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The impact of tomato fruits containing multi-walled carbon nanotube residues on human intestinal epithelial cell barrier function and intestinal microbiome composition†

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Carbon nanomaterials (CNMs) can positively regulate seed germination and enhance plant growth. However, clarification of the impact of plant organs containing absorbed CNMs on animal and human health is a critical step of risk assessment for new nano–agro-technology. In this study, we have taken a comprehensive approach to studying the effect tomato fruits derived from plants exposed to multi-walled carbon nanotubes (CNTs) have on gastrointestinal epithelial barrier integrity and their impact on the human commensal intestinal microbiota using an *in vitro* cell culture and batch human fecal suspension models. The effects of CNTs on selected pure cultures of *Salmonella enterica* Typhimurium and *Lactobacillus acidophilus* were also evaluated. This study demonstrated that CNT-containing fruits or the corresponding residual level of pure CNTs (0.001 μg ml^{-1}) was not sufficient to initiate a significant change in transepithelial resistance and on gene expression of the model T-84 human intestinal epithelial cells. However, at 10 μg ml^{-1} concentration CNTs were able to penetrate the cell membrane and change the gene expression profile of exposed cells. Moreover, extracts from CNT-containing fruits had minimal to no effect on human intestinal microbiota as revealed by culture-based analysis and 16S rRNA sequencing.

Introduction

Nanotechnology is considered one of the best solutions to challenges recognized by the food and agriculture industrial sectors.[1] For example, it was demonstrated that the use of carbon nanomaterials (CNMs) in low doses can offer a new approach to improving the productivity of a wide range of crop species. Thus, different types of CNMs are able to activate seed germination, growth, and fruit production in valuable plants.[2–7] A nanobiotechnological approach to the regulation of plant productivity could be especially applicable for food and non-food sectors of agriculture, including the production of plants for medical applications, biofuels, biodiesel, plastic, fabric, and the ornamental industry. However, the documented ability of CNMs to penetrate seeds or roots and to translocate between the cells and even reach leaves and reproductive organs is raising public and environmental health concerns, since CNMs could potentially enter the food chain *via* many pathways, including human consumption of contaminated plant organs. Besides the intentional application of CNMs as plant growth regulators, CNMs could also be unintentionally released to the environment as nanomaterial waste,[8] absorbed by growing crops, and potentially contained in the food chain.

Since the CNMs are insoluble in water and lipophilic in nature,[9] with limited biodegradability[10] and noted antimicrobial properties,[11] it is essential to understand the potential toxicity of CNMs following ingestion of such residues on the health of consumers. Our previous studies demonstrated that CNMs can be absorbed by plant organs and induce extensive changes in gene expression in different tomato plant organs.[3,5,7] Genomic profiling revealed that a significant number of differently-regulated genes were involved in the major metabolic pathways of CNM-exposed plant organs.[3,7] More recently, we reported that the presence of small amounts

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†Electronic supplementary information (ESI) available: Fig. S1 is bright field microscopy of T-84 cells after exposure to CNT, Fig. S2 is the schematic diagram of NGS data analysis, Fig. S3–5 is the detailed heat map of top significantly altered genes, Tables S1–3 include the list of gene classification, Fig. S6 shows the confirmation of NGS data using real-time PCR, Fig S7–9 show PCA based on time, individuals and doses used. See DOI: 10.1039/c8nr08604d

of multi-walled carbon nanotubes (CNT) in tomato fruits led to the modification of the total tomato fruit metabolome with significant up-regulation of some secondary metabolites that could be potentially harmful to humans.[2] Thus, the toxicity of CNM-contaminated plant-derived food can potentially occur from at least two sources that include traces of CNMs accumulated by plants and bioactive plant metabolites affected by absorbed CNMs.[2]

Many studies have raised concerns about the direct toxicity of different CNMs to animal and human populations. Certain studies have reported a specific adverse effect linked to the ingestion of CNMs. For example, fetuses from pregnant CD-1 dams that were administered a single oral dose of 10 mg kg^{-1} CNMs showed morphological and skeletal abnormalities.[12] Moreover, Folkmann *et al.* reported elevated levels of 8-oxodG in the liver and lung of rats gavaged with fullerene C60 or with single-walled carbon nanotubes.[13] Although such toxicological studies have investigated the effect of CNMs on various model organisms or cell cultures, the consequences of exposing the gastrointestinal microbiota and intestinal epithelial cells to plants contaminated with nanomaterials remain unknown. Nanomaterials can reach the gastrointestinal tract (GIT) through different routes, including mucociliary clearance, oral ingestion of food or water, and nanomaterials found in cosmetics and drug delivery devices.[14–16] While studies on the toxicity of nanomaterials post oral intake are very limited, studies on the toxicity of plant-derived food comprising nanoparticles have not been investigated to date.

The intestinal epithelium, one of the largest immune barriers, protects the body from the external environment. It prevents the passage of harmful substances, including intraluminal toxins, antigens, microorganisms and enteric bacteria,[17,18] to systemic circulation. However, its permeable membrane allows the filtration of certain essential dietary nutrients, electrolytes, and water. The selective barrier of the intestinal epithelium is maintained by a network of proteins that mechanically link adjacent cells and seal the intracellular space. There are three known network complexes, including desmosomes, adherens junctions, and tight junctions,[19–21] which control the permeability of the intestinal barrier. The first route of interaction of an invading substance to the intestinal epithelial layer is called the transepithelial/transcellular pathway, which controls the passage of solutes. The second route is called paracellular permeability, which controls the passage of dietary nutrients between epithelial cells. The latter route is regulated by intercellular complexes localized at the apical–lateral membrane junctions and along the lateral membrane.[19–22]

The intestine is also home to a complex community of microbiota that constitutes a vital function in the human body.[23] However, the presence of potentially pathogenic organisms, toxins, antigens or foreign materials in the GIT leads to a constant dynamic interaction of the microbiota with the host intestinal epithelium.[24] If consumed, CNT residues in food could adversely impact the microbiota in the GIT. The diverse commensal intestinal microbiota encodes genes for essential functions that the human host is incapable of performing, such as vitamin production, metabolism of indigestible dietary polysaccharides, and production of short-chain fatty acids.[23] Indeed, any disturbance to the microbiota can result in altered electrolyte transport and induction of epithelial cell inflammation.

The main goal of this study was to understand whether CNT contaminated fruits can be toxic to epithelial cells and to the human microbiota using an *in vitro* approach. Our aim was two-fold: (a) to understand the effect of CNT-containing tomato fruits on the integrity of GIT epithelial cells; and (b) to demonstrate whether CNT-containing fruits can alter the human intestinal microbiota composition. To achieve these goals, we designed experiments where fruits from tomato plants exposed to CNTs were tested on human epithelial cells and bacterial populations (Fig. 1). Such studies require knowledge about the exact concentrations of CNTs that were absorbed by organs of CNT-exposed plants. Previously, we have estimated the amount of absorbed CNTs in tomato fruits added to a hydroponics system by using advanced microwave-induced heating (MIH) techniques.[2] We found that even after long exposure (10 weeks) to CNTs (50 μg ml^{-1}), tomato plants were able to maintain a low level of CNTs inside the fruits (2.5×10^{-7}% of the total supplied CNT amount). In order to properly design toxicological experiments, extracts from fruit samples previously used for the estimation of the absorbed CNT concentration[2] were applied for experiments presented here. Moreover, a range of concentrations of pure CNTs, including the residue level (0.001 μg ml^{-1}) to high concentrations (10 μg ml^{-1}), were used in this investigation. In this study, we have focused on the analysis of the paracellular permeability of gastrointestinal epithelial cells and identified the gene expression profiles that were affected by exposure to CNT-containing fruits using next-generation sequencing technology. The analysis of predominant phyla (Firmicutes, Bacteroidetes), genera (*Bacteroides*, *Lactobacillus*, *Bifidobacterium*) and family (Enterobacterium) of fecal slurry treated with extracts from CNT-containing fruits was performed using real-time PCR and was correlated with other culture-based studies. Furthermore, 16S rRNA sequencing was done to assess the effect of CNT contaminated fruits on the abundance of different communities of bacteria. Due to the mutual relationship between the microbial composition and the microbial short chain fatty acid (SCFA) production, the level of SCFAs in fecal slurry treated with CNT-containing and non-containing tomato extracts was also quantified to confirm the bacterial abundance shifts. Taken together, the data in this research is the first attempt to provide critical information about the risks associated with the intentional or unintentional introduction of plants contaminated with nanoparticles into the food chain.

Experimental methods

Materials

Multi-walled carbon nanotubes (CNTs), functionalized with –COOH (OD 13–18 nm; length 1–12 μm) were purchased from

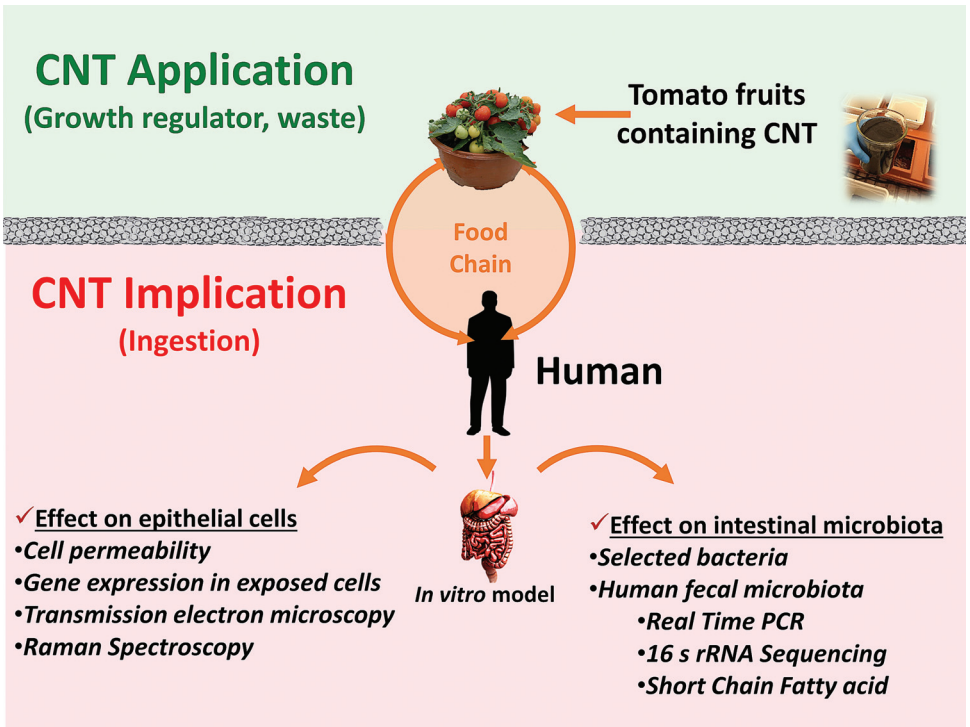

Fig. 1 Schematic diagram of the experimental design evaluating the influence of nanoparticles on the human intestine once introduced into the food chain. In this study, we are using CNT as a representative type of CNM.

