



AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Food-grade titanium dioxide can affect microbiota physiology, adhesion capability, and interbacterial interactions: A study on L. rhamnosus and E. faecium

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1914930

since 2023-08-01T13:28:13Z

Published version:

DOI:10.1016/j.fct.2023.113760

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1	Food-grade titanium dioxide can affect microbiota physiology, adhesion capability, and		
2	interbacterial interactions: a study on L. rhamnosus and E. faecium		
3	Bietto F. ^{1+^*} , Scardaci R. ^{1+°} , Brovia M. ¹⁺ , Kokalari I. ^{2\neq} , Barbero F. ² , Fenoglio I. ² , and Pessione E. ¹		
4			
5			
6	¹ Laboratory of Microbial Biochemistry and Proteomics, Department of Life Sciences and Systems		
7	Biology. University of Turin. Via Accademia Albertina 13, 10123 Torino, Italy.		
8	francescabietto@gmail.com, rossella.scardaci@unito.it, manuel.brovia@gmail.com,		
9	enrica.pessione@unito.it		
10	² Department of Chemistry, University of Turin, via P. Giuria 7, 10125 Torino, Italy.		
11	I.Kokalari@tudelft.nl, francesco.barbero@unito.it, ivana.fenoglio@unito.it.		
12	^ Present address Food Bioscience Department, Teagasc Food Research Center, Moorepark,		
13	Fermoy, Co. Cork, Ireland, P61 NP77.		
14	$^{\circ}$ Present address Department of Molecular Biotechnology and Health Sciences, Molecular		
15	Biotechnology Center, University of Turin, Via Nizza 52, Torino, Italy;		
16	$^{\neq}$ Present address Delft University of Technology, Dept. of Chemical Engineering, Van der		
17	Maasweg 92629 HZ DELFT, The Netherlands.		
18	*Corresponding author: <u>francescabietto@gmail.com</u>		
19	⁺ These three authors have equally contributed		
20			
21	ABSTRACT		
22	Food-grade titanium dioxide (TiO ₂ -FG) is a widespread metal oxide used in food industries.		
23	Recently, the European Food Safety Authority concluded that TiO2-FG cannot be considered safe for		
24	consumption due to its genotoxicity; however, its effect on the gut microbiota has not been completely		
25	unraveled. We studied the effects of TiO2-FG (0.125 mg/mL) on Lactobacillus rhamnosus GG (LGG)		
26	and Enterococcus faecium NCIMB10415 (Ent), in particular some physiological and phenotypic		
27	traits (growth kinetics, bile salts, and ampicillin resistance) and their interactions with the host (auto-		
28	aggregation, biofilm formation, and adhesion on Caco-2/TC7 monolayers) and other gut		
29	microorganisms (antimicrobial activity towards pathogens). The results obtained revealed that TiO2-		
30	FG alters both LGG and Ent growth and lowers their bile resistance (62 and 34.5%, respectively) and		
31	adhesion on Caco-2/TC7 monolayers (34.8 and 14.16%, respectively). The other outcomes were		

32 strictly strain-related: *Ent* showed a lower ampicillin sensitivity (14.48%) and auto-aggregation

(38.1%), while LGG showed a lower biofilm formation (37%) and antimicrobial activity towards
 Staphylococcus aureus (35.73%). These overall results suggest a possible adverse effect of TiO₂-FG

on both the endogenous and exogenously administered probiotics, adding a further piece to the puzzle of the risks of using TiO_2 -FG as a food additive.

37

Keywords: food-grade titanium dioxide, lactic acid bacteria, aggregation, biofilm, host interaction,
antibiotic and bile resistance, bacterial interference.

40

41 **1** Introduction

42 Titanium dioxide (TiO₂) is a widespread white pigment used in architectural engineering, 43 paper, paints, inks, plastics, cosmetics, and food industries, as well as in agriculture in the production 44 of pesticides or fertilizers (Baranowska-Wójcik et al., 2020). As a food additive, it is known as 45 INS171 in North America and E171 in Europe. Food-grade TiO₂ (TiO₂-FG) is employed as a 46 brightening agent for white sauces, skimmed milk, cheese, pastries, ice-creams and confectionery 47 (where they mostly form the coating of chewing gum and sweets) (Mu et al., 2019). It can therefore 48 reach the gastrointestinal tract after ingestion and interact with human cells and the gut microbiota. 49 Moreover, TiO_2 can also be ingested through cosmetic products such as sunscreen and toothpaste 50 (Wang et al., 2013), and through edible fish tissue as a consequence of Ti accumulation (27–43 μ g/L 51 Ti) in colloids present in wastewater treatment plants (Shi et al., 2013). Dietary intake of TiO₂ has 52 been estimated to be between 0.2 and 1 mg/ kg body weight/ day for adults, while children are the 53 most exposed category due to their low body weight and high consumption of sweets (Marucco et al., 54 2020). TiO₂-FG must meet regulatory standards that define the purity level and the crystalline form, 55 but not the size (Commission Regulation 231/2012/EC, 2012). It is mainly produced by sulphate or 56 chlorite processes and the final product is composed of particles in a wide range of size, included a 57 variable fraction of particles in the nano-size range (Peters et al., 2014; Verleysen et al., 2020).

58 A very recent evaluation by EFSA (European Food Safety Authority), ---in part confirming 59 previous toxicity tests on animals (Bettini et al., 2017; Shi et al., 2013)-concluded that TiO₂-FG can 60 no longer be considered suitable for human consumption due to its potential genotoxicity, banning in 61 Europe the use of E171 as a food addictive (EFSA Panel on Food Additives and Flavourings (FAF) 62 et al., 2021). In the gut, TiO₂-FG interacts with epithelial cells of the small intestine causing DNA 63 damage (Jugan et al., 2012; PetkoviĆ et al., 2011; Shukla et al., 2011) and altering the cell cycle, thus 64 inducing constriction of nuclear membranes and apoptosis (Acar et al., 2015; Coccini et al., 2015; Hu et al., 2011; Valdiglesias et al., 2013). A further important aspect of TiO₂-FG toxicity is its moderate 65 66 antibacterial effect used to extend the average storage life of foodstuffs (Helal et al., 2021). The 67 antimicrobial properties of TiO₂ were described over 30 years ago; however, the specific effects of 68 the interaction between TiO₂-FG and the microbiota remain essentially unknown, focusing mainly on 69 E. coli. Some studies suggest that this interaction could occur through electrostatic attraction, owing 70 to their opposite surface charges (Zhukova et al., 2012). Other studies related the antibacterial 71 properties of the particles to their photocatalytic effects (Kumar et al., 2011; Liu et al., 2010) or 72 increased production of reactive oxygen species (ROS) (Tong et al., 2013). However, it has been 73 proven that even in the dark environment of the GI tract, TiO2-FG can display toxic effects (decrease 74in membrane fluidity, damages to the outer lipopolysaccharides) on commensal gut microorganisms 75 (Liu et al., 2016). Therefore, even in the absence of translocation into blood, dysbiosis can be the 76 pivotal event that generates unbalanced homeostasis and a plethora of systemic diseases threatening 77 human health.

78 A question arises: to what extent can TiO₂-FG interfere with the endogenous gut ecosystem 79 affecting resident gut bacteria's metabolic/phenotypic features and causing dysbiosis? The human 80 intestinal microbiota shows long-term stability and resilience to external perturbations (Pessione, 81 2012). However, this community proved to be sensitive to environmental stressors due to diet, drugs, 82 food additives, and sleep deprivation (Karl et al., 2018). In this sense, children represent the most 83 critical population to oral exposure of TiO₂-FG as the main consumers and since they are at a crucial phase in their microbiota's development and diversification. Since data specifically concerning TiO₂ 84 85 effects on the human microbiota are still controversial, further evaluation of TiO₂-FG toxicity on gut 86 bacteria become a priority.

87 In the present investigation, we selected two probiotic bacteria of gut origin, Lactobacillus 88 rhamnosus GG (LGG) and Enterococcus faecium NCIMB10415 (Ent), as models of beneficial gut 89 microbes to be challenged with 0.125 mg/mL TiO₂-FG. This concentration of TiO₂-FG represent the 90 one that reaches the intestinal tract after the intake of 20 g of commercially available chocolate (Khan, 91 2019). LGG is a well-characterized probiotic (Nissilä et al., 2017) effective against epithelial damage 92 (leaky gut syndrome), inflammation, invasiveness, and the proliferation of malignancies (Banna et 93 al., 2017). Ent is a bacteriocin-producing probiotic appreciated for counteracting infections and 94 dysbiosis (Hosseini et al., 2009), and it has been widely used for treating gastrointestinal disorders 95 (Holzapfel et al., 2018). This study aims to analyze the main physiological/phenotypic characters 96 (growth parameters, bile, and antibiotic resistance) that can be affected by TiO₂-FG exposure. In 97 addition, we also investigated the interactive behavior of such strains with other gut bacteria (growth 98 interference) and with the host (based on auto-aggregation, biofilm formation, and adhesion). The 99 results obtained suggest that bacteria can both sense TiO₂-FG and modify their physiology and 100 interactive behavior accordingly, with differences related to the bacterial species.

