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

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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. © *2016 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4972100]

I. 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 (AgNPs) are the most widely used metal NPs in nanoenabled consumer products.[1] In 2014, around 30% of nanotechnology-enhanced commercial products contained AgNPs.[1] Due to their antimicrobial effects on a wide spectrum of microorganisms, such as Gram positive and negative bacteria and yeasts,[2,3] AgNPs are employed in different fields, including food packaging, textile industry, medical devices, water treatments, cosmetics, and coatings.[4–6]

The release of AgNPs from nanoenabled products and treated areas has been observed.[7–10] 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 nonbiocidal concentrations. Thus, low (sublethal) AgNP concentrations are expected to accumulate in both natural and engineered environments following dilution and dispersion phenomena.[11–13] Indeed, predicted environmental concentrations of AgNPs ranging from 1 pg/ml to 10 μg/ml have been found in both solid and liquid environmental matrixes.[14,57–59]

To the best of our knowledge, only a few scientific works have investigated the effects of sublethal AgNP concentrations on microbial systems;[15–17] therefore, their impact on microbial physiology and behavior still remains almost unknown. Moreover, despite the growing body of literature regarding nanoparticles in biosolids,[51–53] 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.*[18–20] 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.[21,22]

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).[47–50] 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.[18] 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.

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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: (1) a Gram-negative bacterium *E. coli* representative of human intestinal flora and responsible for infection, and (2) 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.

II. EXPERIMENT

A. Bacterial strains and growth conditions

E. coli MG 1655 and *B. 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) 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.

B. Silver nanoparticle characterization

AgNPs (10 nm, OECD PVP BioPure Silver Nanoparticles, NanoComposix, San Diego, CA) 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 microscope), 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. The grids were examined by an EFTEM LEO 912AB transmission electron microscope (Zeiss) working at 80 kV. AgNP's diameter was measured by ESIVISION software, and average and standard deviations (SD) were calculated. The Ag concentration in AgNP suspensions was determined by flame atomic absorption spectroscopy (F-AAS; Thermo-Electron Atomic Absorption Spectrometer) after addition of 1% HCl.

C. AgNP 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:

(1) TSB medium.
(2) Luria Bertani medium (LB, Conda, Italy).
(3) Tryptic soy yeast broth (TSYb, Conda, Italy), prepared according to Sproule–Willoughby *et al.*[23] 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 AgNPs were added to a final concentration of 0.005 mg/ml as suggested by the supplier. 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 320–500 nm absorbance range. For each medium, a 1 ml control sample without AgNPs was prepared, and the spectra were 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).

D. Planktonic growth in the 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 ($\pm 2 \times 10^4$) cells/ml for both bacteria. Bacteria were cultured in the presence of different AgNP concentrations (0, 0.01, 0.1, and 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.*[24] Briefly, the A_{600} of suspensions minus the A_{600} of the noninoculated medium were plotted against the incubation time, and the polynomial Gompertz model[25] 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). Each treatment was performed in triplicate.

E. Adhesion assay in the 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.*[26] with some modifications. Briefly,

the cells were resuspended in fresh TSB supplemented with 0 (negative control), 0.01, 0.1, and 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 three washing steps, adhered cells were stained using 10 μg/ml 4,6-diamidino-2-phenylindole (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 an 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.

F. 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.[27] 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, and 1 μg/ml AgNPs, were washed twice with PBS (13 000 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 3 × 30 s. 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 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 were determined by the standard colony counting method.

G. 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.*[28] Briefly, *E. coli* and *B. subtilis* were grown in the TSB medium supplemented with 0 (negative control), 0.01, 0.1, and 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. The 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. The experiments were conducted in quadruplicate.

H. Statistical analysis

The analysis of variance test (ANOVA) was performed using MATLAB software (version R2014b, The MathWorks, Inc., Natick) to statistically evaluate significant differences among samples. The ANOVA was carried out after verifying whether the data satisfied the assumptions of (1) independence, (2) normal distribution, and (3) homogeneity of variance. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine data significance. Differences were considered significant for $p < 0.05$.

