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Effects of silver nanoparticles on *in vitro* gut microbial models and other anaerobic environments

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“E per il resto lasciatevi accadere
la vita.
Credetemi: la vita ha ragione,
in tutti i casi.
Non vi osservate troppo.
Non ricavate conclusioni troppo rapide
da quello che vi accade;
lasciate che semplicemente vi accada.”

Rainer Maria Rilke,
Lettera ad un giovane poeta

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CHAPTER 1

Introduction

1 NANOPARTICLES: FROM USE TO BE EXPOSED

Nanoparticles (NPs) are defined by the International Organization for Standardization (ISO/TS 27687, 2008 - E) as particles with at least one dimension in the nanometer scale (1-100 nm).
NPs can occur naturally in both aquatic and terrestrial environments as finest fractions of mineral systems (e.g. colloidal clays, metal oxide precipitates, dissolved organic matter), but an ever-growing portion of nanomaterials is nowadays released from artificial processes (Batley et al., 2012).
Indeed, due to their special chemical and optical properties humans have utilized NPs early back in history, for instance, to colour glass (Au NPs, Ruvio et al., 2008) and obtain iridescent materials (Cu NPs, Mirguet et al., 2009). Michael Faraday's experiments on the interaction of light with gold NPs dispersions are considered the beginning of modern colloid science and thus nanoscience (Edward and Thomas, 2007).
In recent decades nanotechnology (NT) has largely developed and spread, exploiting chemical and physical peculiar properties of NPS that are not shown in traditional materials (Lagaron et al., 2005). Indeed, engineered nanomaterials, and specifically the NPs, are known to have a higher surface area per unit mass than the bulk material, which means that a higher number of reactive atoms are available for chemical and biochemical reactions, leading to a much higher reactivity (Song et al, 2011). Thus, NPs have been found to be effective catalysts (Roduner, 2006), superconductors (Shi et al., 2012) and showed properties of superparamagnetism (Vatta et al., 2009), ultrahardness (Lamni et al., 2005) and thermal and corrosion resistance (Miyake et al.,2013, Hamdy and Butt 2007). The quantum confinement effect also contributes to their unique electronic and optical properties of semiconductor, such as quantum dots, which fluoresce differently based on size (Roduner, 2006). For these reasons, nanoscience and nanotechnology are receiving growing attention from both industry and research all over the world, in developed countries as well as in fast-growing and developing ones. Moreover, NPs are being increasingly incorporated into consumer products, and employed in a number of agri-food systems applications, enhancing the probability of environmental release and magnification, leading to an ever-growing human exposure through inhalation, ingestion or direct contact.
Of special interest are the possible effects of NPs on complex ecosystems as microbiota associated with the living organisms (Das et al., 2012 a,b). In particular, NP interactions with human microbiota and the finer host-microbiome combined responses to environmental concentration of NPs represent a challenging and still little known field of study and research.

1.1 NANOPARTICLES ECO-TROPHIC FATE THROUGH ENVIRONMENTAL SYSTEMS

Since the manufactured nanomaterials industry began its growth, concern has been raised about the possible entry of nanomaterials into aquatic and terrestrial environments, and their ultimate impact on the biosphere, humans included. The intentional or accidental release of NPs into the environment represents, indeed, a potential toxicity risk to a wide variety of organisms at many trophic levels (Das et al., 2012 a,b). Consequently, ecosystems can become at risk (Costanza et al., 1997; Holden et al., 2012).

NPs enter aquatic and terrestrial environments through air emissions (Cassee et al., 2011), wastewater treatment plants (Kiser et al., 2009) and building facade runoff (Kaegi et al., 2010). Environmental NPS concentrations are still uncertain (Von Der Kammer et al., 2012) and estimates vary depending on considered matrixes, analytical techniques, statistical models of estimations and NPs state of dissolution, agglomeration, sedimentation or other transport processes (Keller et al., 2010, Gottschalk et al., 2011). Indeed, once inside environmental systems, dissolution and aggregation with other nanoparticles (homoaggregation) or with natural mineral and organic colloids (heteroaggregation), as well as chemical and biochemical processes, can change NPS fate, their potential toxicity and persistence in ecosystems (Batley et al., 2012).

As released in the environment, industrial NPs spread following the constant flux of matter and energy across different interconnected levels of biological organization (population, communities, ecosystems) and physical scales. Here NPs can exploit their biological active effects (both toxic or not), undergoing trough biological uptake, tissue accumulation (bioaccumulation) and then concentrate along trophic levels within the food chain (biomagnification) (Hou et al., 2012).

1.1.1 NPS routes through terrestrial environments

In terrestrial environments, microbial communities are the first biological systems concerned by NPs contact and have been found capable to adsorb and concentrate NPs as long as disperse NPs agglomerates (Horst et al 2010). Microbioms play a key-role in ecosystems for both matter and energy management as abundant and versatile catalysts and can be negatively affected by NPs representing the first step of damage to soil ecosystems. For instance, some NPs (e.g. CdSe quantum dots) can associate to bacterial membrane and damage them by means of reactive oxygen species (ROS) production (Priester et al., 2009). Moreover, NPs can then enter and accumulate inside cells, causing further stress, inhibiting growth, and finally biomagnified into protozoan predators and earthworms (Werlin et al., 2011). Nano-TiO_2 and -ZnO have been found able to alter bacterial community structure in a dose-dependent fashion influencing taxa associated with ecosystem processes of N_2 fixation, methane oxidation, and complex C decomposition thus affecting rizosphere health and plant food supply (Ge et al., 2011; Ge et al., 2012).

The importance of uptake and accumulation of NPs by plants is increasingly recognized and some scientific studies have been published on domestic edible plants (Ma et al., 2010). The first report was published by Zhu et al., (2008) showing that iron oxide nanoparticles (Fe_3O_4) was taken up by pumpkin (*Cucurbita maxima*) roots and translocated through the plant tissues. Same results were obtained on uptake and translocation of carbon nanomaterials by rice plants, *Oryza sativa* (Lin et al., 2009). For metallic nanoparticles, Cu nanoparticles (CuNPs) could be taken up and accumulated in the biomass of bean and wheat plants (Lee et al., 2008). The researchers also presented a linear relationship that higher concentrations of CuNPs in the growth media resulted in higher uptake and accumulation of CuNPs in plant tissues (Ma et al., 2010). One more issue corollary to the accumulation of NPs in edible plants is their transmission to the plant's next generation. Lin and colleagues (2009) reported that C70 was detected, although much less frequently, in the leaf tissues of second generation rice plants. If NPs are found in the second-generation plants, there is the possibility that these plants become adapted and more responsive, accumulating more of the respective NPs. Another important issue is the bioavailability of the accumulated NPs to the next trophic level, for example, biomagnification in ruminants and humans (Rico et al., 2011). There are studies showing that NPs in algae and tobacco are transmitted to the next trophic level (Judy et al., 2011; Navarro et al., 2008).

1.1.2 NPs routes through acquatic environments

Despite the higher variability of both fresh and seawater (and relative sediment) ecosystems respect to soil environments, phenomena of concentration, biomagnification and toxicity of NPs through the different levels of food chain have been also found (Holden et al., 2012). The toxicity to different algal species involving adsorption to the cell surface and disruption to membrane transport has been observed (Batley et al., 2012). ZnO NPs have also been found toxic to aquatic mammalian cells, freshwater zebrafish and sea urchin embryos (George et al., 2010; Fairbairn et al., 2011; Xia et al., 2011). The relatively high bioaccumulation and incomplete depuration of NPs in lower trophic level organisms as daphnid, points to the possibility for trophic transfer and biomagnification through the food chain starting from the bottom (Hou et al., 2012). In particular, bacterial communities are at the lower level of food chain in both fresh and marine ecosystems, settled in sediments and on surfaces where NPs can precipitate and, then, bioaccumulate. At this level Werlin et al., (2011) demonstrated that trophic transfer occurs from CdSe QD-contaminated bacteria (*Pseudomonas aeruginosa*) to ciliated protozoa (*Tetrahymena thermophila*). In another study, Holbrook et al., (2008) tested if CdSe–ZnS core–shell QD trophic transfer occurs in a simulated aquatic food web involving bacteria (*Escherichia coli*)- ciliates (*Tetrahymena thermophila*)-rotifers (*Brachionus calycifluorus*). Furthermore, preliminary data on benthic food webs (that support many fisheries and rely on phytoplankton from the overlying water as food) suggest that marine mussels suffer reduced growth and reproduction when they graze upon ZnO NPS-contaminated phytoplankton (Holden et al., 2012). Trophic transfer has also been reported for other high trophic level aquatic food chains, such as QDs transfer from algae to daphnid,

(Bouldin et al., 2008) and QDs or $nTiO_2$ transfer from daphnid to fish (Lewinsky et al., 2011, Zhu et al., 2010).
Considering that current studies used rather simplified food chains and despite additional research is needed to assess biomagnification magnitude of NPs, nanocompounds can eventually reach human via consumption of contaminated water foods (e.g., mussels or fish).

1.2 NANOPARTICLES FATE TROUGH FOOD SYSTEMS

Nanomaterials applications in food industry is growing and appear remarkable for future innovation and economic impact. The food industry, in fact, can benefit from the nanotechnology (NT) with huge potential in terms of safety, quality, food preservation and shelf-life extension (Mahalik et al., 2014). In the agri-food systems NT can be applied along the entire production chain (Martirosyan and Schneider, 2014) with innovations ranging from the improvement of some organoleptic characteristics of the food (e.g. colour, flavour, texture and consistency), obtaining greater absorption and better bioavailability of nutrients and food supplements, to the development of new packaging materials with antimicrobial and/or enhanced mechanical properties. More ambitious applications include the development of nanosensors that would facilitate the "monitoring" of the packaged food during transport and storage (Chaudhry et al., 2008) and new methods for the detection of pathogens (Chen et al., 2006; Maynard et al., 2006). Many products are already on the market and many others are being tested in research field (Bouwmeester et al., 2009).
In March 2016, 118 articles were found in "food and beverage" category of *the Inventory of consumer products based on nanotechnology*, made by the Project on Emerging Nanotechnology (http://www.nanotechproject.org/cpi/) including food, food storage products, dietary supplements, and products used for cooking.
In 2005, on the world market, about 500 were nano-based packaging applications (Taylor et al., 2005); in 2008 more than 200 leading companies used NT in agriculture, as well as in food processing, packaging and food supplements (Chaudhry et al., 2008). The possible applications of NT in the food industry seem to be unlimited, promising rapid growth of nano-enabled ingredients, additives, supplements and packaging materials.

1.2.1 NPS applications in the agri-food sector

In the agri-food sector NT is mainly used on the fallowing fields of application (Mura et al., 2013):
- Agricultural production: nanotechnology, applied to the agricultural sector, could play a key role in order to address global challenges such as population growth, climate change and the

limited availability of important nutrients: nanocapsules, nanoparticles, and even viral capsids, are examples of uses for the detection and treatment of diseases, pesticides delivery, nutrients absorption improvement, active ingredients transport in situ (reducing the damage to the plant non-target tissues and the amount of chemical substances released into the environment) and to processes for the treatment of water and soils (Angel Robles-Garcia et al. 2016; Taylor et al., 2005). Specific nanomaterials and nanostructures are developed in order to obtain highly sensitive biochemical sensors for soil analysis, for easy detection and control of chemical compounds, for the treatment of water resources, for the control of pesticides (Abbas et al., 2009). Pesticides can either nano-enabled in order to improve their solubility and the release in water and soil (Grillo et al., 2016). Furthermore, application of biosolids (semi-solids sewage sludge left over from municipal waste water treatment) containing NPs to fertilize agricultural lands has been recorded (Kiser et al., 2009).

- Food-processing: production and transformation processes of raw food materials can be improved with using NT. Nano-sized ingredients and additives are developed to make food less susceptible to deterioration and better suited to long-distance transport (Chaudhry et al., 2008; Letchford and Burt, 2007). Thanks to nanotechnology, it is also possible to influence the sensory properties, reduce salt and fat contents or increase the bioavailability of nutrients, without compromising taste. Furthermore, the antibacterial effect of some NPs is applied to food surfaces for the elimination of bacteria. Other research goals include modifying the technological characteristics such as agglomeration, fluidity, coatings that create more consistency and maintain food texture (Momin et al., 2013).
- Food supplements and functional foods: micelles, and liposomes act as carriers for the controlled delivery of active ingredients such as essential oils, aromas, antioxidants, coenzyme Q10, vitamins, minerals, phytosterols, proteins, enzymes and antimicrobial ingredients. The integrity of these active substances is maintained by encapsulation which also prevents oxidation and the masking of any unpleasant taste in the final product, improves stability, bioavailability and flavours release in cells and tissues (Taylor et al., 2005; Chen et al., 2006).
- Packaging: Food packaging application is considered the most important of nanotechnologies in the food sector for the foreseeable future (Chaudhry et al., 2008). NPs are added to the packaging materials (e.g. Ag, TiO_2, SiO_2, nano-clay) to ensure a better preservation of food, and a longer shelf-life, improving mechanical properties as heat resistance and barrier effect, limiting the loss of aromatic compounds, blocking the entrance of UV light and reducing the proliferation of bacteria and fungi.

NPs can be easily incorporated in polymers used in Food Contact Materials (FCM) production, with the main objective of food protection against chemical and biological deterioration and physical damage (Juneja and Sofos 2005; Dainelli et al., 2008). In food packaging, nanomaterials are of interest to improve the stability, flexibility and barrier properties of packaging, protecting food against dust,

oxygen, light, moisture, microorganisms and pests, and other environmental risks. Moreover, active antimicrobial or oxygen scavenging properties of NPs can be employed to keep food safe from degradation and contamination. Furthermore, some technologies enable nanomaterials to release useful substances (CO_2, ethanol) or absorb unwanted ones (O_2, ethylene, humidity, off-flavors) (Chau et al., 2007).

The potential advantage of NPs in antimicrobial packaging systems remains undisputed, but their increasing use has raised some concern with regard to environmental and health issues (Arvanitoyannis and Bosnea, 2004; Chaudhry et al., 2008). Indeed, the growing list of products containing NPs components increases the probability of human exposure. Several researches have shown that AgNPs are toxic to eukaryotic cells, altering the normal function of mitochondria, increasing membrane permeability and generating reactive oxygen species (ROS) (Martinez-Gutierrez et al., 2010).

To date, for these uncertainties, the number of FCM present on the market containing Ag is still limited.

1.2.2 Silver nanoparticles

Silver nanoparticles (AgNPs) represent the most widely used metal NPs, found in more than 30% of existent nano-enabled consumer products (Vance et al., 2015). An average of 320 t AgNPs per year are produced and are used in a wide variety of processes and manufactured products, such as coatings, textiles, food, electronics, biomedical, and pharmaceutical industries (Konopka et al., 2009), mainly due to their effective properties against fungi, bacteria, viruses, and other microorganisms (Nowack et al., 2011; Piao et al., 2011; Avalos et al., 2013; Gong et al., 2007; Lloret et al., 2012).

AgNPs are currently found in three fields of applications:

- Biomedical, through coatings or integrated into surgical instruments, prostheses, contraceptives, and dressings to prevent bacterial growth (Chen and Schluesener, 2008; Shenava et al., 2015).
- Alimentary, extending food preservation and assembling packages containing them, due to their fungistatic effects that decelerate the growth of pathogenic microorganisms (Kumari and Yadav, 2014).
- Textiles, specifically in the manufacture of clothing, that magnify ion activity generating anti-odor and anti-bacterial effects (Chen and Chiang, 2008).

Although AgNPs are widely used worldwide by their innovative and promising properties, the fate and impacts of these NPs have not been fully studied. AgNPs could migrate to the environment and therefore into humans. In addition, many products that employ NPs are not labelled to alert consumers about the potential risk, eliminating the right to choose or avoid using these products (Leon Silva et al., 2016).

1.2.3 AgNPs released from food packaging

Among all metallic nanomaterials, AgNPs are the most commonly incorporated in food packaging (Emamifar et al., 2010; Guo et al., 2013; Kanmani and Rhim, 2014; Sadeghnejad et al., 2014) helping to extend and improve the shelf-life of the products. One of the critical points of nano-enabled food packaging is the migration: no material is completely impermeable to atmospheric gases, water vapor or natural substances contained within the packaged food or present in the same packaging material. Migration is a phenomenon of contamination of food by means of mass transfer, which occurs especially in containers and plastic packaging; it is influenced by the laws of diffusion (depending on pH, time and temperature) and affinity of the migrant with the contact phase (chemical interaction between NPs polymers and food composition).

In recent years, researches on the migration of NPs from nanomaterials to food matrices have increased, but are still quite scarce. The knowledge of this mechanism is essential to assess the potential exposition to food-related AgNPs and subsequent impacts on human health (Metak, 2015). The most of studies concerned the migration of AgNPs from plastic packing material (PE, LDPE, PP, PVC, and PLA) investigating the release of NPs into food simulants (water, acid, alcohol and fatty foods) in different experimental conditions of time and temperature. A little number of works investigated and quantified the release of AgNPs on the surface of real-food consumer products (Cushen et al., 2013).

PE bags incubated for 15 days with different simulants (deionized water, 4% acetic acid, 95% ethanol, hexane), revealed the release of AgNPs in direct proportion to time and temperature of contact (Huang et al., 2011). Song et al. (2011) determined the migration after 9 hours at 20, 40, and 70° C showing a higher release of AgNPs in 3% acetic acid than 95% ethanol, enhanced by contact time for both simulants. Von Goetz and colleagues (2013) used PVP food containers cut into pieces and exposed to food simulants, demonstrating that the AgNPs quantities released per unit area were significantly higher than in packaging maintained intact and that the containers have an uneven distribution of Ag. They have also shown that the rate of migration decreased by 10 times fold between the first use and subsequent, this is important since the food containers are often used more than once. Echegoyen and Nerin (2013) studied the release of AgNPs from 3 different containers, using various simulants at high temperatures: after 2 h at 70° C, silver was present in all materials containing acetic acid at 3%, but in none of those with ethanol. Migration from all samples stored at 40° C for 10 days was also found. In a recent study Artiaga et al. (2015) showed the flow of AgNPs from bags of PE stored at 20° C for 10 days. A significant migration, of both NPs and ionic species was observed in distilled water and acid acetic acid at 3%, but no in ethanol.

The results obtained indicate that AgNPs migrate into food simulants solutions, but negligibly (ng/cm^2); the migration occurs easily and acids at high temperatures simulants, since it increases the solubility and therefore the release of AgNPs. However, at present data in the literature are limited, thus placing a significant obstacle to a real assessment of the consumer exposure risks (Bradley et al.,

2011). Recently, considerable efforts are made in the research with the aim of developing standards for analytical protocols to better asses NPs presence and their actual migration in foods (Grombe et al., 2015).

1.3 HUMAN EXPOSURE TO SILVER NANOPARTICLES

Human exposure to AgNPs both from environmental and agri-food systems can occur via three main absorption routes: inhalation, ingestion or direct contact. Once penetrated, NPs can reach the systemic circulation and migrate to different organs and tissues by interacting with biological systems and exert their potential toxicity (Oberdorster et al., 2005).

1.3.1 Exposure routes

Absorption via the respiratory system: inhalation. The respiratory system is the route of absorption of NPs (Maynard et al., 2004). Spray disinfectant containing nanosilver are available on the market (EPA 2012). Once inhaled, the AgNPs are deposited on the mucous membranes and are then translocated into the deeper respiratory tract, in inverse proportion to their size (Geiser et al., 2003). Those more voluminous, in fact, stop in the upper respiratory tree and are subsequently expelled through mechanisms of mucociliary clearance, while those of a smaller diameter, caught at the alveolar level, deposited and are absorbed at lung epithelium level, entering the bloodstream (In et al., 2006).
Major translocation mechanisms of NPs to extra-pulmonary organs are represented by phagocytosis of alveolar macrophages and endocytosis by epithelial and endothelial cells (Hoet et al., 2004).
Several studies were conducted in mice and rats. Takenaka et al. (2001) have shown a rapid decrease of the inhaled AgNPs content in the lungs and an accumulation in the liver, kidney and brain (Kim et al., 2009). The exposure to AgNPs for 28 (Ji et al., 2007) and 90 (Sung et al., 2008) days, showed a dose-dependent increase of AgNPs not just in the lungs, but also in the liver, brain and olfactory bulbs. Furthermore, inhaled NPs can reach the brain (Yang et al., 2010) by cross the blood-brain barrier or through the olfactory nerve, after absorption from the nasal mucosa.
Absoption via dermal exposure. Skin, 1.5 m^2 in adults, is the largest human body surface and represents a potential route of exposure to engineered nanomaterials. Antibacterial tissues and lotions containing AgNPs are present as consumer products and, in medical field, soaked dressings of nanosilver are used to treat burns and damaged skin (Vlachou et al., 2007; Wijnhoven et al., 2009). The healthy skin is a barrier difficult to overcome by NPs (Argyle et al., 2009) but the cutaneous absorption is favored within lacerations and dermal irritations (Larese et al., 2009). Once penetrated the dermis, the NPs can reach the lymphatic vessels and be transported by macrophages and dendritic cells. From

lymphatic system AgNPs can access the bloodstream and be distributed systemically to the whole body (Gwinn et al., 2006).

Absorption via Gastro-intestinal system: ingestion. Nanomaterials can enter the body both by swallowing mucus from respiratory tract that incorporates inhaled NPs or by the direct intake of contaminated foods and water, the use of toothpaste and nano-enabled pharmaceuticals (Lomer et al., 2002; Tiede et al., 2008). Another way of food involuntary contamination by NPs consists in biomagnification trough the food chain (Boxall et al., 2006). Once inside the gastrointestinal tract, AgNPs can be absorbed at the enteric level, move to the systemic circulation and be distributed throughout the body (Jani et al., 1990). In vivo studies on rats treated with AgNPs showed a homogeneous distribution of NPs virtually in all the body, but few have been toxic effects observed, and only at higher concentrations of exposition (Kim et al., 2009; Kim et YS al., 2008). Another study on piglets showed higher concentration inside the liver, but no acute toxic effects (Fondevila et al., 2009). Van der Zande et al., 2012, in a 28-day research on mice, observed the presence of Ag in almost all organs, with higher levels in the liver and spleen.

1.3.2 Toxicity

The most common effect associated with chronic exposure to silver on humans is a bluish-gray discoloration of the skin or eyes called argyria (ATSDR 1990; Drake and Hazelwood, 2005). Exposure to the Ag-soluble compounds can also produce other toxic effects, including liver and kidney damage, irritation of eyes, skin, respiratory and intestinal tract, and changes in blood cells with the specific effect of oxidative stress induction inside mitochondria (Martirosyan and Schneider, 2014). Silver and nanosilver have clearly been shown to have a toxic potential even if, in general, the toxicity in humans appears to be low. In vitro studies on humans and other mammals have shown damage on cells from liver, lung, brain, skin, vascular and reproductive tissues (Ahamed et al., 2010). At high doses, AgNPs have been demonstrated to compromise the blood-brain barrier and induce intestinal and neurotoxic problems in rats and mice. At low concentrations changes were observed in liver cell cycle and chromosome stability due to silver ions release (Kawata et al., 2009). Effects on the immune system have been observed as a result of topical treatments on human patients (Kim et al., 2009). However, the lack of accurate data on the pharmacokinetics and toxicology of AgNPs makes an accurate assessment of the risk to human health impossible. Consequently, there is urgent need for more targeted studies into the problems related to human exposure and intake of AgNPs.

It has been recently recognized that the gut microbiota, the community of organisms living within the gastrointestinal tract is an integral part of the human body (Pietrouisti et al., 2016), and that intestinal bacteria are able to grow as mono- and/or dual-species biofilms (Donelli et al., 2012). There are still few reports on the possible toxicological effects of NPs on microbiota/microbiome, and on their

possible effects but available data suggest that AgNPs may affect the microbiota (Pietrouisti et al., 2016; Marrifield et al., 2013).

2 BIOFILM AND NANOPARTICLES

Biofilms are heterogenic microbial communities embedded in a self- produced polymeric matrix attached to a surface (Hall-Stoodley et al., 2004). The biofilm formation is a nearly universal trait enabling bacteria to develop coordinated architectural and survival strategies (Vlamakis et al., 2013) and is now largely accepted that biofilms constitute the predominant microbial lifestyle in natural and engineered ecosystems (Mc Dougald et al., 2011). Bacteria growing as biofilms are distinct from free-swimming planktonic bacteria in their physiology, in gene expression pattern and even morphology (Landini et al., 2010). While planktonic cells rapidly grow to disseminate and colonize new habitats, the sessile form allows bacteria to settle in that particular habitat. As the bacterial cells adapt to grow in these complex communities, they express phenotypic specific traits that confer to biofilms higher resistance to adverse condition and adaptability to environmental changes (Stewart et al., 2008). Microscope observations of sub-aquatic biofilms (i.e., biofilms growing on a solid surface in contact with a liquid) revealed complex spatial organization with pillars, mushroom-like and tree-like structures with water channels that allow an efficient exchange of nutrients, waste products, and signalling molecules (Stoodley et al., 2002).

2.1 BIOFILM FORMATION AND IMPACTS

According to the largely accepted developmental model, biofilm formation occurs because of a sequence of events, where different stages can be identified (O'Toole et al., 2000). The formation of microbial biofilms begins with the reversible adhesion of a small number of cells to a surface where specific interactions, such as electrostatic, hydrophobic, and van der Waals forces, drive the initial attachment (Van Merode et al., 2008). Upon sensing the contact with the surface, bacteria undergo a cascade of metabolic changes and the alteration in structural components such as membrane proteins and transporters, allowing a transient attachment to the surface (Sauer et al., 2001). Environmental signals can activate cellular mechanisms to strengthen the adhesion, make it irreversible, and cells proliferate in clusters forming a monolayer and then multilayered cell clusters (Hinsa et al., 2003; Ono et al., 2014). Intercellular adhesions require an outer adhesive bacterial surface, requirement that can

be satisfied by the synthesis of an adhesive matrix (Karatan et al., 2009). The matrix is composed of extracellular polymeric substances (EPS), i.e. a mixture of polysaccharides, proteins, and nucleic acids that surrounds the bacterial colony, allowing strong cell-to-cell and cell-to-surface interactions towards the differentiation of a mature biofilm (Karatan et al., 2009). EPS are essential in building the 3D biofilm structure, in retaining nutrients for cell growth, and in protecting cells from dehydration and other cellular stresses (Flemming et al., 2007). The last step of biofilm development is the dispersal. Bacterial cells detached from the biofilm re-enter the planktonic state, and may start a new biofilm formation cycle.

