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

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ABSTRACT

Food-grade titanium dioxide (TiO_2-FG) is a widespread metal oxide used in food industries. Recently, the European Food Safety Authority concluded that TiO_2-FG cannot be considered safe for consumption due to its genotoxicity; however, its effect on the gut microbiota has not been completely unraveled. We studied the effects of TiO_2-FG (0.125 mg/mL) on *Lactobacillus rhamnosus* GG (LGG) and *Enterococcus faecium* NCIMB10415 (*Ent*), in particular some physiological and phenotypic traits (growth kinetics, bile salts, and ampicillin resistance) and their interactions with the host (auto-aggregation, biofilm formation, and adhesion on Caco-2/TC7 monolayers) and other gut microorganisms (antimicrobial activity towards pathogens). The results obtained revealed that TiO_2-FG alters both LGG and *Ent* growth and lowers their bile resistance (62 and 34.5%, respectively) and adhesion on Caco-2/TC7 monolayers (34.8 and 14.16%, respectively). The other outcomes were 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 TiO_2-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.

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

1 Introduction

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

A very recent evaluation by EFSA (European Food Safety Authority), —in part confirming previous toxicity tests on animals (Bettini et al., 2017; Shi et al., 2013)—concluded that TiO_2-FG can no longer be considered suitable for human consumption due to its potential genotoxicity, banning in Europe the use of E171 as a food addictive (EFSA Panel on Food Additives and Flavourings (FAF) et al., 2021). In the gut, TiO_2-FG interacts with epithelial cells of the small intestine causing DNA damage (Jugan et al., 2012; PetkoviĆ et al., 2011; Shukla et al., 2011) and altering the cell cycle, thus 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 TiO_2-FG toxicity is its moderate antibacterial effect used to extend the average storage life of foodstuffs (Helal et al., 2021). The

antimicrobial properties of TiO_2 were described over 30 years ago; however, the specific effects of the interaction between TiO_2-FG and the microbiota remain essentially unknown, focusing mainly on *E. coli*. Some studies suggest that this interaction could occur through electrostatic attraction, owing to their opposite surface charges (Zhukova et al., 2012). Other studies related the antibacterial properties of the particles to their photocatalytic effects (Kumar et al., 2011; Liu et al., 2010) or increased production of reactive oxygen species (ROS) (Tong et al., 2013). However, it has been proven that even in the dark environment of the GI tract, TiO_2-FG can display toxic effects (decrease in membrane fluidity, damages to the outer lipopolysaccharides) on commensal gut microorganisms (Liu et al., 2016). Therefore, even in the absence of translocation into blood, dysbiosis can be the pivotal event that generates unbalanced homeostasis and a plethora of systemic diseases threatening human health.

A question arises: to what extent can TiO_2-FG interfere with the endogenous gut ecosystem affecting resident gut bacteria's metabolic/phenotypic features and causing dysbiosis? The human intestinal microbiota shows long-term stability and resilience to external perturbations (Pessione, 2012). However, this community proved to be sensitive to environmental stressors due to diet, drugs, food additives, and sleep deprivation (Karl et al., 2018). In this sense, children represent the most critical population to oral exposure of TiO_2-FG as the main consumers and since they are at a crucial phase in their microbiota's development and diversification. Since data specifically concerning TiO_2 effects on the human microbiota are still controversial, further evaluation of TiO_2-FG toxicity on gut bacteria become a priority.

In the present investigation, we selected two probiotic bacteria of gut origin, *Lactobacillus rhamnosus* GG (LGG) and *Enterococcus faecium* NCIMB10415 (*Ent*), as models of beneficial gut microbes to be challenged with 0.125 mg/mL TiO_2-FG. This concentration of TiO_2-FG represent the one that reaches the intestinal tract after the intake of 20 g of commercially available chocolate (Khan, 2019). LGG is a well-characterized probiotic (Nissilä et al., 2017) effective against epithelial damage (leaky gut syndrome), inflammation, invasiveness, and the proliferation of malignancies (Banna et al., 2017). *Ent* is a bacteriocin-producing probiotic appreciated for counteracting infections and dysbiosis (Hosseini et al., 2009), and it has been widely used for treating gastrointestinal disorders (Holzapfel et al., 2018). This study aims to analyze the main physiological/phenotypic characters (growth parameters, bile, and antibiotic resistance) that can be affected by TiO_2-FG exposure. In addition, we also investigated the interactive behavior of such strains with other gut bacteria (growth interference) and with the host (based on auto-aggregation, biofilm formation, and adhesion). The

results obtained suggest that bacteria can both sense TiO_2-FG and modify their physiology and interactive behavior accordingly, with differences related to the bacterial species.

2 Materials and methods

2.1 TiO_2-FG particle size distribution

TiO_2-FG E171 was obtained from Kronos (KRONOS 1171 Titanium Dioxide E 171, Kronos, Dallas, TX, USA).

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).

