A POTENTIAL EFFECT OF SILVER NANOPARTICLES (Ag-NPs) ON SOME LACTIC ACID BACTERIA GROWTH

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ABSTRACT

This study aimed to essay what could happen if lactic acid bacteria, especially some antifungal lactobacilli strains, mesophilic and thermophilic starter cultures (G+ve model), were treated with Ag-NPs using standard plate count agar assay. As well, *E coli* (G-ve model) was treated with Ag-NPs in a broth medium. The turbidity was measured for zero, three, six, nine hours and compared to control. The results approved that Ag-NPs had a negligible effect the G+ve strains. On the other hand, Ag-NPs had an aggressive activity against the gram-negative one.

Keywords: antifungal Lactobacilli- Ag-NPs - mesophilic and thermophilic starter cultures- Gram positive and negative strains.

1- INTRODUCTION

Lactobacilli is a main approach under LAB. Lactobacilli strains have employed not only as starter cultures with probiotic functions but also as an antifungal agent. Recently, another consideration took place where silver nanoparticles (Ag-NPs) were revived to be used as an antimicrobial agent **(Kim *et al.,* 2007)**. This approach had a worldwide acceptance because Ag and its compounds were famed as non-toxic, undercover inorganic efficient antimicrobial agents, which can control the growth of about 650 type of microorganisms **(Jeong *et al.,* 2005)**. Notably, silver nanoparticles (Ag-NPs) drew attention as a potential antimicrobial (fungal, and bacterial) agent. This interest was due to the fast progress in research on metal nanoparticles (NPs) and the potential superiority of nano size antimicrobial activity compared to bulk silver metals. Also, Ag-NPs have higher specific surface area and high surface atoms fraction **(Kim *et al.,* 2009; Lee, *et al.,* 2009 and Noorbakhsh *et al.,* 2011)**. Therefore, Ag-NPs present a very promising future to solve one of the biggest problems in dairying, which is the growth of foodborne fungi **(Plocková, *et al.,* 2001; Oliveira *et al,* 2014; Medina and Jordano 1993 and Banjara *et al.,* 2015)**. In this context, it was necessary to investigate the effect of the presence of Ag-NPs as a novel antifungal agent on some individual lactobacilli strains and starter cultures blends, on the other hand.

2- MATERIALS AND METHODS

1- Microorganisms:

1.1. G+ve bacteria:

1.1.1. Lactobacilli strains:

Lb. casei NRRL B-441 (*Lb. casei*) **(4)** *Lb. delbruckii* NRRL B-1024 (*Lb. delbruckii*) **(5)** *Lb. plantarum* NRRL B-531 (*Lb. plantarum*) **(9)** U.V. mutant from original strain *Lb. casei* ssp. *rhamnosus* NRRL B-445 (*Lb. rhamnosus* uv mutant) **(10)** were Supplied as a gift from Dr A. A. Khattab (National Research Center, Gizza, Egypt).

1.1.2. Starter culture blends:

Two freeze-dried lactic cultures for Direct Vat Set (DVS) were used. Mesophilic/ thermophilic culture blend **(Chr-Hansen FRC-60)** containing *Lactococcus lactic, Lactococcus cremoris, Streptococcus thermophilus,* and *lactobacillus bulgaricus*. Thermophilic culture (**Chr-Hansen TTC-3)** containing *Streptococcus thermophilus,* and *lactobacillus bulgaricus.*

1.2. G-ve bacteria:

Two pathogenic bacterial strains *E coli* 076 (G-ve). were a gift from the central laboratory for Microbiology, Faculty of Veterinary Medicine, Kafrelsheikh University Egypt.

2- Materials:

Silver nanoparticles were prepared, characterised as described by **Kim *et al.,* 2007**. (8-11nm) particles suspension with a concentration of (1000 µl/ml) was used in this experiment. A 10000 µl/ml sterilized stock suspension was employed.

