ISSN 2989-0705 (Online)|Vol 2|Issue 1|January 2024

Determination of Antioxidant Activity of *Lactobacillus* Isolated from Yoghurt

Dhivya Praveenaah Vivekanandan[1], Punsisi Rasara Weerasooriya[1*] and Daniel George[1]

[1]School of Science, Business Management School (BMS), Sri Lanka

[*]punsisi@bms.ac.lk

Abstract

Lactobacillus and *Bifidobacterium* are two bacterial genera that produce probiotics, which are microorganisms that are beneficial to health and can be found in fermented dairy products. *Lactobacillus* can offer several health benefits for people who have taken antibiotics, suffer from irritable bowel syndrome or other digestive issues. Consuming a diet rich in antioxidants may help to prevent or reduce the risk of heart disease and certain cancers. Research aimed to identify *Lactobacillus* from different yogurt samples and determine its antioxidant properties. Five different commercially available yogurt samples were purchased from a local market and cultured on MRS agar. Single colonies from each sample were chosen to undergo biochemical assays such as Gram staining, catalase test, acid-fast staining, and endospore staining. Finally, the antioxidant activity of *Lactobacillus* was determined using the DPPH assay in both cell-free and intact cell samples. The presence of *Lactobacillus* in samples was indicated by small opaque, milky-white colony morphology on MRS agar, purple stained, rod-shaped bacteria in Gram staining, the lack of bubbles in the catalase test, blue rods in acid-fast staining, and red rods in endospore staining. Color change was observed for both cell-free and cell-intact samples, and the antioxidant activity was calculated accordingly. The antioxidant activity of the cell-free and cell-intact suspension is not statistically significant ($P > 0.05$). This research revealed that both cell-intact and intracellular cell-free suspensions can inhibit oxidative damage, which emphasize the importance of *Lactobacillus* as a potential source of antioxidants for food supplements.

Keywords: *Lactobacillus*, Antioxidant activity, DPPH, Cell-free, Cell-intact, Probiotic properties

1. Introduction

1.1 Probiotics. Probiotics are particularly in the focus as a consequence of the increasing interest in healthy diet, which is encouraging the food industry to develop novel products in an inventive approach. Probiotics are defined as the supplement that contain live microorganisms intended to maintain normal microflora in the body. They consist of *Saccharomyces boulardi* yeast and lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* species.[1] Fermented foods including curd, cheese, cottage cheese, and yoghurt are the main sources of probiotics. Apart from being used as a starting culture, they are sometimes added as a dietary supplement to dairy products to improve quality. A study which is carried out by Jeong[2] proposed that health benefits attributed to probiotics include enhancement of immune system, cancer prevention and reduction of inflammation and allergies. Probiotics have been viewed as one of the disease control strategies to treat some of the illnesses that might affect the human body, including inflammatory bowel disease, antibiotic-induced diarrhea and irritable bowel syndrome.[3]

1.2 Lactobacillus. This research primarily focuses on *Lactobacillus* which is the traditional probiotics utilized in the food industry. The *Lactobacillus* genus has been recognized as

having several beneficial characteristics among all lactic acid bacteria. They are important members of the healthy human microbiota. *Lactobacillus* is a genus of gram positive, rod shape, catalase negative, non-spore forming bacteria that contains more than ninety species. They are used commercially during the production of cheese, yoghurt, curd and chocolate and they are distinguished by their potential to produce lactic acid as a by-product of glucose metabolism.[5]

Lactobacilli has the ability to survive gastric conditions and colonize the intestine by adhering to intestinal epithelial cells. Thus, it was proposed as a successful probiotic according to study which is carried out by Dempsey.[6] *Lactobacillus* species appear to be promising possibilities for the treatment of intestinal diseases caused by abnormal gut microbiota and altered gut mucosal barrier capabilities. Dempsey and Corr[6] reported the presence of *Lactobacillus acidophilus* and *Lactobacillus salivarius* in human gut which have the capacity to attach to and bind to intestinal brush border tissue, which is regarded to be a critical component that prevents harmful pathogens from entering the gastrointestinal mucosa.[7]

1.3 Yoghurt as a source of Lactobacillus. Since the increased demand in *Lactobacillus*, a variety of products were proposed as supplements for probiotic microorganisms allowing customers to take huge quantities of probiotic cells for the therapeutic benefit. Yoghurt has long been recognized as a *Lactobacillus* containing product which provides the consumers with wide choice of therapeutic benefits. Yogurt is a popular food consumed all over the world today and a mainstay in many diets because of its tasty flavor and possible health advantages. It is a fermented milk product obtained from the fermentation of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.[6] Lactic acid, which is produced during the fermentation process from lactose, acts on milk proteins to give yoghurt its distinctive flavor and texture. Yogurt is available in a variety of flavors and shapes to suit a wide range of tastes. While some yogurts are fruit-flavored or sweetened with different ingredients, others are plain. Greek yogurt, favored for its rich and velvety consistency, has become increasingly well-liked due to its increased protein content and adaptability. There are also dairy-free alternatives, such as coconut, almond, and soy yogurt, to accommodate those with lactose intolerance or dietary restrictions. Yoghurts can be high in Protein, Calcium, Phosphorus, Potassium, Zinc, Magnesium and B Vitamins Riboflavin, Niacin, Vitamin B-6 and Vitamin B-12 and live cultures which can enhance the gut microbiota.[8] It stimulates the gut microbial community as a source of probiotics that prevent intestinal infections, reduce lactose intolerance, and lower the risk of developing cancer. Yoghurt is also thought to have immune-modulating properties, which could help with the treatment of Inflammatory Bowel Disease, which includes gastrointestinal disorders like Crohn's disease. In the case of Crohn's disease, the immune system mistakenly attacks the gastrointestinal tract, leading to chronic inflammation.[11] Some research suggests that certain strains of probiotics found in yogurt may help modulate the immune response, reduce inflammation, and promote a healthier balance of gut bacteria. Yoghurt consumption improves insulin resistance, lowers blood glucose levels, and lowers the risk of Type 2 diabetes. By delaying the digestion and absorption of carbohydrates, yogurt's protein content can improve satiety and aid in blood sugar control. This may lead to a slower release of glucose into the blood, improving glycemic control.[10,11]