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

2.2 Preparation of the TiO_2-FG suspension

A suspension of 1 mg/mL of TiO_2-FG in ultrapure water (MilliQ) was autoclaved and preserved at RT. If not used for a long time, the TiO_2-FG was sonicated for 45 min and vortexed for 3 min at 24000 rpm to ensure good resuspension of the particles, while for daily use, they were sonicated for 7 min and vortexed for 1 minute at 24000 rpm before each test. The stock suspension was then diluted to 0.125 mg/ml in culture media.

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 (≅ 1 or 4 x 10^8 CFU/mL) in control conditions or with the addition of 0.125 mg/mL TiO_2-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 (≅ 17 hours for LGG, ≅ 5 hours for *Ent*).

2.4 Growth curves

Cultures in control conditions or stimulated with TiO_2-FG were diluted to OD_{600} 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: OD_{595} reads every 30 min, 37°C and orbital shaking of 10 seconds before each reading.

2.5 Bile salts tolerance (BST) assay

BST was tested as previously described (Zommiti et al., 2018), with some modifications. Briefly, 1 mL of the early stationary phase of probiotic cultures treated or untreated with the TiO_2-FG, was centrifuged (100000 g, 10 min, RT), and the cells were re-suspended in MRS or BHI containing 0.5% bile salts (Bile, bovine - Sigma Aldrich) to a final concentration of 10^8 CFU/mL. To simulate human intestinal transit, the suspension was incubated in an orbital shaker (100 rpm) at 37°C for 4 hours. Bacteria collected at T0 and T4 were diluted in 0.9% NaCl, and counted on MRS or BHI agar after 48 or 24 hours. We repeated the experiment in four biological replicates. The BST was calculated as a survival rate:

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

The results are expressed as % of control.

2.6 Ampicillin susceptibility assay

To test how TiO_2-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 10^8 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 µg of ampicillin (BD BBL™ Sensi-Disc™, 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 BBL™ Sensi-Disc™ zone diameter interpretive chart, the breakpoints for the *Enterococcus spp.* are ≤ 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).

2.7 Auto-aggregation assay

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

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

2.8 Biofilm Assay

For this assay, the pre-cultures of both bacteria were diluted to OD_{600} 0.4 in control conditions or with TiO_2-FG. Then, 150 µL of each culture was added to a 96-well plate (10 wells for each condition) and incubated with no agitation at 37°C for 24 hours. After 24 hours, the plate was emptied, washed 3 times with MilliQ water, and dried. Next, 150 µL of 0.1% crystal violet (PanReac AppliChem) was added, and the plate was incubated at RT for 15 min. Afterward, crystal violet was carefully removed, and the wells were washed 3 times and completely dried at 37°C. Finally, 150 µL of 100% ethanol (Carlo Erba Reagents, Chau. du Vexin, France) was added to each well, and the A_{595} nm was measured (Filtermax F5, Molecular Devices, LLC.) with an orbital shaking of 10 seconds before reading. The results are expressed as % of control.

2.9 Adhesion on enterocytes

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 in 4.5 g/L glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Euroclone S.p.A., Pero, Italy), 1% Penicillin/Streptomycin (Euroclone S.p.A.), 2 mM Glutamine (Euroclone S.p.A.), and filter sterilized. Cells were maintained in a humidified incubator at 37°C in 5% CO_2 and 95% air atmosphere, and the medium was replaced three times per week. Cells were split after reaching approximately 90% of confluence, seeded in 24-well culture plates, and used at the confluence

(10^6/well). On the day of the experiment, LGG and *Ent* were harvested by centrifugation after incubation with TiO_2-FG and in control conditions. The pellets were concentrated to 10^{10} CFU/mL in DMEM without FBS and antibiotics, diluted 1:100, counted on plates, and applied on confluent cell monolayers for 2 hours. Caco-2/TC7 were washed twice with NaCl (Sigma-Aldrich) 0.9% to remove non-adherent bacteria, and 500 µL of 0.1% Triton 100-X (Sigma-Aldrich) was added to disrupt the cells. The lysates were serially diluted and plated on BHIA to count the number of adherent bacteria. The results are expressed as % of control.

2.10 Antimicrobial activity

The effects of TiO_2 treatments on the antagonistic activity of LGG and *Ent* towards *E. coli* ATCC8739, *S. aureus* ATCC6538, and *L. monocytogenes* CIP55143 were studied with the soft-agar overlay method (Hockett and Baltrus, 2017), with some modifications. Briefly, LB soft agar inoculated with 10^8 CFU/mL of the indicator strains were poured onto BHIA. Once solidified, 10^{10} CFU/mL of the treated or untreated probiotics were spotted on soft agar in triplicate, and the plates were incubated for 24 hours at 37°C. Antimicrobial activity was assessed by measuring the inhibitory zone around the spots: the diameter of the probiotics' spot and the diameter of the total zone of inhibition were measured, and respective areas were calculated. The area of inhibition was computed by subtracting the area of the inoculum from the area of the total zone of inhibition. The results are expressed as % of control.

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 ± standard error (SEM).

