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The Role of Metal Oxide Nanoparticles, *Escherichia coli*, and *Lactobacillus rhamnosus* on Small Intestinal Enzyme Activity

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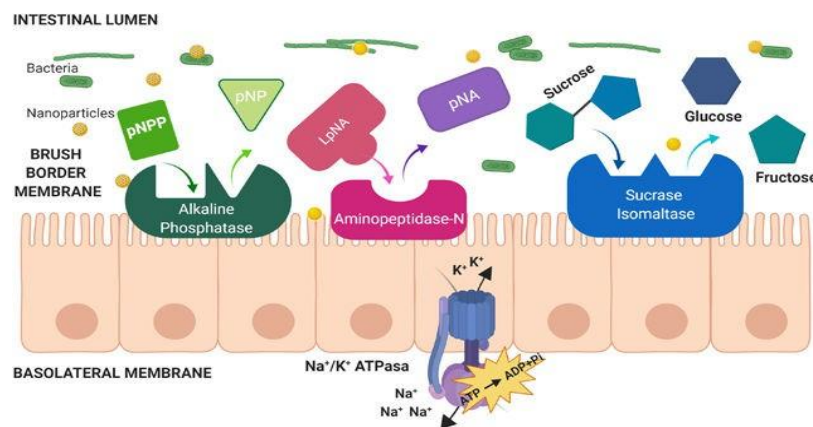
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Abstract

Engineered nanomaterials (ENMs) have become common in the food industry, which motivates the need to evaluate ENM effects on human health. Gastrointestinal (GI) *in vitro* models (e.g. Caco-2, Caco-2/HT29-MTX) have been used in nanotoxicology research. However, the human gut environment is composed of both human cells and the gut microbiota. The goal of this study is to increase the complexity of the Caco-2/HT29-MTX *in vitro* model by co-culturing human cells with the Gram-positive, commensal *Lactobacillus rhamnosus* or the Gram-negative, opportunistic *Escherichia coli*; with the hypothesis that the presence of bacteria would ameliorate the effects of exposure to metal oxide nanoparticles (NPs) such as iron oxide (Fe₂O₃), silicone dioxide (SiO₂), titanium dioxide (TiO₂), or zinc oxide (ZnO). To understand this relationship, Caco-2/HT29-MTX cell barriers were acutely co-exposed (4 hours) to bacteria and/or NPs (pristine or *in vitro* digested). The activity of the brush border membrane (BBM) enzymes intestinal alkaline phosphatase (IAP), aminopeptidase-N (APN), sucrase isomaltase (SI) and the basolateral membrane enzyme (BLM) Na⁺/K⁺ ATPase were assessed. Findings show that (i) the human digestion process alters the physicochemical properties of NPs, (ii) large agglomerates of NPs remain entrapped on the apical side of the intestinal barrier, which (iii) affects the activity of BBM enzymes. Interestingly, some NPs effects were attenuated in the presence of either bacterial strains. Confocal microscopy detected bacteria-NPs interactions, which may impede the NP-intestinal cell contact. These results highlight the importance of improving *in vitro* models to closely mimic the complexities of the human body.

Graphical Abstract



To understand the effects of engineered nanomaterials added intentionally and unintentionally to food, we improved a gastrointestinal *in vitro model* using *in vitro* digested nanoparticles, Caco-2/ HT29-MTX cells and gut microbiota.

INTRODUCTION

In the food industry, engineered nanomaterials (ENMs) are frequently used due to their novel and unique properties^{1–3}. Among their different applications, ENMs are regularly used as food additives and food contact materials^{4–6}. Within the context of European Food and Safety Authority guidelines, and other reports, the term “engineered” is equivalent to the term “manufactured” and/or “processed”^{7,8}. In this case, the term ENMs refers to those nanomaterials (NMs) synthesized and intentionally added to foods for purposes such as: improving food preservation, aspect and quality as well as for nutritional aims^{9,10}. In addition, ENMs are also used in food packaging, where they can indirectly find their way into food, such as the case of nanoparticles (NPs) from cans that migrate into the food matrix⁵. NPs may also be present in foods as a result of food processing procedures, such as homogenization, grinding and cooking¹¹, albeit in some cases not intentionally placed in direct contact with the food but the result of industrial operations. Only certain ENMs are authorized for use in food products and they may vary by country (e.g. Food and Drug Administration, European Food Safety Administration)¹⁰. Examples of authorized materials include TiO₂ (E171), SiO₂ (E155), ZnO (E6) and Fe₂O₃ (E172). The “E number” codes identify substances that can be used as food additives^{12,13}. It has been demonstrated that these materials are present in several consumer

products, fulfilling the strict definition of ENMs regarding primary particle size. For example, it was previously shown that in each consumer product analyzed, 50% of the TiO₂(E171) and up to 33% of the SiO₂(E155) are in the nano-size range^{9,14}. Previously we have shown that the ZnONPs (used in canned foods) from canned asparagus released about 66 mg/kg of Zn into the food, some which may be in the nano-range¹⁵. Iron oxide is commonly used as a food colorant in the United States and can be present at concentrations up to 0.1% by weight of the final product¹⁶. However, no studies have provided information on the size of these iron oxide particles. A summary of the NPs used in this study and their applications and sources is shown in Table 1.

Ingested NPs will potentially interact with human body targets, depending on their physiochemical characteristics (e.g. material composition, molecular structure, ion release capability, reactivity), which could affect human health or organ/tissue function. Therefore, there is interest from industries and regulatory agencies to know the potential consequences associated with the application of nanotechnology in consumer products. Oral ingestion is one of the possible routes for human exposure to ENMs and should be fully understood. Once the ENMs are ingested, they go through the human digestion process which presents three major stages: (i) oral processing, where primary food reduction and bolus formation takes place; (ii) gastric processing, where chemical and enzymatic breakdown begins; and finally (iii) small intestinal processing, where there is degradation of macromolecules and nutrient absorption²⁴. At the end of the human digestion process, ENMs may or may not be further degraded by brush border membrane enzymes (BBM), which are responsible for nutrient hydrolysis occurring adjacent to the cell membrane for a pre-absorptive step, or the ENMs could reach the colon and be excreted²⁵. The human gut environment is composed of both human cells and the gut microbiota. It is well known that the intestinal microbiota, primarily composed of commensal bacteria, forms a complex ecosystem and plays an important role in the health of the host. The gut microbiota has been shown to be involved in basic human biological processes, including the modulation of normal nutrient absorption, drug metabolism and immune responses, as well as protection against pathogens^{26–28}. Changes or imbalances in the microbial community present in the intestine could induce the development of pathological disorders within and beyond the gastrointestinal tract, including mineral absorption deficiencies (e.g. Fe and Zn), inflammatory bowel disease, colorectal cancer, diabetes, or obesity^{29–32}.

In recent years, models of the gastrointestinal tract (GIT) have gained attention as a useful method for evaluating the digestion and absorption of ENMs released from food and food packaging. The *in*

vitro Caco-2/HT29-MTX intestinal model has previously been shown to be a high-throughput, cost-effective and relatively fast experimental process, when compared to *in vivo* models^{33,34}. To develop new biosafety methodology in nanotoxicology, the major goal of this study was to increase the complexity of the Caco-2/HT29-MTX *in vitro* model by co-culturing the human epithelium with two bacterial species that can be found in the duodenum and jejunum, *Lactobacillus rhamnosus* and *Escherichia coli*³⁵. The human-derived beneficial, Gram positive *L. rhamnosus* and opportunistic, Gram negative *E. coli* were selected to compare their roles in an *in vitro* GIT model following exposures to NPs. *L. rhamnosus* is a well-characterized probiotic strain that shows anti-inflammatory effects *in vitro*, adhesion capacity to the lumen mucosa, and can survive in unfavorable GIT conditions³⁶. *E. coli* is a versatile bacterial species and, in the GIT, is found in a broad spectrum of environments and phenotypes (e.g. pathogenic, commensal, planktonic cells and biofilms)³⁷. *Escherichia coli* also presents adhesive capacity to mucus and *in vitro* Caco-2 cells^{38,39}. Therefore, the Caco-2/HT29-MTX barriers were challenged with physiologically relevant doses of pristine or digested NPs including titanium dioxide (TiO₂), silicone dioxide (SiO₂), zinc oxide (ZnO), or iron oxide (Fe₂O₃), which are some of the most common ENMs added to food and food packaging⁵. Upon exposure (4 h) to (i) pristine NPs, (ii) digested NPs, (iii) planktonic bacteria and (iv) digested NPs + planktonic bacteria, we quantified apical BBM enzymatic activity, including intestinal alkaline phosphatase (IAP), aminopeptidase-N (APN) and sucrase isomaltase (SI) (Table 2). The activity of the basolateral sodium/potassium pump (Na⁺/K⁺ ATPase), a vital enzyme for the maintenance of cell osmolarity, was also measured^{40,41}. This model system and functional assays were used to test the hypothesis that the presence of bacteria in the *in vitro* cell culture system would ameliorate the effects of exposure to metal oxide NPs.

METHODS

Nanoparticle dose calculations

In vitro, physiologically relevant doses of NPs were formulated considering the daily human intake of NPs. The doses used for TiO₂NPs, SiO₂NPs (US Research Nanomaterials Inc.

Houston, TX, USA) and ZnONPs (Meliorum Technologies, Inc., Rochester, NY) exposures were identical to the ones previously described by our research group, and are summarized in Table 3 and described in the supplemental information^{15,50,51}. Fe₂O₃NPs were purchased as red iron oxide food colorant (E172) from Town End (Leeds, UK) plc (www.dyes.co.uk). Given that a regular

portion of meat per meal would be around 200 g and that the content of Fe₂O₃ is limited to 0.1% by weight of the finished food⁵², the final amount of Fe₂O₃ ingested would be 0.2 g/meal. Since the purchased food additives present an average diameter of 99.24 nm and a particle density of 1.14 g/cm³, the estimated number of particles in 0.2 g of Fe₂O₃ is 3.43×10^{11} resulting in an intestine exposure to 1.71×10^5 particles/cm² of Fe₂O₃, the physiologically relevant dose used in this work. To represent a range of exposures, additional doses that were generally two orders of magnitude above or below the physiologically relevant dose were also studied. The range of exposures for all four NPs used in the present study are compiled in Table 3.

Digestion of Nanoparticles

In order to further mimic the physiological conditions of human digestion, the four NPs were subjected to an *in vitro* digestion process where they experienced the three primary operational steps: mouth processing, stomach processing and intestine processing. For that purpose, we followed the well-described protocol of Glahn *et al.* (1998) with some modifications previously described in the work of Moreno-Olivas *et al.*^{15,53}. Briefly, NPs were dispersed in 5 mL of 18 MΩ sterile deionized (DI) water. The solution was homogenized in a 20 mL sterile scintillation vial (VWR™ International, LLC) with a Thermolyne Mixer (Maxi Mix II, Type 37600) and sonicated for 30 min with a bath sonicator (VWR® symphony™ Ultrasonic Cleaners, RF-48 W, 35k Hz operation frequency) to break down NPs agglomerates. Consecutively, to simulate the mouth stage, a solution of salts (0.3 mL of 1 M CaCl₂, 0.1 mL of 1 M KCl and 1.4 mL of 2 M NaCl) at 25°C was added to the vial, and the pH was adjusted to 5.5 to 7. To simulate the stomach state, 1 mL of pepsin solution (0.1 gr of pepsin diluted in 0.1 M HCl) was introduced to the NPs suspension after 5 minutes, the pH was decreased to 2.0 and incubated for 1 h at 37 °C. Peristaltic movements were simulated by mixing the vial in a Speci-Mix Thermoline (Dubuque, Iowa, USA) at 55 rpm. To simulate the intestinal stage, 5.25 mL of pre-warmed (37 °C) pancreatin/bile solution (0.05 g of pancreatin from porcine pancreas and 0.3 g of bile extract porcine in 0.1 M NaHCO₃) was added to the NPs suspension and the pH was adjusted to 6.5–7. The final volume was adjusted to 20 mL with 140 mM NaCl / 5 mM KCl solution. The ionic strength of the saliva, stomach and intestine solutions were 6 M, 1.1 M and 0.245 M, respectively. Thus, the final NPs suspension had an ionic strength of 7.345 M. The resulting samples were referred to as 'digested NPs' to differentiate the 'non-digested NPs' or 'pristine NPs'. All the chemicals and enzymes used in this protocol were purchased from Sigma-Aldrich, Inc (St. Louis, MO).

