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PHYSICO-CHEMICAL AND TOXICOLOGICAL BEHAVIOUR OF Al_2O_3 NANOPARTICLES IN FINE PARTICULATE MATTER

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Abstract

The booming production and application of nanoparticles have raised great concern over its effect on environment, due to their increasing release in the environmental systems via intentional or unintentional activities, where their possible physicochemical and toxicological behavior remain unknown. The nanoparticles are released into the different environmental systems like air, soils and waters. However, their interaction with air matrices has been rarely investigated. For this reason, the changes of main physicochemical properties of Al_2O_3 nanoparticles in the presence of fine particulate matter were examined, as well as their toxicity towards gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) using colony counting method was assessed in the presence of fine particulate matter. *E. coli* and *B. subtilis* were the most strongly affected species by the Al_2O_3 nanoparticles under the influence of fine particulate media. Furthermore, the main physicochemical properties (particle size, zeta potential, and surface chemistry) were changed with the exposure of the fine particulate matter. Sulfone groups were detached and nitro groups were attached on nanoparticles surface according to FTIR analysis. Particle size of Al_2O_3 was decreased with the correlation of zeta potentials. This is one of the rare study to evaluate the effect of fine particulate media on Al_2O_3 nanoparticles with the respect of the physicochemical properties and toxicity of Al_2O_3.

Key words: Al_2O_3, bacterial inhibition, nanoparticle toxicity, $PM_{2.5}$, physicochemical transformation

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1. Introduction

Products containing nanoparticles (NPs) are used in various industrial sectors. Aluminium oxide (Al_2O_3) NPs are used in a number of applications including biosensors, foods, textiles, ceramics, electronics, or membrane systems. Due to increasing production and application, the potential for their release into the environmental systems (air, soil, waters) thus posing subsequent impacts on environment and human health have become a great public concern.

In general, NPs behavior largely influenced by small particle size, large surface area, dispersed particles or agglomeration. This properties have ability to produce reactive oxygen species that are believed to play important roles in potential toxicity of the NPs (Bennett and Keller, 2011; Djurisic et al., 2015; Jiang et al., 2009; Pakrashi et al., 2012). One of the most applied methods to investigate the toxicity of NPs are bacteria based viability or bioluminescence assay. Because bacteria play many important roles in the ecosystem and some bacteria (e.g. *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*) can find in the nature. In addition, they are affected from natural or anthropogenic activities in the ecosystem. The interaction between bacteria and NPs may provide significant information about the impact of NPs on the environment, and at the same time, signify that bacteria are good test models to assess the NPs toxicity

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at the cellular level in the ecosystem (Baek and An, 2011; Baysal et al., 2018a, 2018b; 2019; Jiang et al., 2009; Navarro et al., 2008). It is also known that some physicochemical properties or toxicity of NPs can be affected by the environmental system (e.g. water, soil, and air) and its composition (pH, electrolytes, organic compound etc.) (Joo and Zhao, 2017; Liu and Cohen, 2014; Maurer-Jones et al., 2013; Park et al., 2013; Sadiq et al., 2011; Sharifi et al., 2012). Thus, to determine the risk of NPs in environmental systems, the changes in property of NPs must be define with interacted environment. There are increasing research activities focusing on the adverse effects of various NPs on environment systems (Krzyzewska et al., 2016; Li et al., 2011; Navarro et al., 2008; Pakrashi et al., 2012;). Nevertheless, previous studies mostly focused on aqueous systems (seawater, wastewater, etc.) or soil (Amde et al., 2017; Lowry et al., 2012; Maurer-Jones et al., 2014; Zhang et al., 2018). Relatively little information has been generated on the effect of the atmosphere or atmospheric constituents on NPs. On the other hand, inhalation or atmosphere is one of the important pathways for the NP exposure (Sajid et al., 2015). Besides, the studies reported that exposure to NPs via inhalation can lead to adverse effects on health (Liu et al., 2015). The NPs physicochemical state and their toxicological behavior in this system may be changed by the contents and concentration of atmospheric constituents (Amde et al., 2017; Baysal et al., 2018a; 2018b; Kim et al., 2010; Tiwari and Marr, 2010; Tsai et al., 2010; Zhang et al., 2018). The atmosphere or atmospheric constituents include solid or liquid biological and chemical substances that they originate from not only natural but also anthropogenic sources as a result of smelters, fossil fuel combustion, waste incineration, and/or sea effect etc. (Szigeti et al., 2013). NPs can be chemically or physically transformed with the interaction of these substances (Tiwari and Marr, 2010) and can change their physicochemical and toxicological behavior (Hoet and Boczkowski, 2008; Pipal et al., 2014; Sajid et al., 2015). For instance, Liu et al. (2015) investigated the effect of atmospheric aging (e.g. O_3, -OH oxidation) on the cytotoxicity of the single walled carbon nanotubes using human A549 adenocarcinoma-derived alveolar epithelial cells and THP-1 leukemia-derived peripheral blood monocytes. The results showed that oxygen content and adsorption of organic species observed in single walled carbon nanotubes, however viability of cells were not affected. In another study, the toxicity of Ag NPs was investigated using airborne strains of *Staphylococcus spp*, and the strains isolated from air were inhibited by Ag NPs at the concentration of >30 µg/mL (Wolny-Koladka and Malina, 2017). The studies have also investigated some physicochemical properties and toxicological behavior of ZnO, TiO_2 and carbon based nanoparticles in airborne fine particulate matter ($PM_{2.5}$) (Baysal et al., 2018a; 2018b). The bacterial inhibition mostly originated from the surface chemistry of the NPs. Furthermore, bacteria viability and inhibition mechanism showed different response to the various NPs. Inhibited bacteria varied with the exposure of TiO_2 NPs compared to ZnO NPs in $PM_{2.5}$ matrices. In another $PM_{2.5}$ related study, the behavior of some carbon based nanomaterials (multi-walled carbon nanotubes and graphene nanoplatelets) were evaluated in various environmental systems, such as sea water, soil, and airborne fine particulate through the physicochemical properties (size, zeta potential, surface chemistry, morphology and sedimentation) and the toxicity of bacterium (*B. subtilis, S. aureus, E. coli, P. aeruginosa*) (Baysal et al., 2018a). The results indicated that both inhibition levels of bacteria and also the bacterial distribution can change due to their interaction of carbon based nanomaterials in atmospheric media. The viabilities in airborne fine particulates were not changed in graphene nanomaterials compared to multi-walled carbon nanotubes for the tested bacteria. Thus, these results showed that interaction of various NPs with atmosphere or atmospheric constituents needs extra investigation via biological and physicochemical effects. Moreover, Al_2O_3 NPs is one of the most applied particles, their environmental impact (toxicity, transformation etc.) has been examined in limited studies. In their toxicity studies, bacteria such as *B. subtilis, E. coli, P. fluorescens, P. aeruginosa* have been tested and variable toxicity results have been reported (Ansari et al., 2013, 2015; Aruoja et al., 2015; Bhuvaneshwari et al., 2016). For instance, Ansari et al. (2013) found antibacterial effect of Al_2O_3 NPs on *E. coli*, contrarily Aruoja et al. (2015) found no toxic effect on *E. coli* and *S. aureus*. Bhunvaneshwari et al. (2016) stated that gram-negative bacteria (*P. aeruginosa*) and gram-positive bacteria (*B. subtilis)* had high inhibition to the Al_2O_3 NPs than bulk form of Al_2O_3 NPs. Inhibition of Al_2O_3 NPs might be associated with cell wall structure and particle size. Nevertheless, these studies were applied mostly in laboratory conditions, and the effect of environment was not investigated for the Al_2O_3 NPs.

