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RESEARCH ARTICLE

ANTI OXIDANT PROPERTIES OF PROBIOTICS ISOLATED FROM COMMERCIALLY AVAILABLE SOURCES

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ABSTRACT

Reports from human studies indicated that *Lactobacillus rhamnosus* exerted strong antioxidant activity in situations of elevated physical stress. This has benefitted Athletes in the sports arena as these individuals are exposed to oxidative stress during their exercises and activities, hence studies on determining the antioxidant activities of probiotics might benefit humans undergoing stress related activities. The ability of probiotics possessing antioxidant activities will assume greater importance if this hypothesis is tested, since probiotics with antioxidant properties can increase the antioxidant levels and neutralize the effects of reactive oxygen species (ROS) in such individuals. Probiotics are defined as viable microorganisms sufficient amounts of which reach the intestine in active state and exert positive health effects by improving gut health. The aim of this study was to determine the efficacy various probiotic bacteria such as *Bacillus coagulans, Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Bacillus subtilis* isolated from various commercial and domestic sources using standard probiotic testing assays.In-vitro studies on Anti-oxidant activities using standard protocols have shown that *Bacillus coagulans* had the highest (94%) DPPH activity as compared to other probiotics. This investigation could determine that these few test organisms with their additional anti-oxidant properties could be considered as value added useful probiotics due to their ability for not only acid tolerance and bile resistant properties but also having useful antioxidant properties, therefore qualifying as safe commercial probiotic products.

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INTRODUCTION

The age-old quote by Hippocrates, "Let food be thy medicine and medicine be thy food", is certainly the tenet of today. The market for functional foods that promote health beyond providing basic nutrition, is flourishing. Within the functional foods, is the small but rapidly expanding arena of probiotics (Khan and Ansari, 2007). Prebiotics have come to light in more recent years, recognition of probiotic effects dates back the 19th century when the French scientist Louis Pasteur (1822 – 1895) postulated the importance of microorganisms in human life; this was further reinforced by work done by 1908 Nobel Prize winner Elie Metchnikoff (Sanders, 1999). Probiotics are "live micro-organisms regarded as safe (GRAS) status, which when administered inadequate amounts confer a health benefit on the host".

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Probiotics represent probably the archetypal functional food, and are defined as alive microbial supplement, which beneficially affect the host by improving its intestinal microbial balance (Bikila Wedaj *et al.,* 2015). The term Probiotic was derived from the green meaning "the life". The food and Agricultural organisation of the United Nations (FAO) and the World health Organization (WHO) have stated that there is adequate scientific evidence to prove that there is a potential for probiotic foods to provide health benefits and that specific strains are safe for human use (Food and Agriculture Organization of the United Nations and World Health Organization, 2001). An expert panel commissioned by FAO and WHO defined Probiotics as "'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. This is the definition they suggested, should be used and probiotics should not be referred as bio therapeutic agents (McFarland *et al.,* 1995). According to the guidelines of the probiotic organisms, reported by a joint FAO/WHO, there are certain attributes of a probiotic to be fulfilled, based on both survival and growth studies (Soleimanian-Zad *et al.,*

2009; Vizoso Pinto *et al.,* 2006). By assessing the strain's ability in bringing about the change in total viable count and in optical density at the presence of various concentration of acid and bile salt. The time from entrance to release fromthe stomach was reported to be approximately 90 min (Brashears *et al.,* 1998). However the digestion processes have longer residence times thus there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine which contain bile. Hence we are witnessing that besides beneficial health effects, Probiotics have added beneficial effects as they can present antioxidant properties to reduce the damages caused by ROS. Thus the aim of this investigation was to determine whether such antioxidant effects were present in the probiotic organisms tested by us. Scientific evidence suggests that the Oxidative stress is involved in the origination of various diseases and disorders such as Alcoholic induced liver disease, Non-alcoholic liver disease, Ageing and Cancer (Cederbaum, 2004). Oxidative Stress is a result of imbalance between production and elimination of Reactive oxygen species (ROS) and Free radicals, which are generally removed by the endogenous anti-oxidant defence system (Gutteridge and Hallowell, 2000). Hence, consumption of Anti-oxidants, which can quench free radicals and ROS are often prescribed for beneficial effects to human health. The synthetic anti-oxidants are also effective and beneficial, but there remains the concerns in regard to safety and toxicity of the synthetic antioxidants. LAB particularly *Bacillus coagulans* have recently received increasing attention because of the specific role in the maintenance of human health and also decreasing the risk of reactive oxygen species accumulation (Parvez *et al.,* 2006). The lactobacilli are associated with a reduced risk of developing chronic diseases (Chan *et al.,* 2011) these abilities of reducing risk of ROS are mainly focused on viable counts of the organisms and cell-free extracts. Cell free supernatant is also a well-known source of antioxidants. Further, it has been reported that thirty-four strains of lactic-acid-bacteria (seven Bifidobacterium, eleven Lactobacillus, six Lactococcus, and 10 Streptococcus thermophilus) were assayed in vitro for antioxidant activity against ascorbic and linolenic acid oxidation (TAA (AA) and TAA (LA)), trolox-equivalent antioxidant capacity (TEAC). Intracellular glutathione (TGSH), and superoxide dismutase (SOD). Wide dispersion of each of TAA (AA), TAA (LA), TEAC, TGSH, and SOD occurred within bacterial groups, indicating that antioxidative properties are strain specific (Alberto Amaretti *et al.,* 2013).