III. RESULTS

A. AgNP 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 to 17.5 nm. F-AAS data showed

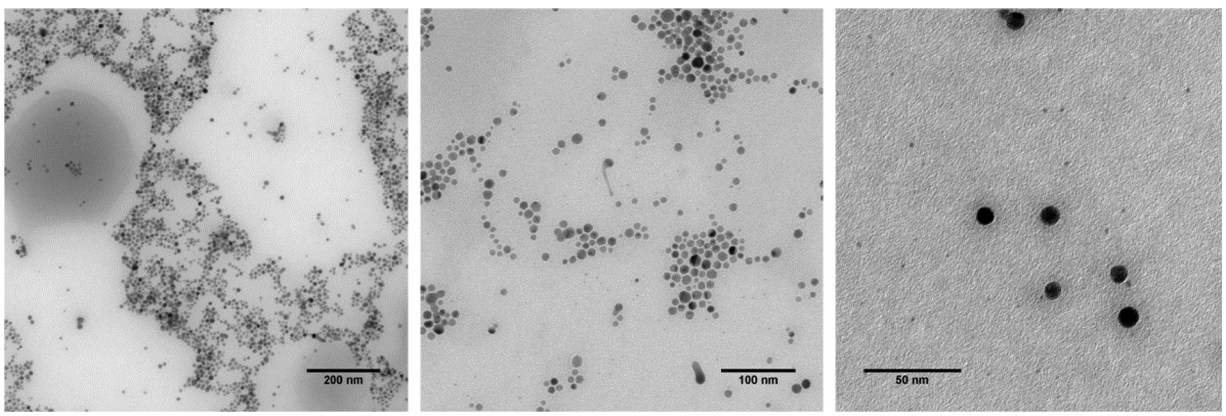


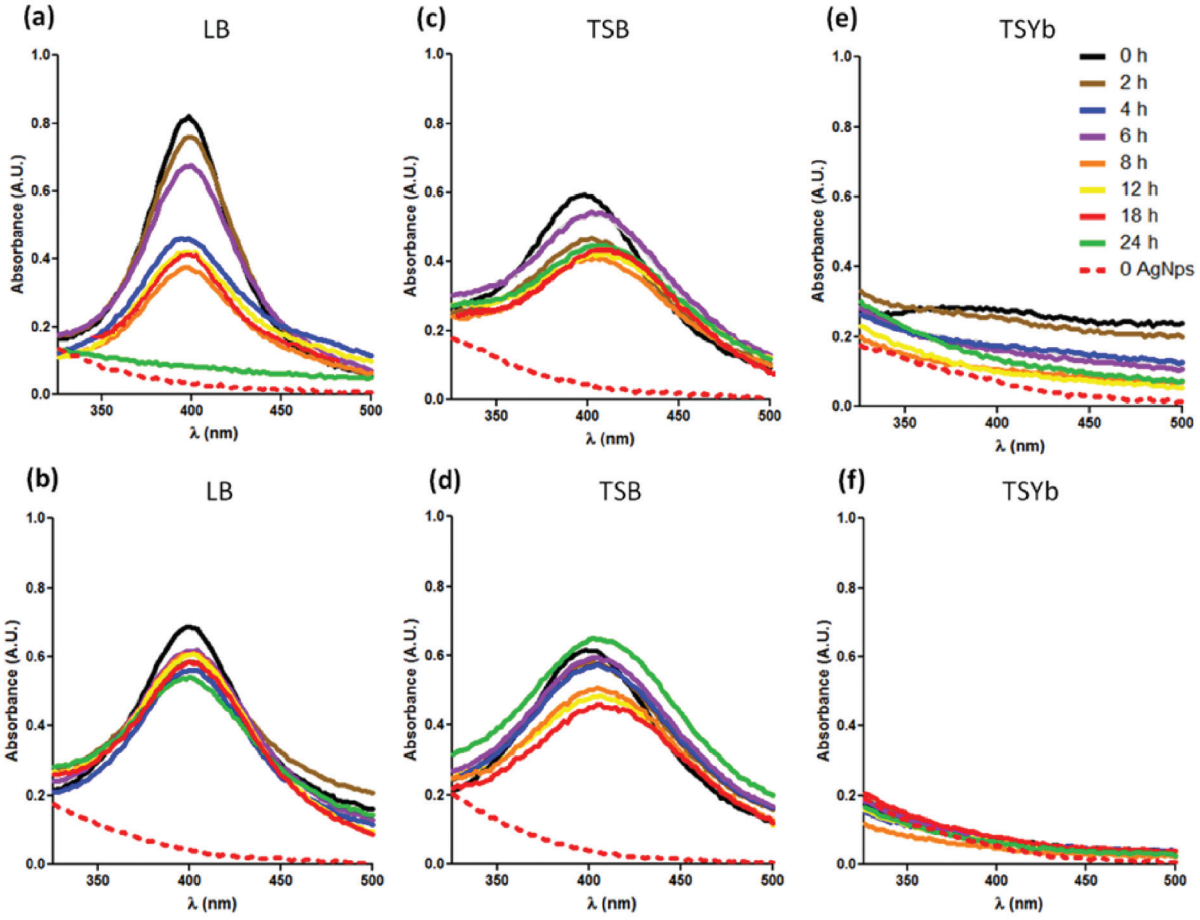
FIG. 1. TEM images of AgNPs at three magnifications.