101

2 Materials and methods

102

2.1 TiO₂-FG particle size distribution

TiO₂-FG E171 was obtained from Kronos (KRONOS 1171 Titanium Dioxide E 171, Kronos,
Dallas, TX, USA).

105

Size distribution in the micrometric range. Analysis was performed by using a Sysmex
 FPIA3000 analyzer equipped with the high magnification objective lens unit. High power field (2×
 secondary lens) was applied. The sample was measured dispersed in in double filtered milli-Q water
 (10 mg/mL).

110 Size distribution in the nanometric range & Z potential measurement. Analysis were 111 performed by the ZetaView® PMX-120 (Particle Metrix GmbH, Germany) nanoparticle tracking 112 analyzer (NTA), equipped with a light source wavelength of 488 nm. Before the measurements, the 113 samples were suspended in double filtered milli-Q water (10 mg/mL) and well vortexed, then stock 114 dispersions were further diluted in double filtered milli-Q water (final concentration 2.5*10-4 115 mg/mL), concentration found suitable for the NTA analysis. After the optimization of the 116 instrumental parameters the sensitivity and the shutter were set at 70 and 100, respectively; 3 x 33 117 videos of 1 second for each sample were recorded analysing ~ 55 particles/video.

118 2.2 Preparation of the TiO₂-FG suspension

119 A suspension of 1 mg/mL of TiO₂-FG in ultrapure water (MilliQ) was autoclaved and 120 preserved at RT. If not used for a long time, the TiO₂-FG was sonicated for 45 min and vortexed for 121 3 min at 24000 rpm to ensure good resuspension of the particles, while for daily use, they were 122 sonicated for 7 min and vortexed for 1 minute at 24000 rpm before each test. The stock suspension 123 was then diluted to 0.125 mg/ml in culture media.

124 2.3 Bacterial cultures

Lactobacillus rhamnosus GG (LGG) and Enterococcus faecium NCIMB 10415 (Ent) were
stored at -80°C in 50% glycerol and cultured respectively in MRS broth (Sigma-Aldrich, St. Louis,
MO, USA) and BHI (PanReach AppliChem, Castellar del Vallès, Spain). Solid cultures were grown
in MRS/agar (1.5%) or BHI/agar (1.5%) or with a lower agar concentration (0.5%). Before each

- experiment, pre-cultures of the strains were incubated overnight at 37°C. The following day the cultures were diluted to OD_{600} of 0.1 or 0.4 (\cong 1 or 4 x 10⁸ CFU/mL) in control conditions or with the addition of 0.125 mg/mL TiO₂-FG. The cultures were treated according to each assay, or, when required, incubated in an orbital shaker (100 rpm) at 37°C to simulate intestinal movements till the early stationary phase (\cong 17 hours for LGG, \cong 5 hours for *Ent*).
- 134 2.4 Growth curves

Cultures in control conditions or stimulated with TiO₂-FG were diluted to OD₆₀₀ 0.1, then 150
µL of each culture was added to the central wells of a 96-well plate (10 wells for each condition).
Growth curves were recorded for 24 hours in a multiplate reader (Filtermax F5, Molecular Devices,
LLC., San Jose, CA, USA) with the following parameters: OD₅₉₅ reads every 30 min, 37°C and orbital
shaking of 10 seconds before each reading.

2.5 Bile salts tolerance (BST) assay

141 BST was tested as previously described (Zommiti et al., 2018), with some modifications. 142 Briefly, 1 mL of the early stationary phase of probiotic cultures treated or untreated with the TiO₂-143 FG, was centrifuged (100000 g, 10 min, RT), and the cells were re-suspended in MRS or BHI 144 containing 0.5% bile salts (Bile, bovine - Sigma Aldrich) to a final concentration of 10⁸ CFU/mL. To 145 simulate human intestinal transit, the suspension was incubated in an orbital shaker (100 rpm) at 37°C 146 for 4 hours. Bacteria collected at T0 and T4 were diluted in 0.9% NaCl, and counted on MRS or BHI 147 agar after 48 or 24 hours. We repeated the experiment in four biological replicates. The BST was 148 calculated as a survival rate:

149

$$(SR) \% = [CFU/mL T_4]/[CFU/mL T_0]*100$$

- 150 The results are expressed as % of control.
- 151 2.6 Ampicillin susceptibility assay

To test how TiO₂-FG might have altered the sensitivity to ampicillin, treated or untreated bacteria at the early stationary phase were diluted to a final concentration of 1 x 10⁸ CFU/mL in 6 mL of MRS or BHI soft agar. This mixture was then poured on MRS or BHI agar. Once solidified, a disc containing 10 μ g of ampicillin (BD BBLTM Sensi-DiscTM, Becton Dickinson, and Company, Franklin Lakes NJ, USA) was added approximately at the center of each plate. Plates were kept at 37°C for 24 hours, and the inhibitory halo was visually detected. According to the BBLTM Sensi-DiscTM zone diameter interpretive chart, the breakpoints for the *Enterococcus spp.* are \leq 16 mm for resistance and ≥ 17 mm for susceptibility. There are not enough data for the definition of a zone breakpoint diameter for LGG (a truly not pathogenic strain), we therefore considered the same values as *Ent*, given the fact that both belong to the lactic acid bacteria family (*Lactobacillacae*). The results are expressed as % of control (untreated bacteria).

163 2.7 Auto-aggregation assay

164 1 mL of each culture (treatments and controls) at the stationary phase was centrifuged at 165 100000 g for 10 min. Supernatants were removed, and the pellets were re-suspended in an equal 166 volume of sterile NaCl 0.9% (Sigma-Aldrich). These suspensions were then diluted to OD_{600} of 0.3 167 in 5 different cuvettes. OD_{600} was measured at T0 (A) and after 4 hours (B) of incubation at 37°C. In 168 the end, for each cuvette, the aggregation percentage was calculated as follows:

169

[(A-B)/A)]*100 (Scardaci et al., 2021).

170 2.8 Biofilm Assay

171 For this assay, the pre-cultures of both bacteria were diluted to OD₆₀₀ 0.4 in control conditions 172 or with TiO₂-FG. Then, 150 µL of each culture was added to a 96-well plate (10 wells for each 173 condition) and incubated with no agitation at 37°C for 24 hours. After 24 hours, the plate was emptied, 174 washed 3 times with MilliQ water, and dried. Next, 150 µL of 0.1% crystal violet (PanReac 175 AppliChem) was added, and the plate was incubated at RT for 15 min. Afterward, crystal violet was 176 carefully removed, and the wells were washed 3 times and completely dried at 37°C. Finally, 150 µL 177 of 100% ethanol (Carlo Erba Reagents, Chau. du Vexin, France) was added to each well, and the A₅₉₅ 178 nm was measured (Filtermax F5, Molecular Devices, LLC.) with an orbital shaking of 10 seconds 179 before reading. The results are expressed as % of control.

180 2.9 Adhesion on enterocytes

181 Human enterocyte-like Caco-2/TC7 cells (colon adenocarcinoma cells) were stocked at -80°C. To start cultures, the stocks were centrifuged at 1000 g for 10 min and the pellets resuspended 182 183 in 4.5 g/L glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland) 184 supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Euroclone S.p.A., Pero, Italy), 185 1% Penicillin/Streptomycin (Euroclone S.p.A.), 2 mM Glutamine (Euroclone S.p.A.), and filter 186 sterilized. Cells were maintained in a humidified incubator at 37°C in 5% CO₂ and 95% air 187 atmosphere, and the medium was replaced three times per week. Cells were split after reaching 188 approximately 90% of confluence, seeded in 24-well culture plates, and used at the confluence

189 (10^{6} /well). On the day of the experiment, LGG and *Ent* were harvested by centrifugation after 190 incubation with TiO₂-FG and in control conditions. The pellets were concentrated to 10^{10} CFU/mL 191 in DMEM without FBS and antibiotics, diluted 1:100, counted on plates, and applied on confluent 192 cell monolayers for 2 hours. Caco-2/TC7 were washed twice with NaCl (Sigma-Aldrich) 0.9% to 193 remove non-adherent bacteria, and 500 µL of 0.1% Triton 100-X (Sigma-Aldrich) was added to 194 disrupt the cells. The lysates were serially diluted and plated on BHIA to count the number of adherent 195 bacteria. The results are expressed as % of control.