Chemical antioxidant activity assays include DPPH radical scavenging activity, Hydroxyl radical scavenging (HRS) method, Reducing power (RP). These assays depend on the capacity of antioxidants to quench or reduce free radicals. However, these chemical assays present several limitations. For example, the DPPH assay is fast and simple way to determine antioxidant activity. The mechanisms of action of antioxidants are not only scavenging free radicals but also inhibiting the production of free radicals and to improve the levels of endogenous antioxidants. At very high concentration free radicals are hazardous to the body and it damages the component of cells, including DNA, Protein and Cell membranes. The damage caused by these free radicals leading to the damage of DNA may play a role in the development of cancer and other diseases. There need to be a balance between Free radical production and Anti-oxidants, which are necessary for proper physiological functions. There is a renewed interest in the search of anti-oxidants, which can be used in food. Role of probiotics as an antioxidant is being keenly investigated. Probiotics is a dietary supplement is also one option. Probiotics are known for their health beneficial effects and are called as Dietary Adjuncts. In view of these findings, and our interest, our aim was to find out the potential of Probiotic strains that can exhibit the Anti-oxidant properties along with acid tolerance and Bile resistant studies.

MATERIALS AND METHODS

Bacterial strain and culture preparation

Bacterial Strains, *Bacillus coagulans, Bacillus subtilis, Lactobacillus plantarum, Lactobacillus rhamnosus,* were isolated from curd, dough and camel milk respectively. These probiotics were tested for their probiotic attributes, and identified using various probiotic attribute assays. All chemicals and media were purchased from HI Media and were of high quality. Probiotics were cultured in broth medium MRS at 37^0C for overnight. The antioxidant properties of the probiotics using their cell lysates were determined by various methods described below. Overnight inoculum (10^6 cfu/ml) was centrifuged at 5000*g for 5min at 4^0C. The supernatant was removed. The precipitate was washed twice with distilled water and was resuspended in buffer. The homogenized sample half of it was collected in a separate vial and sonicated to obtain lysate. Cell walls were destroyed using sonication under mode of the 10 cycles, 25 seconds each, with a pause between each one. Further the solution was centrifuged at 10,000*g for 5min at 4 degrees. The supernatant was collected and used for determination of the Antioxidant activity by Invitro Oxidation methods Diphenyl 1-Picrylhydrazyl, Hydrogen Radical Scavenging activity and Reducing power methods.

Methods / Assays for checking Probiotic attributes

Culturing the organisms and testing Viable Spore Count: 1.0g of sample weighed and transferred to 500 ml sterile standard volumetric flask and approximately 300 ml of sterile isotonic saline solution (0.9%) was added and sonicated for 10-18 minutes. After completion of sonication diluted up to the mark with same sterile isotonic saline solution for further serial dilution. One ml of Stock solution was Transferred into 9.0 ml of sterile isotonic saline solution in a sterile test tube and mixed thoroughly. Serial dilution was continued to get 30- 300 cells/ml in final dilution of test solution. The final solution was allowed to stand in the water bath at 75 degrees for 30 min. Plating was done by adding 1ml of heat treated final dilution into each petridish containing MRS agar medium and incubated in an inverted position for 48 hours.

