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Investigation the inhibitory effects of AgNPs generated by *Bifidobacterium* spp. on bacteria isolated from ready-to-eat foods

Nehan Bahaaldden JAFAR[1], Zainab Hussein FADHIL[1], Evan Mohammed MUSTAFA[1], Ali Mohammed SAADI[2*]

Abstract

Ready-to-eat (RTE) foods including vegetable salads are one of the most preferable foods sold and served in resturants in Kirkuk city-Iraq. Foodborne diseases endure a major problem in developing countries because lacking of personal hygiene and food safety measurements. Silver nanoparticles (AgNPs) are nanomaterials are one of the crucial and fascinating nanomaterials involved in various fields including control of pathogenic bacteria like foodborne pathogens. Therefore, this study aims to synthesize AgNPs by using eco-friendly Bifidobacterium (a silver reducing agent), and studying their effects against a range of foodborne pathogenic microorganisms. The synthesis of the AgNPs was monitored using ultraviolet visible UV- Vis spectroscopy and Fourier transform spectroscopy (FTIR). The results showed that the absorption spectra of biologically-prepared AgNPs was at wave length ranging from 200-600 nm. The AgNPs have also different functional groups such as O-H, -C≡CH, C=C stretching vibration respectively. Antagonism effect of AgNPs on *Staphyloccocus aureus, Pseudomonas aeruginosa, E.coli and Proteus mirabilis* has been tested that isolates from (RTE) vegetable salads. Diameter of growth inhibition zone of these bacteria was determined using agar well diffusion method.

Keywords: *Bifidobacterium* spp.; silver nanoparticles; RTE salads; antagonism activity.

Practical Application: Study the effect of silver nanoparticles synthesized by probiotic bacteria (*Bifidobacterium* spp.) on some pathogenic bacteria isolated from Ready-To-Eat (RTE) salads.

1 Introduction

UV-vis and FT-IR are a widely used technique for nanomaterials, UV-vis is found the best technique to analyzing the optical properties of nanoparticles as with the decrease of the size band gap increase of the materials, while FT-IR measurements were used to identify the functional groups and usually performed to identify and classify probable biomolecules that can be reliable for gapping, leading to proficient stabilization of the gold, silver nanoparticles (Shukla & Iravani, 2019). It is well known that nanomaterial's have different physico-chemical properties from bulk materials having the same chemical composition. As reducing the particle size to nanoscale causes in most cases changes the fundamental properties of selected material and producing new one (AL-Saadi, 2021).

Based on particular features, nanoparticles have new or enhanced features like distribution, morphology and, size (Murphy, 2008). Nanoparticles are made in a variety of ways and are commonly used in a various industrial settings (Loo et al., 2013). Metallic nanoparticles, like; platinum, oxide, gold, silver, zinc and others, have demonstrated applications in medical, water treatment, textile engineering, anti-cancer, and electronics, among other fields (Wong & Liu, 2010; Nithya & Rangunathan, 2012). Silver, on the other hand, has been utilized as an antibacterial agent from ancient times because of its broad range antimicrobial action against viruses, fungi and bacteria. Furthermore, as compared to other metals, silver compounds are considered non-toxic to the human body at low concentrations. This technique led to the enhancement of biological ways that are eco-friendly, cost-effective, and can be scaled for large-scale synthesis due to growing environmental concerns about chemical synthesis. Moreover, biological techniques eliminate the need for high-pressure, high-temperature, and energy-intensive processes, as well as hazardous chemicals (Sathyavathi et al., 2010). The name probiotic comes from the Greek phrase pro bios, which means for life. Probiotics are described by the WHO/FAO as live microorganisms that, when given in sufficient amounts, provide a health benefit to the host (Klaenhammer, 2000; Sanders, 2003; Guarner et al., 2005). Probiotics are bacteria that are comparable to those found naturally in human intestines, as well as those seen in breastfed newborns. *Lactobacillus, Bifidobacterium, Escherichia coli*, and *Bacillus* species are all commonly utilized as probiotics (Haukioja, 2010). Different strains of Bifidobacteria have several health benefits such as; maintaining microbial balance of the intestine, pathogens suppression, enhancing local and systemic immunological responses, vitamin synthesis, and the bioconversion of a variety of food components into bioactive molecules (Ajay et al., 2016).