Nanoparticle characterization

Transmission electron microscopy (TEM) was used to measure the primary particle diameter and morphology of the four NPs (TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs) in their dry form. Briefly, a 10 µl drop of 0.1 mg/mL of NPs suspension in 18 MΩ sterile DI water was dispensed on the top of a 400-mesh copper TEM grid (Ted Pella, Inc.) and allowed to dry. Then, TEM images of random fields of view were taken using a JEOL JEM-2100F (JEOL, Peabody, MA) and processed with Image J software to measure the diameter of 150 NPs. The hydrodynamic size and ζ-potential of all four NPs at all different stages of the *in vitro* digestion process (i.e. water, saliva, stomach and intestine) were assessed by dynamic light scattering (DLS) and laser doppler electrophoresis (LDE), respectively, using a Malvern Zetasizer Nano ZS90 (Malvern Panalytical Ltd). Sample (0.1 mg/mL) measurements were performed in Malvern disposable polycarbonate folded capillary cells with gold plated beryllium-copper electrodes (DTS1070), which were rinsed with 18 MΩ sterile DI water to clean any dust contamination before sample filling. The Refractive Index used for TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs were respectively the following: 2.42, 1.46, 1.95 and 3.32. The samples were equilibrated in the instrument chamber for 120 seconds and measured at 25 °C. Three independent experiments were analyzed (n=3).

Cell culture

Human colon adenocarcinoma Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) at passage 20. HT29-MTX-E12 cell line, also derived from human colon adenocarcinoma, was purchased from Sigma-Aldrich (Saint Louis, MO, USA) at passage 57. Both cell lines were maintained in Dulbecco's modified Eagle's High Glucose medium without sodium pyruvate (DMEM without sodium pyruvate, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (HI-FBS, Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated in a humidified atmosphere of 5% CO₂ at 37 °C. Routinely, Caco-2 and HT29-MTX cell lines were subcultured once a week with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) in 75 cm² flasks (Corning Life Sciences, Corning, NY). Culture medium was changed every 2 days. Both cell lines were further sub-cultured up to 12–15 passages.

For non-transport experimental studies, Caco-2 and HT29-MTX cell lines were trypsinized, mixed at ratios of 75:25 (Caco-2/HT29), and seeded at a cell density of 50,000 cells/cm² in 24 well plates (Corning Life Sciences, Corning, NY) previously coated with rat tail Type I collagen (8 µg/cm²) (BD

Biosciences, San Jose, CA, USA) for 1 h at room temperature (RT). Then, co-cultures were allowed to grow and differentiate for a period of 15 days to present tight junctions between cells, microvilli projections and a mucus layer completely developed that covers the *in vitro* barrier and is approximately 2–10 µm thick⁵⁴. Co-culture medium was changed every 2 days.

Bacterial strains and growth conditions

Major bacterial species/genus that inhabit the human intestine are *Bifidobacterium*, *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium* and *Enterobacteria*⁵⁵. Throughout this study *Escherichia coli* (ATCC® 11775™) and *Lactobacillus rhamnosus* GG (ATCC® 53103™) were purchased from the American Type Culture Collection (ATCC®) and used for further experiments. Both *E. coli* and *L. rhamnosus* overnight cultures were grown on brain heart infusion media (BHI, Becton, Dickinson, Sparks, MD) at 37 °C with 5% CO₂. *E. coli* was grown in agitation at approximate 150 rpm using a Roto Mix (Type 508000, Thermolyne, DE) for 24 h and *L. rhamnosus* was grown under static conditions for 48 h. When inoculating the *in vitro* model, overnight cultures were standardized to 10³ CFU/mL as this was previously found to be the approximate concentration of bacteria in the duodenum⁵⁶.

In vitro model exposure to Nanoparticles, bacteria and ionic controls

For experimental studies, once the *in vitro* model reached the day 15 of differentiation it was subjected, under acute conditions (4 h), to three treatments including:

Pristine NPs. All four NPs were resuspended in 18 MΩ sterile DI water at a concentration of 1mg/mL, homogenized, bath sonicated for 30 min to break down NPs agglomerations, diluted in DMEM+10% Hi-FBS at the desired concentrations (i.e. low, medium and high) (see Table 3), and added at a volume of 0.5 mL to the *in vitro* model.

Digested NPs. NPs were sonicated at a concentration of 1 mg/mL and *in vitro* digested reaching a final concentration of 0.25 mg/mL. NPs were then immediately diluted in DMEM+10% Hi-FBS at the desired concentrations (i.e. low, medium and high) (see Table 3) and added to the *in vitro* model.

Digested NPs co-exposed with *E. coli* or *L. rhamnosus*. *In vitro* models were inoculated with 10³ CFU/mL of *E. coli* or *L. rhamnosus* together with 'digested NPs' (i.e. low, medium and high), both diluted in DMEM+10% Hi-FBS. The control group was inoculated with the bacterial strains but no NPs.

To discern whether any toxicological effects observed in the results are due to the nature of NP or due to ions released from the NP, the *in vitro* model was also subjected, under acute conditions (4h), to

both digested ZnCl₂ and Fe₂O₃NPs (see the methods of supplemental information) in the presence or absence of *E. coli* or *L. rhamnosus*.

Alkaline Phosphatase Activity Assay

Following acute (4 h) NPs exposure, *in vitro* barriers were gently washed three times with 0.5 mL of 1x PBS to avoid any interaction between NPs and the bacteria with the assay reagent. Samples were then bath sonicated for 15 min at RT in 0.2 mL 1x PBS. To recover the cell lysate, each well was scraped and transferred into 1.5 mL centrifuge tubes. The intestinal alkaline phosphatase (IAP) assay detects the presence of p-nitrophenol as the product resulting from the activity of IAP using p-nitrophenyl phosphate (pNPP) as substrate. Concisely, the pNPP solution was made by dissolving one Tris Buffer tablet and one pNPP tablet (product code: N2770 Sigma-Aldrich) in 5 mL of 18 MΩ sterile DI water. IAP hydrolyzes pNPP to p-nitrophenol, which turns into a bright yellow color based on the concentration present. Cell lysate solution (25 µL) from each tube was added to each well of a 96-well plate, followed by pNPP solution (85 µL), and the resulting solution was incubated at room temperature (RT) for 1 h. The concentration of p-nitrophenol was then calculated by measuring the absorbance of the solution at 405 nm (Synergy2, BioTek® Instruments, Inc). The Bradford assay was used to determine the total cell protein concentration. For each assay, a standard curve was created with p-nitrophenol or bovine serum albumin to calculate the unknown concentrations of p-nitrophenol or protein, respectively. Three independent experiments were performed with at least 4 replicates per treatment (n≥12).

Aminopeptidase-N Activity Assay

The aminopeptidase-N *in situ* assay was adapted for Caco-2/HT29-MTX co-cultures from the study of Ferruzza and co-workers⁵⁷. Co-cultures were seeded onto 24 well plates, differentiated for 15 days and exposed for 4 h to all four NPs. Following exposure, the *in vitro* barriers were cautiously rinsed three times with 1x PBS to completely remove NPs and bacteria and avoid any interaction between them and the assay reagent. Then, samples were sonicated for 5 min with 0.2 mL of PBS per well. The cells were scraped and lysed from the bottom of the well by pipetting. Then, 25 µL of the lysate were transferred to a 96-well plates and mixed with 75 µL of L-leucine-p-Nitroanilide (2 mM LpNA) dissolved in 'collection buffer' (30 volumes of PBS pH 7.0 and 1 volume of 10 mM TrisHCl/150 mM NaCl pH 8.0), which works as the enzyme substrate. APN hydrolyzes LpNA to the colored product p-nitroanilide, which turns bright yellow based on the concentration of p-nitroanilide. The solution was

incubated in the dark at 37 °C for 1 h. The absorbance was read at 405 nm in a plate reader (Synergy2, BioTek® Instruments, Inc) to measure the concentration of p-nitroanilide released from the activity of the enzyme. The Bradford assay was used to determine the total cell protein concentration. For each assay, a standard curve was created with the product p-nitroanilide or bovine serum albumin to calculate the unknown concentrations of p-nitroanilide (Sigma-Aldrich, St. Louis, MO) or protein, respectively. Three independent experiments were performed with at least 4 replicates per treatment (n≥12).

Na⁺/K⁺-ATPase Activity Assay

The Na⁺/K⁺-ATPase *in situ* assay was adapted for Caco-2/HT29-MTX co-cultures from the studies of Sarkar and El-Zein *et al.*, where the enzymatic activity of Na⁺/K⁺-ATPase is determined by calculating the difference of inorganic phosphate (PO⁻³₄) liberated in the presence and absence of ATPase enzyme activators (Na⁺, K⁺ and Mg⁺⁺)⁵⁸. Briefly, after the 4 h of exposure, the *in vitro* barriers were washed three times with 18 MΩ filtered sterile DI water to completely remove from the well any residual NPs and bacteria to avoid any interaction with the assay reagent. The 24-well plate was sealed, and bath sonicated for 5 min with 0.2 mL of 18 MΩ filtered sterile DI water per sample at RT (any possible phosphates contaminants such as PBS were avoided). Then, the lysate was homogenized by pipetting up and down, and half of the lysate (0.1 mL) was transferred to a 1.5 mL centrifuge tube where it was mixed with (i) *buffer 1*. Then, the other half was also transferred to another centrifuge tube and mixed with (ii) *buffer 2*. Samples were then incubated for 45 min at 37 °C. 0.2 mM of ATP diluted in 18 MΩ filtered sterile DI water (*buffer 1*), and 0.2 mM of ATP diluted in 'activator buffer' (19.6 mM KCl/ 121.52 mM NaCl/ 3.92 mM MgCl) (*buffer 2*).

Finally, the samples were centrifuged for 5 min at 3000 *xg* and 4 °C to isolate the PO⁻³₄ from the lysate. Following PO⁻³₄ isolation, 10 µL of supernatant were transferred to a 96-well plate, diluted (1/10) in 18 MΩ filtered sterile DI water, mixed with 20 µL of Malachite Green Reagent (Phosphate Assay Kit®, Sigma-Aldrich, St Louis, MO) and incubated at RT for 30 min avoiding light exposure. The absorbance was read at 620 nm in the same 96-well plate to measure the concentration of PO⁻³₄ using a plate reader (Synergy2, BioTek® Instruments, Inc). For each assay, a new standard curve was prepared diluting the PO⁻³₄ premix solution of the Phosphate Assay Kit®. The Bradford assay was used to determine the total cell protein concentration. Results were calculated as following: PO⁻³₄ (µM)/mg of protein (*buffer 2*) - PO⁻³₄ (µM)/mg of protein (*buffer 1*) = Net PO⁻³₄ (µM)/mg of

protein resulted from the Na^+/K^+ -ATPase activity. Three independent experiments were performed with at least 4 replicates per treatment ($n \geq 12$).

Sucrase Isomaltase Activity Assay

The α -glucosidase activity of the Sucrase Isomaltase (SI) enzyme was determined by measuring its ability to break down the sucrose molecule into glucose. For this purpose, the Glucose GO Assay Kit® (product number: GAGO-20, Sigma-Aldrich, St. Louis, MO) was used. Briefly, after the 4 h of NPs exposure, *in vitro* barriers were washed three times with

0.5 mL of sterile PBS to remove the residual NPs and bacteria from the well and avoid any interaction between NPs and the assay reagent or bacteria and the assay reagent. Then, samples were bath sonicated during 5 min at RT with 0.2 mL of PBS and homogenized by pipetting up and down. Following, each sample was mixed with 0.2 mL of 150 mM sucrose during 1 h at 37 °C. Finally, 50 μL of the lysate were transferred to a 96-well plate and mixed with 50 μL of the assay reagent (Glucose Oxidase/Peroxidase + o-Dianisidine) during 30 min at 37 °C. The reaction was stopped by adding 50 μL more of 12 N H_2SO_4 , which

lead the solution to develop a pink color. To measure the amount of glucose generated, the 96-well plate was read at 540 nm using a plate reader (Synergy2, BioTek® Instruments, Inc). For each assay, a new standard curve was prepared diluting the glucose standard solution provided by the kit (product number: GAGO-20, Sigma-Aldrich, St. Louis, MO). The Bradford assay was used to determine the total cell protein concentration. Three independent experiments were performed with at least 4 replicates per treatment ($n \geq 12$).

Immunocytochemistry and Confocal Imaging

The laser scanning confocal microscope (LSCM) (LSM 880, Carl Zeiss Microscopy, LLC) equipped with an oil immersion objective (PL APO 63x/1.40 oil DIC, Carl Zeiss Microscopy, LLC) was used to visualize and colocalize all the elements of the *in vitro* model and observe any NPs-bacteria-cell interaction. Since metal oxide NPs present reflectance properties, they are easily detected using the laser 488 and the reflectance option of the confocal microscope (artificially tagged in white/grey color by the ZEN lite software, Carl Zeiss Microscopy). *E. coli* (ATCC 11775™ strain transformed with pMRP9–1) expressed green fluorescence and *L. rhamnosus* was stained with CellTrace™ CFSE, also visualized in green. In addition, to visualize the cell microvilli actin filaments were stained with Alexa Fluor™ 568 Phalloidin and the cell nucleus with Hoechst 33342, resulting in tagging with red and blue fluorescence, respectively. For confocal imaging, only the high dose of ZnNPs (9.7×10^4 ng/mL) and

Fe₂O₃NPs (3.8×10^4 ng/mL) were used, while the SiO₂NPs (2×10^4 ng/mL) and TiO₂NPs (1.4×10^4 ng/mL) concentrations were increased one and two orders of magnitude respectively, to ensure visualizing of the NPs under the microscope. In general, the sample preparation was the following: both cell lines Caco-2/HT29-MTX were seeded on top of cover slips placed into 6-well plates and left to proliferate and differentiate up to 15 days. After the 4 h of NPs exposure or NPs/bacteria co-exposure, the *in vitro* barriers were fixed with 4% paraformaldehyde (PFA) in 1x PBS for 30 min at RT. After fixation, the samples were permeabilized with 0.1% Triton X-100 for 5 to 15 min at RT. To stain the cell actin and nucleus, both fluorochromes were mixed, diluted in 1x PBS at a final concentration indicated by the manufacturer and applied to all sample surface. The staining solution was incubated in the dark for 20 min at RT. Finally, the coverslips containing the *in vitro* barriers were removed and mounted on a glass slide with ProLong gold mounting medium (Thermo Fisher Scientific). Samples were cured overnight in the dark and imaged the next day. Z Stacks image series were taken with 1 μ m intervals. Comstat2 (www.comstat.dk) analysis was used to quantify the F-actin biomass and thickness, and the bacterial biomass found attached to the epithelium surface.