Biofilms can colonize different surfaces, either biotic or abiotic, causing a beneficial or detrimental effect on environment, industry and human health (Costerton et al., 1987). Biofilm characteristics are beneficially exploited in the wastewater treatment plants (Nicolella, 2000), for bioremediation (Wu et al., 2015; Dash et al., 2013), for the production of biomaterials, or enhance the effect of plant growth promoting rhizobacteria in soil (Rinaudi et al., 2010).Nevertheless, biofilm can also be destructive, causing chronic infections (Bjarnsholt et al., 2013), parasitism phenomena in animals and plants (Rinaudi et al., 2010), biodeterioration of engineered systems and artworks (Cappitelli et al., 2006), fouling of food-processing equipment (Villa et al., 2012). Indeed, biofilms adhesion to metal surfaces promotes corrosion, clogging of pipelines in food processing plants and reduction of heat transfer efficiency, resulting in important economic losses and high risks for health because of the possible food contamination by pathogens, such as *Listeria*, *Pseudomonads, Bacillus* and *Salmonella* spp. (Tan et al., 2014).

2.2 BIOFILM AND NANOPARTICLES

2.2.1 Biofilm interactions with environmental nanoparticles

Microbial biofilms are an omnipresent component in many environments supporting life and represent highly structured and heterogeneous microenvironments, featuring chemical gradients of important parameters, such as oxygen, pH, and nutrients (Flemming et al., 2001). The inherent properties and physical structure of biofilms resemble that of a sorptive sponge capable of capturing various chemical and biological components in their vicinity (Ikuma et al., 2015). Furthermore, microorganisms in biofilms can facilitate the degradation, sorption, sequestration, accumulation, transformation, generation and trophic transfer of environmental contaminants, colloids and NPs (Strathmann et al., 2003). Natural and engineered systems that are significantly impacted by biofilms include soil mineral surfaces, microbial mats, wastewater treatment, and microbiota associated to

living being. In both natural and artificial environments, the persistence and transport of NPs will be modified by interactions with biofilms and hydrodynamic changes due to biofilm presence (Leon-Morales et al., 2004; Peulen et al., 2011).

It is now recognized that environmental biofilms are efficient binding matrices for NPs (Battin et al., 2009; Ferry et al., 2009; Nevius et al., 2012; Kroll et al., 2014), and this can be attributed largely to the extracellular polymeric substances (EPS) that hold biofilm cells together (Flemming and Wingender, 2010; Nevius et al., 2012). Recent studies have shown that significant accumulations of NPs occurred in biofilms of riverine- and marine-mesocosms (Battin et al., 2009; Ferry et al., 2009). These initial studies point to an important role of biofilms for influencing environmental partitioning of NPs within natural systems. In retrospect, this is not surprising since biofilms are efficient chelators for physicaltrapping and binding of dissolved and colloidal forms of metals and organic matter in a wide range of systems such as wastewater treatment (Wuertz et al., 2000; Hu et al., 2005; Hawari and Mulligan, 2006), drinking-water filtration (Lehtola et al., 2004;Berry et al., 2006), and marine and freshwater systems (Schlekat et al., 1998; Decho, 2000; Battin et al., 2009).

The interactions between NPs and the biofilm can be viewed as a three-step process: (1) transport of NPs to the vicinity of the biofilm; (2) attachment to the biofilm surface; and (3) migration within the biofilm. At each of these steps, the interactions are a complex interplay of factors including NP characteristics, the physicochemical and biological makeup of the biofilm matrix, and environmental parameters such as water chemistry, flow, and temperature. The exact fate of the NPs within biofilms is not clear but accumulation of NPs within biofilms has been previously documented (Ferry et al., 2009; Fabrega et al., 2011). Consequently, since biofilms and their associated EPS are readily consumed by grazing animals (see Decho, 1990, 2000, for reviews), the biofilm presents a potentially efficient vehicle for the trophic-transfer of NPs to food webs.

2.2.2 Nanoparticle effects on biofilms

Biofilm formation represents a community-based persistence strategy leading to community-derived resistance against external factors that usually damage microbial cells (Costerton et al., 1999). The biofilm matrix surrounding the bacterial cells protects them from the action of bactericidal compounds, causing drug resistance and has been reported that bacterial cells in biofilms can tolerate up to 1000 times higher antibiotic concentration than their planktonic counterparts (Huh et al.,2011; Hajipour et al., 2012).

It has been reported by the National Institutes of Health that more than 80% of bacterial infections are caused by biofilm formation and that biofilms can impart antibiotic resistance and sometimes become recalcitrant to the host immune system (Qayyum and Khan, 2016). Thus, due to the critical role of biofilms in infections and infective diseases, their environmental persistence and high resistance to

bactericidal compounds have become areas of major concern for research. A huge corpus of studies has flourished to assess new strategies of biofilm eradication, prevention and management in order to protect both human health and productive processes threatened by biofilm formation.

Due to their high reactivity and possibility of targeted drug delivery NPs has been largely studied for their anti-biofilm properties, and their applications are increasing with time (Ansari et al., 2012; Shen et al., 2013).

NPs can penetrate inside the biofilm structure to destroy it, having the advantage over other commonly employed antimicrobials as they do not differentiate between resistant and susceptible microbes (Rai et al., 2009). The non-specific nature of NPs is also one of their demerits, as they can also destroy symbiotic microorganisms. Earlier reports indicated that NPs disturb the biofilm integrity by interacting with EPS, extracellular DNA, proteins, and lipids of biofilms (Nel et al., 2009). Generation of ROS by the interaction of NPs with microbes damages their cell envelopes, cell membranes, cellular structures and biomolecules (Su et al., 2009). NPs are also being utilized for nano-functionalization of the surface of biomedical instruments like catheters, glass surfaces, etc (Stevens et al., 2009).

NPs obtained from different classes of metals have shown antibiofilm potential. The different metal NPs inhibit biofilms via different mechanisms. Metal NPs which have shown antibiofilm activity are silver NPs (AgNPs), silver-based nanocomposites (NMs), iron NPs (FeNPs), copper NPs (CuNPs),

zinc NPs (ZnNPs) and magnesium NPs (MgNPs). In this thesis we considered AgNPs due to their importance in human and environmental exposure as discussed in sections 1.2 and 1.3.

2.2.3 AgNPs effects on biofilm

Several earlier works have reported that AgNPs have the highest efficacy among the investigated metal NPs (Chatterjee et al., 2014). AgNPs are now used in various applications like pharmaceuticals drug delivery systems and development of nanosensors (Chaloupka et al., 2014).

The activity of AgNPs depends on their shape, size, zeta potential and particle chemistry (Panaeck et al., 2006; Pal et al., 2007). Silver nanoparticles probably have multiple mechanisms of antibacterial action, but due to the current dearth of knowledge on this subject, the exact basis for the activity of AgNPs is still uncharacterized (Markowska et al., 2013). Some studies have shown that AgNPs release Ag+ ions in the presence of water (Santoro et al., 2007; Asharani et al., 2008; Damm and Münstedt, 2008). Hence, it was suggested that nano-silver affects bacterial membrane permeability by attaching to the cell membrane surface and modifying the cell potential. Observation of large numbers of nanoparticles inside bacteria suggests that this is important to the antibacterial mechanism (Morones et al., 2005). Proteomic analysis of *E. coli* cells revealed that short-exposure to AgNPs resulted in the accumulation of envelope precursors, which is indicative of the dissipation of the proton motive force.

Proteins whose expression was found to be stimulated by AgNPs over 1.8-fold were the inclusion body binding proteins which serve as molecular chaperones, and 30S ribosomal subunit protein S6 (Lok et al., 2006). Furthermore, AgNPs have been shown to interact with bacterial membrane proteins, intracellular proteins, phosphate residues in DNA, and to interfere with cell division, leading to bacterial cell death (Sondi & Salopek-Sondi, 2004; Xu et al., 2004). Presence of biocidal Ag+ ions released from the nanoparticle surfaces evokes bacterial DNA conglomeration defence mechanisms, which protect the cell from toxic effects, but simultaneously compromises its replication ability. Some studies have reported that nano-silver causes oxidative damage, leading to the production of reactive oxygen species (ROS) (Kim et al., 2007; Hwang et al., 2008), as one of the primary mechanisms of nanoparticle toxicity (Khan, 2012).

The anti-biofilm activity of silver nanoparticles has been demonstrated in a number of studies. Small but significant decreases in the biomass of 24-hour *Pseudomonas putida* biofilms were observed by Fabrega and coworkers (2009) in the first report that discussed interactions between well quantified and characterized bacterial biofilms and silver nanoparticles. AgNPs impregnated on the surface of the disc inhibited the formation of *S. epidermidis* biofilms. The antibacterial efficacy of the disc was retained even after several washings, thus ensuring its reusability (Furno et al., 2004). *Candida albicans* is a fungal pathogen which infects many human organs involving biofilm formation. Several earlier investigators have evaluated the antibiofilm activity of AgNPs against the biofilms of *C. albicans* and *Candida glabrata*. It has been reported that AgNPs were more effective against adhered cells in comparison to biofilm cells except for *C. glabrata* where both the biofilm and adherent cells were reduced by AgNPs (Montero et al., 2011). The antibiofilm activity of three different sized AgNPs (5, 10 and 60 nm) was observed on *C. albicans*. It has been demonstrated that the particle size is not a reason for their efficacy (Montiero et al., 2012). Silva et al. (2013) evaluated the effect of AgNPs and the antifungal agent nystatin on single and dual species biofilms of *C. albicans* and *C. glabrata*. These AgNPs have shown a greater effect on *C. glabrata* biofilm biomass as compared to that of *C. albicans*. Biofilm formation is significant in both medical and industrial setups. In industrial setups, biofilms are responsible for huge loss of money due to biofouling. Biofilm formation occurs under static as well as dynamic conditions in industrial setups. This has also raised another point of concern for scientists, as most of the studies on biofilms are performed under static conditions. Martinez-Gutierrezet al. (2013) studied the effect of AgNPs on biofilms under static and high fluid shear conditions using a bioreactor. The study was performed by taking a panel of microorganisms and the results showed that AgNPs effectively prevented the formation of biofilms because these AgNPs were found to be highly toxic to bacteria in the established biofilms. A comparative study on sensitive and resistant strains demonstrated that antibiotic-sensitive strains were more inhibited than resistant strains by AgNPs (Palanisamy et al., 2014).

It has also been noticed that AgNPs have enhanced the effects of many other antimicrobials when used in combination (Habash et al., 2014; Thomas et al., 2014). The antibacterial activities of chloramphenicol, kanamycin, erythromycin and ampicillin against Gram-positive and Gram-negative bacteria. The activity of cefoperazone against methicillin resistant *S. aureus* (MRSA) were increased in the presence of AgNPs (Souza et al., 2006). Composites of AgNPs with other metals and compounds have been found to enhance their antibiofilm activity. Lungu et al. (2013) demonstrated that Ag–TiO_2 NCs exhibited very strong antibiofilm activity. AgNPs obtained from Nanoparticle Biochem, Inc. (Columbia, USA) inhibited the production of EPS which further led to antibiofilm action against drug-resistant strains of *E. coli* and *K. pneumoniae*. The antibiofilm action of AgNPs against MRSA and methicillin-resistant *S. epidermidis* has already been reported (Asnari et al., 2012, 2015).

Some new anti-biofilm approaches are based on the coating of medical devices or improvement of the properties of biomaterials. Silver has been proposed as a component of coatings that may have potential in combating biofilm formation. AgNP-coated catheters showed in vitro antimicrobial activity and prevented the formation of biofilms of *Escherichia coli*, *Enterococcus* spp., *Staphylococcus aureus*, coagulase-negative *Staphylococci* spp., *Pseudomonas aeruginosa* and *Candida albicans* on their surface (Roe et al., 2008). A monolayer of AgNPs anchored to an amino-silanized glass surface showed antibiofilm activity against *S. aureus* biofilms. Nano-functionalization of the catheter tube is a better approach which can further be employed in many medical and industrial applications (Taglietti et al., 2014). Finally although silver nanoparticles (AgNPs) are used as antimicrobial agents in a wide variety of commercial products, has been demonstrated by Yang and colleagues (2015) that sublethal exposure can counterproductively promote the development of biofilms.

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CHAPTER 2

AIMS OF THE PROJECT

The impacts of AgNPs on both natural and engineered ecosystems is a topic of outstanding importance having socio-economic consequences on medical, industrial and environmental fields**.** Silver nanoparticles are widely used as antimicrobial agents in consumer products for domestic, environmental, medical, and industrial applications. Release of AgNPs from nanoenabled products has been observed, and the potential impacts of such releases on a wide variety of organisms at many trophic levels have been recognized. However, there has been little exploration of the impact of AgNPs on the microflora associated with living organisms and their environments. For instance, of special interest is the effects on the human microbiota considering the range of consumer goods that could be directly or indirectly ingested.

Furthermore, most of the studies concerning toxic effects of AgNPs on biologic systems consider high NPs concentrations, while the effects of real environmental and dietary concentrations are still poorly investigated.

Thus, the principal aim of my PhD project was to provide **science-based evidence needed to elucidate the effects of sub-lethal concentrations of AgNPs on bacterial ecosystems, with the final goal of creating the scientific know-how to master biological processes and develop leading edge methodologies vital for the nanosafety assessment**. In particular, I focused my attention on **studying the response of *in vitro* gut microbial models and other anaerobic ecosystems to acute and chronic AgNPs exposures at vicinity of environmental and human intake concentrations**.

To this end, three different systems have been investigated:

1) Planktonic cultures of two well characterized bacterial strains (Chapter 3). The aim of this work was to compare the impacts of different sub-lethal AgNPs concentrations on the growth kinetic, adhesion ability, oxidative stress, and phenotypic changes of model bacteria under both aerobic and anaerobic conditions. To gain a mechanistic insight, the experiments were conducted using two different microbial model systems: (i) a Gram-negative bacterium *Escherichia coli* representative of human intestinal flora and responsible for infection, and (ii) a Gram-positive bacterium *Bacillus subtilis*, widely distributed in soil, freshwater, marine environments and used as a probiotic. I also established the minimum AgNPs sub-lethal concentration able to evoke effects on planktonic bacteria.

2) Biofilm cultures of the model bacterium *Escherichia coli* and their interplays with CaCo2 cells system (Chapter 4). The goal was to investigate the physiological response of a mono-species gut biofilm to chronic and acute exposure to 1 μg/mL AgNPs, and how this physiological response affected the intestinal epithelial cells. To study the interplays among sub-lethal concentrations of AgNPs, the gut biofilm and its host, a simplified experimental lab model system was designed and tested.

3) Human fecal microbiota in combination with the probiotic *Bacillus subtilis* (Chapter 5). I aimed to explore possible impacts of single and combined treatments of dietary AgNPs and the probiotic *Bacillus subtilis* to the composition, functional performances and microbial metabolites of in-vitro batch fecal fermentation models to mimic the human digestive tract environment. Furthermore, I investigated their potential cytotoxicity and genotoxicity on the human intestinal Caco-2 cell line.

These experimental designs were created to investigate microbial ecosystems of increasing complexity, assessing whether sub-lethal concentrations of AgNPs influence microbial physiology and behavior in such settings.

CHAPTER 3

Effects of sublethal concentrations of silver nanoparticles on *Escherichia coli* and *Bacillus subtilis* under aerobic and anaerobic conditions

The present work is aimed at comparing the effects of sublethal concentrations of silver nanoparticles (AgNPs) on the growth kinetic, adhesion ability, oxidative stress and phenotypic changes of model bacteria (*Escherichia coli* and *Bacillus subtilis*) under both aerobic and anaerobic conditions. Growth kinetic tests conducted in 96-well microtiter plates revealed that sublethal concentrations of AgNPs do not affect *E. coli* growth, whereas 1 μg/mL AgNPs increased *B. subtilis* growth rate under aerobic conditions. At the same concentration, AgNPs promoted *B. subtilis* adhesion, while it discouraged *E. coli* attachment to the surface in the presence of oxygen. As determined by 2,7-dichlorofluorescein-diacetate assays, AgNPs increased the formation of intracellular reactive oxygen species, but not at the highest concentrations, suggesting the activation of scavenging systems. Finally, motility assays revealed that 0.01 and 1 μg/mL AgNPs respectively promoted surface movement in *E. coli* and *B. subtilis* under aerobic and anaerobic conditions. The results demonstrate that *E. coli* and *B. subtilis* react differently from AgNPs over a wide range of sublethal concentrations examined under both aerobic and anaerobic conditions. These findings will help elucidate the behavior and impact of engineered nanoparticles on microbial ecosystems.

1 Introduction

Due to their unique chemical-physical properties, (e.g. reactivity, semiconductor and catalytic properties), nanoparticles (NPs) are today commonly used for commercial and industrial purposes. Silver nanoparticles are the most widely used metal NPs in nano-enabled consumer products (Vance et

al., 2015). In 2014, around 30% of nanotechnology-enhanced commercial products contained AgNPs (Vance et al., 2015). Due to their antimicrobial effects on a wide spectrum of microorganisms, such as gram positive and negative bacteria and yeasts (Morones et al., 2005; Kim et al., 2007), AgNPs are employed in different fields including food packaging, textile industry, medical devices, water treatments, cosmetics and coatings (Choundry et al 2008, Silvestre et al 2011, Reidy et al 2013).

The release of AgNPs from nano-enabled products and treated areas has been observed (Blaser et al., 2008; Kaegi et al., 2008; Gottschalk et al. 2009; Kaegi et al., 2010). Moreover, AgNPs are used as biocides at relatively high concentrations, but downstream from the treated areas there is likely to be a continuum of AgNP dispersion ranging from biocidal to non-biocidal concentrations. Thus, low (sublethal) AgNP concentrations are expected to accumulate in both natural and engineered environments following dilution and dispersion phenomena (Benn et al., 2008; Colman et al., 2013; Khaksar et al., 2015). Indeed, predicted environmental concentrations (PECs) of AgNPs ranging from 1 pg/mL to 10 ug/mL have been found in both solid and liquid environmental matrixes (Gottshalk et al., 2013; Nowalk et al., 2009; Batley et al., 2012; Massarsky et al., 2014).

To the best of our knowledge, only a few scientific works have investigated the effects of sublethal AgNP concentrations on microbial systems (Yang and Alvarez, 2015; Gambino et al., 2015; Wang et al., 2015) therefore their impact on microbial physiology and behavior still remains almost unknown. Moreover, despite the growing body of literature regarding nanoparticles in biosolids (Doolette et al., 2013; Miller et al., 2013; Yuan et al., 2015), little is known about the effects of sublethal concentrations of AgNPs on anaerobic ecosystems. Anaerobic conditions can occur within many natural and engineered ecosystems that act as AgNPs sinks, such as soil and sediments, gut and wounds, as well as wastewater and sludge treatments. According to the literature, AgNPs display different modes of action without and with oxygen. Xiu et al. (2011 and 2012) showed the lack of toxicity of AgNPs on pure bacterial cultures of *Escherichia coli* when synthesized and tested under strictly anaerobic conditions that hinder Ag(0) oxidation and Ag^+ release. Furthermore, the absence of dissolved oxygen precludes the generation of reactive oxygen species (ROS) responsible for a part of AgNP antimicrobial activity (Fabrega et al., 2009; Fu et al., 2015).

However, on studying potential pathways for NP release, and its sinks in the environment, it turns out that engineered NPs are generally released first into aerobic compartments by human activity, from where they can migrate to anaerobic ones (e.g. from water to bottom sediments, from the mouth to the gut) (Gottshalk et al., 2009; Kohler et al., 2013; Sun et al., 2015; Mc Kracken et al., 2016). In addition, in the presence of dissolved oxygen, AgNPs are able to release Ag^+ and promote ROS formation,

amplifying their effects within different environmental and biological compartments. Thus, it is reasonable to expect that an anaerobic environment, which in principle should preclude Ag^+ and ROS formation, might be affected by the reactive species of reactive species introduced by NP modifications and reactivity activated from within aerobic environments. In the light of these considerations, the feedback response of facultative anaerobic microorganisms to AgNPs coming from an aerobic environment (active AgNPs) remains unclear.

The present work tests whether exposure to AgNPs triggers notable changes in the physiology and activity of bacteria under aerobic and anaerobic conditions. To gain a mechanistic insight, the experiments were conducted using two different microbial model systems: i) a gram-negative bacterium *E. coli* representative of human intestinal flora and responsible for many common infections; and ii) a gram-positive bacterium *Bacillus subtilis*, widely distributed in soil, freshwater and marine environments. The effects of sublethal concentrations of AgNPs on the growth kinetic, adhesion ability, oxidative stress and phenotypic changes of the selected model bacteria under both aerobic and anaerobic conditions were investigated.

2 Materials and Methods

2.1 Bacterial strains and planktonic growth conditions

*Escherichia coli M*G 1655 and *Bacillus subtilis (natto)* ATCC 6051 strains were stored at –80°C in phosphate-buffered saline (PBS) solutions containing 20% glycerol. Both microorganisms were routinely cultured in Tryptic Soy Broth medium (TSB, Conda, Italy) at 37°C in aerobic or strict anaerobic conditions. Anaerobic experiments were performed in an anaerobic cabinet (Forma Scientific, Marietta, OH, USA) under N_2:H_2:CO_2 atmosphere (85/10/5, v/v) using TSB medium prereduced in anaerobic conditions for 24 h before experiments began.

2.2 Silver nanoparticle characterization

Silver nanoparticle (AgNPs; 10 nm, OECD PVP BioPure Silver Nanoparticles, NanoComposix, San Diego, CA, USA) stock solutions of 1 mg/mL concentration in aqueous 2 mM citrate were stored at 4°C and resuspended directly in bidistilled water or culture media just before their use in the experiments. According to the supplier, purchased AgNPs have a diameter of 8.5±1.7 nm (JEOL 1010 Transmission Electron Mcroscope), a hydrodynamic diameter smaller than 20 nm and a negative zeta potential of-

27.3 Mv (Malvern Zetasizer Nano ZS). In this study, AgNP size and shape were determined by Transmission Electron Microscopy (TEM) with the following protocol: a drop of 10 mg L-1 AgNPs was placed on formvar/carbon coated nickel grids and dried at room temperature. Grids were examined by an EFTEM LEO 912AB transmission electron microscope (Zeiss) working at 80 kV. AgNP diameter was measured by Esivision software and average and standard deviations were calculated. Ag concentration in AgNP suspensions was determined by flame atomic absorption spectroscopy (F-AAS; Thermo-Electron Atomic Absorption Spectrometer) after addition of 1% HCl.

2.3 AgNPs stability in solution

According to the manufacturer, the simplest way to assess AgNP stability is to monitor the UV/Visible optical spectrum of NPs in solution. As AgNPs support electron oscillations (known as plasmon resonances) they have unique spectra, a function of size, shape and concentration. In this study, the AgNPs had, as indicated by the producer, a wavelength peak of 390 nm in stable conditions, the intensity being proportional to the concentration of the AgNPs in solution.

Three different microbiological growth media were considered:

(i) TSB medium.

(ii) Luria Bertani medium (LB, CONDA, Italy).

(iii) Tryptic Soy Yeast broth (TSYb, CONDA, Italy), prepared according to Sproule-Willoughby et al. (2010) with the addition of D-glucose to reach 0.5% concentration.

For the UV/Visible test 1 mL aliquots of sterile LB, TSYb and TSB media were prepared and AgNP was added to a final concentration of 0.005 mg/mL as the supplier suggested for this analysis. Incubation was conducted in both anaerobic and aerobic conditions at 37°C, and was monitored for up to 24 h. Every 2 h, 100 µL of each solution was diluted in 900 µL of MilliQ water. Spectra were obtained using the JENWAY 7315 Spectophotometer with 320 to 500 nm absorbance range. For each medium a 1 mL control sample without AgNPs was prepared and the spectra recorded under the same experimental conditions. The experiment was repeated in triplicate. The area under the peak was calculated using GraphPad Prism software (version 5.0, San Diego, CA, USA).

2.4 Planktonic growth in presence of AgNPs

Both *E. coli* and *B. subtilis* were grown for 24 h in 100 mL glass vials, each containing 41 mL of TSB. The vials were inoculated with 1 mL (2.4%) of overnight cultures. Inocula absorbance at 600 nm (A_{600}) was measured using the JENWAY 7315 Spectophotometer, the concentrations being assessed by specific calibration curves and then adjusted to gain an initial concentration of 10^5 ($\pm 2x10^4$) cells/mL for both bacteria. Bacteria were cultured in the presence of different AgNP concentrations (0, 0.01, 0.1, 1 µg/mL), in anaerobic and aerobic conditions. Every 2 h, 600 µL of culture were withdrawn from each vial and homogeneously divided into three wells of transparent 96 well- microtiter plates (Greiner bio-one). The absorbance at 600 nm (A_{600}) was measured using the Infinite F200 PRO microtiter plate reader (TECAN, Mannedorf, Switzerland). Absorbance-based growth kinetics were constructed according to Cattò et al. (2015). Briefly, the A_{600} of suspensions minus the A_{600} of the non-inoculated medium were plotted against the incubation time, and the polynomial Gompertz model (Zwietering et al., 1990) was used to calculate the maximum specific growth rate (MSGR, A_{600}/h) and lag phase length (LPL, h) using GraphPad Prism software (version 5.0, San Diego, CA, USA). Each treatment was performed in triplicate.

2.4 Adesion assay in presence of AgNPs

Adhesion assays were performed using the same AgNP concentrations of the planktonic growth curve experiments. *E. coli* and *B. subtilis* adhesion was quantitatively assessed according to Villa et al. (2010) with some modifications. Briefly, the cells were resuspended in fresh TSB supplemented with 0 (negative control), 0.01, 0.1, 1 µg/mL AgNPs in hydrophobic 96-well black-sided plates (Greiner bio-one, Italy). The cells were incubated in anaerobic and aerobic conditions for 18 h at 37°C and, after 3 washing steps, adhered cells were stained using 10 µg/mL 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS for 20 min in the dark at room temperature. Fluorescence intensity was measured using the Infinite F200 PRO microtiter plate reader (TECAN, Mannedorf, Switzerland) at an excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the adhered number of cells/mm^2. Eight replicates of each condition were performed. The experiment was repeated four times. Obtained data were normalized to the negative control and reported as the mean of these data. Percentage reduction in comparison to the control was also calculated.