Acid Tolerance Test: Overnight culture was taken in the ratio of 1:10 into PBS buffer. The sterile PBS adjusted to different pH to study the acid tolerance of the microorganisms. Hence the pH of PBS was adjusted to pH 2,& 3 with Hydro chloric acid (1.0M) and pH7 as a control. The initial bacterial concentration was 10^6 cfu ml and was checked by viable count determination on MRS agar medium as described above. Optical density and viable cell count was done simultaneously for 6hrs on hourly basis. Optical density reading was taken at 660nm and residual viable count was determined after 48 hours of incubation.

Bile Tolerance Test: PBS buffer was inoculated with different bile concentration of 0.15%, 0.3% and 0.6% (ox-bile, Sigma). Overnight cultures of probiotics was taken and inoculated in the PBS buffer which is adjusted with Bile. Growth in control (no bile) and test cultures was monitored hourly by measuring absorbance at 660nm using a spectrophotometer. Growth curves were plotted, and analysis was based on the time required for each culture between control and test culture.

Invitro Oxidative methods: Diphenyl 1-Picrylhydrazyl (DPPH): The method as described by Jiali Xing *et al.* 2015was adopted for determining antioxidants activities of Lactobacilli Cell Free supernatants by Cellular Antioxidant assay: The effect of the cell free supernatant of *Bacillus coagulans, Bacillus subtilis, Lactobacillus plantarum and Lactobacillus rhamnosus* on the free radical DPPH was measured in accordance with, and with slight modifications by Lin and Chang 2000. A sample, (cell free supernatant, PNY media 1mL) and a freshly prepared DPPH solution (0.5mg/100mL Sigma-Aldrich), were mixed. The mixture was vigorously shaken and left to react for 30min in the dark at room temperature. The control sample contained ascorbic acid and DPPH. The scavenged DPPH monitored by determining the absorbance at 517nm using spectrophotometer. The radical scavenging activity was quantified as units/mL by using the following formula:

DPPH activity (U/mL) = (AbsC- AbsS)/S *100

The absorbance of the control and test sample at 517nm, respectively is the volume of the sample.

Hydrogen Radical Scavenging

The generation of hydroxyl radical was performed in solution containing 1,10 –phenanthroline (sigma), 1ml PBS buffer (0.02mM pH 7.4), 1ml distilled water, 1ml FeSo4 (2.5mM). This reaction was initiated by adding 1ml H_2O_2 (20mM), and this mixture was incubated at 37°C for 90 min. HRS activity was monitored by identifying the increase in absorbance at 536 nm by using spectrophotometer. HRS was calculated by using the following equation:

HRS activity (%) = (As-Ac)/ (Ab-Ac)*100%

Where**,** 'As' is the absorbance of the sample, 'Ac' is the absorbance of the control solution, (deionised water was used instead of the sample at the same amount). Ab is the absorbance of the solutions without samples and water.

Reducing Power

The cell free supernatant reducing activity was determined as mentioned by Lin and Yen with slight modification. Cell free supernatant 0.5ml was briefly mixed with Potassium ferricyanide (0.5ml, 1%) and PBS buffer (0.5ml, pH 6.6). Subsequently the mixture was heated at 50°C for 20 minutes and this was allowed to cool. After cooling, 0.5ml of 10% trichloro acetic acid (TCA) was added to the mixture and then centrifuged at 3000g for 5minutes. Upper layer was siphoned and to this upper layer 1ml ferric chloride 0.1% was added and was allowed to mix for 10min. The absorbance of this mixture was obtained at 700nm using a spectrophotometer. Higher absorbance of the mixture indicated the higher reducing power. Ascorbic acid served as the standard.

