tukey’s post-test ($P < 0.05$).

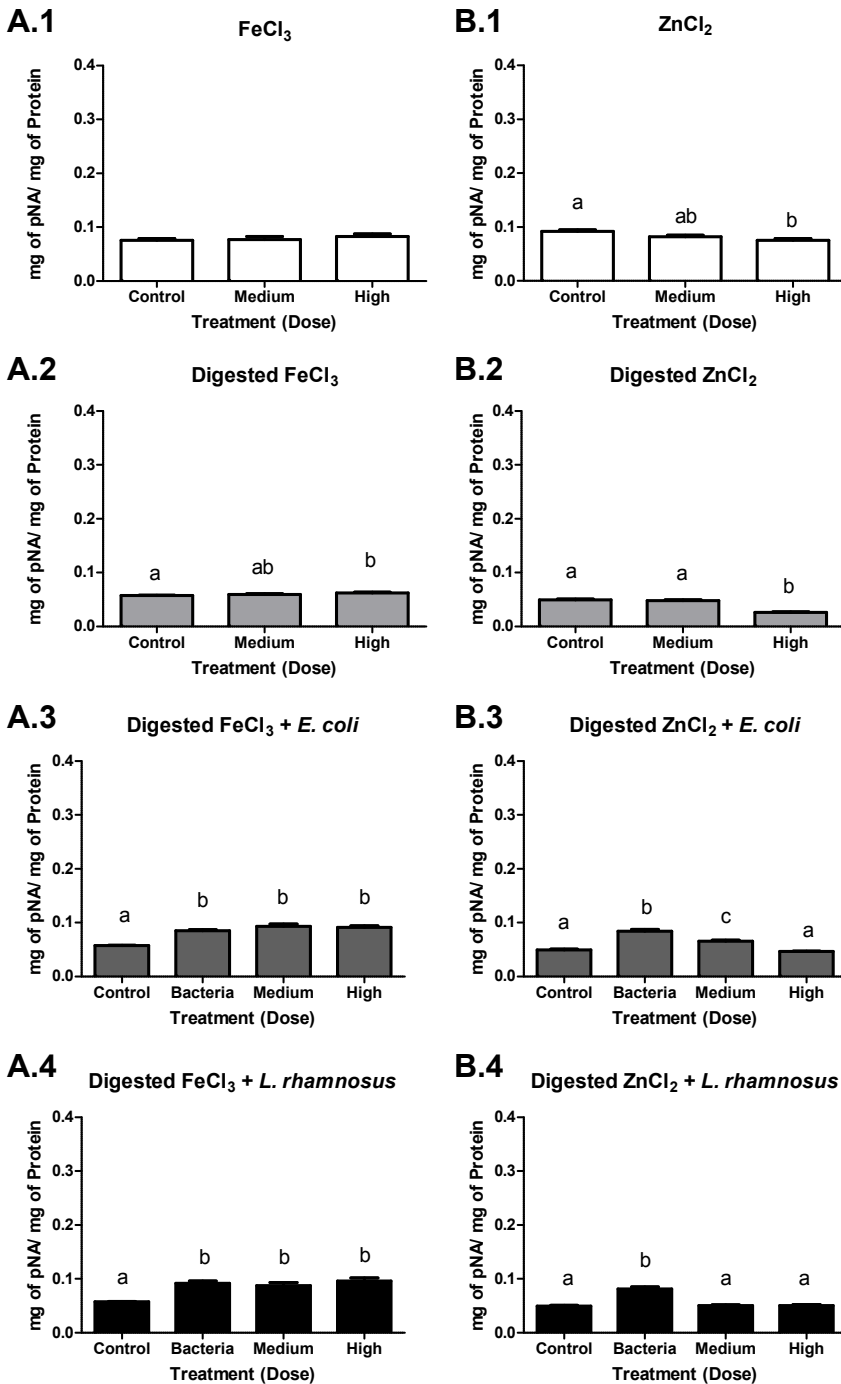


Figure S8. Aminopeptidase-N (APN) activity. The activity (mg of 4-Nitroanilide/mg of Protein) of APN was measured after exposing (4h) the Caco-2/HT29-MTX-E12 barrier to undigested FeCl₃ and ZnCl₂ (A.1 and B.1), *in vitro* digested FeCl₃ and ZnCl₂ (A.2 and B.2), and *in vitro* digested FeCl₃ and ZnCl₂ co-exposed with either *E. coli* (A.3 and B.3) or *L. rhamnosus* (A.4 and B.4). “Control” is the untreated group, and “Bacteria” is the group exposed to bacteria only. Data is represented as mean ± SEM. Bars with no letters in common are significantly different according to a one-way ANOVA with Tukey’s post-test ($P < 0.05$).