In the present study, to understand the physicochemical and toxicological behavior of Al_2O_3 NPs in $PM_{2.5}$, the Al_2O_3 NPs dispersed on $PM_{2.5}$ matrix taken from the urban atmosphere of Istanbul, Turkey, was examined on gram-negative bacteria (*E. coli* and *P. aeruginosa*) and gram-positive bacteria (*B. subtilis* and *S. aureus*), as well as some main physicochemical properties (particle size, surface charge and surface chemistry) of Al_2O_3 NPs were investigated in this environmental matrix. Our results may provide information on the effects of $PM_{2.5}$ fractions on Al_2O_3 NPs toxicity when the latter Al_2O_3 are released into urban aerosols.

2. Materials and method

2.1. Materials

Al_2O_3 NPs were obtained from Torrecid-Turkey (Eskisehir, Turkey). Nutrient agar obtained from Merck (Darmstadt, Germany). All other

chemicals were of analytical grade (Merck; Fluka, Buchs, Switzerland). Ultra-pure water obtained from Milli-Q integral water purification system water quality to Type 1 (Merck KGaA, Darmstadt, Germany). The model organisms used in this study which are gram-negative bacteria (Escherichia coli (E. coli) ATCC 25922 and Pseudomonas aeruginosa (P. aeruginosa) ATCC 27853) and gram-positive bacteria (Bacillus subtilis (B. subtilis) ATCC 6633 and Staphylococcus aureus (S. aureus) ATCC 25923) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cultures activated according to the producer's (ATCC) datasheet: from a single tube, which includes the bacterial culture (5 mL), approximately 0.5 to 1.0 mL of culture was aseptically withdrawn, rehydrated with 2.0 mL of ultra-pure water using Pasteur or micro pipette, and aseptically transferred back into the broth tube. Several drops of the suspension (5-10 µL) used to inoculate plates containing 11-12 mL nutrient agar at 37°C for 24 h using incubator (Thermo-Herathem IGS 100 Incubator, Thermo Fisher Scientific, Langenselbold, Germany).

2.2. Sampling and preparation of $PM_{2.5}$

The preparation and characterization of the $PM_{2.5}$ matrices were explained at below mentioned procedures which used in toxicity assessment of Al_2O_3 NPs, as well as in examination of physicochemical properties of Al_2O_3 NPs. For $PM_{2.5}$ capture, high-volume aerosol samplers with 30 m^3/h air intake and 2880 m^3 total air volume (AirFlow $PM_{2.5}$), equipped with a $PM_{2.5}$ head were used (Analitica Strumenti, Pesaro, Italy). The sampler head was situated approximately 4 m above ground level, 15 m away from a busy road and typical motor vehicle circulation at 3800 items/h in the air quality sampling station of Maslak, Istanbul, Turkey (latitude 41°6.5′ N and longitude 29°1.7′ E). $PM_{2.5}$ samples were collected for 96 h on four consecutive workdays onto quartz fiber filters (Whatman QM-A, Sigma-Aldrich, Taufkirchen, Germany). The $PM_{2.5}$ loaded filters were used to prepare an environmental matrix. To analyze the $PM_{2.5}$ for the various parameters (e.g. major ions, toxicity), the loaded filters extract on a proper solution, such as water (Roper et al., 2015; Szigeti et al., 2013; Watanabe et al., 2017). For this aim, loaded filters extracted on an aqueous solution. The $PM_{2.5}$ extracts were prepared at two concentrations (2 µg/L and 20 µg/L) with ultra-pure water to reflect for low and high levels. Since, $PM_{2.5}$ mass concentration and its composition can be changeable and it was related with the density of traffic, human and industrial activities in sampling area. Furthermore, EU air regulation has limit value for $PM_{2.5}$, for which the limit concentration of $PM_{2.5}$ in air is 25 µg/m^3 (Szigeti et al. 2013). The $PM_{2.5}$ extracts were prepared by weighing the difference between loaded and blank filters, placing the latter into polyethylene flasks (Thermo Fisher Scientific, Waltham, MA, USA), and sonicated using ultrasonic bath for 200 min at 35 kHz frequency (Sonorex, Bandelin Electronic, Berlin, Germany), then filtered . Moreover, it is important to characterize the $PM_{2.5}$ mass concentration, especially with respect to major ions. The major anions of $PM_{2.5}$ were determined and results show in Table 1. Upon filtration, chloride was determined by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) (Szigeti et al., 2013), sulfate (SO_4^{2-}) by UV-Vis spectrophotometry (Libra S70, BioChrom, Cambridge, UK) (Pisal, 2010), nitrate (NO_3^-) determined by sulfanilamide/ethylenediamine with cadmium reduction at 540 nm and ammonia (NH_4^+) determined via the method of nesslerization reaction at 425 nm (Pisal, 2010).

2.3. Preparation of Al_2O_3 NPs for the physicochemical properties and their characterization

In order to examine the effect of $PM_{2.5}$ on the physicochemical properties of Al_2O_3 NPs, NPs were treated with different concentrations of $PM_{2.5}$ extracts (2 µg/L and 20 µg/L); 5, 50 and 100 mg Al_2O_3 NPs mixed in 1.0 L of 2 µg/L and 20 µg/L of $PM_{2.5}$ extracts. Solutions of Al_2O_3 NPs preparing in $PM_{2.5}$ extracts were mixing over 24 h in an orbital shaker at 800 rpm (Stuart SSL1, Staffordshire, UK) at stabilized room temparture (24±2 °C). Then the NPs were centrifuged to remove solution, and dried in a vacuum oven at 24 h until the water fully evaporated. For the reference control, Al_2O_3 NPs were also prepared with ultra-pure water at the same concentration and with the same procedure. The characterization of the Al_2O_3 NPs treated with $PM_{2.5}$ included the investigation of particle size, zeta potential, and surface chemistry. The particle size distributions and zeta potentials were measured using Zeta sizer Nano ZS instruments (Malvern, UK) at 25◦C at a 173◦ scattering angle with 4 mW He-Ne laser. 1.0 mg of treated Al_2O_3 NPs were suspended in 1 mL of ultra-pure water, and then sonicated for five min (Sonorex, Bandelin Electronic, Berlin, Germany) and placed in Standard Malvern zeta potential disposable capillary cells and polystyrene cuvettes (Malvern Instruments Ltd, Malvern, United Kingdom) for zeta potential and size measurements, respectively. All measurements were repeated at least three times.

