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Impact of food grade and nano-TiO_2 particles on a human intestinal community

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ABSTRACT

Titanium dioxide (TiO_2) nanoparticles (NPs) are used as an additive (E171 or INS171) in foods such as gum, candy and puddings. To address concerns about the potential hazardous effects of ingested NPs, the toxicity of these food-grade NPs was investigated with a defined model intestinal bacterial community. Each titania preparation (food-grade TiO_2 formulations, E171-1 and E171-6a) was tested at concentrations equivalent to those found in the human intestine after sampling 1–2 pieces of gum or candy (100–250 ppm). At the low concentrations used, neither the TiO_2 food additives nor control TiO_2 NPs had an impact on gas production and only a minor effect on fatty acids profiles (C16:00, C18:00, 15:1 w5c, 18:1 w9c and 18:1 w9c, $p < 0.05$). DNA profiles and phylogenetic distributions confirmed limited effects on the bacterial community, with a modest decrease in the relative abundance of the dominant *Bacteroides ovatus* in favor of *Clostridium cocleatum* (−13% and +14% respectively, $p < 0.05$). Such minor shifts in the treated consortia suggest that food grade and nano-TiO_2 particles do not have a major effect on human gut microbiota when tested *in vitro* at relevant low concentrations. However, the cumulative effects of chronic TiO_2 NP ingestion remain to be tested.

1. Introduction

Titanium dioxide (TiO_2) is a white metal oxide commonly used as whitening or brightening agents in various applications such as paints, cosmetics and food products. As a food additive, TiO_2 is referred to as E171 in Europe and INS171 in North America. These are added to many foods including cheeses and sauces, skimmed milk, ice-creams and pastries, as well as in sugar confectionary where it constitutes the coating of sweets and chewing-gum (Bachler et al., 2015; Skocaj et al., 2011; Weir et al., 2012). The concentration of TiO_2 incorporated into processed foods depends on the type of product, ranging from 1.25 μg in chocolate-coated candy (M&M's®; Weir et al., 2012) to 2.4–7.5 mg in the coating of a single piece of gum (multiple brands; Chen et al., 2013). Whatever the kind of food, the addition of TiO_2 is limited to 1% of the overall food weight in the United States (USFDA, 2005) and it is used "*at quantum satis*" levels in Europe, which means that although no maximum level is specified it is to be used at levels not higher than necessary to achieve the intended purpose (European Parliament, 1994; EFSA, 2016).

Global per capita TiO_2 ingestion depends on geography with the population of the USA and the UK consuming ~0.2–0.7 mg and ~1 mg TiO_2/kg body weight (bw) per day, respectively (Weir et al., 2012). Due to their lower body mass and their higher consumption of candies and sweets compared to adults, children under 10 are estimated to ingest 1–2 mg and 2–3 mg TiO_2/kg a day in the USA and UK, respectively. This consumption by children is below the lowest "no observed adverse effects levels", recently established at

Abbreviations: TiO_2, Titanium dioxide; NPs, Nanoparticles; MET-1, Microbial ecosystem therapeutic-1; TSA, Tryptic soy agar; CO_2, Carbon dioxide; N_2, Nitrogen; H_2, Hydrogen; GC, Gas chromatograph; FAME, Fatty acid methyl ester; PCR, Polymerase chain reaction; PCR-DGGE, Polymerase chain reaction denaturing gradient gel electrophoresis; TAE, Tris-Acetate-EDTA; NCBI, National center for biotechnology information; ANOVA, Analysis of variance; HSD, Honestly Significant Difference.

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2250 mg TiO_2/kg bw per day (EFSA, 2016).

It is important to note that TiO_2 is considered to have low toxicity (JECFA, 1969). However, recent assessments indicate that food grade TiO_2 can be composed of up to 44% nanoparticles (NPs) (Chen et al., 2013; Weir et al., 2012; Yang et al., 2014; Dudefoi et al., 2017). Since TiO_2 NPs have been classified as potentially carcinogenic for humans via inhalation (Group 2B, IARC), it is important to assess any possible toxicity of TiO_2 NPs associated with ingestion (Tassinari et al., 2014). Toxicity assessments, performed both on epithelial cells and animals, have shown that TiO_2 NPs can accumulate in the intestine, pass through the intestinal barrier inducing toxicity through oxidative stress and genotoxicity, and impair intestinal and systemic immune homeostasis (Böckmann et al., 2000; Jani et al., 1994; Gerloff et al., 2009, 2012; Shi et al., 2013; Bettini et al., 2017). Although exposure to NPs can occur after the deliberate or accidental ingestion of foods, water, and personal care products (Fröhlich and Fröhlich, 2016), there have been only a few investigations on the impact of TiO_2 particles on the gut microbiota (Taylor et al., 2015; Liu et al., 2016).

