Summary and Conclusion
Silver nanoparticles have emerged as an arch product from the field of Nanotechnology.

Green synthesis of AgNP’s is blossoming field of research and it is already well established that living organisms have huge potential for the production of nanoparticles with wide applications.

Use of organisms for AgNP’s with desired shape and size can be produced from simple bacteria to highly complex eukaryotes.

Amongst various microorganisms, probiotic bacteria (LAB) which are GRAS and have high therapeutic values and found abundantly in various dairy products. Thus, to isolate LAB from different samples of dairy products such as milk, yogurt, curd, Khoa and paneer were brought to the lab aseptically, from various local vendors and retail shops from Gulbarga region. Further, the isolates were checked for their ability to mediate AgNP’s synthesis.

Based on visual inspection, Uv-vis spectroscopy, SEM, XRD, AES and EDX analysis the potential LAB was screened and selected amongst the tested isolates.

SEM photographs of isolate VRS-2, VRS-4 and VRS-S, revealed, the presence of AgNP’s embedded over the contour of bacterial ceil wall. The AES analysis depicted that, VRS-4 has recovered 63% of initial AgNO₃ concentration added to the biomass complex, which was found highest, while it was 56 % and 55% for VRS-2 and VRS-5 respectively. However isolate VRS-4 produced strong peaks at 33⁰ which is specific for silver oxides, thus produced reactive silver which cannot be accepted as reduced silver. Thus LAB isolate VRS- 2 was employed for further studies.

XRD analysis was found to be apt technique for confirming the bioreduction of silver ion by LAB.

Potential LAB was identified based on 165 rDNA sequencing and phylogenetic tree construction. The 16 S rDNA sequences of isolate VRS-
2 were deposited in GenBank with Accession No. KF360255 and is similar to the 16 S rDNA sequences of *L. rhamnosus* MH22 (Acc no. FJ409226.1). Thus, based on NCBI BLASTn analysis and phylogenetic tree construction the LAB was confirmed as *Lactobacillus rhamnosus*. The species *L. rhamnosus* is regarded as most beneficial bacteria amongst other probiotic bacteria. These bacteria neither produce toxins nor other harmful bioactive compounds. They are non pathogenic and even unrelated phylogenetically to a pathogen, thus generates enormous attention in the basic research and potential applications.

Experiments conducted in an attempt, to elucidate the mechanism involved in AgNP's synthesis ruled out the involvement of whey (devoid of bacterium) and enzyme NADPH-dependent reductase in AgNP's synthesis.

TEM analysis provided some interesting evidences directing towards the involvement of cell components of bacterial biomass (*L. rhamnosus*) in reduction of silver ions. The obtained results can be deduced that, the bioreduction of ionic silver might have occurred by the bacterial cell surface itself, due to the fact that the reducing sugars of bacterial cells may function as electron donor and reduction of Ag⁺ to Ag⁰.

Further enquiry was made to elucidate the exact interaction of some cell wall components of biomass involved in AgNP synthesis. Thus, EPS was extracted from *L. rhamnosus* and subjected to reduce ionic silver.

FT-IR spectrum of EPS stabilized AgNP's revealed various characteristic peaks ranging from 3455-808cm⁻¹ indicated that the EPS could stabilize AgNP's and strong interaction of Ag with the EPS functional group was found. Thus carbohydrates could most possibly form a coat covering the AgNP’s to prevent agglomeration and stabilization of the particles in the medium.
TEM analysis showed the presence of EPS on the outer surface of *L. rhamnosus*. It is also evident from previous reports, that high concentration of polysaccharide is present over the cell wall of *r. rhamnosus*. A TEM picture clearly reveals the interesting feature of *L. rhamnosus* i.e. a low density, highly extended soft outer layer is detected. TEM image clearly shows that, the AgNP’s were spherical and hexagonal in shape ranging from 5-16nm.

AFM analysis revealed mono dispersed spherical shaped smooth textured AgNP’s.

Uv-vis spectroscopy, XRD spectrum, FT-IR, TEM and AFM analysis cleared depicted that the EPS from *L. rhamnosus* could reduce ionic silver to form AgNP’s.

A drastic shift in the size of AgNP’s from 10-90 nm (*L. rhamnosus* biomass synthesized AgNP’s) to 5-16 nm (EPS stabilized AgNP’s) was observed.