196 2.10 Antimicrobial activity

197 The effects of TiO₂ treatments on the antagonistic activity of LGG and Ent towards E. coli 198 ATCC8739, S. aureus ATCC6538, and L. monocytogenes CIP55143 were studied with the soft-agar 199 overlay method (Hockett and Baltrus, 2017), with some modifications. Briefly, LB soft agar 200 inoculated with 10⁸ CFU/mL of the indicator strains were poured onto BHIA. Once solidified, 10¹⁰ CFU/mL of the treated or untreated probiotics were spotted on soft agar in triplicate, and the plates 201 202 were incubated for 24 hours at 37°C. Antimicrobial activity was assessed by measuring the inhibitory 203 zone around the spots: the diameter of the probiotics' spot and the diameter of the total zone of 204 inhibition were measured, and respective areas were calculated. The area of inhibition was computed 205 by subtracting the area of the inoculum from the area of the total zone of inhibition. The results are 206 expressed as % of control.

207 2.11 Statistical analysis

All experiments were performed at least three times independently. Statistical data analyses were carried out with GraphPad Prism 9 using (two samples) a two-tailed t-test. Significance was considered at ****P ≤ 0.0001 , ***P ≤ 0.001 , **P ≤ 0.01 , *P ≤ 0.05 . Data are expressed as mean \pm standard error (SEM).

3 Results

213 *3.1 Particle size distribution*

214 According to the data sheet provided by the company, the TiO₂-FG is an anatase pigment 215 approved for coloring foodstuffs, therefore meeting safety regulations and standards in terms of 216 purity. However, regulation does not define particle size ranges, that may vary depending upon the 217 provider. For this reason particle size distribution was evaluated here by integrating Nanoparticle 218 Traking Analysis (NTA), that allows to measure NPs, with Flow Particles Image Analysis (FPIA), 219 which detects microparticles (MPs; $>1\mu m$). Figure 1A reports the size distribution obtained by NTA. 220 Particles in the 10-600 nm range were detected, with a mean hydrodynamic diameter of $177,7 \pm 2,1$ 221 nm and the 16.5 % of particles having a diameter < 100 nm. A small number of MPs was detected by 222 FPIA (Figure 1B), which represent a minor fraction of the nanometric/submicrometric one. Z-223 potential (measured at pH 6.8, conductivity 5 µS/cm) was negative, in line with what expected (Zubar 224 et al., 2020).

Based on the analysis performed, the present TiO_2 -FG sample cannot be considered a nanomaterial, according to the European Commission Recommendation (Commission Recommendation 2011/696/EC, 2011), which defines "nanomaterial" a material consisting for 50% or more of solid particles with one or more external dimensions in the range 1 nm to 100 nm. Note however that NTA and FPIA evaluate the particle size in suspension, where the presence of aggregates or agglomerates is likely. Therefore, the amount of nanometric particles possibly released *in vivo* might be underestimated.

3.2 Growth kinetics

233 One of the aims of the present study was to evaluate the effects of TiO₂-FG on the growth 234 profiles of LGG and Ent cultures (along with a control condition) over 24 hours. The bacterial growth 235 curves of LGG and Ent in the presence of TiO₂-FG and untreated conditions are reported in Fig. 1a 236 and 1b, respectively. As shown, compared to the control conditions, the growth curves of both 237 bacteria displayed differences starting from the latency phase, which is slightly longer in the cultures 238 exposed to TiO₂-FG. The highest absorbance values are achieved roughly after 17 and 5 hours of 239 growth for LGG and *Ent*, respectively (beginning of the stationary phase), and tend to remain stable. 240 In this phase, the curve related to the treated microbial cultures settled on lower values than the 241 untreated condition. More in detail, the OD₅₉₅ values at 24 hours for treatment with TiO₂-FG are 0.2 242 \pm 0.06 and 0.06 \pm 0.01 lower than that of the control condition for LGG and *Ent*, respectively.

Moreover, although the biomass yield of the late stationary phase stabilized at values lower than those of the untreated condition, the growth rates (μ ,) were enhanced by TiO₂-FG. In particular, the treatment with TiO₂-FG improved the LGG and *Ent* μ by 11 and 31%, respectively.

246 *3.3 Bile salts tolerance (BST)*

Since bile salt exposure in the human intestine is a stressing event that concerns probiotic bacteria, here we analyzed how treatment with TiO_2 -FG can affect the ability of the two bacteria to tolerate bile salts, comparing the final cell number of exposed and unexposed bacteria. As displayed in Fig. 2a and 2b, the treatment with TiO_2 -FG decreased the bile salts survival rate of LGG and *Ent* by approximately 62 and 35%, respectively.

252 *3.4 Ampicillin susceptibility assay*

The ampicillin susceptibility assay was intended to measure whether TiO₂-FG could affect the susceptibility of LGG and *Ent* to ampicillin. According to the breakpoint zone diameters provided by the antibiotic disks producer, the analyzed strains can be considered sensitive to ampicillin (inihibitory zones ≥ 1.7 cm). As reported in Fig. 3a and 3b, ampicillin susceptibility decreased by more than 14% for *Ent* treated cultures, although still lying in the sensitivity range. Conversely, the sensitivity for LGG was not significantly modified by TiO₂-FG treatment. Table 1 shows the average values of the inhibitory zones diameters found for each antibiotic and culture condition.

260

3.5 Auto-aggregation assay

The ability of LGG and *Ent* to autoaggregate was evaluated for 4 hours (Fig. 4a and b) following exposure to TiO_2 -FG and compared to aggregation in the absence of any treatment. Regarding LGG cultures, no statistically significant differences were detected between TiO_2 -FG treated and control conditions. Conversely, a statistically significant reduction (38.1%) in the autoaggregation ability was observed for *Ent* cultures after TiO_2 -FG treatment compared to the control condition.

267 *3.6 Biofilm formation assay*

The ability of LGG and *Ent* to form biofilms was tested after 24 hours of incubation in the presence of TiO_2 -FG. Fig. 5a and 5b show the results obtained as % of control. As far as LGG cultures are concerned, TiO_2 -FG significantly reduces the microbial capability to produce a biofilm by approximately 36.6% compared to the control condition, whereas for *Ent* cultures, no statistically significant difference was detected between treated and untreated conditions. 273

274 *3.7 Adhesion on enterocytes*

275 LGG and *Ent* treated with TiO_2 -FG were incubated on Caco-2/TC7 intestinal cells for 2 hours 276 to study bacterial adhesion. As shown in Fig. 6a and 6b, TiO_2 -FG treatment reduced the capacity of 277 the two probiotics to adhere to this model of enterocytes. In particular, TiO_2 -FG decreased the 278 adhesion of LGG and *Ent* by 34.87% and 14.16%, respectively.

279 *3.8 Antimicrobial activity*

280 property of probiotics is their antimicrobial One desirable activity against 281 pathogenic/opportunistic microorganisms. Therefore, we evaluated how TiO₂-FG could modulate 282 LGG and Ent antimicrobial activity exerted by direct contact with the indicator strain. The results 283 show that TiO₂-FG could reduce the antimicrobial potential of LGG by 35.73% towards S. aureus 284 (Fig. 7), while no significant changes were found in the antimicrobial activity of LGG towards E. coli and L. monocytogenes and of Ent towards all the three tested pathogens (supplementary material). 285

286 **4 Discussion**

287 Besides the recent evidence of TiO_2 -FG genotoxicity towards human cells (EFSA Panel on 288 Food Additives and Flavourings (FAF) et al., 2021), evidences on possible adverse effects of TiO2-289 FG on gut bacteria likely mediated by reactive oxygen species (ROS) are growing (Khan, 2019; Liu 290 et al., 2016). However, the available data are still controversial, and the mechanisms of interaction 291 between microorganisms and FG nanomaterials not fully elucidated. Among all microorganisms 292 residing in the gastrointestinal tract, some are just commensals, other are beneficial—the probiotics. 293 Probiotics exert an essential role in controlling several physiological functions important to ensure 294 immunological, metabolic and mental health (Pessione, 2012). In our study, we focused on two 295 probiotic strains typically present in the human gut, Lactobacillus rhamnosus GG and Enterococcus 296 faecium NCIMB10415, and their interaction with a commercial sample of food grade TiO₂ (E171). 297 The analyzed FG-TiO₂ sample was composed by submicrometric particles in the 10-600 nm range, 298 with a nanometric fraction of 16.5%.