Cheap Tubes (Brattleboro, VT). Detailed characterization of the CNT used was performed and demonstrated in our early work.[3] The nanoparticles were autoclaved three times to eliminate possible contamination from lipopolysaccharide (LPS) and characterized.[25] Exposure of tomato plants to CNTs (10 weeks of exposure; 50 mg L^{-1} of CNTs) was performed using a hydroponics system as described previously.[2] Control and CNT-exposed fruits were collected previously and used for the quantification of absorbed CNTs.[2] Established fruit samples with the quantified amount of CNTs[2] were used for current work. A total of three biological replicates were used. According to McGehee *et al.*, 3 μg of tomato dry extract will contain approximately 0.0003 μg of CNTs. Due to technical difficulties concerning the detection of CNTs at low levels, we considered 0.001 μg as the residue level per 3 μg of tomato fruits and used this measure as the residue level control. Tomato extract from control and CNT-exposed plants was also prepared as previously described.[2]

Cells and experimental treatments

Human epithelial T-84 cells, obtained from a transplantable human carcinoma cell line derived from a lung metastasis of a colon carcinoma in a 72-year-old male, were initially obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured in T-75 flasks in Dulbecco's Modified Eagle's medium (DMEM/F-12) supplemented with 5% fetal bovine serum (FBS), streptomycin (100 μg ml^{-1}), penicillin (100 U ml^{-1}), and 2 mM glutamine. The cells were grown at 37 °C in the presence of 5% CO_2 and were split by trypsinization when they reached ~80–90% confluence. Typically, T-84 cells were collected, washed, and seeded into cell-culture-grade, collagen-coated Transwell Inserts (Corning, Lowell, MA) at a density of 2.0×10^5 cells per well. Each insert had a 0.33 cm^2 surface and contained 200 μl media. The confluence of cells was checked daily, and the media were changed every other day. The cells were grown for 10–14 days until a complete monolayer of cells was observed, and the TEER was higher than 800 Ω cm^2.

Exposure of pure CNTs and CNT-containing fruit extracts to epithelial cells and measurement of transepithelial resistance

Initially, epithelial T-84 cells were washed with pre-warmed media, and fresh media were replaced inside the inserts. After two hours, the transepithelial electrical resistance (TEER) of all wells was recorded and used as a baseline for subsequent analysis. Immediately before the exposure, pristine CNTs were sonicated in a water bath and added to the epithelial cells at three different concentrations (0.001, 0.1, and 10 μg ml^{-1}). Fruit extracts of wild-type tomato plants and plants exposed to CNTs were vortexed vigorously and then added to the apical chamber of the well at a concentration of 3 μg ml^{-1}. The volume of tested samples, added to the epithelial cells, was constant, and a similar volume of water was added to the unexposed T-84 cells (control). The measurement of TEER was made on monolayers of T-84 cells using an epithelial voltohmmeter (EVOM, World Precision Instruments Inc., Sarasota, FL,

USA). TEER data were measured at 0.25- (15 minutes), 2-, 6-, 24-, and 48-hour post-exposures. The changes in TEER values were expressed as a percentage of the initial values. EGTA was used as a positive control. EGTA is known to decrease the TEER of epithelial cells.[26,27] Images of the cells at 1- and 48-hour post-exposures were obtained using an EVOS cell imaging system (Thermo Fisher Scientific, Waltham, MA).

Detection of CNTs in the epithelial layer using TEM and Raman spectroscopy

The culture wells with unexposed (control) and CNT-exposed T-84 cells (10 μg ml^{-1} of CNT for 24 hours) were rinsed twice at room temperature with 0.1 M PBS pH 7.4 (slowly decant solution and immediately add buffer). After the second PBS rinse, the cells were fixed in the wells at room temperature in 3% glutaraldehyde in 0.1 M Na cacodylate pH 7.4 for 10 minutes prior to scraping. The fixative containing the scraped cells was transferred to 15 ml tubes that were left at room temperature for 20 minutes and then placed at 4 °C overnight. Grids were prepared as previously described.[5] The grids were examined by using a TEM JEOL 2100 FE at Oak Ridge National Laboratory. Raman mapping of samples on TEM grids was conducted using a confocal micro Raman microscope Qontor (Renishaw) in the backscattering configuration through a 50× and 100× objective, using a 532 nm laser for excitation and using 1800 l mm^{-1} grating. The laser power was reduced using neutral density filters to ensure that no damage to the sample occurred during prolonged exposure to laser light. Details of confocal laser mapping are given elsewhere.[28] The Raman map presented in this manuscript was acquired by processing 1372 acquired Raman spectra using WIRE 5.0 software (Renishaw). The laser beam delivered through a 100× objective was scanned over the 15 × 40 μm area of the sample with 0.5 μm steps in the *x*- and *y*-directions while integrating the Raman spectrum (in 100–3200 cm^{-1}) at each of 1372 points over 10 s. The peak fit deconvolution (Fig. 3d) was shown to demonstrate the complexity of peak assignments in the important frequency range.

Next generation sequencing of RNA isolated from T-84 cells exposed to CNT and CNT-containing fruits

RNA from unexposed and exposed T-84 cells was extracted using Trizol® reagent (Molecular Research Center, Cincinnati, OH). Subsequently, RNA was treated with DNase to remove any DNA contamination from samples, using a Turbo DNA-free kit (Ambion, Austin, TX). The quality of RNA was checked by RNA gel electrophoresis and using a QC Bioanalyzer to check the RNA integrity number (RIN). RNA-Seq was performed at the research technology support facility, part of Michigan State University (East Lansing, MI). The library was prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit. After QC and quantitation, the libraries were pooled in equimolar amounts. The pool was loaded on two lanes of the Illumina HiSeq 2500 Rapid Run flow cell (v2) and sequenced in a 1 × 50 bp single end (SE50) format using TruSeq Rapid SBS reagents.

Next-generation sequencing data analysis of exposed and non-exposed T-84 cells

The experiment was designed to understand if the tomato extract containing CNT can affect T-84 cells' integrity differently than control tomato fruits. For this experiment, we had to use 3 types of controls, a negative control, a CNT control, and a wild-type tomato fruit extract control. RNA was isolated from the exposed and non-contaminated T-84 cells and was sequenced using a next-generation Illumina HiSeq 2500 Rapid Run flow cell (v2) in a 1 × 50 bp single end (SE50). The raw output data (fastq format) obtained from the RNA sequencing was analyzed using a novel computational tool termed the BioSignature Discovery System (Seralogix LLC, Austin, TX). This software was used to determine and identify the different gene transcripts. Furthermore, the software allowed the identification of genes that are differentially expressed through a *z*-scoring sliding window threshold or fold change, as was previously described by Lawhon *et al.* (2011).[29] All significantly altered genes were further classified using DAVID software analysis (DAVID Bioinformatics Resources 6.7, NIAID/NIH).[30] Furthermore, the software identified the genes that are differentially expressed through a fold change threshold (a fold change of 0.35 and $p < 0.001$ was required for a difference to be considered significant), as was previously described by Lawhon *et al.* (2011).[29]

Real-time PCR for confirmation of NGS data

Complementary DNA was generated from DNAse treated RNA samples using the SuperScript™ III First-Strand Synthesis System (Invitrogen). The corresponding primers used for the amplification of genes in real-time PCR were as follows:

(a) Claudin 2 (CLDN2): F-5′-GATCCTACGGGACTTCTACTCA-3′ and R-5′-CAGGGAGAACAGGGAAGAAATAA-3′.

(b) Interferon Induced Transmembrane protein 3 gene (IFIT3): F-5′-GAGGATGGTAGTGAGGAAATGG and R 5′-CTCCTCTGTCTCAGTTCAGTTG-3′. 18S was used as a housekeeping gene. The primers of the housekeeping gene are F-5′-TTGGAGGGCAAGTCTGGTG-3′ and R-5′CCGCTCCCAAGATCCAACTA-3′. Relative expression levels were normalized to the internal standard (18S transcript) for each treatment. For each relative quantity determination, two independent biological replicates were used with three technical replicates each.

Bacterial cultures and growth conditions

Salmonella enterica Typhimurium and *Lactobacillus acidophilus* (ATCC®4356) were provided by the Division of Microbiology, NCTR, Jefferson. Both cultures were originally purchased from the American Type Culture Collection (ATCC) (Manassas, VA). *Salmonella enterica* Typhimurium was inoculated in Luria–Bertani (LB) broth and incubated aerobically at 37 °C. *Lactobacillus acidophilus* was inoculated in Man, Rogosa, and Sharpe (MRS) broth, and incubated at 37 °C and 5% CO_2.