The gut microbiota forms a complex ecosystem in the gastrointestinal tract and is of obvious importance for numerous aspects of human physiology from nutritional status to behavior and stress responses, some of which are only just starting to be appreciated (Frank et al., 2007). Since changes to this microbiota can be associated with disease states such as obesity, diabetes, rheumatoid arthritis, and inflammatory bowel disease (Marchesi et al., 2016; Pietroiusti et al., 2016), it is important and timely to assess the impact of food grade TiO_2 on the human gut consortia. However, its complexity and the variability in species composition between individuals, means that assessment of the impact of food additives on this ecosystem is problematic and difficult to interpret. We thus utilized a defined human gut bacterial community known as microbial ecosystem therapeutic-1 (MET-1), which contains 33 different bacterial strains and was originally established from the collected stool of a healthy donor (Petrof et al., 2013). While MET-1 is not a complex community, it does contain a range of bacterial species representative of common human gut autochthonous microbes, which can be cultured as an ecosystem and used for exposure studies. Previously MET-1 has been used to show that silver NPs (200 ppm) have the potential to disrupt the intestinal microbiome (Das et al., 2014). Here the same system has been employed to assess the impact of two commercial food grade TiO_2 additives using a suite of physiological, biochemical and molecular assays to test for toxicity.

2. Materials and methods

2.1. TiO_2 sample preparation

Food-grade TiO_2 (E171-1 and E171-6a) from two European suppliers, and TiO_2 NPs (Degussa P25, Essen, Germany; 100% NPs, with a reported diameter of 25 nm) were employed. E171-1 and E171-6a represent two batches of E171 that have been extensively characterized with respect to size distribution, crystallinity, and surface properties as described elsewhere (Dudefoi et al., 2017). Briefly, E171-1 was chosen as a representative sample of food grade TiO_2 since it contains 17% NPs and is composed of 100% anatase TiO_2, as determined by transmission electron microscopy (Hitachi H-9000 NAR), and by X-ray diffraction (Bruker AXS D8 Advance). Further, it has an isoelectric point of 4.1 and a low specific surface area of 8.6 $m^2\ g^{-1}$ as shown by laser Doppler electrophoresis (Zetasizer Nano ZS) and gas volumetry (BET method, Micromeritics TriStar II 3020 Physisorption Analyzer), respectively. E171-6a (21% NPs) was chosen as an alternative TiO_2 food additive since it showed a different surface chemistry from the other E171 batches, with a lower isoelectric point of 2.2, attributed to the presence of silica at its surface as detected by X-ray photoelectrons spectroscopy (AXIS Nova, Kratos Analytical Company). The P25 NPs used as a reference TiO_2 nanomaterial (OECD, 2009), were also subjected to the same set of characterizations, which confirmed the manufacturer's specifications as previously described (Dudefoi et al., 2017). Particle sterility was interrogated by inoculation of the food additives and the TiO_2 NPs (5 mg each) into sterile 10% tryptic soy culture medium, incubation at 37 °C for 2 days and subsequent plating (100 μL) on 10% TSA (tryptic soy agar) at 37 °C. Stock solutions of the various TiO_2 samples (5 mg/mL) were prepared in sterilized milliQ water in sterile serum bottles.

2.2. Human gut ecosystem culture

The defined MET-1 bacterial community has been described (Petrof et al., 2013). Prior to use, each of the component 33 bacterial strains (see Table 1) were individually cultured on fastidious anaerobe agar (Acumedia) containing 5% defibrinated sheep's blood (Hemostat Laboratories) under anaerobic conditions, and the biomass was used to inoculate a chemostat bioreactor, which was run for 1 day in batch followed by 10 days under flow conditions using a medium approximating the content of the human colon (400 mL/day). Control of both pH and temperature were maintained throughout (pH 6.8, 37 °C), as well as gentle agitation and constant sparging of sterile N_2 gas through the culture to maintain anaerobic conditions. The chemostat set-up and culture, including media components have been fully detailed (McDonald et al., 2013). After 10 days of growth under these conditions, MET-1 attained a

Table 1
Strains present in MET-1, and their closest species matches.

Strain designation	[a]Closest species match	[b]% Homology
14 LG	*Acidaminococcus intestini*	99
3FMU	*Akkermansia muciniphila*	100
5 MM	*Bacteroides ovatus*	99
11 FAA	*Bifidobacterium adolescentis*	99
20 MRS	*Bifidobacterium adolescentis*	99
2 FAA	*Bifidobacterium longum*	99
4 FM	*Bifidobacterium longum*	99
27 FM	*Blautia stercoris*	99
21 FAA	*Clostridium cocleatum*	92
3 FM	*Collinsella aerofaciens*	99
10 FAA	*Dorea longicatena*	99
42 FAA	*Dorea longicatena*	99
3 FM 4i	*Escherichia coli*	100
48 FAA	*Butyricicoccus pullicaecorum*	95
F1 FAA	*Eubacterium eligens*	99
13 LG	*Eubacterium limosum*	97
6 FM	*Eubacterium rectale*	99
29 FAA	*Eubacterium rectale*	99
1 FAA	*Eubacterium rectale*	99
18 FAA	*Eubacterium rectale*	99
47 FAA	*Eubacterium ventriosum*	99
40 FAA	*Faecalibacterium prausnitzii*	99
34 FAA	*Lachnospira pectinoschiza*	95
6 MRS	*Lactobacillus casei*	99
25 MRS	*Lactobacillus paracasei*	99
5 FM	*Parabacteroides distasonis*	99
BF 7	*Enterobacter aerogenes*	100
39 FAA	*Roseburia faecis*	99
31 FAA	*Roseburia intestinalis*	99
11 FM	*Ruminococcus obeum*	99
2 MRS	*Blautia luti*	95
30 FAA	*Ruminococcus torques*	99
9 FAA	*Ruminococcus torques*	99
50 FAA	*Streptococcus mitis*	99

