ISSN: 0067-2904

The Inhibition Activity of Silver Nanoparticles Compared with D-Glycin and Imipenem Effect on the Biofilm Formation by Food-origin *Salmonella* isolates

Marwa H. M. Al-Khafaji
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

ABSTRACT

Since decades silver was depended worldwide as a treatment to a lot of diseases ranging from burn infections, anthrax, and typhoid fever to bacterial conjunctivitis in stillbirth, but its effectiveness against biofilms is still undetermined. *Salmonella* is a major cause of food poisoning outbreaks especially in the third world countries. Thus, in the present study; the antimicrobial activity of silver nanoparticles (Ag-NPs) against *Salmonella enterica* biofilm was examined; their activity was compared with amino acid; D-Glycin and imipenem antibiotic. The result of the study revealed that Ag-NPs exhibited considerable antimicrobial property against *Salmonella enterica* biofilm where the minimum inhibitory concentration (MIC) was found at 50 µg/ml while MIC of D-glycin and imipenem were 50mM and 4µg/ml respectively. The isolates ability to form biofilm was assayed using the tissue culture plate (TCP) assay, all the isolates were biofilm producers but with a different thickness degrees. It was found that both of Ag-NPs and imipenem inhibited *Salmonella* biofilm formation, but the inhibition by Ag-NPs was more than the antibiotic imipenem, whereas D-Glycin increased the ability of *Salmonella* isolates to form biofilm.

Keywords: Silver nanoparticles, Biofilm, *Salmonella*, D-glycin, Chicken meat, Imipenem.

الفعل التثبيطي لجزيئات النانو فضة مقارنة بفعل الكلايسين والإمبينيم على تكون الغشاء الحياتي لبكتريا السالمونيلا غذائية المنشأ

مروى حميد مطشر الخفاجي
قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة

اعتمدت الفضة لعقود من الزمن كعلاج العديد من الامراض من إصابات الحروق والجمرة الخبيثة وحمى التيفوئيد وحتى التهاب ملتحمة العين البكتيري لدى حديثي الولادة، لكن فعاليتها ضد الاغشية الحياتية لازالت غير محددة . ولأهمية جرثومة السالمونيلا كمسبب رئيسي للتسممات الغذائية خصوصا في بلدان العالم الثالث، لذا تم اختبار الفعالية الضد ميكروبية لجزيئات النانو فضة تجاه الغشاء الحياتي لبكتريا السالمونيلا، وتمت مقارنة فعاليتها مع فعالية الحامض الاميني الكلايسين ومضاد الحياة الامبينيم. كشفت نتائج هذه الدراسة ان جزيئات النانو فضة قد أظهرت فاعلية ضد ميكروبية واضحة تجاه الغشاء الحياتي للسالمونيلا إذ كان التركيز المثبط الادنى لها50مايكروغرام\مل بينما كان للحامض الاميني وللمضاد 50 ملي مولر\مل و4 مايكروغرام\مل على التوالي. اختبرت قدرة عزلات السالمونيلا على إنتاج الغشاء الحياتي باستخدام أطباق الزرع

Email: marwa_1981_a@yahoo.com

النسيجي فأظهرت النتائج أن العزلات جميعها كانت منتجة للغشاء الحياتي لكن بدرجات سمك متفاوتة. ثبطت جزيئات النانوفضة ومضاد الامبينيم تكون الغشاء الحياتي للسالمونيلا بينما عمل الكلايسين على زيادة قابلية هذه العزلات لتكوين الغشاء الحياتي.

Introduction

During the last decades, it has become increasingly clear that bacteria, including foodborne pathogens such as *Salmonella enterica*, grow predominantly as biofilms in most of their natural habitats, rather than in planktonic mode. [1] Microbial biofilm develops when microorganisms irreversibly adhere to a surface and produce extracellular polymers that facilitate the adhesion and provide a structural matrix which stabilizes the biofilm [2].