Antimicrobial activity determined by optical density measurement

The effect of CNTs, control fruit extract and fruit extract containing CNTs on the survival of two model bacteria was evalu-

ated. *Salmonella enterica* Typhimurium is a Gram-negative bacterium belonging to the phylum of Proteobacteria and the family of Enterobacteriaceae. *Lactobacillus acidophilus* is a Gram-positive bacteria belonging to the phylum of Firmicutes and the family of Lactobacillaceae. The growth curves of both bacteria were measured after exposure to CNTs, control fruit extract, and fruit extract containing CNTs and using the traditional optical density measurements at $OD_{600\ nm}$. Bacterial cells were allowed to reach mid-log-phase growth each time before any treatment. The growth curve of *Salmonella* was performed by spiking the bacteria in minimum essential medium (pH = 7.4) containing either CNTs (at concentrations of 0.01, 0.1, 1, and 10 μg ml^{-1}) or control fruit extract or fruit extracts containing CNTs (at concentrations of 0.03, 0.3, 3, and 30 μg ml^{-1}). *Salmonella* samples were incubated at 37 °C and the OD was measured using a Cytation3 Cell Imaging Multimode Reader (Biotek, Winooski, VT). *Lactobacillus*, however, was spiked with diluted MRS media containing similar treatments as described above and incubated at 37 °C and 5% CO_2.

Exposure of human fecal slurry to CNTs and CNT-containing fruits

The collection and use of human feces were approved by the NCTR/US-FDA Research Involving Human Subjects Committee (RIHSC #14-061T). All experiments were performed in accordance with the Guidelines of Research Involving Human Subject Committee (RIHSC) of National Center for Toxicological Research, US-Food and Drug Administration. This work was conducted using existing, banked fecal de-identified samples that were self-sampled at extremely minimal risk under RIHSC approval (14-061-T). No data associated with these samples could be used to link them to a living human being. There was no new intervention, interaction or new collections. Under these conditions, the work does not meet the definition of human subject research at 45 CFR 46.102(f)(2), and 45 CFR part 46 does not apply. The Institutional Review Board (IRB) of University of Arkansas at Little Rock also evaluated the research protocol (protocol 16-030 from September 22, 2015), and determined that this research does not qualify as human research based on 45 CFR 46.102. Banked human fecal samples, which were completely de-identified with no accompanying data that can be used to link them to the human subject, were used for the assessment of specific bacterial groups (by real-time PCR) or 16S rRNA sequencing. All three participants were healthy and did not use antibiotics for the last six months. Fecal samples were kept in an anaerobic chamber and used within one day (after collection) for the experiment. The culture conditions of fecal microbiota used in this experiment were previously described by Kim *et al.* (2011).[31] Briefly, fecal samples were diluted with anaerobic Maximum Recovery Diluent (MRD, LbM IDG, Bury, UK) buffer to a final concentration of 25% (w/v) and subsequently cultured in low-concentration carbohydrate medium (LCM, 10 ml) at a final 3% inoculum concentration. All samples were incubated in 50 mL Wheaton Serum Glass Bottles and sealed with a rubber butyl stopper and an aluminum seal. Before the exposure of different treatments to the microbiota culture, an initial sample was extracted to serve as a baseline control. The microbiota cultures were then exposed to different treatments. The untreated samples were spiked with water only and served as a negative control. Pure CNT treated cultures were treated with CNTs at 0.001, 0.1, and 10 μg ml^{-1} concentrations. Others were treated with control fruit extract and CNT-containing fruits at 3 μg ml^{-1}. All samples were incubated anaerobically at 37 °C, and 500 μl of samples was removed using an 18 gauge syringe on days 1, 3, and 7 post-exposure. All samples were stored at −80 °C for future use.

Extraction of DNA from the treated fecal slurry

Bacterial genomic DNA was extracted from the 500 μl stored fecal slurry. The extraction of DNA from the fecal slurry was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Briefly, the fecal slurry was incubated with lysis buffer at 95 °C for 5 min and then centrifuged at full speed for 1 min to pellet stool particles. The supernatant was incubated with one InhibitEX Tablet and followed by incubation with proteinase K at 65 °C for 10 min. After recommended washing steps, DNA was eluted with DNase-free water. DNA quality and quantity were checked using a Qubit™ Fluorometer (Thermo Fisher Scientific, Waltham, MA) and a Cytation3 Cell Imaging Multimode Reader (Biotek, Winooski, VT).

Real-time PCR for identification of bacterial groups

The identification of different bacterial groups was performed as was described previously by Williams *et al.* (2014).[27] Briefly, the real-time PCR (qPCR) was used to amplify the DNA fragments of the predominant phyla (Firmicutes and Bacteroidetes) and representative genera (*Bacteroides*, *Lactobacillus* and *Bifidobacterium*) and the family (Enterobacteriaceae) of bacteria present in the intestinal mucosa. The primers were previously designed by Williams *et al.* (2014).[27] The ABI 7500 machine was used to measure and amplify DNA fragments in real time. A template of the same volume of DNA (3 μl) was used in all experiments, with a 10 μl of SYBR green master mix, 1 μl of (forward and reverse) primers, and 5 μl of DNase-free water. The amplification was carried out with the following steps: 95 °C for 10 minutes followed by denaturation at 95 °C for 15 seconds and annealing and extension at 60 °C for 1 minute. The qPCR reaction was run for 45 cycles and followed by a complete denaturation of the PCR product to obtain melting curves. Melting curve data were obtained from 60 to 95 °C at a rate of 0.5 °C s^{-1} and allowed the confirmation of specificity of the amplicon product. Real-time PCR data were normalized with 18S gene expression in host genomic DNA as described earlier.[32]

16S rRNA gene sequencing of bacterial population

Fecal DNA was also used to conduct community bacterial population analysis by 16S rRNA sequencing. The V4 variable regions were amplified and pooled, purified, and were used to prepare the Illumina DNA library. Sequences were joined, de-

tagged, filtered and denoised. The Operational Taxonomic Units (OTUs) were generated and chimeras were removed. The OTUs were defined by clustering at 97% similarity. These OTUs were assigned taxonomical classification using BLASTn against curated databases RDPII and NCBI.

Quantification of short chain fatty acids (SCFAs) in fecal slurry using HPLC

Nine SCFA calibration standards were prepared at a 1 M concentration for lactic, propionic, isobutyric, butyric, valeric, succinic, isovaleric, hexanoic, and acetic acids. The standards were spiked into control fecal slurry to measure their retention time for a final concentration of 20 mM. The calibration curves were constructed by plotting the relative peak area *versus* the molarity of the solution. Concentrations of SCFAs in studied fecal slurry were determined using HPLC. Briefly, 500 μl of treated and non-treated human fecal slurry at all time points (1, 3, and 7 days) were centrifuged and filtered using a 0.22-micron nylon filter. Afterward, 10 μl of each sample was injected directly into the HPLC System (Agilent, Santa Clara, CA). The column for SCFA separation is an ionic exchange resin, Aminex HPX-87H column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad Laboratories, Richmond, USA), heated at 65 °C. Organic compounds were detected using a UV detector at a wavelength of 210 nm. The mobile phase was composed of an isocratic H_2SO_4 solution (2.5 mM) and was used at a flow rate of 0.5 ml min^{-1} for 50 min.

Statistical analysis

All figures are represented as mean values ± SE (standard errors). All data were analyzed using SPSS® software by performing repeated measure ANOVA for time-effect analysis and ANOVA and *post hoc* analysis using the Tukey test for treatment differences. PCA and heatmap analysis was performed using R software 3.3.1. Statistical significance was determined by $p < 0.05$.

Results and discussion

Effect of pure CNT and CNT-containing tomato fruits on the paracellular permeability of T-84 cell monolayers

The intestinal epithelial cell model used in this study forms a basis for the risk assessment of tested materials in the animal model. Paracellular permeability of intestinal cells is a relevant trait in the assessment of toxicity of xenobiotics applied to epithelial cells.[33] This feature is based on tight junction formation and functions that regulate the movement of solutes, ions, and water across the paracellular space.[22,26,33] Here, we used T-84 cells as a model for clarification of whether pure CNT or CNT-containing fruits can cause toxicity to human gastrointestinal epithelial cells. The paracellular permeability of T-84 cells was studied *in vitro* using a Transwell permeable support system (Fig. 2a). This system has a microporous membrane insert that allows the cells to uptake and secrete molecules on both their basal and apical surfaces. This system was shown to be a valuable tool for the culture of polarized cells[34] (Fig. 2b). In our study, T-84 cells were seeded on the transmembrane and were checked daily until they reach confluency. Baseline resistance reading of each well was recorded and expressed as ohm cm^2. The addition of pure CNTs to the apical chamber of the tight T-84 cell monolayer showed a dose-dependent response (Fig. 2c). The transepithelial electrical resistance (TEER) of T-84 cells exposed to CNTs at concentrations of 0.001 and 0.1 μg ml^{-1} did not show any significant differences compared to the untreated control. In contrast, the TEER of T-84 cells exposed to 10 μg ml^{-1} CNTs was significantly decreased during the first hours of exposure (Fig. 2c). However, after 6 hours, a progressive recovery in TEER values was detected, and the TEER levels in the cells exposed to the highest concentrations of CNTs reached similar values compared to the control. Egtazic acid (EGTA) which was used as a positive control did demonstrate the impairment of the epithelial paracellular membrane, and the TEER levels remained significantly lower than the control.

The effect of CNT-containing fruits on the permeability of the T-84 cell monolayer was also monitored for 48 hours (Fig. 2d). Using the same concentration of fruit extract (3 μg ml^{-1}), TEER values of the cells exposed to CNT-containing fruits were compared to TEER values of the cells exposed to control CNT-untreated fruits, as well as to the untreated control. Both tomato fruit extract samples induced T-84 cells to produce a progressive increase in TEER values. Indeed, after a 48-hour exposure, the TEER values of T-84 cells exposed to either tomato extract, with or without CNTs, were significantly higher than the untreated T-84 cells. The cells exposed to CNT-containing fruits showed a slight but not significant decrease in the TEER value after 48 hours of exposure compared to the cells exposed to control fruits.

