GLOBAL ACADEMIC RESEARCH INSTITUTE

COLOMBO, SRI LANKA

GARI International Journal of Multidisciplinary Research

ISSN 2659-2193

Volume: 07 | Issue: 02

On 30th June 2021

http://www.research.lk

Author: S.N.T. Chamika, Punsisi Weerasooriya

School of Science, BMS, Sri Lanka

GARI Publisher | Food Microbiology | Volume: 07 | Issue: 02

Article ID: IN/GARI/ICHM/2020/161 | Pages: 106-126 (20)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 29.03.2021 | Publish: 30.06.2021

IDENTIFICATION OF LACTOBACILLUS IN COMMERCIALLY AVAILABLE SET YOGHURT SAMPLES AND THEIR APPLICATION IN SYNTHESIS OF SILVER NANOPARTICLES (AGNPS) FROM SILVER NITRATE

[1]S.N.T. Chamika, [2]Punsisi Weerasooriya

School of Science, Business Management School, Sri Lanka

[1]sntchamika1219@gmail.com, [2]punsisi@bms.edu.lk

ABSTRACT

Probiotics are nonpathogenic living microorganisms which confer health benefits on the host. Nowadays, probiotic Lactobacilli are frequently used in green chemistry approach for silver nanoparticle synthesis which is considered as an ecofriendly alternative way to other chemical and physical methods. This study was designed to investigate the biosynthesis of silver nanoparticles using Lactobacillus, isolated from commercially available set yoghurts in local market and to evaluate antibacterial and antioxidant potentials of synthesized silver nanoparticles. Five set yoghurt samples were purchased and cultured on MRS media. Thereupon Lactobacillus was phenotypically and biochemically identified using colony morphologies(on MRS agar) and biochemical tests(Gram staining, acid-fast staining, endospore staining and catalase test), respectively. Then Lactobacillus mediated silver nanoparticle synthesis from silver nitrate was carried out by sunlight irradiation. Nanoparticles obtained were detected by means of UV-visible spectroscopy. Afterwards, DPPH(1,1- diphenyl-2-picrylhydrazine) radical scavenging assay and agar well diffusion assay were carried out to determine antioxidant and antibacterial activity of the synthesized silver nanoparticles. As results, mucoid white colonies with entire margins were observed in all samples. Bacterial colonies from all the samples showed positive results for Gram staining, negative results for acid fast staining, endospore staining and for catalase test. UV-visible absorption spectrum for each sample showed absorbance maximum between 390nm-470nm, which is a characteristic of surface plasmon resonance of silver. In DPPH assay, silver nanoparticles showed significantly high($p<0.05$) antioxidant activity (85.20%±2.6354) compared to Lactobacillus. In agar well diffusion assay, silver nanoparticles displayed considerable antibacterial activity(25.333±0.577mm for Escherichia coli and 21.333±0.577mm for Staphylococcus aureus) whereas Lactobacillus did not show any antagonistic effect against Escherichia coli and Staphylococcus aureus. Considering the findings of this study, it can be concluded that Lactobacillus can be used effectively in the production of silver nanoparticles which consist of antioxidant and antibacterial activity, for commercial and medical applications.

Key words: Lactobacillus, Silver nanoparticles, Antioxidant, Antibacterial

INTRODUCTION

Probiotics

Probiotics are living microorganisms which confer health benefits on the host, when administered in adequate amounts. Probiotics were discovered by Elie Metchnikoff in 1908 and considered as nonpathogenic microorganisms(eg-: Lactobacillus, Bifidobacterium) including some commensal bacterial flora(Morelli and Capurso, 2012). Generally, found in carbohydrate rich environments such as in fermented foods, cosmetic products and in dietary supplements. A study done by Islam(2016), has shown beneficial effects of probiotics on children with acute infectious diarrhea. Furthermore, Goderska, Pena and Alarcon(2018) have demonstrated probiotics induced protective mechanisms of the small intestine mucosal cells, as the basis of using antibiotics combined with probiotics to treat Helicobacter pylori infection. In addition, Abraham and Quigley(2017) have indicated therapeutic applications of probiotics in ulcerative colitis and Chrohn's disease.

Moreover, normalization and colonization of intestinal microbial flora, competitive prohibition of pathogens, bacteriocin production, metabolization of biliary salts, deactivation of xenobiotics, synthesis of short-chain fatty acids(SCFAs) which intern produce wide range of effects; modulating insulin sensitivity, mucin production, cell adhesion and immune modulation are the reported several effects of probiotics on human health(Markowiak and Slizewska, 2017). However, the exact mechanisms of action of probiotics are poorly understood yet.

Characteristics of Lactobacillus

Lactobacillus is a group of rod-shaped, Gram positive, oxidase and catalase negative, non-sporulating, facultative anaerobic lactic acid bacteria(LAB) which is generally recognized as safe(GRAS) (eg- : Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp. bulgaricus) (Angmo, Kumari and Bhalla, 2016). Generally, found in fermented foods, plants and in the normal flora of the human. Lactobacillus is with fermentation ability which responsible for a diversification in the texture and flavor of fermented foods(Han et al., 2016). According to Basso et al.(2014), based on the end product of glucose fermentation, two types of Lactobacilli are present; homofermenters and heterofermenters. Homofermentative Lactobacilli ferment carbohydrates predominantly into lactic acid whereas heterofermentative Lactobacilli ferment carbohydrates predominantly into lactic acid, acetic acid and carbon dioxide.

Recently, several studies have reported that Lactobacillus can exhibit antibacterial activity through different mechanisms including production of anti-microbial metabolites(eg-: organic acids, hydrogen peroxide, bacteriocins), competition with harmful bacteria either for nutrients or for adhesion receptors(Islam et al., 2020). Apart from that Lactobacillus is known to have antioxidant activity due to its extracellular metabolites. Thus, minimize the oxidative stress and play a crucial role in the treatment of free-radicals induced diseases such as cancers.

AgNPs Synthesis

Nowadays, AgNPs are synthesized by various methods namely, chemical methods, physical methods and biosynthesis. Polyol process and precursor injection are the most commonly used chemical methods for AgNPs synthesis(Lin and Cheng, 2020; Nguyen, Tran and Nguyen, 2020). According to Goudarzi et al.(2016), physical methods including condensation, evaporation,

thermal-decomposition are time consuming, require high energy and not economical.

In green synthesis of AgNPs, precursor is reduced by natural products which obtained from living organisms(eg-: bacteria, plant extracts) at room temperature. Jamkhande et al.(2019), has investigated that the plant extracts have low synthesis rate than bacteria. In present study, Lactobacillus has used as biofactories for AgNPs synthesis. According to Siddiqui et al.(2018) Lactobacillus is a best candidate for nanoparticle synthesis due to its ability to reduce heavy metal ions. However, the mechanisms involved in Lactobacillus mediated AgNPs synthesis are not elucidated yet. Majority of literature indicates biosynthesis as a highly economical, low energy consuming, efficient, high yield harvesting and highly reproducible simplest method for AgNPs synthesis(Khan et al., 2018 ; Palomo and Filice, 2016). Apart from that, biosynthesis of AgNPs considered as an eco-friendly approach, due to ability of bacteria to enzymatically degrade hazardious byproducts(Shams, Pourseyedi and Rafsanjani, 2014).

Characteristics and Applications of AgNPs

AgNPs are nanoscale (less than 100nm in size) metallic silver particles which generally contain 20 -15000 silver atoms. Because of the smallest size, surface area: volume ratios of AgNPs are extremely high. Depending on the method which used to synthesized AgNPs, size and shape can be vary (Anandalakshmi, Venugobal and Ramasamy, 2016). Most frequently AgNPs are spherical in shape. Furthermore, AgNPs are chemically stable metallic nanoparticles with catalytic activity and high electrical conductivity(Palomo and Filice, 2016). Moreover, Fahmy et al.(2017), have introduced localized surface plasmon resonance(LSPR) as a most important optical property of AgNPs.