Interestingly, it has been observed that the resistance of biofilm cells to antimicrobials significantly increased compared with what normally seen with the same cells being planktonic [3, 4]. Thus, it is believed that biofilm formation enhances the capacity of pathogenic *Salmonella* bacteria to survive stresses that are commonly encountered both within food processing, as well as during host infection. In food industry, biofilms may create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage [5-9]. Improperly cleaned surfaces promote soil build-up, and, in the presence of water, contribute to the development of bacterial biofilms which may contain pathogenic microorganisms, such as *Salmonella* [1].

Salmonella enterica serovar *Typhimurium* is an important zoonotic gastrointestinal pathogen responsible for foodborne disease worldwide. It is a successful enteric pathogen because it has developed virulence strategies allowing it to survive in a highly inflamed intestinal environment exploiting inflammation to overcome colonization resistance provided by intestinal microbiota [10]. Since around 400 B.C. silver has long been known and documented for its antimicrobial properties when Hippocrates described that it used to enhance wound healing and for preserving water and food specially milk [11] but its medical applications declined with the development of antibiotics [12].

Although, the antimicrobial characters of silver have been well explored but the antibiofilm properties of silver against microorganisms including Salmonella are still unclear. Thus, the current study aimed to study the antibiofilm effect of silver nanoparticles against biofilm formation of food origin *Salmonella*. Furthermore, compare among the silver nanoparticles, antibiotics and D-Glycin effects on biofilm formation.

Materials and Methods

Sample collection

Thirty frozen imported chicken meat samples were collected randomly from local markets in Iraq. The instructions of the Iraqi Standard Criterion No.2/2270 in Sampling, afferent by the Iraqi Central Organization for Standardization and Quality Control (C.O.S.Q.C) were followed [13].

Bacterial Isolation

The Iraqi Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods, [14] was depended in the isolation of *Salmonella* isolates using Salmonella-Shigella (S.S) agar and Xylose Lysin Deoxycholate (XLD) agar. In addition to colonial morphology, identification was carried out by using biochemical tests according to Bergey's Manual [15], Api20E system and Vitik complete system.

Silver nanoparticles

Silver nanoparticles were obtained and prepared as the study of Wajih *et al*., 2015[16]. By using the pulsed laser type Nd-YAG laser of wavelength 1064nm, energy 760mJ and pulse repetition rate 6 Hz.

Preparation of antibiotic stock solution:

A final concentration 1mg/ml of imipenem solution was prepared via dissolving 0.01 g of imipenem antibiotic in 10 ml of normal saline, and then sterilized by filtration using a membrane filter with 0.22 mμ pore size.

Antibiotic susceptibility test

Modified Kirby-Bauer method was followed. Sensitive, intermediate and resistant categories were used to translate the inhibition zone diameter of an individual antimicrobial agent after being compared with the standard inhibition zone [17, 18] according to Clinical Laboratories Standards Institute (CLSI, 2011) [19].

Minimum inhibitory concentration determination

I. MIC of D-Glycin

Minimum inhibitory concentration (MIC) of D-glycine for *Salmonella* planktonic cells was determined by preparing a stock solution 1 M of the amino acid in distilled water, and sterilized by filtration through 0.45 mμ membranes (Billerica, MA. USA). Different amino acid molarities achieved, initiated with 50mM and serially diluted by the medium to the end point concentrations. One hundred microliter of double strength Muller-Hinton broth was added to each test well, in the first well; 100 µl of the stock solution was added and mixed, a series of dilutions was then prepared across the plate, a final inoculum size of $1x10^8$ CFU/ml in each microtiter plate well was achieved by inoculating it with 10 µl of overnight *Salmonella* culture. Positive growth controls contained the bacterial inoculum with nutrient broth without amino acid treatment, while the wells that treated with D-amino acid but without inoculum considered as negative controls. The same experimental conditions were employed on the control wells. The microtiter plates were incubated for 24 h at 37Cº and examined for microbial growth which was detected in the test wells as turbid in relative to the positive and negative controls; the lowest D–amino acid concentration that inhibited ≥ 80% of microbial growth conducted MIC value; MIC determination was carried out in triplicate [20].

