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A study on the minimum inhibitory concentration and minimum bactericidal concentration of Nano Colloidal Silver on food-borne pathogens

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Abstract: In the emerging issue of increased multi-resistant properties in foodborne pathogens, silver nano particles are being used increasingly as antimicrobial agents. Thus, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Nano Colloidal Silver towards food-borne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica Serovar* Typhi, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Bacillus cereus* and *Staphylococcus aureus* were examined in this study. The results obtained suggested that Nano Colloidal Silver exhibit a good bacteriostatic effect but poor bactericidal effect towards all food-borne pathogens tested. Nano Colloidal Silver can be a potential antimicrobial agent due to its low cost of production and high effectiveness in antimicrobial properties, which may find wide applications in various food industries to address food safety issues.

Keywords: nano colloidal silver, MIC, MBC, foodborne pathogens

Introduction

Malaysia is one of the developing countries where the majority of the population is getting more concern of the emerging foodborne diseases. In the process of food production, many kinds of antimicrobial agents are used for preventing and controlling diseases, enhancing growth and increasing production efficiency. The use of antimicrobial agents in them later on promoted to the emergence of resistance in micro-organism (Asai et al., 2005; Olofsson, 2006). Therefore, the resistant bacteria can certainly infect the humans via the food chain causing them to suffer from foodborne illnesses (Phillips et al., 2004). The emergence and the development of antimicrobial resistance in pathogens with its scattering nature therefore turned into a global public health concern (WHO, 2007). Research has been intensively done in antibacterial material containing various natural and inorganic substances to overcome this problem (Kim et al., 2007). Among them, silver or silver ions have known to have strong inhibitory and antibacterial effects as well as a broad spectrum of antimicrobial activities.

Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are now searching for new antibacterial agents. In the present scenario, nano scale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties (Morones et al., 2005; Kim et al., 2007). Nanotechnology is emerging as a rapidly growing field with its application in Science and Technology for the purpose of manufacturing new materials at the nano scale level.

For centuries, the only known method for producing fine silver to treat diseases was to grind it into a fine powder, either manually or chemically. Due to more advanced technical methods of production, today's Colloidal Silver solutions are far superior than those produced prior to 1938, and at a mere fraction of the cost. At the moment, advanced technology also provides us with electro-colloidal solutions that produce even greater results. Little work has been done on evaluation of the inhibition of microbial growth by Ag NPs towards foodborne pathogens. At present, silver has reemerged as a viable treatment option for infections encountered in burns, open wounds and chronic ulcers. Several products have incorporated with silver for use as a topical antibacterial agent are already in market such as silver nitrate, silver sulphadiazine or chlorhexidine and silver sulphadiazine impregnated with lipidocolloid wound dressing (Monafo, 1987). Furthermore, silver nano particles are now widely used as antibacterial

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or antifungal agents in a diverse range of consumer products such as air sanitizer sprays, socks, pillows, slippers, detergents, soaps, shampoos, toothpastes, food storage containers and others (Buzea et al., 2007). Many reports have been published on the prevalence of foodborne pathogens in different types of food in Malaysia (Son et al., 2003; Lesley et al., 2005; Chai et al., 2008; Tan et al., 2008; Tang et al., 2009; Jeyaletchumi et al., 2010; Suzita et al., 2010)

This study was carried out to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Nano Colloidal Silver against foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica Serovar* Typhi, *Vibrio cholerae, Vibrio parahaemolyticus, Bacillus cereus* and *Staphylococcus aureus*.

Materials and Methods

Nano Colloidal Silver

Nano Colloidal Silver Hydrosol (TomiMax) 1-100 nm / 200 ppm were procured from TomiCare Sdn. Bhd (Company Reg No: 562079-W) in solution form. The stock solution was used to prepare ten different concentrations; 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3.125 ppm, 1.56 ppm, 0.78ppm, 0.39 ppm and 0.195 ppm by serial two-fold dilutions.

Purification of bacterial culture and preparation of inoculums

The top of colonies from the NA slant was touched with a swab, and then swirled into one ml of sterile peptone water to obtain a cell suspension. The suspension was incubated with shaking at 37ºC for overnight. The surface of the selective agar medium was streaked with a loop full of the bacterial suspension, and then incubated for 18-24 hours at 37ºC. The bacterial species tested and the agar medium used were: *Listeria monovytogenes* on PALCAM Agar, *Bacillus cereus* on MYP agar, *Staphylococcus aureus* on BP agar, *Escherica Coli* O157:H7 on CHROM agar *E. coli*, *Vibrio parahaemolyticus* on CHROM agar *Vibrio, Vibrio cholerae* on CHROM agar *Vibrio* and *Salmonella entrica Serova*r Typhi on CHROM agar *Salmonella*. Strains used were obtained from the Food Safety Laboratory of Universiti Putra Malaysia, Serdang, Selangor.

A single colony was picked with a sterile loop and transferred into Luria Bertani Broth (LB Broth). The broth was then incubated with shaking at 37°C for overnight. The density of the organism suspensions were adjusted to equal to the 0.5 McFarland standard (0.5 mL of 0.048 M $BaCl_2$ (1.17% m/v $BaCl_2.2H_2O$) was added to 99.5 mL of 0.18M H_2SO_4 (1% v/v)) by adding sterile LB Broth. To aid comparison, test and standard were compared against a white background with a contrasting black line. Final suspensions obtained contain between 10^7 and 10^8 cfu/ml.

The minimal inhibitory concentration (MIC)

Broth Macro-dilution

The minimal inhibitory concentration (MIC) were determined by a broth macro-dilution method, using LB broth and final inocula of 105 and 106 cfu/ml. Ten different concentration of Nano Colloidal Silver prepared were tested against *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica Serovar* Typhi, *Vibrio cholerae, Vibrio parahaemolyticus, Bacillus cereus* and *Staphylococcus aureus*. Sterile centrifuge tubes were arranged in rows of sets for each bacterial inoculum to cover the ten different concentration of Nano Colloidal Silver in duplicate. One mL volumes of each concentration of Nano Colloidal Silver were transferred to the tubes accordingly, containing final inoculums of 10^5 cfu/mL test bacteria. The contents in the tubes were mixed thoroughly and incubated at 37°C for overnight. The MIC endpoint is the lowest concentration of Nano Colloidal Silver at which there is no visible growth in the tubes.