1.4 Probiotic properties of Lactobacillus. *Lactobacillus* may exert probiotic properties in different ways. Certain *Lactobacillus* species have been seen to deconjugate bile acids in the digestive tract through the activity of bile salt hydrolase proteins. By changing bile metabolism, detoxification, and the composition of the gut microbiota, it helps to decrease cholesterol levels.[12] Following ingestion, these beneficial

bacteria establish themselves in the intestines, where they are essential for preserving a well-balanced microbial environment. They adhere to the intestinal lining and compete with harmful bacteria for nutrients, keeping pathogenic microorganisms from gaining hold. This is how *Lactobacillus* strains help ease the symptoms of gastrointestinal disorders like diarrhea and irritable bowel syndrome (IBS) and lower the risk of gastrointestinal infections.[6] Furthermore, it has been demonstrated that *Lactobacillus* improves nutritional absorption and breakdown, which improves digestion. Certain *Lactobacillus* strains generate digestive enzymes that help break down complex proteins and carbs so the small intestine can absorb them more easily. There may be significant effects on overall health and vitality from this increased nutrient availability.[13] *Lactobacillus* enhances intestinal homeostasis by regulating immune response and promoting T-reg cell growth. Perdigon[14] reported that *Lactobacillus Casie* possesses probiotic property by regulating the host immune system. *Lactobacilli* are able to reduce intestinal inflammation through decreased toll-like receptor (TLR) expression, the production of metabolites that may block TNF-α from reaching blood mononuclear cells, and the regulation of NF-κB signaling in enterocytes. A recent study which is carried out by Diaz[15] demonstrated that *Lactobacillus casei and Lactobacillus plantrum* possess probiotic properties by suppressing intestinal inflammation. Apart from these *Lactobacillus* possessed antioxidant activity which can reduce the risk that Reactive oxygen Species (ROS) will be produced during the ingestion of food.[16] Aside from their traditional probiotic qualities, some *Lactobacillus* strains have demonstrated potential in a range of medicinal uses. As an example, some *Lactobacillus* strains have been investigated for their ability to produce lactase, the enzyme required for lactose digestion, which may help relieve the symptoms of lactose intolerance. This opens possibilities for the use of products based on *Lactobacillus* as a supplement to lactose intolerance treatment. In addition, recent studies indicate that specific *Lactobacillus* strains might affect mental health and cognitive function through modifying the gut-brain axis. These results give hope for the use of *Lactobacillus* in holistic approaches to enhance mental health, even if further research in this area is needed.[17]

1.5 Antioxidant activity of Lactobacillus. In the past ten years, it has become abundantly obvious that oxidative stress and antioxidative efficacy are the two key elements influencing the molecular regulation of cellular stress responses. The fundamental understanding of antioxidants is that they are classified as any substance which substantially inhibit the oxidation of the substrate, when present in low concentrations, compared to that of an oxidizable substrate.[18] Hence, its main role is to prevent damage from occurring to cellular components, which may cause a rise in a series of chemical reactions involving free radicals. Similarly, a free radical is defined as a molecular species that is callable of independent existence, consisting of an unpaired electron in an atomic orbital.[19] Oxidative stress is a condition in which DNA, proteins, and lipids are damaged as a result of abnormally high amounts of reactive oxygen species (ROS) production.[20] Reactive oxygen species such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide are certain aggressive oxygen free radicals, which influenced together with Reactive Nitrogen Species (RNS) causing tissue disruption, hence resulting in a condition called oxidative/nitrosative stress. Biological and pathological processes, including aging, inflammation, and carcinogenesis, have been linked, either directly or indirectly, to elevated ROS levels. Cells and organisms have created defense systems to protect themselves against the toxicity caused by oxidative stress. Antioxidants are substance that can reduce the damage and preserve the cells from free radicals.[20]

Zaharani and Shori[20] demonstrated that some *Lactobacilli* possess antioxidant activity,

which can lower the risk that ROS will be formed when food is consumed. ROS can cause oxidative stress and damage to cells if their levels are not properly regulated. The antioxidant activity of Lactobacilli may have potential health effects via lowering oxidative stress. Numerous chronic diseases, such as cardiovascular disease, neurological diseases, and several forms of cancer, have been linked to oxidative stress.[21] Therefore, the antioxidative activity of Lactobacilli may contribute to improving general health and lowering the chance of developing certain diseases. They possess antioxidant activity by chelating metal ions such as Fe^{2+} and Cu^{2+}. Duz[16] reported that *Lactobacillus plantarum* has exhibited hydroxyl scavenging ability by chelating the metal ions utilized to create the hydroxyl free radicals. *Lactobacillus Casie* possesses high antioxidant activity by chelating metal ions[22]. In *Lactobacillus helveticus CNBL1156*, Cappa, Cattiveli, and Cocconcelli[24] discovered the gene *uvrA*, which was involved in oxidative stress responses. The CtsR protein in *Lactobacillus plantarum* is a critical regulator of oxidative stress and also acts as a crucial enzyme for cell development at high temperatures, according to Bove *et al*[23]. *Lactobacillus* can exert antioxidant action via chelating metal ions, according to a study by Lee.[24]