Most of the published research that reported on the effect of CNTs on the integrity of epithelial cells focused on human airway epithelial cells. To our knowledge, this is the first study that demonstrated the impact of pure CNT or CNT-containing fruit exposures on human gastrointestinal epithelial cells. It is important to note that epithelial cells from other body sites could share similar biological mechanisms in response to exposure to foreign materials. For example, Rotoli *et al.* (2008) have demonstrated that CNTs can interfere with the formation of tight junctional complexes and can significantly prevent airway epithelial cells (Calu-3 cells) from the establishment of high resistance epithelial barriers.[35,36] Moreover, it was shown that pure CNTs can impact epithelial cells' resistance restoration if elicited by toxic or infectious factors.[36]

T-84 cells exposed to CNT-containing fruits exhibited similar behavior in the T-84 cell monolayer resistance data compared to control fruits (Fig. 2d). Both fruit extracts (control and CNT-treated fruits) induced an increase in TEER levels in T-84 cells (Fig. 2d). The increase in TEER could be explained by the presence of phenolic compounds in the tomato extract, which were previously shown to enhance the integrity of the epithelial cell barrier.[37,38] However, T-84 cells exposed to the highest dose of pure CNTs (10 μg ml^{-1}) were able to recover after a 48-hour post-treatment (Fig. 2c).

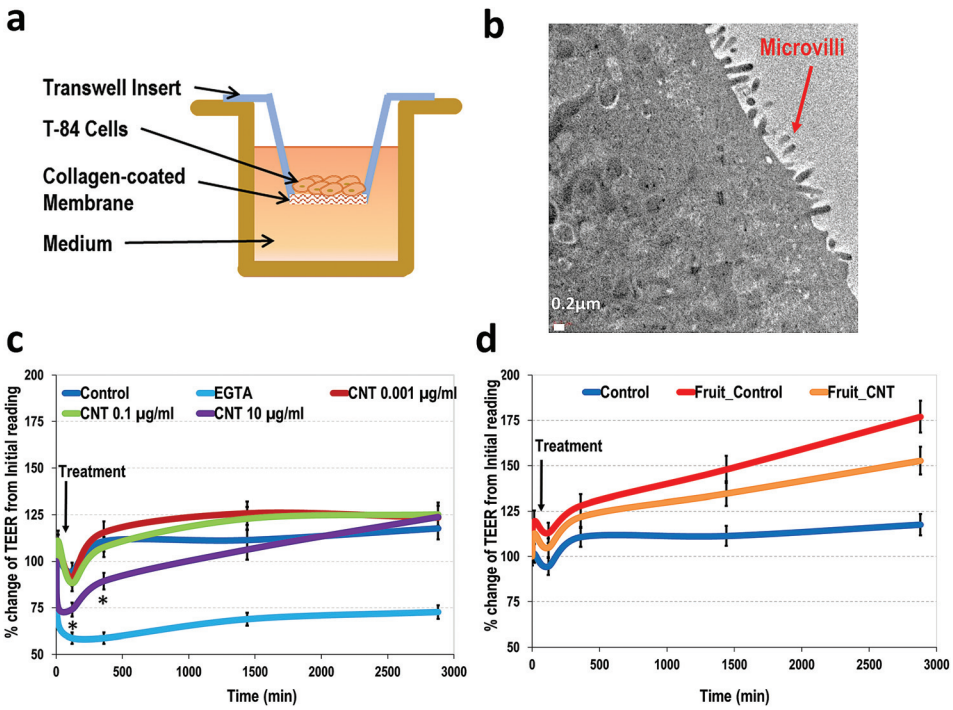

Fig. 2 Time-dependent effects on the TEER levels of T-84 cellular monolayers exposed to CNT-containing fruits. (a) Schematic representation of the *in vitro* T-84 monolayer cell model. (b) TEM micrograph of a T-84 cellular intestinal layer showing microvilli after 10 days in Transwells (bar equal to 0.2 μm). (c) Dose-dependent response of pure CNT treated T-84 cells on TEER readings and compared to the untreated control. CNTs were added to the apical chamber of the culture system at concentrations of 0.001, 0.1 and 10 μg ml^{-1}. EGTA was used as a positive control to demonstrate the impairment of the epithelial paracellular permeability. (d) CNT-containing fruits' response to TEER of T-84 cells and compared to control fruits. Fruit extracts of both treatments were added at a dose of 3 μg ml^{-1}. Error bars represent standard error values. *$p < 0.01$ of 10 μg ml^{-1} CNTs compared to control. "Initial" represents TEER levels of all cultures prior to exposure.

The marked changes in paracellular permeability are usually linked to repression in the expression of tight junction genes, such as zona occludens (ZO)-1 and occludin.[34] However, some research groups have shown that the decrease or increase of TEER could not be associated with the modification of tight junction proteins. For example, the exposure of a human epithelial cell model to asbestos increased cell permeability with no effect on occludin protein expression.[39] Thus, it becomes necessary to question whether CNT or CNT-containing fruits can affect genes related to the integrity of T-84 epithelial cells. The maintenance of the barrier efficiency is an important factor in the protection of the gastrointestinal tract from toxic materials.[40,41] In order to demonstrate the ability of CNTs to penetrate human gastrointestinal epithelial cells, we looked for the presence of CNT in CNT-exposed T-84 cells using microscopic and spectroscopic techniques.

Detection of pure CNTs in T-84 intestinal cells following 48-hour exposure to CNTs

To demonstrate the ability of CNTs to penetrate intestinal cells, we used transmission electron microscopy (TEM) followed by Raman spectroscopy of TEM grids (Fig. 3). TEM analysis confirmed the presence of black CNT clusters in both transversal longitudinal sections of CNT-exposed T-84 cells (Fig. 3a and b). No similar structures were found in sections of control (unexposed) T-84 cells. To confirm that observed black aggregates on TEM images are indeed CNTs, TEM grids were analyzed by Raman spectroscopy for the presence of CNTs (Fig. 3c and d). Raman spectra collected using a 100× objective (NA = 0.85) were analyzed using the WIRE 5.0 software package. Typical G bands associated with the presence of CNTs were found in grids representing sections of T-84 epithelial cells exposed to CNTs (Fig. 3c and d). The integrated intensity of Raman scattering in the 2800–3100 cm^{-1} frequency range, displayed as a color map overlapping the sample's optical image in Fig. 3c, was used for identification of CNTs. Detection and identification of CNTs, especially MWCNTs, in biological media is complicated due to high light scattering and significant biomedia auto-fluorescence, and the small size of nanotubes (a few nm in diameter and length) compared to the large area of the sample. In our samples, we observed a broad fluorescence peak around 2100 cm^{-1}, FWHM = 750 cm^{-1}. Furthermore, the low pH of intestinal media leads to the acidification of short nanotubes, reducing the dimension of graphitic domains, producing signatures of D and G-bands in the 1300–1700 cm^{-1} frequency range and two-photon replicas in the 2200–3000 cm^{-1} frequency range. Chemically modified CNTs may retain similarity to very short nanotubes, but they are composed of isolated areas/patches of sp^2 hybridized conjugated carbon atoms connected/isolated by

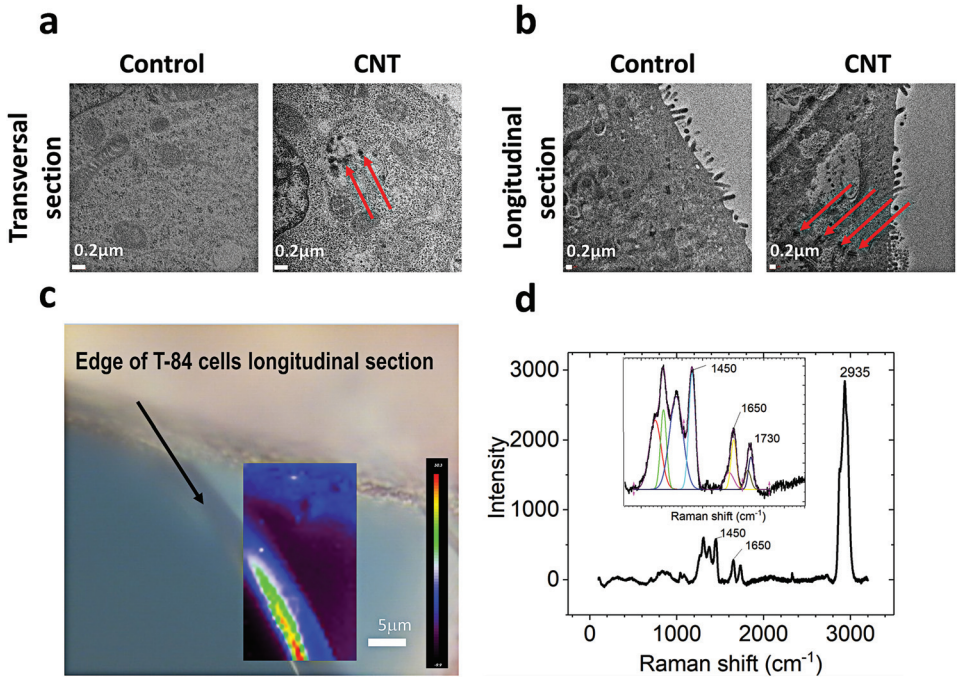

Fig. 3 CNTs were able to penetrate the epithelial layer and translocate within T-84 cells. (a) TEM images of a transversal section of unexposed (control) and CNT-exposed (10 μg ml^{-1} of CNTs for 24 hours) T-84 cells. (b) TEM images of a longitudinal section of unexposed (control) and CNT-exposed (CNT) T-84 cells. (c) Mapping of integrated Raman signal on the longitudinal section of T-84 epithelial cells. (d) Raman spectra of CNTs in epithelial cells (fluorescence background subtracted). Red arrows show a potential area of CNT accumulation inside T-84 epithelial cells.

a network of sp^3 carbons, which break long-range p-conjugation. Structurally and functionally, the modified CNTs may resemble carbon quantum dots (CQDs) or single-walled carbon nanohorns.[42,43]

The confirmation that epithelial T-84 cells can easily absorb pure CNTs raises a question about the possible effect of CNT-containing fruits on the molecular level of exposed epithelial T-84 cells. Previously, we found that the uptake of CNTs by plant organs affected the transcriptome[7] and metabolome[2] of tomato plants exposed to CNTs used as plant growth regulators. It is important to clarify whether such an effect is universal for other biological models and whether the small amount of CNTs located in fruits is sufficient to cause an effect on the transcriptome.