Statistics

All assays were performed in 3 independent experiments, with at least 3 replicates per treatment. Results are expressed as mean $\pm$ standard error. Previous to statistical analysis, data was submitted to the two most recommended normality tests to determine whether the values come from a Gaussian distribution. For that purpose, both (i) D'Agostino-Pearson omnibus normality test, and (ii) Shapiro-Wilk normality test were determined using GraphPad Prism version 5.00. After passing the normality test, one-way ANOVA with Tukey's post-test was used to compare differences between means. Data was analyzed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, <http://graphpad.com>). Differences between means were considered significant at $P < 0.05$.

RESULTS

Characterization of Nanoparticles

Figure 1 shows transmission electron microscopy (TEM) images that demonstrate how TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs that have been dispersed in water and allowed to dry, tend to agglomerate. However, it is still possible to distinguish the primary particle sizes: 24, 19, 39, and 99 nm, respectively. Moreover, dynamic light scattering (DLS), which measures the Brownian motion of

NPs suspended in liquids, was performed to detect the hydrodynamic size of all four NPs. The obtained results demonstrated that TiO₂NPs (177.2±23.11 nm), SiO₂NPs (121.66±15.16 nm), ZnONPs (409.2±62.82 nm) and Fe₂O₃NPs (375.9±7.67 nm) suspended in 18 MΩ deionized (DI) water tended to form agglomerates reaching a hydrodynamic size no bigger than 500 nm (Figure 1. A3, B3, C3, D3). In this case, NP agglomeration seems to be independent of the surface charge and particle stability, since the zeta potential (ζ-potential) values of most NPs [except for SiO₂NPs (−16.9 mV)] were observed to be positively charged, with Fe₂O₃NPs being the most stable (40.3 mV) (Figure 1. E). When measuring the hydrodynamic size of the NPs along the *in vitro* digestion steps, a dramatic increment in NP agglomeration started in the *saliva* stage, reaching almost the μm scale (Figure 1. A3, B3, C3, D3). At the end of the digestion process (*intestine* stage) and due to the high complexity of the solution, the hydrodynamic size of all four NPs increased strikingly, SiO₂NPs being the most agglomerated (4578±718.4 nm), followed by TiO₂NPs (3993±569.6 nm) and Fe₂O₃NPs (2722±138.1 nm), and finally ZnONPs (1434±107.5 nm). These results are supported by the poor stability of the NPs (ζ-potential values < ±30 mV) at the end of the digestion process (Figure S1.C). The polydispersity index values (Pdl) indicated that all four digested NPs suspensions were polydisperse (Pdl >0.05), meaning that the broadness of the NPs' size distribution is extensive (Figure S1.B). Moreover, differences between *in vitro* digested ZnONPs and Fe₂O₃NPs, and non-digested ZnONPs and Fe₂O₃NPs, were evaluated by ICP-MS (Figure S4). NPs suspensions were ultracentrifuged in order to separate the released ions (released into the supernatant) from the NPs (remaining in the pellet) and then analyzed as described in the supplemental information. The amount of Zn content in the supernatant increases proportionally to the ZnONPs concentration in both the digested and undigested ZnONPs. However, more Zn content was detected in the supernatant of the *in vitro* digested samples than the non-digested (Figure S4 B). Similar results were seen when analyzing the ultracentrifugation of digested Fe₂O₃NPs samples (Figure S4 A).

Assessment of the small intestine enzyme's activity

Intestinal Alkaline Phosphatase—As shown in Figure 2, the presence of digested and undigested TiO₂NPs, SiO₂NPs and ZnONPs significantly increased the enzyme activity of intestinal alkaline phosphatase (IAP), except when the barrier was exposed to high doses of ZnONPs (Figure 2 C.1). In our previous studies, TiO₂NPs and SiO₂NPs were also shown to increase the IAP activity after acute (4 hours) and chronic (5 days) exposures^{15,50,51}. No effects on the IAP activity were observed after 4 hours of Fe₂O₃NPs exposure (Figure 2 D.1 and D.2). Interestingly, when the barrier was exposed to bacteria alone, primarily *E. coli*, the IAP activity decreased significantly compared to the untreated

controls. However, bacteria-NPs co-exposure seems to balance and restore the IAP activity to control levels in most of the cases (Figure 2 A.3, B.3 and C.3) except for ZnONPs and Fe₂O₃NPs (Figure 2 C.3 and D.3). Medium and high doses of digested and non-digested ZnCl₂ (ionic control), also affected IAP enzyme by decreasing its activity (Figure S7 B.1 and B.2). In this case, the enzyme activity diminished to control levels with the addition of *E. coli* but not with *L. rhamnosus* (Figure S7 B.3 and B.4). In the case of FeCl₃, no alterations in the enzyme activity were detected (Figure S7 A.1 and A.2), but the introduction of *E. coli* to the system significantly increased the IAP activity (Figure S7 A.3 and A.4).

Aminopeptidase-N. Aminopeptidase-N (APN) activity was also analyzed after acute (4h) exposure to digested and undigested NPs. Similarly to IAP, the presence of digested TiO₂NPs, SiO₂NPs and ZnONPs, but not Fe₂O₃NPs, led to an increase in the APN enzyme activity (Figure 3). However, the addition of bacteria did not diminish the effects of digested TiO₂NPs or digested SiO₂NPs (Figure 3 A.3 and B.3). The co-exposure between *L. rhamnosus* and Fe₂O₃NPs decreased the APN activity (Figure 3 D.3). *E. coli* and *L. rhamnosus* remediated the effects of low and medium doses of digested ZnONPs (Figure 3 C.3). It should be noted that since each fluorometric experiment was performed with a new batch of reagent and using a new standard curve due to the poor stability of the reagents in solution, slight differences in the control samples within each group were detected. ZnCl₂, induced a dramatic decrease in the APN activity, which was mitigated in the presence of bacteria (Figure S8). The addition to digested FeCl₃ to the system increased the APN activity and was further increased in the presence of bacteria (Figure S8).

Sucrase Isomaltase—In Figure 4, it is shown that only digested SiO₂NPs significantly increased the SI activity after 4 h of exposure (Figure 4 B.2). The presence of both *E. coli* and *L. rhamnosus* decreased the SI activity, but bacteria could not mitigate the effects of digested SiO₂NPs (Figure 4 B.3). Both bacteria and co-exposure with ZnONPs dramatically diminished the SI activity of the system (Figure 4 C.3). Different trends were observed in response to ionic controls when the SI enzyme activity was determined. Digested FeCl₃ altered the SI activity, which was ameliorated in the presence of bacteria (Figure S9 A.2, A.3 and A.4). ZnCl₂ effects were altered only in the presence of bacteria (Figure S9 B.3 and B.4).

Na⁺/K⁺ ATPase—As summarized in Table 2, the Na⁺/K⁺ATPase pump is an enzyme that is critical for adequate gut function. Na⁺/K⁺ATPase activity was assessed after 4 h of exposure to undigested

NPs, digested NPs, and digested NPs co-exposed with *E. coli* or *L. rhamnosus* (Figure 5). No significant changes were detected (increase or decrease) in the Na^+/K^+ ATPase activity after 4 h of exposure to the various NPs doses (low, medium and high) or the NPs physiological state (digested or undigested). No significant differences in enzyme activity were detected in the presence of bacteria. Co-exposure to both SiO_2 NPs and *L. rhamnosus* led to a significant decrease in the enzyme activity (Figure 5 B.3). No significant differences were detected when treating the *in vitro* system with ionic controls (ZnCl_2 or FeCl_3), or when co-exposed with bacteria (Figure S10).

Nanoparticles and bacteria localization along and across the Caco-2/HT29-MTX model

In order to further understand the mechanism by which food additives NPs can alter BBM enzymes activity, confocal microscopy was used. F-actin filaments were stained using Phalloidin because they are responsible for the structural shape of the microvilli (seen in red color) in order to clearly localize them along the *in vitro* barrier⁵⁹. In Figure 6 representative ortho images for each treatment are shown. After a 4 h exposure, all digested food additive NPs (artificially targeted in white) were shown to settle on the apical side of the *in vitro* barrier where the microvilli from the enterocytes are facing. A random disposition of TiO_2 NPs (Figure 6 A.2) and SiO_2 NPs (Figure 6 A.3) along the barrier surface and different agglomerate sizes can be observed. Fe_2O_3 NPs were shown to form the largest agglomerates (Figure 6 A.5), but ZnONPs were present in a more particulate form (Figure 6 A.4).

Moreover, no structural damage to the barrier or cell rearrangement were observed after exposing the *in vitro* barrier to both the opportunistic non-pathogenic *E. coli* (Figure 6 B.1) or the commensal *L. rhamnosus* (Figure 6 C.1) for 4 h. Both bacteria (tagged in green) were seen to settle homogeneously on the apical side of the barrier between the microvilli structures. Over the 4 h exposure, both bacterial strains were able to divide and increase in concentration, with *E. coli* dividing faster than *L. rhamnosus*. In addition, the images show that *L. rhamnosus*, unlike *E. coli*, begins forming larger clusters of cells growing into a characteristic filamentous shape (Figure 6 C.1). Typically, *L. rhamnosus* is known to form biofilms on abiotic surfaces and is strongly modulated by culture medium factors and conditions related to the gastrointestinal environments such as low pH, high osmolarity, and presence of bile, mucins, and nondigestible polysaccharides⁶⁰.

When co-exposing the *in vitro* barrier to both bacterial strains and all four digested food additive NPs, no particular patterns of NPs and/or bacteria distribution, neither specific NPs-bacterial adhesions, were clearly detected (Figure 6). Similarly, no significant effects were observed when the *in vitro* barrier was analyzed by confocal microscopy after the co-treatments with ionic controls and the bacterial strains (Figure S12). Following exposures, transversal cuts of the Z stacks were performed

to further study structural interactions (Figure 7). In general, the behavior of all four digested food additive NPs was similar. Large agglomeration of NPs (marked with yellow arrows) was observed to be entrapped on the apical side of the *in vitro* Caco-2/HT29-MTX model, obviously in contact with the cell microvilli and independently of the presence of bacteria. Meanwhile, smaller NPs were more likely to penetrate the barrier (marked with a yellow circle) visibly via intracellular pathways (Figure 7). For the ionic controls, no particulate matter was observed, since ions completely dissolve in the exposure media and do not form agglomerates (Figure S15). In ionic controls only, the co-treatment with ZnCl₂ and *E. coli* was able to structurally alter the *in vitro* barrier (Figure S15 B.2).

Detection of Bacteria-Nanoparticles Interactions with Confocal Microscopy

After 4 hours of co-exposure, not all the bacteria in the medium will settle to the cell barrier surface, and therefore the suspended portion of the *in vitro* system was also analyzed with confocal microscopy (Figure 8). Images in 3D were taken in order to spatially observe any NPs-bacteria interactions. As seen in Figure 8, both bacterial strains (green) were clearly visualized interacting physically (indicated with a red arrow) with all four NPs (white), independently of the NPs molecular composition, size, agglomeration state or concentration. However different trends of interactions were observed; for example, larger agglomerates of SiO₂NPs were observed to be surrounded by numerous *E. coli* cells, while a very large ZnONPs agglomerate surrounded or entrapped a single *E. coli* cell.