Minimal bactericidal concentration MBC

The method used and described below is an amended version of the procedure described in the BSAC Guide to Sensitivity Testing and can be adapted for determining the minimal bactericidal concentration (MBC) of Nano Colloidal Silver by substituting IsoSensitest broth (ISA;Oxoid) with Luria Bertani broth (LB broth). After MIC determination of the Nano Colloidal Silver tested, an aliquot of 10 µl from all tubes in which no visible bacterial growth was observed were seeded in Nutrient Agar (NA) plates not supplemented with Nano Colloidal Silver. The plates were then incubated for overnight at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the NA plates.

Statistical Analysis

MIC and MBC results were analyzed for one-way with Turkey's multiple comparison statistical analysis at a = 0.05 significant level by using MINITAB® Released 14.12.0 software (Minitab Inc. US). The statistical analysis was carried out to determine the significant differences between the food origins isolates used. P values $\leq$ 0.05 were considered significant.

Results and Discussions

A wide variety of synthetic compounds exert antibacterial effect, but just some of them can be used as biocides to develop drugs or coatings. The primary impediment for their use is their toxicity compared with their bactericidal effect; some of them are so toxic for eukaryotic cells that cannot be proposed as antibiotics. Among these materials, silver compounds (salts and colloids) raise as potent antimicrobial agents whose application is restricted to topical creams used to reduce the risk of wound infection and to treat infected wounds. In order to study silver nano particles as novel antimicrobial agents, this research assess the antimicrobial properties of Nano Colloidal Silver against *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica Serovar* Typhi, *Vibrio cholerae, Vibrio parahaemolyticus, Bacillus cereus* and *Staphylococcus aureus*.

The antimicrobial activity of different concentration of Nano Colloidal Silver was determined against food origin isolates of *Escherichia coli* O157:H7 strains (B1, B3, B6, B8); *Listeria monocytogenes* (LM1, LM2, LM3, LM7, LM22); *Salmonella enterica Serovar* Typhi (S7, S8, S9, S10, S11); *Vibrio cholera* (VC1D, VC11D, VC2F, VC10F, VD1G); *Vibrio parahaemolyticus* (VP34, VP35, VP36, VP37); *Bacillus cereus* (BC117, BC122, BC205, BC223, BC224) and *Staphylococcus aureus* (SA2, SA5, SA22, SA25, SA37). Many extravagant, unfounded and/or dubious claims have been made regarding the properties of colloidal silver that it protects against werewolves, guards against respiratory illnesses, and can be used to treat many illnesses including skin cancer. Different food origin isolates showed different sensitivity towards Nano Colloidal Silver.

Table 1 shows the MIC and MBC value of all tested food origin isolates towards Nano Colloidal Silver. The MIC values were in the range of 7 to 25 ppm in average whereas for MBC values, all the tested bacteria exceeded 100 ppm, the highest concentration tested in this study. The results showed good inhibitory effect but poor bactericidal effect of Nano Colloidal Silver against the tested food origin isolates.

Figures 1 to 7 shows the MIC values for individual type of food origin isolates tested against Nano Colloidal Silver. They exert different range of sensitivity against Nano Colloidal Silver. In Figure 1, LM22 strain shows significantly different MIC value from LM3 and LM7 strains. LM7 strain showed the highest MIC value of Nano Colloidal Silver that is 41.67 ppm while LM22 strain showed the lowest MIC value of Nano Colloidal Silver that is 5.21 ppm. S8 and S11 strains show significantly different MIC value from S7, S9 and S10 strains as shown in Figure 2. S 8 strain showed the highest MIC value of Nano Colloidal Silver that is 50.00 ppm while S 9 strain showed the lowest MIC value of Nano Colloidal Silver that is 10.42 ppm.

From Figure 3, it is shown that from all *Staphylococcus aureus* strains used, SA2 strain has significantly different MIC value from SA25 strain. These two strains shows significantly different MIC values from other strains used. SA25 strain have the highest MIC value of Nano Colloidal Silver at 50.00 ppm while SA37 strain showed the lowest MIC value of Nano Colloidal Silver at 2.60 ppm. Figure 4 shows that all *Bacillus cereus* strains used in this study do not have significant different MIC values among the strains tested. BC223 and BC224 strains have the highest MIC values of Nano Colloidal Silver at 16.67 ppm while BC122 strain showed the lowest MIC value of Nano Colloidal Silver at 5.21 ppm. Figure 5 shows B3 and B8 strain for *E. coli* O157:H7 do have significantly different MIC value between B1 and B 6 strain. It also shows B8 strain has the highest MIC value of Nano Colloidal Silver at 25.00 ppm while B1 strain showed the lowest MIC value of Nano Colloidal Silver at 1.30 ppm.

Table 1. MIC and MBC breakpoints for 7 types of food origin isolates

Isolates	Gram	MIC (ppm)	MBC (ppm)
Listeria monocytogenes	-	24.58	> 100
Salmonella Typhi	-	23.75	> 100
Staphylococcus aureus	+	13.85	> 100
Bacillus cereus	+	10.63	> 100
E. coli O157:H7	-	12.43	> 100
Vibrio parahaemolyticus	-	9.64	> 100
Vibrio cholerae	-	7.71	> 100