II. MIC of Silver nanoparticles

The same protocol described above in the previous item was depended in determination of MIC for silver nanoparticles, using micro dilution method in a sterile tissue culture plate, different AgNps concentrations (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) (v/v) were prepared using deionized distilled water. Each microtiter plate well was inoculated with 10 µl of bacteria to achieve a final inoculum size of $1x10^8$ CFU/ml well with overnight culture. Positive growth controls contained the bacterial inoculum with nutrient broth without AgNps treatment, while the wells that treated with AgNps but without inoculum considered as negative controls. After 24 h of incubation at 37°C, the plates were examined and the MIC of each sample was determined.

III. MIC of Imipenem

The same procedure described previously regarding D-glycin and AgNps MIC used to determine the MIC of imipenem.

Biofilm formation assay

A previous study described Tissue culture microtiter plate method as a quantitative test [21]. It is considered as the gold standard technique for biofilm detection. *Salmonella* isolates isolated from fresh nutrient agar plates were inoculated in 10 ml of trypticase soy broth (TSB) with 1% glucose w/v and then incubated at 37Cº for 24 h. A fresh medium then added to the culture to be diluted to 1:100; the wells that filled with sterile broth only considered as negative control. Then the plates were incubated at 37Cº for 24 h, and after incubation, mild tapping was performed to eliminate the content of each well. Planktonic bacteria were eradicated by washing the wells with sterile distilled water once. The formed biofilm by adherent bacteria to the wells was stained using crystal violet (0.1%) w/v. Excess dye was removed by distilled water and plate were left to dry. The optical density (OD) of stained adherent biofilm was obtained using Micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm. The experiment was performed in triplicate Table- 1.

Table 1-Interpretation of Biofilm production

Average OD value	Biofilm production
≤ OD /ODc < ~ ≤ 2x ODc	Non / weak
2x ODc < ~ ≤ 4 x ODc	Moderate
> 4x ODc	Strong

Optical density cut- off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control [22]

Study the effect of Ag-NPs, D-amino acid and antibiotic on Biofilm formation

The method described by Goh and colleagues [23] with minor modifications was performed; using 96 well microtiter plate. Briefly TSB (TSB + 1% w/v glucose) was inoculated with *Salmonella* overnight culture and then it was diluted to 1:100. Each well was loaded with 100 µl of medium and 100 µl of 50 mM of Ag-NPs, while the control well without Ag-NPs. The plate then incubated at 37Cº for 24 h. Planktonic bacteria were removed by washing the wells with sterile distilled water once.

Then crystal violet solution (0.1% w/v) was added to each well and the plate was kept to stain for 10 min at room temperature, thereafter the stain was removed by dipping the plate in a tray filled with water. The microtiter plate was then inverted on paper towels to remove excess liquid and left to air dry. Ninety five percent of ethanol was depended to solubilize the dye in the stained wells; micro ELISA auto reader used to measure optical density at 630nm.

This method was depended with a substitution of 50 mM D-glycin instead of Ag-NPs once and with 4 µg/ml imipenem another time.

Statistical analysis

One-way analysis of variance (ANOVA) in factorial experiment with complete randomized design was performed. Difference between means was analyzed by least significant difference (LSD) at $p<0.05$ using (SPSS) program 2010 and excel application to find results.

Results and Discussion

Food-origin *Salmonella* isolates isolated from chicken meat samples as shown in Figure-1, were tested for their susceptibility to seven antibiotics: imipenem, meropeneme, cefotaxim, azithromycin, amoxicilin, cefalothin, and ciprofloxacin, and most of them were multi-drug resistant (MDR) as shown in Table-2.

