Metal Oxide Nanoparticles Induce Minimal Phenotypic Changes in a Model Colon Gut Microbiota

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Abstract

Nanoparticles (NPs) are becoming prevalent in consumer goods, including foods and cosmetics. Understanding the interactions between NPs and bacteria in an engineered model colon can indicate potential impacts of NP exposure on the gut, and therefore overall human health. Human microbiome health has important implications to overall individual health. This work aims at quantifying the phenotypic response to NP ingestion of a model microbial community within a model colon. Three NPs at environmentally relevant concentrations (0.01 μg/L ZnO, 0.01 μg/L CeO₂, and 3 mg/L TiO₂) were individually introduced into a model colon to identify the subsequent impact on the gut microbial community. Results indicate that NPs cause the microbial community’s phenotype to partition into three distinct phases: initial conditions, a transition period, and a homeostatic phase, with the NP-exposed community displaying significant differences ($p < 0.05$) from the unexposed community in multiple phenotypic traits. Notably, phenotypes, including short-chain fatty acid (SCFA) production, hydrophobicity, sugar content of the extracellular polymeric substance, and electrophoretic mobility, which indicate changes in the community’s stability, were affected by the NPs. TiO₂ NPs led to extended phenotypic transformations for hydrophobicity when compared with the other NPs, likely due to its lack of dissociation and greater stability. Overall, the NPs caused nonlethal, significant changes to the microbial community’s phenotype, which may be related to overall health effects.

Key words: cerium oxide; gut microbiota; human health; model colon; titanium dioxide; toxicity; zinc oxide

Introduction

Nanotechnology offers many positive benefits to human health, such as more efficient drug delivery, sensor development for toxic substances detection, and medical diagnostic techniques (Thess et al., 1996; Kong et al., 2000; Long and Yang, 2001; Martin and Kohli, 2003); however, the increasing popularity of nanotechnology may lead to a rise in potential accidental exposures through ingestion or via environmental release of nanoparticles (NPs). It is estimated that 15,600 metric tons year$^{-1}$ of TiO₂ nanomaterials, 3,700 metric tons year$^{-1}$ of ZnO nanomaterials, and 300 metric tons year$^{-1}$ of CeO₂ nanomaterials enter water systems with the majority of these NPs discharged from wastewater treatment plants (Keller and Lazareva, 2013; Keller et al., 2013).

TiO₂ NPs are used daily by consumers with products such as toothpastes, cosmetics, and sunscreens (Wolf et al., 2003; Kaida et al., 2004) and may easily be ingested. CeO₂ NPs are introduced into water resources through the disposal of coatings, pigments, and paints (Keller et al., 2013). Much like CeO₂ and TiO₂, ZnO NPs can enter bodies of water not only through discarded coatings, pigments, and paints but also from the disposal of cosmetics (Keller and Lazareva, 2013; Keller et al., 2013). These three NPs were chosen based on their potential for human exposures through their widespread use in many consumer products, foods, and because of their potential to be in treated drinking water (Wolf et al., 2003; Kaida et al., 2004; Limbach et al., 2005; Auffan et al., 2009; Gottschalk et al., 2009; Kiser et al., 2009; Weir et al., 2012, 2013; Keller and Lazareva, 2013; Keller et al., 2013, 2014).

NPs selected in this study have been reported to have toxic, nonlethal effects on organisms. Recent work has shown that TiO₂ NPs can cross the epithelial lining in an intestinal model cell line via transcytosis (Koeneman et al., 2010). While TiO₂ did not cause cell death, there are implications of other nonlethal effects to the cells. CeO₂ NPs have caused negative effects in Escherichia coli, reduction in plant germination, and have caused membrane damage in eukaryotic cells (Zeyons et al., 2009; Kim et al., 2010; López-Moreno et al., 2010). In addition, research has also shown the CeO₂ NPs...
may have strain specific antimicrobial effects (Pelletier et al., 2010). CeO₂ particles are found in personal care products, and humans may also risk exposure to CeO₂ NPs through sunscreens and cosmetics (Limbach et al., 2005; Auffan et al., 2009). CeO₂ is also one of the most commonly utilized NPs, and it is used in a wide variety of applications. The disposal of these products into water sources is a potential route of exposure for humans. ZnO NPs exhibit toxicity to eukaryotic cells, decreasing cell viability and proliferation, and disrupting membrane integrity (Kim et al., 2010). ZnO NPs have also shown antimicrobial effects on bacteria; these effects are size and concentration dependent, as well as species specific (Yamamoto, 2001). However, it is important to note that many in vivo studies with higher-level organisms often give mixed results on toxicity (Allon et al., 2009; Chen et al., 2009; Khlebtsov and Dykman, 2011). Therefore, these three NPs were chosen to determine the potential toxicity on the gut microbiota, which is likely to occur through exposure routes such as accidental dosing and ingestion.