2.5 Level of oxidative stress on planktonic cells

The level of oxidative stress in planktonic *B. subtilis* and *E. coli* was determined using the 2,7-dichlorofluorescein-diacetate (H_2DCFDA, Sigma Aldrich Italy) assay (Jakubowsky et al., 2000). Planktonic cells grown at 37°C for 15 h in TSB in both aerobic and anaerobic conditions, with 0 (negative control), 0.01, 0.1, 1 μg/mL AgNPs, were washed twice with PBS (13000 rpm, 15 min) and resuspended in 50 mM PBS. The cells were then broken using glass beads (0.1 μm diameter) and the Precellys 24 (Bertin technologies, France) bead-beater device with a beating profile of 3x30s. After centrifugation, 750 μL of supernatant was incubated with 4 μL 10 mol H_2DCFDA at 30°C for 30 min. The solution was homogeneously divided in three wells of 96 wells black microtiter plates (Greiner bio-one). The relative fluorescence correlated to the reactive oxygen species (ROS) amount was measured with excitation at 490 nm and emission at 519 nm using the Infinite F200 PRO microtiter plate reader (TECAN, Mannedorf, Switzerland). Experiments were conducted in triplicate. The relative fluorescence was normalized against the number of cells, obtained by a viable count of initial cell suspensions: serial dilutions of 0.01 mL cell suspensions were plated on Tryptic Soy Agar (TSA, Fisher Scientific, Italy) and incubated overnight at 30°C. Colony forming units (CFU) were determined by the standard colony counting method.

2.6 Motility assay

Swimming and swarming assays were performed to study the AgNP effects on bacterial motility in both aerobic and anaerobic conditions. Experiments were set up according to the protocol described by Gòmez-Gòmez et al. (2007). Briefly, *E. coli* and *B. subtilis* were grown in TSB medium supplemented with 0 (negative control), 0.01, 0.1, 1 μg/mL AgNPs. Cultures were grown overnight in both anaerobic and aerobic conditions at 37°C. The swimming motility plates were prepared with TSB added with 0.3% Agar (Conda, Italy), the swarming motility plates were prepared with TSB added with 0.7% Agar. A 10 μL drop of each overnight culture was inoculated in the center of TSA plates. Plates were incubated at 37°C in both aerobic and anaerobic conditions and colony diameters were measured after 24, 48 and 96 h of incubation. Experiments were conducted in quadruplicate.

2.7 Statistical analysis

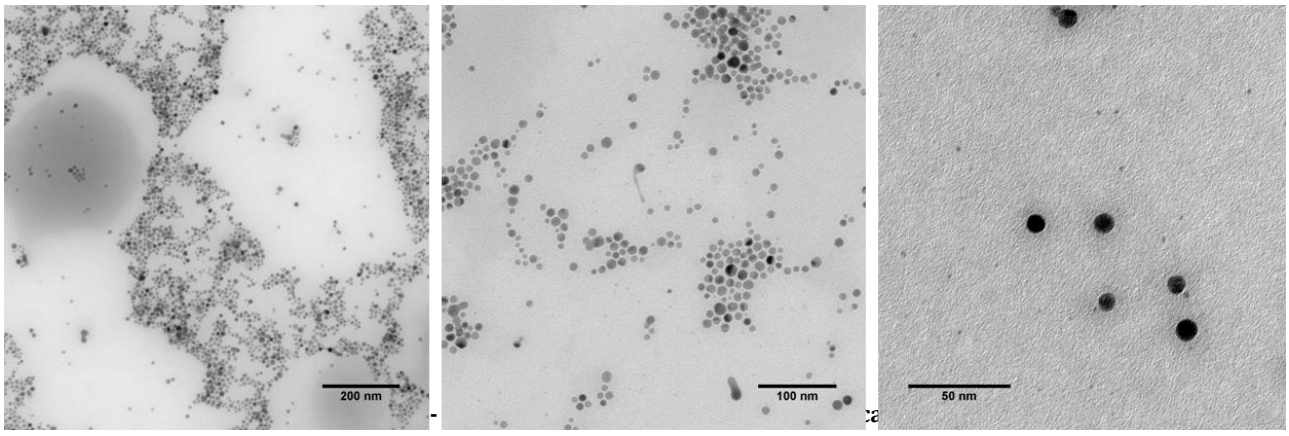
Analysis of variance test (ANOVA) was performed using MATLAB software (Version R2014b, The MathWorks Inc., Natick, USA) to statistically evaluate significant differences among samples. The ANOVA analysis was carried out after verifying whether the data satisfied the assumptions of i)

independence, ii) normal distribution and iii) homogeneity of variance. Tukey's honestly significant different test (HSD) was used for pair-wise comparison to determine data significance. Differences were considered significant for $p<0.05$.

3 Results

3.1 AgNPs characterization

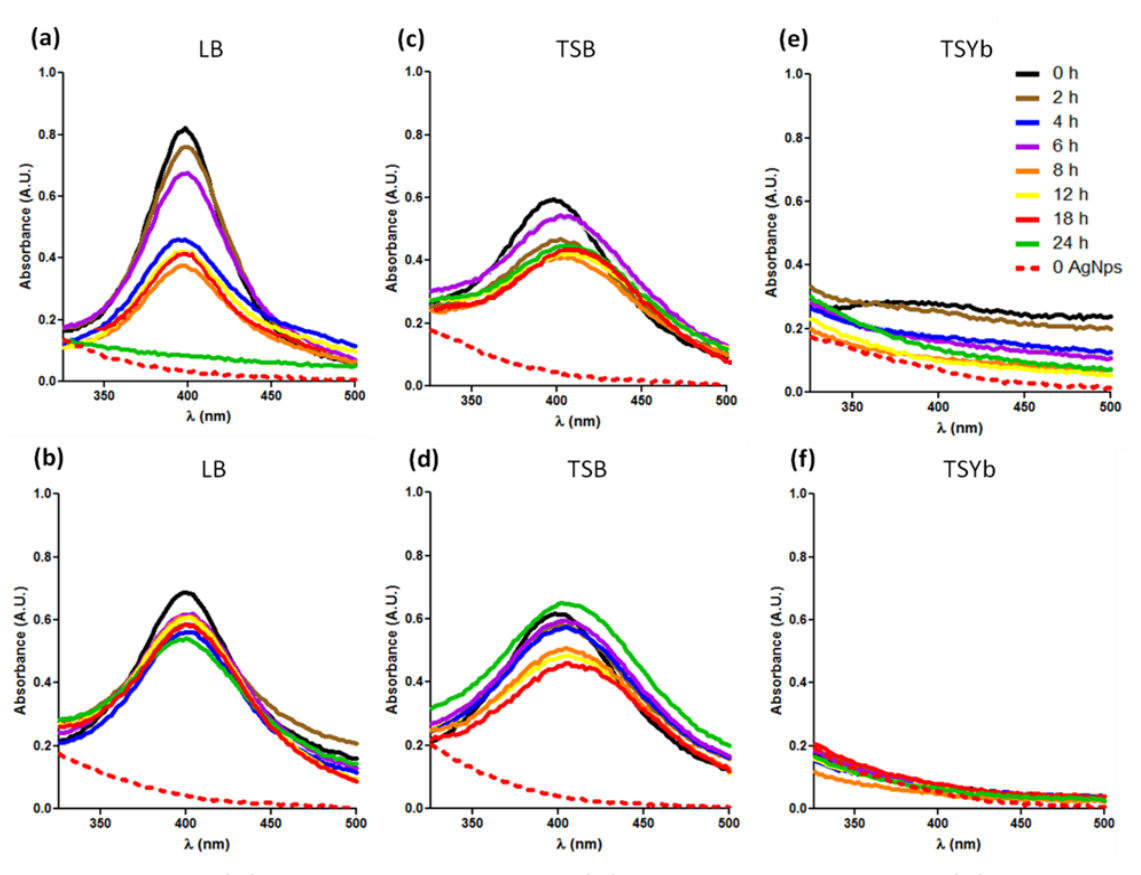
The shape and size of AgNPs were determined by TEM analysis (Fig. 1). The average size of the AgNPs calculated from TEM images was 14 ± 0.3 nm (n=402) with 77% of the particles ranging from 5-17.5 nm. F-AAS data showed that the concentration of Ag in the stock suspension was 1 mg/mL, as reported by the manufacturer.



3.2 AgNPs stability in solution

The stability of diluted AgNPs at a concentration of 50 µg/mL in LB, TSB and TSYb media was investigated to establish the medium with the highest AgNP bioavailability in liquid cultures (Sondi et al,. 2004). Results showed a visible decrease of the expected 390 nm peak in all of the three media during the first 8 h of incubation, both under aerobic and anaerobic conditions, indicating a progressive loss in concentration of suspended AgNPs (Fig. 2). In aerobic conditions, the LB-AgNPs spectra showed a very high 390 nm peak at time 0, indicating a very high AgNP dispersion, but after 18 h of incubation a steady decrease of the 390 nm peak was recorded, indicating a significant loss of

AgNP in suspension (Fig. 2a). In anaerobic conditions, the LB-AgNP spectra showed a lower peak but more stability throughout the experiment (Fig. 1b). The TSB-AgNP spectra showed, in both aerobic and anaerobic conditions, an initial slight decrease of the 390 nm peak that became stable after 8 h of incubation (Fig. 2 c,d). The TSYb–AgNP spectra highlighted a precipitation of the AgNPs immediately after their addition in both aerobic and anaerobic conditions. Indeed, in the TSYb, precipitated AgNPs were even well-visible at the bottom of the tube after 6 h of incubation (Fig. 2 e,f). Areas under the AgNP peak (320-500 nm) were also calculated for all the media up to 24 h of incubation at 37°C (Fig. 2). In aerobic conditions, the LB area values differed significantly from 0 h, 8 h of incubation being comparable to the control at 24 h, showing a severe loss of AgNP solubility.



TIME (h)	AEROBIC			ANAEROBIC		
	LB	TSYB	TSB	LB	TSYB	TSB
Control	5.97±0.54 ●	8.20±3.49 ●	33.89±8.93 ●	7.44±1.57 ●	8.09±0.15	8.74±1.81 ●
0	51.22±7.22	37.97±2.07	86.08±0.73	58.44±6.09	6.85±1.35 o	56.21±4.43
2	59.19±11.43	35.76±2.82	76.81±12	59.15±9.44	7.04±0.37 o	59.02±13.18
4	46.06±0.53	22.75±4.43 ●	92.09±0.61	49.05±7.40	5.85±2.59 o	58.87±9.89
6	39.88±12.20	15.39±7.90 ●o	94.01±7.58	55.83±4.82	6.81±0.27 o	62.08±7.66
8	25.85±0.24 ●	7.16±5.5 ●o	69.71±5.25	53.82±0.33	4.64±0.89 o	51.72±4.41
12	31.36±0.00 ●	11.18±0.00 ●o	79.03±0.00	53.67±1.25	5.66±1.35 o	50.59±10.18
18	26.82±13.54 ●	18.37±0.88 ●o	101.90±30.53	51.86±0.52	7.84±0.46 o	46.82±7.19
24	6.23±1.37 ●o	9.42±2.01 ●o	98.96±26.36	52.86±1.21	6.13±1.75 o	71.15±3.42

Figure 2 - Absorbance spectra measured from wavelengths (λ) 320 nm to 500 nm of 0.05 mg/mL AgNPs within LB (a/b), TSB (c/d), TSYb (e/f) in both aerobic (a/c/e) and anaerobic (b/d/f) conditions, investigated up to 24 h of incubation at 37°C. The peak at 390 nm is proportional to the AgNPs in solution. The table reports areas under spectra (A.U., from 320 to 500 nm) of 0.05 mg/mL AgNPs within LB (a/b), TSB (c/d), TSYb (e/f) in both aerobic (a/c/e) and anaerobic (b/d/f) conditions, investigated up to 24 h of incubation at 37°C. Data represent the means ± standard deviation (SD) of three independent measurements. Asterisks and dots provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing ● show statistical difference to 0 h, and means sharing o show statistical similarities with 0 mg/mL AgNPs negative control.

In contrast, the 0 to 24 h LB area values in anaerobic conditions were statistically similar, different from the AgNP control, in line with spectra observations. In both aerobic and anaerobic conditions the TSB samples always showed a statistical difference in comparison to the control, as well as no differences with respect to the initial concentration of AgNPs in solution, highlighting a constant AgNP concentration during the experiment. A statistical analysis of the TSYb areas showed the absence of AgNPs in solution after 24 h of incubation in both aerobic and anaerobic conditions. Indeed, in the presence of oxygen there was a significant difference between the area values at 0 h and 4 h of incubation, indicating an initial decrease of AgNp solubility in the medium. In the same conditions the samples were similar to the control at 6 h incubation. In contrast, in anaerobic conditions, the samples showed no statistical difference at 0h for the entire experiment, with area values comparable to the control without AgNPs.

Overall, TSB was assessed as the medium providing more stable AgNP concentrations in both aerobic and anaerobic conditions, guaranteeing maximum AgNP stability in cultures. Thus, TSB was the medium used in the subsequent experiments.

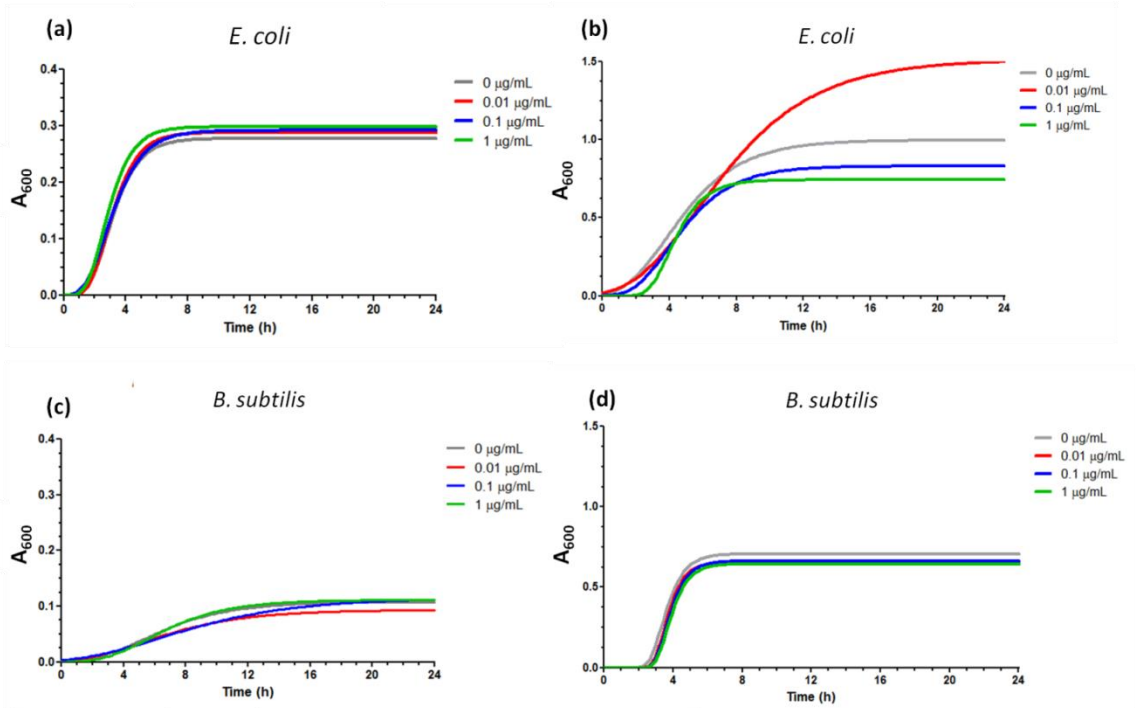
3.3 Planktonic growth in presence of AgNPs

Planktonic growth tests in TSB were performed at different AgNP concentrations (0, 0.01, 0.1, 1 μg/mL), chosen as sublethal environmental concentrations[14,30]. As shown in Fig. 3, *E. coli* and *B. subtilis* were able to grow in all the tested conditions. The presence of oxygen promoted the growth of both bacteria, while the anaerobic environment resulted in shallower growth curve slopes and lower OD 660nm values. Instead, AgNPs seemed to have little effect on bacterial growth compared to the control in both conditions.

To better study planktonic growth the curves were analyzed further, and the Maximum Specific Growth Rate (MSGR) and Lag Phase Length (LPL) were calculated for each condition. For all AgNP

concentrations, the *E. coli* and *B. subtilis* MSGR were significantly higher in aerobic conditions with respect to anaerobic, suggesting a faster cellular metabolism in the presence of oxygen.

For *B. subtilis,* the LPL aerobic values were significantly lower than in anaerobic conditions, showing a faster adaptation of the bacterium to the growth conditions in the presence of oxygen. On the contrary, the *E. coli* LPL values were higher in aerobic conditions than anaerobic conditions, suggesting a longer adaptation time to growth conditions in the presence of oxygen.



BACTERIUM	NPs (µg/mL)	AEROBIC			ANAEROBIC		
		MGR (A_{600}/h)	LPL (h)	R^2	MGR (A_{600}/h)	LPL (h)	R^2
E. coli	0	3.70E-01±1.16E-02[a]	2.368±0.096[a]	0.952	1.00E-01±1.16E-02[a]	1.600±0.152[a]	0.905
	0.01	3.83E-01±2.88E-02[a]	2.627±0.044[a]	0.910	9.03E-02±1.33E-02[a]	1.571±0.126[a]	0.932
	0.1	3.84E-01±2.10E-02[a]	2.605±0.179[a]	0.941	9.03E-02±4.83E-03[a]	1.590±0.126[a]	0.982
	1	3.70E-01±2.12E-02[a]	3.007±0.044[a]	0.929	1.06E-01±1.78E-02[a]	1.604±0.088[a]	0.952
B. subtilis	0	1.64E-01±2.34E-02[a]	2.274±0.429[a]	0.977	7.38E-03±1.26E-03[a]	6.163±1.388[a]	0.949
	0.01	1.51E-01±0.070[a]	1.960±0.070[a]	0.980	6.99E-03±2.29E-03[a]	6.666±0.912[a]	0.883
	0.1	1.79E-01±0.245[a]	2.182±0.245[a]	0.981	7.22E-03±1.66E-03[a]	6.081±1.028[a]	0.905
	1	2.34E-01±0.106[b]	2.775±0.106[a]	0.992	7.09E-03±1.52E-03[a]	6.250±1.011[a]	0.884

Figure 3 - A_{600}-based growth curves of *E. coli* (a,b) and *B. subtilis* (c,d) in presence of different concentrations of AgNPs (0, 0.01, 0.1, 1 µg/mL) in both aerobic (b,d) and anaerobic conditions (a,c). The table provides the growth parameters Lag Phase Length (LPL) and Maximum Specific Growth Rate (MSGR) of both *E. coli* and *B. subtilis* in presence of different concentrations of AgNPs (0, 0.01, 0.1, 1 µg/mL) in both aerobic and anaerobic conditions. Data

represent the means ± SD of three independent measurements. Letters provide the graphical representation for post hoc comparisons. The histogram provides the *p*-values obtained by the ANOVA analysis. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

Comparing the effects of the different AgNP concentrations, significant differences were observed only at the highest concentration used in the presence of oxygen. In such conditions, *E. coli* showed a significantly higher LPL value than at other concentrations, and *B. subtilis* an increased MSGR. In anaerobic conditions, there were no differences in *E. coli* and *B. subtilis* LPL and MSGR at all AgNP concentrations.

3.4 Adhesion assay in presence of AgNPs

After overnight incubation at 37°C, in aerobic and anaerobic conditions, *E. coli* and *B. subtilis* showed similar numbers of adhered cells/mm^2 (Fig. 4), and both bacteria showed aerobic values significantly higher than the anaerobic ones in the control. *E. coli* adhered cells (Fig. 4a) decreased as AgNP concentration increased, showing a descendent trend between 0-0.01 µg/mL and 0.1-1 µg/mL in aerobic conditions and between 0-0.1 µg/mL in anaerobic conditions. The findings also demonstrate that *B. subtilis* adhesion in anaerobic conditions (Fig. 4b) was not affected by AgNPs whilst, in the presence of oxygen, adhered cells increased 3.5-fold with respect to the control at the highest AgNP concentration (1 µg/mL).

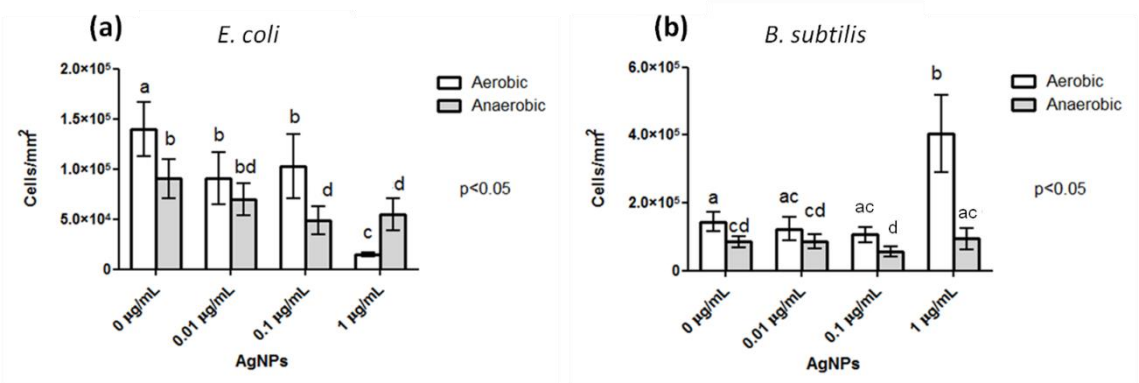


Figure 4 - Adhered cells of *E. coli* (a) and *B. subtilis* (b) in both aerobic and anaerobic conditions in presence of different concentration of AgNPs. Data represent the means ± SD of 4 independent measurements. Letters provide

the graphical representation for post hoc comparisons. The histogram provides the *p*-values obtained by the ANOVA analysis. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

3.5 Level of oxidative stress in planktonic cells

The fluorescence per cell values found in *E. coli* (Fig. 5a) showed a significantly higher oxidative stress level in anaerobic conditions than in aerobic, except at 1 µg/mL AgNP concentration, where the ROS amount was statistically comparable in both the presence and absence of oxygen. The results also demonstrate that none of the AgNP concentrations affected the level of oxidative stress in aerobic conditions, as the fluorescence values were comparable with the negative control without AgNPs. In anaerobic conditions, a decrease in the oxidative stress level was found only at the highest concentration (1 µg/mL AgNPs).

For *B. subtilis* the levels of ROS were higher than in *E. coli* (Fig. 5b) and similar in aerobic and anaerobic conditions, except at 0.01 µg/mL AgNPs. In this case, ROS values were higher in the absence of oxygen. Nevertheless, there was a recognizable drop in the oxidative stress level compared to the control in aerobic conditions for values above 0.01 µg/mL, and in anaerobic conditions at the highest AgNP concentrations.

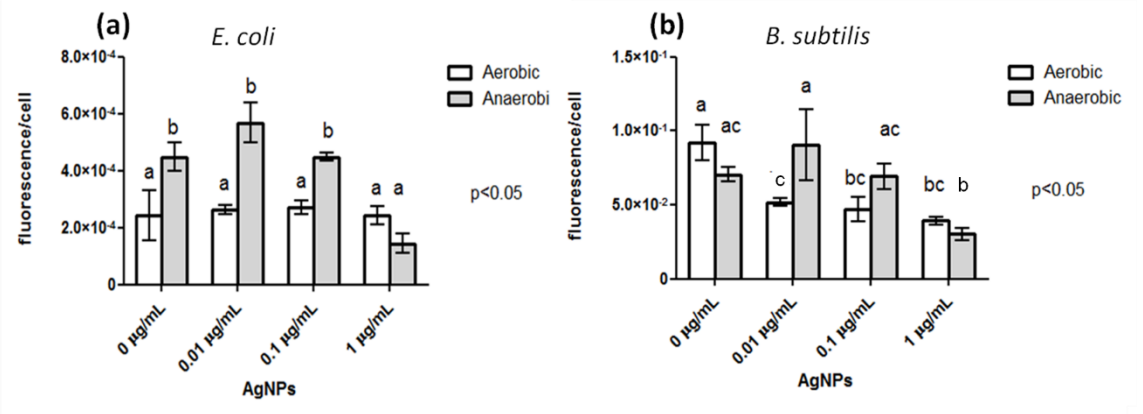


Figure 5 - Amount of fluorescence per cell values for *E. coli* (a) and *B. subtilis* (b) in both aerobic and anaerobic conditions measured in presence of each concentration of AgNPs. Data represent the means ± SD of three independent measurements. Letters provide the graphical representation for post hoc comparisons. The histogram provides the P-values obtained by ANOVA analysis. According to post hoc

analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

3.6 Motility assay

E. coli swimming motility in aerobic conditions (Fig. 6a) was significantly stimulated after 24 h in the presence of 0.01 μg/mL AgNPs, with an increase of migration diameter values with respect to the control. A significant enhancing of swimming motility with respect to the control was also observed at 96 h at 0.1 μg/mL AgNP concentration. In contrast, in anaerobic conditions (Fig. 6b) there was a no significant swimming migration, in neither the presence nor the absence of AgNPs, at different times.

The swarming mobility of the same bacterium did not show any significant difference in samples treated with different AgNP concentrations in both aerobic and anaerobic conditions (Fig. 6c and 6d). Data indicate a small increase in diameter during the three different time-steps in the presence of oxygen and a lack of motility among all the samples in anaerobic conditions.

For *B. subtilis* both swimming and swarming motility was higher than for *E. coli.* In aerobic conditions (Fig. 6a) swimming movement reached the plate diameter (85 mm) between 10 and 5 fold in comparison to the control at 24 h and remained stable over time regardless of the AgNP concentration. Motility in the absence of oxygen (Fig. 7b) was slower. At 24 h and 48 h, 0, 0.01 and 0.1 μg/mL AgNPs showed a statistically comparable diameter value, and only bacteria treated with 1 μg/mL AgNPs was more motile, reaching the plate diameter. At 96 h, all AgNP concentrations gave the same results, reaching the plate diameter. The aerobic swarming results (Fig. 7c) showed a significant effect of AgNPs on motility only at 24 h incubation. At 24 h, only the control reached the plate diameter, while all the treated samples maintained similar diameters around 20 mm.