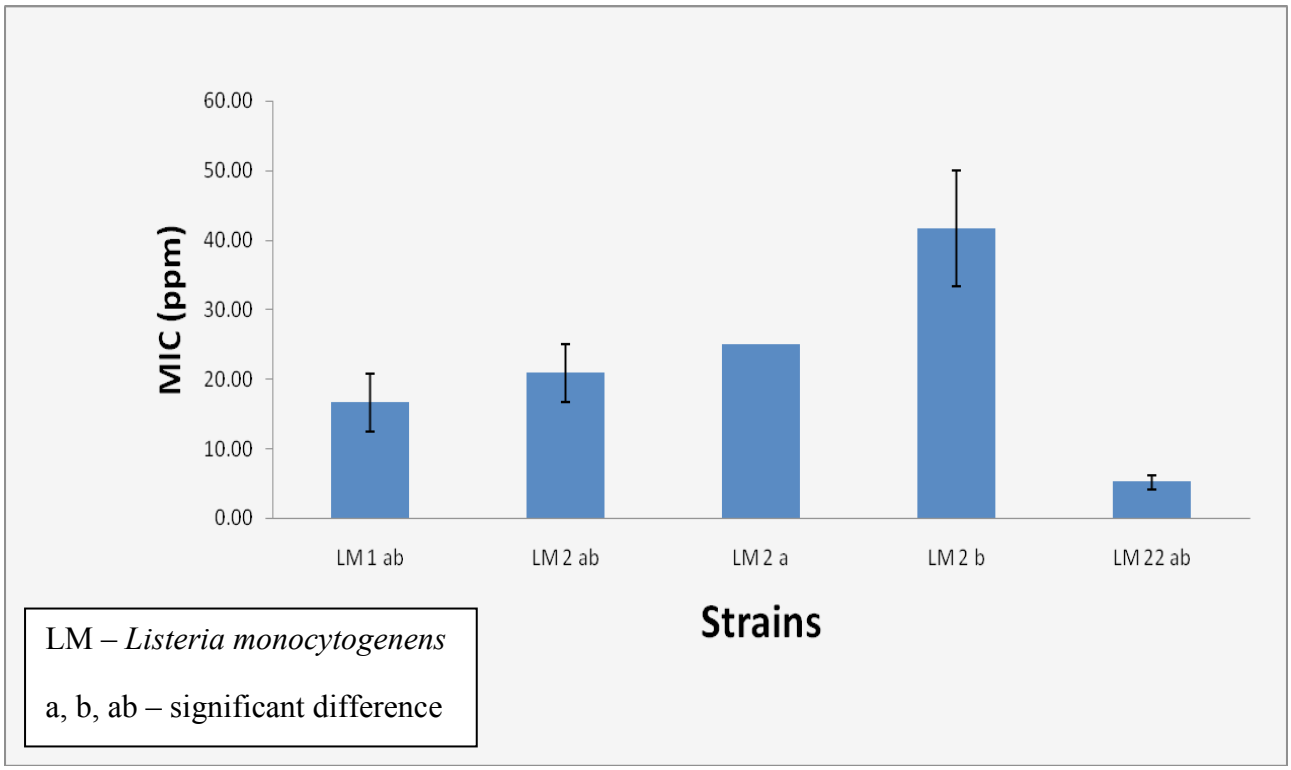

Figure 1. MIC of Nano Colloidal Silver towards *Listeria monocytogenes*. The data bars represented in average of triplicates for each strains used (LM1, LM2, LM3, LM7 and LM22) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

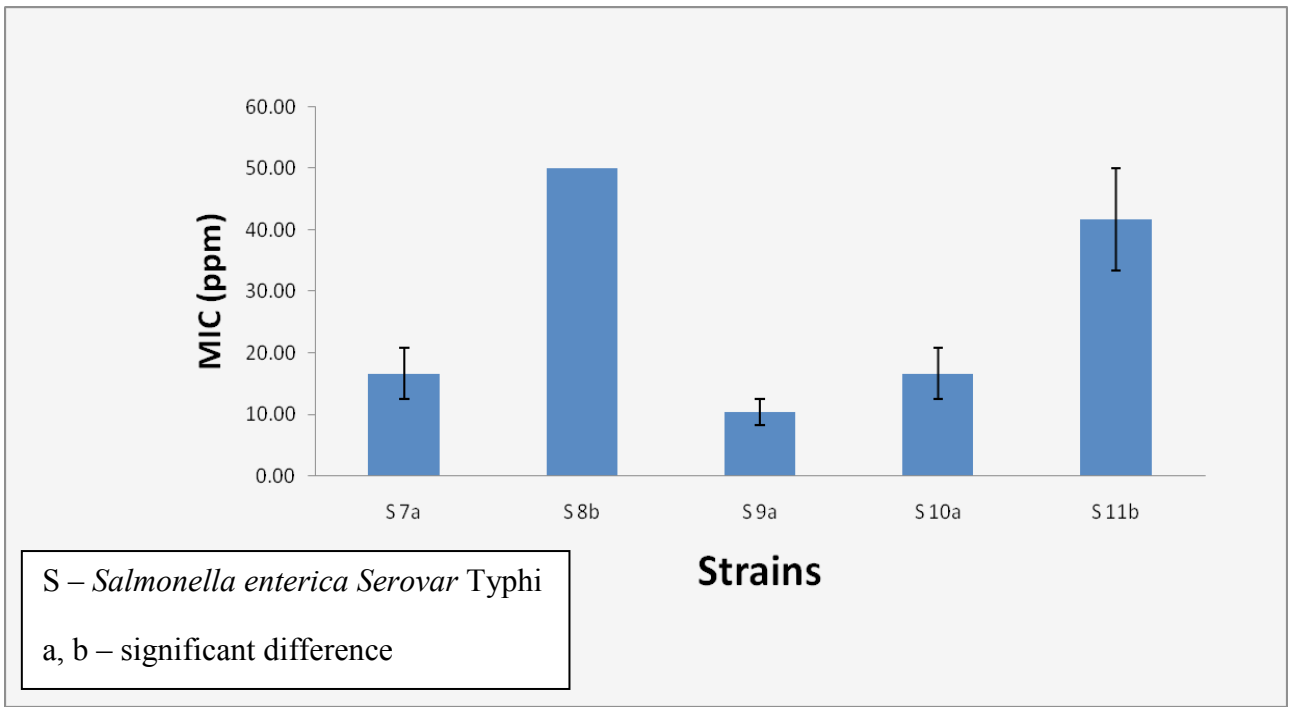

Figure 2. MIC of Nano Colloidal Silver towards *Salmonella enterica Serovar* Typhi. The data bars represented in average of triplicates for each strains used (S7, S8, S9, S10 and S11) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