T-84 cell transcriptome analysis following exposure to pure CNTs and CNT-containing fruits

The experimental design was aimed at gaining a detailed insight into changes in the transcriptional profile of T-84 cell genes affected by CNTs or fruits containing CNT (Fig. S2†). Epithelial T-84 cells were exposed to four different substances. First, the control T-84 cells were treated with tissue culture media only (negative control). Second, T-84 cells were incubated with CNTs at different concentrations (0.001, 0.1 and 10 μg ml^{-1}). Third, control fruit extracts were added to the epithelial cells at a 3 μg ml^{-1} concentration. Fourth, CNT-containing fruits were incubated with T-84 cells at the same 3 μg ml^{-1} concentration. This experimental design was valuable since it allowed us to obtain a better insight on the response of T-84 cells to the pure CNTs, as well as to the tomato extract. Both fruit extracts (with or without CNTs) were compared to the untreated controls.

Table S1† summarizes the numbers of genes that were significantly up- or down-regulated in T-84 cells treated with CNTs, control fruits, or CNT-containing fruits. A modest number of genes were significantly up- or down-regulated in the T-84 exposed cells to CNTs at 0.001 μg ml^{-1} (19 genes) and 0.1 μg ml^{-1} (26 genes). In contrast, a number of genes had their expression perturbed in epithelial cells exposed to the highest concentration of CNTs (10 μg ml^{-1}) (136 genes). The total number of genes significantly altered by the exposure of control fruits to T-84 cells was 1057 genes (60.8% up-regulated and 39.2% down-regulated) compared to the negative control. The exposure of CNT-containing fruits to T-84 cells led to the up-regulation of 129 genes (62.6%) and down-regulation of 77 genes (37.4%). A further comparison of the genes of T-84 cells affected by exposure to CNT-containing fruits *versus* the genes affected by exposure to control fruits resulted in the detection of 614 genes significantly altered compared to the negative control. For each analysis performed (exposure groups 1–4), we selected the top genes significantly perturbed to present as heat maps in Fig. 4a, b, S3, S4, and S5.† In each heat map, the dark red gradient represented significantly activated or up-regulated genes, while green gradient indicated the significantly suppressed or down-regulated genes. To further analyze the common genes within the different groups

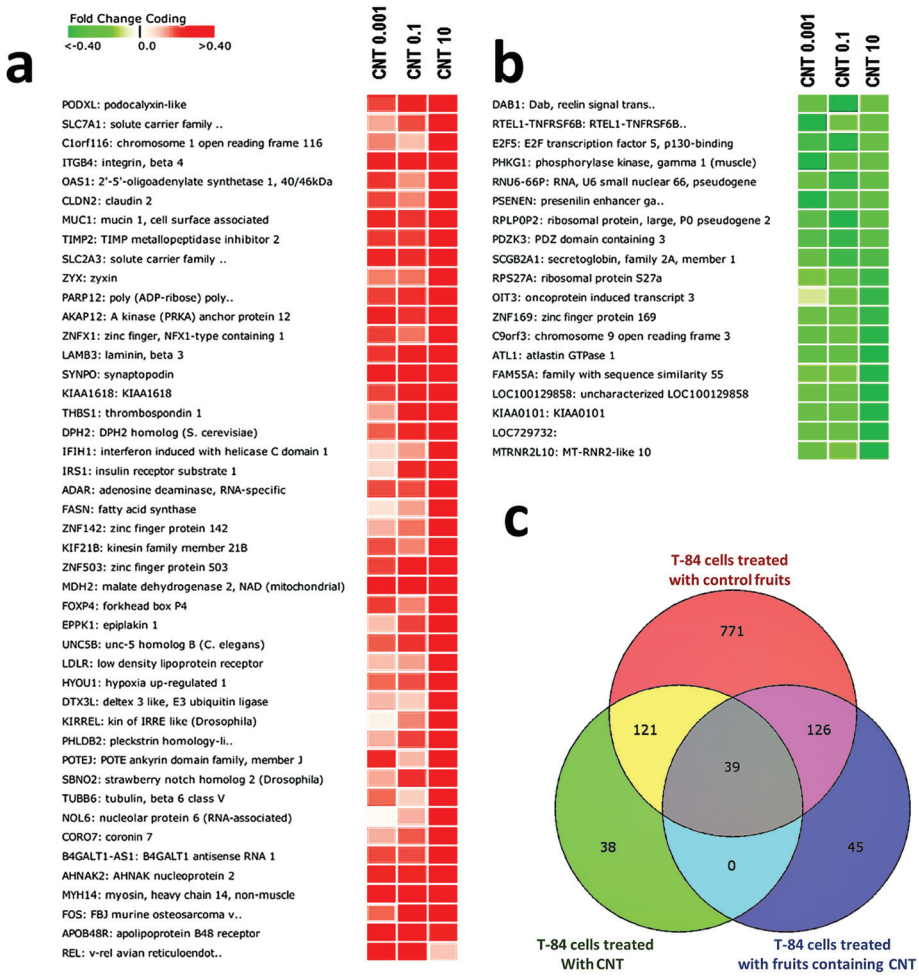

Fig. 4 Gene expression levels perturbed after exposure to pure CNTs or fruit containing CNTs. (a) Heat-map of the top significantly up-regulated genes during exposure of pure CNTs to epithelial cells. (b) Heat-map of the top significantly down-regulated genes.

studied, we calculated the number of genes shared between three groups: a group of T-84 cells treated with control tomato extract, a group of T-84 cells treated with CNT-containing fruits, and a group of T-84 cells treated with CNTs (all concentrations). The shared genes between the three groups are represented in Fig. 4c. We found that there were 39 genes shared between all three groups. The number of genes specifically shared between T-84 cells treated with CNT-containing fruit and the cells treated with pure CNTs was zero. On the other hand, the number of genes specifically shared between T-84 cells treated with control fruits and the cells treated with CNT-containing fruits was 126 genes. These findings show that the response of the epithelial cells toward CNT-containing fruits was more similar to control fruits than to pure CNTs. The presence of CNTs in tomato fruits at low levels could not trigger a pure CNT specific response.

All significantly altered genes were further classified using DAVID software analysis (DAVID Bioinformatics Resources 6.7, NIAID/NIH). Knowing that foreign materials can activate and suppress different pathways in epithelial cells, we focused on cell communication and cell adhesion pathways by looking at genes involved in adherens junctions, tight junctions, and desmosomes. Table S2† summarizes the list of genes that were affected by the exposure of pure CNTs (10 μg ml^{-1}) to T-84 cells and which were involved in adherens junctions, tight junctions, and desmosomes. Interestingly, all these genes were up-regulated in T-84 cells treated with CNTs compared to control cells. Such a finding was in agreement with our previous TEER results (Fig. 2c), which shows that at 48 hours, T-84 cells treated with CNTs at 10 μg ml^{-1} have started to recover their electrical resistance (Fig. 2c).

Early in the exposure (15 minutes), CNTs at a high concentration (10 μg ml^{-1}) affected the integrity of epithelial cells, which in consequence could be due to an effect on the cell communication pathways. However, the ability of T-84 cells exposed to CNTs to rescue the transepithelial electrical resistance and up-regulate genes related to the cell communication pathway could demonstrate that CNTs may interfere minimally with the formation of the high resistance epithelial barrier. On the other hand, the ability of CNTs to agglomerate inside

growth media could explain the observed effects of the decrease of resistance of T-84 cells at early exposure times (15 min) and recovery at late exposure times (48 hours). Indeed, the agglomeration of CNTs inside the media and their deposition on the epithelial cell surface could minimize the toxic effect of the nanostructure on the epithelial cell barrier. In fact, the bright field microscopy image in Fig. S1† shows that the agglomeration of CNTs started to appear as early as the first hour of exposure and continued through 48 hours of exposure. A previous study using dynamic light scattering technology showed that MWCNTs can significantly agglomerate within the first 24 hours after addition to cell culture media[44]

The ontological classification of genes affected by the exposure of T-84 cells to control fruits has resulted in the activation of many different pathways. Table S3† summarizes the list of genes that were significantly altered by the exposure of T-84 cells to control fruit extract and that were involved in processes of tight junctions, adherens junctions, or desmosomes. We found that 5 genes belonged to the tight junction category, 21 genes belonged to the adherens junction category, and 4 belonged to the desmosomes category. Interestingly, all these genes were found to be up-regulated in T-84 cells exposed to control tomato fruits, compared to the untreated control. These results could imply that the extract from tomato fruits can work as enhancers of the epithelial barrier function. Indeed, the previous results, showing the significant increase in TEER readings in epithelial cells exposed to tomato fruit extracts (Fig. 2d), could confirm such an observation. Previously, it was shown that certain phenolic compounds, present in tomatoes, such as ferulic and isofeluric acids, induced the expression of genes encoding the tight junction components in colon epithelial cell monolayers: ZO-1, occludin, and claudin-4.[45] Quercetin, another flavonoid present in tomatoes, was shown to enhance Caco-2 epithelial cell monolayer integrity by the induction of Zo-2, claudin1, and occludin gene expression.[46]

The classification of gene expression alterations in T-84 cells significantly affected by exposure to tomato CNT-containing fruits demonstrated that genes belonging to the cell communication pathway were not affected. In fact, none of the genes significantly perturbed were involved in the tight junctions, adherens junctions, or desmosomes categories. These results could be explained by the high standard deviation between the numbers of transcripts calculated in both replicates of T-84 cells treated with CNT-containing fruits. Indeed, further analysis (data not shown) demonstrated that the genes involved in the cell communication pathway were significantly affected ($p < 0.001$); however, they presented fold change values below the threshold of 0.35. These findings could explain the measured TEER values in T-84 cells exposed to CNT-containing fruits, which were slightly lower than the TEER values in the cells treated with control fruits (Fig. 2d).