3- Microbiological assays:

3.1. Ag-NPs effect on the lactic acid bacterial growth:

3.1.1. The effect on lactobacilli strains:

The standard plate count assay was carried out. Where a standard freshly prepared MRS agar medium was mixed with (10ml the stock Ag-NPs suspension/100ml medium) just before pouring in the Petri dishes. Each plate contained 15 ml with the previous mixture mixed with an individual lactobacilli strain. The same experiment was carried out without adding Ag-NPs to the growth medium as the control negative. All plates were incubated at 37°C/72h. This assay was carried out in three replicates for each strain. The final results were expressed as log CFU for each strain compared to the control plates.

3.1.2. The effect on starter cultures blends:

To evaluate the effect of Ag-NPs on the growth of the thermophilic and mesophilic lactic acid bacterial strains, Bromo cresol green whey agar (BGWA) medium was employed. This medium could

be used to differentiate, and consequently enumerate *Streptococcus thermophiles* and *Lactobacillus bulgaricus* individually on the same plate as described by **Yamani and Ibrahim 1996**. The *Streptococcus thermophillus* colonies were green lenticular with entire edges, while *Lactobacillus bulgaricus* colonies were larger and mostly of light color with greenish centres, irregular in shape and in the form of a mass with twisted or fuzzy filament projections. The same previously mentioned method in **3.1.1.** was carried out.

3.2. The effect of Ag-NPs on *E coli* growth rate:

To study the Ag-NPs effect on the selected G-ve bacterial strain, three 100ml conical flasks containing 50 ml of Macconkey broth medium (Oxoid) were used. Two of them were inoculated with approximately 1×10^6 CFU of freshly prepared broth of *E. coli* **(Agnihotri *et al.,* 2014)**, while the third did not inoculate. The Ag-NPs suspension was added to the first one (Ag) and the final concentration was adjusted to 1000 ppm. In parallel, the second flask was considered a control (C) and the third was a blank (B). Then, the three flasks were incubated in a shaker incubator (Temperaturaturbegrenzer, 3022, Germany (150 rpm) at 37°C/9hr. Growth was measured at three hours intervals by monitoring the optical density at absorbance 600 nm for *E. coli* using spectrophotometer (UV-1800 SPECTROPHOTOMETER) according to **Quigley 2008**. In parallel, the bacterial count was also determined using standard plate count technique in Macconkey agar media incubated at 37ºC for 24hr. The two measurements were plotted together, and a growth curve was obtained for the two strains to estimate the log bacterial count (log CFU) correspondence to the absorbance. This trial was carried out in three typical replicates, and only one curve from each group was taken into consideration.

4. Statistical analysis:

Data from three independent replicate experiments analyzed using Statistical Package for the Social Sciences (SPSS) 16.0 statistical software (Chicago, USA). The data are reported as the mean ± standard error. Significant differences between mean values were located with T-test and Duncan's Multiple Range test ($p<0.05$), followed by one-way ANOVA.

RESULTS AND DISCUSSION

1- Silver nanoparticles (Ag-NPs) potential effect on LAB:

1.1 Ag-NPs effect on antifungal lactobacilli individual strains:

Figure 1 illustrates the Ag-NPs effect on the lactobacilli colonies counts compared to control. The histograms show that there is no significant difference between the strains that had been inoculated into

MRS agar medium containing Ag-NPs when compared to the same strains in MRS agar medium only.

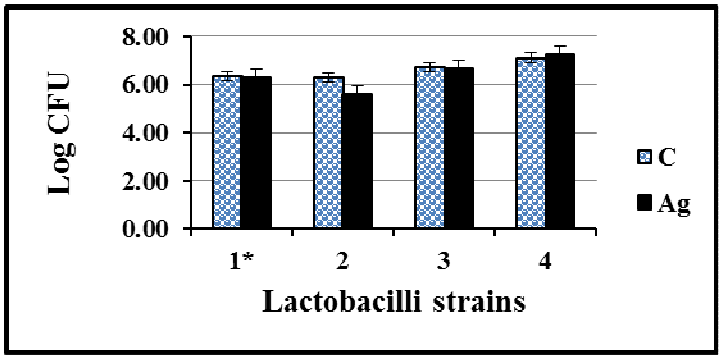

Fig (1): lactobacilli logarithmic colonies count in MRS agar medium with(Ag) and without (C) Ag-NPs after incubation at (37°C/72h).