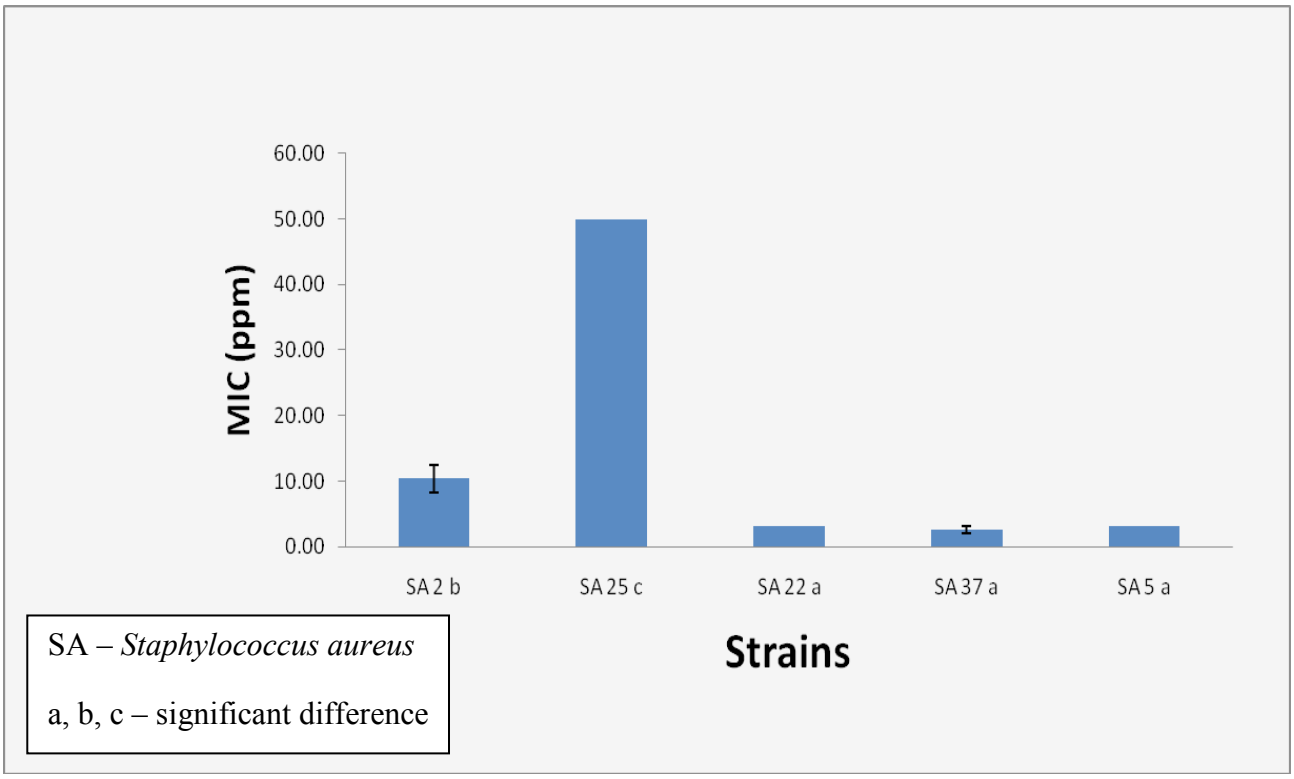

Figure 3. MIC of Nano Colloidal Silver towards *Staphylococcus aureus*. The data bars represented in average of triplicates for each strains used (SA2, SA25, SA22, SA37 and SA5) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

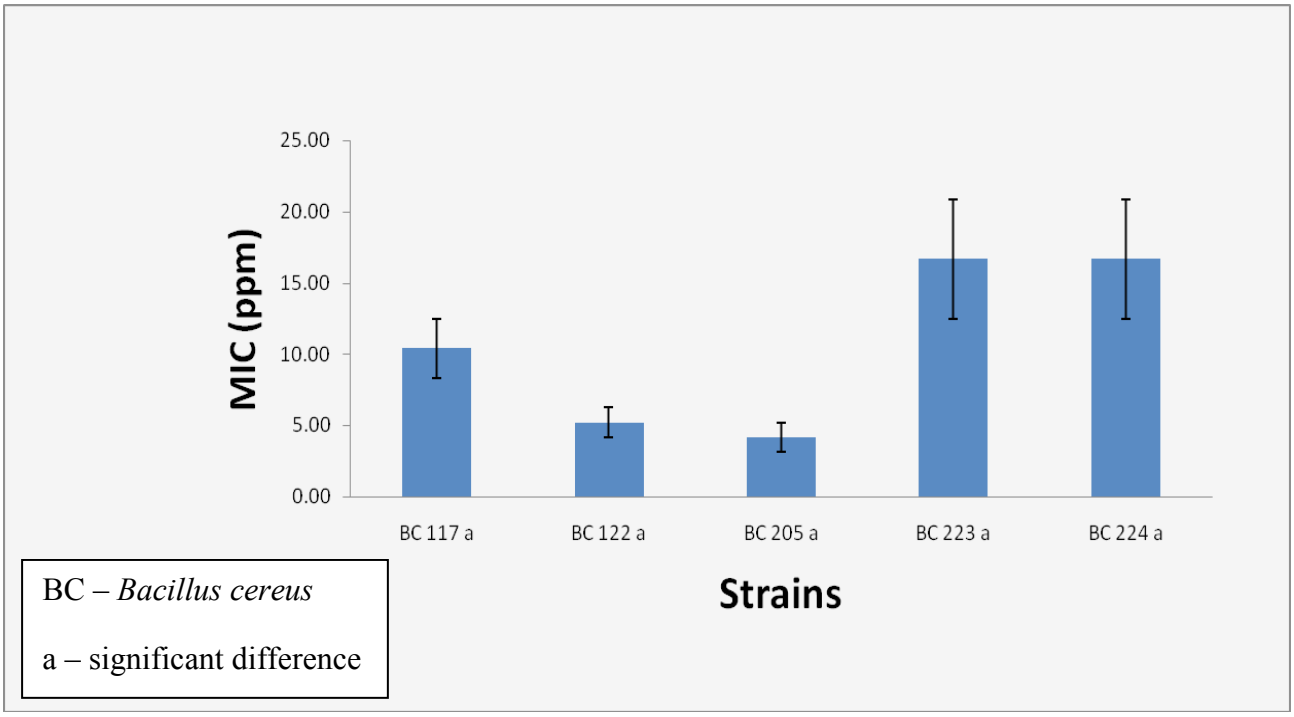

Figure 4. MIC of Nano Colloidal Silver towards *Bacillus cereus*. The data bars represented in average of triplicates for each strains used (BC117, BC122, BC205, BC223 and BC224) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