Antifungal, antiviral, anti-inflammatory, anti-angiogenic, antibacterial and antioxidant properties are some functional properties of AgNPs which currently used in medical field(Burdusel et al., 2018). Furthermore, Foldbjerg, Dang and Autrup (2011), reported about dose-dependent cytotoxicity of AgNPs in human lung cancer cells. Moreover, Acharya et al.(2020), reported that the influence of AgNPs on seed germination of water melon and corn in agricultural field.

Antibacterial and Antioxidant Potentials of AgNPs

Scientists have proposed several mechanisms through which AgNPs exert their antibacterial activity; adhesion of AgNPs onto the bacterial cell wall via bioactive molecules that are present on the surface of AgNPs, which then damage the genetic material, thus ultimately causing bacterial cell death(Duran et al., 2016; Keshari et al., 2018; Gurunathan, 2015). Nevertheless, according to Dakal et al.(2016), Ag+ ions interact with the sulphur or phosphorus containing proteins in bacterial cell wall and then convert it to a nonfunctioning cell wall, which may ultimately cause bacterial cell lysis.

In addition, alteration of permeability of cell membrane, generation of free radicals responsible for the damage of cell membrane, dissipation of the proton motive force resulting in the collapse of the membrane potential, are the other proposed mechanisms for antibacterial activity of AgNPs(Dasgupta and Ramalingam, 2016). However, the exact mechanism has not been fully deciphered. Nowadays, research are focusing on the development of broad-spectrum novel antibiotics to overcome multidrug

resistance using AgNPs(Beretta and Cavalieri, 2016). Furthermore, Kumar et al.(2018), have proved that the antibacterial potential of AgNPs have been used in wound healing.

Bedlovicova et al.(2020), documented that the presence of functional groups on AgNP surface that are with redox properties which allow AgNPs to act as reducing agents, hydrogen donors, and singlet oxygen quenchers may directly contribute to antioxidant action. In addition, study done by Gajbhiye and Sakharwade (2016), has demonstrated about the ability of AgNPs to neutralize reactive oxygen species and thereby protect skin cells from oxidative damage which intern reduce pigmentation and wrinkles. Hence AgNPs are considered to have economical value in cosmetic industry.

Scope and Significance of the study

According to the literature, there is no sufficient evidence about antibacterial and antioxidant potentials of AgNPs synthesized by Lactobacillus isolated from Sri Lankan set yoghurts. Therefore, this is the first study in Sri Lanka that investigates both antibacterial and antioxidant potentials of green synthesized AgNPs which were synthesized by Lactobacillus isolated from set yoghurt samples. The main aim of this study was to investigate the biosynthesis of AgNPs using Lactobacillus which is found in commercial set yoghurts and to evaluate antibacterial and antioxidant potentials of synthesized AgNPs. Apart from that, this study was designed to culture Lactobacilli which found in set yoghurts on MRS(De Man, Rogosa and Sharpe) agar, to identify Lactobacilli using biochemical tests(Gram staining, acid-fast staining, endospore staining and catalase test), to synthesize AgNPs from isolated Lactobacilli via sunlight irradiation, to measure the absorbance of biosynthesized AgNPs using spectrophotometry, to assess antibacterial potential of AgNPs using agar well diffusion assay and to assess antioxidant potential of AgNPs using DPPH (1,1- diphenyl-2-picrylhydrazine) radical scavenging assay. Majority of literature reported about various applications of AgNPs and benefits of Lactobacillus in green synthesis of AgNPs as an eco-friendly alternative way to other methods. Considering all aforementioned facts it can be well perceived that the antibacterial and antioxidant properties of AgNPs are not only with of industrial significance, but also of medical significance in nanomedicine field.

Materials

Reagents

Absolute ethanol(VNR Chemicals), Distilled water, Immersion oil, Gram's iodine(DAYTONA), Gram's decolorizer(DAYTONA), Crystal violet(DAYTONA), Safranin(DAYTONA), Malachite green(DAYTONA), Carbol fuchsin(DAYTONA), Methylene blue(DAYTONA), AgNO3(0.5M – ResearchLab) , DPPH(ALDRICH), Gentamicin(ROTH), MRS(De Man, Rogosa and Sharpe) agar(HIMEDIA), MH(Mueller Hinton) agar(HIMEDIA), MRS broth(HIMEDIA), MH broth(HIMEDIA), BaCl2(LOBA CHEMIE), Absolute H2SO4(VNR Chemicals), Absolute HCl(VNR Chemicals), H2O2(30%-VNR Chemicals)

METHODOLOGY

All the tests were carried out under aseptic conditions.

Sample Collection

Commercially available set yoghurt products of five different brands(A,B,C,D and E), were purchased from local markets in Sri Lanka. In order to obtain highest viability of probiotics from each yoghurt sample, shelf life and the quality of probiotic yoghurts were examined using manufacture dates and expiration dates while purchasing. Apart from that plain yoghurts without any flavoring agents and with the least amount of extra ingredients were selected from each brand. Immediately after purchase, the samples were refrigerated until use.

Sample preparation and homogenization

Initially, 10.0g of each set yoghurt sample was transferred aseptically into 10mL of saline and homogenized thoroughly, until obtained an uniform thickness throughout the sample.

Culturing the samples on MRS agar

First of all, 20mL of prepared MRS media was poured into an each petri plate and allowed to get solidified. Afterwards, the samples were cultured on MRS media using quadrant streaking method. All the cultures were incubated for 48hrs at 370C in the incubator. After 48hrs incubation, individual colonies of Lactobacillus were identified by their phenotypic appearance and observations(colony size, shape and color) were recorded.

Morphological and biochemical identification of isolates

Morphology of the colonies were examined. And colonies were biochemically identified using Gram staining, acid fast staining, endospore staining and catalase test.

Preparation of a bacterial smear

First, a drop of distilled water was placed on the each slide. Next, isolated bacterial colony was transferred from MRS media to the slide and was mixed with water droplet. The smear was air dried and heat fixed(without exposing the dried film directly to the flame). These bacterial smears were subjected to downstream staining procedures.

Gram staining

Initially, bacterial smears were flooded with crystal violet for 1 minute and followed by washing the slides with water. Then the smears were flooded with Gram's iodine for about 1 minute. Once again the slides were washed. Afterwards, few drops of Gram's decolorizer was added onto the smears for 10 seconds and then the slides were washed. Next, the smears were flooded with safranin for 30 seconds and the slides were washed. Finally, smears were air dried and examined under 100× magnification (Nagoba and Pichare, 2012). Observations were recorded.

Catalase test

Each bacterial colony which showed positive results in Gram staining was transferred onto the corresponding slide. A drop of 3% H_2O_2 was added on each bacterial colony and the observations were recorded(Nagoba and Pichare, 2012).

Acid fast staining

First, bacterial smears were flooded with carbol fuchsin. Then the stain was heated until the stain get vaporized and allowed the heated stain to remain on the slide for 5 minutes. The slides were washed. Next, few drops of 3% acid alcohol was added onto smears and kept it for 5 minutes. Once again the slides were washed. Afterwards, the slides were flooded with methylene blue for 1-2 minutes and followed by a washing step.

Finally, smears were air dried and examined under 100× magnification(Nagoba and Pichare, 2012). Observations were recorded.

Endospore staining

The wire gauze was placed on the waterbath with steaming water and the slides were placed on it. Afterwards, small piece of blotting paper was placed on the each smear and the paper was flooded with malachite green for 5 minutes. Then the smears were allowed to cool down to room temperature for 2 minutes. Next, the slides were gently rinsed with water and then the smears were flooded with safranin for 1 minute. Once again the slides were washed. Afterwards, slides were blot dried, followed by air dry. Finally, the smears were observed under 100× magnification and the observations were recorded(Nagoba and Pichare, 2012).

Subculturing the biochemically identified Lactobacillus in MRS broth

First of all, 15mL of prepared MRS broth was transferred into each falcon. Then biochemically identified Lactobacillus pure colonies were subcultured in respective MRS broth. Afterwards, all the subcultures were incubated at 370C for 24 hours.