Lactobacillus species have shown antioxidant activity by producing metabolites like folate, butyrate, and glutathione. According to a study carried by Rodriguez *et al*[25] daily consumption of yoghurt containing *Lactobacillus acidophilus* considerably increased the mean levels of plasma folate, indicating an improved level of oxidative balance. The two antioxidant *Lactobacillus fermentum* strains, E-3 and E-18, were identified by Kullisaar *et al.*,[26] to contain remarkably high quantities of Glutathione. *Lactobacillus* can produce antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) which can breakdown superoxide to bring about antioxidant activity. SOD is the important antioxidant in *Lactobacillus* sp. which can breakdown superoxide into Hydrogen peroxide (H_2O_2) and water, acting as a key regulator of ROS levels. Manganese Superoxide Dismutase (Mn-SOD) could be expressed by *Lactobacillus fermentum* strains E-3 and E-18 to protect against oxidative stress.[27] Low molecular-weight antioxidant compounds also serve as free radical scavengers in various non-enzymatic antioxidant defense mechanisms. Therefore, under normal circumstances, this antioxidant defense system protects the cells from oxidative damage, however, may become insufficient under excessive oxidant-generating conditions.

Some *Lactobacillus* offers antioxidant activity by regulating the enzymes that produce ROS like NOX (NADPH oxidase) and COX (Cyclooxygenase). Adikari[29] reported that antioxidant activity of *Lactobacillus rhamnosus* by inhibiting NOX production by downregulating the mRNA expression. Nrf2–keap 1-ARE, Mitogen activated protein kinase (MAPK), PKC and NF-κB antioxidant signaling pathway are mediated by *Lactobacillus spp.* to possess antioxidant activity. *Lactobacillus rhamnosus* was shown to produce the soluble proteins p40 and p75, which were effective in reducing the breakdown of the epithelial barrier caused by H_2O_2 by a MAPK-dependent mechanism and *Bacillus amyloliquefaciens* reduced the oxidative stress that H_2O_2-induced in IPEC-1 by decreasing ROS levels and regulating Nrf2 expressions.[29]

The main objective of this study was to determine the antioxidant activity of *Lactobacillus* isolated in yoghurt. The presence of *Lactobacillus* in yogurt is important because it can potentially offer therapeutic benefits related to its antioxidant properties. By understanding the antioxidant activity of *Lactobacillus*, researchers aim to explore their potential in treating illnesses induced by oxidative stress. Additionally, manufacturers can use this knowledge to produce high-quality yogurt products that provide consumers with a wide

range of health benefits associated with antioxidants.

2. Methodology

2.1 Sample preparation and culturing on MRS agar. Commercially available, five different yoghurt samples were obtained from local markets and stored aseptically at -4ºC to protect against contamination and deterioration. Samples were labelled as A, B, C, D and E and homogenized in 1mL of autoclaved distilled water in labelled beakers. This was done under aseptic conditions and covered with aluminum foil to avoid contamination. *Lactobacillus*-specific MRS agar medium was prepared. A mass of 3mg of Amphotericin B was dissolved in 12mL of autoclaved distilled water and added to medium. Samples were then streaked on MRS agar using the quadrant streak method under aseptic conditions and incubated at 37°C for 48 hours to form colonies. Subcultures were maintained in MRS broth.

2.2 Gram staining. A single colony of each sample was picked from a culture plate and a thin bacterial smear was prepared on a glass slide. It was allowed to air dry prior to heat setting. Crystal violet was added, and the stain was left for 1 minute. Gram iodine was then added and allowed to stand for 1 minute. The slides were stained using decolorizer and safranin and left for 15-30 seconds. After each staining step, the slides were rinsed with water and dried in the open air. Slides were blotted and observed under a microscope at 100x magnification.

2.3 Test for catalase. Bacterial colonies were picked from those that were used for Gram staining, were mixed with distilled water, and placed on slides. A drop of H_2O_2 was applied and the formation of oxygen bubbles was observed.

2.4 Endospore staining. Bacterial colonies were picked from those that were used for Gram staining, and smear was prepared for each samples separately. A filter paper was placed on the smear and malachite green was added. Then heated over a steam bath for few minutes. Filter paper was removed, and the slide was rinsed with water. Safranin was used to stain the slide for two minutes before being rinsed with water. The slide was finally blotted and observed under 100X magnification.

2.5 Acid fast staining. Slides were labeled as A, B, C, D & E and bacterial smear was prepared from each sample. Carbolfuchsin was added to the slide and heated for 5 minutes. After that 20% sulfuric acid was added to the slide & kept for 30 seconds and Methylene blue was added as a counterstain. After every staining step the slide was rinsed with water and dried in the open air. Finally, the slide was observed under 100X magnification.

2.6 Antioxidant activity using DPPH assay. Cell-free and cell-intact suspension of the *Lactobacillus* strains were initially prepared in order to determine the antioxidant activity using DPPH assay. This method was modified from Azat *et al*, (2016).