*(1) *Lb. casei* NRRL B-441, (2)*Lb. delbrueckii* NRRL B-1024, (3) *Lb. plantarum* NRRL B-531, (4) U.V. mutant of original strain *Lb. casei* ssp*. rhamnosus* NRRL B-445.

a,b,c, Means with unlike small superscript letters within the same column group, are significantly different (P<0.05).

1.2 Ag-NPs effect on starter cultures blends:

Both thermophilic (TTC-3) and mesophilic (FRC-60) starter cultures had the same previous trend of the individual lactobacilli strains. Where shown in fig (2) there is no significant difference between colonies count in control medium compared to the same starter cultures counts in the medium containing Ag-NPs.

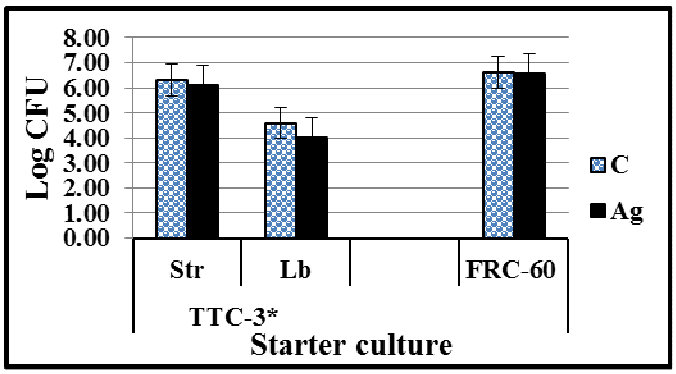

Fig (2) Starter cultures logarithmic colonies count in (BGWA) agar medium with (Ag) and without (C) Ag-NPs after incubation at (37°C/72h) .

*(TT-3) thermophilic starter culture containing: (Str: *Str thermophillus* and Lb: *Lb bulgaricus*) and (FRC-60) mesophilic starter culture.

a,b,c, Means with unlike small superscript letters within the same column group, are significantly different (P<0.05).

2. The effect Ag-NPs on *E coli* growth rate:

Fig (3) is an attempt to elucidate the extent of the bactericidal effect of Ag-NPs concentration on the pathogenic bacteria viability. *E coli* as the Gram-negative "G-ve" model was examined. Fig 3 (A) reveals the growth profile of the strain in the control media (growth media without Ag-NPs). While figure (B) shows that Ag-NPs had a remarkable effect on the G-ve strain viability. *E. coli* had a different trend comparing to the control. Ag-NPs presence led to growth rate rapid decrement.

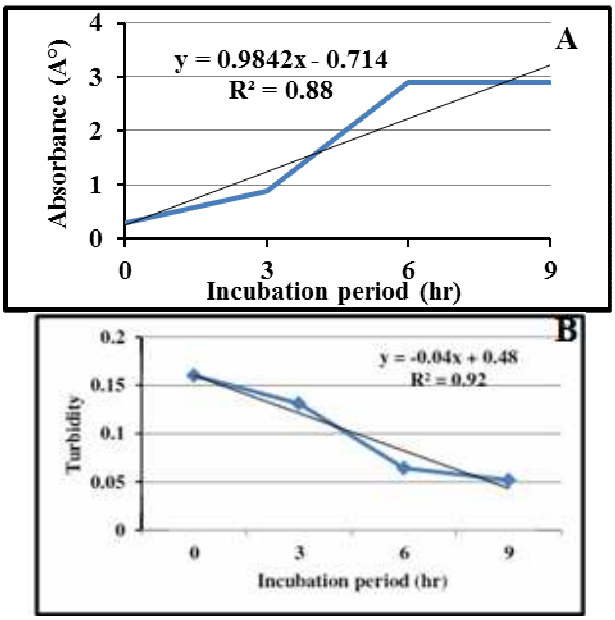

Fig (3): Growth profiles of *E coli* 076. (A) control and (B) in the presence of 1000ppm of Ag-NPs, for (9hr/37°C)

Fig (4) elucidates the media color changes compared to the blank (B). Bacterial strain growth causes medium turbidity in the control (C). Ag-NPs treatment (Ag) got a darker color as a result of silver particles present but didn't show any bacterial growth indicators in Macconkey broth containing *E coli* initial viable cells as much as the control medium.