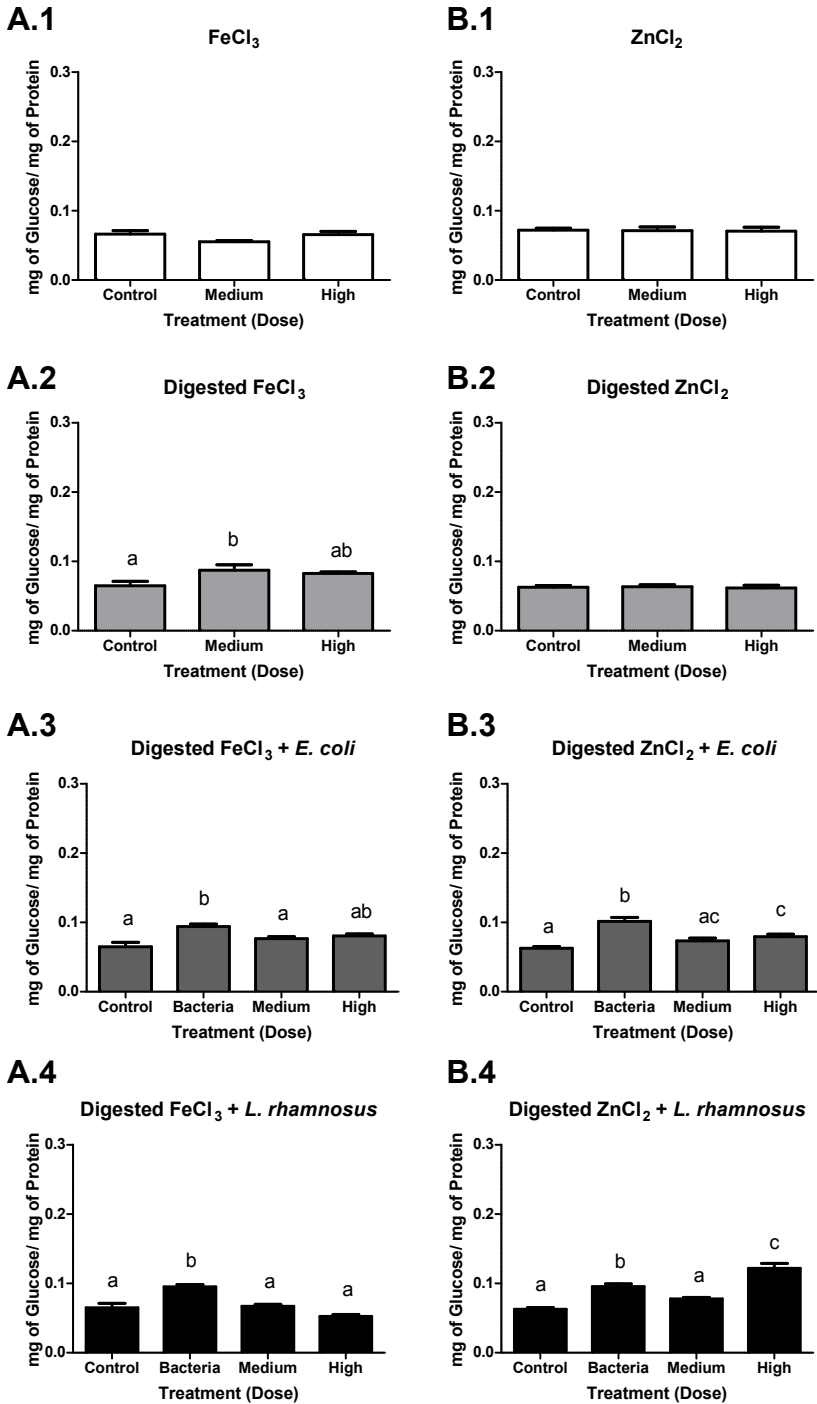


Figure S9. Sucrase Isomaltase (SI) activity. The activity (mg of Glucose/mg of Protein) of SI was measured after exposing (4h) the Caco-2/HT29-MTX-E12 barrier to undigested FeCl_3 and ZnCl_2 (A.1 and B.1), and *in vitro* digested FeCl_3 and ZnCl_2 (A.2 and B.2), *in vitro* digested FeCl_3 and ZnCl_2 co-exposed with either *E. coli* (A.3 and B.3) or *L. rhamnosus* (A.4 and B.4). “Control” is the untreated group, and “Bacteria” is the group exposed to bacteria only. Data is represented as mean \pm SEM. Bars with no letters in common are significantly different according to a one-way ANOVA with Tukey’s post-test ($P < 0.05$).