2.6.1. Preparation of cell-free suspension. A volume of 5 mL of subculture was transferred to the labelled falcon tubes and cells were harvested after centrifuging the tubes at 4000rpm for 10 minutes. Supernatant was discarded and 4mL of autoclave distilled water was added to the pellet followed by incubation at 100ºC for 20 minutes The tubes were immediately transferred to a freezer for 20 minutes at -20ºC. The intracellular cell-free extract was obtained by centrifuging for 10 minutes at 4000 rpm to remove cell debris. Then DPPH assay was carried out with extracted cell-free suspension.

2.6.2 Preparation of cell-intact suspension. For the preparation of intact cells, 5mL of subculture was transferred to labelled tubes followed by centrifugation at 4000 rpm for 10 minutes. Then 4mL of distilled water was added to the pellet and DPPH assay was performed.

2.6.3 DPPH assay. A mass of 5.9148mg of DPPH powder was measured and dissolved in 300mL of ethanol in a conical flask to prepare 50µL/mol DPPH solution. A volume of 1mL of sample mixed with 2mL of 50µL/mol DPPH solution and wrapped with Aluminum foil. Control was carefully covered in aluminum foil and stored in a dark spot. The test tubes were allowed to react for 30 minutes at room temperature (RT) in a dark environment. After the incubation absorbance was obtained at 517nm for both cell-intact and cell-free suspensions, blanks and controls. The percentage of DPPH scavenging activity was calculated using the equation below.

Equation 1: DPPH scavenging activity (Baliyan *et al.*,2022)

$$SA_{DPPH} = [1- (A_{sample} - A_{blank})/A_{control}] \times 100\%$$

2.9 Data analysis. Statistical analysis of antioxidant activity between cell-free and cell-intact suspensions was determined using the one-way ANOVA test in SPSS software. At a 5% level of significance, the p-value was determined. P-values < 0.05 were considered statistically significant.

3. Results

3.1 Isolation of bacterial colonies in MRS agar. The below figure depicts colony morphology of bacterial culture on MRS agar, after 48 hours of incubation at 37°C.

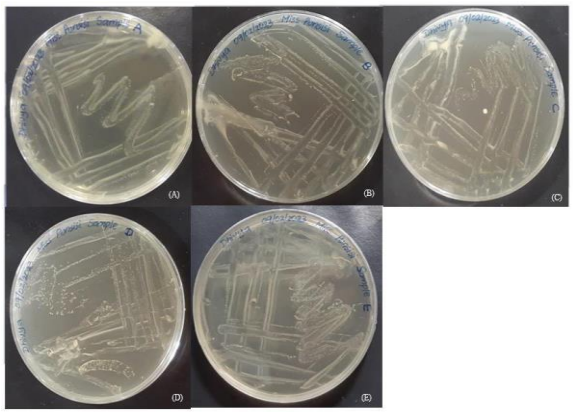

Figure 1. Colony morphology of bacterial culture on MRS agar, after 48 hours of incubation at 37°C.

As observed, until the third streak all culture samples showed growth with isolated colonies present. Creamy white, opaque, and shiny colonies with an entire edge were exhibited in all five samples and they were smooth and moist.

3.2 Gram staining. Gram staining was performed on the isolated bacterial colonies from the culture, and their morphology was observed under 100X magnification.

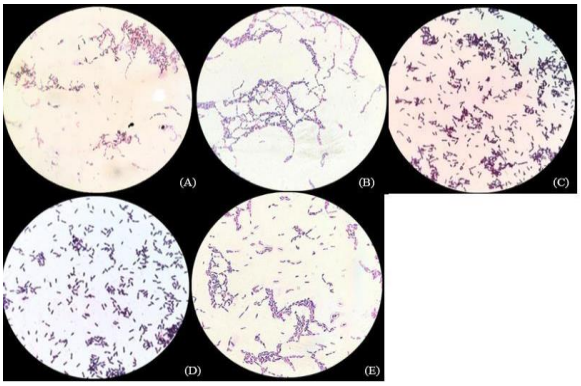

Figure 2. Gram staining of presumptive *Lactobacillus* colonies

The Gram-stain showed the presence of purple rods with round edges which were arranged in a single, double, or short chain in all five samples.

3.3 Catalase test. Figure 3 shows the catalase test results for *Lactobacillus* sample A, B, C, D & E.

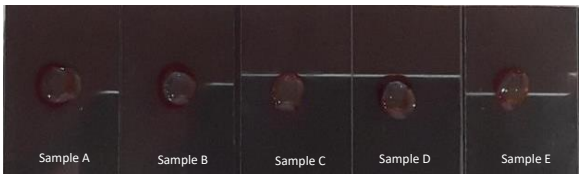

Figure 3. Catalase test for presumptive *Lactobacillus* colonies from all five samples

The colonies were randomly selected from all Petri plates for catalase test. Hence, it was noticed that none of the samples produced any bubbles, which resembled the absence of catalase enzyme.

3.4 Endospore staining. Endospore staining was performed on presumptive bacterial colonies and their morphology was observed under 100X magnification.

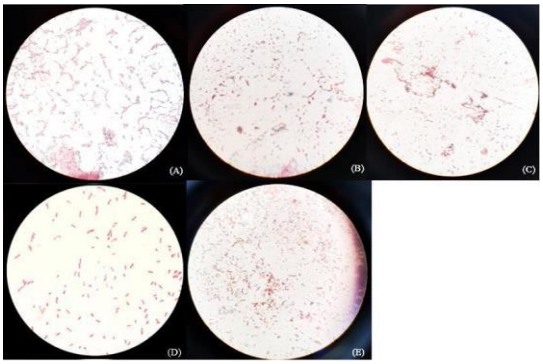

Figure 4. Endospore staining images for presumptive *Lactobacillus* colonies under 100X magnification.