Bifidobacterium microorganism is suitable candidate for the production of silver nanoparticle due to their ability to grow

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[1]*Department of Biology, College of Science, University of Kirkuk, Kirkuk, Iraq*
[2]*Department of Animal Production, Technical Agricultural College, Northern Technical University, Mosul, Iraq*
*Corresponding author: Ali.mohammed@ntu.edu.iq

and proliferate in harsh conditions and their non-pathogenic nature (Denisa et al., 2015).

In recent years, food safety has been a main consideration and focus for many scientists. In addition, public concern over food safety is growing across the world (Food and Agriculture Organization, 2003). Vegetables products and consumption are essential for everyone's overall health; nevertheless, microbial contamination of these vegetables has emerged as a major issue that requires further attention. Salad veggies are a collection of vegetables that constitute a key component of food vending and are frequently implicated in this regard on a global scale. Salads are also good providers of minerals, proteins, vitamins and other nutrients that the human body needs to function properly (Amoah, 2014). Ready-to-eat foods, such as vegetable salads, are, on the other hand, huge potential sources of entropathogens and contaminated disease (Mensah et al., 2002). In the Kumasi metropolis of Ghana, Feglo & Sakyi (2012) found varied amounts of *Staphylococcus aureus, Bacillus species, Klebsiella pneumoniae,* and *Escherichia coli* in several ready-to-eat meals. Clostridium, Staphylococcus, Escherichia coli (E. coli), Shigella, Salmonella, Vibrio and Campylobacter are just a few of the bacteria that can cause food poisoning (Abakari et al., 2018).

Therefore, this study aims to synthesis silver nanoparticles using Gram-positive, branching anaerobic bacteria called Bifidobacterium spp. And then characterize antimicrobial activity of synthesised SNPs against foodborne bacteria isolated from RTEs products.

2 Materials and methods

2.1 Microorganisms and sampling

Bifidobacterium spp. was isolated from ACTIVIA yogurt which purchased from local market in Kirkuk city and cultivated in Man-Rogosa Sharpe Broth (MRS) obtained from HiMedia in test tubes for 24 or 48 hours (De Man et al., 1960).

Two type of salads (Chickpea Dip (Hummus with Tahini) and Coleslaw-carrot with Mayonnaise) were taken from local restaurant in Kirkuk city. Aseptically 25 g collected salad vegetable mixtures out of each vendor that are often served straight to consumers (students) were placed in sterile polythene zip lock bags, stored in an ice chest at temperatures between 0 and 4 degrees Celsius, and then transferred to the Food Microbiology Laboratory of the University for Development Studies within 2 to 4 hours to be tested for microorganisms..

All media were prepared in accordance with the manufacturer's protocol and included MacConkey agar (Oxoid), Cetrimide agar (Oxoid), Xylose Lysine Deoxycholate agar (XLD) (HiMedia), Mannitol Salt Agar (MSA) (Oxoid) to isolate *E.coli, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus* isolation respectively. Morphological Examination, Gram stain, Biochemical Tests, and API 20 (Analytical Profile Index for Enterobacteriaceae) were used for identification and confirmation of bacterial pathogens (Atlas, 2010).

2.2 Preparation of salad samples

Twenty-five grams (25 g) of each salad sample was weighed and transferred into sterile polythene zip lock bags under a laminar flow hood. Salad in each sterile bag was then mixed thoroughly with 225 mL of buffered peptone water. This mixture was homogenized very well by simple hand massaging and constant shaking to obtain a uniform mixture (stock). Ten (10)-fold serial dilutions were also carried out at five (5) levels. Specifically, 0.1 mL each of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were taken aseptically and inoculated into MacConkey, Cetrimide Agar, XLD and Mannitol Salt agars. The inoculated plates were then inverted and incubated at 37 °C (44.5 °C for MacConkey) for 24 h. After 24 h of incubation, bacterial colonies were identified based on the their colours on the surface of each agar plate. Growth of pink colonies were depicted as *Escherichia coli* and growth of yellow goldish color were depicted as *S. aureus*. and growth of black color colonies with Fishy odor were depicted as *Proteus mirabilis*. based on description by Acumedia Manufacturers (2011). The growth of *Ps. aeruginosa* on Cetrimide agar plates were depicted as green colonies with fruity odor.