Table 1. Chemical characterization of tested concentration of $PM_{2.5}$ matrices (extracts) (ultra pure water (control) is free from tested ions (N:3)

Matrix	***Chemical characterization of the $PM_{2.5}$ matrix***			
	SO_4^{2-}, µg/L	***NO_3^-, µg/L***	***NH_4^+, µg/L***	***Cl^-, µg/L***
2 µg/L $PM_{2.5}$	0.24±0.07	0.09±0.11	0.06±0.08	0.02±0.01
20 µg/L $PM_{2.5}$	2.13±1.07	0.85±0.72	0.68±0.22	0.23±0.13

The surface chemistry of the treated and control NPs was investigated using FTIR Spectrometry (Perkin Elmer Inc, Waltham, MA, USA). The morphology was determined using a Scanning electron microscopy (SEM, QUANTA FEG 250, FEI, Thermo Fisher Scientific, Oregon, USA).

2.4. Toxicity procedure

At first, toxicity assessment of Al_2O_3 NPs was performed in controlled condition using colony counting method (Baek and An, 2011; Baysal et al., 2018a; 2018b; Jiang et al., 2009). The toxicity tests conducted in Petri dishes containing nutrient agar culture medium with a specific Al_2O_3 NPs concentration (0, 5, 50, 100 mg/L of Al_2O_3 NPs). Approximately 15 mL of a 2% agar solution was poured into a test unit and immediately hardened in a freezer to avoid the possible precipitation of Al_2O_3 NPs. After appropriate preparation and inoculation of microorganisms, the test units then placed in an incubator (IGS 100, Thermo Fisher) at a controlled temperature of 37°C. Each treatment was prepared at least in three replicates. After a test incubation period (0.5 h, 1h, 2 h, 5 h, 10 h, 24 h), colony forming units (CFU) were counted using a stereoscope. The toxicity of Al_2O_3 NPs in controlled condition was calculated according to the following formula; 'viability rate=N/No', in which, No: CFUs in agar media without NPs, N: CFUs in agar media with NPs.

The toxicity assessment of Al_2O_3 NPs dispersed in $PM_{2.5}$ matrices of urban aerosol was performed with the similar procedure explained above. The stabilized and non-inhibitory duration selected for the incubation period, which is 24 h. The environmental matrix effect on bacteria toxicity was eliminated using control of $PM_{2.5}$ with no Al_2O_3 NPs, in which case: To employ as a control or blank, the 2% agar media was prepared with different concentration of $PM_{2.5}$ extract without Al_2O_3 NPs. To employ Al_2O_3 NPs; the 2% agar solution was prepared by different concentrations of $PM_{2.5}$ extract (low concentration: 2 µg/L $PM_{2.5}$ and high concentration: 20 µg/L $PM_{2.5}$) with Al_2O_3 NPs. The viability calculation explained above and calculated as percentage comparing control.

To examine the toxicity pathway, the amount of protein content and total carbohydrate content were determined in extract of selected bacteria using Bradford assay and phenol-sulphuric acid method (Bhuvaneshwari et al., 2016; Dubois et al., 1956).

2.5. Statistical analysis

The ANOVA with post hoc Tukey used to evaluate the difference between control and each treatment, as well as different treatments. The significance was accepted at the level of $p<0.05$. SPPS 17.0 software applied for the significance and Spearman correlation (two-tailed) tests.

3. Results and discussion

The main physicochemical properties of Al_2O_3 NPs were investigated in the presence of $PM_{2.5}$ matrices. The surface chemistry of the control and $PM_{2.5}$ treated Al_2O_3 NPs characterized with FTIR (Fig. 1). The adsorption of species on the NPs surface and the purification of the surface impurities was also verified in our study; attachment of nitro groups on the surface of Al_2O_3 NPs, and removing the sulfone impurities from Al_2O_3 NPs surface with the increased concentration of matrix. The high intensities were obtained between 700 and 1050 cm^{-1} originated from the sulfonates. However, intensities decreased with the increase concentration of $PM_{2.5}$. The peaks between 1300 and 1600 cm^{-1} observed the nitro groups by the exposure of $PM_{2.5}$. Altering of new chemical components and decreased on the sulfones can break the bound of Al_2O_3 NPs aggregates into smaller particulates, and particle size can decrease with the treatment of $PM_{2.5}$.

Table 2 indicates particle sizes and zeta potentials of Al_2O_3 NPs dispersed in $PM_{2.5}$ matrices and control. Negatively charged and micro level Al_2O_3 NPs was obtained at controlled (normal) condition. The particle size of Al_2O_3 NPs significantly changed in $PM_{2.5}$ matrices. While there were significant differences in particle sizes of Al_2O_3 NPs between in control and tested concentrations of the $PM_{2.5}$ matrices, there was no significant difference between treatment of the low and high concentration of the $PM_{2.5}$ matrices. In addition, the particle size of Al_2O_3 NPs in low concentration of $PM_{2.5}$ matrix was much smaller than high concentration of $PM_{2.5}$ matrix. As well as results showed that there had a high negative correlation between particle size and zeta potentials. Contrary to the significant decrease in particle size, zeta potential slightly changed in high concentration of $PM_{2.5}$ matrix compared to controlled condition. The decrease of particle sizes can explain by the environmental matrix components (sulfate, nitrate, ammonia etc.). They can decrease the NPs agglomeration due to increasing zeta potential (either positive or negative >10-15 mV), and purifying the impurities on the NPs surface. The limited number of studies observed that NPs agglomeration can be affected by environmental media components which change the NPs surface potential (Amde et al., 2017; Joo and Zhao, 2017; Peng et al., 2017). Particle sizes were also good agreement with SEM images of Al_2O_3 NPs obtained in control, and various concentration of $PM_{2.5}$ matrices.

In our study, zeta potentials of NPs changed with the presence of $PM_{2.5}$ (Table 2). The differences can be explained by the presence of the co-ions (sulfate, carbonate etc.) in the environmental matrix (Joo and Zhao, 2017; Peng et al., 2017). Effect of co-ions on the zeta potentials was examined in the limited number of studies.