The Endospore stain showed the presence of brownish red colored short rods with round edges in all five samples. Single, double, or short chains were observed as shown in figure 4.

3.5 Acid fast staining. Acid fast staining of the selected bacterial colonies observed under 100X magnification.

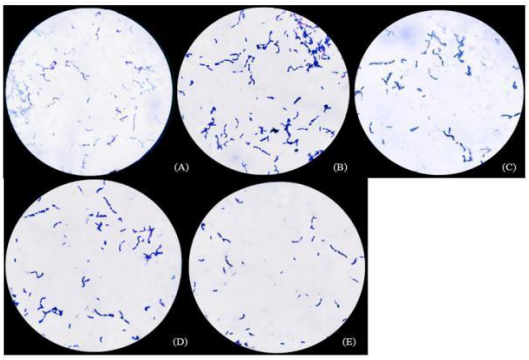

Figure 5. Acid fast staining images of selected bacterial colonies from all five samples

The Acid-fast stain showed the presence of blue colored short rods often present in chains of two or three, for all five samples.

3.6 Antioxidant Activity using DPPH Assay

3.6.1. Cell free suspension. The color of the cell-free suspensions can be observed in the images below both before and after incubation.

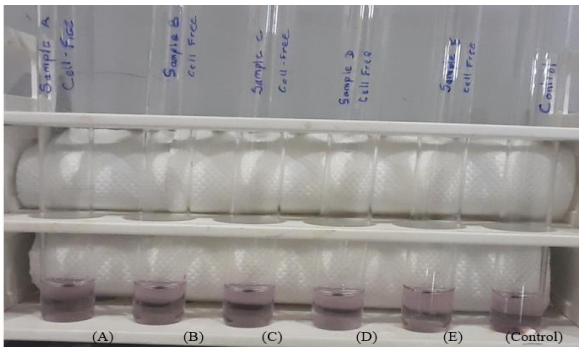

Figure 6. DPPH solution with cell free suspensions before incubation

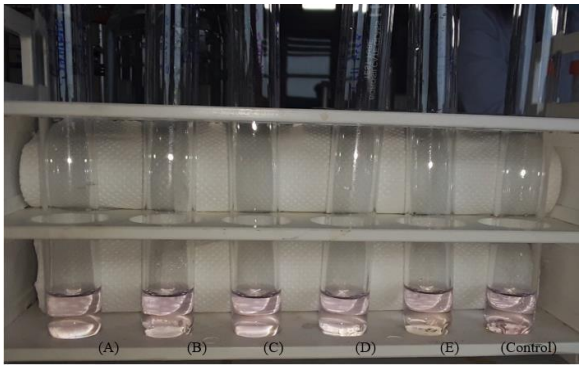

Figure 7. DPPH solution with cell free suspensions after incubation

As shown, after 30 minutes of incubation, the color of all samples changed from purple to pale yellow.

3.6.2 Cell intact suspension. The color of cell-intact suspensions before and after incubation is shown in the images below.

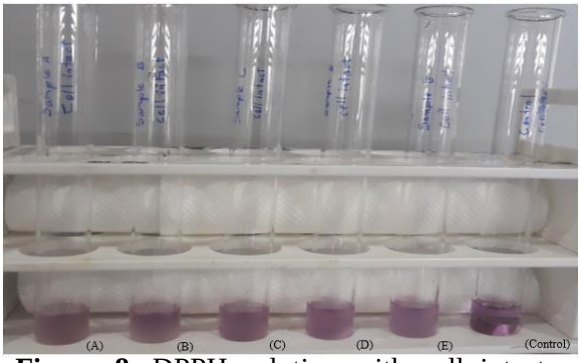

Figure 8. DPPH solution with cell intact suspensions before incubation

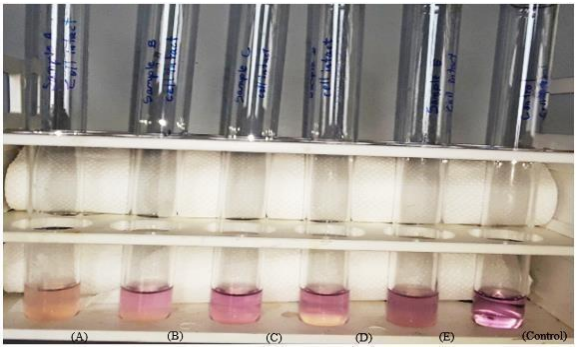

Figure 9. DPPH solution with cell intact suspensions after incubation

After 30 minutes of incubation, it was observed that all samples were changed from purple to pale yellow. However, color change was clearly observed in sample A, B and D while only slight change was observed in sample C and E.

3.7.3 Comparison of Antioxidant activity of Lactobacillus.

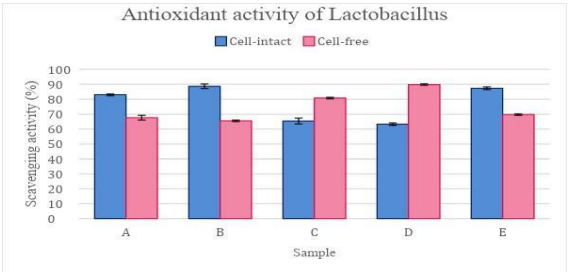

Figure 10. Comparison of antioxidant activity of *Lactobacillus*

Above figure shows the antioxidant activity of cell-free and cell-intact suspensions of Lactobacillus.