3 Results

3.1 Particle size distribution

According to the data sheet provided by the company, the TiO_2-FG is an anatase pigment approved for coloring foodstuffs, therefore meeting safety regulations and standards in terms of purity. However, regulation does not define particle size ranges, that may vary depending upon the provider. For this reason particle size distribution was evaluated here by integrating Nanoparticle Traking Analysis (NTA), that allows to measure NPs, with Flow Particles Image Analysis (FPIA), which detects microparticles (MPs; >1μm). Figure 1A reports the size distribution obtained by NTA. Particles in the 10-600 nm range were detected, with a mean hydrodynamic diameter of 177,7 ± 2,1 nm and the 16.5 % of particles having a diameter < 100 nm. A small number of MPs was detected by FPIA (Figure 1B), which represent a minor fraction of the nanometric/submicrometric one. Z-potential (measured at pH 6.8, conductivity 5 μS/cm) was negative, in line with what expected (Zubar 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

One of the aims of the present study was to evaluate the effects of TiO_2-FG on the growth profiles of LGG and *Ent* cultures (along with a control condition) over 24 hours. The bacterial growth curves of LGG and *Ent* in the presence of TiO_2-FG and untreated conditions are reported in Fig. 1a and 1b, respectively. As shown, compared to the control conditions, the growth curves of both bacteria displayed differences starting from the latency phase, which is slightly longer in the cultures exposed to TiO_2-FG. The highest absorbance values are achieved roughly after 17 and 5 hours of growth for LGG and *Ent*, respectively (beginning of the stationary phase), and tend to remain stable. In this phase, the curve related to the treated microbial cultures settled on lower values than the untreated condition. More in detail, the OD_{595} values at 24 hours for treatment with TiO_2-FG are 0.2 ± 0.06 and 0.06 ± 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 TiO_2-FG. In particular, the treatment with TiO_2-FG improved the LGG and *Ent* μ by 11 and 31%, respectively.

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.

3.4 Ampicillin susceptibility assay

The ampicillin susceptibility assay was intended to measure whether TiO_2-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 TiO_2-FG treatment. Table 1 shows the average values of the inhibitory zones diameters found for each antibiotic and culture condition.

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.

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.

3.7 Adhesion on enterocytes

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

3.8 Antimicrobial activity

One desirable property of probiotics is their antimicrobial activity against pathogenic/opportunistic microorganisms. Therefore, we evaluated how TiO_2-FG could modulate LGG and *Ent* antimicrobial activity exerted by direct contact with the indicator strain. The results show that TiO_2-FG could reduce the antimicrobial potential of LGG by 35.73% towards *S. aureus* (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).

4 Discussion

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

4.1 Effect of TiO_2-FG on probiotics' viability

We evaluated the bacterial viability following exposure to TiO_2-FG by analyzing their growth curves. As reported in the Results section, TiO_2-FG exposure resulted in a much longer latency phase and a slight time delay of the exponential tract than the control. Graphically, following the delay

accumulated in the first phase, the curves seem to recover from the initial disadvantage, showing a remarkable slope, with higher μmax values for the treated bacterial cultures. Finally, TiO_2-FG-treated stationary phase cultures show a dose-dependent decrease in biomass yield and curve profiles compared to the control condition. Given these trends, we can hypothesize that TiO_2-FG may interfere with the initial accumulation of resources (promoting growth in size that prepares for cell replication) perhaps by inhibiting the membrane transporters, thus leading to a delay in growth. Moreover, since the curves settle on lower final absorbance values as the concentration of-FG increases, we can hypothesize that microbial viability is severely hindered by the treatment. All these results agree with previous data from the literature that demonstrated high toxicity of TiO_2-FG against symbiotic intestinal bacteria, including *Acetobacter* species, enterobacteria, and lactobacilli (Liu et al., 2016).

Most of the experiments evaluating TiO_2-FG toxicity focused on the viability of *Escherichia coli*. Pagnout et al. (2012) proved that the toxicity of TiO_2-FG strictly depends on the electrostatic interactions between bacteria and TiO_2-FG, which leads to the particles penetration of the cell surface. Sohm et al. (2015) observed a significant depolarization of the *E. coli* cell membrane with loss of its integrity, and almost irreversible ionic imbalances, together with a substantial decrease in the expression of DNA polymerase III (HolB and DnaX). The authors suggest that this loss of replication ability could explain the reduction in cell number compared to the untreated condition. Interestingly, a remarkable upregulation of osmotically inducible genes, such as osmB, osmC, osmE, and osmY and an increase in trehalose (a molecule widely known for its outstanding osmoprotective properties) synthesis were also recorded in *E. coli* following TiO_2-FG treatment. However, both osmC (Lesniak et al., 2003) and trehalose (Benaroudj et al., 2001; Elbein et al., 2003) have been associated with defense mechanisms against oxidative stress as well. These data fit with the findings of Kumar et al. (2011), which demonstrate that TiO_2-FG induce ROS generation, DNA damage, LDH release, glutathione depletion, along with a significant increase in lipid peroxidation, thus leading to cell death in *E. coli*. Therefore, the cytotoxic effects exerted by these nanomaterials can mainly be ascribed to the increased oxidative stress.