The swift in the size of AgNP’s was explained hypothetically i.e. after interaction of ionic Ag with EPS layer which is the outermost layer, the Ag get reduced and stabilized. Further, other anionic surface components of cell wall (Thick layer of peptidoglycan, teichoic acids, lipoteichoic acids, and proteins) involve in bioreduction of ionic silver. This leads to biosorption followed by accumulation of Ag⁰ over the cell wall, thus forming varying size of silver crystals.

The abundance of polysaccharide and other anionic compounds present on the outer surface of cell wall is expected to determine the surface properties of microorganism and influence the size of the AgNP’s.

In case of EPS stabilized AgNP’s (5-16 nm), which is passive biosorption; we propose a mechanism where, anionic charge present over the EPS has affinity to bind Ag⁺ and form Ag⁰. At certain stage the phenomenon comes to equilibrium and no more charge will be available to further reduce the ionic silver in the reaction mixture. Thus, the size of the
AgNP’s remains the same, adsorbed and entrapped in the EPS without agglomeration and accumulation.

Whey produced during the manufacturing of paneer was employed for culturing of *L. rhamnosus* to make the process economical. Maximum biomass production of 1.3 g/L was observed at pH 6.0 followed by pH 4.0 (0.99 g/L) in whey medium. Increase in pH decreased the biomass production.

Of the different temperature’s 25-45°C studied for biomass production, 35°C was found optimum for maximum biomass production of 1.5 g/L in whey medium. Further increase in temperature decreased the biomass production.

Maximum production of biomass was obtained in MRS medium (1.9 g/L) compared to whey medium (1.5 g/L). Although there was marginal increment of 0.3g/L of biomass was found between MRS and whey, but whey as medium can be considered economical attractive-alternate and cheaper medium replacing costly MRS medium.

AES analysis revealed 136 mg/g of adsorption of Ag on biomass of *L. rhamnosus* at pH 10.0 and least adsorption of 17 mg/g was at pH 2.0, while it was 73.6 mg/g at pH 4.0 and 94 mg/g at pH 6.0 were found intermediate. There was not much noticeable difference in AgNP’s recovery between pH 8.0 (135 mg/g) and 10.0 but at pH10.0 silver reduced and precipitated within 5 hrs, whereas pH g.0 precipitated Ag after 24 hrs. When pH increased both recovery and reduction rates increased.

No detectable nanoparticles were biosorbed over the cell wall at pH 2.0. At pH 4.0 AgNP’s ranging from 35 to 89nm. At pH 10.0 smallest AgNP sizes ranging from 5 to 16 nm were entrapped over the bacterial cell wall are revealed. Thus pH was found critical in Ag ion reduction.

AES analysis revealed maximum biosorption of Ag was obtained at 35°C (137 mg/g) with 5-16 nm size of AgNP’s, followed by 30°C (136.4 mg/g)
where as at 45°C the size increased to 11-55 nm size of AgNP's. Thus increase in temperature was inversely proportional to the absorption of Ag on biomass.

Based on AES analysis highest recovery of AgNP's (136.9 mg/g biomass) was obtained from 11mM of initial concentration of AgNO₃ followed by 10 mM and 9 mM with 136.5 mg/g and 136 mg /g biomass respectively. While, it was 115 mg and 102 mg recovery Ag/g biomass at 8mM and 7 mM concentration. The process was found economical only at 9mM concentration of AgNO₃ and sharp peak of Uv-Vis absorption was observed.

The use of probiotic bacteria, *L. rhamnosus* which is GRAS makes the process of AgNP's synthesis a non-toxin, eco-friendly and economical.

The highest zone of inhibition with 50 µL of AgNP₇ (1mg/ml conc. of AgNP₇ i.e. ~135 µg AgNP's/ml) was observed against *C. albicans* (21 mm) and *Pseudomonas aeruginosa* (20 mm). While for ESBL producing *E. coli, Salmonella typhimurium* and MR-VRSA the zone of inhibition was 19mm, l8 mm and 17mm respectively.

The antibiotic resistance pattern for clinical isolates of *E. coli* strain 566 was phenotypically confirmed as ESBL positive based on double disc diffusion test and was resistant to all tested antibiotics except chloramphenicol while *S. auerus* strain 827 was also resistant to all antibiotics except amikacin and was affirmed as MR and VRSA.

The MBC of AgNP₇ was evaluated against MDR *E. coli* and *S. auerus*. AgNP₇ at concentrations 1.50 mg or ~202 µg AgNP/100 mL and 1.75 mg or ~236 µg AgNP/ 100 mL and higher were found effective bactericides for resistant *E. coli* and *S. auerus* respectively.