[a] As inferred by 16S rRNA gene sequence homology across the full-length gene to the RDP database (Cole et al., 2014).
[b] % Match of full-length 16S rRNA gene sequence to closest species.

steady-state equilibrium. The MET-1 suspension was harvested from the chemostat vessel and 450 μL was aliquoted into 4050 μL of sterile growth medium (1:10) in individual autoclaved glass serum bottles that were then placed in an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2). The serum bottles were amended with one of two concentrations of TiO_2 particles (100 and 250 ppm, referred as low and high exposure concentrations and equivalent to that found in the intestine after ingestion of 1–2 pieces of gum or candy). Experiments included three different TiO_2 particle samples tested individually, including the purchased P25 NPs as a toxicological reference, E171-1 and E171-6a. A mixture of food grade TiO_2 (E171-1, 100–250 ppm) and 50 mg/mL porcine pancreas α-amylase (37.5 U/mL, Sigma Aldrich) was included in one experimental set of bottles in order to model the additional presence of a starch-digesting enzyme, as would be seen in the digestive tract. Negative control cultures were not amended with titania. The final volume of each serum bottle was brought to 5 mL with the addition of sterile, milliQ water and each bottle was then fitted with a sterile rubber stopper and crimped with a metal band. Finally, the culture bottles (n = 30) were placed in an air-tight, double-sealed bag containing anaerobic gas packs (Beckton Dickinson) and allowed to incubate in batch culture for 48 h at 37 °C in the dark, with agitation.

2.3. Physiological assay: gas analysis

Gas production was monitored in the serum bottles after 48 h of incubation, as previously described (Das et al., 2014). Briefly, gas was sampled *in situ* using 10 mL syringes (Becton Dickinson & Co, NJ), immediately injected using a split-less mode into an Agilent Technologies 7890B Gas Chromatograph (GC; Palo Alto, California, USA), equipped with a stainless steel column (50 m × 0.53 mm internal diameter, 10 μm film thickness) and packed with Agilent J&W PoraBOND Q (Palo Alto, California, USA). Total gas production was measured in the syringe and CO_2 and N_2 production were determined using Agilent Technologies Chem Station Integration Software (Palo Alto, California, USA).

2.4. Biochemical assay: fatty acid analysis

Immediately after recording gas production, a sample (1 mL) from each serum bottle was centrifuged at 2000×*g* for 10 min and the pellet was placed into 2 mL cryo-tubes. These were stored at −80 °C until fatty acid methyl ester (FAME) analysis (Keystone Labs; Edmonton, Canada) was conducted. Phospholipid fatty acids were extracted using the MIDI Sherlock Microbial Identification System (Microbial ID Inc., Newark, DE, USA) as previously described (Das et al., 2015). Briefly, each pellet was saponified, methylated, extracted, washed and finally analyzed with a gas chromatograph equipped with an Ultra 2 column containing a flame ionization detector (Agilent). The resulting chromatographic peak areas were converted to the percentage of the total fatty acids (mol %).

2.5. Molecular assays: DNA analysis

Samples (3 × 200 μL) were removed from each serum bottle and placed into 2 mL cryotubes. These were then frozen with liquid nitrogen and stored at −80 °C. DNA was subsequently extracted using a QIAamp DNA stool mini kit (Qiagen Sciences, LLC, MD, USA, Cat° 51504). Briefly, the procedure comprised the lysis of samples in Buffer ASL (a proprietary buffer), adsorption of impurities to an InhibitEX matrix, and the purification of DNA on QIAamp Mini spin columns. The last step of the extraction was modified from the manufacturer's recommended protocol by the substitution of 50 μL of sterile milliQ water, for 200 μL of the proprietary buffer. The extracted DNA was stored at −20 °C until used for polymerase chain reaction (PCR).

Genomic DNA was PCR-amplified in triplicate using a Veriti® 96 Well Thermal Cycler (Applied Biosystems, Burlington, Canada) with the primer pair: 28F (5′- GAGTTTGATCNTGGCTCAG -3) and 519R (5′-GTNTTACNGCGGCKGCTG -3′) as previously described (Fan et al., 2012). PCR reaction mixtures were prepared with 4 μL of DNA template, 2.5 μL of each primer (10 μM), 5 μL of 10× Buffer (Vivantis VSPL1202 containing $MgCl_2$), 0.4 μL of dNTP mix (12.5 mM; Thermo Scientific), 400 ng bovine serum albumin, 0.5 μL of recombinant Taq DNA polymerase (5 U/μL; Vivantis, Malaysia, (Lee et al., 2009)) and 34.1 μL sterile water to a total volume of 50 μL. The PCR conditions included a first step of denaturation for 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C (denaturation), 1:30 min at 50 °C (annealing), 1:45 min at 72 °C (extension) with a final extension for 7 min at 72 °C. Controls with and without DNA were always included. The concentration and integrity of the amplified DNA was subsequently assessed by electrophoresis on 1% agarose gels stained with ethidium bromide and viewed on a UV transilluminator (White, 1993). Primers, dNTPs, unincorporated labeled nucleotides, enzymes, and salts were removed from the amplification products using a GeneJET PCR Purification Kit (Thermo-Scientific, K0702) and the concentration and purity of the amplified DNA was assessed by spectrophotometric analysis using a NanoDrop 1000 (NanoDrop-1000 Ver.3.7.1; Thermo Scientific, Wilmington, USA) (Passalacqua et al., 2009) based on the 260/280 ratio.