As far as probiotic bacteria are concerned, experiments on *Bacillus coagulans*, *Enterococcus faecium*, and *Enterococcus faecalis* suggest that TiO_2-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.

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

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

Here we demonstrated that pre-treatment with TiO_2-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). TiO_2 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.

4.3 Synergies/antagonisms between TiO_2-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).

Since TiO_2-FG contains nanoparticles, we evaluated the effect of TiO_2-FG treatment on the ampicillin sensitivity pattern of LGG and *Ent*. Our experiment shows that exposure to TiO_2-FG at the tested concentrations can decrease *Ent* sensitivity to ampicillin. This result apparently contrasts with a study performed by S. Roy et al. (2010), who reported that TiO_2-FG increased the antibacterial activities of 22 different antibiotics against *Staphylococcus aureus*. However, the authors do not clarify what concentration (mg/mL) of nanomaterials was used in their study. Furthermore, the microbial models, despite being both Gram-positive, consistently differ. *E. faecium* is known for having an intrinsic resistance to most antimicrobials, due to surface characteristics that prevent the antibiotic entering into the cell (Klare et al., 2003), whereas *S.aureus* often bears transmissible plasmids encoding enzymes that degrade the antibacterial molecule (Wright, 2005).

Together with other penicillins and cephalosporins, Amp belongs to beta-lactam antibiotics, which exert their antibacterial action by binding to the penicillin-binding proteins (PBPs) on the bacterial surface and hence blocking cell wall synthesis. The most common mechanisms by which bacteria escape beta-lactam activity are either the production of degrading enzymes (beta-lactamases) or target PBP modifications (Andersen, 1990). Regarding the possible effect of TiO_2-FG on the *Ent* ampicillin sensitivity pattern, although we cannot exclude the enhancement of beta-lactamase production, the alterations of PBPs are the most probable occurrence, since in *Enterococcus* species ampicillin resistance is linked to surface modifications (Klare et al., 2003). Moreover, interactions between TiO_2-FG and bacterial surface structures are largely described in the literature and can contribute to the observed phenomenon (Zhukova et al., 2012). Finally, the sensitivity profile of LGG is not modified by TiO_2-FG treatment because in this strain there is no beta-lactam resistance (either intrinsic or acquired) (Capurso, 2019).

4.4 Effect of TiO_2-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).

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

The auto-aggregation mechanisms exploited by lactic acid bacteria is a self-recognition phenomenon that involves the secretion of extracellular adhesive molecules, mainly proteins and exopolysaccharides, called "autoagglutinin" (Trunk et al., 2018). However, the auto-aggregative phenotype is diversified in different bacterial strains and depends on certain external conditions (Trunk et al., 2018). Several factors can alter the aggregation ability of bacteria, including oxygen availability, stress, or a variation of the temperature, even if not all microorganisms are influenced in the same manner (McLean et al., 2008). Given this variability, we could speculate that TiO_2-FG inhibits the expression of certain auto-agglutinins in *Ent* that are not involved in the auto-aggregation of LGG. Further research is needed to identify the molecules inhibited by the TiO_2-FG exposure and involved in these interactions.

Auto-aggregation is also the initial step in adhesion to surfaces and the formation of biofilms. Biofilms are bacterial communities that involve a strong association of microbial cells and extracellular polymeric substance (EPS), which is mainly composed of a mixture of polysaccharides, lipids, proteins, glycopeptides, and nucleic acids (Donlan and Costerton, 2002). This matrix consists of heterogeneous layers, including nutrients and water transport channels (Flemming and Wingender, 2010). In our study, treatment with TiO_2-FG reduced the biofilm biomass produced by LGG compared to the untreated condition but did not induce significant changes in the biofilm formation of *Ent* cultures.

Very few studies have analyzed the anti-aggregation and anti-biofilm effects of TiO_2-FG, and the molecular mechanisms regarding these processes are still largely unknown. The exposure to TiO_2-FG at higher concentrations than those employed here likely causes the death of some microbial cells within the biofilm, primarily because of the generation of ROS and lipid oxidation at the cell membrane level, as described in some studies on Gram-positive *Staphylococcus aureus* (Shah et al., 2008). Moreover, a study carried out by Zhang and Chen (2009) on *E. coli* indicates that TiO_2-FG may penetrate within the biofilm and directly interact with bacteria through an electrostatic attraction between the positive charges of the metal oxides and some of the negative charges residing on the bacterial outer wall. A study carried out both *in vitro* and *in vivo* by Khan and co-workers (Khan, 2019) demonstrated that TiO_2-FG contained in commercially available chocolate could interfere with the metabolic activity of some probiotics (production of lactic acid) and their ability to form biofilms. Furthermore, TiO_2-FG can be absorbed and interfere with biofilm matrix development by interacting with molecules present in the EPS that bear various functional groups such as hydroxyl, amide, or carboxyl (Gao et al., 2019). How NPs and MPs interact with and move within the matrix depends on several factors, such as the type of particle, the properties of EPS, the pores' size, hydrophobicity, electrical charges, presence of water channels, and chemical gradient of the matrix, and the local environment (Shkodenko et al., 2020). Nevertheless, EPS can be highly variable in terms of chemical composition among bacteria, resulting in a different potential mode of interaction with nanomaterials 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

results suggest that TiO_2-FG may act as biofilm-disrupting agents rather than biofilm formation inhibitors, at least in LGG. Similarly, it is possible to hypothesize that TiO_2-FG can interfere with some surface molecules, essential for cell aggregation but not involved in biofilm formation in this species.