299 4.1 Effect of TiO_2 -FG on probiotics' viability

300 We evaluated the bacterial viability following exposure to TiO_2 -FG by analyzing their growth 301 curves. As reported in the Results section, TiO_2 -FG exposure resulted in a much longer latency phase 302 and a slight time delay of the exponential tract than the control. Graphically, following the delay 303 accumulated in the first phase, the curves seem to recover from the initial disadvantage, showing a 304 remarkable slope, with higher µmax values for the treated bacterial cultures. Finally, TiO₂-FG-treated 305 stationary phase cultures show a dose-dependent decrease in biomass yield and curve profiles 306 compared to the control condition. Given these trends, we can hypothesize that TiO₂-FG may interfere 307 with the initial accumulation of resources (promoting growth in size that prepares for cell replication) 308 perhaps by inhibiting the membrane transporters, thus leading to a delay in growth. Moreover, since 309 the curves settle on lower final absorbance values as the concentration of-FG increases, we can 310 hypothesize that microbial viability is severely hindered by the treatment. All these results agree with 311 previous data from the literature that demonstrated high toxicity of TiO₂-FG against symbiotic 312 intestinal bacteria, including Acetobacter species, enterobacteria, and lactobacilli (Liu et al., 2016).

313 Most of the experiments evaluating TiO₂-FG toxicity focused on the viability of *Escherichia* 314 coli. Pagnout et al. (2012) proved that the toxicity of TiO₂-FG strictly depends on the electrostatic 315 interactions between bacteria and TiO₂-FG, which leads to the particles penetration of the cell surface. 316 Sohm et al. (2015) observed a significant depolarization of the E. coli cell membrane with loss of its 317 integrity, and almost irreversible ionic imbalances, together with a substantial decrease in the 318 expression of DNA polymerase III (HolB and DnaX). The authors suggest that this loss of replication 319 ability could explain the reduction in cell number compared to the untreated condition. Interestingly, 320 a remarkable upregulation of osmotically inducible genes, such as osmB, osmC, osmE, and osmY 321 and an increase in trehalose (a molecule widely known for its outstanding osmoprotective properties) 322 synthesis were also recorded in E. coli following TiO2-FG treatment. However, both osmC (Lesniak 323 et al., 2003) and trehalose (Benaroudj et al., 2001; Elbein et al., 2003) have been associated with 324 defense mechanisms against oxidative stress as well. These data fit with the findings of Kumar et al. 325 (2011), which demonstrate that TiO₂-FG induce ROS generation, DNA damage, LDH release, 326 glutathione depletion, along with a significant increase in lipid peroxidation, thus leading to cell death 327 in E. coli. Therefore, the cytotoxic effects exerted by these nanomaterials can mainly be ascribed to 328 the increased oxidative stress.

As far as probiotic bacteria are concerned, experiments on *Bacillus coagulans, Enterococcus faecium,* and *Enterococcus faecalis* suggest that TiO₂-FG may act externally by damaging the cell membrane or be internalized within microbial cells, where they can interact with DNA. This could also lead to ROS production and the inhibition of cellular respiration (Khan et al., 2015). However, these reports gave no conclusive data, and only more detailed studies and omics investigations can better elucidate what happens at a molecular level outside and inside the bacterial cell.

335 4.2 Effect of TiO_2 -FG on probiotics' bile salts resistance

336 Bile is an aqueous solution of inorganic ions, bile salts, cholesterol, phospholipids, and the 337 pigment biliverdin necessary for fat digestion and hydrophobic vitamin absorption (Barrett and 338 Ganong, 2012). Bile salts have robust antimicrobial activity against gut bacteria since they can disrupt 339 the membranes by solubilizing the phospholipids (Merritt and Donaldson, 2009), inducing oxidative 340 stress and intracellular acidification, and by promoting DNA damage and protein denaturation (Ruiz 341 et al., 2013). To counteract these effects, bacteria have developed several mechanisms, notably the 342 upregulation of chaperones and proteases (Ruiz et al., 2013), changes in membrane composition 343 (Gómez Zavaglia et al., 2002), and the activity of bile-salt hydrolases (BSHs) (Begley et al., 2005).

Here we demonstrated that pre-treatment with TiO₂-FG decreases the natural tolerance of LGG and *Ent* to bile salts. The most probable explanation is that the electrostatic interactions at the surface level with the nanomaterial (Zhukova et al., 2012) induce a membrane rearrangement that disrupts the barrier after bile exposure. Furthermore, in addition to acting as detergents, bile salts are known to produce reactive oxygen/nitrogen species (Begley et al., 2005). TiO₂ can act synergistically by inducing ROS and causing further oxidative stress (Tong et al., 2013) that renders the cell more susceptible to the damaging bile action.

351 4.3 Synergies/antagonisms between TiO₂-FG and antibiotics

An attractive aspect of NPs is their ability to combine with a wide range of antibiotics, either by binding them to their surface or by favoring their internalization (Pissuwan et al., 2011). One of the most used food-grade metal oxides in this field is titanium dioxide alone (Yuan et al., 2010) or in combination with other elements, such as iron oxide (Chen et al., 2008) or silver (Necula et al., 2009).

356 Since TiO₂-FG contains nanoparticles, we evaluated the effect of TiO₂-FG treatment on the 357 ampicillin sensitivity pattern of LGG and Ent. Our experiment shows that exposure to TiO₂-FG at the 358 tested concentrations can decrease *Ent* sensitivity to ampicillin. This result apparently contrasts with 359 a study performed by S. Roy et al. (2010), who reported that TiO₂-FG increased the antibacterial 360 activities of 22 different antibiotics against Staphylococcus aureus. However, the authors do not 361 clarify what concentration (mg/mL) of nanomaterials was used in their study. Furthermore, the 362 microbial models, despite being both Gram-positive, consistently differ. E. faecium is known for 363 having an intrinsic resistance to most antimicrobials, due to surface characteristics that prevent the 364 antibiotic entering into the cell (Klare et al., 2003), whereas S.aureus often bears transmissible 365 plasmids encoding enzymes that degrade the antibacterial molecule (Wright, 2005).

366 Together with other penicillins and cephalosporins, Amp belongs to beta-lactam antibiotics, 367 which exert their antibacterial action by binding to the penicillin-binding proteins (PBPs) on the 368 bacterial surface and hence blocking cell wall synthesis. The most common mechanisms by which 369 bacteria escape beta-lactam activity are either the production of degrading enzymes (beta-lactamases) 370 or target PBP modifications (Andersen, 1990). Regarding the possible effect of TiO₂-FG on the Ent 371 ampicillin sensitivity pattern, although we cannot exclude the enhancement of beta-lactamase 372 production, the alterations of PBPs are the most probable occurrence, since in Enterococcus species 373 ampicillin resistance is linked to surface modifications (Klare et al., 2003). Moreover, interactions 374 between TiO₂-FG and bacterial surface structures are largely described in the literature and can 375 contribute to the observed phenomenon (Zhukova et al., 2012). Finally, the sensitivity profile of LGG 376 is not modified by TiO₂-FG treatment because in this strain there is no beta-lactam resistance (either 377 intrinsic or acquired) (Capurso, 2019).

378 379

4.4 Effect of TiO₂-FG on probiotics' auto-aggregation, biofilm formation, and adhesion on the gut epithelium

Essential features for human host colonization is the ability to aggregate, form biofilms, and adhere to the gut epithelium. These properties are closely related since aggregation allows the microorganisms to reach an adequate biomass essential for biofilm formation and adhesion to the gut epithelium, prerequisites for successful host colonization (Collado et al., 2008; Trunk et al., 2018).

384 Our results showed that, when treated with TiO_2 -FG, LGG cultures had no significant 385 differences in the auto-aggregation capacity, whereas *Ent* cultures revealed a considerably decreased 386 auto-aggregation ability compared to the control.

387 The auto-aggregation mechanisms exploited by lactic acid bacteria is a self-recognition 388 phenomenon that involves the secretion of extracellular adhesive molecules, mainly proteins and 389 exopolysaccharides, called "autoagglutinin" (Trunk et al., 2018). However, the auto-aggregative 390 phenotype is diversified in different bacterial strains and depends on certain external conditions 391 (Trunk et al., 2018). Several factors can alter the aggregation ability of bacteria, including oxygen 392 availability, stress, or a variation of the temperature, even if not all microorganisms are influenced in 393 the same manner (McLean et al., 2008). Given this variability, we could speculate that TiO₂-FG 394 inhibits the expression of certain auto-agglutinins in Ent that are not involved in the auto-aggregation 395 of LGG. Further research is needed to identify the molecules inhibited by the TiO₂-FG exposure and 396 involved in these interactions.