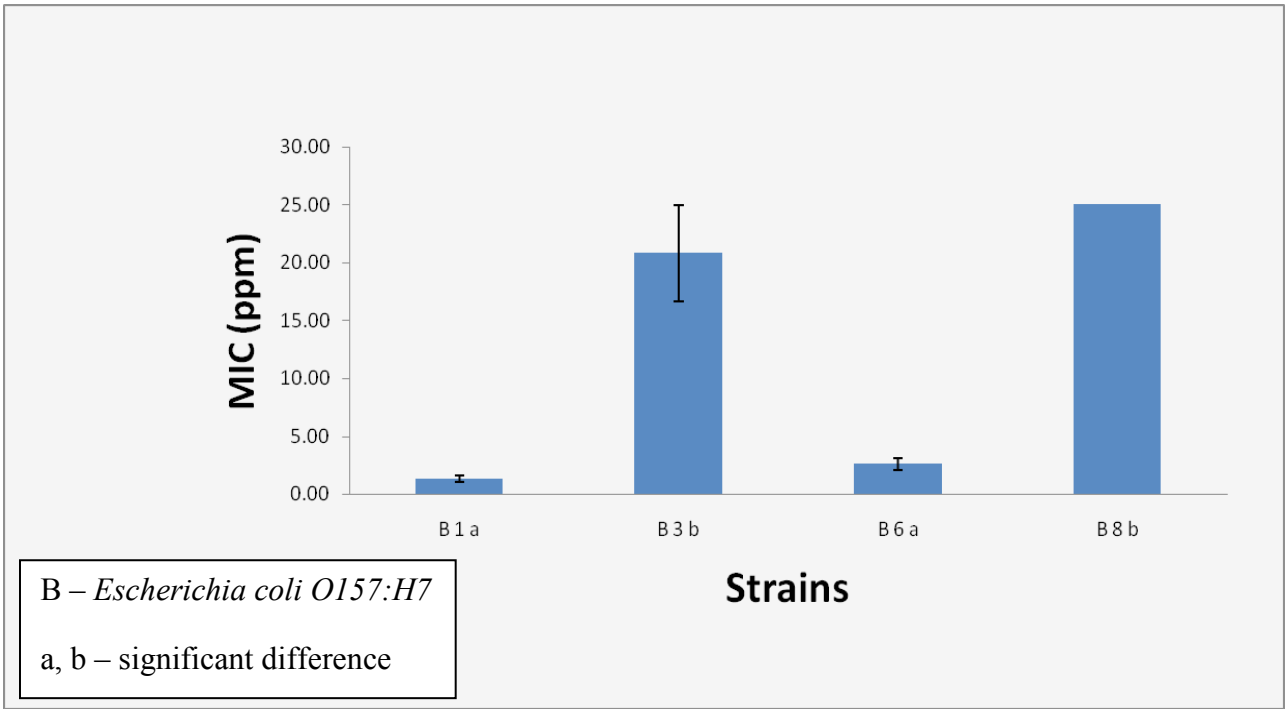

Figure 5. MIC of Nano Colloidal Silver towards *Escherichia coli* O157:H7. The data bars represented in average of triplicates for each strains used (B1, B3, B6 and B8) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