Given these intriguing results, we sought to determine the influence of TiO_2-FG treatment on the adhesion of the two probiotics on the Caco-2/TC7 intestinal cell line. The experimental evidence here reported a decrease in both LGG and *Ent* adhesion after TiO_2-FG treatment. These results could be partly related to the decreased biofilm formation of LGG and auto-aggregation of *Ent* in the presence of TiO_2-FG. Moreover, bacterial adherence to Caco-2/TC7 cells requires various cell wall architectural components such as peptidoglycan, teichoic acids, polysaccharides, and surface proteins (Sengupta et al., 2013). Although we did not investigate all these aspects in detail, we can hypothesize that TiO_2-FG can negatively affect the expression of these adhesive molecules. Alternatively, we can assume that TiO_2-FG present in the culture medium during the experimental procedure could form a layer on the surface of the intestinal cells, thus hindering bacterial adhesion, as it has been observed that the treatment of dental implants with TiO_2 reduces the adhesion of *Staphylococcus* spp. through this mechanism (Del Curto et al., 2005).

4.5 Effect of TiO_2-FG on probiotics' competition with other bacteria

Probiotic bacteria can influence the composition of the microbiota in the gastrointestinal tract by maintaining the correct ratio among species, and by acting against exogenous pathogenic bacteria. They can exert this role in various ways, for example by secreting antimicrobial metabolic end-products (H_2O_2, ethanol, lactic acid, and other organic acids) (Ouwehand and Vesterlund, 2004), competing for nutrients and binding sites (Di Cerbo et al., 2016), and by producing specific weapons called bacteriocins (Ouwehand and Vesterlund, 2004).

In the present investigation, we evaluated the effect of TiO_2-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 TiO_2-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 TiO_2-FG gave similar results to the control conditions.

In general, except for nisin A and mutacin B-Ny266 (Riley and Wertz, 2002), bacteriocins display a narrow spectrum of activity and are generally effective against bacteria related to the

producing strain. This target specificity could explain the difference in the antimicrobial action of both LGG and *Ent* against the tested pathogens: TiO_2-FG could have downregulated the production or secretion of certain LGG bacteriocins active against *S. aureus*, while not affecting the expression/secretion of others.

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.

The evidence obtained from this study indicates that the main probiotic features of two bacterial strains can be negatively affected by treatment with TiO_2-FG. This food additive (0.125 mg/mL) hinders microbial growth and lowers bile resistance and adhesion capabilities of LGG and *Ent* on Caco-2/TC7 cells. As far as other parameters are concerned, the effects are different on the two strains in the study. The ability to form a biofilm and inhibit *S. aureus* growth is impaired in LGG but not in *Ent*. On the contrary, the auto-aggregation ability and ampicillin sensitivity are negatively affected by TiO_2-FG only in *Ent*. The differences in the auto-aggregating and biofilm-forming sensitivity to TiO_2-FG exposure in the strains in the study reflect the different surface compositions of the two genera. This structural diversity could also account for a possible higher perturbing action of TiO_2-FG on *Ent* PBPs, resulting in decreased sensitivity to ampicillin. Conversely, the reduced interfering activity towards *S. aureus* seems linked to the specificity of the bacteriocins produced by LGG.

Taken together, these overall results underline a possible adverse effect of TiO_2-FG on both the endogenous gut microbiota and exogenously administered probiotics. However, the damage exerted by this FG-material can be different due to the structural and functional specificity of each species and due to the complexity of the microbial populations hosted in the gut ecosystem. In addition, further *in vivo* studies are required, since, during the gastrointestinal transit, micro and nanomaterials are exposed to various factors—pH variations or the adsorption of molecules on their surface—that can significantly influence their aggregation and their physicochemical and toxicological properties.

The TiO_2-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

weight in the United States (USFDA, 2005). Conversely, in Europe, E171 was used "*at quantum satis*" (levels not higher than necessary to achieve the intended purpose) (EFSA, 2016). Considering that, an updated evaluation underlines that TiO_2 can no longer be regarded as safe for human health because of genotoxic effects induced following their consumption (EFSA Panel on Food Additives and Flavourings (FAF) et al., 2021), banning E171 from the European market. However, TiO_2–FG effect on the gut microbiota, which plays several essential roles in host health, has not been completely unraveled. This work, can add a further piece to the puzzle of the risks of using TiO_2-FG in food production.

6 Declaration of competing interest

There are no conflicts to declare.

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8 Data availability

Data will be made available on request.