DISCUSSION

Due to the increasing use of nanotechnology in food either as new food ingredients, food additives, or food contact material, an international concern about human health risk has been voiced by regulatory agencies (e.g. FDA, EFSA, WHO). The global production of inorganic nanomaterials is estimated to be approximately 10,000 tons/year for SiO₂, 1,000 tons/year for TiO₂ and Fe₂O₃, and up to 100 tons/year of ZnO^{61,62}. Interestingly, the industry predictions show that the ENMs market has the potential to increase up to USD \$11.8 billion by 2020⁶³. It is estimated that the dietary consumption of ENMs in developed countries is approximately 10¹¹-10¹³ particles/day of TiO₂ and nano-silicates^{17,64}. However, the new applications of ENMs in the agri-food industry may introduce new indirect sources of food contamination, such as nano-sized pesticides and veterinary medicines, as well as, potential migration from food manufacturing instruments and food processing operations^{65,66}. Therefore, the massive production and vast variability of ENMs, mainly inorganic and currently used in the food market, have led to the urgent need for new strategies and methodologies in the nanotoxicology field. When NPs are released into the environment, they have

more capability to interact with the biological matrix/fluid than their bulk counterparts, because of their size-specific physicochemical properties, which confers them high reactivity⁶⁷. This interaction can result in changes (e.g. NPs erosion, ion release, agglomeration, protein corona formation, attractive and repulsive forces, etc) or environmental transformation of the primary structure of the NPs, which can lead to a completely distinct NP behavior and bioavailability⁶⁸. Initially, when NPs are exposed to human biological substances (e.g. human serum, gastric fluids, etc), the absorption of extracellular proteins on the surface of NPs create a protein corona, which can critically affect their interaction with cells, tissues and organs^{69,70}. Recently, Barbalinardo *et al.* demonstrated that the adsorption of serum proteins onto surface of citrate-coated AgNPs is essential to allow them to be internalized by NIH-373 cells, and to develop cytotoxic activity⁷¹. Similar results were seen by Srivastav *et al.*, who showed how ZnONPs enveloped in high amounts of serum proteins elicited a higher immune cell recognition and hepatic inflammation⁷². Also, NPs agglomeration or ion release have been found to play an important role in cytotoxicity. Several studies have shown that agglomeration of TiO₂NPs in aquatic medium inhibits algae growth. In addition, agglomeration of TiO₂NPs in cell culture media (DMEM+FBS) provoke severe structural damages to the *in vitro* Caco-2/HT29 co-culture model^{73,74}. Conversely, it has been shown that metal oxide NPs (e.g. zinc oxide, copper oxide and nickel oxide) with strong cytotoxicity effects, released more soluble metals into the cell culture media and induced a significant intracellular reactive oxygen species (ROS)⁷⁵. Thus, from a toxicological perspective, the environmental transformation of NPs is an important potential pathway to consider in order to understand the potential effects from exposure. Considering these findings, we aimed to assess four NPs *in vitro*, mimicking the human digestion process step by step to understand what the NPs undergo once they are ingested.

As stated previously, all four NPs tended to agglomerate when suspended in DI nano-pure water (Figure 1). This agglomeration can be due to the combined effects of pH, ionic strength (IS), ionic composition, natural organic matter (NOM) and other characteristics of the aqueous media, known to result in particle agglomeration⁶⁸. It is worth noting that even a simple solution of low IS, almost neutral pH and no NOM, such as 18 MΩ deionized water, already impact on the surface atoms of this metal oxides components (composed of oxygen atoms and transition metals), which alter their catalytic properties (Figure 1). It is also relevant to mention that many of the commercially available metal oxide ENPs source powders are synthesized by high temperature vapor phase processes. In these processes, chemical precursors of these NPs react at high temperature, producing droplets of the desired material into the nanoscale⁷⁶. At this point, these hot droplets may collide and coalesce fully

or partially with others, leading to a larger primary particle (fused particles) that are often too strong to break via sonication⁷⁷. As expected, the hydrodynamic size of all four NPs was dramatically increased already during the saliva stage and even more at the intestinal stage (Figure 1 A3, B3, C3, D3). At the saliva stage, only a mix of salts were added, which most likely affected the solution IS, and consequently the relative acidity and basicity of atoms present on the surface of the metal oxide structures⁷⁸. In addition, the four NPs were also found to be negatively charged at the end of the digestion process (Figure 1 E), which could play an important role in the NPs behavior in further processes of the human GIT. Similar results were observed in previous studies that also showed the effect of increasing the media complexity, mainly by the addition of macromolecules, enzymes and serum proteins such as FBS, where the hydrodynamic size of TiO₂NPs and Fe₂O₃NPs used as food additives are dramatically affected when measured by DLS^{11,74,79}. Interestingly, Walczak and co-workers (2013) revealed by SEM-EDX how gastric digestion clustered/ agglomerated the 60 nm AgNPs, while during intestinal digestion these clusters disintegrated back into single 30 nm AgNPs⁸⁰. We observed that the digestion solution can scatter the light (Figure S1 A), most likely because of the amount of proteins present in the solution and its crystallization, which may affect the ζ -potential values⁸¹. DeLoid *et al.*, highlighted the importance of using appropriate analytical methods when working with such complex suspensions, as sometimes light scattering techniques are not accurate enough and have to be supplemented with other methodologies⁷⁹. In the current study, the DLS results (Figure S1 A) were supported by confocal microscopy of the digested NPs. As shown in Figure S11, at the end of the digestion process NPs can be seen forming large agglomerates.

In nanotoxicology, the importance of dose rate and dose range testing has been documented^{10,82}. One goal of this study was to work with realistic and physiologically relevant human exposure doses. Human oral intake of NPs was extrapolated by considering published data of food products containing NPs, regulatory agencies guidance, ICP-MS analysis, and the physiology of the human intestine^{15,18,19}. Once the physiologically relevant doses were estimated (Table 3), a dose range with up to 3 doses (high, medium and low) was established to cover all possible human exposure scenarios. However, assessment of relevant *in vitro* NPs administration also requires a rational time and frequency of exposure. Although human digestion time varies between individual and gender in general, food takes around six to eight hours to pass through the stomach and the small intestine. Concretely, it takes 3 to 5 hours from entry to the duodenum (first part of the small intestine) to exit from the ileum (the last segment of the small intestine)^{83,84}. In this study, the *in vitro* model Caco-2/HT29-MTX was exposed to digested NPs for 4 hours to mimic the time that it would take for one

meal to enter and exit the small intestine.

First, the physiologically relevant dose ranges of each NP were evaluated to determine whether they would confer cytotoxic effects in the complex *in vitro* model. As shown in Figure S2 of the supporting information, after 4 hours of Caco-2/HT29-MTX barrier exposure to all four digested NPs, no cytotoxicity was observed when compared to the negative control. However, when exposing both *E. coli* and *L. rhamnosus* to all four NPs for 4 hours, only the highest dose of ZnONPs significantly affected the viability of both bacterial strains (Figure S3 C1 and C2). ZnONPs are currently used in the food industry as a nutrient source of Zn (e.g. cereals) or as an antimicrobial agent (e.g. food packaging) since ZnONPs possess unique properties and excellent stability with longer life compared with organic-based disinfectants^{15,85}. Toxic effects of ZnONPs onto a wide range of bacterial strains, including Gram-positive and Gram-negative species, has been documented^{86,87}. For example, using scanning electron microscopy (SEM) analysis, Zhang *et al.* showed that nanosized ZnO particles were able to interact with *E. coli* outer membrane resulting in severe wall damage, consequently collapsing the cell membrane⁸⁸. All indications suggest that the dissolution rate of ZnONPs forming Zn²⁺ ions, its photocatalytic activity and the induction of oxidative stress (ROS production) are the most likely mode of action to induce cytotoxicity^{85,89}. Currently, the Zn ion release is the widely accepted antibacterial mechanisms, as it has been shown to inhibit active transport, and to disrupt the amino acid metabolism and enzyme system⁸⁵. The toxicity pathways may vary as the species of dissolved Zn change depending on the medium composition in addition to the physicochemical properties of ZnONPs⁹⁰. It is known that ZnO as well as Fe₂O₃, are amphoteric molecules, meaning that they can behave differently depending on the pH of the solution. They can readily change between electron donor and acceptor and are more prone to release ions when they are in acidic solutions^{91,92}. Despite the extensive literature discussing the mechanisms of action underlying ZnONPs toxicity, more emphasis is needed to cover the gaps in further ZnONPs characterization, biodistribution in complex biological matrices (e.g. tissues and organs), as well as environmental bioaccumulation and trophic transfer (e.g. aquatic and terrestrial)⁹³. Therefore, ICP-MS was used to evaluate further NPs transformations and differences between *in vitro* digested ZnONPs and Fe₂O₃NPs, and non-digested ZnONPs and Fe₂O₃NPs, were evaluated (Figure S4). The amount of Zn and Fe content in the supernatant was higher in *in vitro* digested samples than the non-digested, meaning that the human digestion process and its pH fluctuations can be responsible for partially transforming both ZnONPs and Fe₂O₃NPs by dissolving them into ionic species. In the present work, both ionic controls ZnCl₂ and FeCl₃ were also digested and used for further experiments to elucidate whether the NPs

themselves or their released ions into the media are responsible for the potential toxicity and detrimental effects towards the *in vitro* model. The mass used in the ionic controls are equivalent to the mass of the ions present in the dose of NPs. As shown in Figure S5, no cytotoxicity was observed when the *in vitro* barrier was exposed to medium and high doses of both digested ZnCl₂ and FeCl₃ for 4 hours. In contrast, when *E. coli* and *L. rhamnosus* were exposed to both digested ionic controls for 4 hours (Figure S6) high doses of ZnCl₂ tended to decrease the viable counts (CFU/mL) of *E. coli* and *L. rhamnosus* when compared to the control, albeit not being statistically significant. Thus, a putative combination between nano-Zn and ionic-Zn may be responsible for bacterial toxicity, potentially inducing a misbalance in the intestinal microbiota community as postulated by Feng *et al.* This study demonstrated that exposure to increasing ZnONPs concentration (up to 100 mg/kg) for 9 weeks significantly decreased microbiota diversity of hens [74]. On the other hand, the addition of FeCl₃ to inoculum of *E. coli* promoted a significant increment in its viable counts when compared to the control (Figure S6.A). Similar results were obtained in previous studies where the number of *E. coli* cells significantly increased after the addition of ferrous sulfate and iron⁹⁴. In this case, iron may also be considered as a source of nutrition for bacterial development, like carbon, nitrogen and phosphates⁹⁴. In the case of *L. rhamnosus*, high doses of digested FeCl₃ also tended to increase the viability, albeit not significantly. Notwithstanding that Lactobacilli comprises one of the very few bacteria in which Fe is not an essential growth factor, it has previously been demonstrated that the presence of iron could enhance its probiotic effects^{95–97}.

The registered effects of NPs toxicity upon *in vivo* oral exposures intrinsically include the protective action of the microorganisms that populate the internal surfaces of the body⁹⁸. The intestinal tract contains a huge niche for the co-existence of fungi, virus and bacteria with commensal purposes⁹⁹. Recently, numerous studies have shown that intestinal microbiota are involved in the modulation of human health and disease^{100,101}. It has been shown that in a steady state, commensal microorganisms have a symbiotic relationship with the host and possess critical and distinct functions, including immunomodulation, protection, nutrition, and metabolism¹⁰². Various environmental factors have been reported to imbalance the microbiota, such as environmental contaminants, particulate matter and antibiotics^{103,104}. Numerous gastrointestinal side effects have been attributed to orally administered NPs in *in vivo* studies (e.g. necrosis and intestine inflammation, liver atrophy, reduced body weight, etc)¹⁰⁵. However, little is known about the role that the microbiota plays upon food-borne NPs exposure and its mechanism of action. In 2016, Fröhlich and Fröhlich postulated that, in general the roles of intestinal microbiota on food additive NPs exposures are largely unknown,

including the factors that enhance bacteria-NPs interaction in the GIT⁹⁸.