that the concentration of Ag in the stock suspension was 1 mg/ml, as reported by the manufacturer.

B. AgNP 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.[29] 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. 2(a)]. In anaerobic



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 ○	56.21±4.43 ○
2	59.19±11.43	35.76±2.82	76.81±12	59.15±9.44	7.04±0.37	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	58.87±9.89 ○
6	39.88±12.20	15.39±7.90 ●○	94.01±7.58	55.83±4.82	6.81±0.27	62.08±7.66 ○
8	25.85±0.24 ●	7.16±5.5 ●○	69.71±5.25	53.82±0.33	4.64±0.89	51.72±4.41 ○
12	31.36±0.00 ●	11.18±0.00 ●○	79.03±0.00	53.67±1.25	5.66±1.35	50.59±10.18 ○
18	26.82±13.54 ●	10.37±0.88 ●○	101.90±30.53	51.86±0.52	7.84±0.46	46.82±7.19 ○
24	6.23±1.37 ●○	9.42±2.01 ●○	98.96±26.36	52.86±1.21	6.13±1.75	71.15±3.42 ○

FIG. 2. Absorbance spectra measured from wavelengths (λ) 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. The peak at 390 nm is proportional to the AgNPs in solution. The table reports areas under spectra (AU, 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 ± SD of three independent measurements. Asterisks and dots provide the graphical representation for posthoc comparisons. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing ● show statistical difference to 0 h, and means sharing ○ show statistical similarities with 0 mg/ml AgNPs negative control.

conditions, the LB-AgNP spectra showed a lower peak but more stability throughout the experiment [Fig. 2(b)]. 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 [Figs. 2(c) and 2(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 [Figs. 2(e) and 2(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 (see table in 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. In contrast, the 0–24 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 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 0 h 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.

C. Planktonic growth in presence of AgNPs

Planktonic growth tests in TSB were performed at different AgNP concentrations (0, 0.01, 0.1, and 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 optical density 660 nm 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 MSGR and 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.

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.

D. 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. 4(a)] decreased as AgNP concentration increased, showing a descendent trend between 0 and 0.01 and 0.1 and 1 μg/ml in aerobic conditions and between 0 and 0.1 μg/ml in anaerobic conditions. The findings also demonstrate that *B. subtilis* adhesion in anaerobic conditions [Fig. 4(b)] was not affected by AgNPs, while, in the presence of oxygen, the adhered cells increased 3.5-fold with respect to the control at the highest AgNP concentration (1 μg/ml).