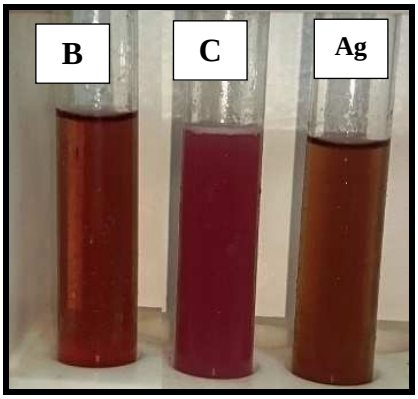

Fig (4): Macconkey broth medium after inoculation with *E coli* 076 (B) blank media, (C) control, and (Ag) in the presence of 1000ppm of Ag-NPs. All tubes were incubated for (9hr/37^{o}C)

Aiming to discuss these results, silver ions are well-known to be effective against a broad variety of microorganisms, including sixteen major bacterial species. This feature makes silver an important choice for the multi-resisting role in food industry field. In the same context, Ag-NPs showed the same behaviour but in tougher ways. This activity is due to the particles huge surface area that the microorganisms expose to. Though Ag-NPs have so many antibacterial applications in different fields, silver nanoparticles antibacterial mode of action is not completely understood **(Prabhu and Poulose 2012)**. In general, low concentrations of Ag-NPs as one of the heavy metals can motivate significant changes in cell functions and structure thus the microorganisms could be destroyed. When used at high concentrations, Ag-NPs behave as a protoplasmic poison; that denature proteins and nucleic acid **(Ogar *et al.,* 2015)**.

In light of these hypotheses, Ag-NPs mode of action could be explained for several prospects. From a morphological standpoint, Ag-NPs have a dimension ranges from (1-100) nm. As a result of this tiny size, Ag-NPs have a huge surface area /volume ratio. So, as mentioned, Ag-NPs have unique physical, chemical, biological characteristics compared to their bulk origin. Thereby, Ag-NPs can physically interact with several bacteria cell surface. This anticipation is important, particularly when discussing Ag-NPs antibacterial activity against *E coli*, where Ag-NPs adhere and accumulate on the bacterial surface. This physical contact leads nanoparticles to attach to lipopolysaccharides layer in cell membranes causing structural changes which in turn make bacterial membrane more permeable. For more elucidation, Ag-NPs direct interaction creates gaps in cell

membrane bilayer causing excessive permeability that leads finally to cell death **(Franci *et al.,* 2015)**.

In our experiment, *E coli* had unusual growth profile when subjected to Ag-NPs compared to the negative control. Where the growth curve showed that it had a slow turbidity decrement during the first 3hr, then it dropped in a fast rate indicating cells death. This growth pattern may be briefed in two explanations: **first;** Ag-NPs have the ability to interact with various microorganisms (such as bacteria) and also impact both the growth of and mature bacterial biofilms and, therefore, could be used as broad spectrum antimicrobials. The antibacterial effect appears to be conferred by their ultra small size and increased surface area, through which they destroy the membrane, cross the body of the microbe and create intracellular damage. Due to the structural difference in the composition of the cell walls of G+ve and G-ve Ag-NPs have significantly less effect on the growth of G+ve bacteria. The Gram-negative bacteria have a layer of lipopolysaccharides on the outside and present below a thin (7 to 8 nanometers) layer of peptidoglycan. Although lipopolysaccharides are composed of lipids covalently bound to polysaccharides, there is a lack of rigidity of the overall structural envelope. The negative charges on the lipopolysaccharides are attracted to the weak positive charge of Ag-NPs. On the other hand, the cell wall of G+ve bacteria (LAB are a clear example) is mainly composed of a thick layer (20 to 80 nanometers) of peptidoglycan consisting of linear polysaccharidic chains cross-linked by short peptides to form a three-dimensional rigid structure. The stiffness and the extensive cross-linking not only reduce the bacterial cell wall anchoring sites for Ag-NPs but also render the wall itself more difficult to penetrate **(Franci *et al.,* 2015)**.