For PCR-denaturing gradient electrophoresis (PCR-DGGE), nested PCR reactions were performed on the amplified DNA using primer 338F (5′-ACTCCTACGGGAGGCAGCAG GC-3′) containing an additional 40-nucleotide GC-rich sequence on the 5'-end as well as the reverse primer 519R (5′-GTNTTACNGCGGCKGCTG -3′) (Bakke et al., 2011). Amplification was achieved after a denaturation step for 5 min at 95 °C, followed by 18 cycles of 1 min at 95 °C, 1:15 min at 67 °C, 1:30 min at 72 °C with a final extension for 7 min at 72 °C. The products were subjected to electrophoresis using a DGGEK-2401 System (CBC Scientific, Del Mar, USA; Tok et al., 1998) on 8% (w/v) polyacrylamide gels containing a denaturing gradient (30%–70% of urea; Sonthiphand and Neufeld, 2013; Myers et al., 1985) at 120 V for 3 min then 70 V for 18 h in Tris-acetate buffer, pH 8.3 containing EDTA (TAE) at 60 °C. After staining (0.5 mg/L ethidium bromide), and subsequent rinsing in TAE 1× buffer (5 min), the gels were visualized using an Alpha Innotech UV transilluminator and photographed using AlphaEase® FC software (Version 6.0, Alpha Innotech, Santa Clara, CA, USA). Finally, intensities of the DGGE-generated images were analyzed using Syngene Genetools software (version 4.03.03, Synoptics Ltd.).

After amplification, DNA samples were sequenced in triplicate using 454 pyrosequencing to identify the different bacterial strains (Table 1). All sequencing was performed by the Research and Testing Laboratory (MR DNA; Shallowater, TX, USA) using a Genome Sequencer Roche 454 FLX Titanium platform (Roche, Nutley, NJ) and following the manufacturer's recommendations. Raw sequence information was clustered into operational taxonomic units (OTUs) based on 97% identity. They were then taxonomically classified using the BLASTn algorithm by comparing the OTUs to a curated database derived from GreenGenes, RDPII and the 16S sequences of the National Center for Biotechnology Information (NCBI) (http://rdp.cme.msu.edu; www.ncbi.nlm.nih.gov; DeSantis et al., 2006). OTUs were then compiled into each taxonomic level into both 'counts' and 'percentage' files, representing the actual number and the relative percentage of sequences within each sample that map to the designated taxonomic classification. Sequences representing less than 0.1% of the reads were routinely discarded since they were frequently found to represent PCR or

sequence errors.

2.6. Statistical analysis

A one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) test calculator was used to compare multiple treatments and to evaluate significant differences in comparison to the control (bilateral Dunnet test) using Xlstat (v 2014.1.08, Addinsoft. 2016. XLSTAT, 2016: Data Analysis and Statistical Solution for Microsoft Excel. Paris, France, 2016). Differences were considered not significant for p values >0.05.

3. Results

3.1. Head space gas analysis

For the most part, after 48 h of incubation, all serum bottles, irrespective of the treatment groups, showed levels of CO_2 and N_2 as well as total gas volumes that were similar to control, non-amended cultures (Fig. 1). This suggests that the addition of food additives and the TiO_2 NPs had no significant influence on bacterial metabolism. In the E171-6a-amended sample there was a small variation in gas volume at both concentrations ($p < 0.05$). However, these p-values were only just within the limit of significance ($p = 0.041$ and 0.046) and only one gas per treatment was involved. Therefore we suggest that the amendments had no overall impact on bacterial respiration.

3.2. Fatty acid analyses

A total of 54 different phospholipid fatty acid peaks were seen in chromatographs obtained from the treated MET-1 cultures. For each sample, the chromatographic peak areas were converted to percentage of total fatty acids (mol %), and of these, 25 dominant fatty acids were selected for comparative analysis and characterized into Gram negative, Gram positive, saturated and unclassified groups (Fig. 2) according to conventional classification (e.g. Bartelt-Ryser et al., 2005; Kumar et al., 2014); but see Suutari and Laakso, 1994 for cautionary interpretive advice). Overall, the food additives and TiO_2 NPs had little to no impact on the overall fatty acid compositions. There were some modest changes that were significant for E171-1 (Fig. 2), with small variations in the saturated fatty acids composition for C12:00, C14:00, C16:00 and C18:00 (−0.3, −0.4, −4.0 and +1.9 percentage points, respectively), at both additive concentrations, Gram negative fatty acids markers (−0.2, +1.0 and −0.9 percentage points for 16:1 w7c/16:1 w6c, 17:0 cyclo and 18:1 w9c, respectively) and four of the unclassified fatty acids (+6.7, −0.1, −0.7 and −2.6 percentage points for 15:1 w5c, 17:00, 18:2 w6, 9c/18:0 ante and 18:1 w9c, respectively). However, most of these represented less than a 1 percentage point change. Only two fatty acids appeared to decrease slightly (−0.4 and −1.3 percentage points for 14:00 and 18:1 w9c, respectively) after incubation with the P25 particles and there were no changes in any fatty acids with the E171-6a additive.