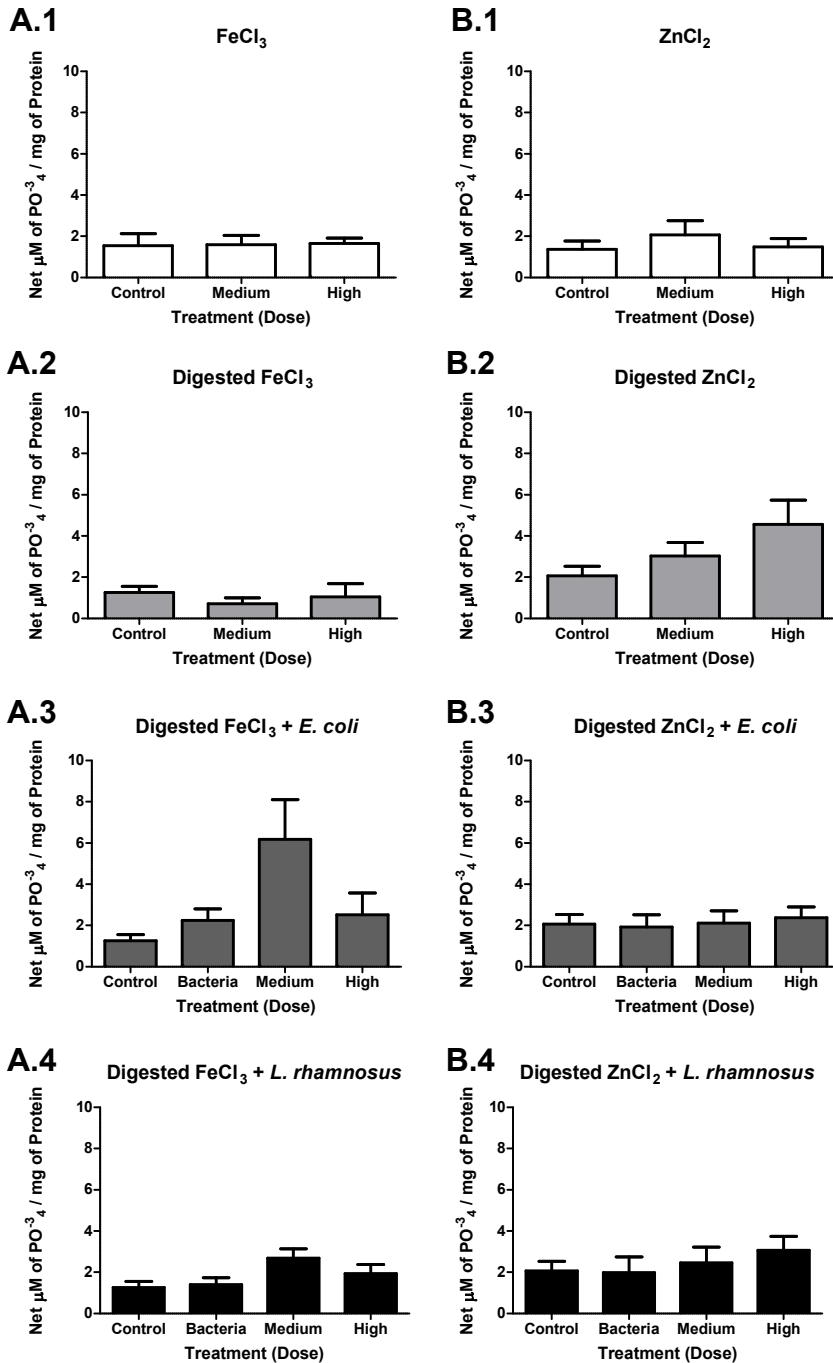


Figure S10. Na^+/K^+ ATPase activity. The activity (net μM of PO_4^{3-} /mg of Protein) of Na^+/K^+ ATPase was measured after exposing (4h) the Caco-2/HT29-MTX-E12 barrier to undigested FeCl_3 and ZnCl_2 (A.1 and B.1), and *in vitro* digested FeCl_3 and ZnCl_2 (A.2 and B.2), *in vitro* digested FeCl_3 and ZnCl_2 co-exposed with either *E. coli* (A.3 and B.3) or *L. rhamnosus* (A.4 and B.4). “Control” is the untreated group, and “Bacteria” is the group exposed to bacteria only. Data is represented as mean \pm SEM. Bars with no letters in common are significantly different according to a one-way ANOVA with Tukey’s post-test ($P < 0.05$).