In vitro function of gut environments has previously been characterized by monitoring enzymatic activity (Van den Abbeele et al., 2010; Maathuis et al., 2012), short-chain fatty acid (SCFA) production (Jiménez-Vera et al., 2008; Van den Abbeele et al., 2010; Maathuis et al., 2012; Thévenot et al., 2013), and by determining microbial community genotypes (van der Werf and Venema, 2000; Rajilic-Stojanović et al., 2010; Van den Abbeele et al., 2010; Maathuis et al., 2012). Gut organisms play an important role with indispensable functions to the human host such as vitamin production, digestion, and immune system activity (Cummings, 1984; Hooper et al., 2002; Kelly et al., 2003).

The role of the gut microbiota is so prominent in human health that researchers have found links between gut microbes and numerous diseases (Bäckhed et al., 2004; Ley et al., 2005, 2006; Manichanh et al., 2006; Frank et al., 2007, 2011; Lepage et al., 2011; Mondot et al., 2011; Hsiao et al., 2013). In fact, changes in diet alone can cause rapid transformations in the activity and structure of the gut microbiota (David et al., 2014), indicating that the microbiota is sensitive with a quick reaction time to changes in the human intestines. This work highlights the importance of studying the effects of environmental contaminants on the gut microbiota.

Understanding the gut microbiome and its importance to human health with techniques such as molecular methods (Collins and Gibson, 1999; Matsuoka et al., 2004; Eckburg et al., 2005; Armougom et al., 2009) will give rise to more treatment and disease prevention options. However, more information is needed than just sequencing data to fully understand the complex and dynamic function of the gut microbiota. Monitoring changes in the community’s characteristics over time, such as the physical-chemical features, may provide a more complete representation of the gut’s function and role in human health. Here, phenotypic characterization techniques based on colloidal (e.g., cell) transport experiments provide valuable insights that may not typically be measured with a microbiota study. To date, this is the first paper analyzing the effects of environmentally relevant concentrations of NPs and their effects on the physical-chemical components of the gut community.

Published work with this model colon design has shown that microbial communities in the gut, wastewater, and groundwater can undergo significant phenotypic and genotypic changes when a perturbation is introduced, such as a pathogen (Marcus et al., 2013). We hypothesize that as compared with pathogen-induced disturbances, the model colon’s microbial community will undergo similar phenotypic changes when the colon is dosed with environmentally relevant concentrations of three NPs: TiO₂, CeO₂, and ZnO.

**Experimental Protocol**

**Microbial community and model colon**

Experimental parameters measured in this work were identical to Marcus et al. (2013) as was the preparation of the microbial community, the microbial medium representing digested food entering the large intestine (Minekus et al., 1999), and the in vitro model colon reactor (Jiménez-Vera et al., 2008; Marcus et al., 2013). The only exception was that the microbial community was donated by and developed (Apajalahi et al., 1998) from a healthy 26-year-old female volunteer who had not received antibiotics in more than 8 months. Briefly, frozen stocks of the microbial community were stored at −80°C and each week a stock community was thawed, inoculated into a 200 mL flask containing colon media (Minekus et al., 1999), and incubated for 24 hrs before being pumped into the dialysis tube inside the custom colon reactor.

A human colon was replicated by using a custom-built reactor (Marcus et al., 2013), which represented conditions inside a proximal colon (Nugent et al., 2001). The model colon ran for consecutive 5-day-long experiments. Colon effluent, or waste, was collected thrice a day during feedings for characterization experiments. The model colon was run for a minimum of two runs (two 5-day-long experiments) per experimental condition to ensure reproducible data. Additional details and images of the model colon set-up are located in the supplementary information (SI) in Supplementary Fig. S1.

**NP selection**

Zinc oxide (ZnO; Meliorum Technologies), titanium dioxide (TiO₂; Evonik Degussa Corporation), and cerium dioxide (CeO₂; Meliorum Technologies) NPs were selected for this work. They have previously been characterized in another study with the primary particle size for each NP reported as 10, 21, and 10 nm, respectively (Keller and Lazareva, 2012). Additional characteristics are listed in the Keller et al. study for the NPs, such as phase and structure, surface area, isoelectric point, purity, and electrophoretic mobility (EPM). Additional studies have also been conducted using these identical NPs to study their transport, aggregation, and effects on soil microbial communities (Ge et al., 2011; Thio et al., 2011; Chowdhury et al., 2013).