2.3 Synthesis of silver nanoparticles

A loop of pure culture of *Bifidobacterium* spp. was inoculated in to 100 mL sterile MRS broth and incubated in shaker at 37 °C for 24 h at 160 rpm. After 24 h of incubation, culture suspension was centrifuged at 10000 x g for 10 min and the supernatant was collected in a separate vial. Extracellular synthesis of silver nanoparticles was accomplished by mixing 50 mL cell free supernatant of *Bifidobacterium* spp. with 50 mL aqueous solution of 1 mM silver nitrate (AgNO3). The mixture was incubated in Erlenmeyer flasks on orbital shaker (200 rpm) at 28 ± 2C in the dark for 24 hours until the pale yellow color change to brown. A flask containing cell free supernatant without AgNO3 was run along with experimental flask and used as a control (Matei et al., 2020).

2.4 Characterization of silver nanoparticles by spectroscopy

UV visible spectroscopy

Silver nanoparticles were characterized using UV-visible spectroscopy, the bio- reduction of AgNO3 by Bifidobacterium sp. Carried out using a double beam ultraviolet visible spectrophotometer (T92+ UV Spectrophotometer, PG INSTRUMENTS). Samples were loaded into 1 cm optical-path-length quartz cuvettes for analysis. The UV-VIS spectrophotometric readings were recorded at a scanning speed of 2 nm intravels were scanned from 190-900 nm. Presence of Ag NPs was confirmed by comparing obtained peaks with surface Plasmon resonance of Ag (Priyaragini et al., 2013).

Fourier Transform Infrared spectrum (FTIR) spectroscopy

The supernatant from the maximum time point of production (of silver nanoparticles) was freeze-dried and the dried powder was mixed with potassium bromide and pressed using hydraulic press to form pellets then recorded the spectrum in Fourier

Transform Infrared spectrum (FTIR) (NICOLET IR100 FT-IRSPECTROPHOTOMETER) in the range of 4000–400 cm−1 at a resolution of 4 cm−1 by using diffuse reflectance accessory (Silambarasan & Jayanthi, 2012).

2.5 Antimicrobial activity of nano silver particles

The antibacterial activity of biosynthesized silver from *Bifidobacterium* spp. was tested against foodborne bacterial isolates (*E. coli, Ps. aeruginosa, P.mirabilis, S. aureus*) by agar well diffusion method (Thomas et al., 2014). The pure cultures of organism were sub cultured on Muller Hinton broth medium at 35 °C on rotary shaker at 200 rpm. Wells of size 6 mm have been made on Muller- Hinton agar (MHA) plates using cork borer which used to punch holes on an agar plate. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Using micropipette, 20 µl (20 mg) of the sample of nanoparticles solution was poured into wells on all plates. After incubation at 35 °C for 18 hours, the different levels of zone of inhibition were measured using a ruler (Silambarasan & Jayanthi, 2012).

3 Results and discussion

3.1 Identification of microorganisms

In this, study Bifidobacterium spp. was isolated From ACTIVIA yogurt on MRS medium under anaerobic condition using morphological, microscopic and biochemical tests (**catalase, oxidase, motility, indole**) to confirm this genus. The result showed branching shape, Bacilli, Gr+ bacteria (Figure 1)

Four species of foodborne bacteria were isolated from RTE salads from local restaurants in Kirkuk city (*E. coli, Ps. aeruginosa, P. mirabilis and Staph. aureus*) and identification morphological, microscopic, biochemical tests **(catalase, oxidase, motility, IMViC**) and API 20 System used for confirmed these pathogenic bacteria (Figure 2, 3, 4)

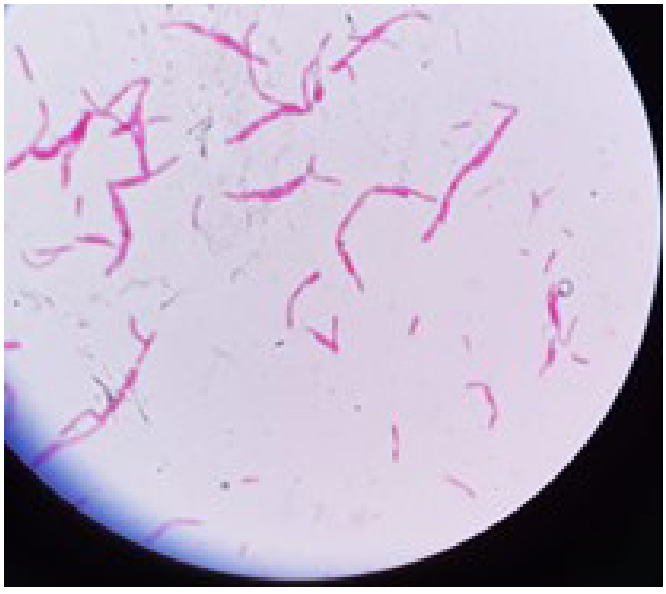

Figure 1. *Bifidobacterium* spp.