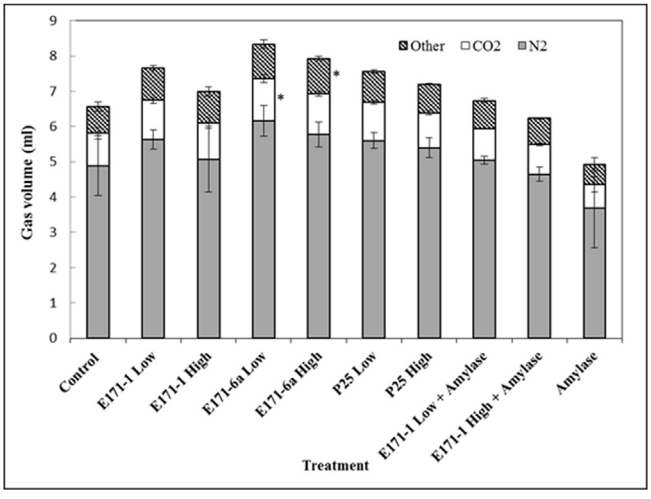

Fig. 1. Gas volume and analysis after culture. Carbon dioxide (CO_2), nitrogen (N_2) and other gases recovered from the total gas generated by the MET-1 communities are shown after 48 h exposure to food grade (E171-1 and E171-6b) and P25 TiO_2 particles at 100 (Low) and 250 (High) mg/L, and control (with no TiO_2 particles). Star symbols indicate significance differences ($p < 0.05$) in gas composition (volume) compared to the unamended controls.

In contrast with these very limited effects, the addition of pancreatic amylase, as well as the mixture of amylase and E171-1, to MET-1 induced significant shifts in all classes of fatty acids when compared to controls ($p < 0.05$; Fig. 2). Amylase addition reduced some Gram positive (−0.4 percentage points for 15:0 3OH) and Gram negative signatures (−0.2, −1.2 and −2.2 percentage points for 16:1 w7c/16:1 w6c, 16:0 3OH and 18:1 w7c) and shifted saturated fatty acids profiles (−0.4, −0.6, −1.3, +2.7 and +1.4 percentage points for 10:00, 12:00, 14:00, 18:00 and 20:00) and unclassified fatty acids proportions (−0.6 and +1.6 percentage points for 17:0 2OH and 18:2 w6,9/18:0 ante), indicating that the batch MET-1 cultures were physiologically responsive.

3.3. DNA analysis

After TiO_2 amendment, PCR-DGGE banding patterns appeared identical to the controls, with the regular appearance of 6 major bands (Fig. 3), suggesting that TiO_2 NPs at the concentrations used had no impact on the MET-1 ecosystem. In contrast, consistent differences in banding patterns and intensities were seen in treatment groups containing amylase compared to controls. In particular, one band (numbered 4; Fig. 3) disappeared in three treatment groups containing pancreatic amylase additions (amylase controls and the E171 food additive containing the enzyme).

After incubation, the cultured consortium was dominated by 8 strains, and thus although results were obtained for the entire MET-1 community, only the most abundant bacteria were examined in detail since any modest perturbations of very minor species contributors would be of no interest to consumers or regulators. Consistent with the PCR-DGGE analysis, phylogenetic distributions obtained from 454 pyrotag 16S rRNA gene sequencing showed that there were no substantial differences between the E171-6a treatment groups and controls. In the E171-1 (250 ppm) food additive group there was an 8 percentage point decrease in the OTUs attributed to *Bacteroides ovatus* (strain 5 MM; Fig. 4), but there were no other significant changes. The 7 and 13 percentage point decrease in *B. ovatus* in the P25 treatment groups, at 100 and 250 ppm, respectively, was correlated with a 11 and 14 percentage point increase in the abundance of strain 21FAA (closest database relative *Clostridium cocleatum;* $p < 0.05$; Fig. 4). When the taxonomic distribution was normalized to total fatty acids (Fig. S1), there appeared to be no negative impact on consortium viability after TiO_2 treatments. P25 and E171-6a treatments induced no significant differences when compared to controls, and E171-1 amendment resulted in some small increases in the relative proportions of *Acidaminococcus intestini*, *Eubacterium ventriosum* and *Eubacterium rectale* strains (4.4, 0.5 and 2.6 percentage points, respectively, $p < 0.05$) in the groups (Fig. S1).