Environmentally relevant NP concentrations were chosen to emulate human exposures to NPs through ingestion of both food and drinking water at 0.01 μg/L ZnO NP, 0.01 μg/L CeO₂ NP, and 3 mg/L TiO₂ NP (Gottschalk et al., 2009; Kiser et al., 2009, 2013; Weir et al., 2012; Keller and Lazareva, 2013). Recent work has also indicated that adults in the USA ingest 5 mg TiO₂ per day, half of which is in the nano-size range (Lomer et al., 2000; Powell et al., 2010). Exposure routes and reliable dosing information of NPs that are
embedded in solid matrices are difficult to predict, and this is often a limitation of analytical techniques (Nowack et al., 2012; Yang and Westerhoff, 2014). The exposure levels used in this study were predominately selected from literature values that give predictions on amount of NPs in water and food sources (Gottschalk et al., 2009; Kiser et al., 2009; Weir et al., 2012; Keller and Lazareva, 2013; Keller et al., 2013).

NPs were added in bulk to the colon media before autoclaving and without alteration to achieve the concentrations mentioned earlier; this eliminated any contamination issues that may have occurred by adding NPs to the colon reactor independently of the food source. The addition of the NPs to the sterile food source also provided a realistic exposure scenario that can provide the possibility for representative NP transformations (Albanese and Chan, 2011; Levard et al., 2012, 2013; Lowry et al., 2012a, 2012b; Lombi et al., 2013; Reidy et al., 2013). During the week-long experiments, the sterile colon media containing the NPs was continually stirred, and thrice a day, 100 mL was added to the model colon by pumping the sterile colon media into the reactor. During each feeding, 100 mL of colon effluent was also removed for characterization experiments.

As a control, the model colon was run without NPs for two runs (two 5-day-long experiments) to determine the gut microbiota phenotype in the absence of NPs. All NP experiments were run for a minimum of two runs (two 5-day-long experiments) to ensure reproducibility, and all colon experiments were run under dark conditions to eliminate light effects. Additional controls were conducted to test the EPM and hydrophobicity of the NPs as a function of the extreme range of pH in the gastrointestinal tract. The purpose of this was to account for possible transformations of NPs and their subsequent changes in physicochemical properties. These tests are listed in the SI and mentioned in the Discussion section.

**Phenotypic characterization of microbial community**

Changes in the gut microbiota phenotype were examined with the following analyses: cell concentration, sugar and protein content of the extracellular polymeric substance (EPS), EPM (an indicator of the relative surface charge), hydrophobicity, and cell size following published methods (Marcus et al., 2012a, 2012b, 2013). Briefly, on daily sampling, bacteria from the model colon were washed with centrifugation (3,700 g) and then suspended in a 10 mM KCl solution before all phenotypic characterizations. All samples collected for all characterization methods were measured in three replicates twice a day at the same time points to eliminate additional variables. Additional details on these methods can be found in the SI.

**Biochemical characterization of microbial community**

Changes in SCFA production, pH, and conductivity of the gut environment were monitored to determine the effects of the NPs on the microbial community and gut environment. pH (Thermo Scientific™ Orion™ Model GD9156BNWP) and conductivity (YSI 3200 Conductivity Instrument Model # 3200 115V) of the colon effluent were measured twice a day at the same time points with three replicates each for all control and NP experiments. Conductivity is an indirect measurement of the metal ion dissociation from the NPs; measuring ionic content of the colon effluent gives approximations of changes in ionic strength (Griffin and Jurinak, 1973). Samples for SCFA analysis were collected in three replicates twice a day at the same time points from the colon effluent and polyethylene glycol (PEG) solution and were stored at −20°C until analysis with the gas chromatography flame ionization detector (GC-FID) (Agilent, Santa Clara, CA) using previously published methods (Venema et al., 2003; Marcus et al., 2013). The total concentration of the SCFAs was determined based on both the colon effluent and PEG samples. Butyric acid and acetic acid were analyzed by integration under a fitted flame ionization detection curve. Propionic acid is not reported due to the inability to integrate under the curve and account for accurate measurements of this SCFA.

**Statistical analyses**

All data was tested for normality and equal variance and analyzed with a one-way analysis of variance (ANOVA) and a Student’s t test in Excel (v.14.0; Microsoft, Redmond, WA) to determine the phenotypic variation of the microbial community. Results were considered significant if $p < 0.05$.

**Results**

All data presented in the results are based on a minimum of two 5-day-long experimental runs in the model colon. All data points collected are a culmination of a minimum of three replicates per measurement. Additional details are found in the SI regarding control experiments used in this study.