Preparation of Lactobacillus subcultures for AgNPs synthesis

Initially, 5mL of Lactobacillus subculture was transferred into respective falcon. Then 5mL of newly prepared MRS broth was added into each subculture. Afterwards, all the subcultures were incubated at 370C for 24 hours.

Preparation of Lactobacillus cell intacts using Lactobacillus subcultures

After incubation, all the subcultures were centrifuged at 4000rpm for 10 minutes. Supernatants were discarded and 1mL of autoclaved distilled water was added into each pellet. Then water was discarded and once again aforementioned washing step was repeated. Afterwards, 1mL of autoclaved distilled water was added into each pellet and the pellets were mixed with water. Next, the number of Lactobacillus bacterial cells was adjusted to 108 CFU/mL by obtaining the absorbance of 0.2 at 600nm using a spectrophotometer (Azat et al., 2016).

AgNPs synthesis

First of all, 900µL of autoclaved distilled water was added into each test tube. Next, 100µL of Lactobacillus cell intact was transferred into respective test tube. Then 9mL of AgNO3(1mM) was added into each test tube. The initial color of AgNO3 solutions with Lactobacillus cell intacts was observed and then all the test tubes were placed under sunlight for 24 hours(Garmasheva et al., 2016). After 24hrs, observations were recorded and the absorbance values for each sample at 340nm, 370nm, 400nm, 430nm, 460nm, 490nm, 520nm, 550nm wavelengths were obtained. Finally, UV-visible absorption spectrum for each sample was plotted using excel.

Assessing antioxidant activity

Preparation of Lactobacillus subcultures for DPPH radical scavenging assay

First, 5mL of Lactobacillus subculture was transferred into respective falcon. Then 5mL of newly prepared MRS broth was added into each subculture. Afterwards, subcultures were incubated at 370C for 24 hours.

Preparation of Lactobacillus cell intacts using Lactobacillus subcultures

After incubation, all the subcultures were centrifuged at 4000rpm for 10 minutes. Supernatants were discarded and 4mL of autoclaved distilled water was added into each pellet. Then the pellets

were mixed with water and the number of Lactobacillus bacterial cells was adjusted to 108 CFU/mL by obtaining the absorbance of 0.2 at 600nm (Azat et al., 2016).

DPPH (1,1- diphenyl-2-picrylhydrazine) radical scavenging assay

This assay for each sample was performed with triplicates. First, 1mL of each cell intact and AgNPs were transferred into the corresponding "cell intact" and "AgNPs" test tubes, respectively. Then 2mL of 50µmol/L DPPH was added into each test tube. All the reaction mixtures were stored in the dark place for 30 minutes. The color change was observed and absorbances for each DPPH reaction mixture at 571nm were recorded. Next, DPPH radical scavenging activity for each reaction mixture was calculated according to following given formula (Azat et al., 2016) (A_{sample} – Absorbance of sample, A_{blank} - Absorbance of blank, $A_{control}$ - Absorbance of control).

DPPH scavenging activity (SA_{DPPH}) = $[1- (A_{sample}- A_{blank})/A_{control}] \times 100\%$ (Azat et al., 2016)

Statistical interpretation by analysis of variance (ANOVA)

The statistical analysis was carried out using SPSS software (version 21.0). The comparison between the means of the antioxidant activity were performed by one way analysis of variance (ANOVA) at significance level of $p<0.05$.

Assessing antibacterial activity

Subculturing the Escherichia coli and Staphylococcus aureus

First of all, 5mL from each Escherichia coli and Staphylococcus aureus subcultures were transferred into respective falcon. Then 5mL of newly prepared Mueller Hinton broth was added into each subculture. Finally, the subcultures were incubated at 370C for 24 hours.

Preparation of inoculums of indicator for agar well diffusion assay

First, 3mL of autoclaved distilled water was added into two test tubes. Then Escherichia coli and Staphylococcus aureus (from 24hour subculture) were inoculated into respective test tube until the turbidity of both inoculums get equal to 0.5 McFarland standards' turbidity.

Preparation of Lactobacillus subcultures for agar well diffusion assay

First of all, 5mL of Lactobacillus subculture was transferred into respective falcon. Then 5mL of newly prepared MRS broth was added into each subculture. Afterwards, subcultures were incubated at 370C for 24 hours.

Preparation of Lactobacillus cell intacts using Lactobacillus subcultures

After incubation, all the subcultures were centrifuged at 4000rpm for 10 minutes. Supernatants were discarded and 4mL of autoclaved distilled water was added into each pellet. Then the pellets were mixed with water and the number of Lactobacillus bacterial cells was adjusted to 108 CFU/mL by obtaining the absorbance of 0.2 at 600nm (Azat et al., 2016).

Agar well diffusion assay

This assay was performed with triplicates to examine antimicrobial effectiveness of whole bacteria culture (cell intact) and synthesized AgNPs. Escherichia coli and Staphylococcus aureus were used as inoculums of indicator. The detailed procedure was as

follows: First, 20mL of prepared Mueller-Hinton media was poured into each petri plate and allowed to get solidified. After solidification, Mueller-Hinton media in each petri plate was inoculated with Escherichia coli and Staphylococcus aureus inoculums. Next, in each petri plate four wells (for cell intact, AgNPs, positive control and negative control) were created in the media. Afterwards, 100µL of autoclaved distilled water and 100µL of gentamicin(1g/mL) were loaded into "negative control" and "positive control" wells, respectively. Then 100µL of corresponding AgNPs were filled into respective wells. Next, 100µL of each Lactobacillus cell intact was filled into corresponding wells. Finally, all the cultures were incubated at 370C for 24 hours(Benakashani et al., 2016). After incubation, diameter of each inhibition zone was measured and antimicrobial activity was characterized.

RESULTS

Sample culturing

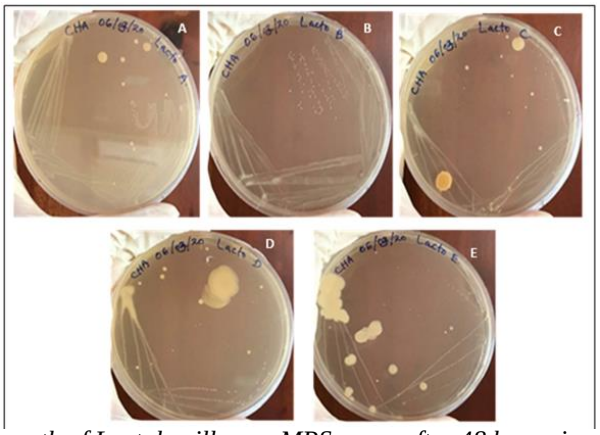

Figure 1.0: Growth of Lactobacillus on MRS agar, after 48 hours incubation at 37^0C

Observations –: Small convex, smooth glistening mucoid white colonies with entire margins were observed in all samples.

Gram staining

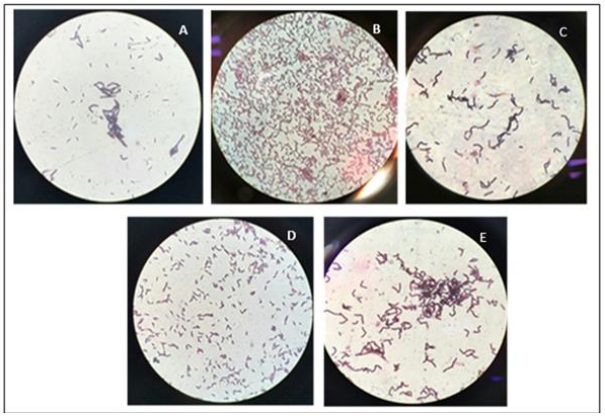

Figure 2.0: Photomicrographs of Gram-stained Lactobacillus, under 100x magnification

Observations-: Gram positive (purple color), rod shaped bacilli arranged in a single, pairs or short chains were observed.