These studies observed that presence of the co-ions in the matrix can be adsorbed on the NPs surface or they clean the some impurities on the NPs surface (organic carbon, carbonate and hydroxyl group etc.) (Afshinnia et al., 2017; Baysal et al., 2018a; 2018b; Hiroki and LaVerne, 2005; Metreveli et al., 2016). As shown in Table 1, the co-ions (sulfate, nitrate etc.) were determined in $PM_{2.5}$ matrices. The zeta potentials of Al_2O_3 NPs were changed in $PM_{2.5}$ matrices compared to control. It increased with the existence of the co-ions in the low concentration of $PM_{2.5}$ than control. However, relatively low zeta potentials were obtained with treatment of high concentration of $PM_{2.5}$. It can be observed that the intensities of negatively charge ions, such as sulfone groups, were decreased in the treatment of high concentration of $PM_{2.5}$.

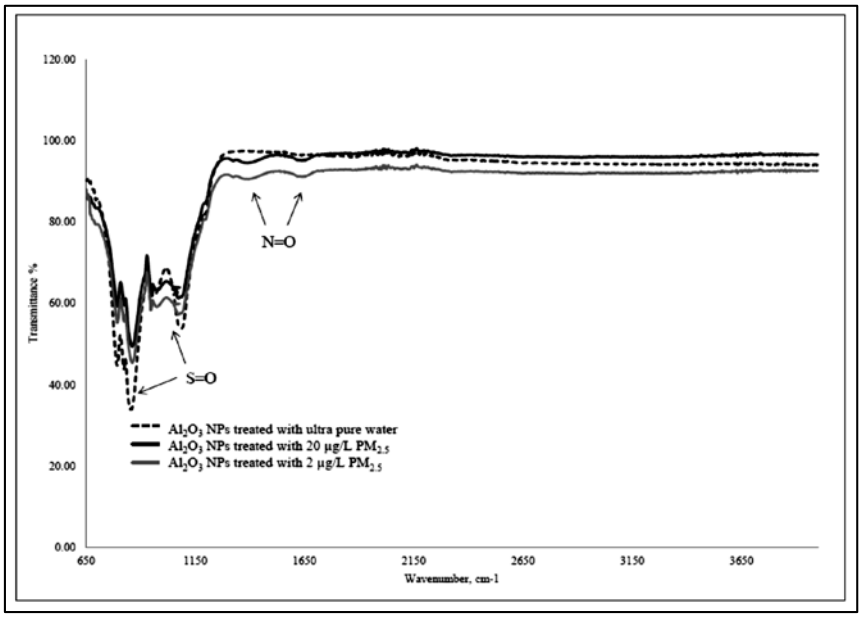

Fig. 1. FTIR spectra of the Al_2O_3 NPs with the exposure of different concentration of $PM_{2.5}$ and control (Exposure duration: 24 h)

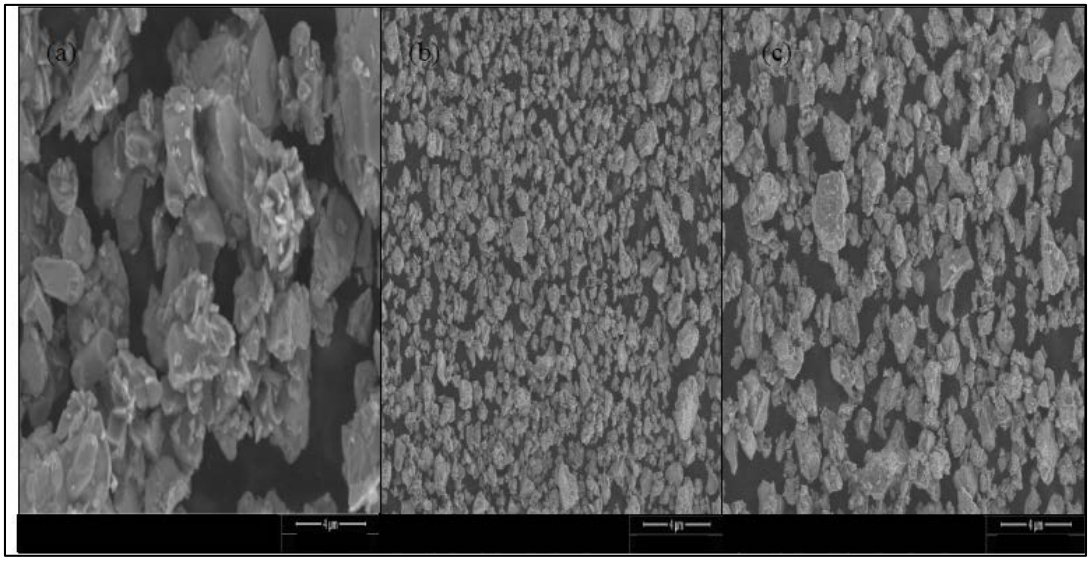

Fig. 2. SEM images of (a) Al_2O_3 NPs treated with ultra-pure water, (b) Al_2O_3 NPs treated with 2 µg/L $PM_{2.5}$, (c) Al_2O_3 NPs treated with 20 µg/L $PM_{2.5}$ (Exposure duration: 24 h)

Table 2. Some physicochemical properties of tested NPs dispersed in $PM_{2.5}$ matrices (extracts) and ultra-pure water (control). N:3

Nanoparticle	***Physicochemical characterization of the nanoparticles***	
	Zeta potentials, mV	***Size, nm***
Al_2O_3 NPs treated with ultrapure water	-16.6±5.3	1450±210
Al_2O_3 NPs treated with 2 µg/L $PM_{2.5}$	-20.9±8.3	99±20
Al_2O_3 NPs treated with 20 µg/L $PM_{2.5}$	-10.8±3.7	120±47

In order to understand the inhibition impact of Al_2O_3 NPs in the presence of urban ultra fine particulate matter and control, bacteria model was assessed. For this purposes, first of all, dose and time-dependent normal growth of selected bacteria to the Al_2O_3 NPs were examined in controlled condition, which is shown in Fig. 3. The Al_2O_3 NPs did not show any significant difference in toxicity on selected gram-negative bacteria (*E. coli* and *P. aeruginosa*), however, some changes observed in the viability at exposure time of 1 h in 100 mg/L Al_2O_3 NPs to *E. coli,* at exposure time of 5 and 10 h in all tested oncentrations of Al_2O_3 NPs to *P. aeruginosa*. Additionally, no significant inhibition were observed for gram-positive bacteria *S. aureus*, except at exposure time of 1h in 100 mg/L Al_2O_3 NPs. On the other hand, Al_2O_3 NPs were more effective on gram-positive bacteria *B. substilis* in the early stages of growth. 50 mg/L and 100 mg/L Al_2O_3 NPs were more inhibited concentration for *B. substilis* than 5 mg/L until at the exposure time of 10 h. Response of the bacteria against the activity is mostly dependent on the cell wall properties, e.g. cell wall strain, cell wall composition, and its thickness. Due to potential differences between the bacteria cell wall and the Al_2O_3 NPs surface charge, the cell can allow the intake of NPs.