397 Auto-aggregation is also the initial step in adhesion to surfaces and the formation of biofilms. 398 Biofilms are bacterial communities that involve a strong association of microbial cells and 399 extracellular polymeric substance (EPS), which is mainly composed of a mixture of polysaccharides, 400 lipids, proteins, glycopeptides, and nucleic acids (Donlan and Costerton, 2002). This matrix consists 401 of heterogeneous layers, including nutrients and water transport channels (Flemming and Wingender, 402 2010). In our study, treatment with TiO₂-FG reduced the biofilm biomass produced by LGG 403 compared to the untreated condition but did not induce significant changes in the biofilm formation of Ent cultures. 404

405 Very few studies have analyzed the anti-aggregation and anti-biofilm effects of TiO₂-FG, and 406 the molecular mechanisms regarding these processes are still largely unknown. The exposure to TiO₂-407 FG at higher concentrations than those employed here likely causes the death of some microbial cells 408 within the biofilm, primarily because of the generation of ROS and lipid oxidation at the cell 409 membrane level, as described in some studies on Gram-positive Staphylococcus aureus (Shah et al., 410 2008). Moreover, a study carried out by Zhang and Chen (2009) on E. coli indicates that TiO2-FG 411 may penetrate within the biofilm and directly interact with bacteria through an electrostatic attraction 412 between the positive charges of the metal oxides and some of the negative charges residing on the 413 bacterial outer wall. A study carried out both in vitro and in vivo by Khan and co-workers (Khan, 414 2019) demonstrated that TiO2-FG contained in commercially available chocolate could interfere with 415 the metabolic activity of some probiotics (production of lactic acid) and their ability to form biofilms. 416 Furthermore, TiO₂-FG can be absorbed and interfere with biofilm matrix development by interacting 417 with molecules present in the EPS that bear various functional groups such as hydroxyl, amide, or 418 carboxyl (Gao et al., 2019). How NPs and MPs interact with and move within the matrix depends on 419 several factors, such as the type of particle, the properties of EPS, the pores' size, hydrophobicity, 420 electrical charges, presence of water channels, and chemical gradient of the matrix, and the local 421 environment (Shkodenko et al., 2020). Nevertheless, EPS can be highly variable in terms of chemical 422 composition among bacteria, resulting in a different potential mode of interaction with nanomaterials 423 of each microbial species.

As described above, cell aggregation is a crucial step in biofilm formation. Consequently, reduced microbial auto-aggregation should decrease a microbe's ability to form a biofilm. Nevertheless, a significant decrease in the biofilm thickness is observed in TiO_2 -treated LGG cultures, whose auto-aggregation potential was not influenced by the treatment. In contrast, no significant changes were observed in the biofilm-forming capability of *Ent*, whose auto-aggregation was decreased by the presence of TiO_2 -FG. Together with the evidence available in the literature, these 430 results suggest that TiO_2 -FG may act as biofilm-disrupting agents rather than biofilm formation 431 inhibitors, at least in LGG. Similarly, it is possible to hypothesize that TiO_2 -FG can interfere with 432 some surface molecules, essential for cell aggregation but not involved in biofilm formation in this 433 species.

434 Given these intriguing results, we sought to determine the influence of TiO₂-FG treatment on 435 the adhesion of the two probiotics on the Caco-2/TC7 intestinal cell line. The experimental evidence 436 here reported a decrease in both LGG and Ent adhesion after TiO2-FG treatment. These results could 437 be partly related to the decreased biofilm formation of LGG and auto-aggregation of Ent in the 438 presence of TiO₂-FG. Moreover, bacterial adherence to Caco-2/TC7 cells requires various cell wall 439 architectural components such as peptidoglycan, teichoic acids, polysaccharides, and surface proteins 440 (Sengupta et al., 2013). Although we did not investigate all these aspects in detail, we can hypothesize 441 that TiO₂-FG can negatively affect the expression of these adhesive molecules. Alternatively, we can 442 assume that TiO₂-FG present in the culture medium during the experimental procedure could form a 443 layer on the surface of the intestinal cells, thus hindering bacterial adhesion, as it has been observed 444 that the treatment of dental implants with TiO₂ reduces the adhesion of *Staphylococcus* spp. through 445 this mechanism (Del Curto et al., 2005).

446 4.5 Effect of TiO₂-FG on probiotics' competition with other bacteria

447 Probiotic bacteria can influence the composition of the microbiota in the gastrointestinal tract 448 by maintaining the correct ratio among species, and by acting against exogenous pathogenic bacteria. 449 They can exert this role in various ways, for example by secreting antimicrobial metabolic end-450 products (H₂O₂, ethanol, lactic acid, and other organic acids) (Ouwehand and Vesterlund, 2004), 451 competing for nutrients and binding sites (Di Cerbo et al., 2016), and by producing specific weapons 452 called bacteriocins (Ouwehand and Vesterlund, 2004).

In the present investigation, we evaluated the effect of TiO₂-FG on the antimicrobial activity exerted by LGG and *Ent* against Gram-positive and Gram-negative bacteria, using *S. aureus, L. monocytogenes,* and *E. coli* as indicator microorganisms. Our results show that treatment with TiO₂-FG reduced the antimicrobial potential of LGG towards *S. aureus,* whereas no significant changes were found towards *L. monocytogenes and E. coli*. As far as *Ent* is concerned, all the experiments with TiO₂-FG gave similar results to the control conditions.

459 In general, except for nisin A and mutacin B-Ny266 (Riley and Wertz, 2002), bacteriocins 460 display a narrow spectrum of activity and are generally effective against bacteria related to the 461 producing strain. This target specificity could explain the difference in the antimicrobial action of 462 both LGG and *Ent* against the tested pathogens: TiO_2 -FG could have downregulated the production 463 or secretion of certain LGG bacteriocins active against *S. aureus*, while not affecting the 464 expression/secretion of others.

465 **5** Conclusions

In food toxicology, there is growing interest in studying the impact of foodborne particles on the human gut microbiota since the antimicrobial effect of micro- and nanomaterials could induce changes both in the composition of commensal microorganisms and in their metabolic activities at a single cell level, thus affecting host health.

470 The evidence obtained from this study indicates that the main probiotic features of two 471 bacterial strains can be negatively affected by treatment with TiO₂-FG. This food additive (0.125 472 mg/mL) hinders microbial growth and lowers bile resistance and adhesion capabilities of LGG and 473 Ent on Caco-2/TC7 cells. As far as other parameters are concerned, the effects are different on the 474 two strains in the study. The ability to form a biofilm and inhibit S. aureus growth is impaired in LGG 475 but not in *Ent*. On the contrary, the auto-aggregation ability and ampicillin sensitivity are negatively 476 affected by TiO₂-FG only in Ent. The differences in the auto-aggregating and biofilm-forming 477 sensitivity to TiO₂-FG exposure in the strains in the study reflect the different surface compositions 478 of the two genera. This structural diversity could also account for a possible higher perturbing action 479 of TiO₂-FG on Ent PBPs, resulting in decreased sensitivity to ampicillin. Conversely, the reduced 480 interfering activity towards S. aureus seems linked to the specificity of the bacteriocins produced by 481 LGG.

482 Taken together, these overall results underline a possible adverse effect of TiO₂-FG on both 483 the endogenous gut microbiota and exogenously administered probiotics. However, the damage 484 exerted by this FG-material can be different due to the structural and functional specificity of each 485 species and due to the complexity of the microbial populations hosted in the gut ecosystem. In 486 addition, further in vivo studies are required, since, during the gastrointestinal transit, micro and 487 nanomaterials are exposed to various factors-pH variations or the adsorption of molecules on their 488 surface-that can significantly influence their aggregation and their physicochemical and 489 toxicological properties.

The TiO₂-FG used in this study corresponds to the food additive E171 in Europe, and INS171
in North America. In every type of food, the addition of INS171 is limited to 1% of the product's final

492 weight in the United States (USFDA, 2005). Conversely, in Europe, E171 was used "at quantum 493 satis" (levels not higher than necessary to achieve the intended purpose) (EFSA, 2016). Considering 494 that, an updated evaluation underlines that TiO₂ can no longer be regarded as safe for human health 495 because of genotoxic effects induced following their consumption (EFSA Panel on Food Additives 496 and Flavourings (FAF) et al., 2021), banning E171 from the European market. However, TiO₂-FG 497 effect on the gut microbiota, which plays several essential roles in host health, has not been 498 completely unraveled. This work, can add a further piece to the puzzle of the risks of using TiO₂-FG 499 in food production.

500

Declaration of competing interest

- 501 There are no conflicts to declare.
- 502 **7 Funding**

6

503 The University of Torino (local research funding ex 60%) has supported the present project.

- 504 8 Data availability
- 505 Data will be made available on request.