8.1 CRediT authorship contribution statement

Francesca Bietto: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; **Rossella Scardaci**: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing; **Manuel Brovia**: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft; **Ida Kokalari**: Data curation, Methodology, Conceptualization, Funding acquisition, Supervision, Writing – review & editing; **Francesco Barbero**: Data curation, Formal analysis, Investigation; **Ivana Fenoglio**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing; **Enrica Pessione**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Table 1

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

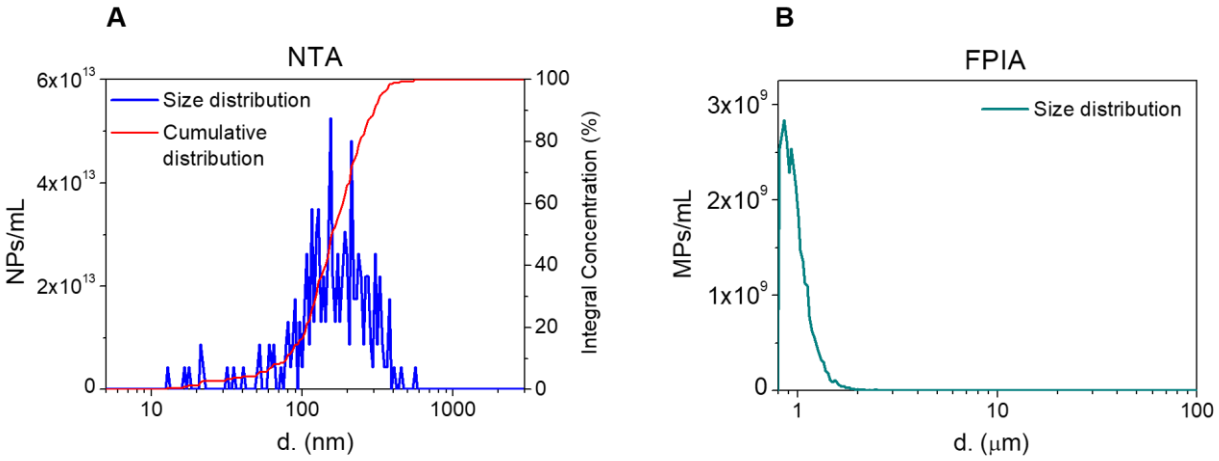
Tab.1. ζ-potential, mean size, NPs < 100 nm %, NPs / g, MPs / g of 10 mg/mL of TiO_2-FG water dispersion

Table 2

Strain	**Control (cm)**	**TiO_2-FG (cm)**
***Lactobacillus rhamnosus* GG**	2.62±0.02	2.62 ± 0.04^{ns}
***Enterococcus faecium* NCIMB10415**	2.66±0.04	$2.27\pm0.01^{**}$

Tab. 2. The average diameter of the Ampicillin inhibitory zones for TiO_2-FG (0.125 mg/mL)-treated LGG and *Ent*. Results are expressed in cm ± SEM. **P≤ 0.01, ns P> 0.05.

Figures



Z potential (mV)	Mean Size (nm)	NPs % by number < 100 nm	NPs (<0,8 μm) /g	MPs (>0,8 μm)/g
-10.7 ± 3.1	177.7 ± 2.1	16.5	9.0×10^{16}	3.2×10^{12}

Fig. 1. Size distribution and cumulative distribution of 10 mg/mL TiO_2-FG water dispersion obtain by Nanoparticle Tracking Analysis (A) and by Flow Particles Image Analysis (B).

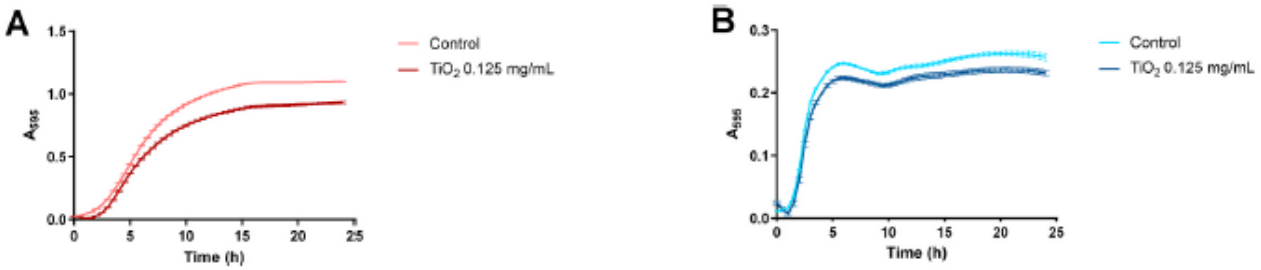


Fig. 2. Effect of TiO_2-FG (0.125 mg/mL) on the growth of LGG (A) and Ent (B). TiO_2-FG treatment reduces the growth pattern of the two probiotics.