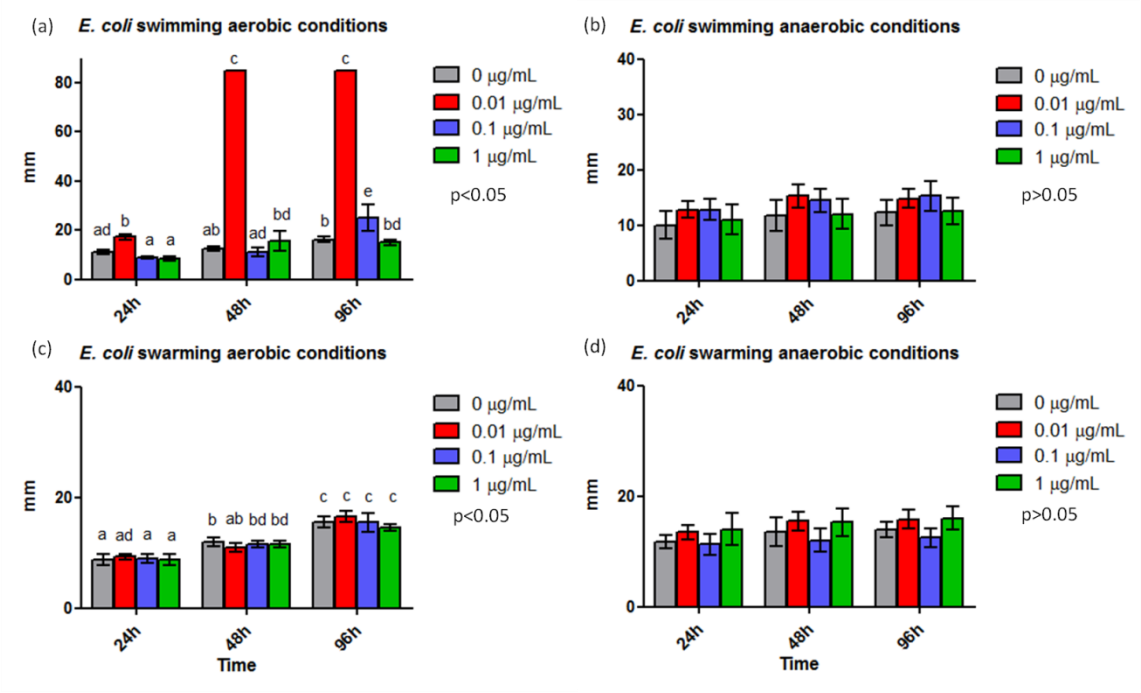


Figure 6 - Swimming and swarming expansion radius of *B. subtilis* previously grown in presence of sublethal concentrations of AgNPs. Experiments were performed in both anaerobic and aerobic conditions and data collected at 24, 48 and 96 h of incubation. Data represent the means ± the SD of four independent measurements. The histogram provides the *p*-values obtained by ANOVA analysis. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

At 48 h and 96 h, all the samples reached plate diameters with no statistical differences. In anaerobic conditions (Fig. 7d), swimming motility decreased compared to the anaerobic and significant differences among AgNP concentrations were observed. Cultures grown in the presence of the AgNP concentrations of 0.01 and 1 µg/mL showed the highest values (around half in comparison to the equivalent aerobic condition). Other tested concentrations remained statistically similar throughout the experiment.

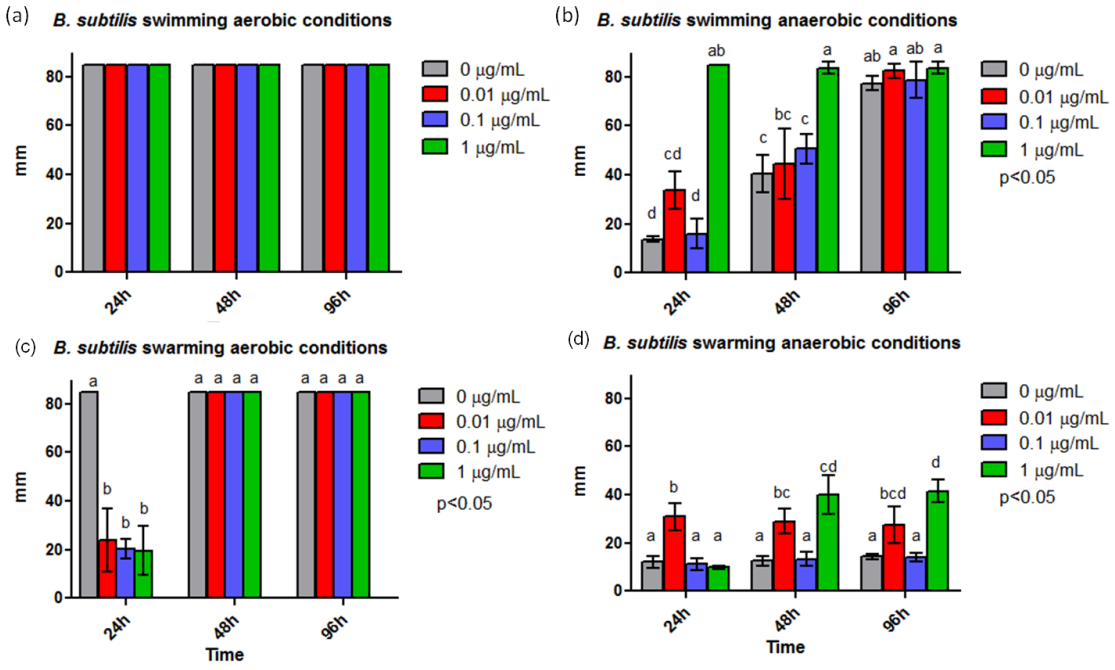


Figure 6 - Swimming and swarming expansion radius of *B. subtilis* previously grown in presence of sublethal concentrations of AgNPs. Experiments were performed in both anaerobic and aerobic conditions and data collected at 24, 48 and 96 h of incubation. Data represent the means ± the SD of four independent measurements. The histogram provides the P-values obtained by ANOVA analysis. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

4 Discussion

It has been known for quite some time that high concentrations of AgNPs have antimicrobial properties; for instance, they are able to inhibit the adherence of microorganisms to a surface, the first step in biofilm formation (Allaker et al., 2010; Kalishwaralal et al., 2010; Dror-Ehre et al., 2010). However, low (sublethal, rather than biocidal) AgNP concentrations are expected to predominate in both natural and engineered ecosystems, following dilution and dispersion pathways. Furthermore, the dominant route of AgNPs in the environment is likely to be mobilization from an aerobic compartment to an anaerobic one. Literature concerning AgNP effects in anaerobic conditions always considers these to be very strictly anaerobic. In these closed systems NPs never meet molecular oxygen since their production and microbial survival is used as a toxicity bio-indicator (Xiu et al., 2011- 2012). Although this approach is useful to evaluate the different mechanisms of action, it does not resemble real systems where anthropogenic NPs are usually released in aerobic environments and

oxidation processes take place. Such oxidized AgNPs are then diluted and transferred to other environmental compartments where the oxygen tenor can fluctuate and anaerobic conditions can occur.

Scientific literature has started to address important questions about the impact of nanoparticles on microbial systems (Du et al., 2012; Yang et al., 2013; Demirel et al., 2016; Dinesh et al., 2012). However, none take into consideration the effects of sublethal concentrations of nanoparticles under aerobic and anaerobic environments in the same experimental design. What happens when sublethal concentrations of AgNPs coming from an aerobic environment meet a bacterial community under anaerobic conditions? And what are the effects of sublethal AgNP concentrations on growth kinetics, adhesion ability, oxidative stress and phenotypic changes of facultative bacteria under both aerobic and anaerobic conditions? These are critical gaps in the knowledge needed for a better understanding of the impact of engineered nanoparticles on ecosystems. To investigate these phenomena, we compared the response to sublethal concentrations of AgNPs of two facultative bacteria growing under oxygenic or anoxic conditions in a medium that could guarantee AgNP stability.

In fact, it is well known that the effects of most metal nanoparticles depend on their stability, namely resistance to aggregation, dissolution and reprecipitation (Schacht et al., 2012). Although the influence of the medium's chemistry on silver nanoparticle toxicity is a crucial issue, most available studies assess stability in the laboratory in deionized water (Jiang et al., 2009; Jin et al., 2010). After we had determined that TSB was the best medium to preserve physical and chemical properties of AgNPs, we adopted growth kinetic data to evaluate the susceptibility of *E. coli* and *B. subtilis* to different sublethal concentrations of active AgNPs (Schacht et al., 2012).

In order to survive and thrive, in both the presence and absence of oxygen, facultative aerobic bacteria have to regulate physiological processes to obtain the maximum benefit from the environmental conditions. For instance, in aerobic conditions these microorganisms can activate specific methabolic pathways to undergo oxygenic respiration while restraining oxidative stress (Fu et al., 2015). Such acclimation to aerobic environments can affect bacterial growth differently from under anaerobic conditions, in terms of both oxidative damage and energy balance, resulting in growth kinetics changes. *E. coli* and *B. subtilis* maximum specific growth rates (MSGR) were higher in aerobic conditions, highlighting a more active metabolism in the presence of oxygen, while lag phase length (LPL) of *E. coli* indicated a longer acclimation time of the bacterial strain to the aerobic conditions rather than the anaerobic. In aerobic conditions, 1 μg/mL was the only effective concentration of AgNPs on planktonic growth. This threshold dose caused a significant increase of MSGR in *B. subtilis*.

Recently, Gambino and colleagues (2015) reported that within the sublethal range 0.01-1 μg/mL AgNPs, there was nearly constant *B. subtilis* growth. However, the authors did not calculate the specific growth rate, which makes comparison with our study difficult. The finding that specific concentrations of nanoparticles in the sublethal range might stimulate bacterial growth is not new. The inoculation of *E. coli* with AgNPs over 24h revealed large differences in growth within the sublethal range 0-0.09 pmol/L. In this range, enhanced growth was observed, indicating the stochastic effects of stimulation[48]. Furthermore, Schacht et al. (2012) observed that AgNP treatment resulted in higher maximum growth rates of *Cupriavidus necator* after extended lag phases at the sublethal concentrations tested between 20 and 40 μg/mL. In the light of previous observations, it is possible to argue that microorganisms might experience partial growth stimulation under moderate stress conditions, compared to cultures without Ag(0) treatment.

Bacterial surface adhesion is the key step in the transition from planktonic lifestyle to biofilm lifestyle. Adhesion assays allowed us to evaluate whether sublethal concentrations of active AgNPs affected the early stage of biofilm development. Here, the number of *E. coli* adhered cells decreased, along with increased AgNP concentrations under both aerobic and anaerobic conditions. The reduction was even more evident in the presence of oxygen, where 1 μg/mL active AgNPs led to an 89% reduction in the number of adhered cells. This finding demonstrates an interesting antibiofilm effect of AgNPs at sublethal concentrations, suggesting that mechanisms subtler than simple killing activity occur at subinhibitory levels (Lara et al., 2015; Gurunathan et al., 2014; Martinez-Gutierrez et al., 2013). By contrast, 1 μg/mL AgNPs promoted adhesion in *B. subtilis* under aerobic conditions, the same condition that increased the MSGR in the planktonic growth tests. Recently, Yang and Alvarez (2015) reported that sublethal exposure of *Pseudomonas aeruginosa* PAO1 to AgNP enhanced biofilm development and upregulated quorum sensing, lipopolysaccharide biosynthesis, and antibiotic resistance (efflux pump) genes.

The different behavior of *E. coli* and *B. subtilis* demonstrates how differently sublethal concentrations of active AgNPs might affect the behavior and surviving strategies of natural microbial community taxa, altering the ecosystem equilibrium, especially in aerobic conditions. A huge corpus of studies is flourishing on oxidative stress, showing how the presence of free oxygen can enhance NP bactericidal effects (Xu et al., 2011; Yang et al., 2013; Lu et al., 2013), and new light has been shed on bacterial oxidative stress response to AgNP-induced ROS (Fabrega et al., 2009; Fu et al., 2015; Gambino et al., 2015). Consequently, intracellular levels of ROS were assessed in the presence and absence of oxygen and at different concentrations of active AgNPs.

In *E. coli* we observed higher levels of ROS under anaerobic conditions, and no significant effect of sublethal AgNP concentrations under aerobic conditions. The fast penetration of Ag inside the cell, and the subsequent production of ROS, may have generated a cascade activation of the scavenging system, e.g. SoxRS system activated by superoxide radical and regulating for superoxide dismutase and other scavenging enzymes, guaranteeing a negative feedback on the radical abundance itself (Fu et al., 2015).

Lower ROS levels in aerobic conditions and higher levels in an anaerobic atmosphere can be interpreted as the consequence of a scavenging system, constantly activated in the presence of oxygen, engaged in maintaining ROS concentrations at harmful levels. Interestingly, 1 μg/mL AgNPs under anaerobic conditions provided the lowest level of ROS in *E. coli*, suggesting the activation of dose-dependent scavenging systems (Gambino et al., 2015). A similar explanation could apply to the *B. subtilis* results where the lowest ROS levels were observed at the highest sublethal concentrations of active AgNPs.

Many scientific works have demonstrated how flagella-driven motility types, swimming and swarming, are deeply linked to the ability of the microorganism to colonize a surface and develop antimicrobial resistant phenotypes, these being strategies to survive in the presence of adverse conditions (Lai et al., 2008; Overhage et al., 2008; Butler et al., 2010). Nevertheless, up until now bacterial motility data in anaerobic conditions are still poor and show controversial results (Che et al., 2006; Poggio et al., 2007; Nachin et al., 2005; Kan et al., 2004), indicating a very complex physiological and regulative scenario.

AgNPs have been proved to both inhibit bacterial motility at high concentrations and enhance negative taxis responses at sublethal concentrations (Ortega-Calvo et al., 2011; Lee et al., 2013). Villa et al. (2012) demonstrated how sublethal levels of oxidizing biocides can lead to increased swimming and swarming motility in the soil bacteria *Azotobacter vinelandii,* a strategy to escape adverse conditions. Our studies revealed that 0.01 μg/mL AgNPs increased swimming movement of *E. coli* under aerobic conditions 6-fold. Under anaerobic conditions both swimming and swarming migrations were not affected, in agreement with the study of Che (2006), who reported a decrease of both flagella-driven motility types of *P. aeruginosa* under anaerobic conditions, the result of fewer flagellated cells in the population.

In contrast, *B. subtilis* had a completely different behavioral response to AgNP exposure, showing constitutive high swimming and swarming motility rates, especially under aerobic conditions. In the aerobic swarming assay, the temporary inhibition effect caused by AgNP pre-exposure agrees with

data of bacterial motility inhibition at sublethal AgNP concentrations reported by Ortega-Calvo (2011)[45]. While there was a reduction of motility in the absence of oxygen, we observed an increase in swimming migration in the presence of AgNPs, particularly at the highest concentration, before the bacterium can reach the maximum diameter. In this case, AgNPs promoted an active motility, probably as a chemotactic response to escape from stress, as previously reported by Villa et al. (2012) and Butler et al. (2010). Swarming migration under anaerobic conditions was promoted at the highest AgNP concentration tested, corresponding to the most bio-active doses in all the experiments.

5 Conclusions

The results obtained from this study demonstrate that both *E. coli* and *B. subtilis* reacted very differently to AgNPs over the wide range (100-fold) of sublethal concentrations examined under aerobic and anaerobic conditions. The findings showed that exposure to AgNPs under aerobic conditions triggers the most notable changes in the physiology and activity of the selected bacteria, affecting their growth kinetics, adhesion ability, oxidative stress and inducing phenotypic changes on model bacteria. Indeed, *B. subtilis* seemed to react positively to 1 μg/mL AgNPs by increasing its growth rate and the ability to colonize a surface, thanks also to its increased motility. By contrast, the same concentration of AgNPs reduced *E. coli* adhesion, suggesting that mechanisms subtler than the simple killing activity occur at subinhibitory levels. Overall, the present work demonstrates that different physiological processes occur within the sublethal range of AgNP concentrations.

However, it is unclear to what extent silver ions played a role in the observed responses. Future work will be devoted to investigating the contribution of dissolved silver vs. silver NPs in our experimental conditions, in order to clarify particle-related and ion-related effects and modes of action on biological systems. These findings are an initial contribution to elucidate the behavior and impact of sublethal engineered nanoparticles on microbial ecosystems, issues still little explored by current literature.

6 References

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CHAPTER 4

Effects of acute and chronic exposition to sub-lethal concentrations of silver nanoparticles on a simulated gut system.

From the current state-of-the-art, it is clear that nanotechnology applications are expected to bring a range of benefits to the food sector aiming at providing better quality and conservation. The present work consisted in investigating the physiological response of a mono-species gut biofilm to chronic and acute exposure to 1 μg/mL AgNPs, and how this physiological response affected the intestinal epithelial cells. To study the interplays among sub-lethal concentrations of AgNPs, the gut biofilm and its host, a simplified experimental lab model system was designed. The developed *in vitro* gut system operated in a semi-batch mode, allowing the fine control of experimental conditions in two different compartments: (i) an aerobic compartment composed by a mono layer of CaCo-2 cells to mimic the intestinal mucosa and (ii) an anaerobic and anaerobic compartment characterized by a mono-species *E. coli* biofilm to simulate a simplified gut biofilm. To this end, transwell permeable supports were used to set up the system, and interactions between the two compartments were reproduced by putting in contact biofilm basolateral permeates with CaCo-2 cells.

Under anaerobic conditions, chronic exposures to 1 μg/mL AgNPs promoted biofilm growth, and in acute experiments, a significant decrease in culturable cells count, but not in the total biofilm biomass, leaded us to hypothesize a shift to the dormant state, in which cells are still viable but unable to proliferate. This hypothesis was also confirmed by studying GFP expression along the sections of fully hydrated biofilms.

Under anaerobic conditions, both chronic and acute samples showed higher percentages of GFP-positive cells in comparison to aerobic biofilms, suggesting a more active bacterial subpopulation in absence of oxygen. In contrast to the control, chronic experiments under anaerobic conditions showed high cell activity at the bottom of the biofilm, being the region directly exposed to nanoparticles.

Biofilms exposed to both acute and chronic AgNPs treatments under aerobic conditions experienced higher level of oxidative stress than under anaerobic environments. In presence of oxygen, levels of reactive oxygen species (ROS) were similar in both chronic and acute treatments. ROS levels increased in samples exposed to AgNPs acute treatments in absence of oxygen.

Comet assays demonstrated a protective role of biofilms against the genotoxic effect of 1 μg/mL AgNPs on intestinal epithelial cells represented by CaCo-2 cells monolayer.

1 Introduction

Taking advantage of their unique physico-chemical properties, nanoparticles (NPs) are widely used in the agri-food industry as agrochemicals for controlling environmental pests and pathogen microbes (Grillo et al., 2016; Polo et al., 2011), delivery of active ingredients (Chen et al., 2006), nanosized ingredients and additives (Chaudry et al., 2008). Another interesting and growing application of nanomaterials is for functionalizing food processing surfaces and packaging, mostly to improve their mechanical and antimicrobial properties (Dainelli et al., 2008).

Given these widespread applications, exposure to NPs represents a potential toxicity risk for human health. Of special interest are the effects of NPs on the human gut microbiota, considering the range of consumer goods that could be intentionally or accidentally ingested. However, most published literature regarding the effects of NPs on human health concerns lung cells *in vitro* cultures (Geary et al., 2016). Only few animal studies are available about gastrointestinal intakes and impacts of NPs, insufficient to allow a clear safety assessment of ingested NPs (Pietroiusti et al., 2016; Joneset al., 2015; Arbor and Witzman, 2013; Tran & Chaudhry, 2010) and understanding of their impacts on the intestinal ecosystem (Frohlich and Frohlich, 2016).

A major reason for the scarcity of relevant literature on the effects of NPs on the gut ecosystem can be related to the lack of effective and simplified models systems to study the nature of these complex interactions. Furthermore, most of these studies have been conducted with relatively high (bactericidal) NP concentrations, and the effects of sub-lethal (low concentrations) exposure are poorly understood. This is a critical knowledge gap because low (rather than high) NP concentrations are expected to predominate following dilution and dispersion along the food chain and gastrointestinal system (Benn and Westerhoff, 2008; Colman et al., 2013).

In addition, there is a growing recognition that the intestinal microflora exists as a biofilm showing different characteristics from those of the planktonic counterpart (Donelli et al., 2012). Despite the evidence of human microbiome existence as a biofilm on gut mucosa (Donaldson et al., 2016; Donelli et al., 2012), the complexity of intestinal biofilms and their interactions with low concentrations of NPs (the actual human consumption) and the epithelial intestinal cells are still largely unknown.

In this study, we investigated the physiological response of a mono-species gut biofilm to chronic and acute exposure to sub-lethal concentrations of silver nanoparticles (AgNPs), being the most commonly used metal nanoparticles across diverse applications, due to their antimicrobial properties, and how this physiological response affects the intestinal epithelial cells represented by CaCo-2 cells monolayer. To study the interplays among sub-lethal concentrations of NPs, the gut biofilm and its host, a simplified experimental model that can be easily manipulated and controlled was developed.

2 Materials and Methods

2.1 Bacterial strains and planktonic growth conditions

The well characterized *Escherichia coli MG 1655* and green fluorescent protein (GFP)-*Escherichia coli MG 1655* were stored at –80°C in phosphate-buffered saline (PBS, Medicago AB, Uppsala, Sweden) solutions containing 20% of glycerol. Both microorganisms were routinely cultured in liquid Tryptic Soy Broth medium (TSB, Conda, Italy) with an addiction of 100 mg/L ampicillin (Sigma Aldrich) for the GFP strain, at 37°C in aerobic or anaerobic atmosphere. Anaerobic experiments were performed in an anaerobic box (Forma Scientific, Marietta, OH, USA) under N_2:H_2:CO_2 atmosphere (85/10/5, v/v) using TSB pre-reduced in anaerobic conditions for 24h before experiments began .In a previous research, TSB was chosen as the best nutritive medium that could guarantee a satisfactory stability of dispersed AgNPs in time (Garuglieri et al., 2016).

2.2 Silver nanoparticles

Silver nanoparticles (AgNPs; 10 nm, OECD PVP BioPure Silver Nanoparticles, NanoComposix, San Diego, CA, USA) stock solutions at a concentration of 1 mg/mL concentration in aqueous 2 mM citrate were stored at 4°C, and resuspended directly in bidistilled water or culture media just before their use in the experiments. According to the supplier, purchased AgNPs have a diameter of 8.5±1.7 nm (JEOL 1010 Transmission Electron Microscope), a hydrodynamic diameter smaller than 20 nm and a negative zeta potential of-27.3 Mv (Malvern Zetasizer Nano ZS). AgNP size was assessed by Transmission Electron Microscopy (TEM) by our team in a previous works 14±0.3 nm (Garuglieri et al., 2016).

2.3 Transwell biofilm cultures

A sterile polycarbonate membrane (PC, Whatman Nucleopore, diameter 2.5 cm, pores diameter 0.2 μm) was carefully placed on a sterile TSA plate and inoculated at its center with 0.05 mL of an overnight TSB culture of *E.coli*/GFP-*E.coli* grown at 37°C in aerobic/anaerobic conditions. Inocula were normalized trough optical density (OD) measurements at 600 nm with an JENWAY 7315 Spectophotometer, to obtain a final concentration of 10^8cells/mL. The membrane was left on the agar plate until the inoculum resulted completely dried. Then it was carefully put inside the transwell (ThinCert™ Cell Culture Inserts with translucent PET membrane - Greiner bio-one) inlaid in a 6 wells culture plate (Greiner bio-one). One mL of (pre-reduced if working in anaerobic condition) TSB medium was added in the plate well (basolateral compartment). Biofilm formation was performed at 37°C in both aerobic and anaerobic conditions. To guarantee a continuous growth, every 24 h

transwells were transferred in new plate wells with fresh TSB. Basolateral media were collected every 24 h and stored at -80°C to be used in further experiment of CaCo-2cells exposure in the aerobic part of the model.

2.4 Biofilm growth curves under chronic AgNPs exposure

Biofilms of *E. coli* were cultured using transwell setups as described in 2.3. In the chronic experiments, biofilms were grown for 96 h with 1µg/mL AgNPs (chronic samples) dispersed in the basolateral medium. Control samples without AgNPs were run simultaneously. Experiments were conducted in triplicate under both aerobic and anaerobic conditions. Every 24h, PC membranes with adherent biofilms were removed from transwell setups, washed inside tubes containing 1mL of sterile PBS, vortexed and sonicated for 3 min in the sonication bath (Sonica Ultrasonic Cleaner, Soltec, Milano, Italy). Using this procedure, all the cells were dislodged from the membranes and clumps of cells were broken apart. Serial dilutions of 0.01 mL of the cell suspension were plated in triplicate on Tryptic Soy Agar (TSA, Fisher Scientific) to perform a plate count viability assay as reported by Gambino et al. (2015). All plates were incubated overnight at 30°C. Colony forming units (CFUs) were determined by standard colony counting method.

2.5 Biofilm growth curves under acute AgNPs exposure

Biofilms of *E. coli* were cultured using transwell setups as reported in 2.3. Acute samples were obtained by growing the biofilm for 72 h without AgNPs and then exposing the biofilm for 24 h to 1µg/mL AgNPs. Control samples without AgNPs were run simultaneously. Experiments were conducted in triplicate under both aerobic and anaerobic conditions. Every 24 h, PC membranes with adherent biofilms were removed from transwell setups and treated as described in paragraph 2.4 above.

2.6 Biofilm Sectioning and imaging by Confocal Laser Scanning Microscopy

E. coli-GFP biofilms were grown in triplicate under both chronic and acute AgNPs exposure as described in sections 2.4 and 2.5. At 96h, biofilms adherent to the PC membranes were carefully covered with a layer of Killik cryo-stat embedding medium (Bio-Optica, Milan, Italy) and placed at -80°C until completely frozen. Frozen samples were sectioned at 19°C using a Leica CM1850 cryostat, and the 5-µm thick cryosections were mounted on Superfrost/Plus microscope slides (Fisher Scientific). Samples were observed using a Nikon Eclipse E800 microscope with a 10X or 20X dry objective. The sections were viewed both in bright-field and in the epifluorescence mode.