RESULTS

This investigation was designed to evaluate the potential of probiotics consumed normally by human to see if there are any value added properties in these organisms. Hence the probiotic attributes of the four organisms *Bacillus coagulans, Bacillus subtilis, Lactobacillus rhamnosus and Lactobacillus plantarum* were checked by taking the MRS broth of each individual organisms after overnight incubation and directly subjecting them to each of the two stress factors: low pH and Bile salts. The importance of acid tolerance is an important criterion to be a good source of probiotic as these organisms have to survive in the highly acidic environment of the human stomach. The viability of the probiotics was recorded at pH 2, 3 and 7. The count was good at pH 3 and pH 7 even after 6 hrs of incubation. However at pH 2 no growth of the organisms was seen, suggesting at harsh pH (pH2) the cells were killed (Figure 1). According to Zavaglia et al acid such as the Hydrochloric acid (HCl) that is found in the human stomach is a strong oxidiser. Thus it can oxidize many important biomolecular compounds in the cells and disrupt them while it will undergo reduction. The acid can destroy fatty acids, proteins (Wolvers *et al.,* 2010) like cholesterol. The number of cell counts remained significantly unchanged at pH 7. Based on the results obtained, it was evident that the survival of probiotic organisms was from pH 3 to pH 7 (Figure-1). Probiotics that are tolerant to bile are considered as the most promising ones. Bile is a viscous alkaline fluid it consists of bile salts, pigments and bile acids, cholesterol and phospholipids. Higher inhibition of growth of probiotics was seen as the bile concentrations increased. 0% bile acted as control for the experiment. At a bile concentration of 0.15% growth was not observed after 3 hours, but at 0.3% and 0.6% the growth was observed even after 4hours of incubation (Figure 2).

Anti-oxidation: DPPH: 1,1 Diphenyl 2 picrylhydrazyl (DPPH) free radical scavenging method is the first approach for the evaluation of the anti-oxidant potential of a compound, an extract or a biological source. This is a simplest method where in the test compound or extract is mixed with DPPH solution and absorbance is recorded (Figure-3).

HRS activity: HRS activity results showed that the cell free supernatant of the test organism can inhibit the formation of two radicals. Antioxidant activities is directly related to its concentration in the cell free supernatant. The reducing power of cell free supernatant of Probioticsis based on the kinetics of the reduction of Fe 3+ to Fe 2+ to prevent the oxidation reaction and to control the transition metal ions. Among all the Probioticstested *Bacillus coagulans* showed highest antioxidation (Figure-4).

Reducing Power

This applies that -the more antioxidant compounds present in the extract the more is converted from the oxidation form of iron (Fe+3) in ferric chloride to Ferrous (Fe+2) chloride. The results of this assay are presented in Figure-5. *Bacillus coagulans* showed thehighest Reducing power. Previous reviews suggested that the potential of probiotics is extendable to lower the frequency and duration of diarrhea; stimulate humoral and cellular immunity; prevent colon cancer; and decrease unfavorable metabolites, including ammonium and procancerogenic enzymes in the colon.