In the present study we also aimed to understand both the protective role of the intestinal microbiota and the effects of the NPs biotransformation after the digestion process, by challenging an *in vitro* model Caco-2/HT29-MTX with either *E. coli* or *L. rhamnosus* in addition to food additive NPs. The functionality of the small intestinal epithelium was assessed by measuring the activity of digestive enzymes in the lack or presence of the aforementioned bacteria. As summarized in Table 2, IAP plays an essential role in intestinal homeostasis and health through interactions with the resident microbiota, diet, and the gut. It also presents the ability to detoxify pathogens toxins, which is essential to protect the host from sepsis during acute and chronic inflammatory conditions¹⁰⁶. Altered IAP expression has been related to several chronic inflammatory states such as irritable bowel disease (IBD), celiac disease, and obesity. IBD patients seem to produce less *ALPI* gene expression in inflamed tissues¹⁰⁷. Also, it has been demonstrated that the oral administration of IAP for murine colitis reduces colonic inflammation by decreasing mRNA levels of the pro-inflammatory cytokine TNF- α and induced nitric oxide synthase (iNOS)¹⁰⁸. In healthy tissues, IAP molecules can also be present at high concentrations within luminal vesicles secreted by enterocytes on the brush border of the microvilli¹⁰⁶. These findings are in agreement with our results (Figure 2), as an increased enzyme activity could be a mechanism of self-defense against the adverse effects of food additive NPs. Furthermore, the IAP activity returned to the basal levels with the addition of both bacterial strains, which could be acting as a mechanical barrier against foreign particulate matter preventing their implantation or interaction in the small intestinal epithelium. However, the presence of *E. coli* alone led to a decrease of the IAP activity, which seems to counteract and balance the NPs effects (Figure 2). An *in vivo* study with pigs already detected that the presence of two non-pathogenic *E. coli* strains, O86 and O83, resulted in a significant decrease of APN activity compared to germ free pigs¹⁰⁹. Moreover, Liévin Le Moal *et al.*, also reported that the pathogenic *E. coli* C1845 strain can adhere to enterocyte cells and induce a dramatic alteration in the distribution of the major apical cytoskeleton associated proteins, including F-actin, affecting the microvilli architecture and in consequence also the expression of brush border enzymes such as IAP and SI¹¹⁰. However, further studies would be necessary to elucidate whether *E. coli* can specifically decrease the IAP activity in presence of regular human microbiota. In this case, the *E. coli* is also more likely to adhere to the surface of the intestinal cells than *L. rhamnosus*, although no significant differences were detected on their biomass quantification (Figure S15). Moreover, no significant differences in the F-actin biomass were observed when the *in vitro* model was treated either with *E. coli* or *L. rhamnosus*, suggesting that there was no microvilli damage or rearrangement (Figure S15). Alternatively, several studies have demonstrated the beneficial role of different *Lactobacillus*

strains on intestinal ALP activity^{111,112}. Furthermore, Jang *et al.*, found that bacteria and hosts competed for fatty acid absorption in the intestine, resulting in decreased weight gain, body fat mass, and hepatic lipid accumulation¹¹³. Similar studies have previously demonstrated the detrimental effects that food additives NPs can cause to the intestinal *in vitro* model when the microbiota is not present. For example, different shaped TiO₂NPs were able to settle on top of the barrier, penetrate the cell cytoplasm, cross paracellularly the barrier, and compromise the barrier structure and integrity after 24 and 48 h of exposure⁷⁴. SiO₂NPs were also seen entrapped in the apical part of the Caco-2/HT29 model and internalizing into the cells after 24 h of exposure, decreasing the transepithelial resistance and altering the levels of nutrient transport proteins located in the microvilli of the enterocytes after chronic exposures (5 days)^{50,114}. Similarly, physiologically relevant doses of ZnONPs were reported to cause damage to the microvilli of the intestinal cells and decreasing the gene expression of mineral transporters also placed in the microvilli¹⁵. Fe₂O₃NPs were detected precipitating on the top of the *in vitro* barrier and provoked morphological alterations to the co-culture after 3 hours of treatment¹¹⁵.

Although differences in cell culture methodologies (*e.g.* cell clones, ratio, exposure time, etc) could trigger outcome variabilities, these results reinforce the idea that microbiota present an innate role of protecting the human intestine by either being the first physical barrier in contact with the intestinal lumen or by symbiotically interacting with the intestinal epithelium and neutralizing physiological alterations.

Similarities were observed when the APN activity was analyzed (Figure 3). As mentioned above, mainly low and medium doses of digested TiO₂-, SiO₂-, and ZnONPs significantly increased the APN activity. However, high doses of ZnONPs and ZnCl₂ notably diminished the enzyme activity. APN is the most studied enzyme from the M1-family of metalloenzymes, which are zinc-dependent aminopeptidases containing a zinc-binding HEXXH motif⁴⁶. Although in these circumstances zinc would act as a catalytic and structural cofactor for the metalloenzymes activity, it has been proved that ionic zinc can also inhibit enzymes in general, including zinc metalloenzymes^{116,117}. The high amount of ionic zinc released from ZnONPs dissolution in water and further enhanced by *in vitro* digestion could inhibit the activity of the intestinal APN. In general, our results indicate that food additive NPs increase the intestinal APN activity, which would intensify the peptide breakdown by removing the amino acids from the N-terminus and consequently promoting its availability. High intake and digestion of proteins has been associated with disorders of bone and calcium homeostasis (*e.g.* hypercalciuria), renal malfunction and precipitated progression of coronary arterial disease¹¹⁸.

Beyond its key role in digestion of protein and metabolic balance, APN has also been shown to function as a regulator of inflammatory responses. It has been postulated that APN can be used as a pathologic indicator of cytomegalovirus infection, hepatocellular carcinoma and intestinal lesions^{119,120}. In this case, the addition of microbiota to the system did not decrease the APN activity by itself and only counteracted the effects of low and medium doses of ZnONPs, but not the effects of TiO₂NPs nor SiO₂NPs. Although neither *E. coli* nor *L. rhamnosus* affect the APN activity, it has been reported that the monocolonization of piglets intestines with the non-pathogenic *E. coli* O83 resulted in a remarkably increased expression of APN on the tips of the villi¹⁰⁹. Moreover, Willing *et al.* have shown an up-regulation of APN gene expression in response to *E. coli* but not *Lactobacillus fermentum*¹²¹. These findings suggest that the APN expression and activity could vary with the bacterial strain.

Sucrase isomaltase (SI) is another BBM enzyme important for the final step of carbohydrates digestion, such as starch (Table 2). The α -glucosidase activity of SI can hydrolase α -1,6 linkages of di- and oligosaccharides (e.g. sucrose and maltose) into monosaccharides (e.g. glucose and fructose)¹²². In the present study, only the exposure to digested SiO₂NPs significantly increased the SI activity (Figure 4). Although the carbohydrate digestion and absorption processes rely on a larger number of enzymes such as saliva α -amylases, as well as, glucose transporters (e.g. SGLT1, GLUT5, GLUT2), also expressed by the small intestinal enterocytes, a higher activity of SI would facilitate the availability of monosaccharides to be transported¹²². Kishi *et al.*, showed that in rats, fructose increased mRNA expression of SI as well as mRNA of SGLT1, GLUT5, and GLUT2¹²³. Moreover, our previous study demonstrated that acute exposures of SiO₂NPs could significantly upregulate the expression of GLUT2, but not SGLT1⁵⁰. Thus, chronic consumption of high glycemic index foods combined with the acceleration of glucose availability because of food additives NPs such as SiO₂, could be extremely unhealthy.

Increases of blood sugar levels correlates to Type 2 diabetes and coronary heart disease and has been implicated as a factor in the development of obesity¹²⁴. In some cases, the addition of either *E. coli* or *L. rhamnosus* significantly decreased the SI activity correlating with the results from Liévin-Le Moal *et al.*, previously mentioned¹¹⁰.

The Na⁺/K⁺ATPase pump activity was also evaluated in this work (Figure 5). Na⁺/K⁺ATPase is an integral protein present in the basolateral membrane (BLM) of mammalian epithelial cells. In the small intestine, Na⁺/K⁺ATPase is not only essential for cell homeostasis maintenance (Table 2), but also

plays a general role in the proper functionality of intestinal nutrient transporters dependent of the Na⁺ co-factor (e.g. Na-glucose transporter SGLT1)¹²⁵. It has been suggested that the decrease of Na⁺ and nutrient absorption in diarrheal diseases characterized by chronic intestinal inflammation is likely due to a down-regulation of Na⁺/K⁺ATPase in the BLM of enterocytes¹²⁶. Thus, the proper functioning of Na⁺/K⁺ATPase is essential for efficient nutrient absorption and subsequent maintenance of good intestinal health. The results of this study showed that, in general, none of the NPs, nor the ionic controls (Figure S10), significantly altered the activity of the Na⁺/K⁺ATPase pump. This is most likely due to the physical localization (in the BLM) of the integral protein. Na⁺/K⁺ATPase is less exposed to probable mechanical interaction with NPs or NPs agglomerates than those enzymes located on the apical surface of the cell barrier. However, for the proper trafficking of Na⁺/K⁺ATPase to the BLM of enterocytes, an ankyrin-spectrin skeleton is necessary⁴¹. Ankyrins are a family of polypeptide implicated in the targeting, stability, and regulation of select ion channels, transporters, pumps, and cytosolic proteins¹²⁷. If, hypothetically, NPs were able to penetrate the cell, the chances to interact with the cytoskeleton and consequently alter Na⁺/K⁺ATPase conformation and functionality would also increase.

In order to clarify and reinforce these findings, confocal laser scanning microscopy was used. As described in previous studies, the versatility of this technique allows for the straightforward detection of metal oxide NPs due to their refractive capability and ability to differentiate NP from the fluorescently tagged tissue culture^{74,114,128}. As previously mentioned, all four NPs, used in this study, were clearly distinguished along and across the *in vitro* model (Figure 6 and 7). As anticipated, larger agglomerates of NPs tended to remain entrapped on the apical side of the barrier. Although this tendency was seen for all four NPs (data not shown), the transversal cuts in Figure 7 only depicts large agglomerates of TiO₂NPs and F₂O₃NPs indicated by yellow arrows. In this case, the mucus layer plays an important role in protecting the *in vitro* intestinal epithelium from nano- and micro-particulate matter. The characterization of the mucus layer formed by the *in vitro* Caco-2/ HT29-MTX model demonstrate the presence of apical acidic and neutral mucosubstances (e.g. sialic acid) forming a continuous, thick layer (2–10 μm)⁵⁴. Our previous studies detected that exposures to TiO₂NPs diminish the mucus thickness but not the amount, suggesting that the *in vitro* model is actively secreting and replenishing mucosubstances.

Moreover, we showed that the presence of either *E. coli* or *L. rhamnosus* stimulates the mucus production, which counteract the detrimental effects of TiO₂NPs¹²⁹. However, smaller agglomerates

of all four NPs had the ability to cross the cell plasma membrane and begin crossing the *in vitro* intestinal barrier (Figure 7). Similar results have been previously observed by confocal microscopy, where following 24 h of exposure to NPs, large agglomerates of TiO₂- and SiO₂NPs were observed to settle down in the apical part of the Caco-2/HT29 barrier¹¹⁴. This could potentially result in alterations of the microvilli structure and consequently affect the enzymatic function of the brush border membrane¹⁵. However, internalization of NPs could also lead to further detrimental effects towards the general functionality of the barrier⁷⁴. Several cytotoxic mechanisms have been reported due to cell exposure to inorganic NPs¹³⁰. In 2006 Nel *et al.*, described the intrinsic ability of metal oxide NPs to generate damaging radicals (e.g. reactive oxygen species, ROS), participate in redox reactions, catalyze Fenton reactions producing ·OH, and alter cellular behavior¹³¹. However, the available *in vitro* toxicological data suggest that each NP induces toxicity via a different pathway. For example, ROS generation by TiO₂NPs, the protein corona formation in amorphous SiO₂NPs and the ion release of ZnONPs are the most common mechanism of toxicity described^{20,132,133}. However, it is still unclear whether iron oxide NPs are generally safe or should be used more careful in consumer products¹³⁴. It has been suggested that hematite (α-Fe₂O₃), a type of iron oxide NPs mainly known as a pigment mineral, can be metabolized and release iron ions easily transported and stored by the body¹³⁵. Transition metal ions have the capability to generate ROS and stimulate the peroxidation of cell membrane lipids¹³⁶. In the current study, the results indicate that Fe₂O₃NPs generally tended to agglomerate after the *in vitro* human digestion process (Figure 1), although a small amount also dissolved and resulted in ion release (Figure S4). However, neither the nano-iron (Figure S2 and S3), nor the ionic iron (Figure S5 and S6) at physiologically relevant doses were found to be cytotoxic to human cells or bacteria. The Fe₂O₃NPs formed large agglomerates that were able to come in contact with the microvilli of the enterocytes (Figure 6 and 7), but they did not disrupt the digestive function of the Caco-2/HT29-MTX *in vitro* model (Figures 2, 3, 4 and 5). A previous study by Kalive *et al.*, indicated that smaller hematite NPs (17 nm) were more toxic to the epithelial integrity than larger NPs (53 or 100 nm), and those effects were aggravated with time (up to 28 days)¹³⁷. Moreover, Zhang *et al.*, found out that after 1 hour of exposure, hematite NPs did not affect the enterocyte structure. However, at longer exposure times (up to 5 days) NPs were able to structurally disrupt the enterocyte microvilli¹³⁸. Altogether, these results lead us to conclude that when agglomerated, Fe₂O₃NPs lose reactivity and consequently turns into a more biologically inert material. However, the Fe₂O₃NPs bioaccumulation and biopersistence could aggravate the impact of iron oxide NPs in cell cultures or *in vivo*.

Finally, as described in the results section, all NPs regardless of their primary structure, agglomeration state and concentration, were found getting in contact with both types of bacteria (Gram-positive and Gram-negative) (Figure 8). It has been described that in bacterial cells, lipopolysaccharides (LPS) and lipoteichoic acids (LTA), proteins, and phospholipids are the principal biomolecules highly capable to interact with NPs when they approximate to the cell wall¹³⁹. In 2010, Jiang *et al.*, demonstrated that metal oxide NPs (e.g. Al₂O₃NPs, TiO₂NPs and ZnONPs) binds to bacterial LPS and LTA present in the cell envelope through hydrogen bonding and ligand exchange¹⁴⁰. Biomolecules on the bacterial envelope dominate cell adhesion through their highly charged structure, functional groups, and/or the bridging effect to the surface^{141,142}. The electrostatic attraction between NPs and bacteria would be another possible explanation. This occurs when the NPs are positively charged promoting attractive forces to the bacterial cell wall, which is negatively charged¹⁴³. However, this could not explain the NPs adhesion observed under confocal microscopy since our DLS results concluded that, after the *in vitro* digestion, NPs are negatively charged (Figure 1E). Furthermore, the bacterial cell abundance as well as external bacterial cell structures (e.g. fimbriae, pilus and flagella), can influence the bacteria-NPs adsorption rate. In the present case, the fast growth rate of *E. coli* (Figure S3) could increase the rate of NPs adsorption compared to the slow growth of *L. rhamnosus*. However, further experiments are needed to elucidate the factors and mechanisms of bacteria-NPs interaction and determine the differences between species. Here, the observed tendency of both bacterial strains is to adsorb the digested NPs to its surface potentially diminishing the number of NPs that interact with the *in vitro* epithelium of the small intestine. This could be one mechanism by which the intestinal microbiota could protect the small intestinal and modulate the toxic effects of food additives NPs.