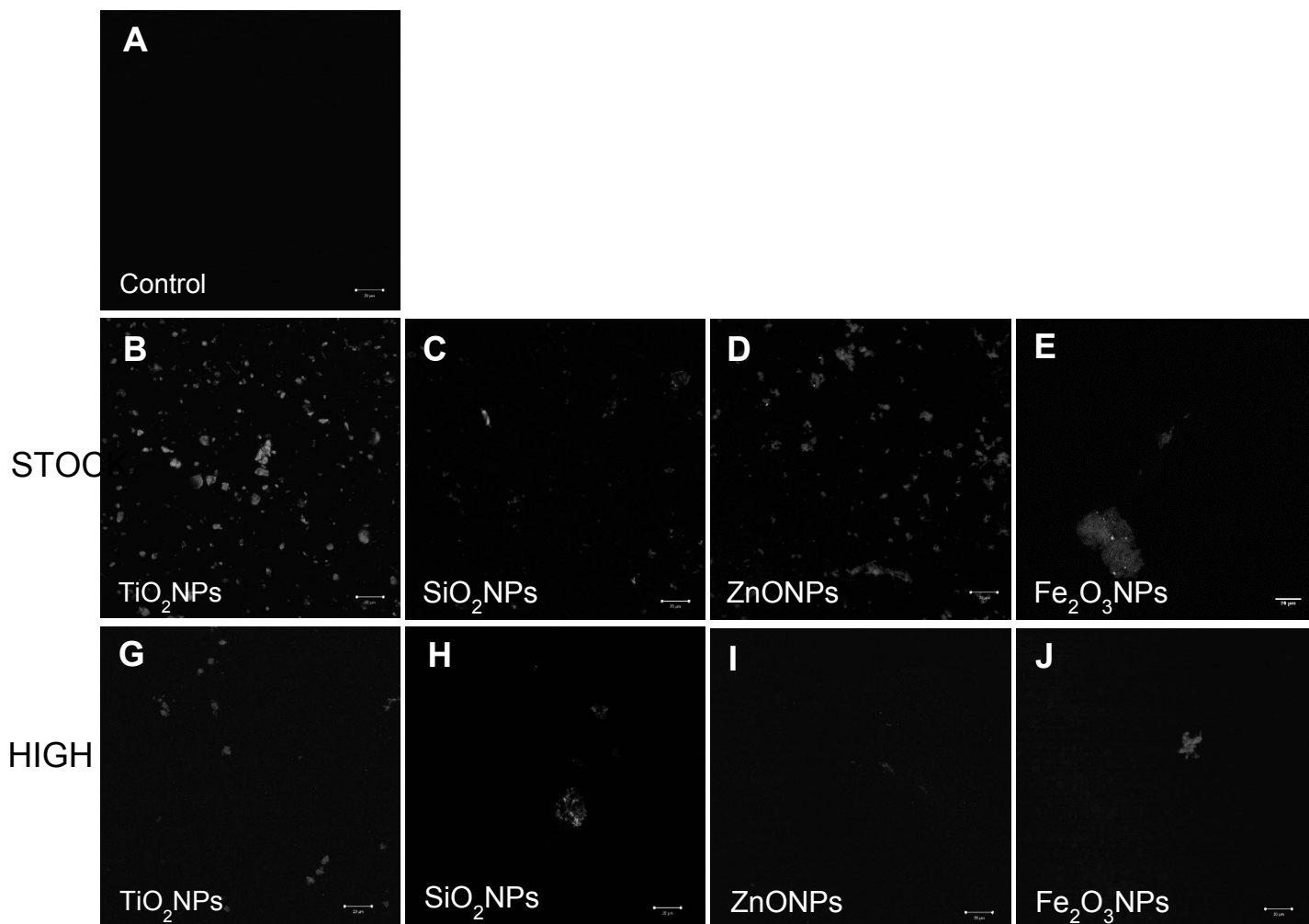


Figure S11. Confocal images (2D) of the NPs reflection. A drop (100 μL) of the NPs stock solution (**B, C, D and E**) and the highest concentration (HIGH []) treatment (**G, H, I and J**) was analyzed by confocal microscopy using the 488 nm laser and the reflection settings. Nanoparticles are shown in a white-grey color (Scale bars of 20 μm).