Figure 1- *Salmonella* colonies with black center on XLD agar (left) and on S.S agar (right) after 24 h incubation at 37˚C aerobically.

Table 2- Antibiotic susceptibility of *Salmonella* strains

Isolate No.	Antibiotics						
	IPE	MEM	KF	CIP	SAM	CTX	AZM
S2	S	S	R	R	R	R	R
S15	S	S	R	R	R	R	R
S26	S	S	R	R	R	R	R
S30	R	S	R	R	R	R	R
S37	S	R	R	R	R	R	R
S38	S	S	R	R	R	R	R

I: Intermediate; R: Resistant ;S: Sensitive

The obtained results in this study revealed that 16.6% of *Salmonella* isolates were imipenem resistant, and the same percent of the isolates were meropeneme resistant, while all the isolates the other antibiotics. The researchers in a previous study conducted that the levels of antibiotic resistance are similarly elevated among food-borne pathogens such as *Salmonella* [24]. It is not certain to prove a straight role of antibiotic resistance in bacteria contaminating food stuffs with elevated clinical cases of resistant infections, but the incidence of such bacteria in food stuffs and food environment could play a vital role in the extent of antimicrobial resistance among food-borne pathogens [25]. Production of carbapenemases causes resistance to imipenem that refers to carbapenems group which used to treat infections caused by MDR even those extended spectrum β-lactamases producers [26].

Results showed that the MICs of the silver nanoparticles, D-glycin and imipenem suppressing the growth of *Salmonella* strains were determined as 50mM of AgNps, D-glycin, and 4µg/ml for imipenem.

Regarding biofilm production, *Salmonella* isolates were tested for ability to produce biofilm by tissue culture plate assay, and all of them produced biofilm but with a diverse thickness grades as measured by ELIZA reader at 630 nm. Pathogenic or spoilage microorganisms badly affect the food quality and safety via the formation of biofilm in food and its environment, by its acting as a persistent contamination source, leading to transmission of diseases or food spoilage which may end in economic losses [27, 28].

In this study, adding Ag-Nps caused biofilm inhibition for all the tested isolates of *Salmonella*, so as the treatment with imipenem inhibited the formation of biofilm, but its effect as compared with Ag-Nps was less than the latter. An amazing result concerning D-Glycin, that the amino acid addition increased the biofilm thickness [Table-3, Figure-2].

Table 3- Biofilm formation by food-origin *Salmonella* isolates using TCP assay.

Eliza results of Biofilm formation by six *Salmonella* isolates						
Isolate number / Treatment	S2	S15	S26	S30	S37	S38
Without treatment	0.090	0.090	0.092	0.098	0.098	0.097
Without treatment	0.091	0.092	0.094	0.100	0.100	0.096
Without treatment	0.092	0.088	0.091	0.098	0.101	0.097
Imipenem	0.090	0.083	0.071	0.079	0.092	0.089
Imipenem	0.090	0.081	0.070	0.079	0.091	0.089
Imipenem	0.090	0.082	0.072	0.079	0.090	0.085
D-glycin	0.153	0.115	0.115	0.105	0.120	0.127
D-glycin	0.155	0.117	0.113	0.105	0.118	0.131
D-glycin	0.152	0.118	0.112	0.108	0.123	0.129
Silver nanoparticles	0.074	0.067	0.054	0.063	0.076	0.043
Silver nanoparticles	0.072	0.067	0.050	0.061	0.073	0.040
Silver nanoparticles	0.070	0.062	0.051	0.060	0.070	0.041