The differences in transcript abundance noted by Next Generation Sequencing (NGS) data were further confirmed by real-time PCR (Fig. S6†). We found that the trend of expression of two selected genes was in agreement when confirmed by either method (NGS or real-time PCR). For example, NGS revealed that the Claudin 2 (CLDN2) gene was up-regulated in T-84 cells exposed to CNTs at 10 μg ml^{-1} (Fig. 4). The up-regulation of CLDN2 in the 10 μg ml^{-1} CNT exposed T-84 cells was also confirmed by real-time PCR (Fig. S6a†). Interferon-Induced Protein with Tetratricopeptide repeats 3 (IFIT3) was up-regulated in both T-84 cells treated with control fruit extract or CNT-containing fruits, as revealed in the heat map analysis (Fig. S3 and S4†). The observation of up-regulated IFIT3 gene expression was also confirmed by real-time PCR (Fig. S6†).

Previously, it was demonstrated that the fruits obtained from plants exposed to CNTs were found to contain nanomaterials.[2] The low amount of absorbed CNTs (0.001 μg ml^{-1}) was not enough to initiate a significant distress in the *in vitro* epithelial cell model used in our study. However, the ability of pure CNTs to penetrate T-84 cells and change gene expression could provide health risks to human and other organisms. Many reports have shown that even a small amount of CNT could affect the growth and survival of certain gut bacteria,[11,47–49] and subsequently, CNT-containing fruits could induce epithelial cell inflammation. For this reason and as a next step, we decided to study the impact of CNT-containing fruits and pure CNTs on two model bacterial species, as well as on human fecal slurry using an *in vitro* batch culture system.

Effect of pure CNT and CNT-containing fruits on representative bacterial populations

Culture-based analysis. To evaluate whether pure CNTs and tomato extracts containing CNT could have an impact on the growth rate and survival of bacteria, two model bacteria were chosen for a 24-hour exposure study. The first bacteria were a Gram-negative *Salmonella enterica* Typhimurium. The second bacteria were a Gram-positive *Lactobacillus acidophilus*. The antibacterial activities of CNTs or tomato containing CNTs were estimated by measuring the growth rate of *Salmonella* and *Lactobacillus* after exposure to either treatment at $OD_{600\ nm}$. CNTs had a dose-dependent antimicrobial effect on both microorganisms. Fig. 5 presents the growth curves of *L. acidophilus* and *S.* Typhimurium when incubated with CNTs at 0.001, 0.01, 0.1, 1, and 10 μg ml^{-1}. A lower concentration of CNTs had no significant impact on the growth of both model bacteria. In contrast, we noticed a delay in the growth of bacteria (2 hours in *L. acidophilus* and 4 hours in *S.* Typhimurium) when exposed to the highest concentration of CNTs used (10 μg ml^{-1}). Moreover, the exponential growth phase in *Salmonella* treated with CNTs at 1 and 10 μg ml^{-1} occurred for a shorter period than the untreated control group. The shorter exponential period resulted in a lower number of bacteria in the stationary phase. The pattern of results indicated a greater antimicrobial activity with increasing concentrations of CNTs. These findings confirm previous findings that CNTs could have an antimicrobial effect on the growth of certain gut microbes.[49] The toxic effect of CNTs was found to be dependent on the size, shape, purity, and functional groups

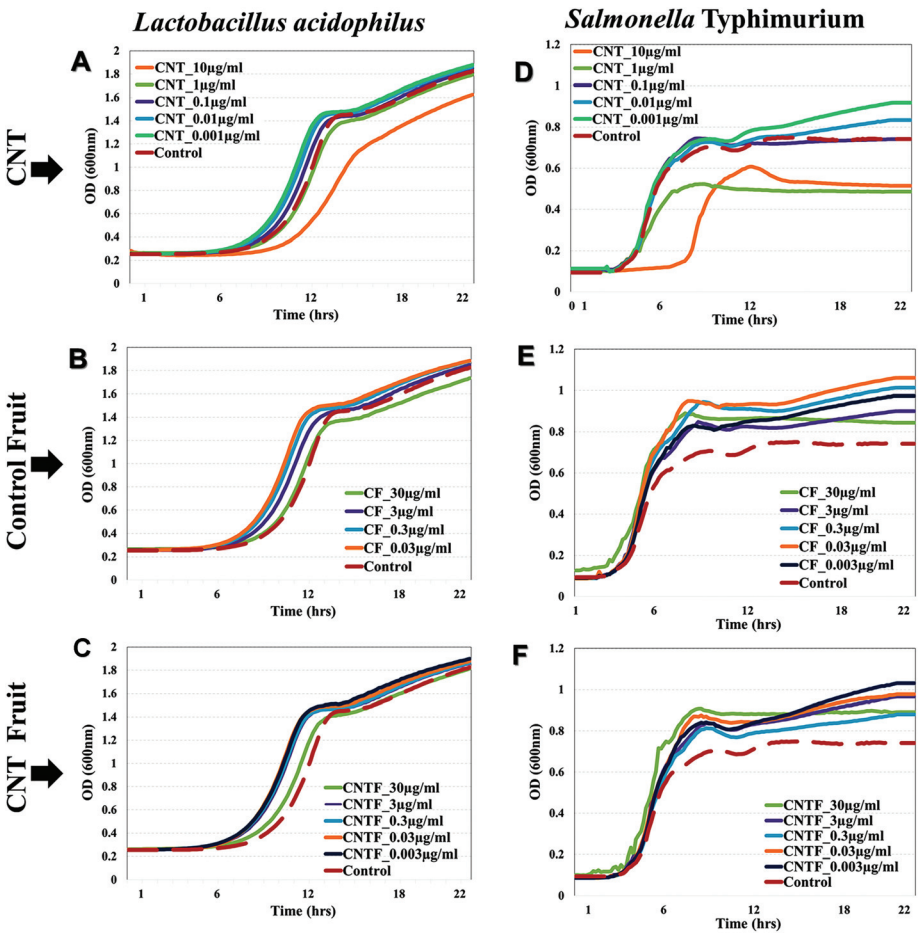

Fig. 5 Growth curves of *L. acidophilus* (left column) and *S.* Typhimurium (right column) cells treated with pure CNTs (a, d), control fruits (b, e), or CNT-containing fruits (c, f). CNTs were added at concentrations of 0.01, 0.1, 1 and 10 μg ml^{-1} to media. Control fruits (CF) were added at concentrations of 0.03, 0.3, 3, and 30 μg ml^{-1}. CNT-containing fruits (CNTF) were added at concentrations of 0.03, 0.3, 3, and 30 μg ml^{-1}. The results were compared to the negative control (Control). The study was performed for 22 hours. *S.* Typhimurium was incubated in minimal essential media at 37 °C. *L. acidophilus* was incubated in MRS media at 37 °C and 5% CO_2.

of CNTs, as well as the growth media used for the study.[50–53] The mechanism by which CNTs could be toxic to bacteria was linked to physical damage to the bacterial membrane through piercing and inducing the release of certain intracellular components such as DNA or RNA.[49]

To study whether CNT-containing tomato extracts could affect the growth of both model bacteria, we incubated the fruit extract with bacteria at 0.003, 0.03, 0.3, 3 and 30 μg ml^{-1} CNTs and compared the results to the growth of bacteria incubated with control fruit extract. The pattern of growth of both bacteria was similar when exposed to either tomato extracts with or without CNTs (Fig. 5b, c, e and f). No significant antimicrobial effect was noticed in comparison to the negative control. In contrast, untreated *Salmonella* and *Lactobacillus* had a longer exponential growth phase, which resulted in a higher number of bacteria. Moreover, *Lactobacillus* have entered the exponential growth phase earlier when incubated with either tomato fruit extract compared to the untreated control (Fig. 5b and c). Interestingly, the effect of tomato on the growth of *Lactobacillus* was first observed in 1925 by Mikle and Breed.[54] The investigators observed that the addition of tomato juice to growth media enhanced the bacterial development and growth of *L. acidophilus*. On the other hand, the growth of *Salmonella enterica* in tomato has been observed in many bacterial food-borne illnesses where *S. enterica* colonized tomato plants.[55]

Results on both model bacteria encouraged us to study the impact of CNT-containing fruits on gut commensal bacterial populations. Indeed, CNTs have shown an antimicrobial activity toward both Gram-positive and Gram-negative bacteria (Fig. 5). Knowing that tomato fruit extracts contained a small amount of CNTs, it was not surprising that the direct effect of CNT-containing fruits on bacterial growth and survival was minimal. However, gut microbiota comprises, at least, several hundred to a thousand different species of bacteria.[56–58] Since direct analysis of the intestinal microbiota in the human colon is challenging, a study was performed with human fecal specimens using an *in vitro* batch culture approach.

Effects of CNTs on Firmicutes and Bacteroidetes proportions

The dominant phyla (Firmicutes and Bacteroidetes) from fecal slurry were quantified using real-time PCR after exposure to a 3% inoculum concentration of specimens to water (negative control), CNTs (at three concentrations), control fruit extracts, and CNT-containing fruit extracts (Fig. 6). The results indicated a dose-dependent and time-dependent response of both phyla abundance and ratio during exposure to CNTs. During early exposure (1–3 days) of CNTs to the fecal slurry, no significant differences in abundance in both phyla were noted in cultures treated with the lowest concentrations of CNTs (0.001 and 0.1 μg ml^{-1}) compared to the negative control (Fig. 6). In contrast, during the same period, CNTs at 10 μg ml^{-1} induced an increase in abundance in both phyla. After 7 days of exposure to CNTs, we noticed that the abundance of Firmicutes and Bacteroidetes was similar between all concentrations of CNTs. Fig. 6 (panel C) is a recapitulative figure, where the *y*-axis shows a 100% stack column to emphasize the ratio of both phyla in comparison to the negative control. This figure demonstrates how the ratio of both phyla that was significantly impacted by the different concentrations of CNTs and the length of exposure reached a similar ratio of 40% Firmicutes and 60% Bacteroidetes in both concentrations of 0.001 μg ml^{-1} and 10 μg ml^{-1} on day 7. However, the ratio was inverted with 60% in Firmicutes and 40% in Bacteroidetes after exposure of CNTs (0.1 μg ml^{-1}) to the fecal slurry.