CONCLUSIONS

The goal of this study was to realistically recreate an entire digestive system and analyze the role of intestinal microbiota following ingestion of food additive NPs. The well-established *in vitro* Caco-2/HT29-MTX model was exposed to four types of NPs commonly added to food (TiO₂NPs, SiO₂NPs, Fe₂O₃NPs) and used in food packaging (ZnONPs). NPs were first assessed for biotransformation after subjecting them to an *in vitro* human digestion. DLS measurements supported by confocal images demonstrated that NPs arrive to the duodenum primarily in the form of large agglomerates. The physiologically relevant doses of all four NPs did not confer cytotoxic effects to the *in vitro* intestinal barrier after 4 hours of treatment except for high doses of ZnONPs, which are a known antimicrobial agent.

Although no cytotoxicity was observed, TiO₂NPs, SiO₂NPs, and ZnONPs were shown to modulate

the digestive function of the *in vitro* intestinal barrier by altering the IAP and APN enzyme activities, but not the Na⁺/K⁺ATPase pump. SI activity was only significantly altered after the exposure to high doses of SiO₂NPs. Differences between BBM enzymes responses following NPs and bacteria exposures may be explained by differences in regulatory mechanisms of expression and/or receptor affinity. Confocal images of NP localization in the *in vitro* model would support the hypothesis that enzymes on the apical side of the BBM were more likely to physically interact with agglomerates of NPs than those located on the BLM, and consequently the BBM enzymes IAP, APN and SI were the most affected. Fe₂O₃NPs were shown to be the most inert compound even though the concentrations of Fe₂O₃NPs were the largest and their agglomerates were also observed to come in contact with the *in vitro* barrier and both bacterial strains. Finally, the addition of *E. coli* or *L. rhamnosus*, ameliorated some of the enzyme activities that were affected by the presence of food additives NPs. Confocal images reinforced the hypothesis that the intestinal bacteria could adsorb metal oxide NPs, diminishing the chance for NP to interact with the human intestinal epithelium. Overall, these results show that food additives NPs can cause significant effects to the functionality of the small intestine. However, the intestinal microbiota plays a critical role in protecting the human intestinal barrier from foreign particulate matter. A more realistic environment of the gastrointestinal tract has been achieved, in this work, by supplementing the *in vitro* Caco-2/HT29-MTX model with intestinal bacteria and an *in vitro* digestion process. Although further experiments are needed (e.g. testing other species that colonize the human intestine, long term experiments, etc), this system has been demonstrated to be a high-throughput, cost-effective, relatively fast experimental process and a more physiologically realistic methodology when compared to *in vivo* models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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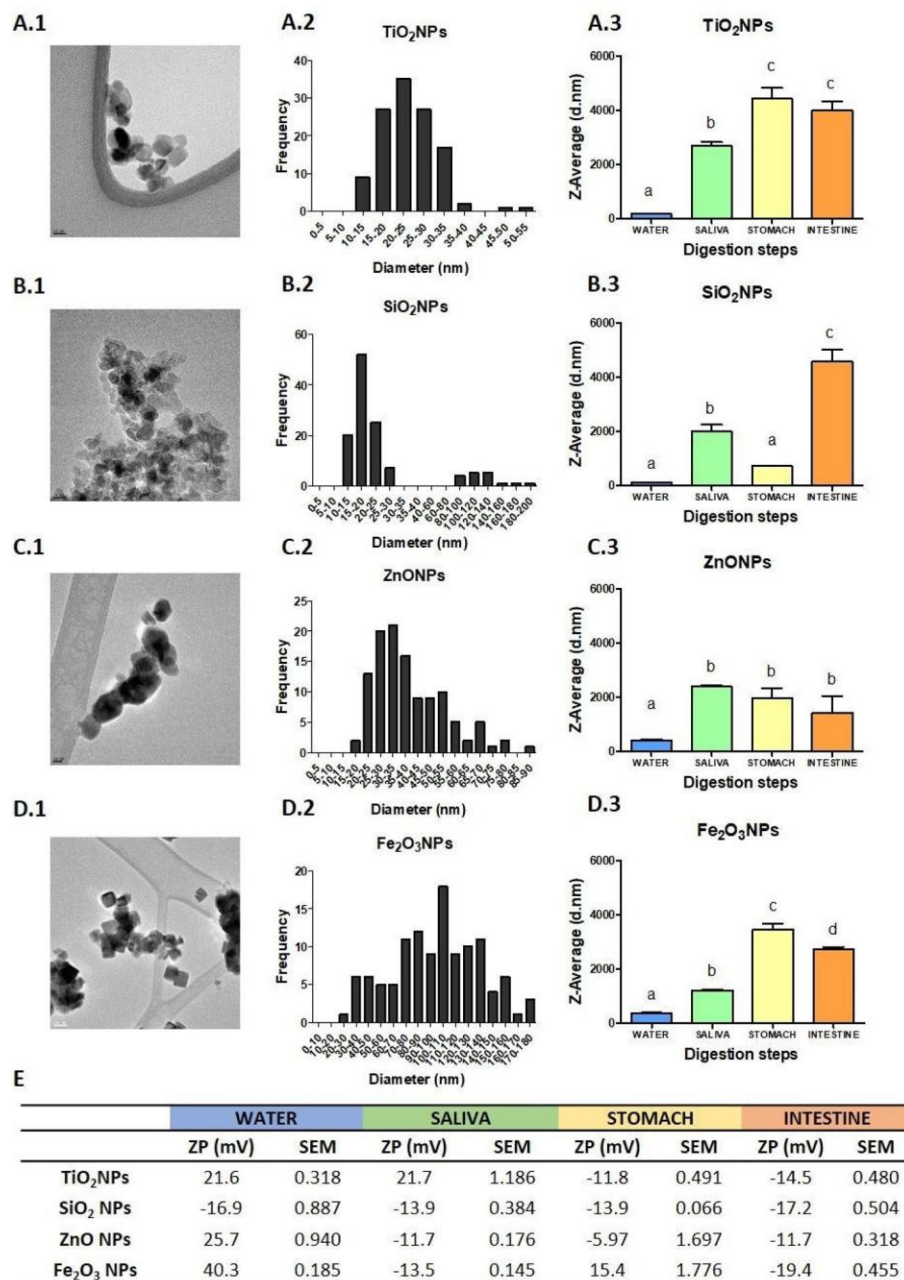


Figure 1. Nanoparticles (NPs) Characterization. Transmission electron microscopy (TEM) images of TiO₂NPs (**A.1**), SiO₂NPs (**B.1**), ZnONPs (**C.1**) and Fe₂O₃NPs (**D.1**), respectively, in dry form (20 nm scale bar). Frequency histogram of the primary particle diameter (nm) measured with Image J software (**A.2**, **B.2**, **C.2** and **D.2**). Dynamic light scattering (DLS) measurements of the NPs hydrodynamic size in each stage of the digestion process (saliva, stomach and intestine) (**A.3**, **B.3**, **C.3** and **D.3**). Results were analyzed according to the one-way ANOVA with Tukey's post-test and

represented as mean $\pm$ SEM. Bars that do not share a letter are significantly different ($P < 0.05$). (E) Table summarizing the zeta potential (ZP) values of the NPs in each stage of the digestion process. Data represented as mean $\pm$ SEM.

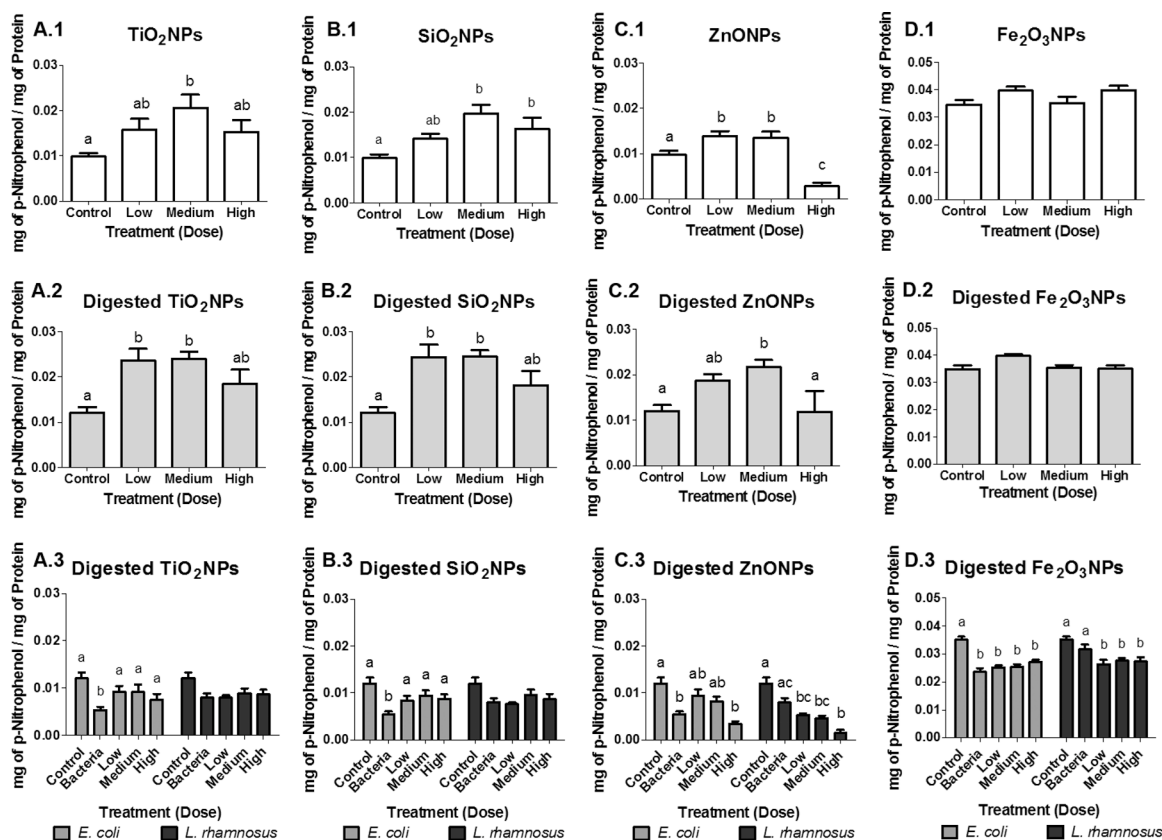


Figure 2. Intestinal alkaline phosphatase activity. The activity (mg of p-Nitrophenol/mg of Protein) of IAP was measured after exposure (4h) of the Caco-2/HT29-MTX-E12 barrier to undigested TiO_2NPs , SiO_2NPs , ZnONPs and $\text{Fe}_2\text{O}_3\text{NPs}$ (A.1, B.1, C.1 and D.1), *in vitro* digested TiO_2NPs , SiO_2NPs , ZnONPs and $\text{Fe}_2\text{O}_3\text{NPs}$ (A.2, B.2, C.2 and D.2), and *in vitro* digested TiO_2NPs , SiO_2NPs , ZnONPs and $\text{Fe}_2\text{O}_3\text{NPs}$ co-exposed with either *E. coli* or *L. rhamnosus* (A.3, B.3, C.3 and D.3), respectively. “Control” consists of untreated samples, and “Bacteria” of samples 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 ($P < 0.05$).