The significant rate of bacterial contamination in ready-to-eat salads found in this study might be attributed to a lack of fundamental sanitation standards for processing items that do not require pre-heating before consumption. Another reason might be the use of low-quality water for washing and pre-disinfection of fresh fruits and vegetables during salad preparation. (Majolagbe et al., 2012).

3.2 Visual analysis and spectrophotometric characterization of silver nanoparticles

After 24 hours of incubation with AgNO3, the color of the cell free culture progressively changed to dark brown, suggesting

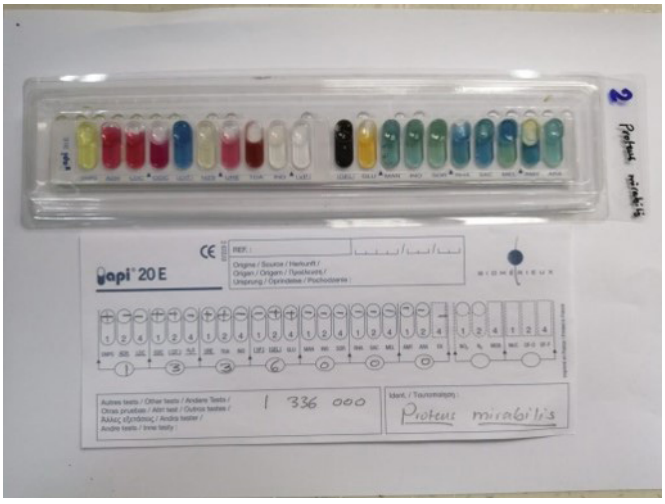

Figure 2. *P. mirabilis*

Figure 3. *Proteus* on KIA

the creation of silver nanoparticles (due to Ag+ to Ag0 reduction mediated by enzyme nitrate reductase), while there was no color change in the control without silver ion Ag+. (Figure 5) (Shareef et al., 2019).

Surface Plasmon vibration in silver nanoparticles caused the biosynthesis nanoparticles' dark brown hue. According to spectrophotometric measurement, the greatest absorption peak (optical density) happened at 420 nm wavelength. (Figure 6)

The result in agreement with the finding of Kavakebi et al. (2021) which exhibited the widening peak of AgNPs was performed at 430 nm and showed the strong wide peak was detected at 430 and 475 nm.

Figure 4. *Ps. aeruginosa*

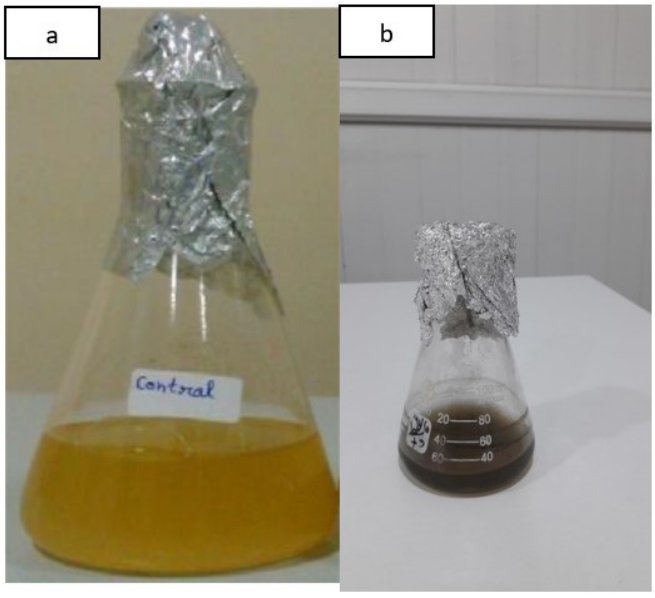

Figure 5. Biosynthesis of silver nanoparticles (a) Control Flask without change colour (b) SNPs synthesized by Bifidobacterium spp. With colour change to brown