Cytotoxic effects of varying concentration of AgNP₇ against HeLa cells cultured reveal that the inhibitory Concentration (IC₅₀) value of AgNP₇ was 12.5 mg/100 mL which was toxic to cell lines, while the MBC of AgNP₇ for *E. coli* and *S. aureus* 1.5 and 1.75 mg/ 100mL respectively.
where, these concentrations of AgNP were found nontoxic and thus confirmed its biocompatibility.

The major hypothesis proposed form our studies focused that if oral dose of AgNP (nontoxic concentrations) given to treat the patient suffering from gastroenteritis caused by enteric pathogens, it does not lets the cell wall bounded nano silver into the blood stream, otherwise lead to accumulation of AgNP’s in various organ systems. Any research in this direction is encouraging.

Orthopaedic PMMA bone cement loaded with 1% EPS stabilized AgNP's and 5% vancomycin each were evaluated for antibiofilm activity against strong biofilm forming S. auerus strain g27.

Amongst the three types of bone cement tested, biofilms were formed over plain PMMA and PMMA with vancomycin, only PMMA discs loaded with AgNP's were devoid of biofilm growth of S. auerus strain 827.

Plain PMMA cement could not inhibit proliferation of S. auerus MTCC-96 (MS & VSSA), whereas, vancomycin loaded bone cement led to inhibition of bacterial proliferation with inhibition zone of 30 mm on MHA plates. Cement with a concentration of 1% of nanosilver also inhibited the proliferation of S. auerus MTCC-96 but with only 11 mm zone of inhibition.

Neither plain nor vancomycin loaded cement discs were found effective against s. auerus strain 827 (MR & VRSA) on MHA plates. Bone cement with of AgNP’s was effective against s. auerus strain 827 and showed 11 mm zone of inhibition on MHA plates.

Further, the viability/ non viable bacteria over the surfaces of PMMA materials was confirmed by recording the optical density after dislodge biofilm formative bacteria by vortexing the PMMA types and incubating in TSB medium for 24 hrs. Results clearly revealed the complete inhibition of bacterial proliferation in TSB medium was only found with 1% of AgNP’s loaded bone cement.
The larger zone of inhibition of PMMA loaded with vancomycin against *S. auerus* strain MTCC-96 (VSSA) when compared to AgNP’s loaded PMMA may be attributed to the diffusion of vancomycin antibiotic from the PMMA material in to the surrounding medium, whereas the strongly bounded AgNP’s to PMMA formed sterile zone within its restricted range. Moreover, no biofilm was formed over AgNP’s loaded PMMA indicating superior antibacterial activity over the surface of bone cement.

Though the zone of inhibition of PMMA with AgNP’s was less than PMMA loaded with vancomycin, antibiotic resistant *S. aureus* formed biofilm over PMMA with vancomycin and on contrary failed to establish over the AgNP’s loaded PMMA material. Thus these finding paves the way to adopting a non antimicrobial approach which may become a future technology to combat biofilm forming antibiotic resistant pathogens in orthopedic implants especially in PMMA bone cement based implants.

PMMA with AgNP’s was more radiopaque than those without nanosilver. As expected, PMMA with AgNP’s possessed increased X-ray intensity compared to PMMA alone and PMMA with vancomycin. Thus, increased radiopacity of bone cements will allow for a reduced patient’s exposure to X-rays and consequently lead to a lower incidence of cancer.

Since such rapid silver reduction rates are typically achievable only while using chemical processes, our method combines the eco-friendliness of biological AgNP’s production with the speed of chemical processes. Moreover, the chemically synthesized nanoparticles require another step for the prevention of aggregation of the particles. But, biological synthesis of AgNP’s uses harmless, eco-friendly reducing agents and the nanoparticle structure is stabilized by the biomolecules present in the environment eliminating the extra step in preventing the aggregation of chemically synthesized nanoparticles by stabilizers.

Since EPS are eco-friendly, the pollution due to chemicals and byproducts can be prevented from reaching the environment. The size
and shape of the particle can also be completely regulated by controlling the environment where the nanocrystal growth occurs (pH and temperature). Furthermore, biological methods have the greater advantage of easy bulk synthesis which can be exploited for industrial scale production too.

The association of the nanoparticles with the bacterial biomass facilitates the concentration of the nanoparticles by centrifugation and further these biocomposite can be used in various applications.

Whether AgNP's are an option to confront the transmission of infection caused by drug-resistant bacteria remains to be determined.

It is imperative that more studies be carried out in future to assess the toxic effect of nanosilver *in vivo* before a conclusion on its toxicity is reached.