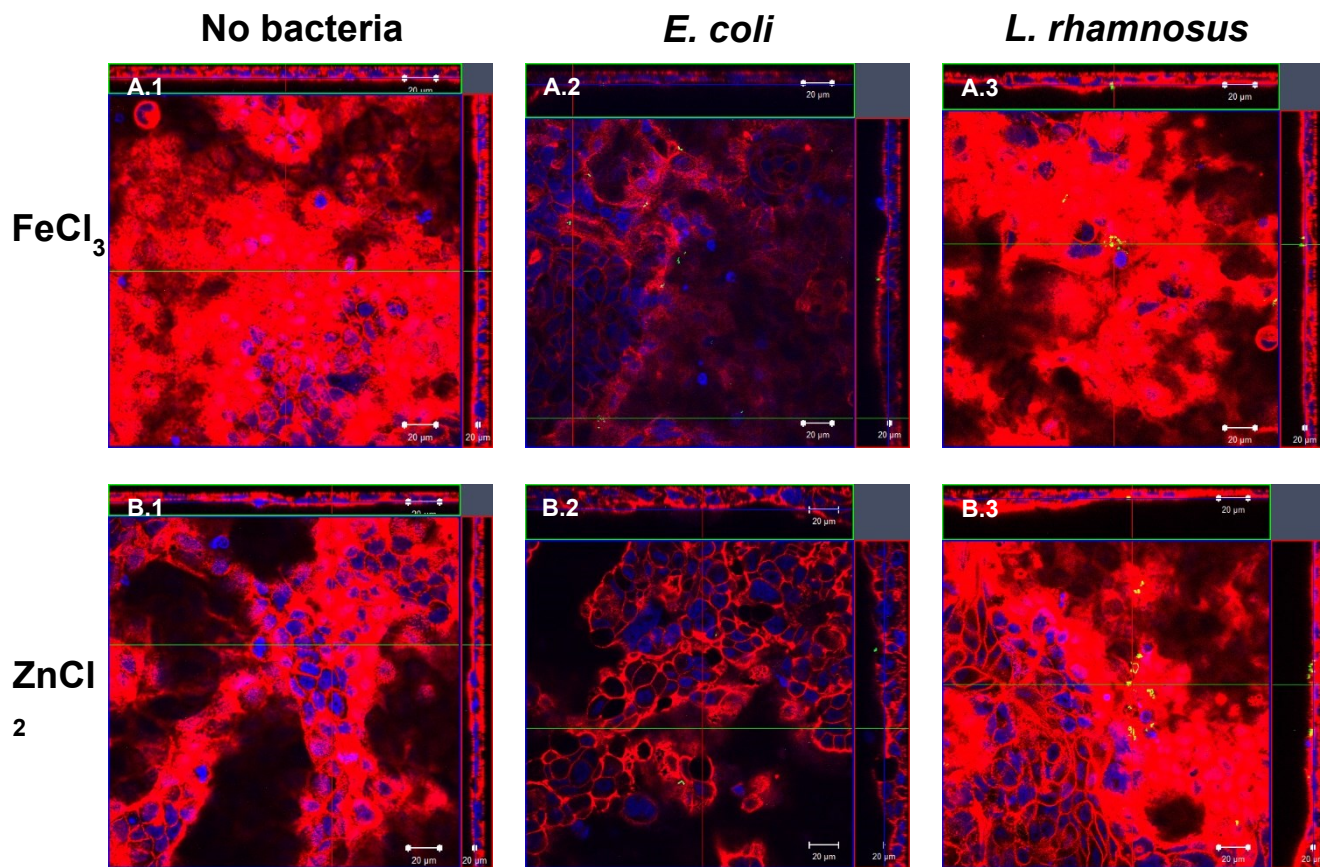


Figure S12. Confocal images (*ortho* images) of the Caco-2/HT29-MTX-E12 *in vitro* barrier. Z-scans were performed after exposing the barrier during 4 h to both digested ionic controls FeCl₃ and ZnCl₂ without bacteria (**A.1 and B.1**), co-exposed with *E. coli* (**A.2 and B.2**) or with *L. rhamnosus* (**A.3 and B.3**). Filaments of actin were marked in red, cell nucleus in blue and both *E. coli* and *L. rhamnosus* in green (Scale bars of 20 μm).