Furthermore, gram-positive *S. aureus* have more glycerol and ribitol chains on teichoic acids polymers in cell wall than gram-positive *B. subtilis* which resulted in less toxic effects of Al_2O_3 NPs on *S. aureus* (Brown et al., 2013). As a result, the most inhibited gram-positive bacterium was *B. subtilis* in the early stage of growth. Gram-negative *E. coli* strains showed more tolerance to Al_2O_3 NPs compared to gram-negative *P. aeruginosa* due to *E. coli* have more disaccharide units on the cell wall than *P. aeruginosa* (Huang et al., 2008). Also in some cases fluctuation and high inhibition ratios were observed in early stages of the controlled conditions. With the increased exposure duration, the inhibition was decreased and stabilization and high viability was obtained with the adaptation to the tested concentration of Al_2O_3 NPs (Fig. 3). Difference between adaptation can be originated from surviving behavior of the organisms to the toxic substance (Nascarella and Calabrese, 2012). Consequently, the adaptation was conducted at the end of the 24 h for all tested bacteria, and they were stable against the various concentration of Al_2O_3 NPs in controlled condition.

Figs. 4 and 5 show the viability ratio of gram-positive and gram-negative bacteria in the presence of $PM_{2.5}$ against Al_2O_3 NPs. As seen in Fig. 4a-b, the viability of *E. coli* significantly decreased at the applied concentration of Al_2O_3 NPs in the presence of low and high concentration of $PM_{2.5}$ compared to their control. However, *P. aeruginosa* as another gram-negative bacteria did not show any toxic effect to Al_2O_3 NPs dispersed in $PM_{2.5}$ matrices, except the exposure of 5 mg/L Al_2O_3 NPs in the presence of high concentration of $PM_{2.5}$ (Figs. 4c-d). For the gram-positive *S. aureus*, Al_2O_3 NPs were not observed any decrease in high concentrations of $PM_{2.5}$, but significant inhibitions on *S. aureus* were observed with the exposure of low concentration of $PM_{2.5}$ at 50 and 100 mg/L Al_2O_3 NPs addition (Figs. 5a-b). For the other tested gram-positive bacterium which is *B. subtilis*, significant changes were obtained by Al_2O_3 NPs after exposure to both concentrations of the $PM_{2.5}$ matrices (Figs. 5c-d). No inhibition observed on the *P. aeruginosa* against Al_2O_3 NPs in the presence of $PM_{2.5}$ matrices. Because there is no potential differences between Al_2O_3 NPs (surface charge: -20.9±8.3 and -10.8±3.7 mV treated with low and high concentration of $PM_{2.5,}$ respectively) and the negatively charged surface of the bacteria (*E. coli*, *P. aeruginosa* etc.). This may avoid interaction between bacteria and Al_2O_3 NPs. The inhibition of gram-positive *B. subtilis* was observed due to the charge difference between bacteria and Al_2O_3 NPs which caused their interaction (Jiang et al., 2009; Simon-Deckers et al., 2009). On the other hand, as briefly mentioned before, the difference of the inhibition on each gram strain is caused by their cell wall properties (Brayner et al., 2006; Gonzalez et al., 2011; Silhavy et al., 2010). Not only gram-positive but also gram-negative bacteria have peptidoglycan layer, however their structure are different. Gram-positive bacteria cell wall has peptidoglycan layers and they include lipotechoic and techoic acid molecules. This part of the cell wall is important for the cell surface, and they play a central role in cell growth, division, morphology, adhesion, and envelope integrity. Thus, inhibition results of *B. subtilis* and *S. aureus* can be attributed to usage of different sets of enzymes to make teichoic acid (Brown et al., 2010). Besides that, they use different number of phosphate unit to make cell wall teichoic acids (Brown et al., 2013). For example, *S. aureus* have more glycerol and ribitol chains on teichoic acids polymers which resulted in less toxic effect compared to *B. subtilis*. The results showed that more teichoic acid polymers in cell wall of *S. aureus* can be protected the bacteria against Al_2O_3 NPs with the exposure of $PM_{2.5.}$

Gram-negative bacteria cell wall is much complex than gram-positive bacteria cell wall. They have an outer membrane situated above a thin peptidoglycan layer. Sandwiched between the outer membrane and the inner (plasma) membrane (Beveridge, 1999; Silhavy et al., 2010). Their outer membrane includes different saccharide groups, for example *E. coli* have ~20–30 disaccharide units, and *P. aeruginosa* has 2 disaccharide units (Huang et al., 2008; Matias et al., 2003; Ramphal et al., 1991). In controlled conditions, high viability responses were obtained in *E. coli* compared *to P. aeruginosa.* However, *E. coli* strains showed more inhibition for Al_2O_3 NPs in $PM_{2.5}$ matrices than *P. aeruginosa*. It can be said that Al_2O_3 NPs with the exposure of $PM_{2.5}$ influenced on the long disaccharide units in the cell wall.