Figure 10 shows that samples A, B, and E had higher levels of scavenging activity in intact cells than in free cells, whereas samples C and D had higher levels in cell-free suspension. Comparing cell intact suspensions to cell free suspensions generally reveals higher scavenging activity.

3.8 Statistical analysis. Table 1 shows the outcomes of the one-way ANOVA analysis performed with the SPSS software to compare the antioxidant activity of cell-free and cell-intact suspensions.

Table 1. Analytical outcomes for antioxidant activity between cell-free and cell-intact suspensions

ANOVA

antioxidant activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.003	1	.003	9.525	.005
Within Groups	.008	28	.000		
Total	.010	29			

According to the outcome, the p-value is 0.05. The cell-free and cell-intact suspensions does not have statistically significant difference in its antioxidant activity.

4. Discussion

Lactobacillus spp. have received a lot of consideration for their possible probiotic effects in human health, such as regulating intestinal flora balance, lowering serum cholesterol, preventing, and lowering the risk of cancers, and revitalizing the immune system, among other things. They have been found to have antioxidative activity and can reduce the likelihood of ROS accumulation after food ingestion[30]. This research studied the antioxidant properties of *Lactobacillus* bacteria isolated from

yoghurt samples purchased from the Sri Lankan local market.

The identification of bacteria in samples was carried out using culture-based methods, which may be imprecise. Thus, the samples were first cultivated on *Lactobacillus* specific MRS agar supplemented with Amphotericin B to prevent fungal growth. Until the third streak, all cultures showed growth, with isolated colonies present. Similar to the *Lactobacillus* colony morphology described by Adikari *et al*[29]. The isolated bacteria exhibited a small circular, milky white, shiny, smooth, colony with entire margins and flat elevations when *Lactobacillus* was cultivated on MRS agar. As a result, the colonies seen in this study could be *Lactobacillus* colonies. *Lactobacillus* can be isolated, enumerated, and identified using MRS agar. Other bacterial flora is efficiently repressed by the low pH and high acetate concentrations, which promote the growth of *Lactobacillus.*[31]

Certain biochemical assays, including the Gram staining, catalase test, acid fast staining, and endospore staining, were carried out on the samples after they had been isolated in order to characterize the presence of *Lactobacillus* was present. In the Gram stain, all samples exhibited short purple rod-shaped bacteria with varying lengths (Figure 2) were detected under the microscope and it was determined that the bacteria were gram-positive. The Gram stain simply distinguishes between Gram-positive and Gram-negative bacteria based on cell wall and cell membrane permeability, thus gram-positive bacteria preserve the CV-I complex and remain purple.[31] Safranin has no effect on the color of purple gram-positive bacteria. These purple-colored rod-shaped bacteria were presumptive to be *Lactobacillus* since these observations were consistent with a previous study by Mojgani.[32] *Lactobacillus fermentum* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* with comparable morphology were found in fermented yoghurt by Tan *et al.*[33] In this study, the appearance of purple rods with rounded edges grouped into single, double, or short chain formations indicates the presence of Gram-positive bacteria in the samples. The bacteria may belong to the Lactobacillus genus, which is recognized for its rod-shaped morphology and propensity to form chains, based on their arrangement in pairs or short chains.

According to Amelia *et al.,*[34] *Lactobacilli* are selective anaerobes that favor anaerobic conditions. *Lactobacillus* is catalase negative because it detoxifies hydrogen peroxide with non-oxygen evolving peroxidase. Oxygen is occasionally used in the formation of hydrogen peroxide [H_2O_2], which is harmful to *Lactobacillus* because they lack the catalase enzyme, which breaks it down. As a result of the catalase enzyme's deficiency, *Lactobacillus* emit H_2O_2. They are released into the host's extracellular space and can be detected using proper qualitative and quantitative techniques. As a result of this study, all samples were found to be devoid of catalase enzyme (Figure 3) similar to the research carried out by Amanulla[30] in yoghurt samples for five different *Lactobacillus* spp. Thus, strains in the samples were identified as belonging to the species *Lactobacillus*.

The Endospore stain showed the presence of brownish red colored short rods with round edges in all five samples (Figure 4). Similar observation was reported in the study which was carried out by Boyanova.[35] Chamika and Weerasooriya[36] also reported the reddish rods when they were performing endospore staining on *Lactobacillus* which isolated from yoghurt. Bacterial endospores are alternative life forms that some Gram-positive bacteria develop in order to survive in unfavorable environmental conditions.[37] These endospores stained with the malachite green appear green, while vegetative cells stained with the counter dye seem red or pink, according to Rohde.[38] Therefore, the brownish red rods which were observed in figure 4 were identified as *Lactobacillus.*

The Acid-fast stain showed the presence of blue colored short rods in all five samples (Figure 5). They were observed as single, double, or short chains. According to a study which was carried out by Bayonova,[35] the observation of blue colored rods in acid fast stain indicates the acid-fast negative bacteria. Lack of lipoidal material in the cell walls of non-acid fast bacteria causes them to be quickly decolorized, resulting in colorless cells. To identify them, a counterstain, such as Methylene blue, is used, which stains the non-acid fast bacteria and allows them to appear as blue in color under the microscope. In Bergey's manual[39], *Lactobacillus* is described as an acid-fast negative. According to Chamika and Weerasooriya[40] they reported similar observation for acid-fast staining which was performed on *Lacobacillus* isolated from set yoghurt. Although *Lactobacillus* was discovered as blue colored bacillus in acid fast staining, according to Gurung *et al*[40.] Thus, the bacillus with a blue color that were seen in the acid-fast staining were identified as *Lactobacillus*. Isolated colonies from yoghurt samples were found to be gram positive, non-spore forming and catalase negative, similar to the research carried out by Mojgani, *et al.*,[32]. *Lactobacillus*-specific MRS broth promoted *Lactobacillus* growth in particular. However, this does not preclude the growth of other bacteria, alongside the *Lactobacillus* culture.