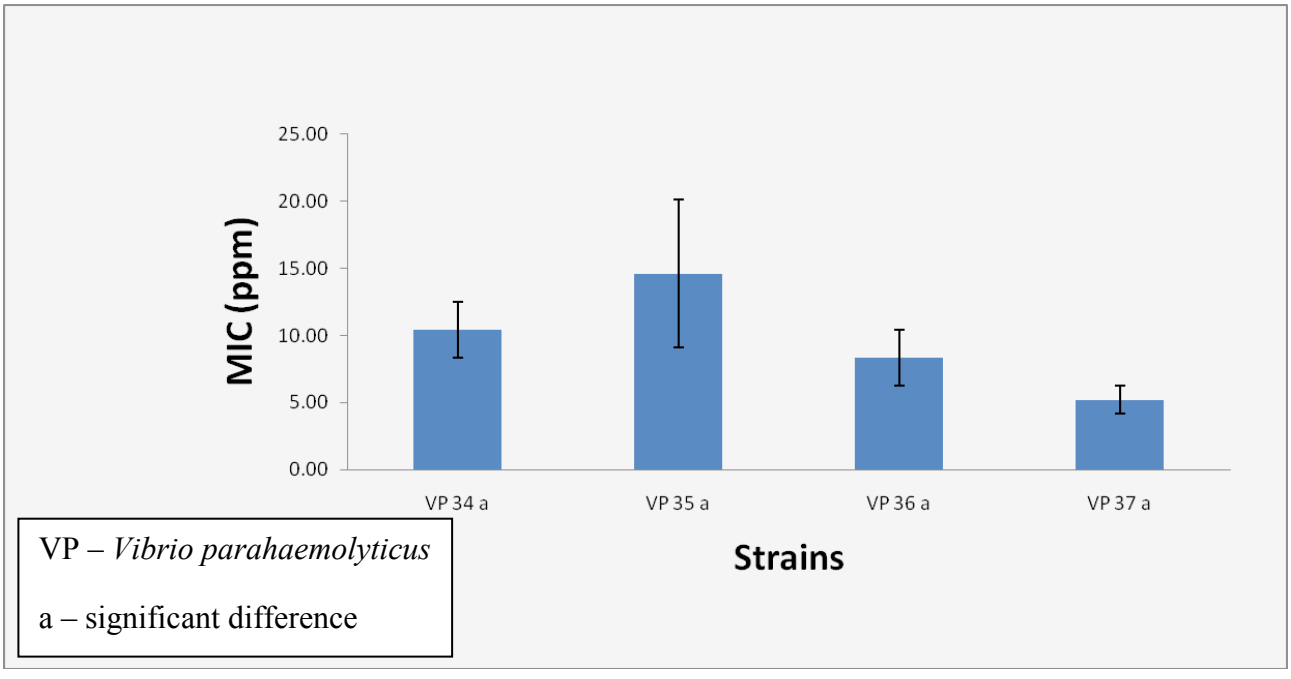

Figure 6. MIC of Nano Colloidal Silver towards *Vibrio parahaemolyticus*. The data bars represented in average of triplicates for each strains used (VP34, VP35, VP36 and VP37) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

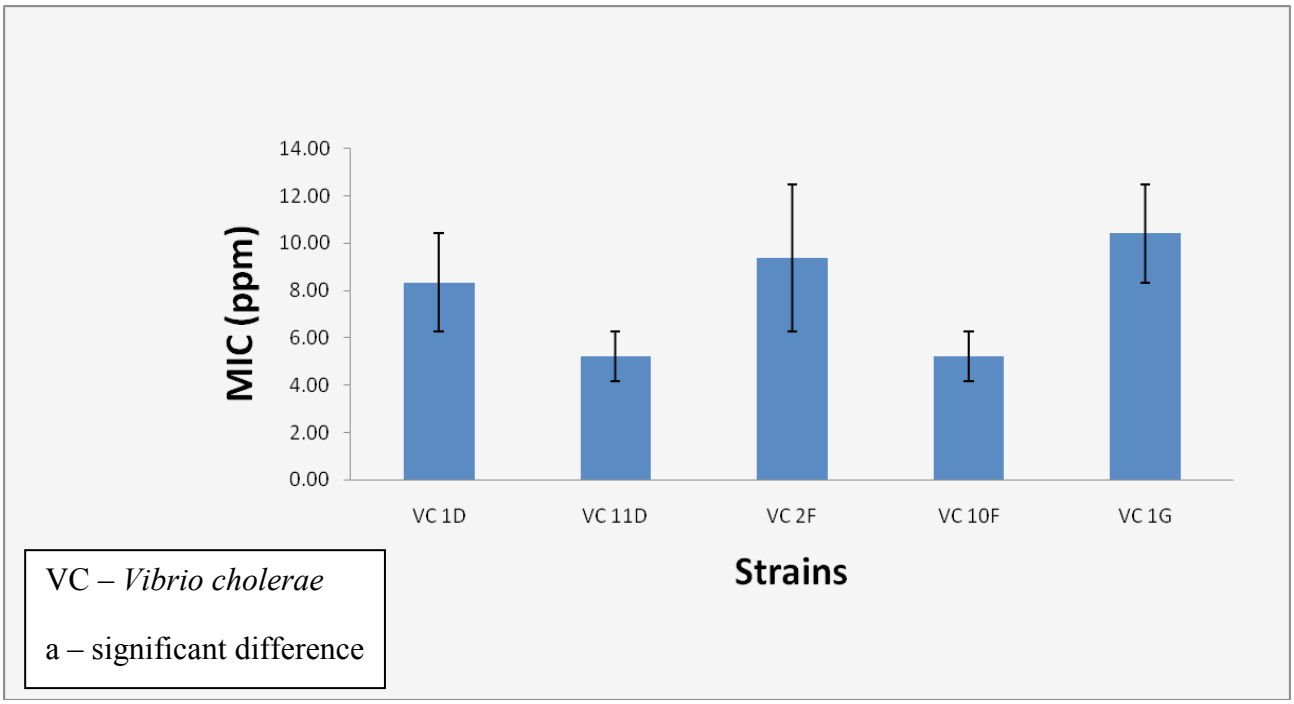

Figure 7. MIC of Nano Colloidal Silver towards *Vibrio cholerae*. The data bars represented in average of triplicates for each strains used (VC1D, VC11D, VC2F and VC1G) respsectively. MIC values indicated by different letters are significantly different P< 0.05)