The FTIR spectrum of silver nanoparticles was used to discover potential interactions between silver and bioactive compounds present in cell free extract of Bifidobacterium sp, which is accountable for silver nanoparticle production and stability (capping material). Broad O-H stretching of alcohols and phenols and vibration of quinone oximes was ascribed to the peaks at 3397.97 cm-1. 1636.44 cm-1, (-NH-C=O) indicates the presence of N-H bended primary amines (C=C stretching vibration of alkenes), a weak band at 666.89 cm-1 due to the occurrence of vibrations of alkyl halides as shown in (Figure 7). Different peaks in the FTIR spectra of silver nanoparticles produced from were discovered by Elbeshehy et al. (2015). According to the FTIR spectrum of silver nanoparticles, proteins and phenolic compounds may function as silver nanoparticle capping agents, and various biological molecules are responsible for silver nanoparticle production (Ajay et al., 2016).

3.3 Antagonistic activity of silver nanoparticles by using Bifidobacterium spp.

Silver nanoparticles made from Bifidobacterium sp. culture filtrate had an inhibiting impact on all foodborne pathogens examined. On MH agar plates, the efficacy (inhibition zone) is determined using agar well diffusion method as shown in (Table 1).

The results revealed that cell free cultures of Bifidobacterium spp. have strong inhibitory effects on RTE bacterial isolates (*E. coli, Ps. aeruginosa, P. mirabilis and Staph. aureus*) (Figure 8).

Table 1. Antagonistic effect of AgNPs.

Test microorganism (RTE isolates)	Inhibition zone of AgNPs (mm)
Escherichia coli	17
Pseudomonas aeruginosa	15
Proteus mirabilis	R
Staphylococcus aureus	17

R: Resistance.

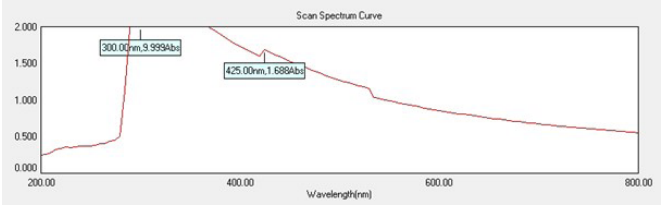

Figure 6. UV.VIS spectra of AgNO3 obtained using *Bifidobacterium* spp.

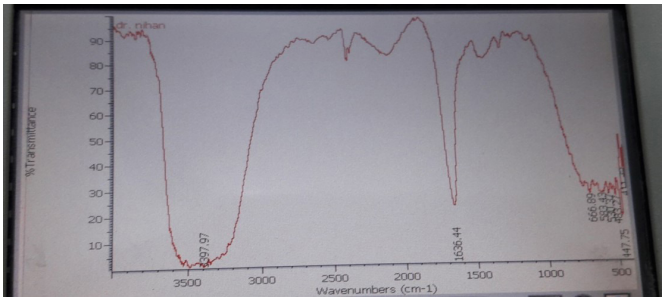

Figure 7. FTIR spectra of AgNO3 obtained using *Bifidobacterium* spp.