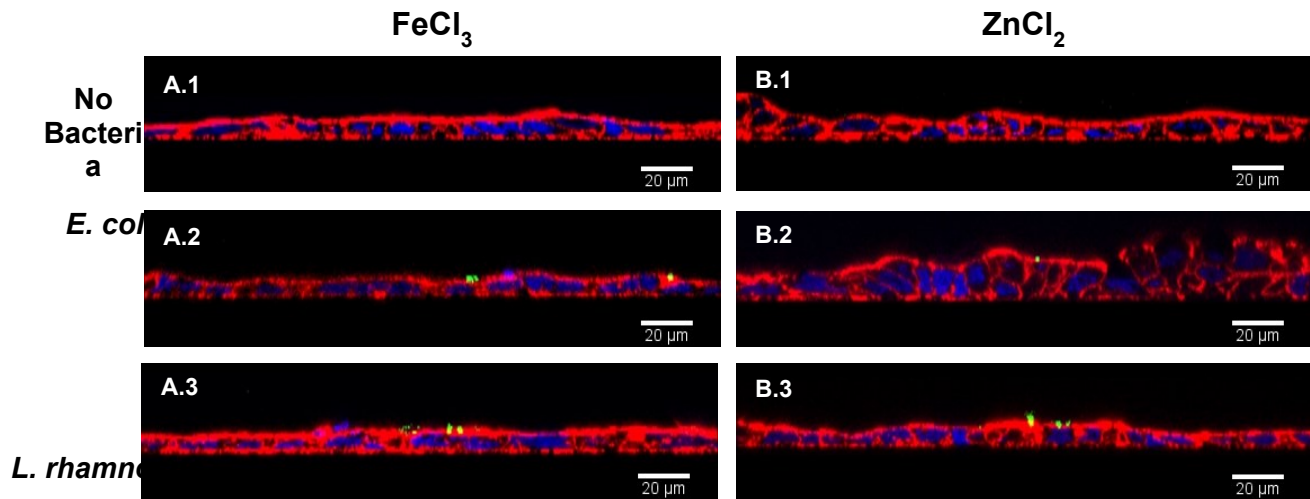


Figure S13. Transversal cuts of the Caco-2/HT29-MTX-E12 *in vitro* barrier. Z-scans were performed after exposing the barrier during 4 h to both digested ionic controls FeCl₃ and ZnCl₂ without bacteria (**A.1 and B.1**), co-exposed with *E. coli* (**A.2 and B.2**) or with *L. rhamnosus* (**A.3 and B.3**). Filaments of actin were marked in red, cell nucleus in blue and both *E. coli* and *L. rhamnosus* in green (Scale bars of 20 μm).