The software ImageJ (Schneideret al., 2012) performed the image analysis and biofilm thickness measurements of the control and treated samples. More than five images per sample were taken for microscope analysis. For each picture, the biofilm thickness was measured at 3 different locations randomly selected along the profile. These measurements were used to calculate the average thickness and the associated standard deviation.

Relative quantification of green signals in biofilm sections was carried out by using the standard tools "segmentation and quantification of cellular structures" of ImageJ software.

Average intensity measurements of the fluorescence were collected from the periphery and the center within biofilm clusters. The regions were square regions with dimensions 875 μm^2. Therefore, the total areas analyzed at the periphery, and center of each cluster were equal. Intensity values were normalized by dividing the fluorescence intensity of the AgNPs treated samples (I) by the fluorescence intensity values of the control samples (I_0) obtained at the same locations. This normalized intensity (I/I_0) was used to compare values of green fluorescence among samples.

In addition, the ratios fluorescent intensity aerobic samples vs. fluorescent intensity anaerobic samples were used to compare the expression of GFP in presence and in absence of oxygen.

2.7 Level of oxidative stress in biofilm cells

The level of oxidative stress in *E. coli* biofilms was assessed using the 2,7-dichlorofluorescein-diacetate (H_2DCFDA, Sigma Aldrich Italy) assay (Jakubowski et al., 2000). Control, chronic and acute biofilms samples of *E. coli* were cultured, in both aerobic and anaerobic conditions. Experiments were conducted in triplicate. Every 24 h, three PC membranes with adherent biofilms were removed from transwell insert, washed inside tubes containing 1mL of sterile PBS, then vortexed and sonicated for 3 min in the sonication bath (Sonica Ultrasonic Cleaner, Soltec, Milano, Italy). The obtained cellular suspensions were washed twice with PBS (13000 rpm, 15 min) and resuspended in 50 mM PBS. Cells were then broken using glass beads (0.1 μm diameter) and the Precellys 24 (Bertin technologies, France) bead-beater device with a beating profile of 3x30s. After centrifugation (13000 rpm, 15 min), 750 μL of supernatant were incubated with 4 μL of 10 M H_2DCFDA at 30°C for 30 min. The solution was homogeneously divided into three wells of 96 wells black microtiter plates (Greiner bio-one). The relative fluorescence correlated to the reactive oxygen species (ROS) amount was measured with excitation at 490 nm and emission at 519 nm, using the Infinite F200 PRO microtiter plates reader (TECAN, Mannedorf, Switzerland). The relative fluorescence was normalized against the number of cells, obtained by a viable count of initial cell suspensions.

2.8 Cell line

Human CaCo-2 cells were obtained from the European Collection of Animal Cells Culture (UK). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM,Sigma Aldrich) supplemented with 10% heat inactivated (30 min at 56°C) fetal bovin serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM non-essential amino acids, in incubator with an atmosphere of 95% air and 5% carbon dioxide. The culture medium was routinely changed every two days, and always the day before the exposure to AgNP. All cell culture reagents were purchased from Sigma Chemical Co (St Louis, Mo) and chemicals from Merck (Darmstad, Germany).

2.9 Cell line maintenance and subculturing protocol

When cells reached the subculturing density of 70% confluence, they were detached by means of trypsinization: the medium was removed from the flask (75cm^2), cells were washed and treated with 2.5 mL of fresh trypsin–EDTA solution in the incubator. Trypsin action, lasted 4 min, was arrested by the addition of 4 mL of complete medium and cell suspension was transferred in a 15 mL tube and centrifuged5 min at 600g. After removing supernatant, cell pellet was resuspended in complete medium and seeded at 10^4 cells/cm^2 (Natoli et al., 2012).

2.10 Cell differentiation protocol

For the differentiation experiments, cells were seeded on 24-wellplates (Cellstar, Greiner) at a density of 10^5cells per well and, after confluence, maintained for 10 days in complete medium; the medium was changed three times a week (Sambuy et al., 2005).

2.11 Citotoxicity and genotoxicity analysis

After being cultured ten days, differentiated CaCo-2 cells monolayer were incubated with: 1) TSB without AgNPs (control), 2) TSB with1 µg/mL AgNPs (TNP), 3) all the basolateral media from anaerobic control and chronic exposure biofilm samples taken at 24, 48, 72 h, diluted 1:1 with medium. The treatments lasted for 1 h at 37 °C, in incubator with 95% humidity and 5% CO_2. Every treatment was performed in triplicate and negative (PBS) and positive (H_2O_2 50µM) controls were included in each experimental batch to verify the reliability of the Comet assay procedure (Venturi et al., 1997).
After the incubation, cells were detached by trypsinization and an aliquot of this cell suspension was used to assess citotoxicity of treatment, by measuring cell viability, with Trypan Blue exclusion test (expressed as percentage of viable cells) (Strober, 2001). The Trypan Blue is a dye capable of

selectively coloring only the dead cells: this occurs thanks to the extreme selectivity of the cell membrane. Indeed, the live cells, having an intact membrane, do not allow the dye penetrating into the cytoplasm. In contrast, in dead cells the dye penetrates easily and they are therefore distinguishable from the other cells.

Another aliquot of cell suspension was used for the Comet assay, to assess genotoxicity of the treatments. Briefly, cells were centrifuged (11000g, for 15s), re-suspended in 1% low-melting point agarose, and spread on microscope slide previously covered with 1% normal-melting point agarose layer. Embedded cells were lysed, DNA was allowed to unwind in electrophoresis buffer (pH 10) and then electrophoresis was performed at 25 V and 300 mA for 20min. After this step, the slides were immersed in neutralization buffer for 15 min, stained with ethidium bromide and analysed using a fluorescence microscope (BX60 Olympus, Japan) equipped with Image-Pro Plus software (Immagini & Computer, Bareggio. Milano, Italy). Fifty images were analysed for each slide and tail moment registered: DNA damage was expressed as percentage of DNA in the tail (Tice et al., 2000).

2.11 Statistical analysis

To evaluate statistically significant differences among samples analysis of variance test (ANOVA) was performed trough MATLAB software (The MathWorks Inc., Natick, USA). The ANOVA analysis was carried out after verifying whether the data satisfied the assumptions of i) independence, ii) normal distribution and iii) homogeneity of variances. Tukey's honestly significant different test (HSD) was used for pair wise comparison to determine data significance. Differences were considered significant for $p<0.05$.

3 Results

3.1 Model System

In our model (Fig. 2) the effect of nanoparticles on biofilm and CaCo-2 cells can be analyzed separately and their interaction can be assessed putting in contact biofilm basolateral media with CaCo-2 cells. CaCo-2 cells need oxygen to live. Inside the intestine oxygen concentrations decrease precipitously to anoxia moving inward from the mucosa surface to the lumen along the radial axis and both facultative anaerobic and aerotolerant microbes are found associated to the mucosal surface (Espey, 2013). Consequently, biofilms were grown in both aerobic and anaerobic conditions and submitted to chronic and acute AgNPs exposure. Thus the model offers the advantage of a flexible workflow that allows to

work independentely with compartements with incompatible atmosphere conditions: aerobic conditions for the CaCo-2 cells and anaerobic for the human gut biofilm.

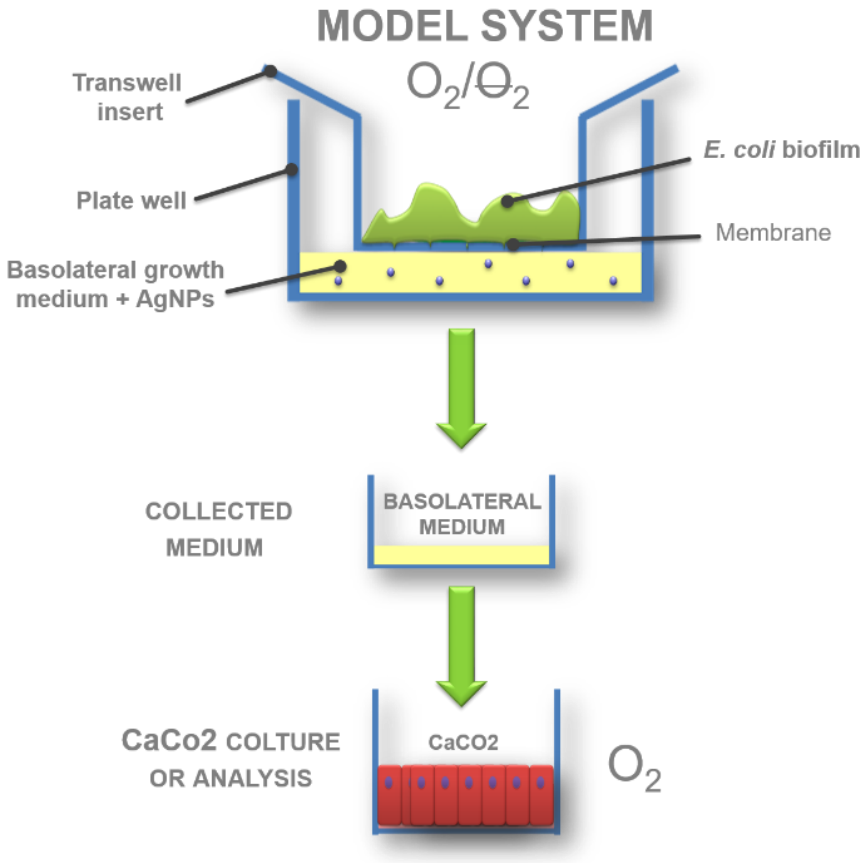

Figure 2 – In-vitro model outline: transwell biofilm culture exposed to AgNPs and *in vitro* system workflow.

3.2 Biofilm growth under chronic AgNPs exposure

Aerobic trends of curves showed an exponential phase up to 24h when the growth reached a stationary phase (Fig 3a). In anaerobic conditions the same trend was observed but at 72 h when a second growth phase of the biofilm was initiated (Fig 3b).

Biofilm growth curve in aerobic conditions showed no differences between chronic samples and control except at 24 h when biofilms grown in absence of AgNPs resulted in a 40% lower number of viable cells respect to the chronic samples (Table 1). In anaerobic conditions, statistically significant higher growth rates characterized the chronic samples at 24, 48 and 72 h in comparison to the control (Table 1).

Control values in aerobic versus anaerobic conditions showed no statistically significant differences except at 96 h when 70% more growth was observed in anaerobic conditions. Chronic anaerobic values were found higher than the aerobic counterparts except at 24 h.

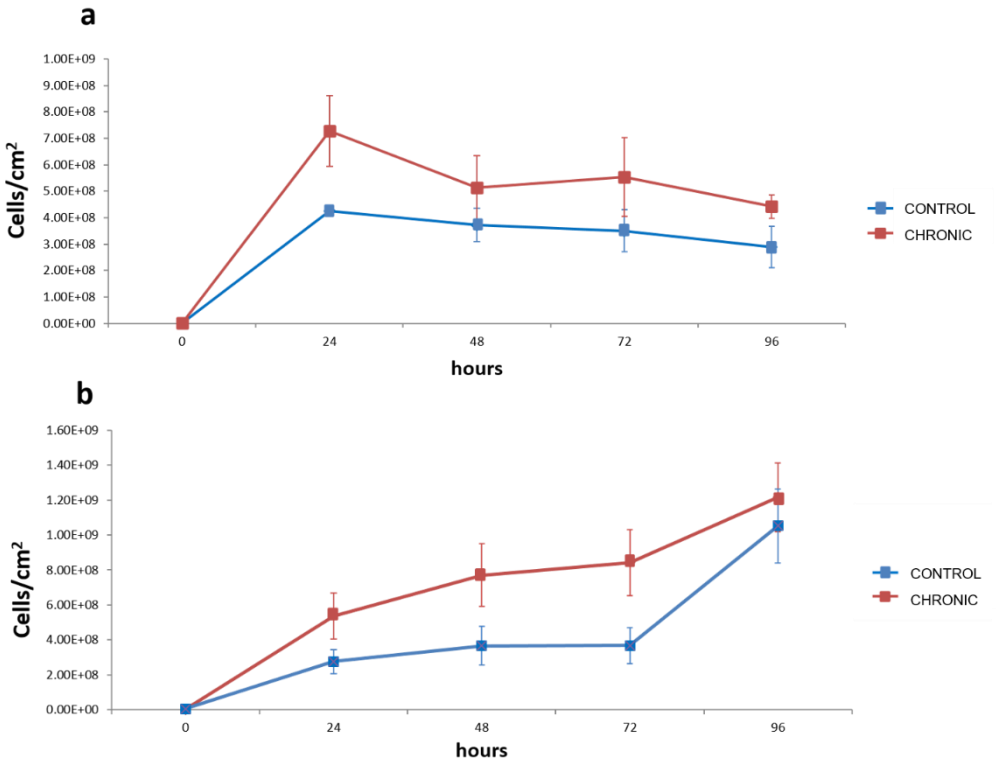

Figure 3– Growth curves of *E. coli* biofilm based on viable counts in presence of 0 (control) and 1 µg/mL (chronic samples) AgNPs in aerobic (a) and anaerobic (b) conditions during a 96h incubation.

	TIME (h)	AEROBIC	ANAEROBIC
CONTROL	24	4.26E+08±1.13E+08	3.66E+08±1.00E+08
	48	3.72E+08±6.37E+07	3.67E+08±1.10E+08
	72	3.51E+08±8.03E+07	3.68E+08±1.03E+08
	96	2.89E+08±7.79E+07	1.05E+09±2.59E+09 o
CHRONIC	24	7.29E+08±1.33E+08●	5.37E+08±1.31E+08●
	48	5.13E+08±1.21E+08	7.71E+08±1.80E+08● o
	72	5.54E+08±1.50E+08	8.42E+08±1.33E+08● o
	96	4.42E+08±4.43E+07	1.22E+09±1.96E+08 o

Table 1 – Cell counts values of *E. coli* biofilms in presence of 0 (control) and 1 µg/mL (chronic) AgNPs in aerobic and anaerobic conditions at 24, 48, 72 and 96 h of incubation. Data represent the means ± standard deviation (SD) of three independent measurements. Dots provide the graphical representation for post hoc comparisons.

According to post hoc analysis (Tukey's HSD, P<0.05), chronic means sharing ● show statistical difference to the relative control, and anaerobic means sharing o show statistical difference to the aerobic counterparts.

3.3 Biofilm growth under acute AgNPs exposure

In aerobic conditions (Fig 4a) control and acute samples resulted statistically similar at each time point. In anaerobic conditions (Fig. 4b), samples showed a statistically significant reduction respect to control (35%) at 96 h after the acute treatment, similar values at 72h and statistical differences at 24 and 48h. Statistical significant reduction (55%) was also shown at 96h in acute treated samples respect to chronic samples in anaerobic conditions (Tab 2).

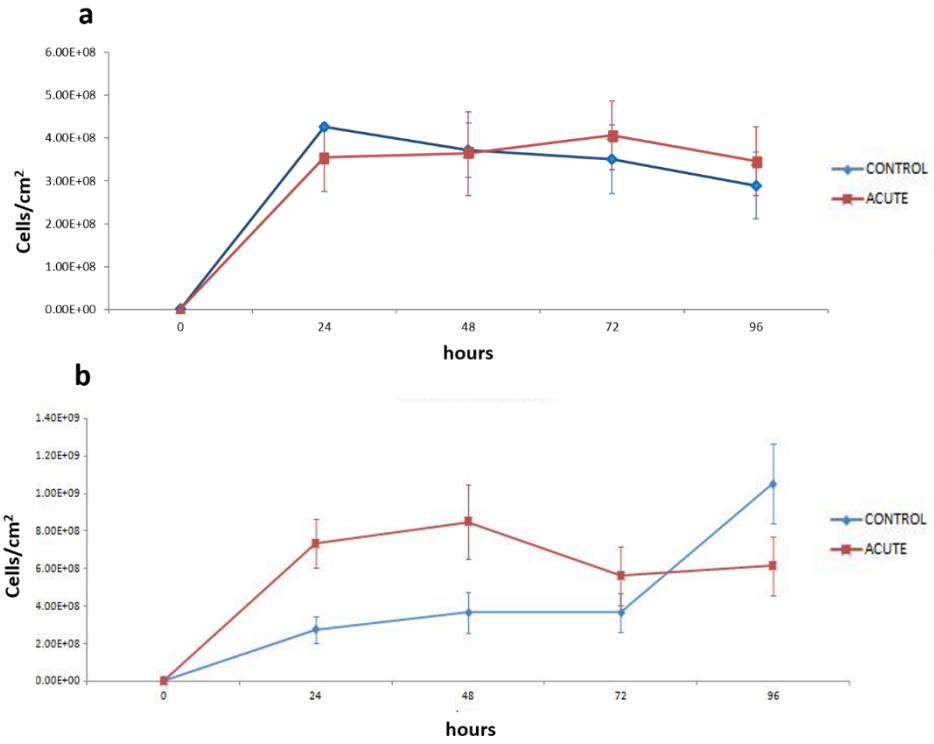

Figure 4 – Growth curves of *E. coli* biofilm based on viable counts in presence of 0 (control) AgNPs and after acute treatment (acute) in aerobic (a) and anaerobic (b) conditions during a 96h incubation.

	TIME (h)	AEROBIC	ANAEROBIC
CONTROL	96	2.89E+08±7.79E+07	1.05E+09±2.59E+09
CHRONIC	96	4.42E+08±4.43E+07	1.22E+09±1.96E+08
ACUTE	96	3.46E+08±8.01E+07	6.15E+08±1.58E+08 ●o

Table 2 – Cell counts values of *E. coli* biofilms at 96 h timepoint in presence of 0 (control), 1 µg/mL (chronic) AgNPs and after the acute treatment, in aerobic and anaerobic conditions. Data represent the means ± standard deviation

(SD) of three independent measurements. Dots provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing ● show statistical difference to the control, and ACUTE means sharing o show statistical difference to the chronic counterparts.

At 96 h of incubation in anaerobic conditions viable counts resulted statistically higher in both control and chronic samples respect to aerobic conditions (Table 2). In contrast, all acute samples showed statistically similar values in both presence and absence of oxygen.

3.4 Biofilm sectioning and imaging by Confocal Laser Scanning Microscopy

Cryosectioning combined with microscopy revealed that *E. coli* aerobic biofilms exposed to AgNPs responded by reducing their thickness by 49.8% and 42.9% in the acute and chronic treatments, respectively (Table3). By contrast, in the anaerobic environment no statistically significant differences in biofilm thickness were observed between the control and the samples treated with AgNPs (Table3).

Sample	**Thickness_aerobic environment**	**Thickness_anaerobic environment**
Control	325 ± 32 μm	201 ± 26μm
Chronic exposure	186 ± 45 μm ●	181 ± 41 μm
Acute exposure	163 ± 55 μm ●	228 ± 58 μm
Sample	**% GFP-positive cells_aerobic environment**	**% GFP-positive cells_anaerobic environment**
Control	72%	81%
Chronic exposure	66%	75%
Acute exposure	69%	62%●

Table 3- Biofilm thickness and percentage of GFP-positive cells in both aerobic and anaerobic environments. Data represent the means ± standard deviation (SD) of at least fifteen independent measurements. Dots provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing ● show statistical difference to the control.

The percentage of active cells under aerobic conditions decreased when biofilms are subjected to acute exposure to 1 μg/mL AgNPs. Anaerobic conditions, surprisingly increased the amount of active cells in biofilms compared to aerobic experiments, and the % of GFP-positive cells significantly decreased under chronic exposure to 1 μg/mL AgNPs.

In control samples, after 96 h of growth under both anaerobic and aerobic conditions, GFP-positive population was located predominantly along the biofilm-air interface (top of the biofilm). The cells at

the bottom of the biofilm were generally inactive with respect to the expression of the GFP protein (Fig 5a,d). These results demonstrate that these biofilms contained at least two distinct subpopulations, an actively growing population of cells near the air-biofilm interface and a population of cells with a very low growth rate in the deeper regions of the biofilms.

Under aerobic condition, chronic exposure did not show significant differences from control whilst acute exposure to 1 μg/mL AgNPs induced a different trend with respect to the expression of the GFP protein. The population at the biofilm-membrane interface showed a significant diminished fluorescence (-40%) respect to the control, while at the top of these *E. coli* biofilm GFP was expressed at high levels (+20%).

Under anaerobic condition, chronic fluorescence trends were overturned respect to aerobic ones. As showed by I/I_0 ratios (table 4), the chronic exposure increased the fluorescence at the membrane level of the biofilm by 20% in comparison to the control, and reduced the fluorescent signal by 40% at the apical part. Acute treatments showed a significant decrease (30%) of fluorescence at membrane level.

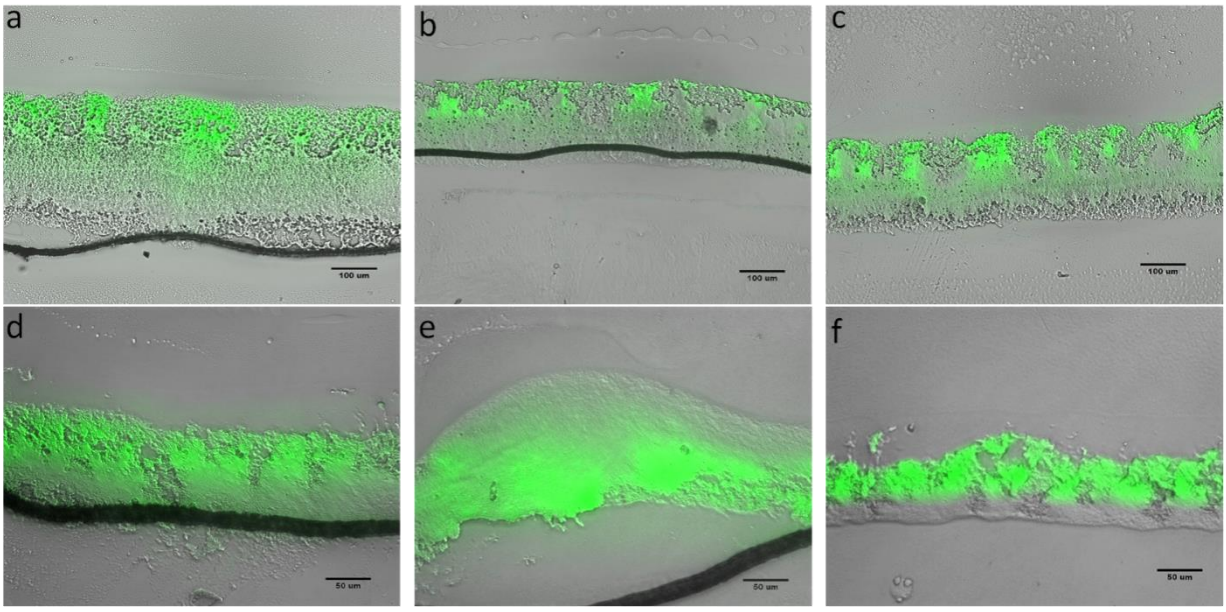

Figure 5–Merged bright-field and fluorescence images of representative 5-μm-thick cryosections of GFP-*E. coli* biofilms under both aerobic (a-c) and anaerobic (d-f) atmosphere. Images were collected after 96h incubation growth under the following conditions: control (a,d, grown 96 h in absence of AgNPs), chronic AgNPs exposition (b,e, grown for 96 h in presence of 1 μg/mL AgNPs) and acute AgNPs exposition (c,f, grown for 72 h in absence of AgNPs and then treated with 1 μg/mL AgNPs for additional 24 h).

Atmosphere	Sample	Sampling zone	I/Io average	
Aerobic	Chronic	Apical	0.88 ± 0.07	
		Middle	0.89 ± 0.10	
		Membrane	0.87 ± 0.04	
Aerobic	Acute	Apical	1.29 ± 0.18	
		Middle	0.92 ± 0.05	
		Membrane	0.58 ± 0.01	*
Anaerobic	Chronic	Apical	0.55 ± 0.10	*
		Middle	0.93 ± 0.08	
		Membrane	1.29 ± 0.12	
Anaerobic	Acute	Apical	0.98 ± 0.13	
		Middle	0.89 ± 0.08	
		Membrane	0.75 ± 0.07	*

Table 4 – Values of fluorescence intensity normalized on controls of GFP-*E. coli* biofilms chronic and acute samples, under both aerobic and anaerobic atmospheres. Data represent the means ± standard deviation (SD) of three independent measurements. Asterisk provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing * show statistical difference to the controls.

As showed in Table 5 fluorescence values resulted significant higher in anaerobic respect to aerobic conditions in both controls and treatments. Aerobic control samples resulted in a diminished fluorescence of -80% respect to anaerobic ones in all the sampling zones. Both chronic and acute samples under aerobic conditions showed a drop of GFP expression especially at membrane level (-85%), where the direct exposure to AgNPs took place.

Sample	Sampling zone	Aerobic/Anaerobic
Control	Apical	0.24 ± 0.02
	Middle	0.21 ± 0.03
	Membrane	0.22 ± 0.04
Chronic	Apical	0.39 ± 0.07
	Middle	0.22 ± 0.05
	Membrane	0.14 ± 0.01
Acute	Apical	0.37 ± 0.07
	Middle	0.22 ± 0.03
	Membrane	0.15 ± 0.02

Table 5 – Values of aerobic versus anaerobic fluorescence intensity of GFP-*E. coli* biofilms control, chronic and acute samples. Data represent the means ± standard deviation (SD) of three independent measurements. Asterisk provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing * showed statistical similarities between aerobic and anaerobic data.

3.5 Level of oxidative stress in biofilm under chronic and acute AgNPs exposure

In aerobic condition, the oxidative stress of chronic samples (Fig 6a) was constant and similar to the control until 72h of growth, with a sudden decrease (80%) at 96h in the control samples compared to the chronic. Under anaerobic condition, the oxidative stress of both chronic samples and control resulted statistically similar at 24 and 48h (Fig 6b). At 72h a high drop of fluorescence (55%) in chronic samples was recorded.

At 96 h in aerobic condition, chronic and acute samples showed values statistically significantly higher respect to the control (Fig 6c). Under anaerobic condition, the control had the lowest levels of fluorescence followed by chronic and acute samples each statistically different from the other (Fig 6d).