506 8.1 CRediT authorship contribution statement

507 Francesca Bietto: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing; Rossella Scardaci: Data curation, Formal 508 509 analysis, Investigation, Methodology, Visualization, Writing - review & editing; Manuel Brovia: 510 Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft; 511 Ida Kokalari: Data curation, Methodology, Conceptualization, Funding acquisition, Supervision, Writing - review & editing; Francesco Barbero: Data curation, Formal analysis, Investigation; 512 513 Ivana Fenoglio: Conceptualization, Funding acquisition, Supervision, Writing – review & editing; 514 Enrica Pessione: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

515 9 Acknowledgments

516 The authors express their appreciation for the support received by the University of Turin.

10 References

- 518 Acar, M., Bulut, Z., Ateş, A., Nami, B., Koçak, N., and Yıldız, B. (2015). Titanium dioxide
- 519 nanoparticles induce cytotoxicity and reduce mitotic index in human amniotic fluid-derived cells.
- 520 Hum Exp Toxicol *34*, 74–82. https://doi.org/10.1177/0960327114530742.
- 521 Andersen, B.M. (1990). [Bacterial resistance against beta-lactam antibiotics]. Tidsskr Nor
- 522 Laegeforen 110, 3233–3239. .
- 523 Banna, G.L., Torino, F., Marletta, F., Santagati, M., Salemi, R., Cannarozzo, E., Falzone, L.,
- 524 Ferraù, F., and Libra, M. (2017). Lactobacillus rhamnosus GG: An Overview to Explore the
- 525 Rationale of Its Use in Cancer. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00603.
- 526 Baranowska-Wójcik, E., Szwajgier, D., Oleszczuk, P., and Winiarska-Mieczan, A. (2020). Effects
- 527 of Titanium Dioxide Nanoparticles Exposure on Human Health—a Review. 12. .
- Barrett, K.E., and Ganong, W.F. (2012). Ganong's review of medical physiology (New York:
 McGraw-Hill Med).
- Begley, M., Gahan, C.G.M., and Hill, C. (2005). The interaction between bacteria and bile. FEMS
 Microbiol Rev 29, 625–651. https://doi.org/10.1016/j.femsre.2004.09.003.
- 532 Benaroudj, N., Lee, D.H., and Goldberg, A.L. (2001). Trehalose Accumulation during Cellular
- 533 Stress Protects Cells and Cellular Proteins from Damage by Oxygen Radicals *. Journal of
- 534 Biological Chemistry 276, 24261–24267. https://doi.org/10.1074/jbc.M101487200.
- 535 Bettini, S., Boutet-Robinet, E., Cartier, C., Coméra, C., Gaultier, E., Dupuy, J., Naud, N., Taché, S.,
- 536 Grysan, P., Reguer, S., et al. (2017). Food-grade TiO 2 impairs intestinal and systemic immune
- 537 homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat

colon. Scientific Reports 7, 40373. https://doi.org/10.1038/srep40373.

- 539 Capurso, L. (2019). Thirty Years of Lactobacillus rhamnosus GG: A Review. Journal of Clinical
- 540 Gastroenterology 53, S1–S41. https://doi.org/10.1097/MCG.00000000001170.
- 541 Chen, W.-J., Tsai, P.-J., and Chen, Y.-C. (2008). Functional Fe3O4/TiO2 core/shell magnetic
- 542 nanoparticles as photokilling agents for pathogenic bacteria. Small 4, 485–491.
- 543 https://doi.org/10.1002/smll.200701164.
- 544 Coccini, T., Grandi, S., Lonati, D., Locatelli, C., and De Simone, U. (2015). Comparative cellular
- 545 toxicity of titanium dioxide nanoparticles on human astrocyte and neuronal cells after acute and
- 546 prolonged exposure. NeuroToxicology 48, 77–89. https://doi.org/10.1016/j.neuro.2015.03.006.

- 547 Collado, M.C., Meriluoto, J., and Salminen, S. (2008). Adhesion and aggregation properties of
- 548 probiotic and pathogen strains. Eur Food Res Technol 226, 1065–1073.
- 549 https://doi.org/10.1007/s00217-007-0632-x.
- 550 Commission Recommendation 2011/696/EC (2011). Commission Recommendation of 18 October
- 551 2011 on the definition of nanomaterial.
- 552 Commission Regulation 231/2012/EC (2012). Commission Regulation (EU) No 231/2012 of 9
- 553 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation
- (EC) No 1333/2008 of the European Parliament and of the Council (Publications Office of the
- 555 European Union).
- 556 Del Curto, B., Brunella, M.F., Giordano, C., Pedeferri, M.P., Valtulina, V., Visai, L., and Cigada,
- 557 A. (2005). Decreased Bacterial Adhesion to Surface-Treated Titanium. Int J Artif Organs 28, 718–
- 558 730. https://doi.org/10.1177/039139880502800711.
- 559 Di Cerbo, A., Palmieri, B., Aponte, M., Morales-Medina, J.C., and Iannitti, T. (2016). Mechanisms
- and therapeutic effectiveness of lactobacilli. J Clin Pathol 69, 187–203.
- 561 https://doi.org/10.1136/jclinpath-2015-202976.
- 562 Donlan, R.M., and Costerton, J.W. (2002). Biofilms: Survival Mechanisms of Clinically Relevant
- 563 Microorganisms. Clinical Microbiology Reviews 15, 167–193.
- 564 https://doi.org/10.1128/CMR.15.2.167-193.2002.
- 565 EFSA Panel on Food Additives and Flavourings (FAF), Moldeus, P., Passamonti, S., Shah, R.,
- 566 Waalkens-Berendsen, I., Wölfle, D., Corsini, E., Cubadda, F., De Groot, D., FitzGerald, R., et al.
- 567 (2021). Safety assessment of titanium dioxide (E171) as a food additive. EFS2 19.
- 568 https://doi.org/10.2903/j.efsa.2021.6585.
- 569 Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a
- 570 multifunctional molecule. Glycobiology *13*, 17R-27R. https://doi.org/10.1093/glycob/cwg047.
- 571 Flemming, H.-C., and Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology 8,
- 572 623–633. https://doi.org/10.1038/nrmicro2415.
- 573 Gao, J., Xi, B., Chen, K., Song, R., Qin, T., Xie, J., and Pan, L. (2019). The stress hormone
- 574 norepinephrine increases the growth and virulence of *Aeromonas hydrophila*. MicrobiologyOpen 8,
- 575 e00664. https://doi.org/10.1002/mbo3.664.

- 576 Gómez Zavaglia, A., Kociubinski, G., Pérez, P., Disalvo, E., and De Antoni, G. (2002). Effect of
- 577 bile on the lipid composition and surface properties of bifidobacteria. Journal of Applied
- 578 Microbiology *93*, 794–799. https://doi.org/10.1046/j.1365-2672.2002.01747.x.
- Helal, M., Sami, R., Khojah, E., Elhakem, A., Benajiba, N., Al-Mushhin, A.A.M., and Fouda, N.
- 580 (2021). Evaluating the coating process of titanium dioxide nanoparticles and sodium
- tripolyphosphate on cucumbers under chilling condition to extend the shelf-life. Sci Rep 11, 20312.
- 582 https://doi.org/10.1038/s41598-021-99023-3.
- 583 Hockett, K.L., and Baltrus, D.A. (2017). Use of the Soft-agar Overlay Technique to Screen for
- 584 Bacterially Produced Inhibitory Compounds. Journal of Visualized Experiments : JoVE
- 585 https://doi.org/10.3791/55064.
- 586 Holzapfel, W., Arini, A., Aeschbacher, M., Coppolecchia, R., and Pot, B. (2018). Enterococcus
- 587 faecium SF68 as a model for efficacy and safety evaluation of pharmaceutical probiotics. Beneficial
- 588 Microbes 9, 375–388. https://doi.org/10.3920/BM2017.0148.
- 589 Hosseini, S.V., Arlindo, S., Böhme, K., Fernández-No, C., Calo-Mata, P., and Barros-Velázquez, J.
- 590 (2009). Molecular and probiotic characterization of bacteriocin-producing Enterococcus faecium
- 591 strains isolated from nonfermented animal foods: Probiotic E. faecium strains. Journal of Applied
- 592 Microbiology 107, 1392–1403. https://doi.org/10.1111/j.1365-2672.2009.04327.x.
- 593 Hu, R., Zheng, L., Zhang, T., Gao, G., Cui, Y., Cheng, Z., Cheng, J., Hong, M., Tang, M., and
- Hong, F. (2011). Molecular mechanism of hippocampal apoptosis of mice following exposure to
- titanium dioxide nanoparticles. Journal of Hazardous Materials 191, 32–40.
- 596 https://doi.org/10.1016/j.jhazmat.2011.04.027.
- Jugan, M.-L., Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Sauvaigo, S., Douki, T., and
- 598 Carriere, M. (2012). Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair
- 599 activity in A549 cells. Nanotoxicology *6*, 501–513. https://doi.org/10.3109/17435390.2011.587903.
- 600 Karl, J.P., Hatch, A.M., Arcidiacono, S.M., Pearce, S.C., Pantoja-Feliciano, I.G., Doherty, L.A.,
- and Soares, J.W. (2018). Effects of Psychological, Environmental and Physical Stressors on the Gut
- 602 Microbiota. Front. Microbiol. 9, 2013. https://doi.org/10.3389/fmicb.2018.02013.
- 603 Khan, S.T. (2019). Survival of probiotic bacteria in the presence of food grade nanoparticles from
- 604 chocolates: an in vitro and in vivo study. Appl Microbiol Biotechnol 12. .