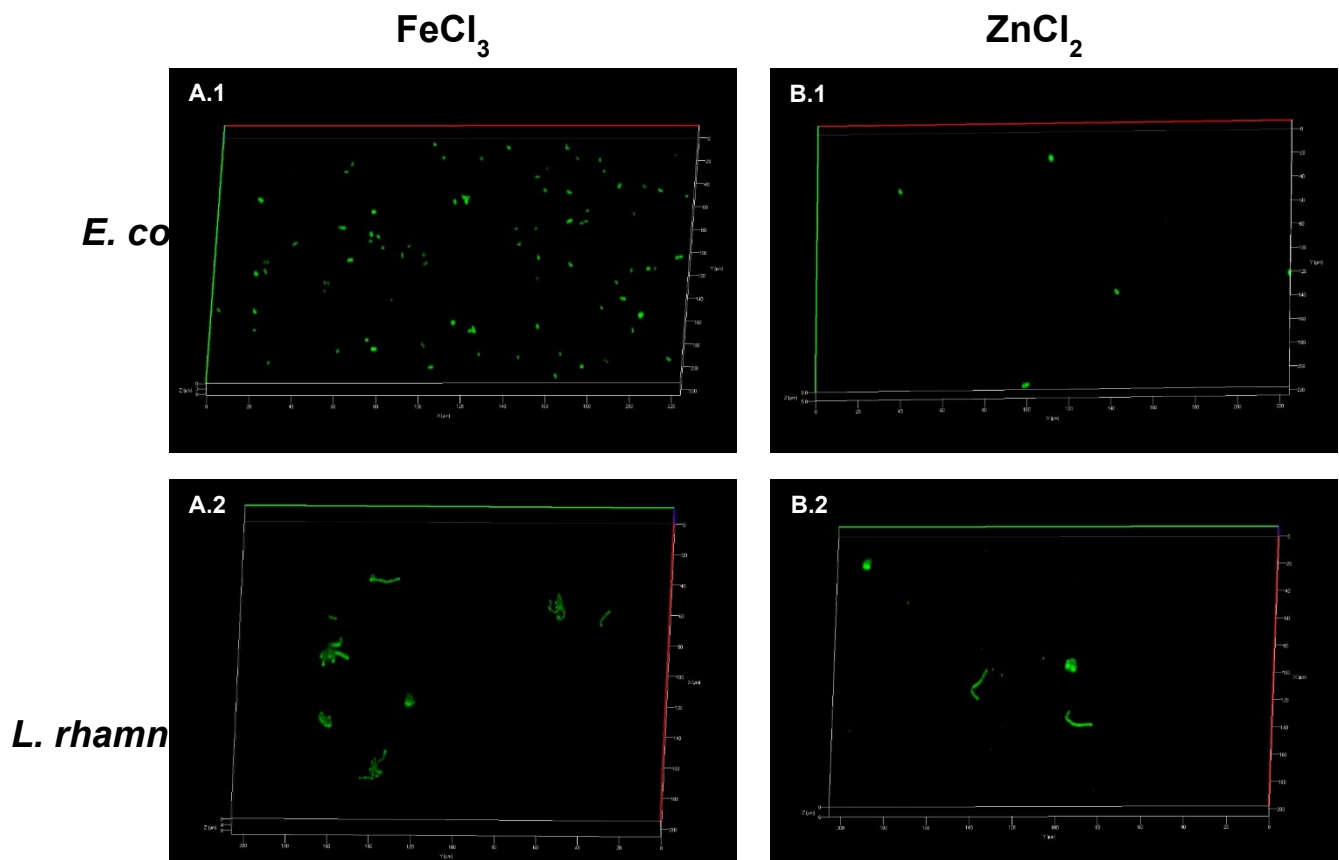


Figure S14. Confocal 3D images of bacteria and ionic controls co-exposures. The Caco-2/HT29-MTX-E12 *in vitro* barrier was co-exposed for 4 h to both digested ionic controls and bacteria. Images of the supernatant containing both FeCl₃ and ZnCl₂ and *E. coli* (**A.1 and B.1**) or *L. rhamnosus* (**A.2 and B.2**), respectively, were taken at the end of the exposure. Both *E. coli* and *L. rhamnosus* are localized in green (Axis marks separated every 20 μm).