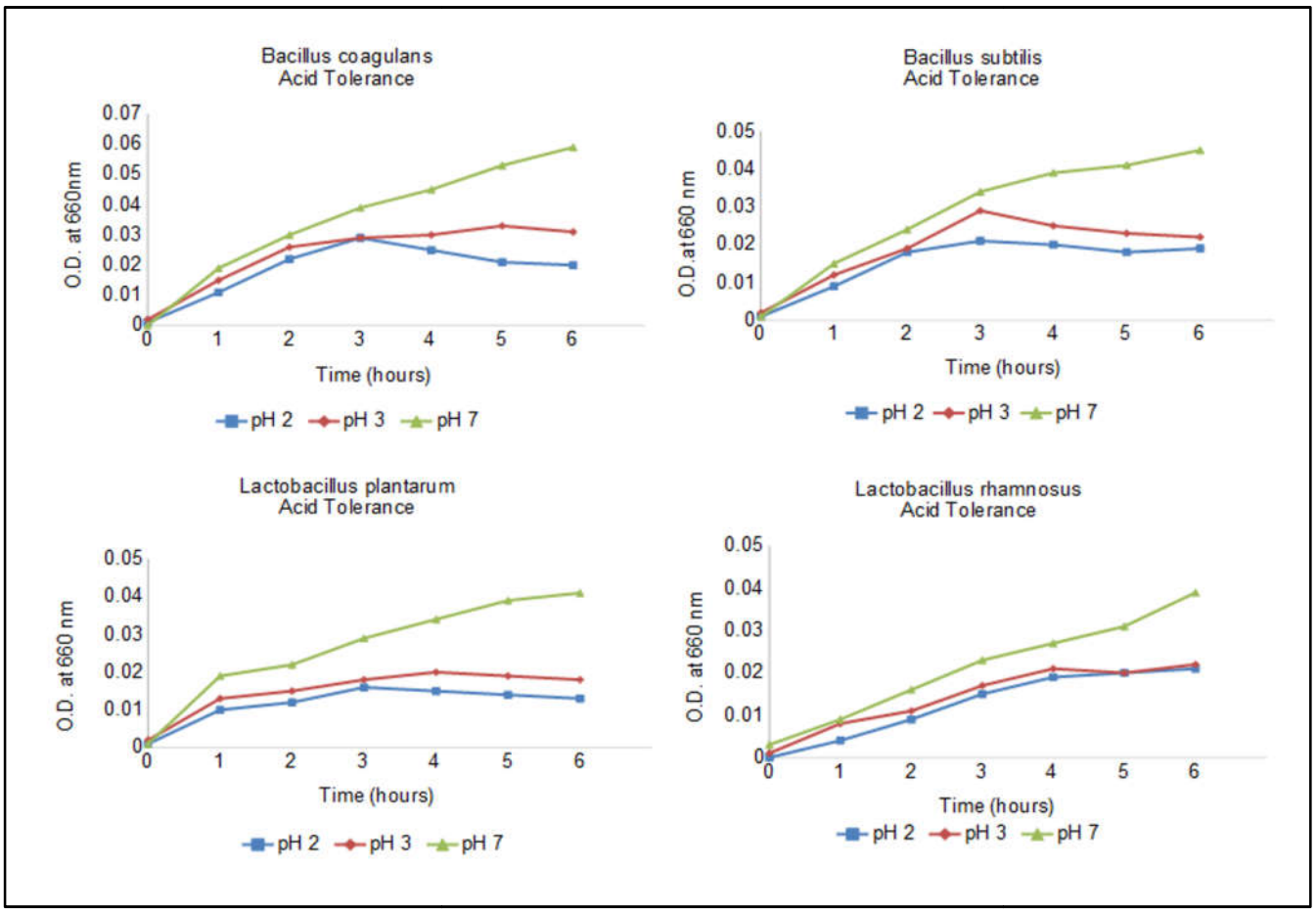

Figure 1. Acid tolerance test; -Figures1a; 1b; 1c; 1d; showing the viability of the Probiotics at various pH ranges (pH-2,pH-3, pH-7)