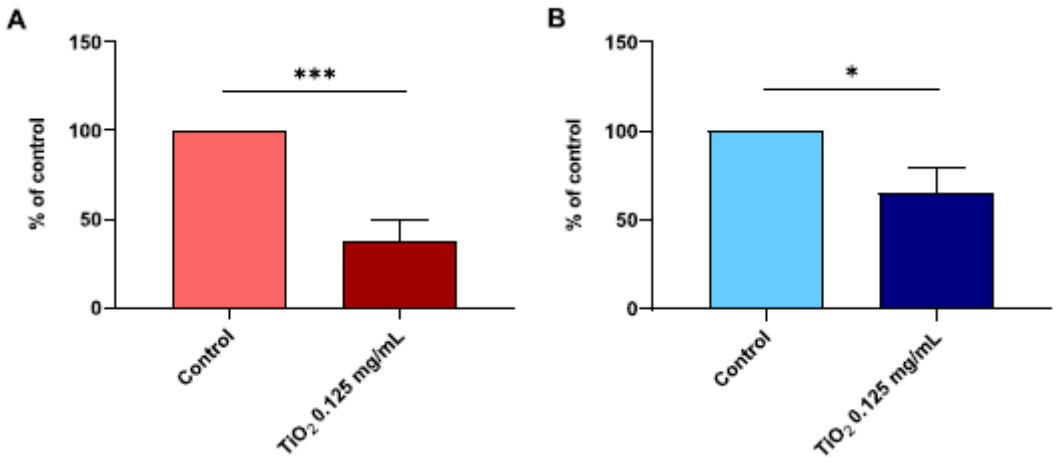


Fig. 3. Bile salts (0.5%) tolerance of LGG (A) and *Ent* (B) after TiO_2-FG (0.125 mg/mL) treatment. TiO_2-FG decreases the bile salts survival rate of LGG and *Ent* by 62% and 34.6%, respectively. The results are expressed as % of control. ***P ≤ 0.001, *P≤ 0.05.

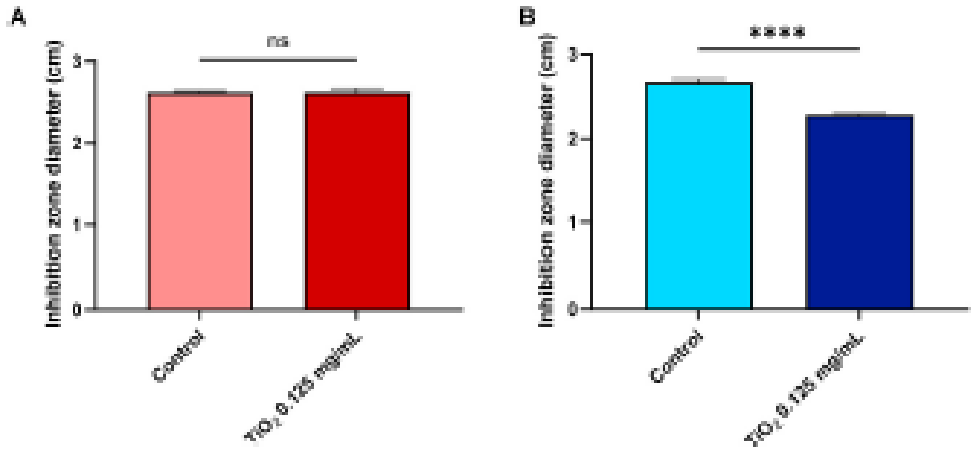


Fig. 4. Ampicillin susceptibility of LGG (A) and *Ent* (B) untreated and treated with TiO2-FG (0.125 mg/mL). Treatment with TiO_2-FG lowered ampicillin sensitivity by 14.48% in the *Ent* treated cultures. The results are expressed as % of control. ***P ≤ 0.00,1, ns P>0.05.

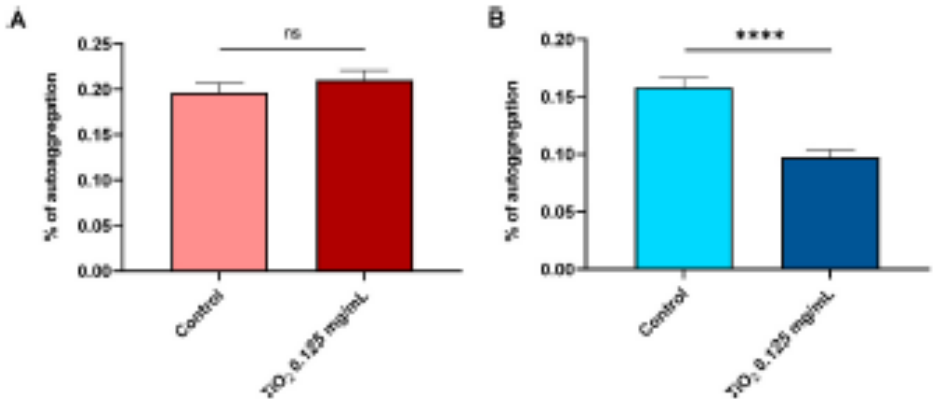


Fig. 5. Effect of TiO_2-FG (0.125 mg/mL) on the aggregation ability of LGG (A) and *Ent* (B). Auto-aggregation of *Ent* is reduced by 38.1% after the treatment. The results are expressed as % of control. ****P ≤ 0.0001, ns P>0.05.