**Phenotypic characterization**

Extensive phenotypic testing of the microbial community was not only chosen based on known human health indicators such as SCFA production but also used common evaluation techniques, such as surface charge, hydrophobicity, cell concentration, and EPS content, that are associated with environmental microbial community sampling (Topping and Clifton, 2001; Wong et al., 2006; Tazehkand et al., 2008; Bolster et al., 2009, 2010; Marcus et al., 2012a). These nontraditional testing methods were selected to further characterize the gut environment and microbial community and the changes that may occur during perturbances.

Data for cell hydrophobicity, EPM, and the sugar and protein content of the EPS were partitioned into three statistically different phases ($p < 0.05$) throughout the 5-day-long experiments. Tuesday and Wednesday (days 2–3) data were statistically the same ($p > 0.05$), and Thursday and Friday (days 4–5) data were also statistically the same ($p > 0.05$). Therefore, weekdays were grouped in the following manner as three significantly different time points: an initial phase on Monday (day 1), a transition phase on Tuesday and Wednesday (days 2–3), and a homeostatic phase on Thursday and Friday (days 4–5). Data for all of the phenotypic characterization tests are also displayed per daily values and can be found in the SI (Supplementary Figs. S2–S7).

For cell hydrophobicity, all three metal NPs altered cellular hydrophobicity when compared with the control, ranging from a 0% to 10% increase during the course of the 5-day experiments (Fig. 1). All day 1 values (for all three NPs and control) were statistically the same (1–5% ± 0.8–5.3%, $p > 0.05$). CeO$_2$ and ZnO showed significant increases in hydrophobicity ($p < 0.05$) between day 1 and days 2–3 (CeO$_2$ increased from...
0.9% ± 5.4% to 19.0% ± 5.3%, ZnO increased from 8.4% ± 5.3% to 15.0% ± 8.2%), whereas all three metals led to significant increases in cellular hydrophobicity from day 1 to days 4–5 (CeO2 from 0.9% ± 4.4% to 34% ± 16.8%, ZnO from 8.4% ± 5.3% to 31.0% ± 14.1%, and TiO2 from 4.5% ± 0.6% to 30% ± 14.8%).

EPM (an indicator of the relative surface charge) of the microbial community was partitioned into three distinct phases. EPM became significantly less negative and was near neutral during the mid-week phase (days 2–3) for all three NPs when compared with the control (control = −1.1 ± 0.2 [μm/s]/(V/cm)], NPs range from −0.7 ± 0.2 to −0.9 ± 0.3 [μm/s]/(V/cm)], Fig. 1). EPM then became significantly more negative during the homeostatic phase (days 4–5, control = −0.9 ± 0.6 [μm/s]/(V/cm)], NPs range from −0.97 ± 0.5 to −1.2 ± 0.4 [μm/s]/(V/cm)]) (p < 0.05).

Sugar and protein content of the EPS was also partitioned into three significantly distinct data points (Fig. 2). The sugar content of the EPS significantly peaked mid-week during the transition phase (days 2–3, TiO2 and CeO2 6–8.5 ± 1.7–3.5 mg/cell×10^10 for TiO2 and CeO2 NPs (p < 0.05), whereas the protein content had no significant trends.

Cell concentration decreased from 4–5.5×10^10 cells/mL to 1–3×10^10 cells/mL during the course of the 5-day-long experiments for all conditions (data not shown); no significance was noted between the control (without NPs) and the three metal NPs, indicating that NPs did not have an effect on cell concentration in the model colon.

There was no significant difference (p > 0.05) in cell size (radius) within the control experiments during the course of the 5-day-long experiment (Table 1), indicating that in the absence of NPs the cell size remains constant inside the model colon. However, significant changes (p < 0.05) were seen in cell size during exposure to all three metal NPs. Data did not partition into the three distinct phases seen with the other cell phenotypes. CeO2 caused cells to decrease significantly for the entire duration of the experiment when compared with the controls; specifically, cells were significantly smaller during the CeO2 exposure on days 4 and 5 (control 0.60 ± 0.02–0.62 ± 0.02 μm, CeO2 days 4 and 5 0.46 ± 0.02–0.47 ± 0.02 μm). Of the three NPs, CeO2 caused the most significant deviations from the control cell size. TiO2 NPs caused a decrease in cell radius on days 3 and 5, exhibiting a smaller cell size, 0.59 ± 0.01 and 0.55 ± 0.05 μm, when compared with the control. Of the three NPs, TiO2 caused the least amount of changes in cell size. When compared with the control, ZnO caused significant (p < 0.05) decreases in cell size for days 1, 3, and 5 (0.49 ± 0.02, 0.59 ± 0.05, and 0.53 ± 0.04 μm). In summary, the phenotypic changes occurring