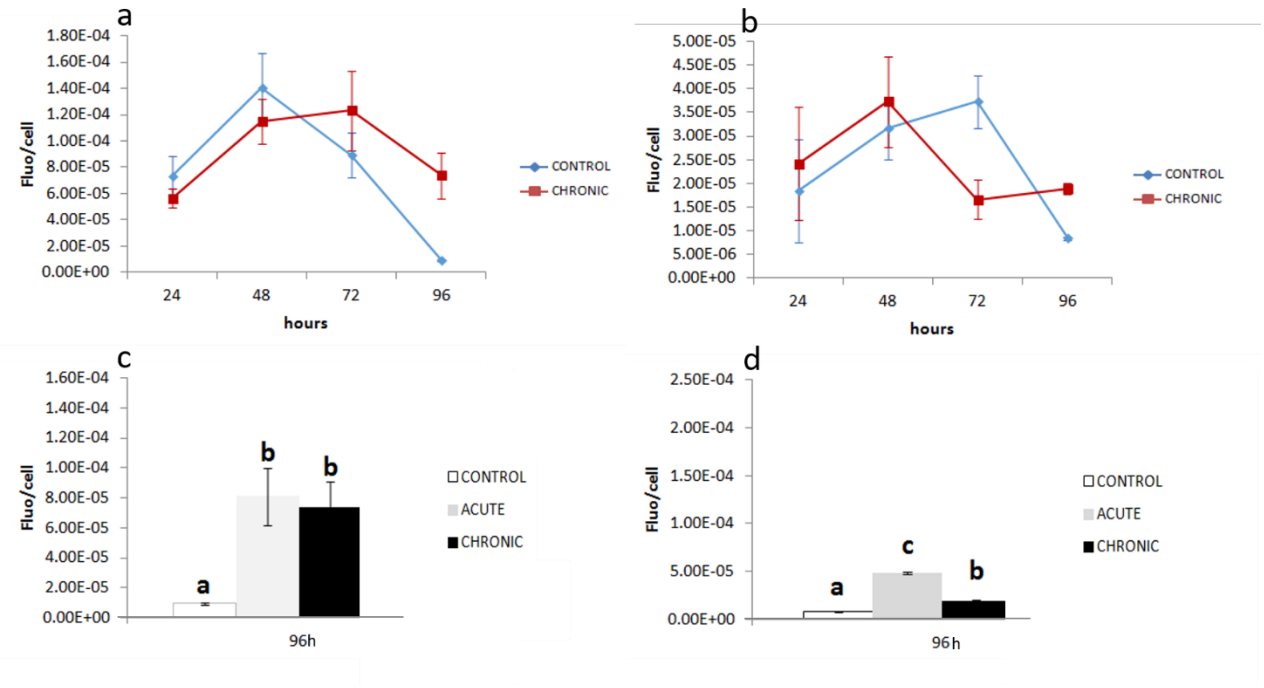

Figure 6 – Fluorescence per cell values trends in both under aerobic (a) and anaerobic (b) atmosphere of *E. coli* biofilms every 24 hours during 96 h incubation growth in presence (chronic) and absence (control) of 1 µg/mL AgNP. Histograms provide amount of fluorescence per cell under aerobic (c) and anaerobic (d) atmosphere under three different AgNP exposition treatments: control, acute and chronic. Data represent the means ± SD of 3 independent measurements. Letters provide the graphical representation for post hoc comparisons. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

3.6 Citotoxicity and Genotoxicity

In cytotoxicity assay neither TNP nor basolateral media samples were found to exert cytotoxic effects on CaCo-2 cells (Fig 7). The viability of the treated cells was consistently >70% and not significantly different from that of cells treated with TSB; consequently, all treated cell suspensions were processed for Comet Assay.

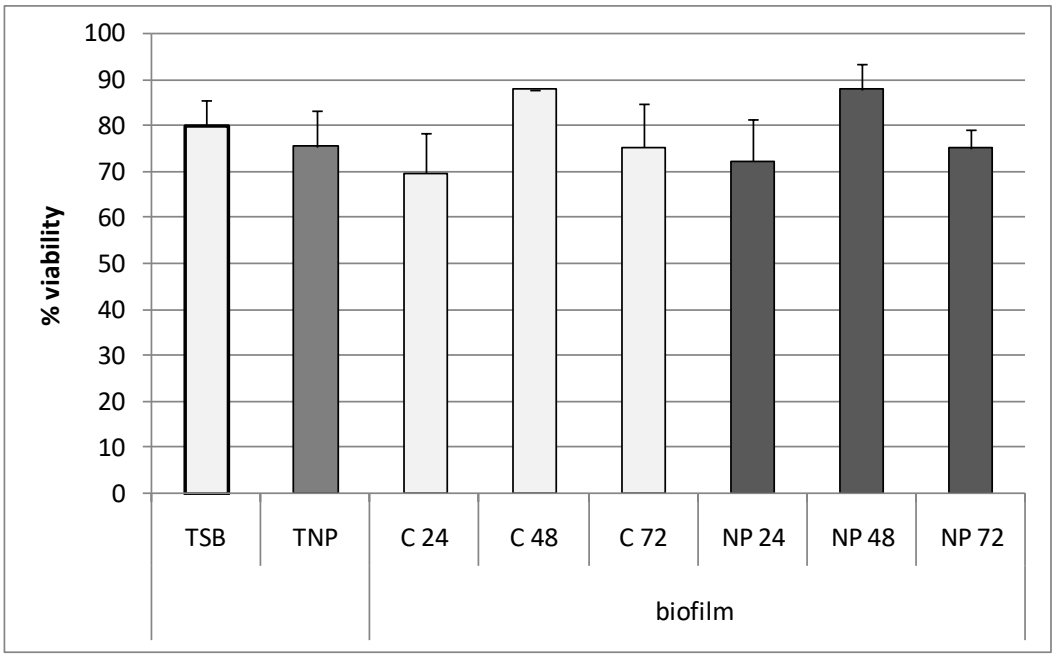

Figure 7 - Viability of CaCo-2 cells exposed, for 1h at 37°C , to Tryptic Soy Broth (TSB), TSB added with 1 µg/mL AgNP (TNP), basolateral solutions collected after 24h, 48h and 72h from control biofilm (C24,C48 and C72) and basolateral solutions collected after 24h, 48h and 72h from biofilm grown in presence of 1 µg/mL AgNP (NP 24, NP48 and NP72).

DNA oxidative damage was assessed using the Comet assay. After electrophoresis, the formation of a comet-like tail implies the presence of a damaged DNA single strand: the length of the tail increases with the extent of DNA damage (AshaRani et al., 2009).

Tail moment of control cells was compared with treated cells, the extent of damage was assessed and the results of genotoxicity were expressed in percentage (Fig. 8). Data are expressed as mean of three independent experiments for each different treatment.

TSB treatment resulted in a very low damage (<5%), whilst in cells treated with TNP, the DNA oxidative damage significantly increase of about 10-fold. All the basolateral media treatments generated a moderate oxidative DNA damage (<30%). Cells treated with basolateral media collected at 24 and 48 h, from both control (C24, C48) and chronic (NP24 and NP48) samples, resulted statistically similar to each other and showed a significant increase of DNA oxidative damage (16 - 19 %) respect to TSB.

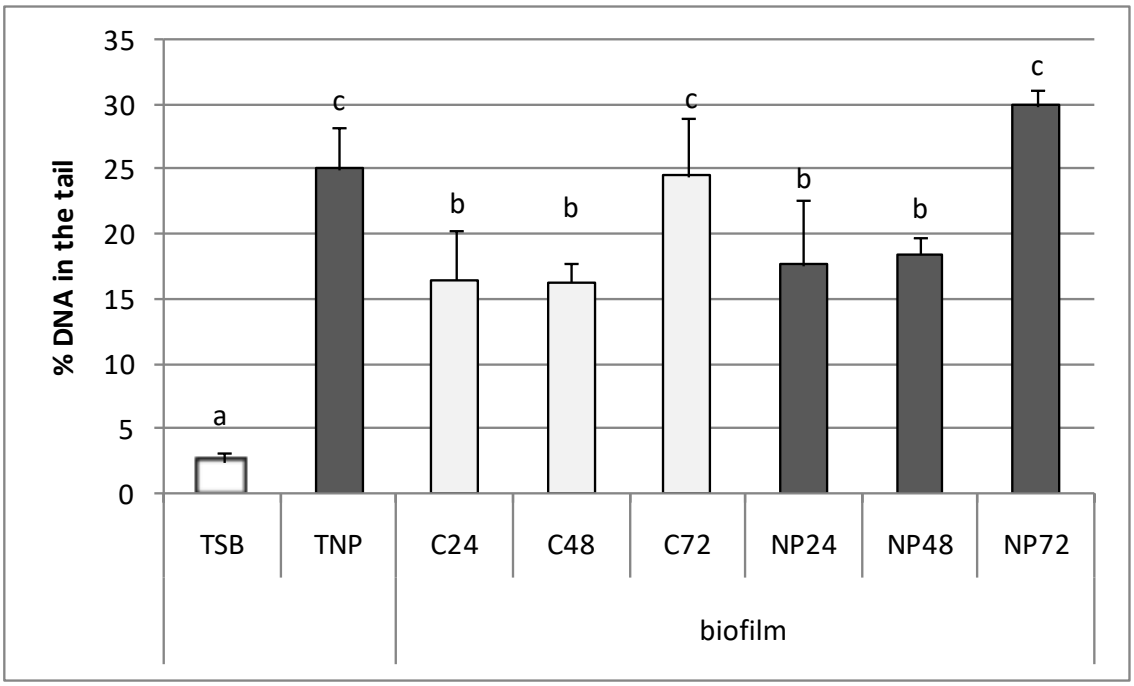

Figure 8 –Comet Assay: genotoxicity, expressed as % of DNA in the tail (mean ± SD), measured after 1h of treatment with: Tryptic Soy Broth (TSB), TSB added with 1 µg/mL AgNP (TNP), basolateral solutions collected after 24h, 48h and 72h from control biofilm (C24,C48 and C72) and basolateral solutions collected after 24h, 48h and 72h from biofilm grown in presence of 1 µg/mL AgNP (NP 24, NP48 and NP72). Data not sharing common letter are significantly different, p <0.05.

Moreover, cells treated with basolateral media collected at 72 h from both control and chronic samples (C72, NP72) resulted statistically similar to each other and showed values of DNA oxidative damage (more than 25%) statistically higher than those found for 24 and 48 h samples.
Indeed, the DNA damage was not significantly affected by AgNPs presence in basolateral media collected from chronic samples respect to those collected from control samples at the same time.
Therefore, it was observed that until 48h DNA oxidative damage of chronic basolateral media resulted significantly less respect to the TNP treatment.

4 Discussion

A variety of*in vitro* models have been proposed to simulate and study the microbiome of the human gastrointestinal system environment. These can be divided in (i) batch and (ii) dynamic systems (Marchesi, 2014). Batch systems are reactors that represent one single segment of the gastrointestinal tract. They can be used for short incubation times (usually a maximum of 48 h) due to the changing in pH, redox potential and nutrient content caused by bacteria during their growth (Gibson and Fuller, 2000; Centanni et al.,2013). In these simple systems, diluted bacteria from fecal samples and

eukaryotic cells are not cultured together but put in contact. Dynamic systems as Enteromix (Makivuocco et al., 2006), TIM-2 (Aguirreet al., 2015) andS HIME™ (Molly et al., 1993) are composed by two or more chambers connected by vessels or membranes, simulating the lower or complete digestive tract. These systems allow a continuous flux of fluids and fine adjustment of the experimental conditions. An example is the custom-made bipartite chamber host-microbiota interaction (HMI) module(Marzorati et al.,2014) that allows investigating cross-interactions between gut microbiota (under anaerobic conditions) and mucosal cells (under aerobic condition) located each in a different compartment.

Using two compartments, in this study we addressed the issue that the luminal gut microbiota needs an anaerobic atmosphere and the intestinal mucosa an aerobic environment, but using a device that is widely available on the market and easy to use, the plastic transwell permeable support. This is a well-known system, commonly used for *in vitro* tests to simulate intestinal absorption of drugs and other substances (Hilgers et al., 1990) that has been widely used to test nanoparticles effects on intestine mucosal model (Williams et al., 2016). The system allows working in a semi-batch mode. In comparison to the batch static models, our system has the additional advantages of fine control of experimental conditions in different compartments, constant nutrient support and the possibility to collect metabolites of cultured cells in long term experiments.

The increasing use of AgNPs has been the subject of concern regarding both environmental and human health issues (Chaudhry et al., 2008). Several reports indicated that AgNPs are toxic to eukaryotic cells and can alter their normal physiological pathways (AshaRani et al., 2009; Martinez-Gutierrez et al., 2010). However, all these studies considered high concentrations of AgNPs (>10 mg/L) and this may not always be relevant to real exposure ranging from 10 mg/L to less than 0.1 ng/L (Kuorwel et al., 2015). Here, biofilm studies were performed in the presence of 1 µg/mL AgNPs chosen as representative of both environmental and food-related sub-lethal concentrations of human exposure (Gottshalk et al., 2013, Lu and al. 2013, Kuoruel et al., 2015). In addition,1 µg/mL AgNPs is the minimal concentration to cause visible effect on the planktonic growth of *E. coli* (Garuglieri et al., 2016).

In our study, *Escherichia coli* MG 1655was chosen as model bacterium because: 1) *E. coli* is widely used in toxicological studies related to AgNPs (Sondi et al., 2004, Li et al., 2010, Pal et al., 2007); 2) it is an intestinal and well characterized strain (Tenaillon et al., 2010); 3) it is a facultative anaerobic bacterium which allows to investigate aerobic conditions experienced when the gut mucosal surface releases oxygen in the luminal compartment (Albenberg et al., 2014). Furthermore, *E. coli* MG 1655 has a number of other important advantages, namely a well-developed literature base, being genetically tractable and amenable to molecular technique such as mutagenesis and “omics” based approaches, and existence of *in silico* metabolic models (Blattner et al., 1997; Fong et al., 2003; Hayashi et al., 2006).

Our results showed that *E. coli* biofilm growth is affected by 1 μg/mL nanoparticles mainly in the absence of oxygen. Samples grown in presence of 1μg/mL AgNPs resulted in an initial higher number of cells in both aerobic and anaerobic condition respect to controls. Such difference was maintained only for the first 24 h of incubation in aerobic conditions, while stably persisted until 72h in anaerobic atmosphere. Moreover, anaerobic chronic samples resulted always in higher number of cells than their aerobic counterparts, underlying a biofilm growth promoting action associated to the presence of nanoparticles joint to absence of oxygen. Furthermore, after a first acclimation period until 72h, anoxic conditions promoted a second exponential phase of biofilm growth (absent in aerobic samples) in both control and chronic samples, increasing the number of viable cells.

In acute experiments, the same magnitude for number of cells was found in both aerobic and anaerobic conditions after 96h treatments. Acute values were much lower respect to both control and chronic values in absence of oxygen while in aerobic conditions no statistically significant differences were found. Working at proven sub-lethal doses of AgNPs (Garuglieri et al., 2016),a decrease in cell number leaded us to hypothesize that acute treatment may cause a change in the number of dormant cells: viable bacterial cells in an inactive physiological state, not forming colony units, allowing cells to survive unfavorable conditions and to return to the active state when environmental parameters improve (Woodet al., 2013;Lewis, 2010;Lewiset al., 2008). They thereby evade detection by conventional culture-based methods. This hypothesis was also confirmed by biofilm cryosections, where the subpopulation of active cells (with respect to the expression of the GFP protein), in acute treatments under anaerobic conditions is reduced by 24% in comparison to the control samples.

As GFP fluorescence is proportional to cellular metabolic activity (Sholtz et al., 2000), it is possible to localize and semi-quantify active or inactive cells within the biofilm. It is known that dormant cell ratio can increase in association with intracellular oxidative stress augmentation induced for example by biocides presence and starvation (Nguyenet al., 2011; Wuet al., 2012; Kuczynska-Wisnsky, 2015). Under anaerobic conditions, both chronic and acute samples showed higher percentages of GFP-positive cells in comparison to aerobic biofilms, suggesting a more active bacterial subpopulation in absence of oxygen. This finding might result incorrect as a potential problem associated with the use of GFP in anaerobic bacteria is the requirement of oxygen for posttranslational folding of the GFP to generate the fluorophore (Cubitt et al., 1995). However, it has been reported that very low levels of residual oxygen might be experienced under anaerobic environments, which is sufficient to allow GFP maturation for facultative anaerobes such as *E. coli* or oxygen-tolerant bacteria (Hansen et al., 2000).

In contrast to the control, chronic experiments under anaerobic conditions showed intense activity in correspondence of the bottom surface exposed to nanoparticles. Chronic exposition to 1 μg/mL AgNPs has a stimulating effect on *E. coli* biofilm activity in absence of oxygen. In contrast to control and chronic samples, in acute treated biofilms apical active cells and a thick layer of GFP-negative cells on

the bottom surface was observed. All together these findings suggested that the effects of AgNPs strongly depend on the presence or absence of oxygen.

It is nowadays well known the relationship between exposition to AgNPs and cellular oxidative stress, in presence of oxygen and the consequent intracellular ROS formation (Fabrega et al., 2009; Fu et al., 2015). In natural systems dispersed silver nanomaterials, even if of anaerobic derivation, sooner or later come into contact with oxygen, undergoing oxidation processes. Such activated nanoparticles can then be released in both aerobic and anaerobic compartments. With this perspective, oxidative stress quantification H_2DCFDA assay was performed in both aerobic and anaerobic conditions. As expected, oxidative stress was higher in aerobic than in anaerobic conditions, due to limitation to ROS release, and controls were statistically different from AgNPs treated biofilms. In presence of oxygen, all chronic and acute samples resulted significantly similar. In anoxic atmosphere, we found higher ROS values in acute samples and lower in chronic ones. Likely, in chronic growth, intracellular scavenging systems acclimate to AgNPs constant presence becoming more efficient in maintaining low oxidative stress levels. In acute treatment, high oxidative stress was sudden established resulting in a ROS increase that scavenging systems may not be capable to contrast efficiently in only 24h.The same acclimation phenomenon can occur in aerobic condition where scavenging system is acclimated to very high levels of oxidative stress due to the presence of oxygen, higher than those caused by AgNPs. Thus, due to the absence of oxygen, difference between chronic and acute treatments became appreciable only in anaerobic conditions.

Considering the application of AgNPs in supplements, products for personal care or cosmetics and in materials in contact with food (i.e. packaging or in household products) (Vance et al 2015), it seems reasonable hypothesize that human gut is exposed to very low concentrations of nanoparticles, although chronically. Therefore, the evaluation of AgNPs effects on Caco-2 compartment of the model, was performed only considering basolateral solutions collected from biofilm grown in the presence of AgNPs.

The results obtained with the *in vitro* model set up in this study, clearly sustained a protective effect of biofilm against the genotoxic effect exerted by a concentration of 1 μg/mL of AgNPs on CaCo-2 cells. Notwithstanding, at 72 h results showed an increase of DNA damage regardless of AgNPs presence (C72 and NP72): this damage may be attributed to unidentified metabolites produced by the bacterial biofilm, able to exert cell genotoxicity.

Sahu et al.(2014) demonstrated that exposure to AgNPs of 20 nm size (concentrations from 1 to 15 μg/mLfor 24h) decreased the viability of CaCo-2 cells, starting from 10 μg/mL, but was not able to exert a genotoxic effect, estimated as presence of micronuclei by flow cytometry, whatever the concentration tested. The present study is consistent with the work of Sahu et al. (2014) in sustaining the lack of a severe genotoxic effect following the exposure to AgNPs at a concentration of 1 μg/mL. Moreover, in our work the mild DNA oxidative damage caused by TNP treatment was significantly

reduced by the preliminary interaction of AgNPs with biofilm basolateral media, at least for 24 and 48h.

Aueviriyavit and collaborators (2014) exposed undifferentiated CaCo-2 cell cultures to increasing concentrations (from 5 to 1000 μg/mL) of uncharacterized AgNPs for 24 h. In line with our results, a decrease in cell viability (80% compared to control) was found only at exposure concentrations higher than 10 μg/mL AgNPs. Nanoparticles internalization at cytoplasm level was also assessed (15 μg/mL) andthe authors concluded that the AgNPs are capable of inducing cytotoxicity in CaCo-2 cells through the induction of oxidative stress.

In literature, few studies have investigated the effect of supplementation of silver nanoparticles in cell culture (Kim et al., 2009; Piao et al., 2011; Mukherjee et al., 2012), and even fewer are those who used CaCo-2 cells (Aueviriyavit et al., 2014; Sahu et al., 2014). Considering only papers that have been conducted on CaCo-2 cells, so remarkable differences are found in the different experimental protocols, such as nanoparticles size, exposure times solution media, characteristics of cell culture (confluenced or differentiated cells, cells in adhesion or in suspension), that it becomes difficult to make a comparison with the results obtained in this work .It is possible to conclude that the results obtained with the *in vitro* model set up in this study, clearly sustain a protective effect of biofilm against the genotoxic effect exerted by a concentration of 1 μg/mL of AgNPs (TNP).

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CHAPTER 5

Impacts of dietary silver nanoparticles and probiotic administration on the microbiota of an in-vitro gut model.

1 Introduction

The wide-ranging antibacterial, antiviral, and antifungal properties of silver nanoparticles (AgNPs), along with their intrinsic catalytic and optical signatures, make these engineered nanoparticles the most popular and commonly used in consumer products for domestic, environmental, medical, and industrial applications (Klasen, 2000; Simpson, 2003; Ip et al., 2006). More than 400 products containing AgNPs are currently inventoried, comprising ~51% of all marketed nano-based consumer products (Vance et al., 2015).

Release of AgNPs from nanoenabled products has been observed (Blaser et al., 2008; Gottschalk et al., 2009; Kaegi et al., 2010; Kaegi et al., 2011), and the potential toxicity of such releases on different natural and engineered ecosystems has been investigated (Kim et al., 2011; Colman et al., 2013; Yang et al., 2013; Wang et al., 2013; Priester et al., 2014).

Because of their broad use and accumulation in the environment, humans are widely and increasingly exposed to AgNPs. Oral exposure is one of the main routes of human exposure to AgNPs, as there are numerous NP applications that directly or indirectly have potential for ingestion. Such applications include flavor enhancers, food pigments, or health supplements (Wijnhoven et al., 2009; Frohlich and Roblegg, 2012; Tulve et al., 2015). Some non-edible products may release NPs over time – examples are nanosilver-coated toothbrushes, plastic food and drink packaging, and even baby bottles and pacifiers (Benn et al., 2010; Echegoyen and Nerín 2013; Mackevica et al., 2017). Medical applications include oral drug delivery vehicles or therapeutic molecules (Ge et al., 2014; Benyettou et al., 2015). In addition to direct ingestion, a proportion of inhaled particulate materials are eventually removed via the gastrointestinal tract, after being mobilized up the trachea via the mucociliary escalator (Bergin and Witzmann, 2013). It has been estimated that a range of 0.032-12.6 μg/mL of metal NPs can be ingested every day (Fröhlich and Fröhlich, 2016).

Because of the increased potential for consumer exposure to AgNPs, it appeared urgent to assess the possible impact on the gut microbiota, which is now considered as an entire metabolic organ with

numerous physio(patho)logical functions. Few studies have investigated this issue, focusing on the in-vivo impact of oral exposure to AgNPs in weaned pigs, rats and mice (Fondevila et al., 2009; Hadrup et al., 2012; Han et al., 2014; Williams et al., 2015; Wilding et al., 2016; van den Brule et al., 2016). Das and colleagues (2014) studied the effects of 25, 100 and 200 mg/L of AgNPs on a synthetic human intestinal microbiota. The results showed a shift in the community structure and a significant reduction in culture-generated gas production at the two highest AgNP concentrations compared to controls, as well as significant changes in fatty acid methyl ester profiles even at the lowest concentration. These findings suggested that AgNPs ingestion could have negative consequences on the human intestinal microbiota.

However, AgNP doses administered in experiments greatly exceed those estimates of actual human consumption which are currently available. In fact, most of the studies have been conducted with relatively high (bactericidal) AgNP concentrations, and the effects of sub-lethal (low concentrations) exposure are poorly understood. This is a critical knowledge gap because low (rather than high) AgNP concentrations are expected to predominate in the gastrointestinal tract (McCracken et al., 2016). Furthermore, none of these studies included the effects of probiotic interventions on the intestinal microbiota exposed to AgNPs. Probiotics are being used with increasing frequency as a treatment for several medical conditions, including gastrointestinal disorders (Kristensen et al., 2016).

In this study, potential interactions among sub-lethal concentration of AgNPs, the intestinal microbiota and a probiotic bacterium were tested using in-vitro batch fermentation models inoculated with human fecal matter to mimic the human digestive tract environment (Payne et al., 2012; Long et al., 2015). Therefore, this study first characterized the composition and phylogenetic distribution of the fecal microflora responding to singular and combined exposures of 1 μg/mL AgNPs (Garuglieri et al., 2016) and the probiotic *Bacillus subtilis* (Cutting, 2011) through next generation sequencing (NGS) techniques and fluorescence in situ hybridization (FISH) analysis. We further defined the functional capabilities associated with the untreated and treated fecal bacterial communities using metagenomic inference. Finally, we investigated the effects of AgNPs and the probiotic on the fecal metabolic profile (short-chain fatty acids, SCFA) using gas chromatography, and its potential cytotoxicity and genotoxicity on the human intestinal Caco-2 cell line, using cell viability assays (Trypan blue dye exclusion method) and DNA strand breaks assay (Comet test), respectively.

2 Experimental design, outcomes and discussion

2.1 Effects of AgNPs and probiotic on the composition and phylogenetic distribution of the fecal microflora

Fecal samples were obtained from 4 healthy human volunteers who had not been treated with antibiotics and pro or prebiotics for more than 3 weeks prior to sampling, and were selected randomly

from 2 youth and 2 middle aged people. After collection, fecal samples were immediately placed under anaerobic conditions, pooled together and fermentation was initiated by inoculation of each fermentation medium-containing vessel (Simon et al., 2005) with 10% of 10 g/L fecal suspension. The vessels were inoculated in triplicate with i) the probiotic *B. subtilis* to gain a final concentration of 10^7 cells/mL (Cutting, 2011), ii) AgNPs to gain a concentration of 1 μg/mL (Garuglieri et al., 2016) and iii) both the probiotic and the NPs at the same concentrations reported for single exposure experiments. According to previous characterization, AgNPs have an average size of 14± 0.3 nm with 77% of the particles ranging from 5 to 17.5 nm (Garuglieri et al., 2016). Control samples without any treatments were run simultaneously. The fermentation cultures were incubated anaerobically at 37° C for 24 hours, shaking them every hour. Aliquots of the fermentation cultures were sampled at 0 and 24 h. Whole genomic DNA from each fermentation culture was extracted using the PowerFecal DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), and high-throughput sequencing analysis of the V3–V4 region of the bacterial 16S rRNA gene was performed by using a MiSeq platform (Illumina) with v3 chemistry providing 2x300 paired-end reads. The obtained 16S reads were taxonomically assigned using in-built functions of Qiime v and SILVA database.