Catalase test

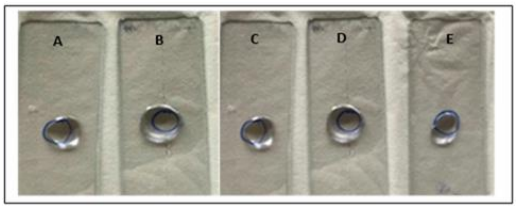

Figure 3.0: Catalase test results for sample A, B, C, D and E

Observations -: All the samples were catalase negative. Bubbles were not observed.

Acid fast staining

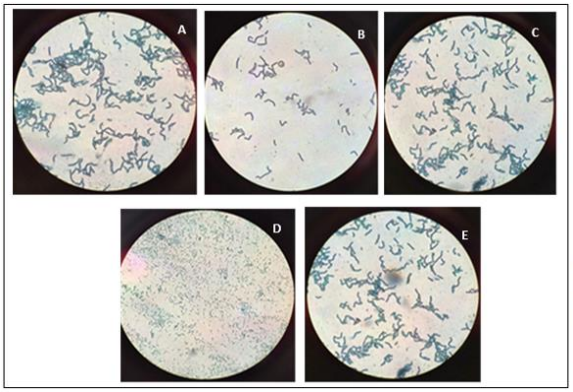

Figure 4.0: Photomicrographs of acid-fast stained Lactobacillus, under 100x magnification.

Observations-: Acid fast negative (light blue color), rod shaped bacilli arranged in a single, pairs or short chains were observed.

Endospore staining

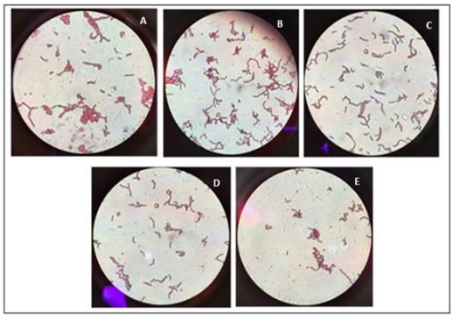

Figure 5.0: Photomicrographs of endospore stained Lactobacillus, under 100x magnification

Observations-: Endospore negative(reddish pink color), rod shaped bacilli arranged in a single, pair or short chains were observed. Only the vegetative cells were observed and endospores were not observed.

AgNPs synthesis

Figure 6.0: *Appearance of $AgNO_3$ solution (with Lactobacillus), before exposure to sunlight.*

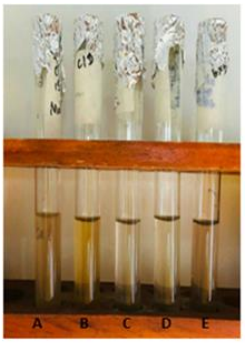

Figure 7.0: *Appearance of $AgNO_3$ solution (with Lactobacillus), after exposure to sunlight for 24hrs.*

Observations-: Brownish color change was observed in sample A and E. Sample B was with yellowish color. Sample C and D were with mild pinkish color.

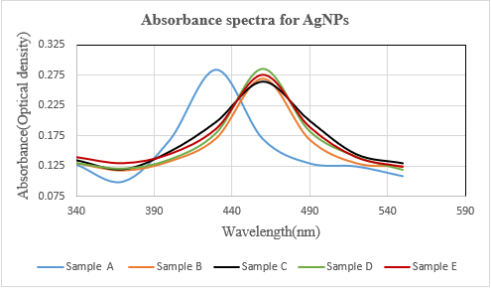

Figure 8.0 : UV-visible absorption spectra for AgNPs of sample A,B,C,D and E.

In UV-visible absorption spectra for AgNPs, all the peaks were observed between 390-470nm and it confirmed the presence of Lactobacillus mediated biosynthesis of AgNPs in all the samples.

Statistical Analysis

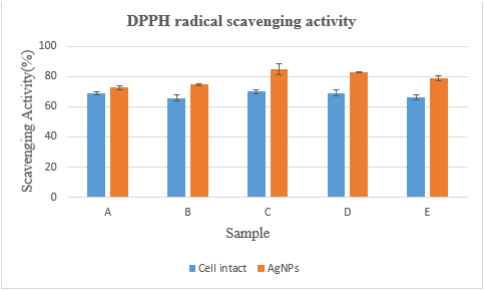

Figure 9.0: Bar chart depicting the comparison of antioxidant potential between Lactobacillus and AgNPs, including error bars representing standard error of the mean

Table 1.0: Statistical analysis by the POST HOC test for statistical significance of the difference between subjects effects by SPSS (version 21.0) using one-way ANOVA.

ANOVA

Antioxidant activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1180.014	1	1180.014	70.669	.000
Within Groups	467.535	28	16.698		
Total	1647.549	29			

The obtained p value which was less than 0.05 indicated that there is a significant difference between the antioxidant activity of Lactobacillus cell intacts and AgNPs.

Agar well diffusion assay
Against Staphylococcus aureus

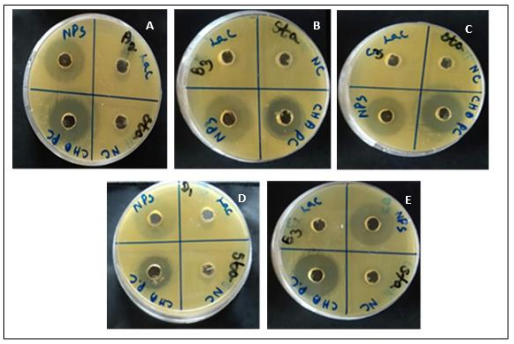

Figure 10.0 : Antibacterial activity of Lactobacillus and AgNPs against Staphylococcus aureus. PC- positive control, NC- negative control, NPs- AgNPs, Lac- Lactobacillus cell intact.

Observations-: Inhibition zones were observed around AgNPs and positive control wells. No inhibition zones were observed around Lactobacillus and negative control wells.

Table 2.0: Mean inhibition zone diameters against Staphylococcus aureus

Sample	AgNPs(mm)	Positive control(mm)
A	18.333±0.577	18.000±1.732
B	17.667±0.577	17.000±1.000
C	18.333±1.528	16.667±1.528
D	19.667±0.577	16.667±1.155
E	21.333±0.577	19.000±2.646

Against Escherichia coli

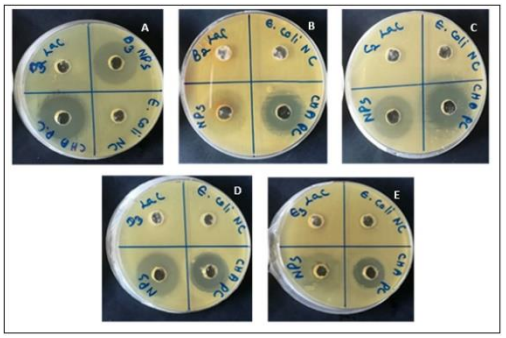

Figure 11.0 : Antibacterial activity of Lactobacillus and AgNPs against Escherichia coli. PC- positive control, NC- negative control, NPs- AgNPs, Lac- Lactobacillus cell intact.

Observations-: Inhibition zones were observed around AgNPs and positive control wells. No inhibition zones were observed around Lactobacillus and negative control wells.

Table 3.0: Mean inhibition zone diameters against Escherichia coli

Sample	AgNPs(mm)	Positive control(mm)
A	25.333±0.577	21.333±3.055
B	18.667±0.577	17.667±0.577
C	18.667±0.577	17.333±2.082
D	18.667±0.577	17.667±1.528
E	19.333±0.577	20.333±4.726

DISCUSSION

As most commonly found probiotic in set yoghurts, Lactobacillus is with vast number of probiotic properties; bile tolerance, antibacterial and antioxidant potentials etc(Jiang et al., 2016). Majority of literature indicated that the Lactobacillus found in dairy products are with ability to biosynthesize AgNPs(Sani, Aminu and Mukhtar, 2017; Gomora et al., 2017). This study was carried out to investigate the biosynthesis of AgNPs, using Lactobacillus found in five commercial set yoghurt samples and to evaluate antibacterial and antioxidant potentials of synthesized AgNPs.