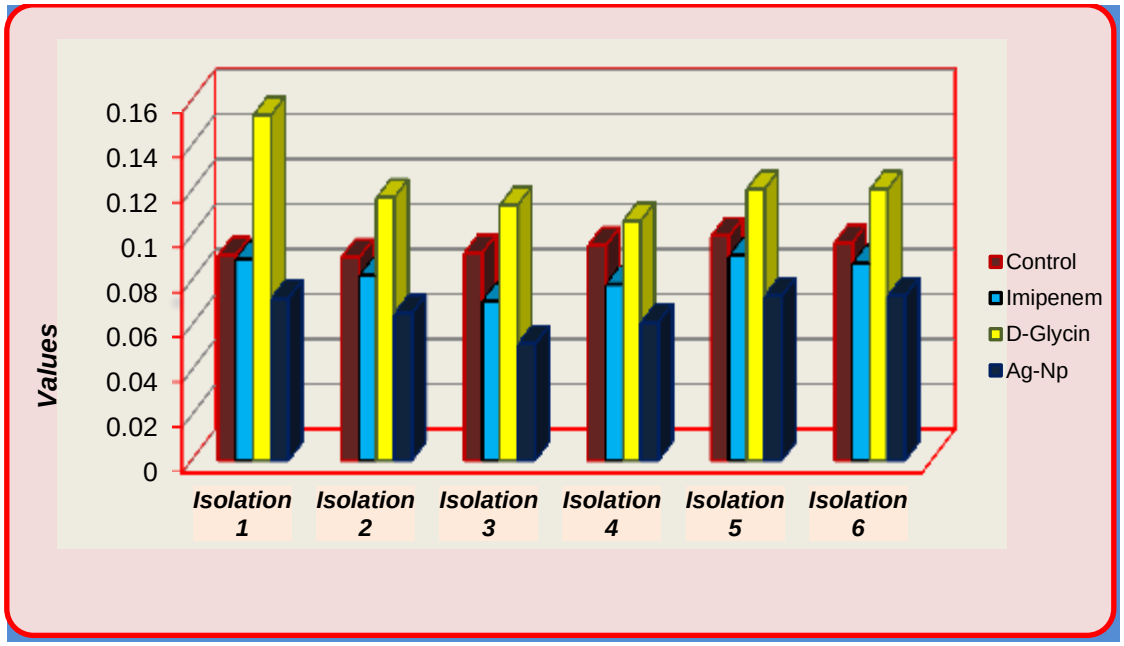

Figure 2-The effect of Ag-Nps compared with that of Imipenem and D-Glycin on biofilm formation by food origin *Salmonella* isolates in terms of absorbance means.

It is clear from the figure above that both of silver nanoparticles and imipenem inhibited the biofilm formation by *Salmonella* isolates isolated from frozen chicken meat samples.

In another studies it was reported that the biofilm formation by *E. coli* isolates inhibited by glycine and this effect was concentration-dependent [23, 29]. There is an opinion that AgNPs have the same mechanism of the antimicrobial agents, which include; Inhibition of cell wall synthesis, nucleic acid, protein synthesis and metabolic pathway [30]. Previous studies stated that the silver ion positive charge is essential for its effectiveness as an antimicrobial agent via electrostatic attraction between their positive charge and the bacterial cell membrane negative charge. [31-33]. AgNps penetrate inside the bacterial cell and have affinity to react with phosphorous and sulfur groups, so that sulfur containing proteins of the cell membrane and DNA are the favored sites for AgNPs [34, 35].

Conclusions

Our results revealed that the ability to initiate biofilm on the polystyrene surface by food origin *Salmonella* isolates increased in the presence of D-amino acid, while silver nanoparticles inhibited this ability to form biofilm more than even imipenem antibiotic. So this study conveys a promise solution for the contamination of food environment by bacterial biofilms, thus sequestering the uncontrolled antibiotic usage which contribute in the wide spread of antibiotic resistance among food origin *Salmonella* isolates. Further studies on the effectiveness of nanoparticles of other metals against biofilms and new combinations will be useful to overcome the antibiotic resistance problem concerning biofilms in food environments and biofilm associated infections.