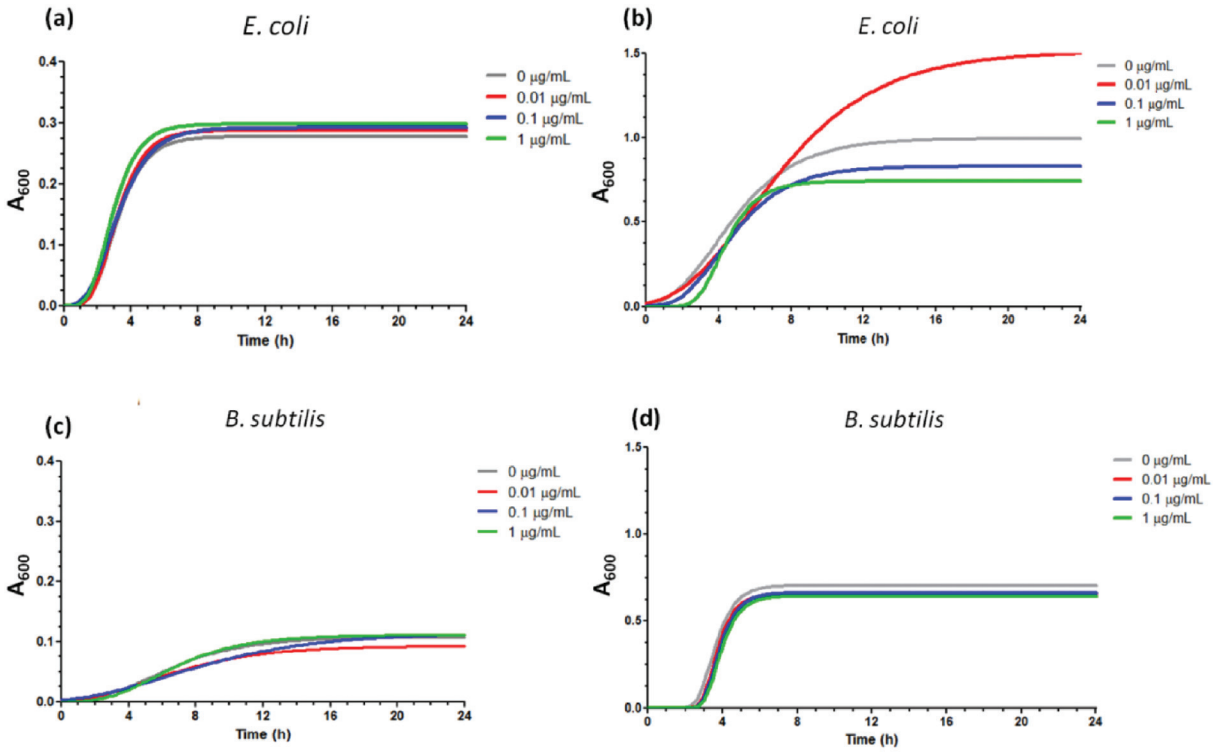
E. Level of oxidative stress in planktonic cells

The fluorescence per cell values found in *E. coli* [Fig. 5(a)] showed a significantly higher oxidative stress level in anaerobic than in aerobic conditions, 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. 5(b)] 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.