- 605 Khan, S.T., Al-Khedhairy, A.A., and Musarrat, J. (2015). ZnO and TiO2 nanoparticles as novel
- antimicrobial agents for oral hygiene: a review. J Nanopart Res 17, 276.
- 607 https://doi.org/10.1007/s11051-015-3074-6.
- Klare, I., Konstabel, C., Badstübner, D., Werner, G., and Witte, W. (2003). Occurrence and spread
- 609 of antibiotic resistances in Enterococcus faecium. International Journal of Food Microbiology 88,
- 610 269–290. https://doi.org/10.1016/S0168-1605(03)00190-9.
- 611 Kumar, A., Pandey, A.K., Singh, S.S., Shanker, R., and Dhawan, A. (2011). Engineered ZnO and
- 612 TiO2 nanoparticles induce oxidative stress and DNA damage leading to reduced viability of
- 613 Escherichia coli. Free Radical Biology and Medicine 51, 1872–1881.
- 614 https://doi.org/10.1016/j.freeradbiomed.2011.08.025.
- 615 Lesniak, J., Barton, W.A., and Nikolov, D.B. (2003). Structural and functional features of the
- 616 Escherichia coli hydroperoxide resistance protein OsmC. Protein Sci 12, 2838–2843.
- Liu, L.-Y., Sun, L., Zhong, Z.-T., Zhu, J., and Song, H.-Y. (2016). Effects of titanium dioxide
- 618 nanoparticles on intestinal commensal bacteria. NUCL SCI TECH 27, 5.
- 619 https://doi.org/10.1007/s41365-016-0011-z.
- 620 Liu, P., Duan, W., Wang, Q., and Li, X. (2010). The damage of outer membrane of Escherichia coli
- in the presence of TiO2 combined with UV light. Colloids and Surfaces B: Biointerfaces 78, 171–
 176. https://doi.org/10.1016/j.colsurfb.2010.02.024.
- 623 Marucco, A., Prono, M., Beal, D., Alasonati, E., Fisicaro, P., Bergamaschi, E., Carriere, M., and
- 624 Fenoglio, I. (2020). Biotransformation of Food-Grade and Nanometric TiO2 in the Oral–Gastro–
- 625 Intestinal Tract: Driving Forces and Effect on the Toxicity toward Intestinal Epithelial Cells.
- 626 Nanomaterials *10*, 2132. https://doi.org/10.3390/nano10112132.
- 627 McLean, J.S., Pinchuk, G.E., Geydebrekht, O.V., Bilskis, C.L., Zakrajsek, B.A., Hill, E.A.,
- 628 Saffarini, D.A., Romine, M.F., Gorby, Y.A., Fredrickson, J.K., et al. (2008). Oxygen-dependent
- autoaggregation in Shewanella oneidensis MR-1. Environ Microbiol 10, 1861–1876.
- 630 https://doi.org/10.1111/j.1462-2920.2008.01608.x.
- 631 Merritt, M.E., and Donaldson, J.R. (2009). Effect of bile salts on the DNA and membrane integrity
- 632 of enteric bacteria. Journal of Medical Microbiology 58, 1533–1541.
- 633 https://doi.org/10.1099/jmm.0.014092-0.

- Mu, W., Wang, Y., Huang, C., Fu, Y., Li, J., Wang, H., Jia, X., and Ba, Q. (2019). Effect of Long-
- 635 Term Intake of Dietary Titanium Dioxide Nanoparticles on Intestine Inflammation in Mice. J.
- 636 Agric. Food Chem. 67, 9382–9389. https://doi.org/10.1021/acs.jafc.9b02391.
- 637 Necula, B.S., Fratila-Apachitei, L.E., Zaat, S.A.J., Apachitei, I., and Duszczyk, J. (2009). In vitro
- 638 antibacterial activity of porous TiO2-Ag composite layers against methicillin-resistant
- 639 Staphylococcus aureus. Acta Biomater 5, 3573–3580. https://doi.org/10.1016/j.actbio.2009.05.010.
- 640 Nissilä, E., Douillard, F.P., Ritari, J., Paulin, L., Järvinen, H.M., Rasinkangas, P., Haapasalo, K.,
- 641 Meri, S., Jarva, H., and de Vos, W.M. (2017). Genotypic and phenotypic diversity of Lactobacillus
- 642 rhamnosus clinical isolates, their comparison with strain GG and their recognition by complement
- 643 system. PLoS ONE 12, e0176739. https://doi.org/10.1371/journal.pone.0176739.
- 644 Ouwehand, A.C., and Vesterlund, S. (2004). Antimicrobial components from lactic acid bacteria.
- 645 Lactic Acid Bacteria: Microbiology and Functional Aspects 375–395.
- 646 Pagnout, C., Jomini, S., Dadhwal, M., Caillet, C., Thomas, F., and Bauda, P. (2012). Role of
- 647 electrostatic interactions in the toxicity of titanium dioxide nanoparticles toward Escherichia coli.
- 648 Colloids and Surfaces B: Biointerfaces 92, 315–321. https://doi.org/10.1016/j.colsurfb.2011.12.012.
- 649 Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and
- shadows. Frontiers in Cellular and Infection Microbiology 2.
- 651 https://doi.org/10.3389/fcimb.2012.00086.
- 652 Peters, R.J.B., van Bemmel, G., Herrera-Rivera, Z., Helsper, H.P.F.G., Marvin, H.J.P., Weigel, S.,
- Tromp, P.C., Oomen, A.G., Rietveld, A.G., and Bouwmeester, H. (2014). Characterization of
- Titanium Dioxide Nanoparticles in Food Products: Analytical Methods To Define Nanoparticles. J.
- 655 Agric. Food Chem. 62, 6285–6293. https://doi.org/10.1021/jf5011885.
- 656 PetkoviĆ, J., Žegura, B., StevanoviĆ, M., Drnovšek, N., UskokoviĆ, D., Novak, S., and FilipiČ, M.
- 657 (2011). DNA damage and alterations in expression of DNA damage responsive genes induced by
- TiO2 nanoparticles in human hepatoma HepG2 cells. Nanotoxicology 5, 341–353.
- 659 https://doi.org/10.3109/17435390.2010.507316.
- 660 Pissuwan, D., Niidome, T., and Cortie, M.B. (2011). The forthcoming applications of gold
- 661 nanoparticles in drug and gene delivery systems. Journal of Controlled Release 149, 65–71.
- 662 https://doi.org/10.1016/j.jconrel.2009.12.006.
- Riley, M.A., and Wertz, J.E. (2002). Bacteriocins: Evolution, Ecology, and Application. Annual
- 664 Review of Microbiology 56, 117–137. https://doi.org/10.1146/annurev.micro.56.012302.161024.

