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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.