MRS agar consists of nutrients and favourable pH(6.5) for Lactobacillus growth(Yang et al., 2018). Therefore, for sample culturing MRS media, a selective media for Lactobacillus was used. Nevertheless, according to Renschler et al.(2020) other lactic acid bacteria(eg-: Streptococcus, Pediococcus) also grow in MRS media. Therefore, Greppi et al.(2017) suggested that the supplement the media with CaCO3 may enhance the selectivity of media. The optimal growth temperature for Lactobacillus is ranging from 300C- 400C(Bengoa et al., 2018). According to Yang et al.(2018), growth time of Lactobacillus is between 25 to several hundred minutes. Hence, in order to obtain sufficient bacterial growth, cultures were incubated at 370C for 48 hours.

In present study, identification of Lactobacillus by phenotypic traits were proceeded by assessing colony morphology. Riley(2017) has defined convex, mucoid white colonies with entire margins, as colony morphology for Lactobacillus which was grown on MRS media. Therefore, observed bacterial colonies on MRS media indicated the presence of Lactobacillus in selected samples.

In the current study, biochemical identification of Lactobacillus was performed by Gram staining, endospore staining, acid-fast staining and catalase test. Gram staining categorises bacteria on the basis of cell wall composition; the bacteria with thick layer of peptidoglycan in cell wall retain primary dye(crystal violet) and stained in purple color(positive) whereas the bacteria with thin layer of peptidoglycan are not able to trap primary dye and stained with secondary dye(safranin) in red/pink(negative) (Boyanova, 2018; Rohde, 2019) . A study done by Herreros et al.(2003) has observed Lactobacillus as purple colored bacilli in Gram staining. Therefore, in Gram staining, purple colored rods were identified as Lactobacillus.

Catalase test differentiates bacteria based on the presence or absence of catalase enzyme activity which catalyses the breakdown of H2O2 into H2O and O2. Lactobacillus lack catalase enzyme(Konig and Frohlich, 2017). Therefore, the bacteria do not produce bubbles in catalase test(negative). In this study absence of bubbles during catalase test confirmed the presence of Lactobacillus in each sample.

Jain, Jain and Jain(2020) demonstrated that the acid-fast staining differentiates bacteria based on the presence or absence of mycolic acid in cell wall; the bacteria with mycolic acid allow primary stain(carbol fuchsin) to enter the cytoplasm and stained with red(positive) whereas the bacteria lack of mycolic acid get easily decolorized and stained in blue with secondary dye(methylene blue)(negative). According to Gurung et al.(2018) Lactobacillus was observed as light blue colored bacilli in acid fast staining. Therefore, light blue colored bacilli which was observed in acid fast staining was recognized as Lactobacillus.

Atrih and Foster(2002) defined bacterial endospores as alternative life forms that are produced by some Gram-

positive bacteria, which support bacteria to endure the unfavourable environmental conditions. Furthermore, Logan et al.(2011) introduced genus bacillus as the most commonly studied endospore producing bacteria. Nevertheless, Petrova et al.(2017) revealed that the Lactobacillus is non-sporulating. According to Beveridge, Lawrence and Murray(2007), in endospore staining, primary dye(malachite green) stained endospores in green while vegetative cells are stained in red/pink with counter stain(safranin). Shen and Zhang(2017) has elucidated that the Lactobacillus stained in reddish pink color in endospore staining(negative). Hence, the reddish pink colored bacilli which observed in endospore staining was identified as Lactobacillus. Further, no endospores were observed in endospore staining. Selected bacterial colonies from all the samples, rendered positive results for Gram staining, negative results for endospore staining, acid fast staining and catalase test. Therefore, all the obtained biochemical test results confirmed that all the samples were rich in Lactobacillus.

In biosynthesis of AgNPs by sunlight irradiation, the color of AgNO3 solution turned into brown. This color change has been previously observed by Mathew, Prakash and Radhakrishnan(2018) and suggested that the color change appeared due to surface plasmon resonance of deposited AgNPs. In current study, the brownish color change was observed in sample A and E. Sample B was with yellowish color whereas sample C and D were with mild pinkish color. A study done by Lee and Jun(2019) observed that AgNPs which are <100nm were with brownish or yellowish color, while AgNPs which are >100nm were with pinkish color or colorless. In the same concern, Zhang et al.(2016) demonstrated that the pinkish color could be due to destabilization of nanoparticles or aggregation or precipitation of nanoparticles which then leads to elevation in diameter of nanoparticle. Raj et al.(2016) reported that the UV-visible absorption spectrum for AgNPs is in the range of 390-470nm and this may vary due to size, shape and distribution of nanoparticles. Considering, UV-visible absorption spectra for AgNPs, all the peaks were lyed between 390-470nm and it confirmed the presence of AgNPs.

In this study it was found to that the AgNPs were able to act as antibacterial and antioxidant agents more efficient than Lactobacillus. Further, Duran et al.(2016) demonstrated that the AgNPs possess greater antibacterial and antioxidant activity due to the presence of bioactive molecules on the surface of AgNPs and high surface area. DPPH reaction mixtures with Lactobacillus were with mild yellow color, whereas DPPH reaction mixtures with AgNPs were with yellowish orange color. In DPPH assay, antioxidants' reaction with the stable DPPH radicals may cause either color change from violet to yellow or discoloration of the solution. Among five samples, AgNPs and Lactobacillus cell intact of sample-C showed highest antioxidant potential. However, among those two AgNPs rendered higher scavenging activity(85.20%±2.6354) than Lactobacillus(70.96%±0.5194). Similarly, AgNPs of other samples showed greater antioxidant potentials compared to respective cell intact. Bedlovicova et al.(2020), documented that the presence of functional groups on AgNP surface that are with redox properties, may contribute directly to antioxidant action. Furthermore, unlike Lactobacillus, AgNPs are thermally stable and chemically inert which facilitate exploiting the immobilization of antioxidants(Xu et al., 2012).

Moreover, degree of antioxidant activity varied among Lactobacillus cell intact samples. This can be due to genetic variations among different Lactobacillus species which resulting in variations of

cell membrane components(Wang et al., 2017). Further, antioxidant activity of AgNPs of different samples also varied. This can be due to alterations in chemical composition, surface charge, particle size, shape and surface to volume ratio(Bhakya et al., 2016).

In agar well diffusion assay, AgNPs showed considerable inhibition of Escherichia coli and Staphylococcus aureus growth. In general, inhibition zone diameter ranged from 16-26mm, which represents the strength of antibacterial activity. Sample-E AgNPs were with highest (21.333±0.577mm) antibacterial activity against Staphylococcus aureus, whereas sample A-AgNPs were with highest (25.333±0.577mm) antibacterial activity against Escherichia coli. Similar to these observations Ghiuta et al.(2018) observed zones of inhibition when the synthesized AgNPs were tested against Escherichia coli, Salmonella typhii, Pseudomonas aeruginosa. No antagonistic activity for Lactobacillus against Escherichia coli and Staphylococcus aureus was detected in contrast to Shah et al.(2016). Further, inhibition zone diameter among AgNPs of different samples were vary. This also could be due to alterations in nature, chemical composition, surface charge, surface to volume ratio(Bhakya et al., 2016). Additionally, technical errors(eg-: loading errors) could be a possible reason for these variations. Moreover, Raza et al. (2016), investigated that the antibacterial activity of the AgNPs is dose and size dependent. In fact, according to Garmasheva et al.(2016), smaller AgNPs possess greater antibacterial potential than larger nanoparticles.

Many authors have reported that, due to the small size and crystallographic structure surface, the interaction of the nanoparticle is better with the targeted pathogen. Thus compared to Lactobacillus, AgNPs are more effective as antibacterial agents (Keshari et al.,2018). Apart from that, high affinity of silver for sulfur or phosphorus compounds which are abundantly found throughout the cell membrane of pathogens, could be a possible reason for its greater antibacterial activity(Dakal et al., 2016). Further, Liao et al.(2019) reported that the AgNPs are with ability to penetrate bacterial cell wall due to small size and ultimately cause DNA damage to pathogenic bacteria. According to Kang et al.(2017) large size(compared to nanoparticles) and depletion of cell viability with the time could be possible reasons for absence of antibacterial activity and low antioxidant activity of Lactobacillus cell intacts.