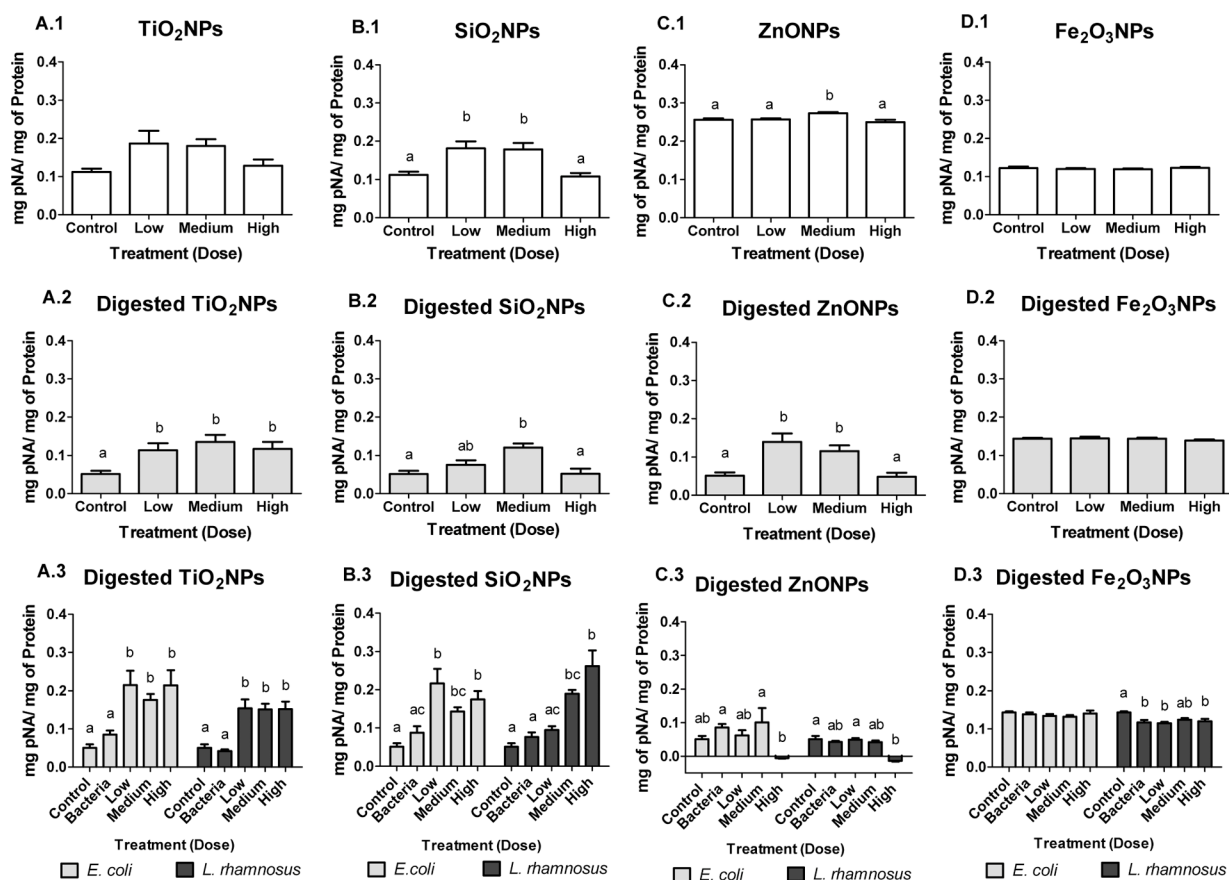


Figure 3. Aminopeptidase-N activity.

The activity (mg of 4-nitroanilide/mg of Protein) of APN was measured after exposure (4h) of the Caco-2/HT29-MTX-E12 barrier to undigested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (A.1, B.1, C.1 and D.1), *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (A.2, B.2, C.2 and D.2), and *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs co-exposed with either *E. coli* or *L. rhamnosus* (A.3, B.3, C.3 and D.3), respectively. “Control” consists of untreated samples, and “Bacteria” of samples 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 ($P < 0.05$).

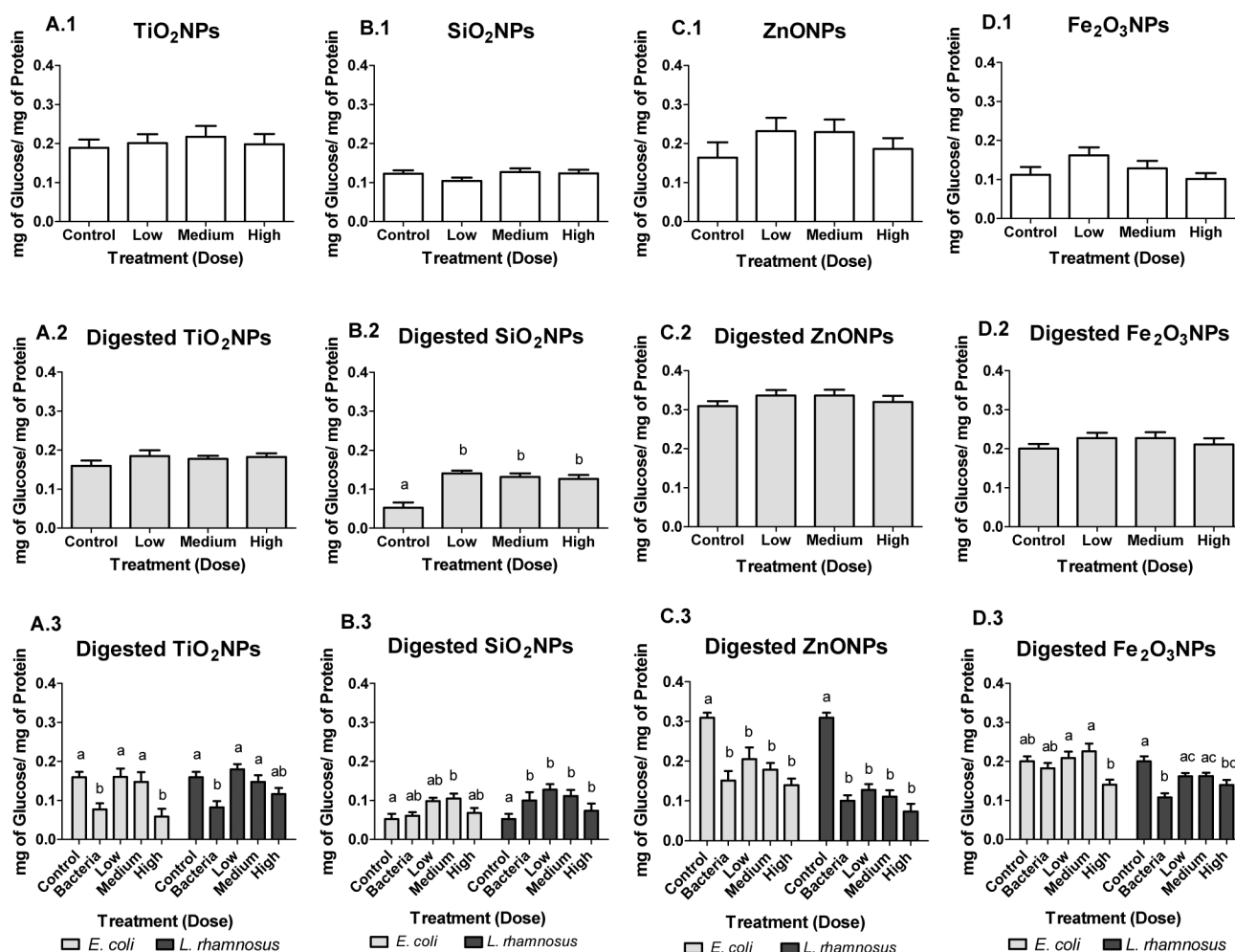


Figure 4. Sucrase Isomaltase activity. The activity (mg of glucose/mg of Protein) of SI was measured after exposure (4h) of the Caco-2/HT29-MTX-E12 barrier to undigested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (A.1, B.1, C.1 and D.1), *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (A.2, B.2, C.2 and D.2), and *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs co-exposed with either *E. coli* or *L. rhamnosus* (A.3, B.3, C.3 and D.3), respectively. “Control” consists of untreated samples, and “Bacteria” of samples 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 ($P < 0.05$).

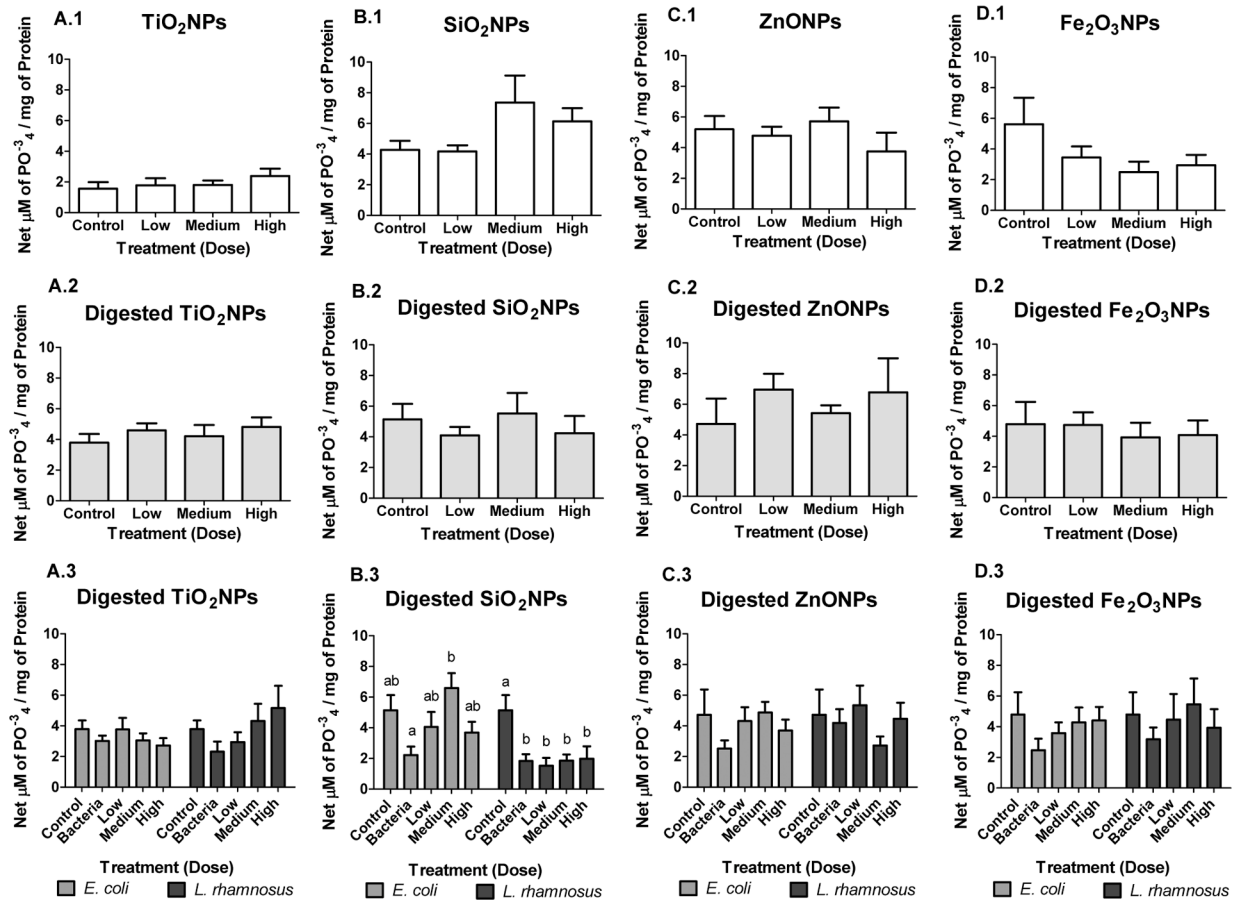


Figure 5. Na⁺/K⁺ ATPase activity. The activity (net μM of PO_4^{3-} /mg of Protein) of Na⁺/K⁺ ATPase was measured after exposure (4h) of the Caco-2/HT29-MTX-E12 barrier to undigested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (**A.1, B.1, C.1 and D.1**), *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs (**A.2, B.2, C.2 and D.2**), and *in vitro* digested TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs co-exposed with either *E. coli* or *L. rhamnosus* (**A.3, B.3, C.3 and D.3**), respectively. “Control” consists of untreated samples, and “Bacteria” of samples 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 ($P < 0.05$).