The antioxidant activities of isolated *Lactobacillus* were studied in this research using the DPPH assay. DPPH is mostly used to evaluate the antioxidant activity as it is a stable compound that can be reduced by accepting hydrogen or electrons showing color change. Figure 6, 7, 8 and 9 show the color change of cell-free and cell-intact suspensions observed before and after 30 minutes incubation under dark condition. The degree of discoloration of DPPH solution indicates the scavenging potential of the antioxidant compounds. The DPPH solution is light sensitive which turns the solution from purple to yellow in the presence of light. Therefore, it is necessary to incubate the samples in dark condition to maintain the accuracy of the result. Also, the DPPH scavenging ability of intact cells and cell free suspensions of *Lactobacillus* is shown in Figure 10. According to this, samples A, B, and E have higher scavenging activity in cell intact than in cell free suspension. Remarkably, Sample C and D showed a different result in DPPH radical scavenging activity of the cell free extract (80.78%, 89.79%, respectively) compared to that of the cell intact extract (63.34%, 65.23%, respectively). Cell free suspensions could exert higher antioxidant activity due to the enhanced accessibility of antioxidant metabolites to the substrate with oxidative properties. Cell- free suspensions exert their activity by producing various metabolites that include glutathione, butyrate, and folates or by chelation of metal ions required to initiate the oxidative stress induced oxidation.[41] Apart from these, previous studies suggest antioxidant activity of cell free suspensions is also due to the production of NADH, NADPH, Mn^{2+} and bioactive compounds. According to a study carried out by Kullisaar *et al*[26], NADH oxidase, NADH peroxidase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH) and many other internal enzymes are likely obtained after the bacterial cells are isolated and decomposed into cell-free extracts. It denotes the antioxidant capacity of bacteria.[26] In comparison to cell-free extracts, whole cells were higher at scavenging DPPH. The extracellular antioxidant components most likely to be involved are polysaccharide, peptidoglycan, and teichoic acid. The peptidoglycan layer is made up of several different glycosyl chains. These generally parallel strands are joined together by peptide bonds formed between the side chains of amino acids. The cell walls of gram-positive bacteria frequently contain the amino acid teichoic acid, which is provided. A study which was carried out by Herreros.[42] further supported the antioxidant activity of *Lactobacillus* cell-surface proteins or

polysaccharides. Previous studies suggest that cell intact suspensions could exert antioxidant activity against 4NQC, a potent carcinogen that can cause DNA damage and cytotoxicity. This proves that the presence of *Lactobacillus* bacteria as viable bacteria in food products is also essential in certain circumstances. Cell free suspensions of *Lactobacillus* can exhibit antioxidant properties in vitro and in vivo despite the acidic environment of the gut. A study by Kaizu *et al.*,[43] demonstrated that cell free suspensions of *Lactobacillus* are potent enough to exert anti oxidative effects in rats who are deficient in an anti-oxidative metabolites of cell free suspensions beyond cell intact suspensions for their anti-oxidative effect.[43] The antioxidant activities of lactic acid bacteria would be helpful in the dairy food industry. They could beneficially influence the customer by providing *Lactobacillus* with the potential of producing antioxidants during the period of growth in the intestinal tract or providing another dietary source of antioxidants.

The p-value was 0.05, according to the statistical analysis provided in table 1. This could imply that the antioxidant activity mediated by cell-free, and cell intact suspension techniques was not statistically significant. However, both suspensions showed antioxidant activity.[48] Intake of foods which contain *lactobacillus* with antioxidant properties might work against the production of free radicals to prevent harmful diseases such as cancers, cardiovascular diseases and neurologic diseases.

5. Conclusion

In conclusion, the presence of *Lactobacillus* in all yoghurt samples was demonstrated using biochemical, staining, and molecular techniques. Cell intact suspensions and cell-free suspensions contain the unique antioxidant properties of *Lactobacillus*. These antioxidant properties which serve to neutralize free radicals, can be considered as a promising treatment for oxidative stress induced diseases.

Acknowledgements

Authors would like to acknowledge Business Management School (BMS) for the opportunity to conduct the research project.