One of the limitations of this study is the number of samples used. Therefore, the study can be further improved by using more number of samples to draw more solid. Further, in addition to biochemical identification of Lactobacillus, molecular biological detection of Lactobacillus can also be carried out by using PCR(Polymerase Chain Reaction) and sequencing. Furthermore, further characterization of synthesized AgNPs can be performed by scanning electron microscopy(SEM) and transmission electron microscopy(TEM).

CONCLUSION

The aim of this research was to investigate the biosynthesis of AgNPs using Lactobacillus isolated from set yoghurts and to evaluate antibacterial and antioxidant potentials of synthesized AgNPs. Biochemical tests results confirmed that all the selected samples were rich in probiotic Lactobacillus. Considering DPPH radical scavenging assay results, it can be concluded that, AgNPs are with significantly high($p < 0.05$) antioxidant potentials compared to Lactobacillus. Out of five samples, sample C-AgNPs showed highest antioxidant activity. Moreover, in agar well diffusion

assay, AgNPs of all the samples displayed considerable antibacterial activity against Escherichia coli and Staphylococcus aureus whereas Lactobacillus did not showed any antagonistic effect against Escherichia coli and Staphylococcus aureus. Sample A-AgNPs and sample E-AgNPs showed highest antibacterial activity against Escherichia coli and Staphylococcus aureus, respectively. AgNPs showed more effective properties compared to Lactobacillus in this study. However, more research is needed to exploit other potential properties of AgNPs. The knowledge gained from this study can be used in industrial and nanomedicine fields ; to enhance the probiotic qualities of dairy products, to develop antibiotics against multidrug resistance and to develop nano-antioxidant mediated treatments, respectively.

Acknowledgement

I kindly acknowledge the assistance and guidance of my supervisor, Ms. Punsisi Weerasooriya and the laboratory staff of the School of Science, Business Management School (BMS), Colombo 06. And I would like to take this opportunity to thank Dr. Mathi Kandiah and Dr. Michelle Benedict for guidance and advice. Further, I would also like to express my gratitude to the Northumbria University and Business Management School for providing the opportunity to conduct this project. Moreover, I would like to thank Business Management School for all the resources and funding for the research. At last but not the least, I wish to thank my family and friends who have provided much needed help and support throughout this process.

REFERENCES

Abraham, B.P., Quigley, E.M.M. (2017) 'Probiotics in inflammatory bowel disease', Gastroenterology Clinics, 46(4), pp.769-782 Theclinics [Online]. Available at : https://www.gastro.theclinics.com/article/S0889-8553(17)30084-5/abstract (Accessed : 28 March 2020)

Acharya, P., Jayaprakasha, G.K., Crosby, K.M., Jifon, J.L. (2020) 'Nanoparticle-mediated seed priming improves germination, growth, yield and quality of watermelons(Citrullus lanatus) at multi-locations in Texas' Scientific Reports, 10(1), pp.1-16 Nature [Online]. Available at : https://www.nature.com/articles/s41598-020-61696-7 (Accessed : 11 April 2020)

Anandalakshmi, K., Venugobal, J., Ramasamy, V. (2016) 'Characterization of silver nanoparticles by green synthesis method using Pedalium murex leaf extract and their antibacterial activity', Applied Nanoscience, 6(3), PP.399-408 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s13204-015-0449-z (Accessed : 07 April 2020)

Atrih, A., Foster, S.J. (2002) 'Bacterial endospores the ultimate survivors', International Dairy Journal, 12(3), pp.217-223 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0958694601001571 (Accessed : 20 July 2020)

Azat, R., Liu, Y., Li, W., Kayir, A. (2016) 'Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese', Journal of Zhejiang University, 17(8), pp.597-609 Springer [Online]. Available at : https://link.springer.com/article/10.1631/jzus.B1500250 (Accessed : 21 March 2020)

Basso, T.O., Gomes., F.S., Lopes, M.L., Amorim, H.V.D. (2014) 'Homo and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation', Antonie Van Leeuwenhoek, 105(1), pp.169-177 Springer [Online]. Available at : https://link.springer.com/article/10.

1007/s10482-013-0063-6 (Accessed : 09 April 2020)

Bedlovicova, Z., Strapac, I., Balaz, M., Salayova, A. (2020) 'A brief overview on antioxidant activity determination of silver nanoparticles', Molecules, 25(14), pp.3191-3195 MDPI [Online]. Available at : https://www.mdpi.com/1420-3049/25/14/3191 (Accessed : 21 August 2020)

Benakashani, F., Allafchian, A.R., Jalali, S.A.H. (2016) ' Biosynthesis of Silver nanoparticles using Capparis spinosa L. leaf extract and their antibacterial activity', Karbala International Journal of Modern science, 2(4), pp.251-258 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S2405609X16302159 (Accessed : 26 March 2020)

Bengoa, A.A., Llamas, M.G., Iraporda, C., Duenas, M.T. (2018) 'Impact of growth temperature on exopolysaccharide production and probiotic properties of Lactobacillus paracasei strains isolated from kefir grains', Food Microbiology, 69(7), pp.212-218 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0740002017302939 (Accessed : 20 July 2020)

Beretta, G.L., Cavalieri, F. (2016) 'Engineering nanomedicines to overcome multidrug resistance in cancer therapy', Current Medicinal Chemistry, 23(1), pp.3-22 Ingentaconnect [Online]. Available at : https://www.ingentaconnect.com/content/ben/cmc/2016/00000023/00000001/art00003 (Accessed : 11 April 2020)

Beveridge, T.J., Lawrence, J.R., Murray, R.G.E. (2007) 'Sampling and staining for light microscopy', Methods for General and Molecular Microbiology, 22(5), pp.19-33 Wiley [Online]. Available at : DOI : https://onlinelibrary.wiley.com/doi/abs/10.1128/9781555817497.ch2 (Accessed : 20 July 2020)

Bhakya, S., Muthukrishnan, S., Sukumaran, M., Muthukumar, M. (2016) 'Biogenic synthesis of silver nanoparticles and their antioxidant and antibacterial activity', Applied Nanoscience, 6(5), pp.755-766 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s13204-015-0473-z (Accessed : 23 August 2020)

Boyanova, L. (2018) 'Direct Gram staining and its various benefits in the diagnosis of bacterial infections', Postgraduate Medicine, 130(1), pp.105-110 Tandfonline [Online]. Available at : DOI : https://www.tandfonline.com/doi/abs/10.1080/00325481.2018.1398049 (Accessed : 26 July 2020)

Burdusel, A.C., Gherasim, O., Grumezescu, A.M., Mogoanta, L. (2018) 'Biomedical applications of silver nanoparticles : an up-to-date overview, Nanomaterials, 8(9), pp.681-685 MDPI [Online]. Available at : https://www.mdpi.com/2079-4991/8/9/681 (Accessed : 07 April 2020)

Dakal, T.C., Kumar, A., Majumdar, R.S., Yadav, V. (2016) 'Mechanistic basis of antimicrobial actions of silver nanoparticles', Frontiers in Microbiology, 7(2), pp.1831-1836 Frontiersin [Online]. Available at : https://www.frontiersin.org/articles/10.3389/fmicb.2016.01831/full (Accessed : 07 April 2020)

Dasgupta, N., Ramalingam, C. (2016) 'Silver nanoparticle antimicrobial activity explained by membrane rupture and reactive oxygen generation', Environmental Chemistry Letters, 14(4), pp.477-485 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s10311-016-0583-1 (Accessed : 20 August 2020)

Duran, N., Duran, M., Jesus, M.B.D., Seabra, A.B. (2016) 'Silver nanoparticles : a new view on mechanistic aspects on antimicrobial activity',

Nanomedicine : Nanotechnology, Biology and Medicine, 13(2), pp.789-799 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S1549963415006000 (Accessed : 05 April 2020)