In vitro Caco-2/HT29-MTX-E12 Barrier

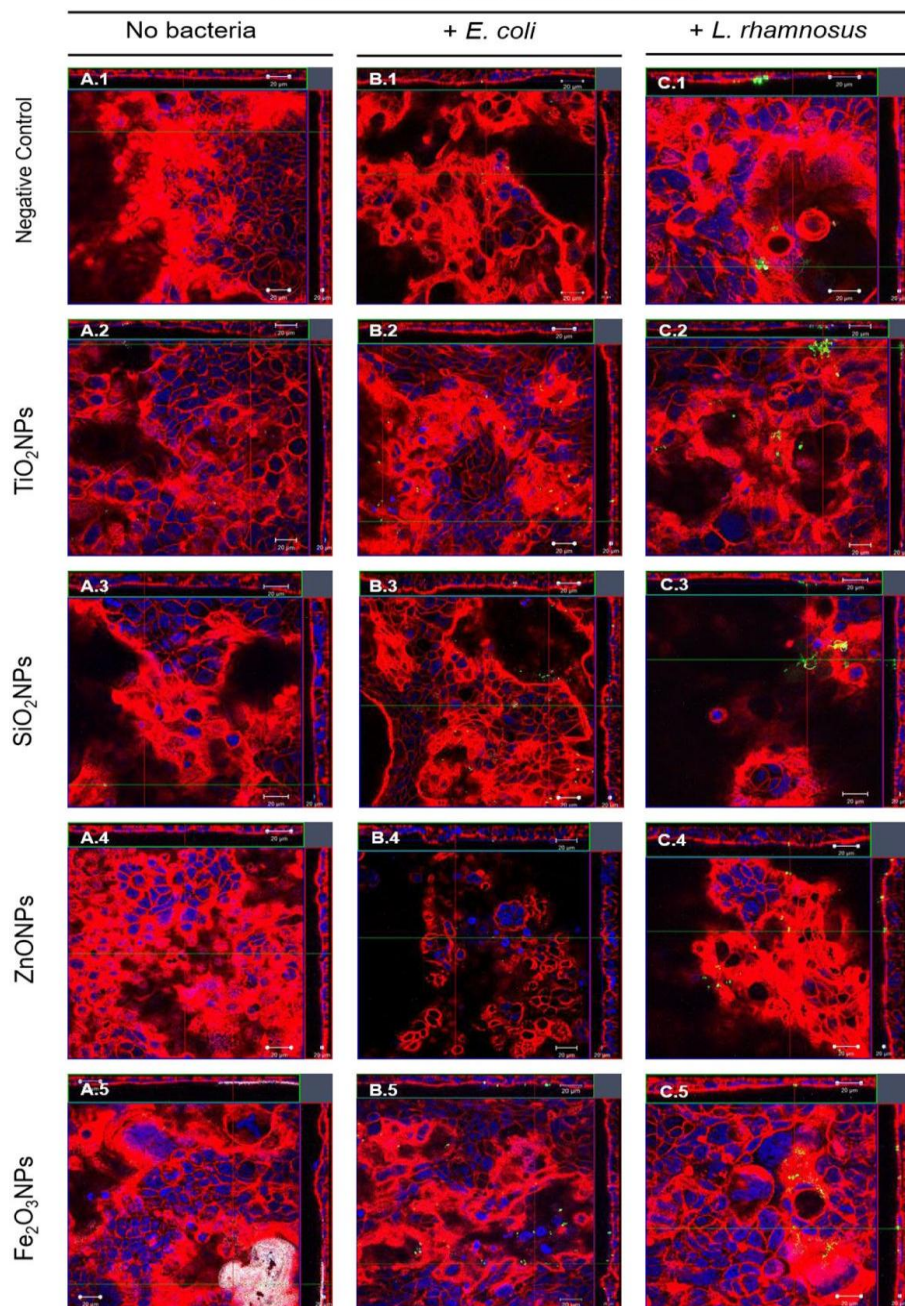


Figure 6. Confocal images (*ortho* images) of the Caco-2/HT29-MTX-E12 *in vitro* barrier.

Z-scans were performed after exposing the barrier for 4 h to (A) digested NPs, (B) digested NPs and *E. coli*, and (C) digested NPs and *L. rhamnosus*. Filaments of actin were marked in red, cell nucleus in blue, both *E. coli* and *L. rhamnosus* in green, and NPs reflection in white (Scale bars = 20 μm). Images are representative of 9 images analyzed.

In vitro Caco-2/HT29-MTX-E12 Barrier

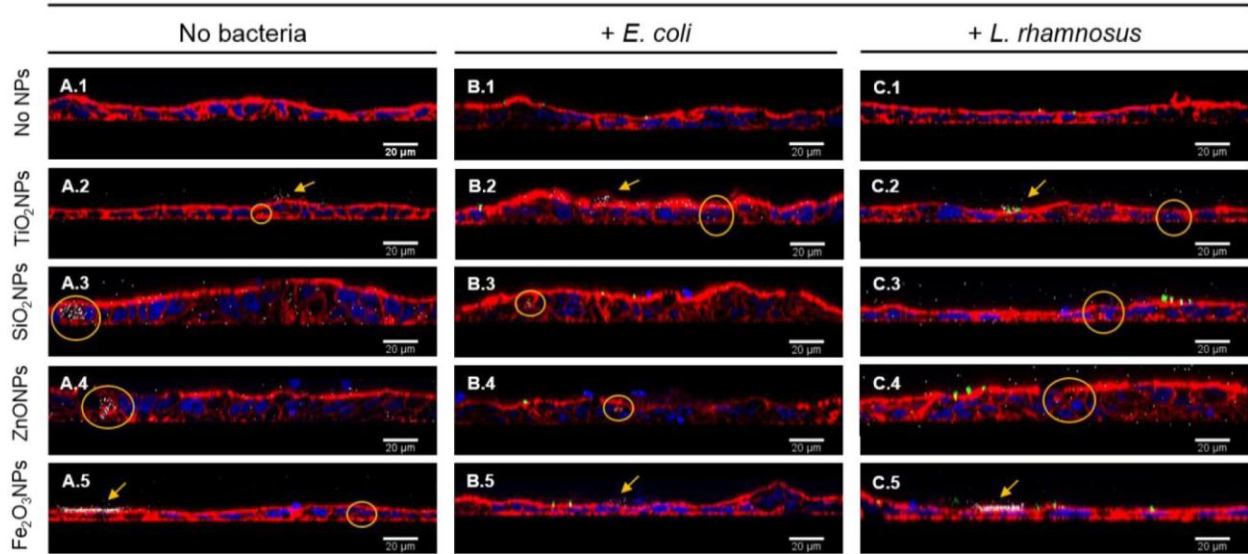


Figure 7. Transversal cuts of the Caco-2/HT29-MTX-E12 *in vitro* barrier.

Further analysis of the Z-scans was performed to study the NPs fate (high dose) across the Caco-2/HT29-MTX-E12 *in vitro* barrier. **(A)** *In vitro* barrier + digested NPs, **(B)** *in vitro* barrier + digested NPs + *E. coli*, and **(C)** *in vitro* barrier + digested NPs + *L. rhamnosus*. Filaments of actin are marked in red, cell nucleus in blue, both *E. coli* and *L. rhamnosus* in green, and NPs reflection in white (Scale bars = 20 μm). Yellow arrows indicate large NPs agglomerations entrapped on the apical side of the *in vitro* barrier. Yellow circles indicate smaller particles and/or NPs agglomerations crossing the *in vitro* barrier. Images presented are representative of 9 images analyzed.

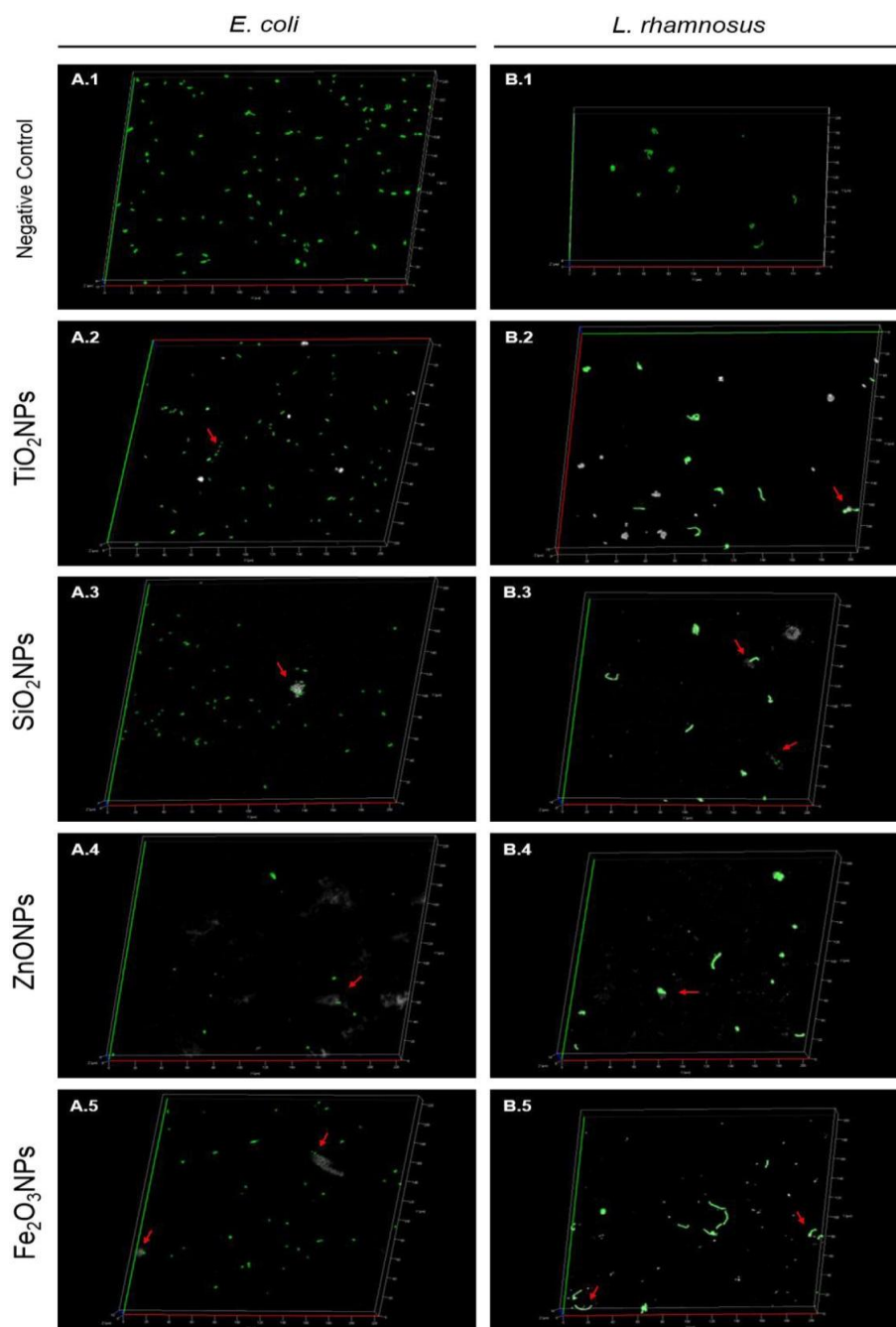


Figure 8. Confocal 3D images of bacteria-NPs interactions.

After 4 h of Caco-2/HT29-MTX-E12 *in vitro* barrier co-exposure with NPs and bacteria, the supernatant was collected and analyzed by confocal microscopy. **(A.1, A.2, A.3, A.4 and A.5)** *E. coli* and **(B.1, B.2, B.3, B.4 and B.5)** *L. rhamnosus* exposed to DMEM (negative control), TiO₂NPs, SiO₂NPs, ZnONPs and Fe₂O₃NPs, respectively. Bacteria was marked in green and NPs reflection in white. Red

arrow indicates a physical bacteria-NP interaction (Axis marks = 20 μ m). Images presented are representative of 9 images analyzed.

Table 1.

Most common ENMs used as food additives or food contact materials

Food additives	Properties	Food or food contact sources	References
Titanium dioxide	Whitening and reflectance	Powdered donuts, candies, and glazed bakery items	17,18
Silicon dioxide	Defoaming, anticaking and stabilizer	Multivitamins, powdered ingredients and industrial bakery items in general	14,19
Zinc oxide	Nutrition ¹ and Antimicrobial ²	Breakfast cereals ¹ , nutrition drinks ¹ and bars ¹ , and food packaging ² and cans ²	20,21
Iron oxide	Nutrition ¹ and Pigmenting ²	Supplemental capsules ¹ , meat ² , sauces ² , flavored drinks ² and chewing gums ²	16,22,23

Table 2.

Assayed small intestinal enzymes, location, and major function.

Enzyme	Location	Functions	References
Intestinal Alkaline Phosphatase	Apical brush border membrane of the small intestine	<ul style="list-style-type: none"> • Regulation of pH • Regulation of bicarbonate secretion • Long chain fatty acid absorption • Protection against pathogens 	42-44
Aminopeptidase-N	Apical brush border membrane of the small intestine	<ul style="list-style-type: none"> • Final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases 	45,46
Sodium-Potassium ATPase Pump	Basolateral side of the small intestine epithelium	<ul style="list-style-type: none"> • Regulation of ionic homeostasis • Maintenance of cell membrane potential • Maintenance of cell osmolarity 	40,41,47
Sucrase Isomaltase	Apical brush border membrane of the small intestine	<ul style="list-style-type: none"> • Digestion of carbohydrates (e.g starch), sucrose and isomaltose 	48,49

Table 3.

Dose range of NPs used to expose the Caco-2/HT29-MTX model. (*) Physiologically relevant doses extrapolated from the real human intake.

Dose	Nanoparticles per meal							
	TiO ₂ NPs		SiO ₂ NPs		ZnONPs		Fe ₂ O ₃ NPs	
	ng/mL	Particles/cm ²	ng/mL	Particles/cm ²	ng/mL	Particles/cm ²	ng/mL	Particles/cm ²
High	140	1×10 ¹⁰	2000	5.4×10 ¹⁰	9.7×10 ⁴	1×10 ¹³	3.8×10 ⁴	1.71×10 ⁷
Medium	*1.4	1×10 ⁸	*20	5.4×10 ⁸	970	1×10 ¹¹	*380	1.71×10 ⁵
Low	0.014	1×10 ⁶	0.2	5.4×10 ⁶	*9.7	1×10 ⁹	3.8	1.71×10 ³