In contrast to the results for the titania amendments alone,

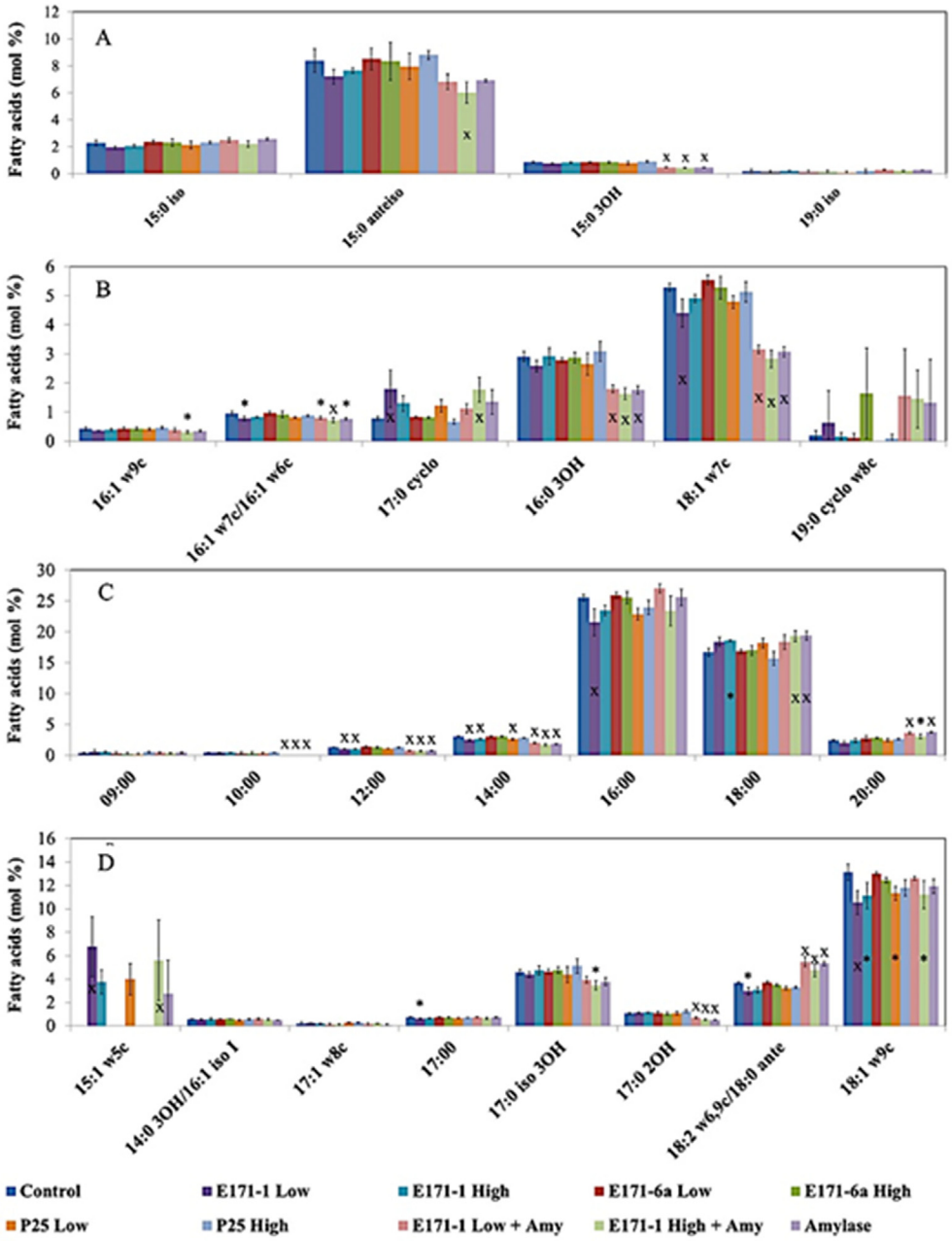

Fig. 2. Fatty acid analysis after culture. Relative amounts (mol %) of extracted methylated fatty acids in control (no TiO_2) and TiO_2 treatments at 100 mg/L (Low) and 250 mg/L (High) concentrations are shown. The bars represent the means of three independent fatty acid assessments and standard errors. Fatty acids (≥0.15 mol%) were classified as (A) Gram positive, (B) Gram negative, (C) saturated, and (D) unclassified fatty acids for ease of analysis, however, note that assignments to specific groupings may be problematic (see Results). Stars and letter x symbols indicate significant differences at $p < 0.05$ and $p < 0.01$ levels, respectively, compared to the controls.

treatment groups containing pancreatic amylase, in the presence or absence of E171-1, resulted in a consistent and significant 4.8 percentage point reduction of *Eubacterium rectale* (represented by four strains), in favor of the predominant bacteria in the consortium, *B. ovatus*, which increased by 13 percentage points ($p < 0.01$; Fig. 4; Fig. S1).

4. Discussion

Here we show that TiO_2 food whitening and brightening additives containing ~20% NPs do not have a major impact on an *in vitro* model of the human gut microbiota when used at concentrations that mimic the concentration in the adult intestine after chewing a single piece of gum. The minor impact of the two additives is, nevertheless, different between E171-1 and E171-6a samples of TiO_2, which may be due to their different chemistries, with E171-6a's silica coat possibly maximizing contacts with water molecules and thus the potential to reduce the formation of adhesive interactions between the particles and the bacteria. Our results are in agreement with the conclusions reached after a TiO_2 food additive similar to E171-1 was introduced in a model colon reactor containing a microbial community extracted from human fecal material (Waller et al., 2017). Exposure to 36 mg/L per day of the additive for 5 days resulted in little effect on the composition and phenotype of the microbiota.