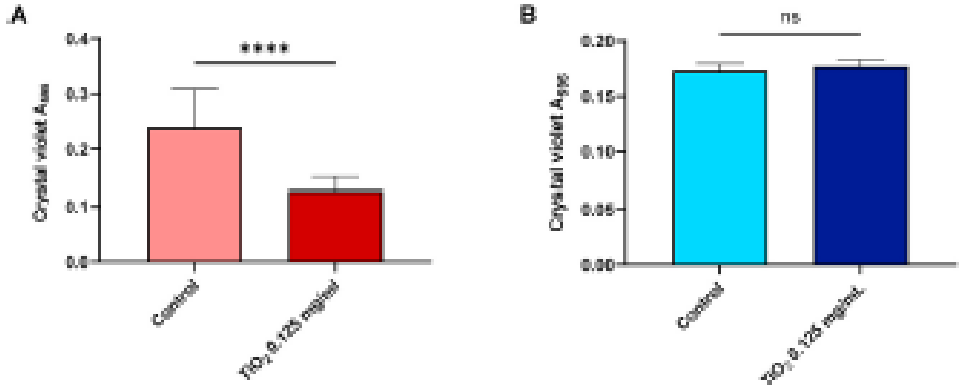


Fig. 6. Effect of TiO_2-FG (0.125 mg/mL) on LGG (A) and *Ent* (B) biofilm biomass formation. TiO_2-FG treatment reduces LGG biofilm formation by 37%. The results are expressed as % of control. ***P ≤ 0.00,1, ns P>0.05.

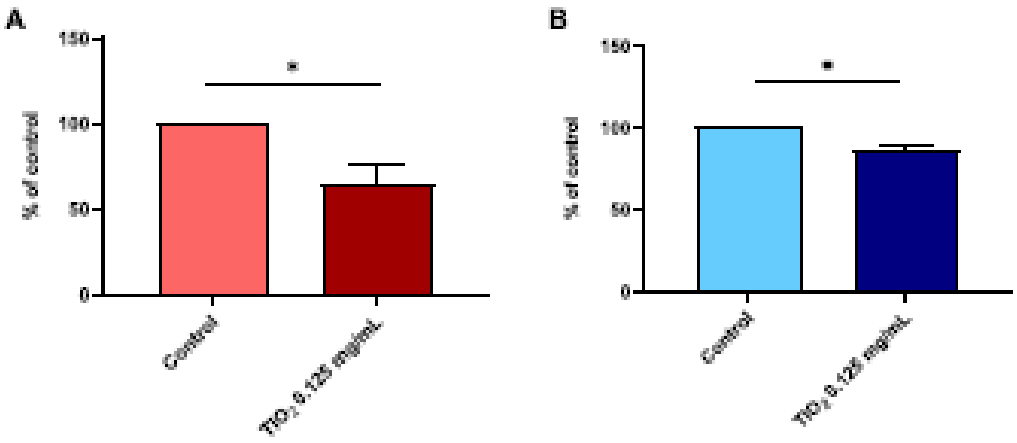


Fig. 7. LGG (A) and *Ent* (B) adhesion on Caco-2/TC7 cells in control condition or stimulated with TiO_2-FG (0.125 mg/mL). The treatment decreased the adhesion of LGG and *Ent* by 34.87% and 14.16%, respectively. The results are expressed as % of control. P≤0.05.

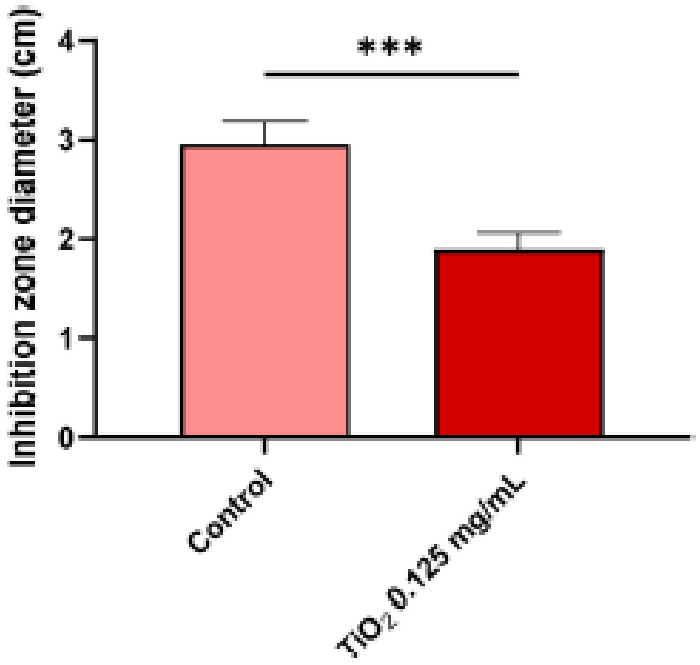


Fig. 8. Effect of TiO_2-FG (0.125 mg/mL) on the antimicrobial activity of LGG towards *S. aureus* ATCC6538. The treatment reduces the antimicrobial potential of LGG by 35.73%. The results are expressed as % of control. ****P ≤ 0.0001.