According to the obtained sequences, a total of 15 phyla and 258 genera were identified. Bacterial species belonging to the phyla Bacteroidetes and Firmicutes have been reported to dominate human feces (Eckburg et al., 2005; Qin et al., 2010; Takagi et al., 2016) and NGS analysis revealed that these two phyla represented 56% and 19% of the total phyla identified in all samples, respectively (Figure 1). Species belonging to other phyla, Proteobactera, Actinobacteria and Verrucomicrobia, were present in minor proportions but had been detected in the fermentation cultures.

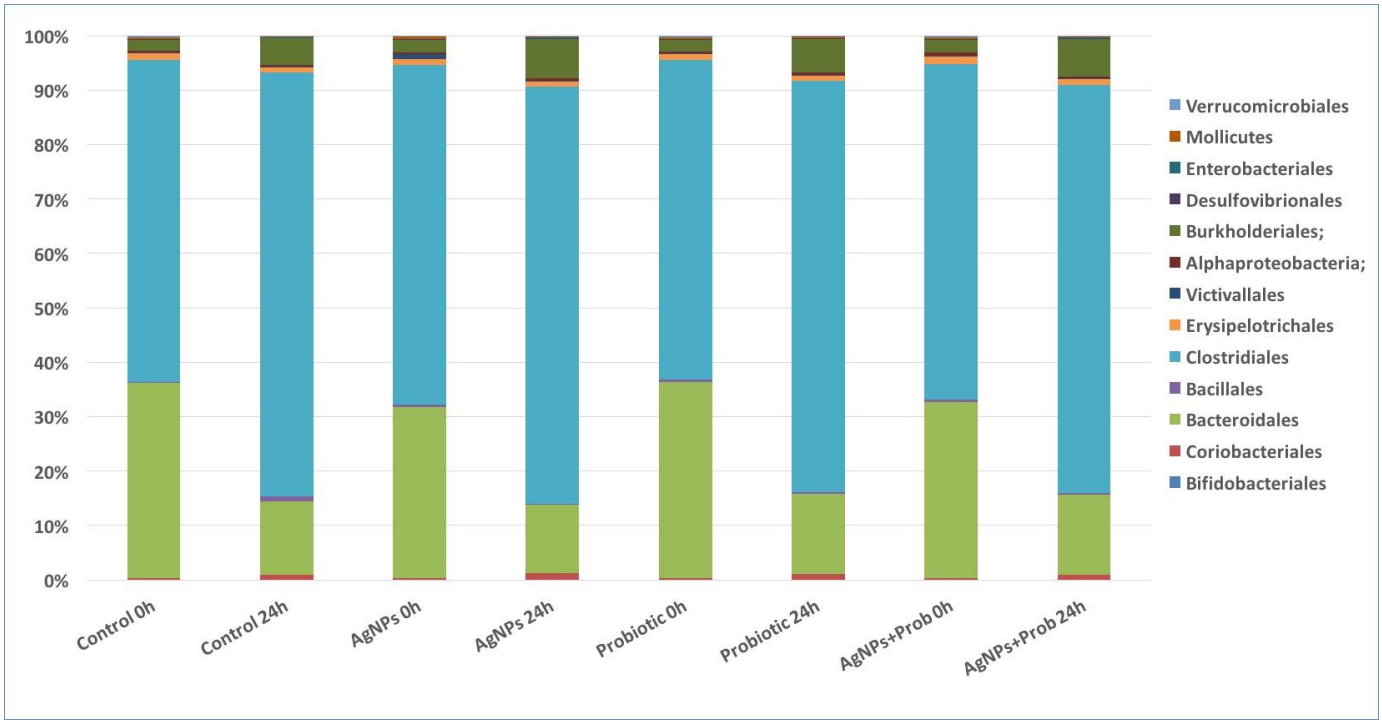


Figure 1 - Taxonomic classification of bacterial reads retrieved from different untreated and treated fermentation cultures. Relative abundance of bacterial at phylum level.

Firmicutes/Bacteroidetes ratio was constant in both untreated and treated fermentation cultures (Table 1).

	Control	AgNPs	Probiotic	AgNPs + Prob
0 h	1.7	2	1.7	2
24 h	5.4	5.3	5.2	5.2

Table 1 - Firmicutes/Bacteroidetes ratios in the untreated and treated fermentation cultures.

After 24 hours of fermentation, species belonging to the orders Bacteroidales, Coriobacteriales and Enterobacteriales decreased in number, being replaced primarily by those of the orders Clostridiales and Burkholderiales. Proportions of species of the orders Bifidobacteriales, Bacillales, Erysipelotrichales, Victivallales, Alphaproteobacteria, Enterobacteriales, Mollicutes and Verrucomicrobiales were largely unchanged in all the fermentation cultures. No significant differences were observed among the different treatments at order level.

To appreciate the impacts on fecal microflora of single and combined exposure to AgNPs and probiotic, the microbial composition of the main order Clostridiales was further examined at the lowest taxonomic level (Figure 2).

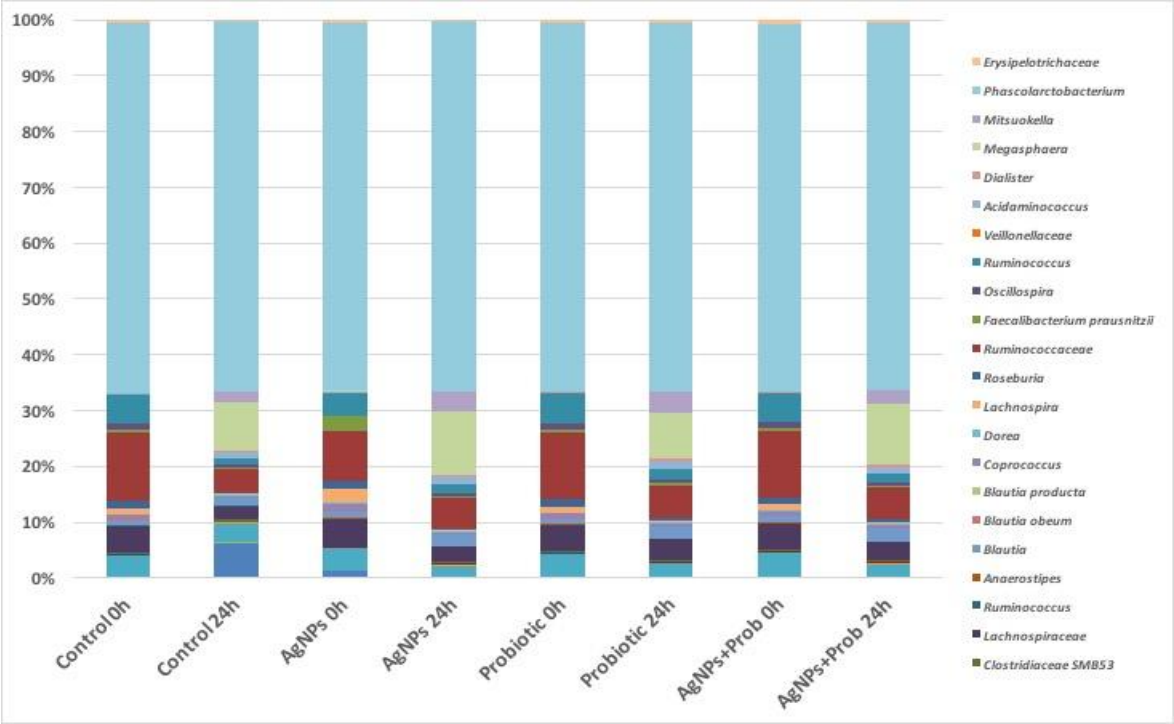


Figure 2 - Taxonomic classification of bacterial reads retrieved from different untreated and treated fermentation cultures. Relative abundance of bacterial at lowest taxonomic level for the order Clostridiales.

In all the cases, the majority of the species of order Clostridiales belonged to genus *Phascolarctobacterium* in both the control samples and the treated batch fermentation cultures. *Phascolarctobacterium* spp. produce high amounts of the short chain fatty acids (SCFA) acetate and propionate and it is specialized in the utilization of succinate produced by other bacteria (Watanabe et al., 2011). Furthermore, all the treated fermentation cultures maintained and simulated proportions of the majority of the bacterial taxa that dominated the control samples, including Clostridiales, Lachnospiraceae, Ruminococcaceae and *Ruminococcus*. In contrast, the genus *Megasphaera* increased by 35% in the fecal microflora treated with AgNPs and the combined action of AgNPs and the probiotic. *Megasphaera* is a genus of Firmicutes bacteria that exhibits antibiotic resistance and efficient stress response systems (Shetty et al., 2013).

Principal coordinate analysis (PCoA) based on the relative abundance of OTUs revealed that the fecal microflora shifted over time in both control and treated fermentation cultures. In fact, in Figure 3 it is possible to observed two main clusters on the PC2 axis composed by both treated and untreated samples at 0h and at 24h.

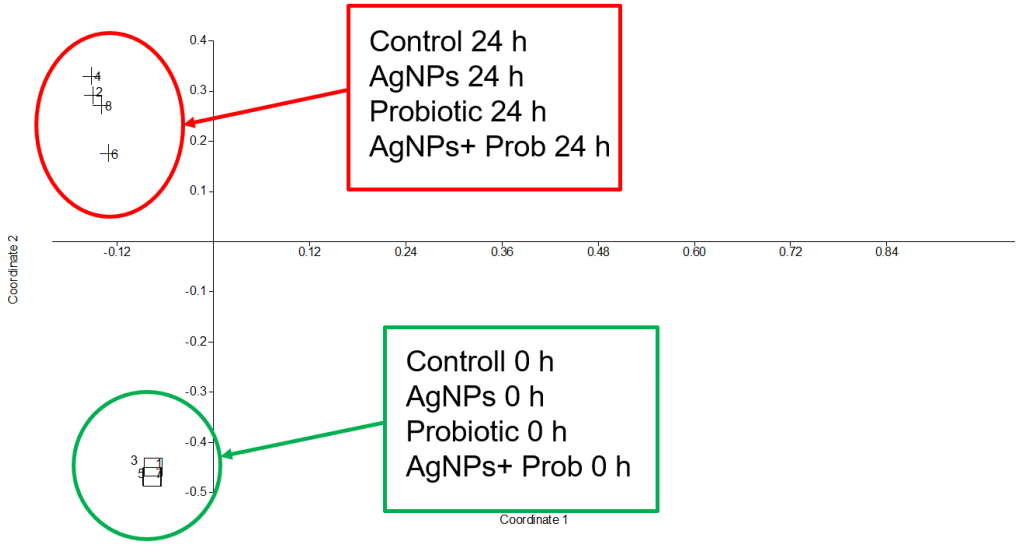


Figure 3- Principal coordinate analysis (PCoA) plot of the untreated and treated fecal microflora based on the relative abundance of OTUs. The first two PC axis explained 86.2% of the variability associated with the data.

As shown in the PCoA score plot, which explained 86.2% of the variation, single and combined treatments with sub-lethal concentration of AgNPs and prebiotic did not change the overall fecal microflora in the fermentation system. These indicated that most of the microbial species in all samples were not affected by the presence of nanoparticles and the probiotic, although the proportions/numbers of some species changed during the in-vitro cultivation.
Fluorescence in situ hybridization (FISH) analyses were conducted on specific bacterial groups (Table 2) following the protocol of Manz et al. (1992).

LABEL	TARGET	SEQUENCE	% FA	REFERENCE
Eub338 mix I, II, III	Dominio *Bacteria*,	GCT GCC TCC CGT AGG AGT	35	Van der Waaij et al., 1996
Lab158	*Lactobacillus-Enterococcus*	GGT ATT AGC AYC TGT TTC CA	0	Harmsen et al., 1999
Bac303	*Bacteroides-Prevotella*	CCA ATG TGG GGG ACC TT	0	Manz et al., 2006
Bif164	*Bifidobacterium*	CAT CCG GCA TTA CCA CCC	0	Langendijk et al., 1995
Chis150	*Clostridium hystolyticum*	TTA TGC GGT ATT AAT CTY CCT TT	0	Franks et al., 1998
Erec482	*Clostridium coccoides* *Eubacterium rectale*	GCT TCT TAG TCA RGT ACC G	0	Kempf et al., 2000

Ec1513	*E. coli*	CAC CGT AGT GCC TCG TCA TCA	35	Poulsen et al., 1994
Fpau645	*Faecalibacterium prausnitzii*	CCT CTG CAC TAC TCA AGA AAA AC	30	Suau et al., 2001
Rbro730	*Ruminococcus*	TAA AGC CCA GYA GGC CGC	30	Kempf et al., 2000

Table 2: details of FISH labels used in experimentation.

Results reported in Figure 4 showed that the exposure to sub-lethal concentration of AgNPs negatively affected the Bacteroides-Prevotella (Figure 4a), *Faecalibacterium prausnitzii* (Figure 4b) and *Clostridium coccoides/Eubacterium rectale* taxa (Figure 4c).

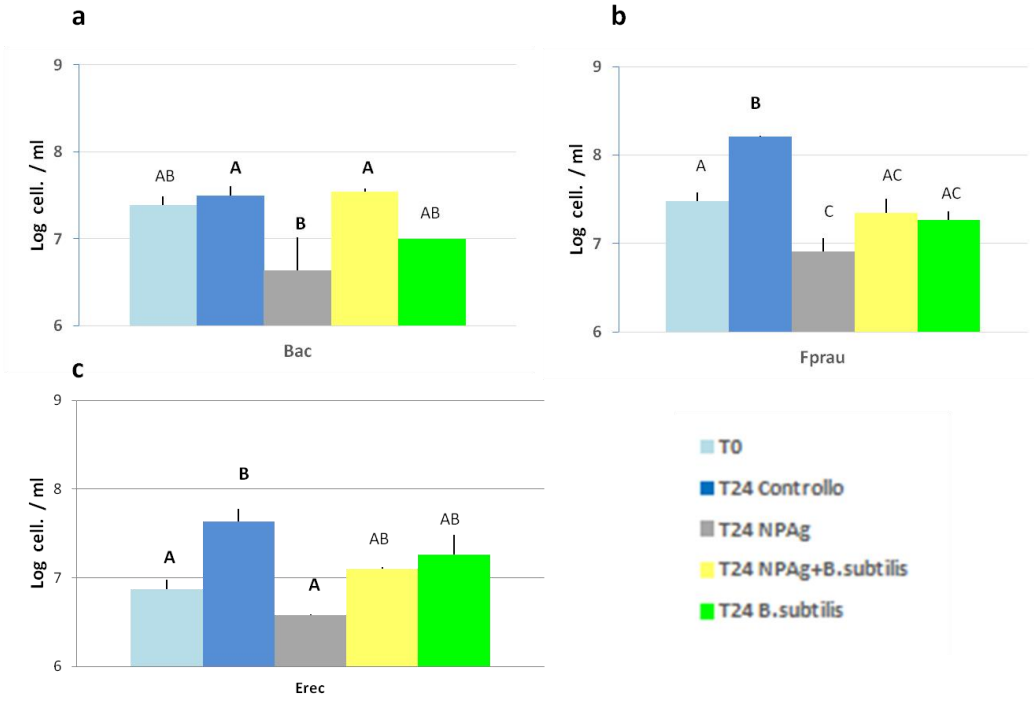


Figure 4 – Values of bacterial abundance of Bacteroides-Prevotella (a), *Faecalibacterium prausnitzii* (b) and *Clostridium coccoides/Eubacterium rectale* taxa (c) different fermentation batch samples. Letters provide the graphical representation for post hoc comparisons. The histogram provides the P-values obtained by ANOVA analysis. According to post hoc analysis (Tukey's HSD, P<0.05), means sharing the same letter are not significantly different from each other.

Studies focused on the composition of the microbiota showed that AgNPs of 14 nm did not alter the ratio of Bacteroides to Firmicutes after oral exposure of rats (Hadrup et al., 2012). The lack of obvious changes in the microbiota composition has also been reported after exposure of mice to 20 and 110 nm AgNPs (Wilding et al., 2015). In contrast, Williams et al. (2015) have detected size- and dose-dependent changes in ileal-mucosal microbial populations after oral gavage of rats with 10, 75 and 110 nm AgNPs. After treatment with 10 nm AgNPs greater proportions of Firmicutes phyla, along with

a decrease in the *Lactobacillus* genus were observed. In the absence of morphological damage to enterocytes, the population of lactic acid bacteria was increased in the guts of Japanese quail that received colloidal 25 mg/kg AgNPs in their drinking water (Sawosz et al., 2007). Exposure to 110 nm AgNPs caused a decrease in Firmicutes at the highest concentration of 36 mg/kg. 60–100 nm AgNPs also reduced coliforms in the gut microbiota of weaning pigs (Fondevila et al., 2009). When using in-vitro exposures of the porcine microbiota samples effects were even more pronounced; coliforms were markedly and lactobacilli slightly reduced. In synthetic stool mixtures of 33 different isolates from a healthy human donor polyvinylpyrrolidone-capped 10 nm AgNPs increased the abundance of *Escherichia coli* (Das et al., 2014). Recently, Pietroiusti et al. (2016) and Frölich and Frölich (2016) has attempted to elucidate AgNP mechanisms of action and explain discrepancies between studies. Discrepancies between studies could be explained by differences in species, modes of administration (gavage vs diet), doses, NP size and coating, in-vitro or in-vivo exposure and duration of exposure. The differences of results between studies could also be related to the techniques used to analyze the microbiota (Goodriche et al., 2014). This includes the type of sample and site of collection, culture-dependent or -independent approach, and techniques to analyze the microbiota (FISH, quantitative (q) PCR or NGS).

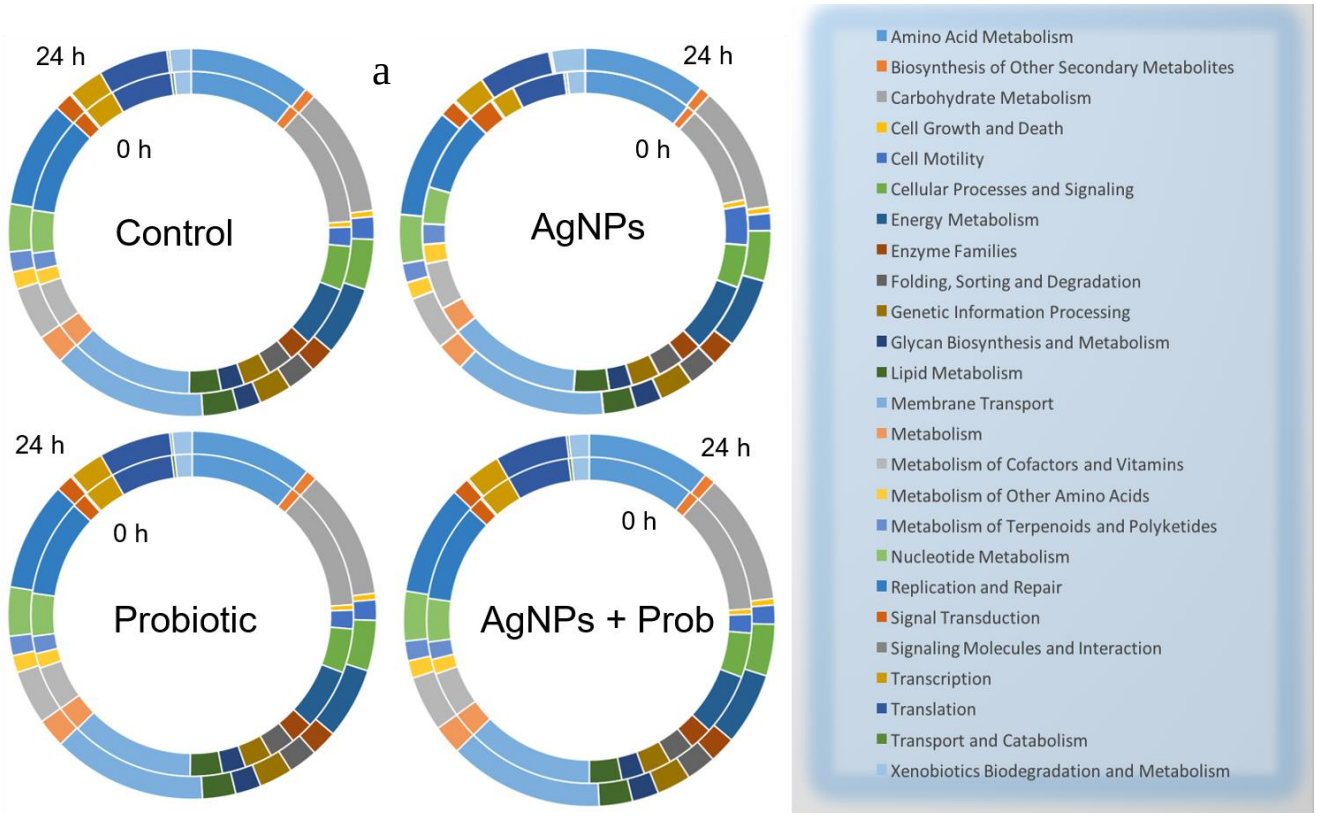
2.2 Effects of AgNPs and the probiotic on functional profiles of the fecal microflora

The molecular investigations indicated that single and combined exposure to AgNPs and *B. subtilis* did not significantly affect the overall compositions and phylogenetic distributions of the fecal microflora. However, functional differences might occur, reflecting dramatically altered performances of the gut ecosystem. To gain insight into the molecular functions of bacterial microbiota across untreated and treated fermentation cultures, we used PICRUSt to predict the metagenomic contribution of the communities observed. PICRUSt predicts metagenomic potential by imputing the available annotated genes within a known sequenced database, such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the Clusters of Orthologs Groups (COGs) catalogue, based on the presence/absence of OTUs in a 16S rRNA survey (Langille et al., 2013).

Using PICRUSt as a predictive exploratory tool, the present study inferred that 25 gene families were identified in both control and treated samples. Of the 25 gene families, the majority of the genes belonged to membrane transport (12.61% at 0h and 13.55% at 24h), carbohydrate metabolism (11.66% at 0h and 11.35% at 24h), amino acid metabolism (10.63% at 0h and 10.76% at 24h), replication and repair (9.26% at 0h and 9.52% at 24h) energy metabolism (5.93% at 0h and 6.21% at 24h) and translation (5.92% at 0h and 5.97% at 24h) (Figure 5). This is consistent with the general metabolic functions (such as carbohydrate, protein and amino acid metabolism) being essential for microbial survival (Lamendella et al., 2011; Erickson et al., 2012), and it is in line with the

observations of other metagenomic studies in mice and humans (Arumugam et al., 2011; Ridaura et al., 2013; Lu et al., 2014; Mao et al., 2015).

Of the 25 gene families, 4 gene families in AgNPs-treated microbiota at 24h had significantly different abundances in comparison to the respective controls at 0h: i) cell motility (-60.1%), ii) replication and repair (+17.7%), iii) signal transduction (-42.7%) and iv) xenobiotics degradation and metabolism (+39.9%). Noteworthy was the observation that the combined treatment with AgNPs and the probiotic did not exert any effects on the functional capability of the fecal microflora, suggesting a protective role of the probiotic *Bacillus subtilis* against sub-lethal concentrations of AgNPs.



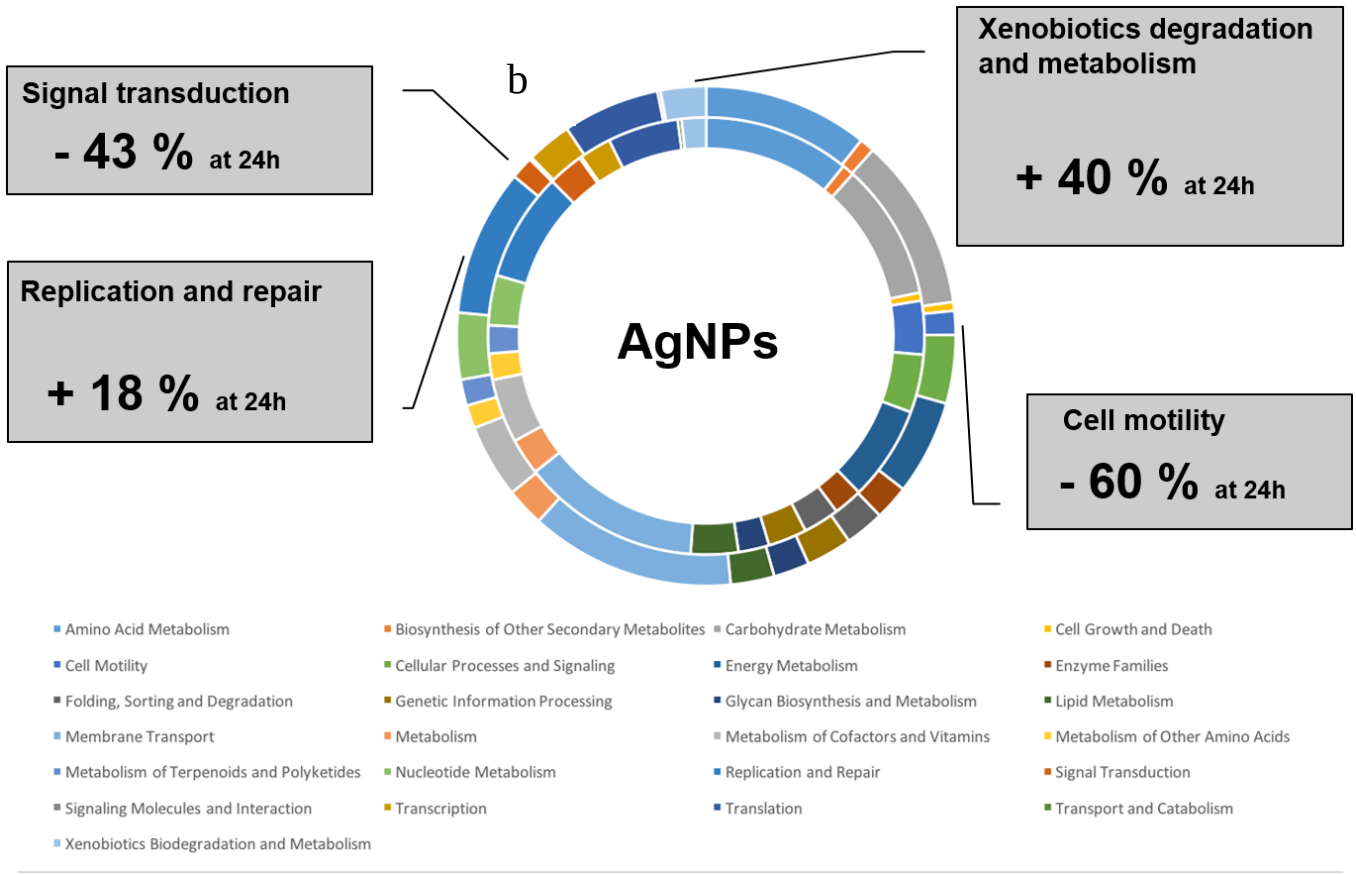


Figure 5 - (a) Metabolic pathway of untreated and treated fermentation cultures obtained using metagenomic inference. (b) Gene families in the AgNPs-treated microbiota at 24h that showed significantly different abundances in comparison to the respective controls at 0h.