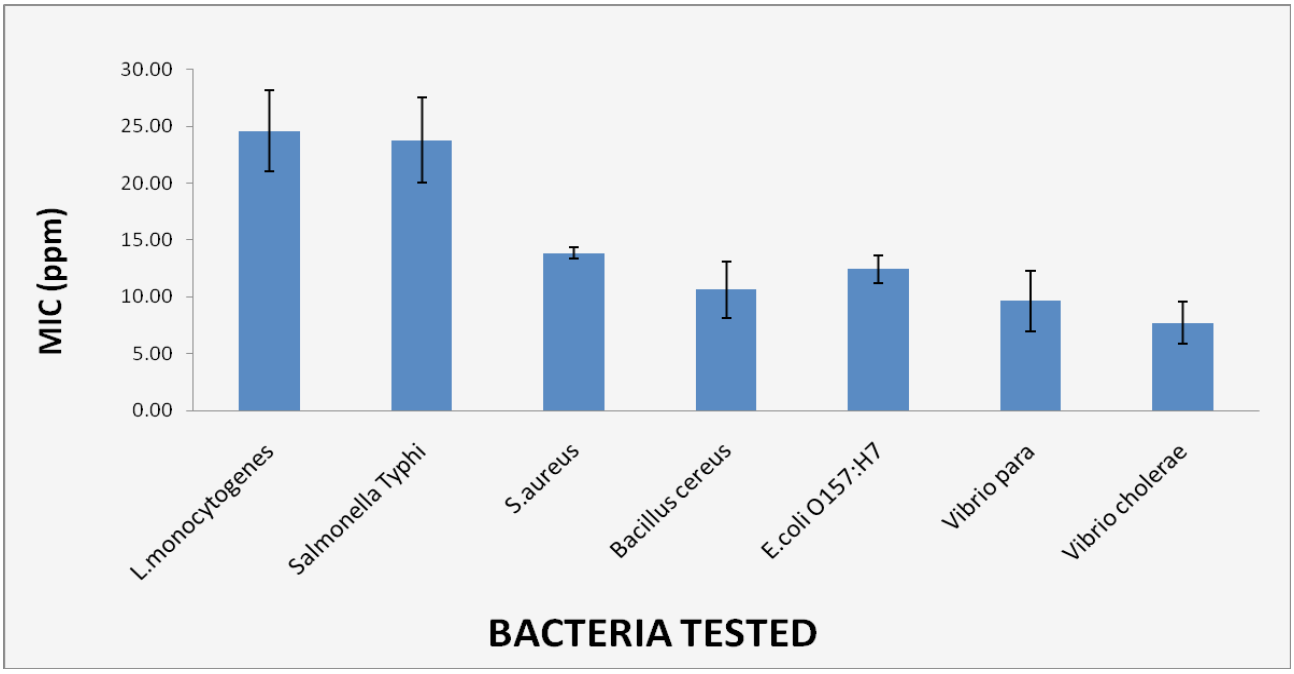

Figure 8. Antimicrobial activity of Nano Silver Colloidal towards all the food origin isolates tested. The data bars represented in average of triplicates for each types of food origin isolates used (*L. monocytogenes, Salmonella* Typhi*, S. aureus, Bacillus cereus, E. coli* O157:H7, *Vibrio parahaemolyticus* and *Vibrio cholerae*) respsectively

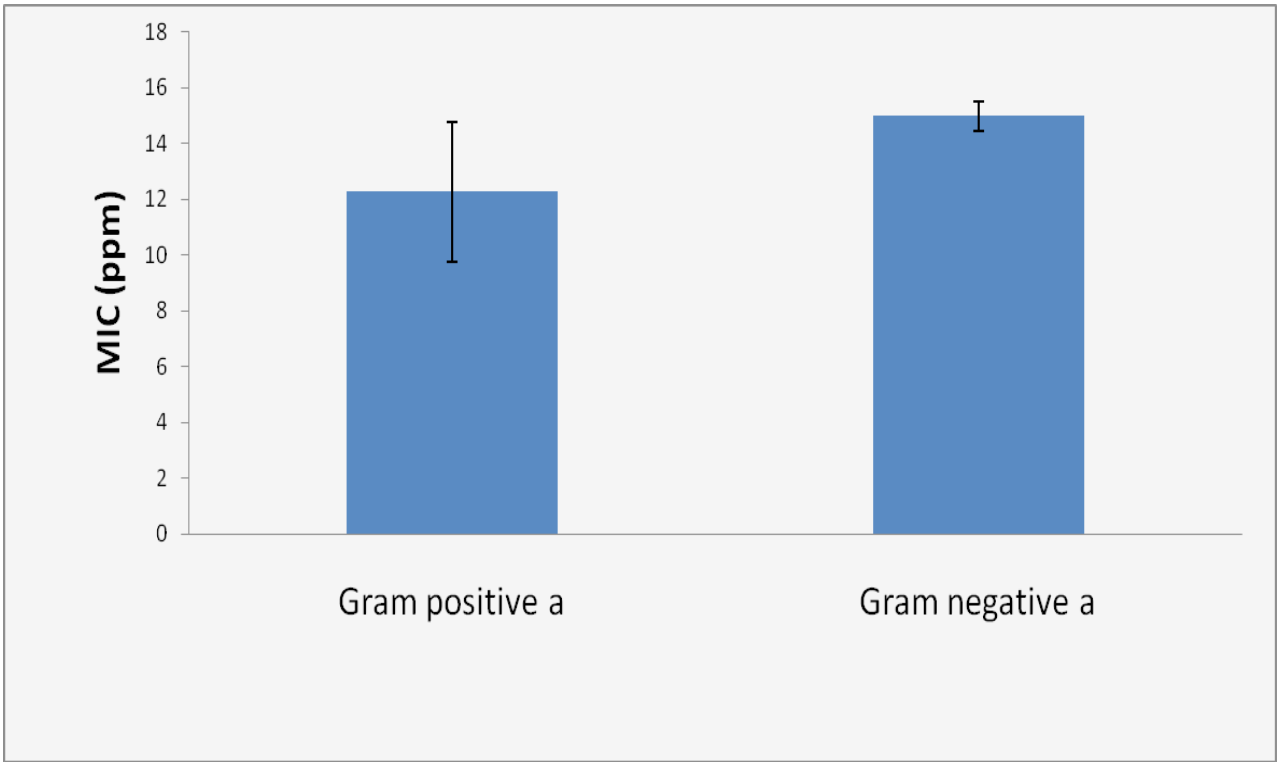

Figure 9. Antimicrobial activity of Nano Silver Colloidal towards gram negative and gram positive food origin isolates. The data bars represented in average of triplicates for each types of food origin isolates used (*B. cereus and S. aureus; L. monocytogenes, Salmonella* Typhi, *E. coli* O157:H7, Vibrio parahaemolyticus and Vibrio cholerae) for gram positive and negative respsectively. MIC values indicated by different letters are significantly different P< 0.05)

From Figure 6, all the *Vibrio parahaemolyticus* strains used do not have significant difference in their MIC values, with VP35 strain having the highest MIC value of Nano Colloidal Silver at 14.58 ppm and VP37 strain showed the lowest MIC value of Nano Colloidal Silver at 5.21 ppm. Figure 7 shows that all the *Vibrio cholerae* strains used in this study do not exert significantly different MIC values among the strains tested. VC1G strain have the highest MIC value of Nano Colloidal Silver at 10.42 ppm while VC11 D and VC10F strains were found to have the lowest MIC value of Nano Colloidal Silver at 5.21 ppm. Figure 8 shows the MIC value for all types of food origin isolates tested in this study, which shows that the MIC value for *Listeria monocytogenes* and *Salmonella enterica Serovar* Typhi were the highest while *Vibrio cholerae* was the least. Figure 9 shows the antimicrobial activity of Nano Silver Colloidal towards gram negative and gram positive food origin isolates used in this study which shows that there is no significant different between gram positive and negative bacteria tested.