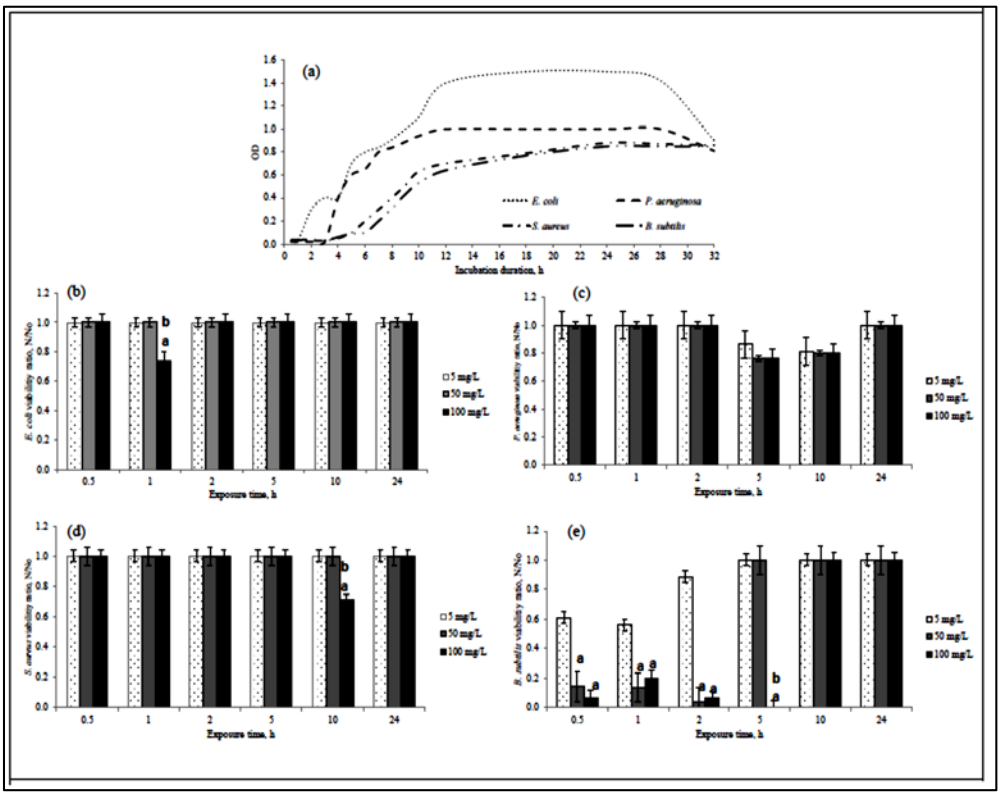

Fig. 3. Bacterial growth (a) and changes in bacterial growth with respect to time and dose associated impact of Al_2O_3 NPs under controlled conditions, (b) *E. coli*, (c) *P. aeruginosa*, (d) *S. aureus*, (e) *B. subtilis*. Different letters for the bars indicate statistically significant results; (a) Significantly different from 5 mg/L Al_2O_3 NPs dispersed in ultrapure water ($p<0.05$). (b) Significantly different from 50 mg/L Al_2O_3 NPs dispersed in ultrapure water ($p<0.05$)

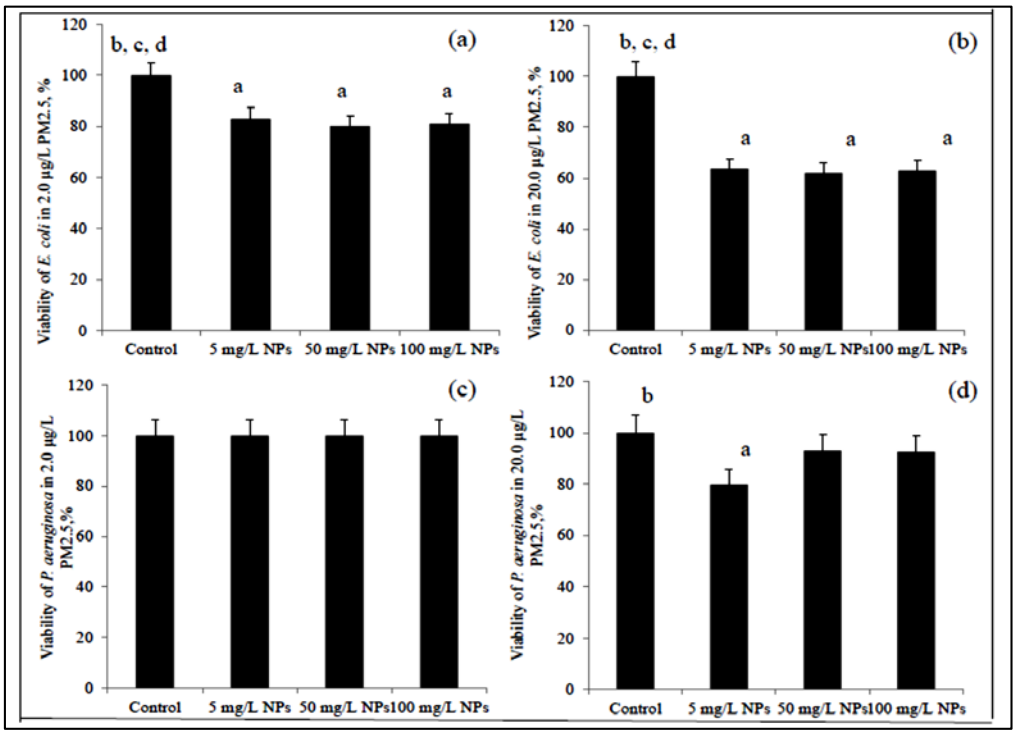

Fig. 4. Viability percentages of gram-negative bacteria exposed to Al_2O_3 NPs with dose associated impact (a) viability of *E. coli* in 2.0 µg/L $PM_{2.5}$, (b) viability of *E. coli* in 20.0 µg/L $PM_{2.5}$, (c) viability of *P. aeruginosa* in 2.0 µg/L $PM_{2.5}$, (d) viability of *P. aeruginosa* in 20.0 µg/L $PM_{2.5}$. Different letters for the bars indicate statistically significant results; (a) in relation to control ($p<0.05$). (b) in relation to 5 mg/L Al_2O_3 NPs ($p<0.05$). (**c**) in relation to 50 mg/L Al_2O_3 NPs ($p<0.05$). (d) in relation to 100 mg/L Al_2O_3 NPs ($p<0.05$). Viability calculation of the control of $PM_{2.5}$ was performed with control of controlled condition. Exposure duration: 24 h