Foldbjerg, R., Dang, D.A., Autrup, H. (2011) 'Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549', Archieves of Toxicology, 85(7), pp.743-750 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s00204-010-0545-5 (Accessed : 09 April 2020)

Gajbhiye, S., Sakharwade, S. (2016) 'Silver nanoparticles in cosmetics', Journal of Cosmetics, Dermatological Sciences and Applications, 6(1), pp.48-53 SCIRP [Online]. Available at : https://m.scirp.org/papers/65036 (Accessed : 16 April 2020)

Garmasheva, I., Kovalenko, N., Voychuk, S., Ostapchuk, A. (2016) ' Lactobacillus species mediated synthesis of silver nanoparticles and their antibacterial activity against opportunistic pathogens in vitro', BioImpacts: BI, 6(4), pp.219-223 NCBI [Online]. Available at : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5326670/ (Accessed : 18 March 2020)

Ghiuta, I., Cristea, D., Croitoru, C., Kost, J. (2018) 'Charaterization and antimicrobial activity of silver nanoparticles, biosynthesized using bacillus species', Applied Surface Science, 438(9), pp.66-73 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0169433217328118 (Accessed : 23 August 2020)

Goderska, K., Pena, S.A., Alarcon, T. (2018) 'Helicobacter pylori treatment : antibiotics or probiotics', Applied microbiology and biotechnology, 102(1), pp.1-7 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s00253-017-8535-7 (Accessed : 26 March 2020)

Gomora, A.E.H., Carrillo, E.L., Navarro, J.B.R., Vilchis, R.J.S. (2017) 'Biosynthesis of silver nanoparticles on orthodontic elastomeric modules: evaluation of mechanical and antibacterial properties', Molecules, 22(9), pp.1407-1410 MDPI [Online]. Available at : https://www.mdpi.com/1420-3049/22/9/1407 (Accessed : 20 July 2020)

Goudarzi, M., Mir, N., Kamazani, M.M., Bagheri, S. (2016) 'Biosynthesis and characterization of silver nanoparticles prepared from two novel natural precursors by facile thermal decomposition methods', Scientific Reports, 6(2), pp.327-339 Nature [Online]. Available at : https://www.nature.com/articles/srep32539 (Accessed : 29 March 2020)

Greppi, A., Hemery, Y., Berrazaga, I., Almaksour, Z. (2017) 'Ability of lactobacilli isolated from traditional cereal-based fermented food to produce folate in culture media under different growth conditions', LWT 86, 86(5), pp.277-284 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S0023643817305765 (Accessed : 21 July 2020)

Gurunathan, S. (2015) 'Biologically synthesized silver nanoparticles enhances antibiotic activity against Gram- negative bacteria', Journal of Industrial and Engineering Chemistry, 29(5), pp.217-226 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S1226086X15001264 (Accessed : 02 April 2020)

Gurung, R., Shrestha, R., Poudyal, N., Bhattacharya, S.K. (2018) ' Ziehl Neelsen vs Auramine staining technique for detection of acid fast bacilli', Journal of BP Koirala Institute of Health Sciences, 1(1), pp.59-66 NEPJOL [Online]. Available at :

https://www.nepjol.info/index.php/jbpkihs/article/view/19755 (Accessed : 25 March 2020)

Han, X., Yang, Z., Jing, X., Yu, P. (2016) ' Improvement of the texture of yogurt by use of exopolysaccharide producing lactic acid bacteria', BioMed Research International, 2016(67), pp.85-91 Hindawi [Online]. Available at : https://www.hindawi.com/journals/bmri/2016/7945675/ (Accessed : 19 March 2020)

Herreros, M.A., Fresno, J.M., Prieto, M.J.G., Tornadijo, M.E. (2003) ' Technological characterization of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese)', International Dairy Journal, 13(6), pp.469-479 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0958694603000542 (Accessed : 19 March 2020)

Islam, R., Hossain, M.N., Alam, M.K., Uddin, M.E. (2020) 'Antibacterial activity of lactic acid bacteria and extraction of bacteriocin protein', Advances in Bioscience and Biotechnology, 11(2), pp.49-59 SCIRP [Online]. Available at : https://www.scirp.org/journal/paperinformation.aspx?paperid=98497 (Accessed : 20 July 2020)

Islam, S.U.I. (2016) 'Clinical uses of probiotics', Medicine, 95(5), pp.22-29 NCBI [Online]. Available at : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4748908/ (Accessed : 26 March 2020)

Jain, A., Jain, R., Jain, S. (2020) 'Staining methods-simple staining, negative staining, Gram's staining and acid-fast staining', Basic Techniques in Biochemistry, 45(9), pp.111-116 Springer [Online]. Available at : https://link.springer.com/protocol/10.1007/978-1-4939-9861-6_31 (Accessed : 23 July 2020)

Jamkhande, P.G., Ghule, N.W., Bamer, A.H., Kalaskar, M.G. (2019) 'Metal nanoparticles synthesis : an overview on methods of preparation, advantages and disadvantages, and applications', Journal of Drug Delivery Science and Technology, 53(7), pp.123-126 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S1773224718308189 (Accessed : 02 April 2020)

Jiang, M., Zhang, F., Wan, C., Xiong, Y. (2016) 'Evaluation of probiotic properties of Lactobacillus plantarum WLPL04 isolated from human breast milk', Journal of Dairy Science, 99(3), pp.1736-1746 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S0022030216000771 (Accessed : 20 August 2020)

Kang, M.S., Lim, H.S., Oh, J.S., Lim, Y.J. (2017) 'Antimicrobial activity of Lactobacillus salivarius and Lactobacillus fermentum against Staphylococcus aureus', Pathogens and Disease, 75(2), pp.175-186 OUP [Online]. Available at : https://academic.oup.com/femspd/article/75/2/ftx009/2966468 (Accessed : 24 August 2020)

Keshari, A.K., Srivastava, R., Singh, P., Yadav, V.B. (2018) ' Antioxidant and antibacterial activity of Silver nanoparticles synthesized by Cestrum nocturnum ', Journal of Ayurveda and Integrative Medicine, 23(4), pp.16-22 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S097594761730390X (Accessed : 26 March 2020)

Khan, A.U., Malik, N., Khan, M., Cho, M.H. (2018) 'Fungi-assisted silver nanoparticle synthesis and their applications', Bioprocess and Biosystems Engineering, 41(1), pp.1-20 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s00449-017-1846-3 (Accessed : 29 March 2020)

Konig, H., Frohlich, J. (2017) 'Lactic acid bacteria', Biology of Microorganisms on Grapes, in Must and in Wine, 25(4), pp.03-41 Springer [Online]. Available at : https://link.springer.com/chapter/10.

1007/978-3-319-60021-5_1 (Accessed : 27 July 2020)

Kumar, S.S.D., Rajendran, N.K., Houreld, N.N., Abrahamse, H. (2018) 'Recent advances on silver nanoparticle and biopolymer-based biomaterials for wound healing applications', International Journal of Biological Macromolecules, 115(4), pp.165-175 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S014181301830518X (Accessed : 10 April 2020)

Lee, S.H., Jun, B.H. (2019) 'Silver nanoparticles: synthesis and application for nanomedicine', International Journal of Molecular Sciences, 20(4), pp.865-869 MDPI [Online]. Available at : https://www.mdpi.com/1422-0067/20/4/865 (Accessed : 20 July 2020)

Liao, S., Zhang, Y., Pan, X., Zhu, F. (2019) 'Antibacterial activity and mechanism of silver nanoparticles against multidrug-resistant Pseudomonas aeruginosa', International Journal of Nanomedicine, 14(3), pp.1469-1472 NCBI [Online]. Available at : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6396885/ (Accessed : 25 August 2020)

Lin, S.K., Cheng, W.T. (2020) 'Fabrication and characterization of colloidal silver nanoparticle via photochemical synthesis', Materials Letters, 261(5), pp.425-447 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0167577X19317094 (Accessed : 29 March 2020)