The abundance of Firmicutes and Bacteroidetes after exposure of fecal slurry to fruits containing MWCNT or control fruits showed no significant differences between both treatments during the first three days of exposure (Fig. 6) compared to the negative control. However, the abundance of both phyla was significantly increased during the 7th day of exposure in comparison to the negative control. After a seven-day exposure, the ratio of both phyla in fecal slurry treated CNT-containing fruits (28% in Firmicutes and 72% in Bacteroidetes) was slightly but not significantly different compared to samples treated with control fruits (33% in Firmicutes and 67% in Bacteroidetes).

The differences observed regarding the abundance and ratio between all the fecal slurry treated with different treatments (CNT, control fruits or CNT-containing fruits) in comparison to the negative control could be due to shifting in the subpopulations that represent both phyla. Thus, we assessed the effect of CNTs and CNT-containing fruits on the bacterial population representing a major genera in the gut microbiota.

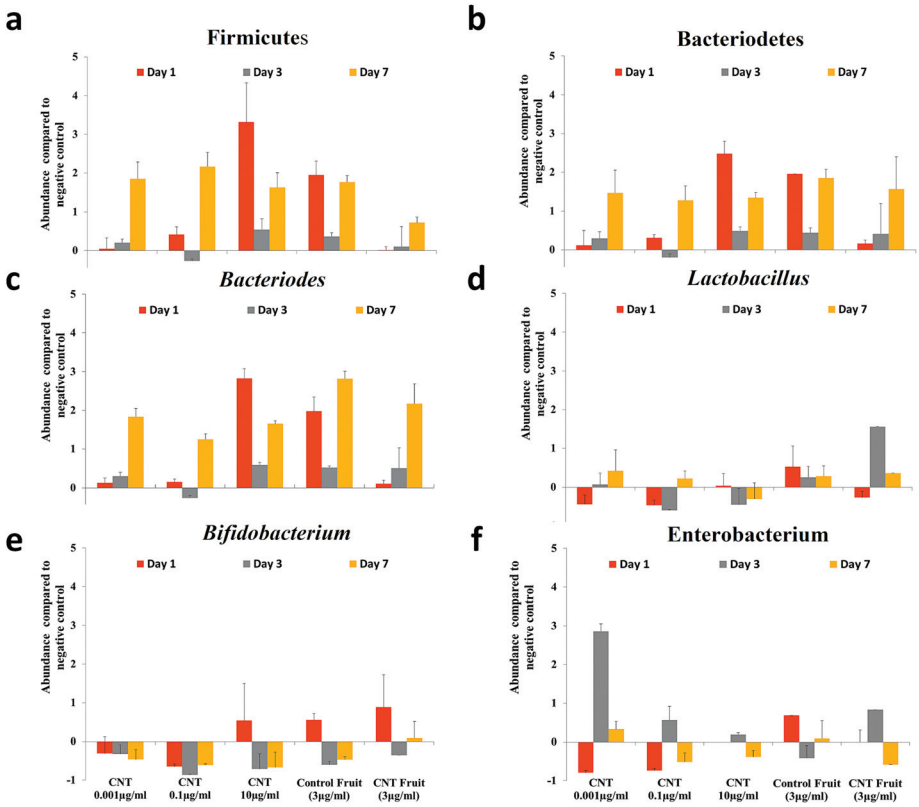

Fig. 6 The relative abundance of Firmicutes (a), Bacteroidetes (b), *Bacteroides* (c), *Lactobacillus* (d), *Bifidobacterium* (e), and Enterobacterium (f) after treatment with pure CNTs (0.001 μg ml^{-1}, 0.1 μg ml^{-1}, and 10 μg ml^{-1}), with both control fruits and CNT-containing fruits. The relative abundance is expressed as fold change compared to the untreated control and shown for 1-, 3- and 7-day(s) post-treatment. Error bars represent standard error values (n = 6).

The relative expression of genes for specific genus: *Bacteroides*, *Lactobacillus*, and *Bifidobacterium*

The abundance of both genera representing Firmicutes and Bacteroidetes (*Lactobacillus* and *Bacteroides*) showed a similar pattern compared to their representative phyla. Interestingly, the abundance of the *Lactobacillus* genus was significantly impacted when the fecal specimen was exposed to the highest concentration of CNTs (10 μg ml^{-1}) (Fig. 6). *Lactobacillus* abundance in fecal slurry treated with CNTs at the lowest concentrations recovered by day 7, and it remained significantly lower in fecal slurry treated with the highest concentration of CNTs compared to the negative control. This observation confirmed the preliminary growth data of *Lactobacillus acidophilus* when exposed to CNTs, which showed a distress of growth at the highest concentration of 10 μg ml^{-1} (Fig. 5). Overall, the populations of all three studied genera showed similar levels of abundance in fecal slurry treated with CNT-containing fruits in comparison to control fruits. This observation correlated with the growth study of *Lactobacillus acidophilus* or *Salmonella enterica* Typhimurium when exposed to control fruits or CNT-containing fruits (Fig. 5).

The abundance of the *Bifidobacterium* genus, which encompasses beneficial symbiotic bacteria,[59] was assessed (Fig. 5f). The results indicated that all different treatments caused a slightly decreased abundance of *Bifidobacterium* compared to the negative control. However, no significant differences were recorded.

The relative quantitation of Enterobacteria family-specific genes

As Proteobacteria represent one of the most abundant phyla, the presence of one of its family members (Enterobacteria family) was assessed by real-time PCR analysis of the Enterobacteria family-specific gene expression. Members of this family are usually opportunistic and show higher abundance during dysbiosis. As expected, the relative abundance of Enterobacteria was not affected by CNTs or CNT-containing fruits (Fig. 6d) ($p > 0.001$). It should be noted, however, that a significant increase in the expression of the Enterobacteria family-specific gene was observed on day 3 (Fig. 6d) in fecal slurry exposed to CNTs at 0.1 μg ml^{-1}. This level eventually decreased and normalized to the negative control group levels by day 7 post-exposure.

The design of this experiment required taking into account different variables that could have contributed to some of the effects observed during our analysis. In fact, fecal specimens of three individuals were used in our analysis with repeated measures at 3 time points (1, 3, and 7 days), and a total of 5 treatments were performed. Thus, using the relative abundance values compared to the negative control ($2^{(-\Delta\Delta Ct)}$ values) obtained from the real-time PCR data, we performed multivariate analysis using principle component analysis (PCA). Fig. S7† represents the different clusters of treatments performed in our analysis. The results showed that the clusters of all 5 treatments overlapped during all days of samplings. Moreover, most of the variability in the data between treatments was contributed by the data on the abundance of Firmicutes and *Bifidobacterium* during day 1; Firmicutes and *Lactobacillus* during day 3; and *Bifidobacterium* during day 7. These results confirm our obtained results that no significant impact of CNTs or CNT-containing fruits on fecal slurry was noted (no separate cluster was observed).

Considering that the fecal slurries were sampled at three time points, we wanted to know the importance of the sampling's time (1, 3, and 7 days) in this experimental study. Fig. S8† shows a PCA of different samples clustered based on their time of sampling. Each cluster shows the 95% confidence limit, calculated using all samples belonging to the same group defined by the sampling date. Based on the cluster sizes, we can conclude that the variability between all samples was much higher compared to the 3rd and 7th day of sampling. In fact, the 7th day of exposure had the smallest cluster size, which corresponds to the smallest variability between treatments. Interestingly, such observation confirms our previous data where differences between treatments previously noted on days 1 and 3 become equivalent on day 7 post-exposure.

In this study, we used three healthy individuals' fecal specimens to investigate the effect of CNTs and CNT-containing fruits on representative bacterial populations in the human gut microbiota. It is well known that the initial profile of bacterial populations can differ from one person to another.[59,60] Thus, we wanted to see whether the differences between individual specimens used in this study could contribute to the observed variable effect. Fig. S9† represents a PCA analysis of different bacterial populations organized based on the origin of the specimen (individual A, individual B, or individual C). The results have shown that during day 1 and day 3, all three clusters representing all three individuals overlapped. This is an indication that the differences between individuals contributed less to the differences observed between time of sampling (days 1, 3, and 7). By contrast, on day 7 we could clearly see that all clusters started to separate, which implies that the bacterial populations from each individual had a unique pattern of response during the exposure to the different treatments studied.

The abundance of different taxonomic classes after exposure to pure CNTs and CNT-containing fruits

Sequencing the 16s rRNA also revealed a dominance of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, along with the very limited presence of some other bacterial phyla (Lentisphaerae, Cyanobacteria, Spirochaetes, Tenericutes, Euryarchaeota, Verrucomicrobia, and Synergistetes); however, no statistically significant differences were observed among any experimental groups when one-way ANOVA was applied to the data (data not shown). Upon 16s rRNA analysis, a clear separation was observed for the abundance of the bacterial population at the genus level on day 1 or day 7 (Fig. 7a). The one-way ANOVA showed a significant difference in the abundance of bacterial genera *Senegalimassilia*, *Paraprevotella*, *Blautia*, *Bacillus*, *Phascolarctobacterium*,