**FIG. 1.** (A) Comparison of production of short-chain fatty acids (SCFAs, acetic and butyric acids) over 5-day-long experiments in the model colon in the presence and absence of TiO2, CeO2, and ZnO NPs. SCFA samples were collected twice a day for a minimum of 2 weeks and analyzed in triplicate with GC-FID. Each data point is an average of a minimum of 60 values averaged that were collected over the course of two experimental runs (two 5-day-long experiments) with samples in triplicate collected twice a day. (B) Hydrophobicity of bacterial cells is displayed as a function of NP exposure in the model colon during the 5-day-long experiments. Hydrophobicity was measured twice a day in triplicate. (C) Electrophoretic mobility (EPM), an indicator of the relative surface charge, of bacterial cells was measured in triplicate twice a day during the course of the 5-day-long experiments for a minimum of two runs. All measurements were made on washed cells from colon effluent. Error bars indicate standard deviation.

**FIG. 2.** Average protein (A) and sugar content (B) of the extracellular polymeric substance (EPS) was measured in triplicate once a day during the course of the 5-day long experiments for a minimum of two runs (two 5-day-long experiments). EPS samples were collected once a day from the colon effluent and analyzed for sugar and protein content in triplicate. Error bars indicate standard deviation.
Day 1 pH values for the controls and the TiO₂, CeO₂, and ZnO comparisons among pH values for all experimental conditions. –0.2 (day 2) and 6.6 showed a significantly lower pH in the presence of TiO₂ and compared with the control for days 2–3. Model colon expressed a lower pH for each NP when compared with the control and all NP conditions (Fig. 1). The only significant result occurred during exposure to CeO₂ and showed a decrease in butyric acid production (p<0.05, control = 105.8 ± 13.9 mM, butyric acid = 78.0 ± 16.0 mM).

Changes in pH were monitored twice a day from the colon effluent. During the three individual NP exposures, the pH was significantly lower during the transition phase condition. The effluent. During the three individual NP exposures, the pH was significantly lower during the transition phase condition. The system also exhibits small variation within the control population. However, work has shown that even pure cultures of genetically identical bacteria can behave in radically different ways under the same experimental conditions (Kulasekara et al., 2013). This may explain some of the variation seen within the same conditions.

Increasing conductivity trends (Fig. 3) were noted for CeO₂ exposures, with only day 2 data significantly higher than the control (p=0.02, control = 5.9 ± 0.1 μS/cm, CeO₂ = 6.7 ± 0.5 μS/cm). ZnO on day 3 (p=0.002) and day 5 (p=0.008) had a lower conductivity for both days when compared with the control (control = 6.2 ± 0.1 μS/cm, ZnO = 5.0 ± 0.3 μS/cm). TiO₂ did not cause a statistical change in conductivity. Overall, biochemical characterization demonstrated that slight changes occurred within the model colon reactor due to the NPs.

**Biochemical characterization**

SCFA production remained relatively constant between the control and all NP conditions (Fig. 1). The only significant result occurred during exposure to CeO₂ and showed a decrease in butyric acid production (p < 0.05, control = 105.8 ± 13.9 mM, butyric acid = 78.0 ± 16.0 mM).

**Discussion**

Techniques used in this work offer unique insights into alterations to the microbial metabolic processes inside of a model colon caused by NPs. An important consideration when studying the gut microbiota and interpreting results is that one human sample does not represent the high variability and diversity of gut microbiomes present in the human population. However, work has shown that even pure cultures of genetically identical bacteria can behave in radically different ways under the same experimental conditions (Kulasekara et al., 2013). This may explain some of the variation seen within the same conditions.

Here, the NPs added into the system clearly affected the phenotypic characteristics of the microbial community as well as the gut microenvironment. It is also important to note that the system also exhibits small variation within the control conditions on a weekly basis, specifically, with the cell size (no significant difference during control conditions, 0.59 ± 0.05–0.62 ± 0.02 μm) and the EPM (~0.90 ± 0.2 to ~1.1 ± 0.2 (μm/s)/

**Table 1. Cell Size Measurements as a Function of Nanoparticle Exposure**

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.59 (0.05)</td>
<td>0.61 (0.02)</td>
<td>0.61 (0.02)</td>
<td>0.60 (0.02)</td>
<td>0.62 (0.02)</td>
</tr>
<tr>
<td>CeO₂</td>
<td>0.53 (0.03)</td>
<td>0.54 (0.02)</td>
<td>0.54 (0.02)</td>
<td>0.46 (0.02)</td>
<td>0.47 (0.02)</td>
</tr>
<tr>
<td>TiO₂</td>
<td>0.63 (0.02)</td>
<td>0.62 (0.02)</td>
<td>0.59 (0.01)</td>
<td>0.61 (0.03)</td>
<td>0.55 (0.05)</td>
</tr>
<tr>
<td>ZnO</td>
<td>0.49 (0.02)</td>
<td>0.53 (0.02)</td>
<td>0.59 (0.05)</td>
<td>0.56 (0.05)</td>
<td>0.53 (0.04)</td>
</tr>
</tbody>
</table>