F. Motility assay

E. coli swimming motility in aerobic conditions [Fig. 6(a)] was significantly stimulated after 24 h in the presence of 0.01 μg/ml AgNPs, with an increase of migration diameter



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

FIG. 3. A_{600}-based growth curves of *E. coli* [(a) and (b)] and *B. subtilis* [(c) and (d)] in presence of different concentrations of AgNPs (0, 0.01, 0.1, and 1 μg/ml) in both aerobic [(b) and (d)] and anaerobic conditions [(a) and (c)]. The table provides the growth parameters LPL and MSGR of both *E. coli* and *B. subtilis* in presence of different concentrations of AgNPs (0, 0.01, 0.1, and 1 μg/ml) in both aerobic and anaerobic conditions. Data represent the means ± SD of three independent measurements. Letters provide the graphical representation for posthoc comparisons. The histogram provides the *p*-values obtained by the ANOVA. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

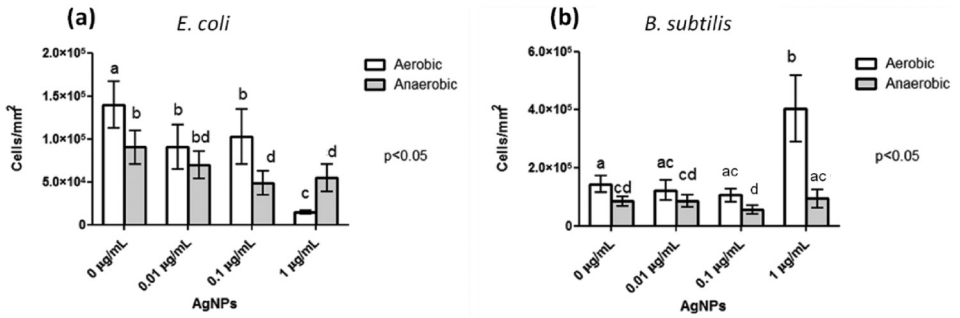


FIG. 4. Adhered cells of *E. coli* (a) and *B. subtilis* (b) in both aerobic and anaerobic conditions in presence of different concentration of AgNPs. Data represent the means ± SD of four independent measurements. Letters provide the graphical representation for posthoc comparisons. The histogram provides the *p*-values obtained by the ANOVA analysis. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

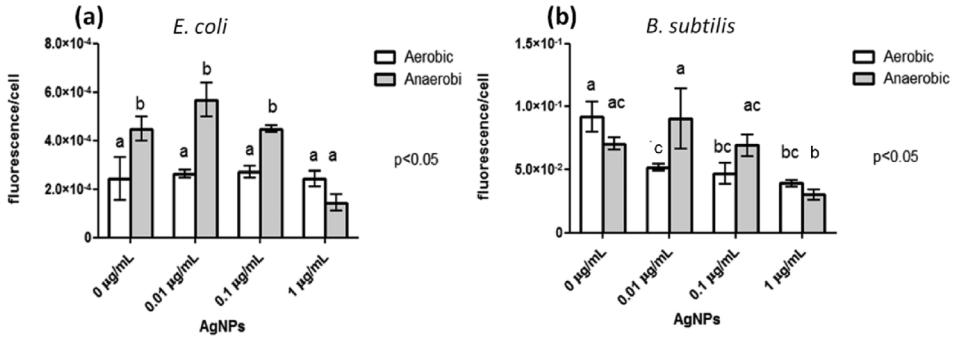


FIG. 5. Amount of fluorescence per cell values for *E. coli* (a) and *B. subtilis* (b) in both aerobic and anaerobic conditions measured in presence of each concentration of AgNPs. Data represent the means ± SD of three independent measurements. Letters provide the graphical representation for posthoc comparisons. The histogram provides the *p*-values obtained by ANOVA analysis. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

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. 6(b)], 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 [Figs. 6(c) and 6(d)]. 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. 6(a)] swimming movement reached the plate diameter (85 mm) between ten- and fivefold 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. 7(b)] was slower. At 24 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. 7(c)] 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.