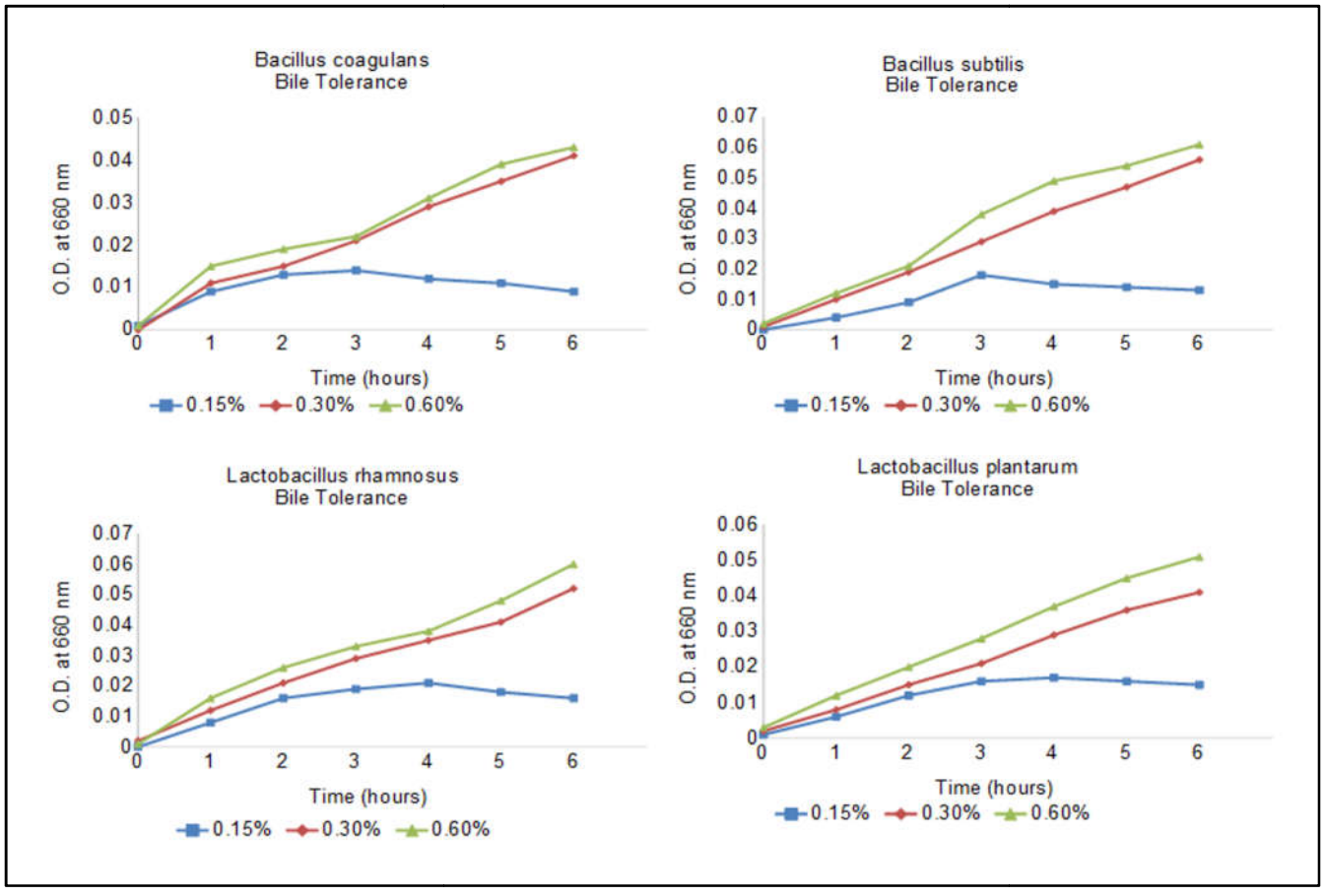

Figure 2. Bile tolerance test, Fig 2a; 2b; 2c; 2d; showing bile tolerance at various time periods