Even if P25 is not an optimal model to mimic the nano-sized

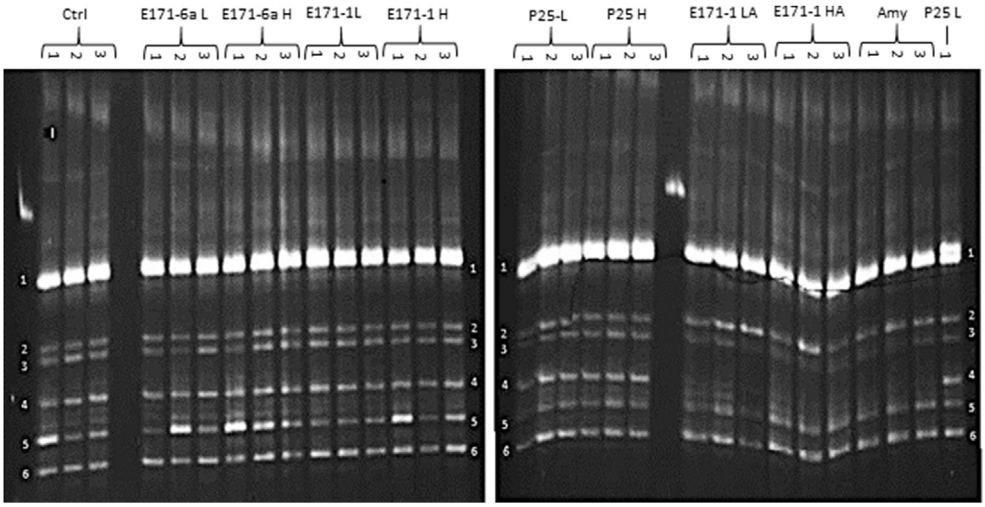

Fig. 3. PCR-DGGE gel analysis. Representative DNA banding patterns seen in controls (Ctrl) and after exposure to E171 food grade TiO_2 particles (E171-1 and E171-6a) and to TiO_2 NPs (P25) at 100 ppm (low; L) and 250 ppm (high; H) concentrations, without and in the presence of amylase (Amy). Porcine pancreatic amylase (37.5 U/ml) was added alone or as a mixture with E171-1 at both concentrations (E171-1 LA and E171-1 HA). Numbers at the end of the descriptors (1, 2, 3) indicate replicate samples. Numbers next to the bands mark those that were analyzed for intensity variations. Unmarked lanes contained marker DNA.

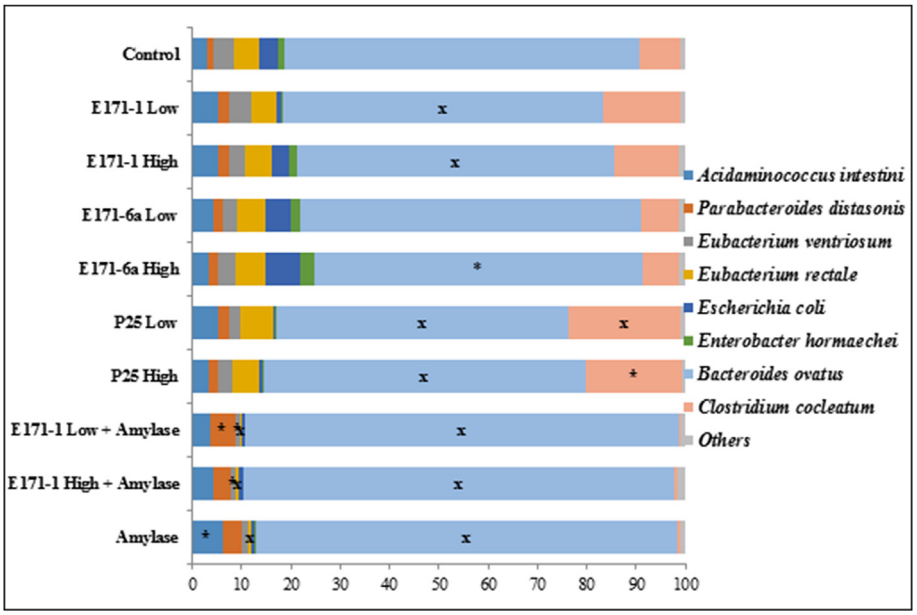

Fig. 4. Phylogenetic composition in the MET-1 consortium after various amendments. Strains are represented as distribution percentages after 48 h exposure to 100 ppm (Low) and 250 ppm (High) concentrations of food grade E171-1 and E171-6a, as well as to P25 TiO_2 NPs (P25), compared to controls (Control). Treatment groups containing pancreatic amylase (37.5 U/ml; Amylase) are indicated. Distribution percentage of each strain (indicated on the color key) is based on the means of triplicate samples of those contributing ≥1% relative abundance (for figure clarity less abundant strains are indicated as 'Others'). Significant differences ($p < 0.05$ and $p < 0.01$, respectively) compared to the control cultures are indicated by stars and the letter x, respectively, on the bars.