Data represent the means ± the SD of four independent measurements. The histogram provides the *p*-values obtained

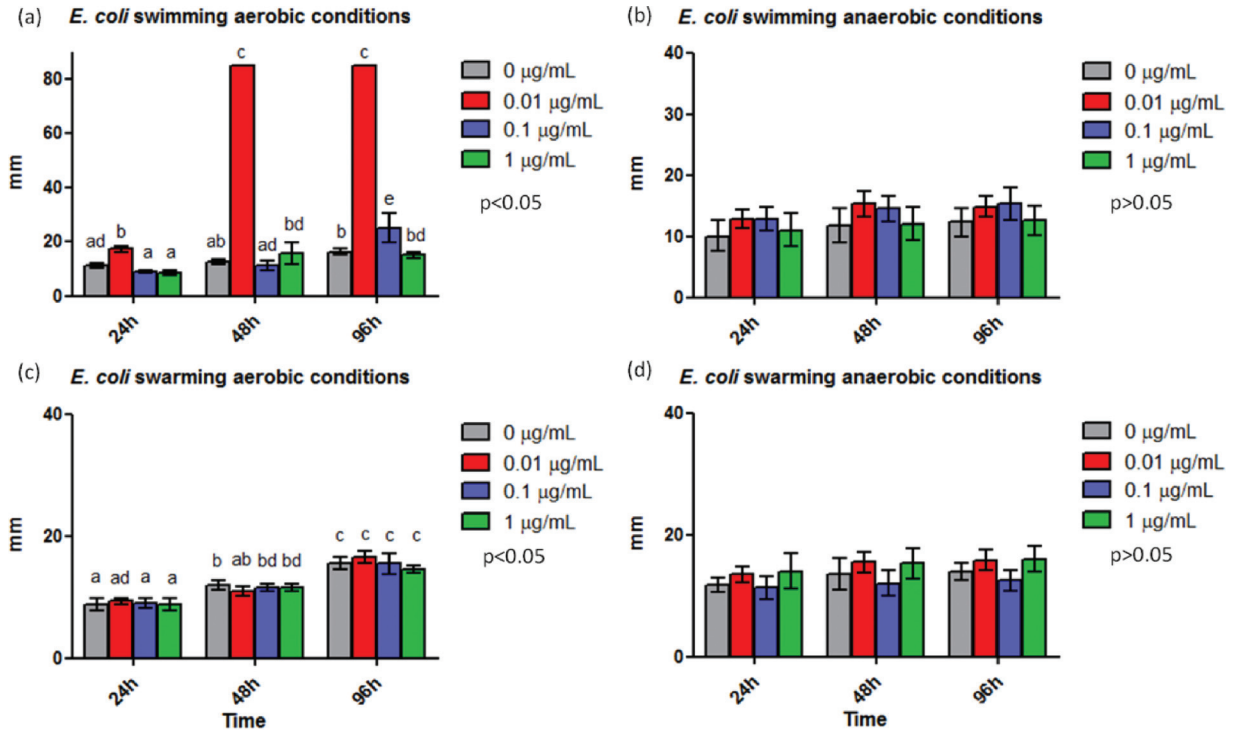


FIG. 6. Swimming and swarming expansion radius of *B. subtilis* previously grown in presence of sublethal concentrations of AgNPs. Experiments were performed in both anaerobic and aerobic conditions and data collected at 24, 48, and 96 h of incubation.