Cell size as bacteria radius (μm) was measured in triplicate twice a day during the course of the 5-day-long experiments for a minimum of two runs (two 5-day-long experiments). Values in parentheses indicate standard deviation. *Indicates significant changes in cell size (p < 0.05) as a function of NP treatment when compared with the control. †Indicates significant changes in cell size within individual weeks (p < 0.05) specific to NP treatments.

**FIG. 3.** (A) Change in pH during the course of the 5-day-long experiments for the control and three NPs: TiO₂, CeO₂, and ZnO. pH measurements were taken from the colon and were measured in triplicate twice a day during the course of the 5-day-long experiments for a minimum of two runs (two 5-day-long experiments). (B) Change in conductivity (μS/cm) during the course of the 5-day-long experiments for the control and three NPs: TiO₂, CeO₂, and ZnO. Conductivity measurements were taken from the colon effluent in triplicate twice a day for a minimum of two runs, resulting in a minimum of 60 data points. Error bars indicate standard deviation.
(V/cm)]. Therefore, the system may accurately reflect a human gut microbial community.

**Phenotypic characterization**

Changes in cellular hydrophobicity have been linked to the formation of biofilms (Schäfer et al., 1998; Marcus et al., 2012b), which are aggregates of microorganisms. Over the course of the experiments, the cells experienced an increase in hydrophobicity in all NP conditions. Therefore, increases in hydrophobicity may limit the cell’s surface area for interactions with the colon media and NP solution inside of the model colon, possibly reducing contact between cells and NPs.

Bacterial cells during the NP exposures that presented near-neutral EPM when compared with the control may be attributed to the NPs coating the surface of the cell and therefore changing its EPM (Jiang et al., 2009). EPM has been linked to the attachment and stability of microorganisms, with higher absolute values of EPM linked with more stable, or mobile, microorganisms (Elimelech et al., 1998; Hermansson, 1999; Marcus et al., 2013). This suggests that the microbial community exposed to the NPs is the least stable during the mid-week transition phase, and it is more stable in the initial and homeostatic phases. This indicates there is an increased chance for the community to undergo attachment and form aggregates during less stable conditions. In addition, this study had more near-neutral EPM values associated with the microbial community when compared with previous research with this system that used a microbial community that had more negative values and was donated from a healthy male (Marcus et al., 2013). This demonstrates that microbial communities among individuals will also vary in the overall surface charge.

Sugar and protein content of the EPS was measured, because they have been utilized as indicators of cellular conditions (Eboigbodin and Biggs, 2008). Increased amounts of sugar compared with the protein content of the EPS are related to cell aggregation and biofilm formation (Marcotte et al., 2007), which may be a mechanism of the cell to limit exposure to NPs inside the model colon; less surface area of the cells may be exposed to the colon media/NP solution. Here, the sugar content relative to the protein increased mid-week, indicating a greater potential for the cells to undergo aggregation during the transition (mid-week) phase. However, the protein portion of the EPS did not show distinct trends; this indicates that this testing method may not be ideal for determining changes within a colon microbial community.

Establishing changes in the distribution of bacterial cell size can give an indication whether NPs are causing phenotypic changes to the microbial community within the model colon; radius measurements indicate that morphological changes are occurring in bacteria width and length. Multiple changes in eukaryotic cellular morphology, which include changes in cell size such as cell rounding, nuclear membrane blebbing, chromatin condensation, and alterations in cytoplasmic organelles, have been associated with cytotoxicity caused by quantum dots and single-walled carbon nanotubes (SWCT) (Shvedova et al., 2003; Lovrić et al., 2005). Decreases in bacteria cell size have been attributed to stress, with one example being starvation. Stressed cells undergo phenotypic changes in size to increase the likelihood of survival. This includes reductive division to increase cell numbers and increasing the production of hydrophobic molecules that favor aggregation (Morita, 1986; Jürgens et al., 1987). However, it should be mentioned that while community structure was not determined here with DNA sequencing, changes in the distribution of cell size may be attributed to changes in the microbial community structure (Hahn and Höfle, 1999; Jürgens et al., 1999; Hueso et al., 2012). Regardless of this, this measurement gives an indication that the community’s size distribution is changing over time in response to the NP exposures.