fraction of E171 (Dudefoi et al., 2017), the comparison with this widely-studied reference material is of interest. Previously it was reported that P25 TiO_2 NPs had a low impact on Gram negative species (Rincón and Pulgarin, 2005), and here we show a modest decrease in the proportion of the Gram negative *B. ovatus* and an increase in Gram positive *C. cocleatum* strains after P25 amendment (Fig. 4). *Bacteroides* species are important for carbohydrate metabolism and with the capacity to synthesize conjugated linoleic acid, which has antidiabetic, antiatherogenic, antiobesogenic, hypolipidemic and immunomodulatory properties (Devillard et al., 2007, 2009; Baddini Feitoza et al., 2009). These species also play a role in intestinal immunity by the activation of dendritic cells (He et al., 2007). In contrast, *C. cocleatum* is thought to affect immune responses, and higher numbers have been found in patients with irritable bowel syndrome (Kassinen et al., 2007). Our results are also in agreement with the small increase in extracellular polymeric sugar content in a model colon community after treatment with P25 TiO_2 NPs (Taylor et al., 2015). The fact that P25 amendments have a slightly higher impact than food additives may be attributed to the higher surface reactivity of the smaller particles (P25) than the larger ones (E171) and/or their different surface properties (Dudefoi et al., 2017).

The limited impact of TiO_2 particles on the MET-1 consortium contrasts with results obtained after amending this community

with silver NPs, which induced major changes in the ecosystem as indicated by a significant reduction in gas production, changes in fatty acid methyl ester profiles and shifts in the community structure (Das et al., 2014). Thus titania particles clearly have little impact on the microbiome in comparison to silver NPs.

To ensure that the MET-1 community remained sensitive to amendment, pancreatic amylase was added to the consortium, as well as to the E171-1 treatment groups. The results clearly showed that the addition of the enzyme alone or in combination with titania had a significant impact on the consortium. Pancreatic amylase would have hydrolyzed a portion of the starch substrate, which in turn would have modified the nutrient availability in the culture media. The result was a shift in some fatty acids when compared to controls ($p < 0.05$), the reduction of some identified Gram negative and Gram positive signatures, and changes in the saturated and unclassified fatty acids profiles (Fig. 2). Correspondingly, DNA analysis demonstrated the disappearance of a band in PCR-DGGE (Fig. 3), and the significant reduction of sequences corresponding to the four strains of *E. rectale* in favor of the abundant Bacteroidetes member of the model gut consortium, *B. ovatus* ($p < 0.01$; Fig. 4), two bacteria known for their starch-utilization pathways (Cockburn et al., 2015; Degnan et al., 1997). The fact that the proportion of *B. ovatus* increased at the expense of *E. rectale* can be attributed to the ability of *B. ovatus* to synthesize several starch-hydrolyzing enzymes with different specificities and activities, conferring a significant competitive advantage in the colonic ecosystem (Degnan et al., 1997). When exogenous amylase was added, the remaining starch would be utilized by *B. ovatus* rather than *E. rectale*. This perturbation of the microbial consortium in response to the addition of the amylase clearly demonstrates the ability of the MET-1 cultures to respond to modification and thus helps establish this ecosystem as an appropriate model system to assess food additives, including TiO_2 NPs.

Although nano-toxicity mechanisms are unknown, it has been suggested that NPs interact with bacteria to produce reactive oxygen species (ROS), which in turn can damage DNA, RNA and proteins (Cabiscol et al., 2000). Thus Gram positive bacteria, with their thick peptidoglycan and lipoteichoic cell walls may be better protected against ROS damage. Further, nano-titania absorbs ultraviolet light and is in consequence an important photocatalyst, generating ROS which then can oxidize organic compounds (Barnes et al., 2013). Indeed, TiO_2 NPs appear to be significantly less toxic in the dark (Tsuang et al., 2008; Bonnet et al., 2015). Notably, our experiments with MET-1 were conducted in the absence of oxygen and also in the dark, possibly explaining the very low toxicity observed here. These results further suggest that TiO_2-containing food additives could be of lesser concern for consumers since these two conditions are found in the human gut.

Although these results on food grade TiO_2 should be greeted as good news for consumers, these studies must be validated *in vivo* as there may be different effects. For example, TiO_2 NPs (10, 50 and 100 nm) inhibited the growth of intestinal commensal bacteria *in vitro* in an insect model, but not *in vivo* (Liu et al., 2016).

5. Conclusions

Titanium dioxide is a white metal oxide commonly employed as a pigment in food products that have been found to contain up to ~40% NPs. Based on our investigations using a defined anaerobic gut bacterial community MET-1, the addition of relevant concentrations of commercial food additives (100–250 mg/L) had little impact as assessed through bacterial respiration, fatty acid profiles and phylogenetic composition. Taken together, our results suggest that food grade TiO_2 particles do not significantly alter the human gut microbiota. However, we caution that the cumulative effects of chronic ingestion and the impact of higher concentrations of nano-titania remain to be assessed as well as *in vivo* experimentation before there can be assurance that there is no significant toxicity to our microbiome by these ubiquitous food additives.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.05.050.

Transparency document

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