These results support notion that the antibacterial activity of Ag-NPs might be their adsorption on bacterial surface. In general, Ag ions, which have antimicrobial activity, were used as an antibacterial agent. The antibacterial activity of Ag ion is inhibition of intracellular enzyme activity. Therefore, another possibility could be that the remaining Ag ions in Ag-NPs solution or dissolved Ag ions might affect on bacterial growth. Silver has an oligodynamic effect, that is, silver ions are capable of causing a bacteriostatic (growth inhibition) or even a bactericidal (antibacterial) impact.

The antibacterial form of silver is the ions. Minute sub-particles continuously emit a sufficient number of positively charged ions. These destroy the enzymes of the bacteria, destabilize the cell membrane, the cell plasma or the cell wall and prevent their reproduction. The bacteria do not survive this concentrated attack. In real, medical devices coated in silver therefore remain germ-free. The "attack points" for the silver are varied which of particular significance with this are the enzymes that are responsible for the control of the cell metabolism. These are blocked and can no longer fulfill the vital functions for the bacteria cells.

In this study, the Nano Colloidal Silver show efficient bacteriostatic property rather than bactericidal property towards all tested food origin isolates. Silver antimicrobial property compared to other salts is very high due to their extremely large surface area, which provides better contact with microorganisms. The silver nano particles in Nano Colloidal Silver get attached to the cell membrane and also penetrate inside the bacteria. The bacterial membrane contains sulfur-containing proteins and

the silver nano particles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. When silver nano particles enter the bacterial cell, it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerates thus, protecting the DNA from the silver ions. This gave reason on why the silver nano particles have a higher tendency to inhibit the growth of the bacteria and not to fully kill them.

An interesting finding by other researchers (Feng et al., 2000; Sondi and Salopek-Sondi, 2004; Morones et al., 2005; Song et al., 2006) stated that the nano particles release silver ions in the bacterial cells, which enhance their bactericidal activity. The mechanism of action of silver is linked with its interaction with thiol group compounds found in the respiratory enzymes of bacterial cells. Silver binds to the bacterial cell wall and cell membrane and inhibits the respiration process (Klasen, 2000). In the case of *E. coli*, silver acts by inhibiting the uptake of phosphate and releasing phosphate, mannitol, succinate, proline and glutamine from *E. coli* cells (Rosenkranz and Carr, 1972; Haefili et al., 1984; Yamanaka et al., 2005).

The mechanism for the antimicrobial action of silver ions is not properly understood, however, the effect of silver ions on bacteria can be observed by the structural and morphological changes. It is suggested that when DNA molecules are in relaxed state the replication of DNA can be effectively conducted. But when the DNA is in condensed form it loses its replication ability hence, when the silver ions penetrate inside the bacterial cell the DNA molecule turns into condensed form and loses its replication ability leading to cell death. Also, it has been reported that heavy metals react with proteins by getting attached with the thiol group and the proteins get inactivated (Liau et al., 1997; Feng et al., 2000). Thus, further studies need to be done to investigate more on the bactericidal property of Nano Colloidal Silver towards bacteria causing harm to humans.

In this study, it is also observed that Nano Colloidal Silver do not have significantly different antimicrobial activity towards Gram positive and negative bacteria. *Bacillus cereus* and *Staphylococcus aureus* are the two Gram positive bacteria strains used in this study. Silver nano particles act primarily in three ways against Gram-negative bacteria (Curtis et al., 2001). Silver nano particles mainly in the range of 1-10 nm attach to the surface of the cell membrane and drastically disturb its proper function, like permeability and respiration; (Waren et al., 1998). Nano particles release silver ions, which have an additional contribution to the antimicrobial effect of the silver nano particles (Feng et al., 2000). Although bacterial cell lysis could be one of the reasons for the observed antibacterial property, nano particles also modulate the phosphotyrosine profile of putative bacterial peptides, which could thus affect bacterial signal transduction and inhibit the growth of the organisms. The effect is dose dependent and is more pronounced against Gram negative organisms than Gram-positive ones. Overall, there is consensus that surface binding and damage to membrane function are the most important mechanisms for the inhibition of bacteria by AgNPs (Clement et al., 1994).

The results of this study strongly suggest that Nano Colloidal Silver demonstrate the ability to inhibit growth of MRSA with significantly lower MIC value. MRSA can be fatal and cause serious infections even when treated with antibiotic. These results indicate Nano Colloidal Silver demonstrate a good bacteriostatic effect towards MRSA strains tested. This may also be applied in treating wounds by reducing the bacterial infection wound healing improves by approximately three times and reduces pain in the process. This reduction in pain and improvement to healing can be attributed to the fact that infection, inflammation, and tissue damage are reduced when using Nano Colloidal Silver. This is also supported by George et al. (1997) where silver sulfadiazine and chlorhexidine (Silvazine) were proven to be effective against MRSA in vitro when tested against 50 strains of MRSA.

There is an need to investigate more on the antimicrobial activity for these food origin isolates that are causing problems to human to study the morphology changes in order to understand the reaction mechanism of Ag NPs, not only on Nano Colloidal Silver. Further studies are highly recommended to study the variation of antimicrobial activity exert by Ag NPs toward Gram positive and Gram negative bacteria as the number of these bacteria used in present study do not equivalent.

Conclusion

The results of the minimum inhibitory concentration and minimum bactericidal concentration of Nano Colloidal Silver against *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica Serovar* Typhi, *Vibrio cholerae, Vibrio parahaemolyticus, Bacillus cereus* and *Staphylococcus aureus* indicated that Nano Colloidal Silver's antibacterial effect is independent of acquisition of resistance by the bacteria against antibiotics and thus be a potential antimicrobial agent to be used. However, further studies are

highly recommended to be conducted to verify if the bacteria may develop resistance towards the nano silver particles and to examine cytotoxicity of nano particles towards human cells before proposing their potential as antimicrobial agent to be used in the food industry.

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