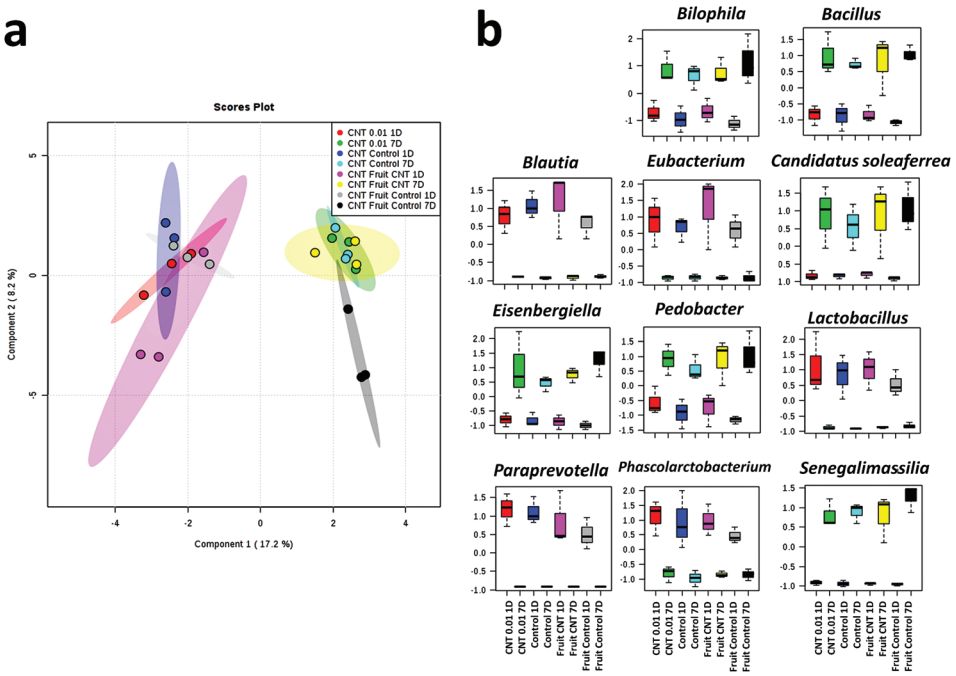

Fig. 7 Analysis of 16S rRNA sequencing data from human fecal slurries treated with CNT-containing fruits on day 1 and day 7, compared to controls (untreated, control fruits, and pure CNTs at a residual level). (a) Principal component analysis based on all taxonomic classes between all tested samples. (b) The average abundance of species showing significant differences between groups studied (based on ANOVA and the *post-hoc* Tukey test. The groups included in this analysis are samples taken on day 1 (1D) and day 7 (7D) and comprise the following: untreated fecal slurry (control) and fecal slurry treated with pure CNTs at 0.001 μg ml^{-1} (CNT 0.001); control fruit (Fruit Control); and CNT-containing fruits (Fruit CNT).

Eisenbergiella, *Lactobacillus*, *Bilophila*, *Eubacterium*, *Pedobacter*, and *Candidatus soleaferrea* (Table S4†). In general, bacteria representing genera *Bacillus*, *Bilophila*, *Candidatus Soleaferrea*, *Eisenbergiella*, *Pedobacter*, and *Senegalimassilia* showed lower abundance on day 1 as compared to day 7. However, bacteria representing genera *Blautia*, *Eubacterium*, *Lactobacillus*, *Paraprevotella*, and *Phascolarctobacterium* had a higher abundance on day 1 as compared to day 7 (Fig. 7b). There was also an increased abundance of bacteria that belong to genera *Blautia* and *Eubacterium* during the exposure of intestinal bacteria to the fruits grown with CNTs on day 1. However, the exposure of intestinal bacteria to the fruits grown with CNTs for 7 days induced a decrease in the abundance of genera *Bilophila* and *Eisenbergiella*, as compared to the group exposed to the control fruit on day 7. Some of the bacteria that belong to these genes play very important roles in the production of SCFA.[61–63] These results indicate that individual responses of bacterial populations to pure CNTs or CNT-containing fruits over the long term could differ. For this reason, *in vivo* chronic exposure studies of longer duration should be performed for a better understanding of the safety of CNTs in fruits exposed to the nanomaterials. It was shown that changes in microbial composition are usually linked with changes in microbial SCFA production.[64] To further confirm the previous results obtained using real-time PCR and 16S rRNA sequencing, we measured the levels of SCFAs in fecal cell cultures treated with CNT-containing fruits and compared them to wild-type fruits. Moreover, we measured the SCFA concentration in samples treated with pure CNTs at all concentrations (0.001, 0.1, and 10 μg ml^{-1}).

The analysis of SCFAs in human fecal slurry exposed to CNT-containing fruits

Fig. 8 shows a heat-map analysis of SCFA concentrations as measured by HPLC. The results show that the time of sampling was the major contributor to the differences between samples. On day 1, the levels of SCFAs (propionic, isobutyric, butyric, valeric, succinic, isovaleric, hexanoic, and acetic acid) in all studied treatments were lower compared to those on day 3 and day 7. In contrast, lactic acid levels decreased significantly after day 1. The addition of carbon nanotubes only at the highest concentration studied (10 μg ml^{-1}) showed a significant decrease of butyric, valeric, succinic, and hexanoic acids on day 3, which subsequently recovered and equaled the negative control on day 7. However, the levels of acetic and propionic acid remained significantly low compared to the control until day 7 (Fig. 8). The fruit extract containing CNTs did not significantly alter SCFA levels in comparison to wild-type tomato fruits; the dendrogram (Fig. 8) reveals the grouping of samples based on their time of sampling (1, 3, and 7 days) rather than on their different treatments. These results are in agreement with our previous findings using real-time PCR of the different phyla and genera where no significant dysbiosis in the treated fecal cultures was observed.

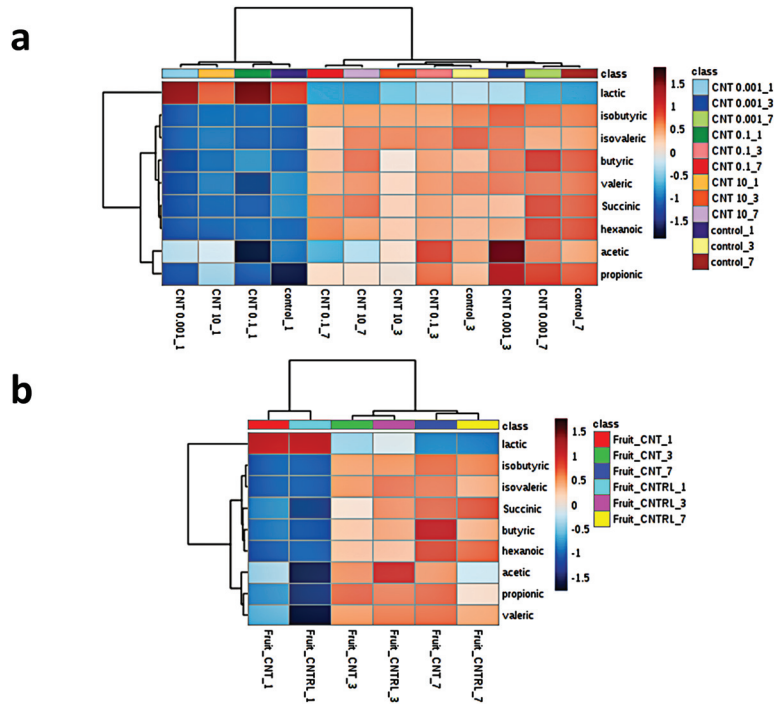

Fig. 8 Heat-map analysis of SCFAs quantified in batch cultures treated with fruit containing CNTs. (A) Heat-map of SCFAs in CNTs only 0.001 μg ml^{-1} (CNT_0.001), 0.1 μg ml^{-1} (CNT_0.1), and 10 μg ml^{-1} (CNT_10) treated fecal slurry on 1, 3, and 7 days. (B) Heatmap of SCFAs in CNT-containing fruits (Fruit_CNT) and compared to control fruit (Fruit_CNTRL) on 1, 3, and 7 days. Data shown are normalized values of concentrations measured using HPLC.

Conclusions

A number of research groups have attempted to elucidate the biological effect of different nanoparticles on epithelial cells and the gastrointestinal tract.[65–69] Because of the unique physicochemical characteristics of nanoparticles and differences in experimental research protocols used, investigations associated with the biological effects of nanoparticles on *in vitro* animal/human models demonstrated high variability and generated many contradicting results. The assessment of the effects of extracts from plant organs that absorbed CNMs as a result of the use of nanomaterials as plant growth regulators, using *in vitro* or *in vivo* toxicological systems, has not been performed yet. Such a comprehensive risk assessment is needed because of the high number of CNM applications currently described as promising nanomaterials for use in agriculture.[14] We have reported previously that the exposure of CNTs to tomato plants resulted in low level accumulation of the nanoparticles in fruits and modification of fruit metabolome.[2] The possible introduction of CNTs into the food chain in contaminated fruits requires that a comprehensive toxicological assessment be performed.

In this work, the impact of the CNT-containing fruits on the integrity of the intestinal epithelial cell barrier and commensal gastrointestinal microbiota was compared to the impact of wild-type tomato fruit control and to the equivalent amount of CNTs previously found in the contaminated fruits.[2] No major toxic effect of extracts from CNT-containing fruits was observed at the cellular or gene expression levels of human epithelial cells. Moreover, the residue level of CNTs present in the tomato extract was too low to show any significant impact on epithelial cells. However, higher doses of pure CNTs were toxic (10 μg ml^{-1}), disturbed the epithelial cell barrier (decrease in TEER during the early exposure and 48 hours after exposure recovery), and changed the gene expression pattern in exposed cells. On the other hand, CNT-containing fruits were not toxic to the growth of *L. acidophilus* and *S. enterica* Typhimurium. Furthermore, following 7 day exposure of the CNT-containing fruits to human fecal slurries under the experimental conditions defined in this study, the results showed a subtle impact on the specific bacterial population studied. Different assays were used to confirm that the CNT-containing fruits did not cause any significant shift in the abundance of bacterial communities after exposure to CNT-containing fruits in comparison to control fruits. However, the abundance of different bacterial populations studied was disturbed after exposure to high doses of pure CNTs (10 μg ml^{-1}) during the first day of exposure and recovered after 7 days.

To our knowledge, this is the first report on the effects of CNT-containing fruits on the human intestinal microbiota composition and gastrointestinal epithelial cell barrier function. Future *in vivo* experiments with high throughput analysis of the gastrointestinal impact of CNT-containing fruits could

aid in elucidating the influence of nanoparticles on human health once introduced into the food chain.

Conflicts of interest

There are no conflicts to declare.

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