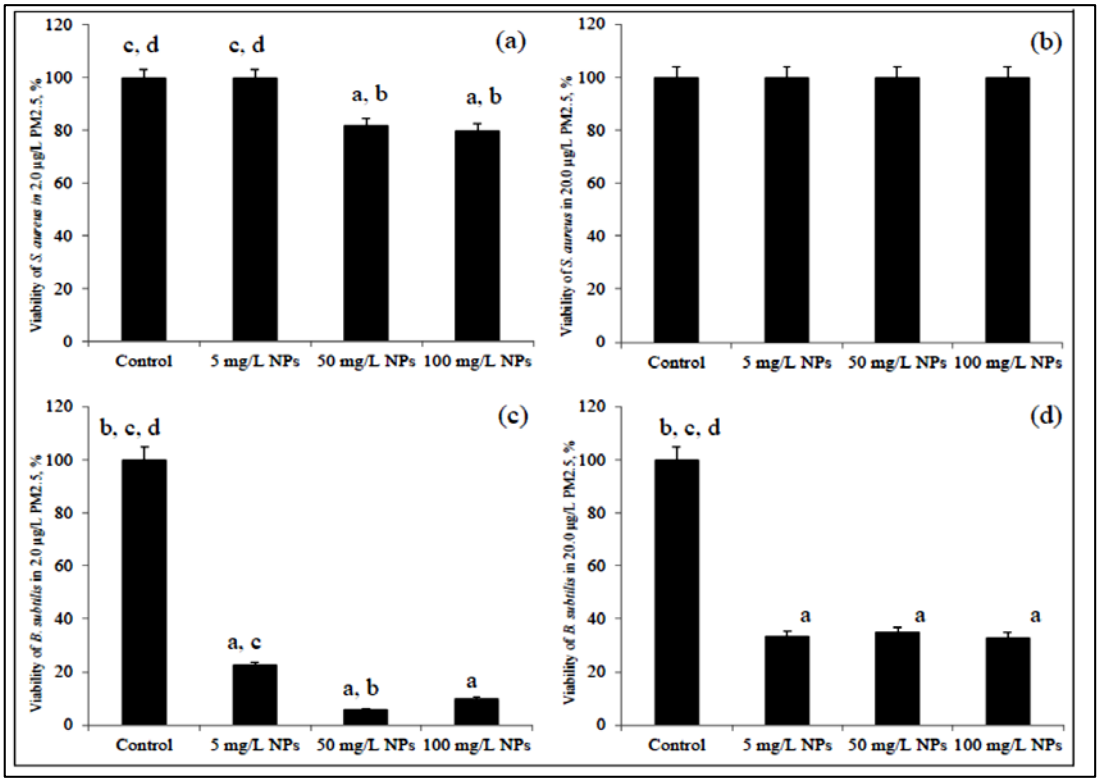

Fig 5. Viability percentages of gram-positive bacteria exposed to Al_2O_3 NPs with dose associated impact (a) viability of *S. aureus* in 2.0 µg/L $PM_{2.5}$, (b) viability of *S. aureus* in 20.0 µg/L $PM_{2.5}$, (c) viability of *B. subtilis* in 2.0 µg/L $PM_{2.5}$, (d) viability of *B. subtilis* in 20.0 µg/L $PM_{2.5}$. Different letters for the bars indicate statistically significant results; (a) in relation to control ($p<0.05$). (b) in relation to 5 mg/L Al_2O_3 NPs ($p<0.05$). (c) in relation to 50 mg/L Al_2O_3 NPs ($p<0.05$). (d) in relation to 100 mg/L Al_2O_3 NPs ($p<0.05$). Viability calculation of the control of $PM_{2.5}$ was performed with control of controlled condition. Exposure duration: 24 h

On the other hand, the increased inhibition on *E. coli* with the increased concentration of $PM_{2.5}$ are not clearly explained using cell wall properties (Fig. 4). Furthermore, the increased viability on *S. aureus* and *B. subtilis* with the incresed concentration of $PM_{2.5}$ needs to be clarified by the physicochemical properties (Fig. 5). The results obtaining physicochemical characterization showed that the inhibition of gram-positive bacteria increased with smaller Al_2O_3 NPs in the treatment of low concentration of $PM_{2.5}$. Contrarily, bigger Al_2O_3 NPs increased the inhibition of gram-negative bacteria. Smaller Al_2O_3 NPs upon treatment with $PM_{2.5}$ can be explained with the altering nitro groups on the NPs surfaces and purifying the surface impurities.

In general, the toxicity mechanism of NPs can occur by different pathways; like, i) chemically; interactions between NPs and the components of the cell wall, ii) physically; interactions between NPs and the cell wall structure, iii) a combination of the physical and chemical interaction (Baysal et al., 2018b; Chang et al., 2012; Djurisic et al., 2015; Fu et al., 2014; Ninganagouda et al., 2014; Sirelkhatim et al., 2015). Few studies reported that toxicity pathway of Al_2O_3 NPs towards bacteria (Bhuvaneshwari et al., 2016; Fajardo et al. 2014).

It has been demonstrated that Al_2O_3 NPs are toxic to gram-positive and gram-negative bacteria since they damaged bacteria cell wall due to reactive oxygen production, Al ion release, or adhesion. However, the detailed mechanism on bacteria has not been well understood. Since carbohydrate and protein are one of the dominant biochemical components of self-defense mechanism of cell and cell structure, the toxicity mechanism investigated using carbohydrate and protein contents (Fig. 6 and Fig. 7).

The carbohydrate release increased in *E. coli* as a gram-negative bacteria and *B. subtilis* as a gram-positive bacterium with the treatment of Al_2O_3 NPs exposure in $PM_{2.5}$ due to self-defense mechanism of the bacteria. On the other hand, *E. coli* includes more carbohydrates in the cell wall structure, therefore the increase on the carbohydrate contents were more significant in *E. coli* than *B. subtilis* (Bhuvaneshwari et al., 2016). Upon treatment to $PM_{2.5}$ of Al_2O_3 NPs, protein release of *E. coli* and *B. subtilis* was greater than control due to self-defense mechanism of bacteria (Fig. 7).

However, the increase on the protein was more in *B. subtilis* than *E. coli*, because cell wall structure of *B. subtilis*. The results are showed that inhibition pathway can be occurred towards carbohydrate for gram-negative bacteria and protein for gram-positive bacteria.