Acknowledgement

Author would like to acknowledge Dr. Ayyad Al Shahwany for his assistance in offering silver nanoparticles.

References

1. Giaouris, E., Chorianopoulos, N., Skandamis, P., and Nychas, G**. 2012**. Attachment and Biofilm Formation by Salmonella in Food Processing Environments, Salmonella - A Dangerous Foodborne Pathogen, Dr. Barakat S M Mahmoud (Ed.), ISBN: 978-953-307-782-6.
2. Namasivayam, S. K. R., Preethi, M., Bharani, A. R. S., Robin, G. and Latha, B. **2012**. Biofilm Inhibitory Effect of Silver Nanoparticles Coated Catheter against Staphylococcus aureus and Evaluation of Its Synergistic Effects with Antibiotics. *International Journal of Biological & Pharmaceutical Research*, **3**: 259-265.
3. Gilbert, P., & Mcbain, A. J. **2003**. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clinical Microbiology Reviews*, **16**(2): 189e208.
4. Mah, T.-F.C. & O'Toole, G.A**. 2001**. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, **9** (1): 34-39.
5. Brooks, J. D., & Flint, S. H. **2008**. Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science & Technology*, **43**(12): 2163e2176.
6. Carpentier, B., & Chassaing, D. **2004**. Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. *International Journal of Food Microbiology*, **97**(2): 111e122.
7. Ganesh Kumar, S. & Anand, S.K. **1998**. Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*, **42**: 9-27.
8. Lindsay, D. & von Holy, A. **2006**. What food safety professionals should know about bacterial biofilms. *British Food Journal*, **108**(1): 27-37.
9. Zottola, E. A. & Sasahara, K.C. **1994**. Microbial biofilms in the food processing industry –Should they be a concern? *International Journal of Food Microbiology*, **23**: 125-148.
10. Drumo, R., Pesciaroli, M., Ruggeri, J., Tarantino, M., Chirullo, B., Pistoia, C., Petrucci, P., Martinelli, N., Moscati, L., Manuali, E., Pavone, S., Picciolini, M., Ammendola, S., Gabai, G., Battistoni, A.,Pezzotti, G., Alborali, GL., Napolioni, V., Pasquali, P., and Magistrali, CF. **2016**. Salmonella enterica Serovar Typhimurium Exploits Inflammation to Modify Swine Intestinal Microbiota. *Front Cell Infect Microbiol*. **5**, 106.
11. Magner, L. N. **1992**. *Hippocrates and the Hippocratic Tradition: A History of Medicine*. Marcel Dekker, Inc., New York.
12. Sharma, B. K., Saha, A., Rahaman, L., Bhattacharjee, S. and Tribedi, P. **2015**. Silver Inhibits the Biofilm Formation of *Pseudomonas aeruginosa*. *Advances in Microbiology*. **5**: 677-685.
13. Iraqi Central Organization for Standardization and Quality Control (C.O.S.Q.C). **2006.** Iraqi Standard Criterion No.2/2270 in Sampling.