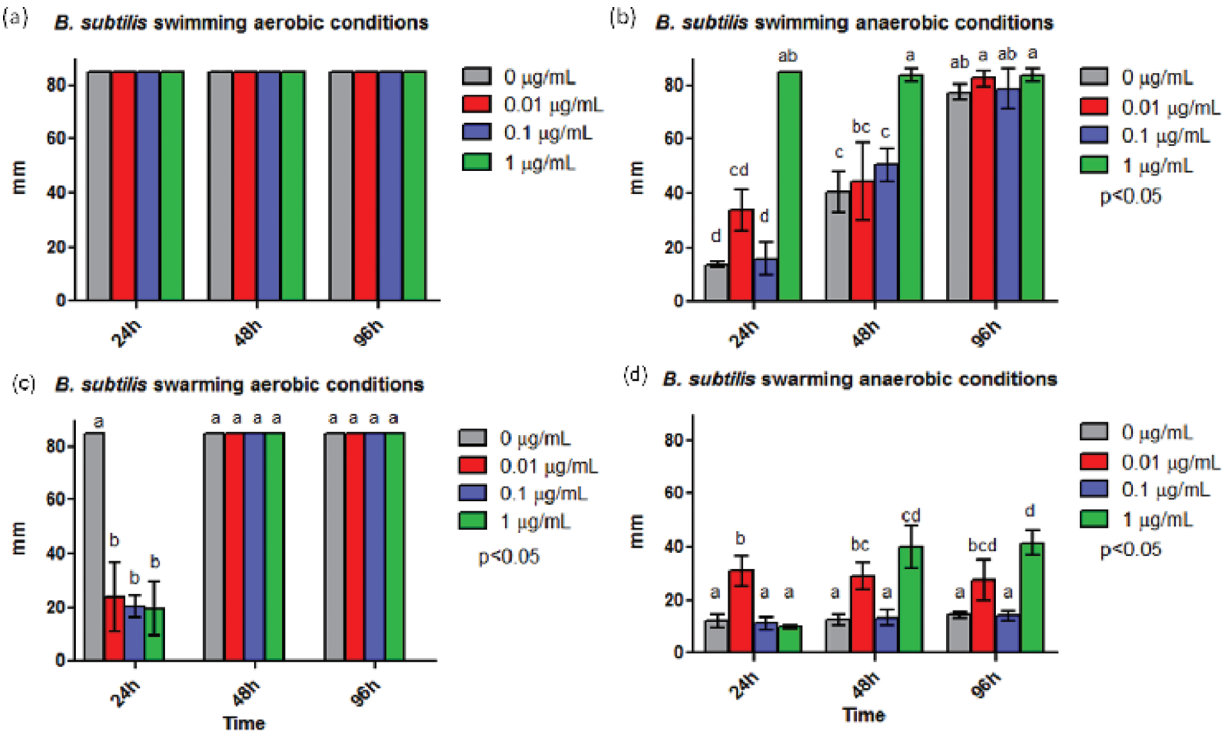


FIG. 7. Swimming and swarming expansion radius of *B. subtilis* previously grown in presence of sublethal concentrations of AgNPs. Experiments were performed in both anaerobic and aerobic conditions and data collected at 24, 48, and 96 h of incubation. Data represent the means ± the SD of four independent measurements. The histogram provides the *p*-values obtained by ANOVA analysis. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

by ANOVA. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

At 48 and 96 h, all the samples reached plate diameters with no statistical differences. In anaerobic conditions [Fig. 7(d)], 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.

IV. 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.[54–56] 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 bioindicator.[18–20] 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.[60–63] 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.[31] 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.[32,33] After determining 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.[31]

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 metabolic pathways to undergo oxygenic respiration while restraining oxidative stress.[22] Such acclimation to aerobic environments can affect bacterial growth differently from that under anaerobic conditions, in terms of both oxidative damage and energy balance, resulting in growth kinetics changes. *E. coli* and *B. subtilis* MSGRs were higher in aerobic conditions, highlighting a more active metabolism in the presence of oxygen, while 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 reported that within the sublethal range of 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 24 h 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.*[31] 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.[31]

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.[34–36] 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[15] 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,[20,30,37] and new light has been shed on bacterial oxidative stress response to AgNP-induced ROS.[16,21,22] 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.[22]

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.[16] 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.[38–40] Nevertheless, up until now, bacterial motility data in anaerobic conditions are still poor and show controversial results,[41–44] 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.[45] Villa *et al.*[46] 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 sixfold. Under anaerobic conditions, both swimming and swarming migrations were not affected, in agreement with the study of Che,[41] 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.[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.*[46] and Butler *et al.*[40] Swarming migration under anaerobic conditions was promoted at the highest AgNP concentration tested, corresponding to the most bioactive doses in all the experiments.

V. 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 versus silver NPs in our experimental conditions, in order to clarify particle- 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.

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