**Biochemical characterization**

Butyric acid is largely produced by the microbial breakdown of dietary fiber in the gut; increased levels of butyric acid inside the gut have been linked to protective measures against colorectal cancer such as cell proliferation reduction, decreases in tumor mass, and maintenance of a normal microbial population inside of the intestines (Ames, 1983; Boffa et al., 1992; Harris and Ferguson, 1993; McIntyre et al., 1993; Topping and Clifton, 2001). A decrease in butyric acid inside the gut not only results in increased susceptibility to inflammation but may also cause a decrease in the barrier function of the gut (Sunkara et al., 2011). This indicates that NPs could have a two-fold impact on the intestine by first affecting SCFA production, then leading to systemic circulation of the NPs via an inhibited intestinal barrier.

Under all conditions tested (control and the three individual NP exposures), the pH of the colonic fluid decreased with time. This decrease is a natural phenomenon of the microbial community as it ages and digests the nutrients it is supplied during the course of the experiments (McLauchlan et al., 1989; van Herwaarden et al., 1999). The presence of the NPs did not result in a significant difference from the control, with all experiments exhibiting the same gradual decline in pH. Therefore, it is likely that the addition of the NPs did not alter the pH, but induced a change in the microbes that may have altered the pH.

pH did not partition into three distinct phases unlike the other data: hydrophobicity, sugar and protein content, and EPM. During the course of the experiments, the colon bioreactor mimicked conditions found inside of a proximal colon with an expected pH range between 5.5 and 7 (Nugent et al., 2001) for control days 1–3, which demonstrates that the model colon is stable during this period. Control days 4–5 have a pH below 5.5; therefore, the changes noted in the NP experiments during days 4–5 (homeostatic phase) cannot be exclusively credited to the presence of the NPs. While decreases in a complex media with a neutral pH have been attributed NP dissolution (Bian et al., 2011), it is unlikely that this occurred in the model colon due to the similar trends between the control and the three NP trends. Changes in pH may also be caused by acidic metabolites and the available substrates within the intestines (Louis et al., 2007). A study in the literature has shown that there are circadian fluctuations within the stomach and intestinal pH (McLauchlan et al., 1989; van Herwaarden et al., 1999). Since pH did not show significant differences between the control and the NP exposures for 3 out of the 5 experimental days, overall, the decrease in pH within this system is a valid trend (McLauchlan et al., 1989; van Herwaarden et al., 1999).

Here, conductivity is used as an indirect measurement of the metal ion dissociation from the NPs; and it gives...
approximations of changes in the ionic strength of the colon effluent (Griffin and Jurinak, 1973). Compared with the control, the increase in day 2 CeO₂ conductivity may be attributed to metal ion release from the NP at this specific pH, while the decrease in conductivity for ZnO days 3 and 5 may be attributed to cellular uptake of ions, or ions being bound and made unavailable by the complex colon matrix (Bian et al., 2011).

Additional controls with NPs were conducted to simulate the range of pH exposure that may occur, from ingestion of NPs in food and water at a neutral pH, to a low pH in a stomach environment, and, finally, to a pH of 5.5–7 in the proximal intestine. The purpose of these controls was to determine whether changes in pH would affect the physicochemical properties of the NPs, and therefore have the possibility of altering the effects of NPs in the gut environment. As previous work has shown, changes in pH cause alterations in the physical-chemical behavior of NPs such as the surface charge, aggregation rate, and size (Wang and Keller, 2009; Chowdhury et al., 2011, 2013). Similar results were noted in the additional NP controls used in this study (data not shown) and may indicate that NP behavior may change on entering the low pH stomach environment, specifically the surface charge and size.

It is essential to note that interactions between NPs and bacteria in the intestines may be dependent on numerous factors: the surface charge of the NPs and bacteria, the chemical composition and surface charge of the digested food, and variability in diet. These factors may ultimately correlate to effects seen in humans on an individual basis. In fact, similar work has demonstrated that exposing common NPs found in food to stomach-like conditions will change their surface chemistry from negative to neutral or positive, causing the NPs to interact with negatively charged mucus proteins in the gastrointestinal tract and, in turn, affecting the transport of NPs within the intestine (McCracken et al., 2013). The purpose of this work was to measure responses of the microbial community during the NP exposures. Based on previous research, it is anticipated that the NPs altered by stomach-like conditions would also cause changes in the gut environment (McCracken et al., 2013).

Changes measured in the microbial community when the model colon was dosed with environmentally relevant concentrations of NPs demonstrate that not only will the NPs only minimally affect phenotype but also the particles may also elicit other nonlethal effects. Such effects may include stress to the microbial community. This may imply that other undesired effects on the human gut occur due to the NPs and, therefore, some alterations in overall human health could occur. Other studies have indicated that intestinal exposure to metal NPs has numerous outcomes, such as alterations in the enteric microbiota secretory response of serotonin, and NPs having increased retention time within the gut from entrapment in the intestinal folds, which led to amplified changes to the intestinal physiology (Özel et al., 2013, 2014).