References

1. M.U. Hassan, H.Nayab, T.U.Rehman, M.P.Williamson, K.U.,Haq, N. Shafi and F.Shafique. *BioMed Research International*. 2020.
2. J.J. Jeong, H.J. Park, M.G. Cha, E.Park, S.M. Won, R.Ganesan, H. Gupta, Y.A. Gebru, S.P. Sharma, S.B. Lee, and G.H. Kwon, *Microorganisms*, 2022;**10**(2);288.
3. A.A. Amara and A. Shibl, Saudi *Pharmaceutical Journal*, 2015;**23**(2);107-114.
4. L.Rudzki, L. Ostrowska, D.Pawlak, A. Małus, K. Pawlak, N. Waszkiewicz, and A. Szulc. *Psychoneuroendocrinology*, 2019;**100**;213-222.
5. S. Karami, M. Roayaei, H. Hamzavi, M.Bahmani, H. Hassanzad-Azar, M. Leila and M. Rafieian-Kopaei. *Int J Pharm Investig.* 2017;**7**(3);37-141.
6. E. Dempsey and S.C. Corr. *Front Immunol*, 2022;**6**(13).
7. R.M.Martinez, K.G. Hulten, U.Bui, and J.E. Clarridge. *Journal of Clinical Microbiology*, 2014;**52**(1);30-36.
8. Z. Zhai, S. Hu, L. Zhong, Z. Lu, X. Bie, H. Zhao, C. Zhang, and F. Lu. *Journal of Food Protection*, 2019;**82**(8);1292-1299.
9. J. Plaza-Diaz, C. Gomez-Llorente, L.Fontana, and A. Gil. *World Journal of Gastroenterology*, 2014;**20**(42).
10. A.B. Shori, G.S. Aljohani, A.J. Al-zahrani, O.S. Al-sulbi, and A.S. Baba. *LWT*, 2022;**153**.
11. S.M. Lim, N.K. Lee, K.T. Kim and H.D.Paik. *Microbial Pathogenesis*, 2020;**147**.
12. J.Minj, P. Chandra, C. Pauln and R.K. Sharma. *Food Science and Nutrition*, 2020.
13. W. Fong, Q. Li, and J. Yu. *Nature News*.2020.
14. C. M. Galdeano and G. Perdigón. *Clin Vaccine Immunol.* 2006;**13**(2);219-226.
15. J. Plaza-Diaz, F.J. Ruiz-Ojeda, M. Gil-Campos, and A. Gil. *Adv-Nutr*, 2019.
16. M. Duz, Y.N. Dogan and I. Dogan. *An Acad Bras Cienc*. 2020;**7**(92).
17. X. Zhao, X. Zhong, X. Liu, X. Wang and X. Gao. *Nature Communications*, 2021.
18. I.S. Young and J.V. Woodside. *Journal of Clinical Pathology*, 2021;**54**(3);176-186.
19. X. Luan, M. Feng and J. Sun. *Food Research International*, 2021;**144.**
20. A.J.A. Zahrani and A.B. Shori. *LWT*, 2023;**176.**
21. J. Feng, Y. Jiang, M. Li, S. Zhao, Y. Zhang, X. Li, H. Wang, G. Lin, H. Wang, T. Li and C.Man. *SpringerLink*, 2018.

22. F. Cappa, D. Cattivelli, and P.S. Cocconcelli. *Research in Microbiology*, 2005;**156(**10); 1039–1047.
23. P. Bove, A. Gallone and P. Russo. *Appl Microbiol Biotechnol*, 2012;**96**;431–441.
24. A.L.C.N. Lee, M.N. Lani, R. Alias and Z. Hassan. *UMT University, Malaysia Terengganu Journal of Undergraduate Research*, 2019;**2**;1-7.
25. L.G.R. Rodriguez, F. Mohamed, J. Bleckwedel, R. Medina, L.D. Vuyst, E.M. Hebert and F. Mozzi. *Frontiers in Microbiology*, 2019.
26. T. Kullisaar, M. Zilmer, M., Mikelsaar, T. Vihalemm, H. Annuk, C. Kairane and A. Kilk. *Int J Food Microbiol*., 2002;**72(**3);215-224.
27. T. Feng and J. Wang. *Gut Microbes*, 2020;**12**(1).
28. W. Tang, Z. Xing and W. Hu. *Appl Microbiol Biotechnol*, 2016;**100**;7193–7202.
29. A.M.M.U. Adikari, H. Priyashantha, J.N.K. Disanayaka, D.V. Jayatileka, S.P. Kodithuwakku, J.M.A.S. Jayatilake and J.K. Vidanarachchi. *CellPress*, 2021;**7**(10).
30. M. Amanullah, M. Kabir, M. Rahman, S. Hossain, P Halder and M. Samad. *Bangladesh Journal of Livestock Research*, 2021.
31. R. Fevria and I. Hartanto. *Journal of Physics: Conference Series, IOP Science*, 2019.
32. N. Mojgani, F. Hussaini and N. Vaseji Jundishapur. *J Microbiol*., 2015;**8(**2).
33. R. Yi, F. Tan, X. Zhou, J. Mu, L. Li, X. Du, Z. Yang and X. Zhao. *Front Microbiol*, 2020;**3**(11),573586.
34. R. Amelia, K. Philip, Y.E. Pratama and E. Purwati. *E. Food Sci Technol*, 2020.
35. L. Boyanova. *Postgraduate Medicine*, 2018; **130**(1);105-110.
36. S.N.T. Chamika and P.R. Weerasooriya. *GARI International Journal of Multidisciplinary Research*, 2021;**7**(2).
37. Oktari, Y. Supriatin, M. Kamal and H. Syafrullah. *Journal of Physics: Conference Series*, 2017.
38. M. Rohde. *Wiley*, 2019;**40**(2);3-18.
39. D.H.I. Bergey and J.G. Holt. *Bergey's manual of determinative bacteriology*, 1993, 9th ed.
40. R. Gurung, R. Shrestha, N. Poudyal and S.K. Bhattacharya. *Journal of BP Koirala Institute of Health Sciences*, 2018;**7**(2);59-66.
41. S. Baliyan, R. Mukherjee, A. Priyadharshini, A. Vibhuti, A. Gupta, R.P. Pandey and C.M. Chang. *Molecules*, 2022;**27**(4);1326.
42. M.A. Herreros, J.M. Fresno, M.J.G. Prieto and M.E. Tornadijo. *International Dairy Journal*, 2003;**13(**6);469-479.
43. H. Kaizu, M. Sasaki, H. Nakajima and Y. Suzuki. *Journal of Dairy Science*, 1993;**76(**9);2493-2499.