The second explanation is helping to understand *E coli* unusual growth rate as it is related to the cells age, according to the fact that *E coli* generation time ranges between 27-30 min. Where at the first 3hr of incubation, the domain cells are the mother cells where cell membrane is thicker and harder to be affected by the Ag-NPs presence. So it needs much more time to be damaged. As time goes by, each mother cell divides into two daughter cells where they are weaker and easier to be affected by the nanoparticles exposure. Returning to the antibacterial Ag-NPs mode of action possibilities, another point of view is that the Ag-NPs render a permanent damage on bacterial cells by inhibiting DNA replication, modifying intracellular ATP levels and damaging cytoplasm membranes. Theories explaining this depend on a hypothesis that Ag-NPs can release silver ions and free radicles. Silver ions interact with the thiol groups (-SH), which are a constituent of many vital enzymes, leading to blocking them. Therefore, several functions will be inhibited causing irreversible cell

damage. One of these inhibited functions is suppressing the respiratory enzymes causing generating reactive oxygen species that attack the cell itself. Also, citrate-capped Ag-NPs tend to be a weak acid which in turn tends to react with weak bases. Sulfur and phosphorus are weak bases, and they are the major cell components. Consequently, the reaction among these elements and Ag-NPs in the cell causes its death. In the same context, DNA major components are sulphur and phosphate, which explain why Ag-NPs complex with it; causing replication problems and eventually terminating the microbe **(Agnihotri *et al.,* 2014; Prabhu and Poulose 2012)**.

These approaches may have occurred in parallel which elucidates the G-ve quick response towards Ag-NPs. The Ag-NPs antibacterial activity results in this work go with the previous studies work **(Kim *et al.,* 2007; Agnihotri *et al.,* 2014; Franci *et al.,* 2015)**. On the other hands, others confirmed that Ag-NPs had a remarkable inhibiting effect on both G-ve and G+ve strains. This contradiction may also retune to the fact that the antibacterial mechanism(s) of Ag-NPs is not entirely recognized **(Devi and Bhimba 2014; Noorbakhsh *et al.,* 2011)**. Therefore, Ag-NPs antibacterial activity against G-ve and G+ve bacteria needs more profound investigations.

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الملخص العربي

تهدف هذه الدراسة إلى دراسة تأثير وجود جسيمات الفضة النانمترية على نشاط بكتيريا حمض اللاكتيك (كنموذج موجب لجرام) خاصة اللاكتوباسيلس المضادة للفطريات ومخاليط مزارع البادئات المحبة للحرارة المتوسطة وكذلك المحبة للحرارة المرتفعة. وقد أظهرت النتائج ان جسيمات الفضة النانومترية لم يكن لها تأثير ملحوظ على جميع سلالات بكتريا حمض اللاكتيك موضع الدراسة. وأخيرا، ولتعزيز نتائجنا، تم اختبار تأثير جسيمات الفضة النانومترية على إيشيريشيا كولاي (كنموذج سالب لجرام) حيث تم قياس درجة التعكير في بيئة سائلة مناسبة لكل سلالة لمدد صفر، 3، 6، 9 ساعات وبالمقارنة مع الكنترول لكل سلالة؛ أكدت النتائج أن جسيمات الفضة النانومترية ليس لديها تأثير يذكر على البكتريا الموجبة لجرام بالمقارنة بتأثيرها الواضح على السالبة لجرام.