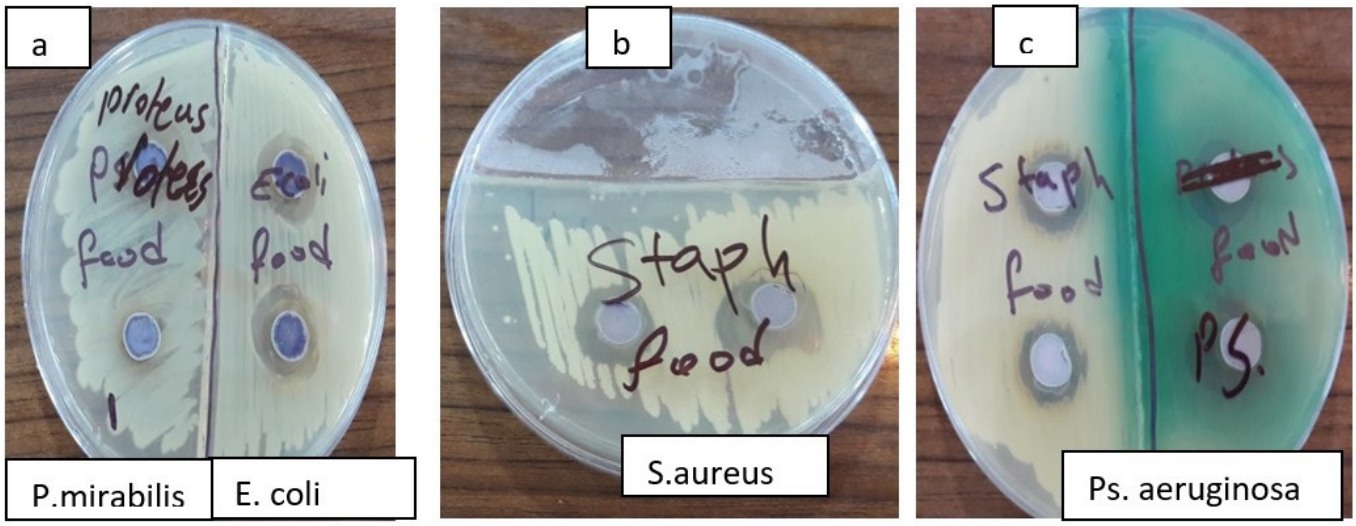

Figure 8. Bionanoparticles against (a) *E. coli* and *P. mirabilis* (b) *Staph. aureus* (c) *Ps. Aeruginosa.*

The results obtained in this study indicate that biologically synthesized silver nanoparticles possess tremendous antimicrobial properties. The broadest inhibition zone was found to be for both *E. coli* and *Staph. aureus* (17 mm) followed by *Ps. aeruginosa* (15 mm) but did not showed any effect on *P. mirabilis*. Owing to the broad spectrum antibacterial activity of SNPs which synthesized from probiotic bacteria (Bifidobacterium spp.) can be considered to have an effective inhibition action against both Gram positive and negative pathogens. Among other related findings Silvan et al. (2018) demonstrated the antibacterial activity of Ag-NPs against MDR Campylobacter strains obtained from poultry food chain and clinical patients. Another study found that nanoparticles made from the fruit water extract of Forsythia suspensa had antibacterial activity against the most prevalent foodborne pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, Salmonella typhimurium and *Vibrio parahaemolyticus* (Du et al., 2019). Many studies showed diverse results in terms of inhibition zone diameter, which might be due to variations in AgNO3 concentrations as well as changes in diffusion effects caused by agar medium composition (Zorraquín-Peña et al., 2020).

4 Conclusion

Biosynthesis of silver nanoparticles was achieved utilizing Bifidobacterium spp. culture filtrates and a 1 mM AgNO3 aqueous solution. The presence of SNPs was verified by a shift in the color of the supernatant from pale yellow to brown. With a UV-Vis spectrophotometer, silver nanoparticles displayed peaks between 200 and 600 nm. The presence of protein in silver nanoparticle samples, as well as amino acid for its stability, was revealed by the FTIR spectrum, which revealed broad O-H stretching vibrations, -CCH stretching vibrations, C=C stretching vibrations, C-H deformation vibrations, and C-H symmetrical deformation vibrations. The present study revealed that ready-to-eat salads served by one of Kirkuk city restaurants are contaminated with different kinds of foodborne pathogens, this contamination might be attributable to improper food handling, unhygienic food preparation and processing, source contamination of the vegetables from production sites and generally along the value chain and environmental conditions. According to Schuh et al. (2020), who studied (8) types of minimally processed vegetables, including lettuce, cabbage, alfalfa sprouts, kale, Italian salad (mix), tropical salad (mix), carrot, and fruit salad, the most significant contamination was related to the count of mesophilic aerobic microorganisms, molds, This might have occurred as a result of a greater bacterial load in the raw material, insufficient storage in some of the production phases, or an insufficient sanitation procedure. These conditions can cause vegetables to deteriorate, lowering their shelf life and rendering the food unsuitable for eating. According to the findings of this study, there is an urgent need to enhance food safety and quality standards of ready-to-eat meals at all cafeterias at Kirkuk city so that we studied the effect of silver nanoparticles on foodborne pathogens which isolated from RTE salads and have shown higher antibacterial activity against pathogens, The width of the zone of inhibition varies according to the bacterial type.

The results suggest that silver nanoparticles biosynthesized utilizing Bifidobacterium spp. culture could be used as antibacterial agents in biotechnological applications.

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