Ortega-Calvo and colleagues (2011) demonstrated a significant inhibition of cell motility at a concentration of AgNPs above 0.1 µg/mL and a negative tactic response in bacteria at low but environmentally relevant, sub-lethal AgNPs concentrations. It has also been found that the nanoparticles can modulate the signal transduction in bacteria. It is a well-established fact that phosphorylation of protein substrates in bacteria influences bacterial signal transduction. Nanoparticles dephosphorylate the peptide substrates on tyrosine residues, leading to signal transduction inhibition and thus the stoppage of growth (Prabhu and Poulose, 2012; Dakal et al., 2016). Another fact is that DNA has sulfur and phosphorus as its major components; NPs can act on these soft based activating replication and repair systems (Butler et al., 2015). Indeed, xenobiotics biodegradation and metabolism pathways were activated by the presence of metal nanoparticles. In addition, NPs can influence the growth profile of degrading microorganisms to augment the biodegradation rate (Bhatia et al., 2013; Kumari and Singh, 2016).

2.3 Effects of AgNPs and probiotic on SCFA profiles of the fecal microflora

We sought to define the response of a gut microbiome to AgNPs and their interaction with probiotics, and better to understand the ability of this complex system on microbial metabolites of physiological interest like short-chain fatty acids (SCFA).

SCFA are metabolic products of the human gut microbiota that are absorbed by the host; these metabolites have been associated with benefits for host health, by inducing widespread effects on gut, brain, and behavior (Rastall and Gibson, 2015). Therefore, qualitative and quantitative profiles of SCFA in fermentation cultures were obtained by gas chromatography (Weaver et al., 1997) to explore any possible influence of AgNPs and the probiotic on phenotypes of the fecal community. In all the fermentation samples, SCFAs consisted mainly of acetate, propionate, and butyrate (Figure 6).

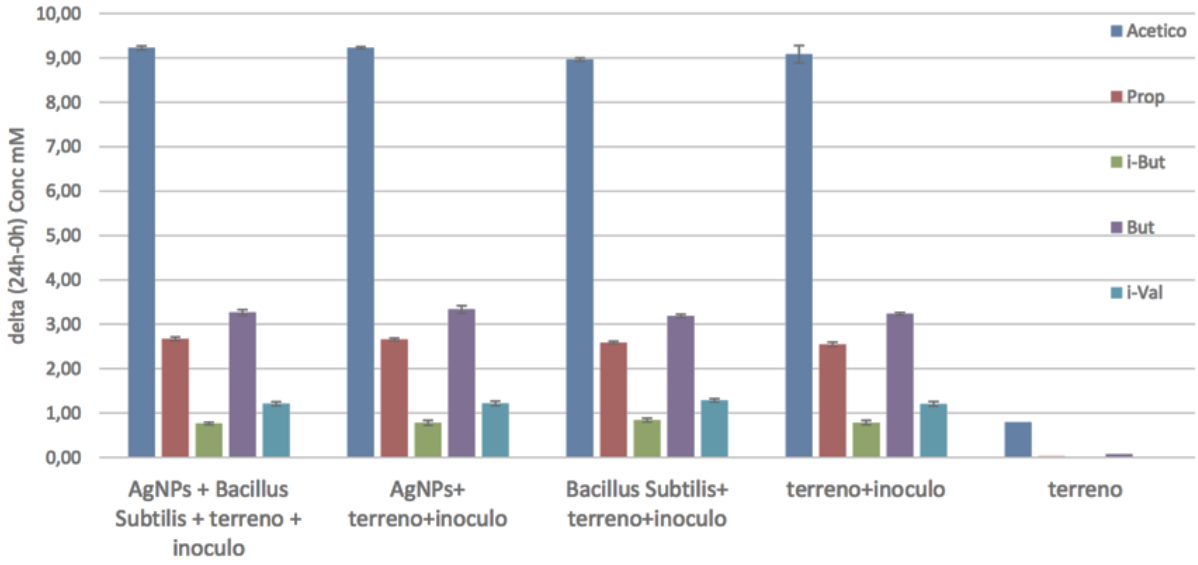


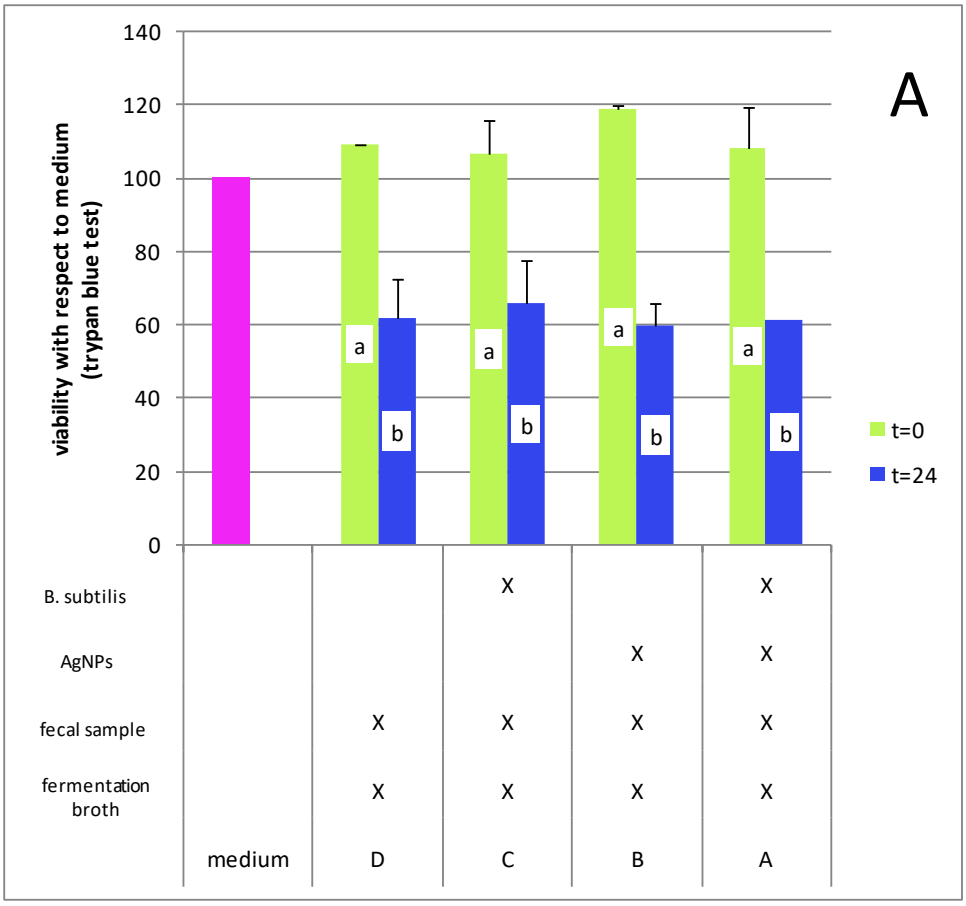
Figure 6 - SCFAs values in different fermentation batch cultures after 24h of fermentation.

Acetate, propionate, and butyrate are the most abundant (≥ 95%) SCFAs in the human colon and stool, and are present in an approximate molar ratio of 60:20:20 (den Besten et al., 2013). Figure 6 shows that there were not significant differences ($p>0.05$) in SCFA profiles among untreated and treated fermentation cultures, as predicted by inferred metagenomic with PICRUST that saw the same representation of fatty acid biosynthesis pathways along samples.

Previous investigations reported that metal NPs such as zinc oxide, cerium oxide and titanium oxide caused non-lethal but significant changes to the phenotype of the microbial community, including fatty acid production (Taylor et al., 2015; Antisari et al., 2016; Oberemm et al., 2016; Ramelingam et al., 2016).

2.4 Citotoxicity and genotoxicity on Caco-2 cell monolayers

Differentiated Caco-2 cells grown as monolayers were incubated under the different treatments for 30 min at 37 °C, with 95% humidity and 5% CO_2. Every treatment was analyzed in triplicate and controls, either negative (medium and fermentation broth) and positive (medium with H_2O_2 50 µM) were included in each batch to check the procedure. After the incubation, an aliquot of this cell suspension was used to assess citotoxicity of treatments by measuring cell viability with Trypan Blue exclusion test (Strober, 2001). The Trypan Blue is a dye capable of selectively coloring only the dead cells: this occurs thanks to the extreme selectivity of the cell membrane. Indeed, having an intact cell membrane, the viable cells do not allow the dye penetrating the cytoplasm. In contrast, the dye penetrates in dead cells easily and they are therefore distinguishable from the other cells. The viable and total cells are then counted within the grids on the hemocytometer, and viability expressed as percentage of viable cells on total cells; the viability of cells treated with medium, is arbitrarily fixed to 100% (Figure 7A) (Gratzl et al., 2011; Tice et al., 2000; Venturi et al., 1997).



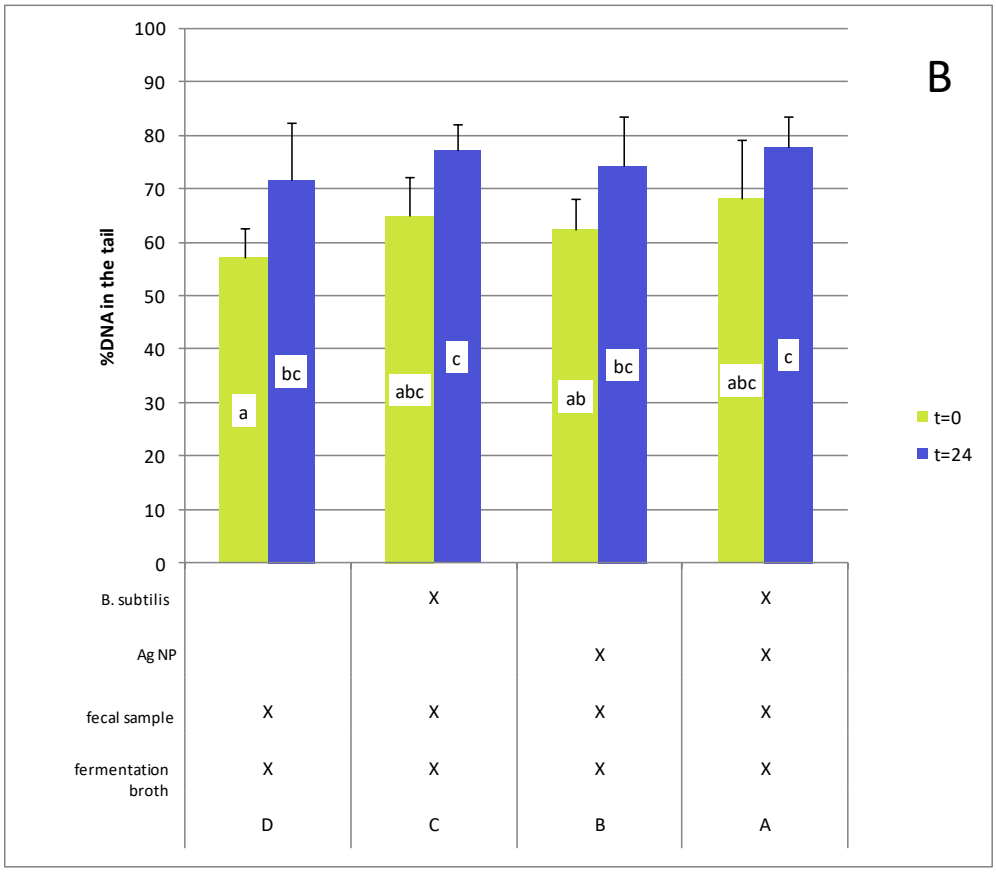


Figure 7- Viability by Trypan Blue test (A) and genotoxicity by Comet Assay (B) of differentiated Caco-2 cells, grown on monolayer, treated with supernatant of fecal fermentation broth with or without *B. subtilis* and/or 1 µg/mL AgNPs.

Statistical analyses revealed that the metabolic profiles of the fecal microflora exposed to both AgNPs and the probiotic alone and in combination exerted neither cytotoxic nor genotoxic effects on Caco-2 cell monolayers.

The scientific literature reports discrepancies between studies on the cytotoxic and genotoxic effects of AgNPs. Martirosyan et al. (2013) indicated that AgNPs were cytotoxic for Caco-2 cells with an EC50 of ca. 40 µg/mL, a concentration much higher in comparison to the one used in this study. Panda and colleagues (2011) showed that cell death and DNA damage induced by silver nanoparticles (AgNPs) were prevented by Tiron and dimethyl thiourea, which scavenge superoxide anions (O_2^-) and H_2O_2 respectively, demonstrating the role of ROS in AgNP-induced cell death and DNA damage. In another study (Asare et al., 2011), 200-nm AgNPs caused a concentration-dependent increase in DNA strand breaks in NT2 human testicular embryonic carcinoma cells. Although in another study (Rim et al., 2013) no significant induction of DNA damage in AgNP-treated mouse lymphoma cells was observed in the standard Comet assay, the AgNP treatments induced a dose-responsive increase in oxidative DNA damage in an enzyme-modified Comet assay in which oxidative lesion-specific endonucleases were added. These AgNPs were taken up by cells, decreasing cell viability in a dose- and time-dependent manner at 6.25–100 µg/mL, and decreasing the activities of SODs and GSH peroxides.

Levels of malondialdehyde, a lipid peroxidation end product, were also increased in the AgNP-exposed cells (Song et al., 2012). In a study conducted by Piao et al. (2011), AgNPs reduced cell viability, as demonstrated by the formation of apoptotic bodies, sub-G1 hypo-diploid cells, and DNA fragmentation. According to Piao et al. (2011) silver nanoparticles cause cytotoxicity by oxidative stress-induced apoptosis and damage to cellular components.

Researchers have also shown that AgNPs impair mitochondrial function, mainly owing to altered mitochondrial membrane permeability, which results in an uncoupling effect on the oxidative phosphorylation system (Teodoro et al., 2011; Singh and Ramarao, 2012; Bressan et al., 2013; Dakal et al. 2016). In L929 fibroblasts, but not in RAW 264.7 macrophages, 20-nm AgNPs were shown to be more cytotoxic than Ag ions in L929 fibroblasts but not in RAW264.7 macrophages. Collectively, these results indicate that the effects of AgNPs on different toxicities may be a consequence of their ability to inflict cell damage. In addition, the tendency of Ag to induce greater cell damage when in the NP form rather than in the ion form is cell type- and size-dependent (Park et al., 2011). AgNP cytotoxicity was also shown to depend on NP size and dosage in human lung fibroblast cells (Li et al., 2012; Zhang et al., 2016). DNA strand breaks (Comet assay) in human lymphocytes revealed that AgNPs at concentration 25 μg/mL can cause genotoxicity. In-vivo experiments on plants (*Allium cepa* and *Nicotiana tabacum*) and animal (Swiss albino male mice) showed impairment of nuclear DNA (Ghosh et al., 2012). A recent study carried out by Butler et al. (2015) assessed the genotoxicity of AgNPs by mutagenicity, clastogenicity and DNA strand-break-based DNA damage as measured in the Comet assay. AgNPs of all sizes tested (10, 20, 50 and 100nm), were negative for mutagenicity (reverse mutation assay) in five bacterial strains of *Salmonella typhimurium* and *Echerichia coli*. Clastogenicity (flow cytometry-based micronucleus assay) and intermediate DNA damage (DNA strand breaks as measured in the Comet assay) were assessed in two mammalian white blood cell lines. It was also observed that micronucleus and Comet assay end points were inversely correlated with AgNP size, with smaller NPs inducing a more genotoxic response.

All these results suggest that AgNPs might induce cytotoxicity and genotoxicity in a concentration-size- and coating-dependent manner (Guo et al., 2016).

3 Conclusions

This in-vitro study was designed to unravel how the composition, functional performances and microbial metabolites of the human gut microbiota change in response to single and combined treatments of AgNPs, at a concentration relevant for currently estimated daily human intake, and the probiotic *B. subtilis*. Our findings suggested that 1 μg/mL AgNPs and the probiotic did not substantially affect the composition and phylogenetic distribution of the fecal microflora. However, functional differences, even in only few critical pathways, occurred in AgNPs-treated fermentation cultures,

reflecting altered performances of the gut ecosystem in presence of sub-lethal concentrations of AgNPs.

We also demonstrated that while not altering the overall structure of the fecal microflora, the probiotic administration has a chemopreventive role by protecting against the impacts of AgNPs on some key metabolic pathways.

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CHAPTER 6

Products

PAPERS

Garuglieri, E., Cattò, C., Villa, F., Zanchi, R., & Cappitelli, F. (2016). Effects of sublethal concentrations of silver nanoparticles on Escherichia coli and Bacillus subtilis under aerobic and anaerobic conditions. Biointerphases, 11(4), 04B308.

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POSTERS

Human Gut Microbiome and desease Conference 2015, 25-26 June 2014, Milano -"NanoGut. Unraveling the effects of food-related engineered NANOparticles on the GUT interactive ecosystem." Garuglieri E., Cattò C., Villa F., Cappitelli F.

*20th Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology,*23-25 September 2015, Perugia "Unrevealing the effects of food-related engineered nanoparticles on the intestinal biofilm." Garuglieri E.

3rd International conference on microbial diversity. The challenge of complexity, 27-29 October 2015 Perugia – "Unraveling the effects of food-related engineered NANOparticles on the GUT interactive ecosystem (NanoGut)" Garuglieri E., Zanchi R., Cattò C., Troiano F., DeVincenti L., Cappitelli F.

XIV Congresso FISV – Federazione Italiana Scienze della Vita, 20-23 September 2016 Roma - "Unraveling the effects of food-related engineered nanoparticles on the GUT interactive ecosystem" Domingo G., Bracale M., Mansoni M., Erba D., Garuglieri E., Cappitelli F., Vannini C.

SPEECHES

NanoGut Conference,15 September 2016, Milano - "*Microbiologic analysis on biofilms*"

CHAPTER 7

Conclusions

Silver nanoparticles (AgNPs) are the most prevalent nanomaterials in consumer products due to their strong antimicrobial action. While AgNP toxicity at high concentrations has been thoroughly investigated, their effects on microbial ecosystems at sub-lethal levels are relatively unknown.
In the light of the previous consideration, I focused my attention on the effects of low (rather than high) concentrations of AgNPs on three different systems:

1. Planktonic cultures of the model bacteria *Escherichia coli* and *Bacillus subtilis* (Chapter 3);
2. Biofilm cultures of the model bacterium *Escherichia coli* and their interplays with CaCo2 cells system (Chapter 4);
3. Human fecal microbiota in combination with the probiotic *Bacillus subtilis* (Chapter 5).

These experimental designs were created to investigate microbial ecosystems of increasing complexity, assessing whether sub-lethal concentrations of AgNPs influence microbial physiology and behavior in such settings.
Planktonic and biofilm experiments were performed under both aerobic and anaerobic conditions to simulate complex natural systems showing gradients in oxygen distribution, such as the intestinal mucosa.

My project started by characterizing the physical and chemical properties of AgNPs used throughout this study, and by individuating the minimum AgNPs sub-lethal concentration able to evoke effects on planktonic bacteria (Chapter 3). To this end, I compared the impacts of different sub-lethal AgNPs concentrations on the growth kinetic, adhesion ability, oxidative stress, and phenotypic changes of model bacteria under both aerobic and anaerobic conditions. To gain a mechanistic insight, the experiments were conducted using two different microbial model systems: (1) a Gram-negative bacterium *Escherichia coli* representative of human intestinal flora and responsible for infection, and (2) a Gram-positive bacterium *Bacillus subtilis*, widely distributed in soil, freshwater, marine environments and used as a probiotic.

Growth kinetic tests conducted in 96-well microtiter plates revealed that sub-lethal concentrations of AgNPs do not affect *E. coli* planktonic growth, whereas 1 µg/mL AgNPs increased *B. subtilis* growth rate under aerobic conditions. At the same concentration, AgNPs promoted *B. subtilis* adhesion, while

it discouraged *E. coli* attachment to the surface in the presence of oxygen. As determined by 2,7-dichlorofluorescein-diacetate assays, AgNPs increased the formation of intracellular reactive oxygen species, but not at the highest concentrations, suggesting the activation of scavenging systems. Finally, motility assays revealed that 0.01 and 1 μg/mL AgNPs, respectively, promoted surface movement in *E. coli* and *B. subtilis* under aerobic and anaerobic conditions.

Thus, 1 μg/mL AgNPs resulted the most interesting concentration able to evoke effects on planktonic microorganisms, and for this reason chosen to be used in further experiments.

A second part of my project (Chapter 4) consisted in investigating the physiological response of a mono-species gut biofilm to chronic and acute exposure to 1 μg/mL AgNPs, and how this physiological response affected the intestinal epithelial cells. To study the interplays among sub-lethal concentrations of AgNPs, the gut biofilm and its host, a simplified experimental lab model system was designed. The developed *in vitro* gut system operated in a semi-batch mode, allowing the fine control of experimental conditions in two different compartments: (i) an aerobic compartment composed by a mono layer of CaCo2 cells to mimic the intestinal mucosa and (ii) an anaerobic and microaerophilic compartment characterized by a mono-species *E. coli* biofilm to simulate a simplified gut biofilm. To this end, transwell permeable supports were used to set up the system, and interactions between the two compartments were reproduced by putting in contact biofilm basolateral permeates with CaCo2 cells.

Biofilms were exposed to acute and chronic treatments of AgNPs under both aerobic and anaerobic conditions. Under anaerobic conditions, chronic exposures to 1 μg/mL AgNPs promoted biofilm growth. In acute experiments, a significant decrease in culturable cells count, but not in the total biofilm biomass, leaded us to hypothesize a shift to the dormant state, in which cells are still viable but unable to proliferate. This hypothesis was also confirmed by studying GFP expression along the sections of fully hydrated biofilms.

Under anaerobic conditions, both chronic and acute samples showed higher percentages of GFP-positive cells in comparison to aerobic biofilms, suggesting a more active bacterial subpopulation in absence of oxygen. In contrast to the control, chronic experiments under anaerobic conditions showed high cell activity at the bottom of the biofilm, being the region directly exposed to nanoparticles.

Biofilms exposed to both acute and chronic AgNPs treatments experienced higher level of oxidative stress than under anaerobic environments. Under aerobic conditions, levels of reactive oxygen species (ROS) were similar in both chronic and acute treatments. ROS levels increased in samples exposed to AgNPs acute treatments in absence of oxygen.

Comet assays demonstrated a protective role of biofilms against the genotoxic effect of 1 μg/mL AgNPs on intestinal epithelial cells represented by CaCo2 cells monolayer.

I also explored possible impacts of single and combined treatments of dietary AgNPs (at a sub-lethal concentration relevant for currently estimated human intake) and the probiotic *Bacillus subtilis* to the composition, functional performances and microbial metabolites of in-vitro batch fecal fermentation models to mimic the human digestive tract environment (Chapter 5). Furthermore, I investigated their potential cytotoxicity and genotoxicity on the human intestinal Caco-2 cell line. Molecular investigations showed that 1 μg/mL AgNPs and the probiotic did not substantially affect the compositions and phylogenetic distributions of the fecal microflora. However, the metabolic pathway of AgNPs-treated samples obtained using metagenomic inference revealed functional differences in few critical pathways such as cell motility (-60.1%), replication and repair (+17.7%), signal transduction (-42.7%) and xenobiotics degradation and metabolism (+39.9%). Notably, when the probiotic was used AgNPs did not exert any effects on the functional capability of the fecal microflora, suggesting a protective role of *Bacillus subtilis*. Gas-chromatography and inferred metagenomic indicated no significant differences in short chain fatty acid profiles among untreated and treated fermentation cultures. Finally, the metabolic fecal profile of treated samples was neither cytotoxic nor genotoxic against human intestinal Caco-2 cell line.

In conclusion, my results demonstrate that *E. coli* and *B. subtilis* reacted very differently to sub-lethal concentrations of AgNPs examined under both aerobic and anaerobic conditions. These findings show that exposure to AgNPs under aerobic conditions triggers the most notable changes in the physiology and activity of the selected bacteria, affecting their growth kinetics, adhesion ability, biofilm development, oxidative stress, and inducing important phenotypic changes on model bacteria.
In addition, *in vitro* batch fecal fermentation models, mimicking the human digestive tract environment, suggest that 1 μg/mL AgNPs do not substantially affect the compositions and phylogenetic distributions of the fecal microflora. However, functional differences, even in only few critical pathways, occurred in AgNPs-treated fermentation cultures, reflecting altered performances of the gut ecosystem in presence of sub-lethal concentrations of AgNPs.
I also demonstrated that the probiotic administration, while not altering the overall structure of the fecal microflora, have a chemopreventive role by protecting against the impacts of AgNPs on some key metabolic pathways.
Overall, the present work demonstrates that different physiological processes occur at low AgNP concentrations, suggesting that mechanisms subtler than the simple killing activity occur at these levels.
These findings will contribute to elucidate the behavior and impact of sub-lethal concentrations of engineered nanoparticles on microbial ecosystems, issues still little explored by current literature.

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