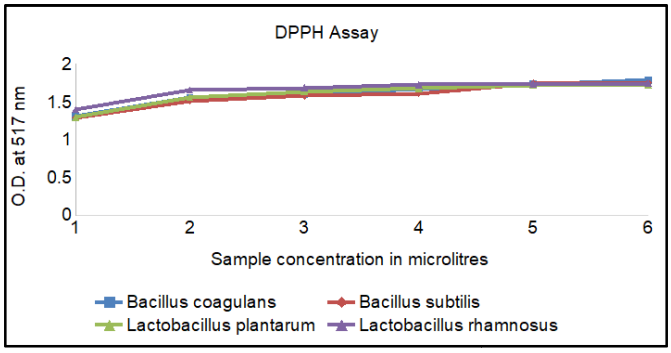

Figure 3. Antioxidant properties of the probiotics using DPPH assay

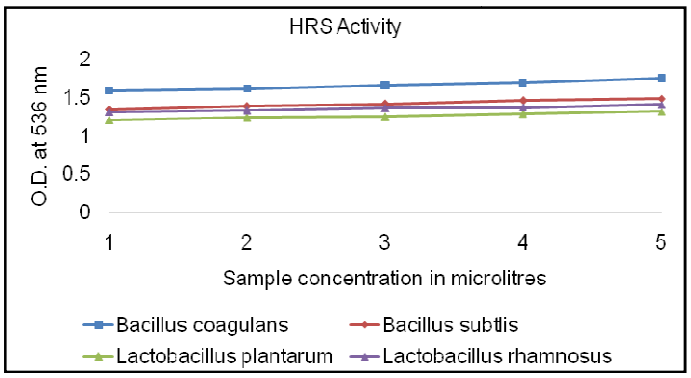

Figure 4. The antioxidant properties of the 4 probiotics using HRS assay

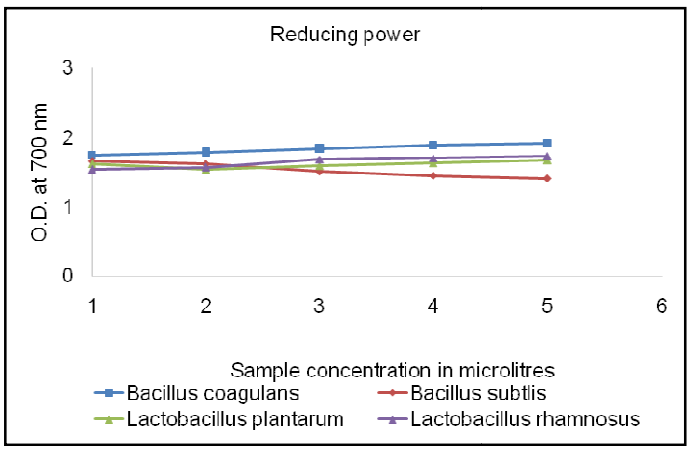

Figure 5. Bioassay results showing the reducing power test of the probiotics

DISCUSSION

In this study *Bacillus coagulans* in the presence of Bile salts could survive at 0.3% and 0.15% for 4 hours. Studies on *invitro* anti-oxidation showed that cell free supernatant of *Bacillus coagulans* exhibited antioxidant activity. The DPPH activity showed that 93% and Hydrogen radical scavenging activity and Reducing power activities had highest antioxidation properties. The cellular antioxidant activity method is a relatively reliable and sensitive method compared other chemical assays. Generally Oxidative stress refers to elevated intracellular levels of oxygen radicals that cause damage to lipids, proteins, and DNA. Reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, are one of the highly active oxygen free radicals. During evolution, most living organisms possess enzymatic defenses (superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR)), non-enzymatic antioxidant defenses (glutathione, thioredoxin, Vitamin C, Vitamin E), and repair systems to protect them against oxidative stress. However, these native antioxidant systems are generally not enough to prevent living organisms from oxidative damage. Antioxidant additives using substances that delay or prevent the oxidation of cellular substrates have demonstrated the capacity to protect the human body against oxidative damage. Although several synthetic antioxidants, including butylated hydroxyanisole and butylated hydroxytoluene, have been widely used in retarding lipid oxidation, their safety has recently been questioned due to liver damage and carcinogenicity. Therefore, in recent years, finding safer and natural antioxidants from bio-resources to replace synthetic antioxidants has received a great deal of attention. Chan et al reported that Hydrochloric acids (HCl) found in human stomach, disrupts the biomolecules of cells such as protein, DNA and fatty acids. Other studies confirm that exposing to gastric acid with pH $\leq$ 2 after 3 hours of incubation caused reduction in the viability count of the bacteria intensively (Mandal *et al.,* 2006). According to Prasad et al and Chan et al the threshold point to acid resistance in this research was set at pH 2 and pH 3 for 3 hours of incubation, as it stimulates bacterial residence in the stomach (Shen *et al.,* 2011; Asemi *et al.,* 2013). This is in accordance with the findings from Liong and Shah which stated that resistance at pH 3 is the standard for acid tolerance of probiotics. Results in this study indicate the strong inhibition on the viable bacteria number at pH 2 and growth was observed at pH 3 and pH7. In this study the bacterial growth decreased at pH 3 after 3 hours and remained constant at higher pH 7. After the bacteria gets exposed to bile salts, disruption of cellular homeostatic occurs resulting in bacterial content leakage and finally leads to death of the cell. Probiotics are proved to show hypercholesteraemic activity (Bao *et al.,* 2012). Probiotics from new sources like camel milk which have cholesterol lowering property have been studied (Prasad *et al.,* 1998) and the useful effects have also been studied like use of probiotics in metabolic syndromes, Urinary tract infections, Intestinal bowel syndrome (IBS) etc. (Prasad *et al.,* 1998; Ratna Sudha *et al.,* 2010) and also probiotics helps in Hypercholesterolemia (Ratna Sudha *et al.,* 2009). One possible mechanism could be that the nitric acid produced by Lactic acid bacteria (LABs) protects mucosa for damages and excessively permeability Payne et al 1993.When Kaizu and co-workers in 1993 discovered anti-oxidative activity of Lactic acid bacteria, a few of them had effects in clinical human trials. *Lactobacillus fermentum* ME-3 had shown both anti-oxidative-anti Atherogenic and anti-microbial activity and this was patented from Tartu University by the name LfME-3.This strain is of human origin and has proven its safety. Thus, the complete picture of the interaction between probiotics and antioxidant capacity may come into view soon. Probiotics modulate the redox status of the host via their metal-ion chelating ability, antioxidant systems, regulating signaling pathways, enzyme producing ROS, and intestinal microbiota. However, there are still many unsolved questions. It is controversial whether the in-vitro results are transferable to humans. However it can be concluded that Probiotic strains which are capable to limit excessive amounts of reactive radicals in vivo may contribute to prevent and control several diseases associated with oxidative stress.

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Compliance with Ethics Requirements: NA

Conflict of Interest:Authors declare - no conflict of interest.

Ethical Standards: This article does not contain any studies with human or animal subjects.

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