14. Iraqi Central Organization for Standardization and Quality Control (C.O.S.Q.C). **2006**. The Iraqi Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods.
15. Holt, J. G., Krieg, R., Sneath, P., Staley, J., Williams, S. **1994**. *Bergey's manual for determinative Bacteriology*. 9th ed., Williams &Wilkins. USA.
16. Wajih, Z. A., Al-dahan1, Z. T. and Al shahwany, A. **2015**. Antimicrobial Effects of Silver Nanoparticles Produced by Laser Ablation. *Iraqi Journal of Science,* **56** (3B): 2170-2186.
17. Benson, J.M. **2001**. Microbiological applications: Laboratory manual in general microbiology. 8th ed. 2001. McGraw Hill. New York.
18. Morello, J. A., Mizer, H. E., Granato, P. A. **2006**. *Laboratory manual and workbook in microbiology applications to patient care*. 18th ed. McGraw Hill. New York.
19. Wayne, P. A. **2011**. Clinical and laboratories standards institute. Performance standards for antimicrobial susceptibility testing. Clinical and laboratories standards institute.
20. Bardawell,S. K. **2014**. D-amino acids: Prospects for new therapeutic agents. *J Med Bioeng,* **3**: 197.
21. Christensen, D., Simpson, A., Younger, J. A. **1995**. Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices. *Clin Microbiol,* **22**: 996-1006.
22. Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A., Iqbal, M. **2011**. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz. J. Infect. Dis.*, **15**: 305-11.
23. Goh, N., Fernandez, A., Ang, S. Z., Lau, W.Y., Ng, L. **2013**. Effect of different amino acids on biofilm growth, swimming, motility and twitching motility in *Escherichia coli* BL 21. *J. Biology Live Sci.,* **4**: 111.
24. Farzana, K., Akhter, S., Jabeen, F. **2009**. Prevalence and antibiotic resistance of bacteria in two ethnic milk based products. *Pak. J. Bot.,* **41**: 935-943.
25. Hassan, A., Farooqui, A., Khan, A., Yahya, A., Kazmi, S. **2010**. Microbial contamination of raw meat and its environment in retail shops in Karachi, Pakistan .*J Infect Dev Ctries,* **4**(6): 382-388.
26. Chakraborty, D.; Basu, S.; Das, S. **2010**. A study on infections caused by Metallo Beta Lactamase Producing Gram negative Bacteria in Intensive Care Unit Patients. *Am J infect Dis* **6**: 34-39.
27. Blaschek, P., Wang, H., Agle, M. E. **2007**. *Biofilms in the Food Environment*. 1st ed. Blackwell publishing. *IFT press*. USA.
28. Brooks, J. D., Flint, S. H. **2008**. Biofilms in the food industry: problems and potential solutions. *Int. J. Food Science Technol,* **43**: 2163-2176.
29. Alkhafaji, M. H. M., Tawfeeq, H. K., Mahdii, B. A. **2016**. Effect of combination of D-glycin and antibiotics on biofilm formation by clinical, food and environmental isolates of *Escherichia coli*. *World J. Exp. Bio. sci.,* **4**: 112-117.
30. Guzman, M., J. Dille and S. Godet, **2012**. Synthesis and antibacterial activity of silver nanoparticles against Gram-positive and Gram-negative bacteria. *Nanomedicine: Nanotechnology, Biology and Medicine,* **8**: 37-45.
31. Parameswari, E., C. Udayasoorian, S. P. Sebastian and R. M. Jayabalakrishnan, **2010**. The bactericidal potential of silver nanoparticles. *International Research Journal of Biotechnology,* **1**(3): 044-049.
32. Tiwari, D. K., J. Behari and Sen, P. **2008**. Time and dose dependent antimicrobial potential of Ag nanoparticles synthesized by top-down approach. *Current Science,* **95**(5): 647-655.
33. Vijayakumar, M., K. Priya, F.T. Nancy, A. Noorlidah and A.B.A. Ahmed, **2013**. Biosynthesis, characterization and a nti-bacterial effect of plant-mediated silver nanoparticles using *Artemisia nilagirica*. *Industrial Crops and Products,* **41**: 235-240.
34. El-Batal, A. I., Amin, M. A., Shehata, M. K. Hallol, A. **2013**. Synthesis of Silver Nanoparticles by *Bacillus stearothermophilus* Using Gamma Radiation and Their Antimicrobial Activity. *World Applied Sciences Journal,* **22**(1): 01-16.
35. Dehkordi, S. H., Hosseinpour, F. and Kahrizangi, A. E. **2011.** An *in vitro* evaluation of antibacterial effect of silver nanoparticles on *Staphylococcus aureus* isolated from bovine subclinical mastitis. *African Journal of Biotechnology,* **10**(52): 10795-10797.