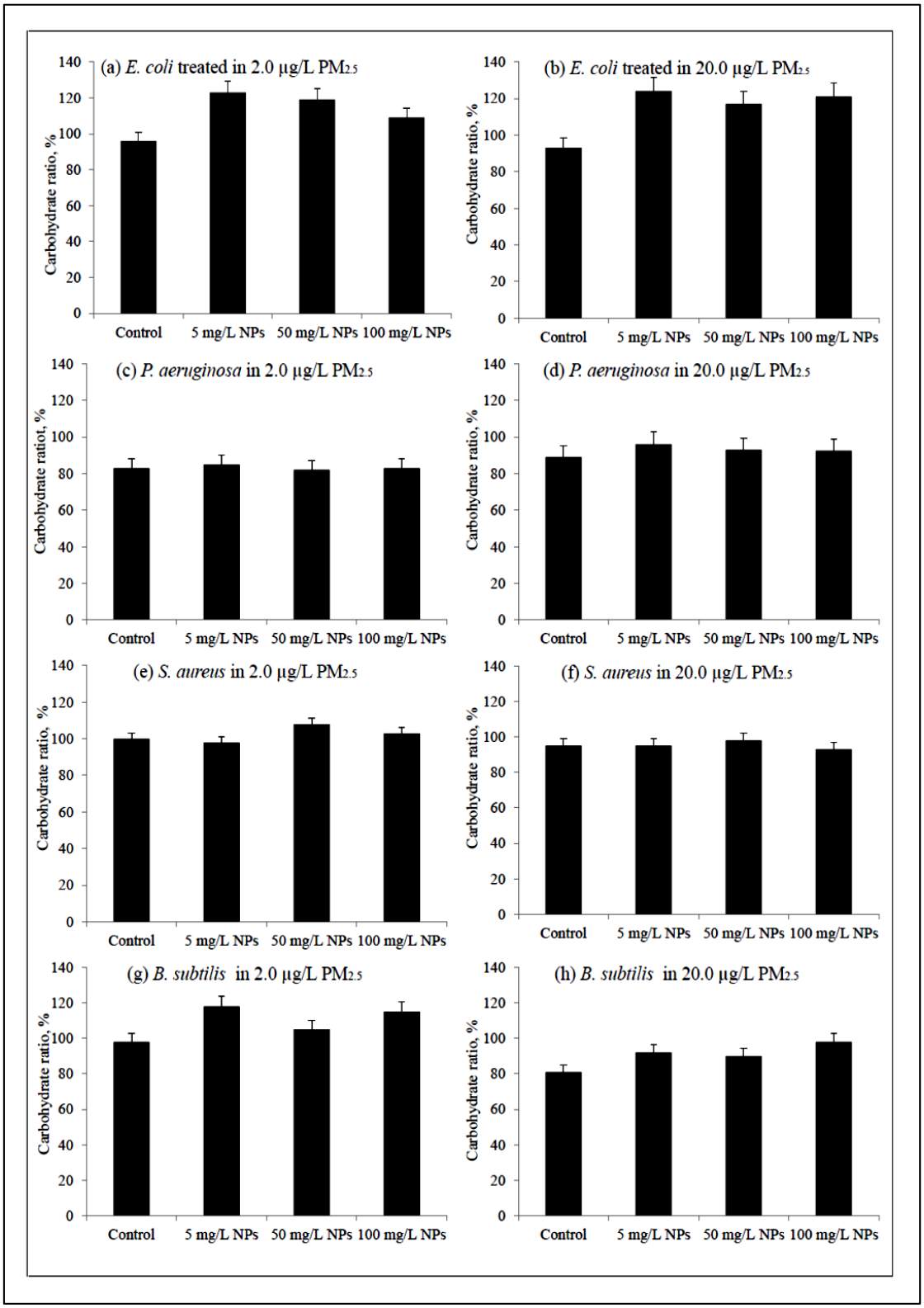

Fig 6. Carbohydrate ratio of gram-positive bacteria and gram-negative bacteria exposed to Al_2O_3 NPs with dose associated impact in 2.0 µg/L and 20.0 µg/L $PM_{2.5}$.

4. Conclusions

This study is one of the few studies to examine physicochemical and toxicological changes of Al_2O_3 NPs due to co-existence of $PM_{2.5}$ fractions of urban aerosols. Al_2O_3 NPs did not exhibit toxicity to selected bacteria in controlled condition, with the exception of early growth stages of *B. subtilis*. However, viability of *E. coli* and *B. subtilis* decreased when $PM_{2.5}$ were also present.

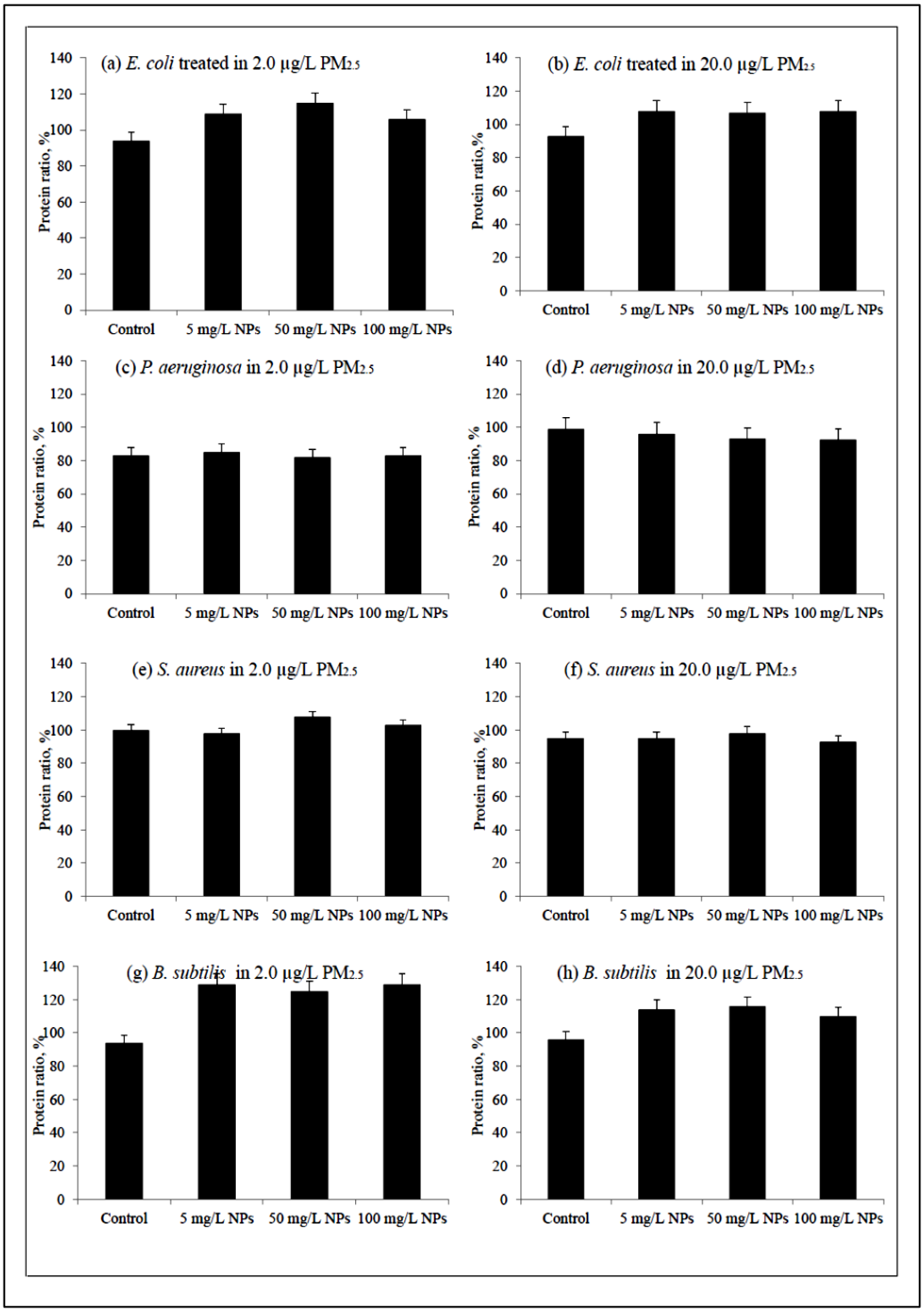

Fig 7. Protein ratio of gram-positive bacteria and gram-negative bacteria exposed to Al_2O_3 NPs with dose associated impact in 2.0 µg/L and 20.0 µg/L $PM_{2.5}$

The main physicochemical properties such as surface chemistry and zeta potential of NPs changed during the exposure of $PM_{2.5}$; attachment of nitro groups on the surface of Al_2O_3 NPs, and cleaning the sulfone impurities on the surface. Furthermore, the decrease on the size of Al_2O_3 NPs was significant due to the co-ions of $PM_{2.5}$ matrices. Thus, our present findings show that the inhibition of Al_2O_3 NPs on the selected bacteria is caused by obtaining lower particle sizes of the Al_2O_3 NPs with the influence of co-ions in the $PM_{2.5}$ matrix.

In addition, cell wall chemistry, number of disaccharides in gram-negative bacteria and proteins in gram-positive bacteria, has also impact on Al_2O_3 NPs with the influence of $PM_{2.5}$ matrix. Consequently, the study showed that after Al_2O_3 NPs being released to the atmosphere, NPs can interact with different atmospheric components, undergo transformations

and exhibit different behavior from its basic or control form.

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