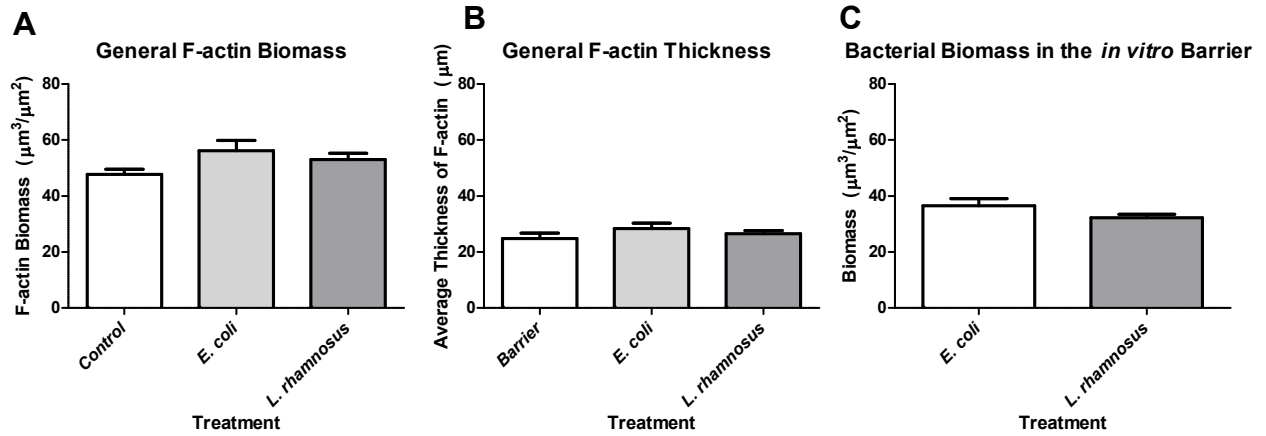


Figure S15. Comstat2 measurement of; (A) F-actin biomass ($\mu\text{m}^3/\mu\text{m}^2$) from the *in vitro* barrier untreated (control) and after 4 h of exposure to *E. coli* and *L. rhamnosus*; (B) F-actin thickness (μm); and (C) bacterial biomass ($\mu\text{m}^3/\mu\text{m}^2$) found attached on the *in vitro* barrier after the 4 h of treatment.