In addition, ambient air particulate matter (<10 μm), which are particles three orders of magnitude larger than the model NPs investigated, have also been shown to cause changes inside of the mice intestine. Specifically, the colloidal material was observed to enhance gut permeability and cytokine secretion, altering SCFA production (decrease in butyric acid), and shifting the microbial community composition (Kish et al., 2013). This example provides evidence that additional and alternative exposure routes should be considered when designing NP studies. Particulate matter, which can contain particles on the nanoscale, has been shown to have adverse consequences on the gastrointestinal tract and is associated with increased risks for many diseases in which the gut microbiota also play a significant role (Gubéran et al., 1992; Kaplan et al., 2009; Orazzo et al., 2009; Ananthakrishnan et al., 2011; Beamish et al., 2011; Kish et al., 2013). Therefore, another route of exposure to NPs in the gut may be through inhalation of ambient air, in addition to the ingestion exposure route and relevant doses accounted for in this study. The inhalation exposure route demonstrates that atypical exposure scenarios should be considered when designing future NP exposure experiments and this is a relevant pathway for exposure given the current knowledge on ultrafine particle matter and its impacts on health (Bakand, 2012 #638; Grassian, 2007 #637).

Furthermore, NPs can undergo transformations in the environment or in consumer products such as speciation, dissolution, or aggregation that can alter the NP toxicity, reactivity, and physicochemical properties. These changes are not accurately represented with testing “as-received” particles (Levard et al., 2012, 2013; Lowry et al., 2012a, 2012b; Lombi et al., 2013; Reidy et al., 2013). Studying NPs in realistic scenarios is of the utmost importance. Here, the concern of a realistic exposure and subsequent transformation was addressed by dosing the sterile colon media with the NPs. The NPs remained in the colon media for the duration of the 5-day-long experiments. Unfortunately, one disadvantage is the difficulty in characterizing NPs after introduction into complex media due to limitations in analytical techniques (Levard et al., 2012; Reidy et al., 2013). While the NP exposures were designed to mimic realistic scenarios that would involve NP transformation, it was beyond the scope of this particular paper to evaluate the degree of transformation occurring in the colon environment. However, complementary studies have similarly used bacteria in both realistic settings and idealized laboratory studies to determine the effects of NP toxicity on organisms without measuring the fate or transformation of the particles in the systems (Ge et al., 2013; Ivask et al., 2013).

Our initial hypothesis, that NPs induce phenotypic changes in a gut microbial community, was affirmed through significant measurable effects seen in the data. Tests that supported that NPs caused changes in the phenotype included hydrophobicity, EPM, sugar content of the EPS, cell size, conductivity, and SFCA (specifically butyric acid) production. Data for cell concentration and the protein content of the EPS demonstrated no significant results. Data were inconclusive for pH. With such a complex biological system, it is very likely that the phenotypic and biochemical changes observed are combinations of responses happening in parallel. The effects seen may be attributed to both changes induced by the NPs and natural phenomena associated with microbial community activity and other metabolic processes in a multifaceted environment such as the gut. Some examples of natural processes that could also influence the phenotypic and biochemical parameters are osmolarity, active metabolites, and electrolyte concentrations (Miller and Wood, 1996; Record et al., 1998).

In addition, not only the complexity of the microbiota but also the intricacy of a living colon environment is extremely
difficult to predict and monitor. Since the human colon microbiota plays a large and diverse role in overall human health, particularly with immunity and disease development (Guarner and Malagelada, 2003; Wallace et al., 2011; Clemente et al., 2012), and can vary greatly per individual basis, having an understanding of how the microbiota is affected at the phenotypic level may provide crucial information to better characterize an unhealthy or stressed colon microbial community.

Conclusions

This work highlights the relevance of studying a complex matrix and microbial community in vitro rather than individual microbial species in situ rather than individual microbial analysis such as phenotypic measurements, are also needed to further characterize changes in microbial communities. These tests can provide a depth of information that may complement microbial community sequencing data and other traditional colonic enzyme assays. Here, significant changes in hydrophobicity, EPM, sugar content of the EPS, cell size, conductivity, and SCFA demonstrated that representative NPs found in consumer products and water sources with the potential to be ingested can minimally impact the gut microbial community. The techniques used and presented here offer a novel combination of indicators for identifying NP-induced perturbances within the gut microbiota.

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Author Disclosure Statement

The authors declare no conflict of interest.

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