- Ruiz, L., Margolles, A., and Sánchez, B. (2013). Bile resistance mechanisms in Lactobacillus and
- 666 Bifidobacterium. Frontiers in Microbiology 4, 396. https://doi.org/10.3389/fmicb.2013.00396.
- 667 S. Roy, A., Parveen, A., R. Koppalkar, A., and Prasad, M.V.N.A. (2010). Effect of Nano Titanium
- 668 Dioxide with Different Antibiotics against Methicillin-Resistant Staphylococcus Aureus. JBNB 01,
- 669 37-41. https://doi.org/10.4236/jbnb.2010.11005.
- 670 Scardaci, R., Varese, F., Manfredi, M., Marengo, E., Mazzoli, R., and Pessione, E. (2021).
- 671 Enterococcus faecium NCIMB10415 responds to norepinephrine by altering protein profiles and
- 672 phenotypic characters. Journal of Proteomics 231, 104003.
- 673 https://doi.org/10.1016/j.jprot.2020.104003.
- 674 Sengupta, R., Altermann, E., Anderson, R.C., McNabb, W.C., Moughan, P.J., and Roy, N.C.
- 675 (2013). The Role of Cell Surface Architecture of Lactobacilli in Host-Microbe Interactions in the
- 676 Gastrointestinal Tract. Mediators of Inflammation 2013, 1–16.
- 677 https://doi.org/10.1155/2013/237921.
- 678 Shah, R.R., Kaewgun, S., Lee, B.I., and Tzeng, T.-R.J. (2008). The Antibacterial Effects of
- 679 Biphasic Brookite-Anatase Titanium Dioxide Nanoparticles on Multiple-Drug-Resistant
- 680 Staphylococcus aureus. Journal of Biomedical Nanotechnology *4*, 339–348.
- 681 https://doi.org/10.1166/jbn.2008.324.
- 682 Shi, H., Magaye, R., Castranova, V., and Zhao, J. (2013). Titanium dioxide nanoparticles: a review
- of current toxicological data. Particle and Fibre Toxicology *10*, 15. https://doi.org/10.1186/17438977-10-15.
- 685 Shkodenko, L., Kassirov, I., and Koshel, E. (2020). Metal Oxide Nanoparticles Against Bacterial
- 686 Biofilms: Perspectives and Limitations. Microorganisms 8, 1545.
- 687 https://doi.org/10.3390/microorganisms8101545.
- 688 Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S., and Dhawan, A. (2011). ROS-
- 689 mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells.
- 690 Toxicology in Vitro 25, 231–241. https://doi.org/10.1016/j.tiv.2010.11.008.
- 691 Sohm, B., Immel, F., Bauda, P., and Pagnout, C. (2015). Insight into the primary mode of action of
- 692 TiO₂ nanoparticles on *Escherichia coli* in the dark. Proteomics 15, 98–113.
- 693 https://doi.org/10.1002/pmic.201400101.

- Tong, T., Shereef, A., Wu, J., Binh, C.T.T., Kelly, J.J., Gaillard, J.-F., and Gray, K.A. (2013).
- 695 Effects of Material Morphology on the Phototoxicity of Nano-TiO2 to Bacteria. Environ. Sci.
- 696 Technol. 47, 12486–12495. https://doi.org/10.1021/es403079h.
- 697 Trunk, T., S. Khalil, H., C. Leo, J., and Bacterial Cell Surface Group, Section for Genetics and
- 698 Evolutionary Biology, Department of Biosciences, University of Oslo, Oslo, Norway (2018).
- 699 Bacterial autoaggregation. AIMS Microbiology 4, 140–164.
- 700 https://doi.org/10.3934/microbiol.2018.1.140.
- 701 Valdiglesias, V., Costa, C., Sharma, V., Kiliç, G., Pásaro, E., Teixeira, J.P., Dhawan, A., and
- Laffon, B. (2013). Comparative study on effects of two different types of titanium dioxide
- nanoparticles on human neuronal cells. Food and Chemical Toxicology *57*, 352–361.
- 704 https://doi.org/10.1016/j.fct.2013.04.010.
- Verleysen, E., Waegeneers, N., Brassinne, F., De Vos, S., Jimenez, I.O., Mathioudaki, S., and Mast,
- J. (2020). Physicochemical Characterization of the Pristine E171 Food Additive by Standardized
- and Validated Methods. Nanomaterials 10, 592. https://doi.org/10.3390/nano10030592.
- 708 Wang, Y., Chen, Z., Ba, T., Pu, J., Chen, T., Song, Y., Gu, Y., Qian, Q., Xu, Y., Xiang, K., et al.
- 709 (2013). Susceptibility of Young and Adult Rats to the Oral Toxicity of Titanium Dioxide
- 710 Nanoparticles. Small 9, 1742–1752. https://doi.org/10.1002/smll.201201185.
- 711 Wright, G.D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification.
- 712 Adv Drug Deliv Rev 57, 1451–1470. https://doi.org/10.1016/j.addr.2005.04.002.
- Yuan, Y., Ding, J., Xu, J., Deng, J., and Guo, J. (2010). TiO2 nanoparticles co-doped with silver
- and nitrogen for antibacterial application. J Nanosci Nanotechnol *10*, 4868–4874.
- 715 https://doi.org/10.1166/jnn.2010.2225.
- 716 Zhang, H., and Chen, G. (2009). Potent Antibacterial Activities of Ag/TiO2 Nanocomposite
- 717 Powders Synthesized by a One-Pot Sol–Gel Method. Environ. Sci. Technol. 43, 2905–2910.
- 718 https://doi.org/10.1021/es803450f.
- 719 Zhukova, L.V., Kiwi, J., and Nikandrov, V.V. (2012). TiO2 nanoparticles suppress Escherichia coli
- cell division in the absence of UV irradiation in acidic conditions. Colloids and Surfaces B:
- 721 Biointerfaces 97, 240–247. https://doi.org/10.1016/j.colsurfb.2012.03.010.
- Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., Ferchichi, M.,
- and Connil, N. (2018). Evaluation of Probiotic Properties and Safety of Enterococcus faecium

- 724 Isolated From Artisanal Tunisian Meat "Dried Ossban." Front. Microbiol. 9, 1685.
- 725 https://doi.org/10.3389/fmicb.2018.01685.
- 726 Zubar, T., Fedosyuk, V., Tishkevich, D., Kanafyev, O., Astapovich, K., Kozlovskiy, A., Zdorovets,
- M., Vinnik, D., Gudkova, S., Kaniukov, E., et al. (2020). The Effect of Heat Treatment on the
- 728 Microstructure and Mechanical Properties of 2D Nanostructured Au/NiFe System. Nanomaterials
- 729 *10*, 1077. https://doi.org/10.3390/nano10061077.

Table 1

ζ-potential (mV)	Mean Size (nm)	NPs < 100 nm %	NPs (<0.8 µm) / g	MPs (>0.8 μ m) /
				g
-10.7±3.1	177.7±2.1	16.5	9.0 X 10 ⁶	$3.2 \ge 10^{12}$

Tab.1. ζ -potential, mean size, NPs < 100 nm %, NPs / g, MPs / g of 10 mg/mL of TiO₂-FG water dispersion

Table 2

Strain	Control	TiO ₂ -FG
	(cm)	(cm)
Lactobacillus rhamnosus GG	2.62±0.02	2.62±0.04 ^{ns}
Enterococcus faecium NCIMB10415	2.66±0.04	2.27±0.01**

Tab. 2. The average diameter of the Ampicillin inhibitory zones for TiO₂-FG (0.125 mg/mL)-treated LGG and *Ent*. Results are expressed in cm \pm SEM. **P \leq 0.01, ns P> 0.05.

Figures



Fig. 1. Size distribution and cumulative distribution of 10 mg/mL TiO₂-FG water dispersion obtain by Nanoparticle Tracking Analysis (A) and by Flow Particles Image Analysis (B).



Fig. 2. Effect of TiO₂-FG (0.125 mg/mL) on the growth of LGG (A) and Ent (B). TiO₂-FG treatment reduces the growth pattern of the two probiotics.



Fig. 3. Bile salts (0.5%) tolerance of LGG (A) and *Ent* (B) after TiO₂-FG (0.125 mg/mL) treatment. TiO₂-FG decreases the bile salts survival rate of LGG and *Ent* by 62% and 34.6%, respectively. The results are expressed as % of control. ***P \leq 0.001, *P \leq 0.05.



Fig. 4. Ampicillin susceptibility of LGG (A) and *Ent* (B) untreated and treated with TiO2-FG (0.125 mg/mL). Treatment with TiO₂-FG lowered ampicillin sensitivity by 14.48% in the *Ent* treated cultures. The results are expressed as % of control. ***P \leq 0.00,1, ns P>0.05.



Fig. 5. Effect of TiO₂-FG (0.125 mg/mL) on the aggregation ability of LGG (A) and *Ent* (B). Autoaggregation of *Ent* is reduced by 38.1% after the treatment. The results are expressed as % of control. ****P \leq 0.0001, ns P>0.05.



Fig. 6. Effect of TiO₂-FG (0.125 mg/mL) on LGG (A) and *Ent* (B) biofilm biomass formation. TiO₂-FG treatment reduces LGG biofilm formation by 37%. The results are expressed as % of control. ***P \leq 0.00,1, ns P>0.05.



Fig. 7. LGG (A) and *Ent* (B) adhesion on Caco-2/TC7 cells in control condition or stimulated with TiO₂-FG (0.125 mg/mL). The treatment decreased the adhesion of LGG and *Ent* by 34.87% and 14.16%, respectively. The results are expressed as % of control. $P \le 0.05$.



Fig. 8. Effect of TiO₂-FG (0.125 mg/mL) on the antimicrobial activity of LGG towards *S. aureus* ATCC6538. The treatment reduces the antimicrobial potential of LGG by 35.73%. The results are expressed as % of control. ****P \leq 0.0001.