Logan, N.A., Hoffmaster, A.R., Shadomy, S.V., Stauffer, K.E. (2011) 'Bacillus and other aerobic endospore-forming bacteria', Manual of Clinical Microbiology, 11(2), pp.381-402 Asmscience [Online]. Available at : https://www.asmscience.org/content/book/10.1128/9781555816728.chap24 (Accessed : 24 July 2020)

Markowiak, P., Slizewska, K. (2017) 'Effects of probiotics, prebiotics and synbiotics on human health', Nutrients, 9(9), pp.1021-1025 MDPI [Online]. Available at : https://www.mdpi.com/2072-6643/9/9/1021 (Accessed : 28 March 2020)

Mathw, S., Prakash, A., Radhakrishnan, E.K. (2018) 'Sunlight mediated rapid synthesis of small size range silver nanoparticles using Zingiber officinale rhizome extract and its antibacterial activity analysis', Inorganic and Nano-Metal Chemistry, 48(2), pp.139-145 Tandfonline [Online]. Available at : DOI : https://www.tandfonline.com/doi/abs/10.1080/24701556.2017.1373295 (Accessed : 21 August 2020)

Morelli, L., Capurso, L. (2012) 'FAO/WHO guidelines on probiotics: 10 years later', Journal of Clinical Gastroenterology, 46(9), pp.11-22 IWW [Online]. Available at : https://journals.lww.com/jcge/Citation/2012/10001/FGV9Jm2u7rmsCe65wKzPTw5jtS38n2tVEGi0_Years_Later.2.aspx (Accessed : 21 March 2020)

Nagoba, B.S., Pichare, A. (2012) Medical microbiology: preparation manual for undergraduates. 02nd edn. Indraprakash bulding, 21 Barakamba Road, New Delhi

Nguyen, C.M.T., Tran, Q.K., Nguyen, V.T. (2020) 'Polyol synthesis of sliver nanoparticles and deposition on carbon vulcan for 4-nitrophenol reduction catalysis', Oriental Journal of Chemistry, 36(1), pp.139-143 Orientjchem [Online]. Available at : http://www.orientjchem.org/vol36no1/polyol-synthesis-of-silver-nanoparticles-and-deposition-on-carbon-vulcan-for-4-nitrophenol-reduction-catalysis/ (Accessed : 29 March 2020)

Palomo, J.M., Filice, M. (2016) 'Biosynthesis of metal nanoparticles : novel efficient heterogeneous nanocatalysts',Nanomaterials, 6(5), pp.84-87 MDPI [Online]. Available at : https://www.mdpi.com/2079-

4991/6/5/84 (Accessed : 02 April 2020)

Petrova, M.I., Reid, G., Vaneechoutte, M., Lebeer, S. (2017) 'Lactobacillus iners: friend or foe?', Trends in Microbiology, 25(3), pp.182-191 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0966842X16301810 (Accessed : 25 July 2020)

Raj, D.R., Prasanth, S., Vineeshkumar, T.V., Sudarsanakumar, C. (2016) 'Surface plasmon resonance based fiber optic dopamine sensor using green synthesized silver nanoparticles', Sensors and Actuators, 224(9), pp.600-606 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S0925400515305712 (Accessed : 20 July 2020)

Raza, M.A., Kanwal, Z., Rauf, A., Sabri, A.N. (2016) 'Size- and shape-dependent antibacterial studies of silver nanoparticles synthesized by wet chemical routes', Nanomaterials, 6(4), pp.74-78 MDPI [Online]. Available at : https://www.mdpi.com/2079-4991/6/4/74 (Accessed : 10 April 2020)

Renschler, M.A., Wyatt, A., Anene, N., Hill, R.R. (2020) 'Using nitrous acid-modified de Man, Rogosa and Sharpe medium to selectively isolate and culture lactic acid bacteria from dairy foods', Journal of Dairy Science, 103(2), pp.1215-1222 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/pii/S0022030219310781(Accessed : 20 July 2020)

Riley, P.A. (2017) ' Principles of microscopy, culture and serology-based diagnostics', Medicine, 45(10), pp.639-644 ScienceDirect [Online]. Available at : https://www.sciencedirect.com/science/article/abs/pii/S1357303917301913 (Accessed : 22 March 2020)

Rohde, M. (2019) 'The Gram-positive bacterial cell wall', Gram-Positive Pathogens, 40(2), pp.3-18 Wiley [Online]. Available at : DOI : https://onlinelibrary.wiley.com/doi/abs/10.1128/9781683670131.ch1 (Accessed : 26 July 2020)

Sani, N.J., Aminu, B.M., Mukhtar, M.D. (2017) 'Eco-friendly synthesis of silver nanoparticles using Lactobacillus delbrueckii subsp. bulgaricus isolated from kindrimo(locally fermented milk) in Kano State, Nigeria', Bayero Journal of Pure and Applied Sciences, 10(1), pp.481-488 AJOL [Online]. Available at : https://www.ajol.info/index.php/bajopas/article/view/165665 (Accessed : 25 July 2020)

Shah, N., Patel, A., Ambalam, P., Holst, O. (2016) 'Determination of an antimicrobial activity of Weissella confusa, Lactobacillus fermentum and Lactobacillus plantarum against clinical pathogenic strains of Escherichia coli…', Annals of Microbiology, 66(3), pp. 1137-1143 Springer [Online]. Available at : https://link.springer.com/article/10.1007/s13213-016-1201-y (Accessed : 24 August 2020)

Shams, S., Pourseyedi, S., Rafsanjani, H.H. (2014) 'Green synthesis of silver nanoparticles : eco-friendly and antibacterial', International Journal of Nanoscience and Nanotechnology, 10(2), pp.127-132 Ijnnonline [Online]. Available at : http://www.ijnnonline.net/article_6118.html (Accessed : 05 April 2020)

Shen, C., Zhang, Y. (2017) ' Staining technology and bright-field microscope use', Food Microbiology Laboratory for the Food Science Student, 5(1), pp.9-14 Springer [Online]. Available at : https://link.springer.com/chapter/10.1007/978-3-319-58371-6_2 (Accessed : 25 March 2020)

Siddiqui, M.N., Redhwi, H.H., Achilias, D.S., Kosmidou, E. (2018) 'Green synthesis of silver nanoparticles and study of their antimicrobial properties', Journal of Polymers and the Environment, 26(2), pp.423-433 Springer [Online]. Available at :

https://link.springer.com/article/10.1007/s10924-017-0962-0 (Accessed : 29 March 2020)

Wang, Y., Wu, Y., Wang, Y., Xu, H.(2017) 'Antioxidant properties of probiotic bacteria', Nutrients, 9(5), pp.521-526 MDPI [Online]. Available at : https://www.mdpi.com/2072-6643/9/5/521 (Accessed : 23 August 2020)

Xu, H., Qu, Feng, Xu, Hong, Lai, W. (2012) 'Role of reactive oxygen species in the antibacterial mechanism of silver nanoparticles on Escherichia coli 0157: H7', Biometals, 25(1), pp.45-53 Springer [Online]. Available at : https://link.springer.com/article/10.1007%2Fs10534-011-9482-x (Accessed : 22 August 2020)

Yang, E., Fan, L., Yan, J., Jiang, Y. (2018) 'Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria', AMB Express, 8(1), pp.10-15 Springer [Online]. Available at : https://link.springer.com/article/10.1186/s13568-018-0536-0 (Accessed : 22 July 2020)

Yazdi, M.K.S., Davoodabadi, A., Zarin, H.R.K., Ebrahimi, M.T. (2017) 'Characterization and probiotic potential of lactic acid bacteria isolated from Iranian traditional yogurts', Italian Journal of Animal Science, 16(2), pp.185-188 Tandfonline [Online]. Available at : DOI : https://www.tandfonline.com/doi/full/10.1080/1828051X.2016.1222888 (Accessed : 20 August 2020)

Zhang, X., Liu, Z.G., Shen, W., Gurunathan, S. (2016) 'Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches', International Journal of Molecular Sciences, 17(9), pp.1530-1534 MDPI [Online]. Available at : https://www.mdpi.com/1422-0067/17/9/1534 (Accessed : 23 July 2020)