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Assessment of Probiotic Attributes and Antioxidant Profiling of Bacteria Isolated from Probiotic Milk "YAKULT"

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Abstract - Probiotic beverages such as Yakult are well-known for their health-promoting properties, primarily due to the presence of beneficial bacteria. In this study, six bacterial isolates were successfully obtained from the commercial probiotic drink Yakult. These isolates were subjected to a series of evaluations to determine their antioxidant capabilities, a key attribute in combating oxidative stress and supporting overall health. Among the six isolates, isolate 3 demonstrated significantly higher antioxidant activity compared to the others, suggesting its potential as a strong candidate for therapeutic and functional food applications. Additionally, isolate 3 exhibited promising probiotic characteristics, including the ability to survive in acidic and bile salt conditions, which are essential traits for colonization and activity in the gastrointestinal tract. These preliminary findings highlight the potential of isolate 3 as a functional probiotic strain with notable antioxidant properties. However, to validate its efficacy and safety for human consumption, further in vivo studies are required. These future investigations will help confirm its physiological benefits and potential for incorporation into health-promoting products. The results of this study provide a foundation for the development of enhanced probiotic formulations with added antioxidant benefits.

***Key Words*:** Probiotic, Yakult, Bacterial isolates, Antioxidant activity, Isolate 3, In vivo studies

1.INTRODUCTION

Probiotics are live microorganisms that, when administered in adequate amounts, provide health benefits to the host. Many strains exert these effects by temporarily colonizing the human gut, where they interact with existing intestinal microorganisms and the host's mucosal surfaces. The human gut contains a vast population of microorganisms, collectively known as the gut microbiota (Lorena Ruiz et al., 2016). Probiotics are defined as viable microorganisms that reach the intestine in sufficient quantities and in an active state, thereby promoting health. Numerous probiotic strains, such as Lactobacillus rhamnosus GG, L. reuteri, bifidobacteria, and certain strains of L. casei and L. acidophilus group, are commonly found in probiotic foods, particularly in fermented milk products (Michael de Vrese and J. Schrezenmeir, 2008).

Probiotics are gaining significance in both basic and clinical research and are of growing commercial interest due to their increasing popularity. Unlike conventional drugs, probiotics may be useful in healthy individuals to reduce the risk of disease or enhance physiological functions (F. Pace et al., 2015). Recognized for their health benefits, probiotics are now widely used as dietary supplements. There is growing interest in identifying probiotic strains with antioxidant properties alongside their other health-promoting attributes (Vijendra Mishra et al., 2015).

Fermented milk products, such as kefir, have drawn attention from researchers in healthcare, industry, and pharmaceuticals. Studies have shown that dietary probiotics like kefir, a fermented milk originating from the Caucasus and Tibet, may have cancer-preventive and therapeutic effects (Mohammadreza Sharifi et al., 2017).

Oxidative stress arises when the balance between pro-oxidants and antioxidants in cells is disrupted, leading to damage to DNA, proteins, lipids, and even cell death. Probiotics have been shown to mitigate oxidative stress by exhibiting antioxidant activity, thus helping to prevent oxidative damage (Yang Wang et al., 2017). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated during aerobic metabolism or inflammation, are key contributors to oxidative stress. The skin, as a protective barrier, plays a critical role in

defending against such oxidative damage (Jinok Baek and Min-Geol Lee, 2016).

ROS—including superoxide anions, hydroxyl radicals, and hydrogen peroxide—can damage cellular components. Although organisms have enzymatic (e.g., superoxide dismutase, glutathione peroxidase) and non-enzymatic (e.g., vitamin C, vitamin E) antioxidant defenses, these systems are often insufficient to fully prevent oxidative damage. While synthetic antioxidants like butylated hydroxyanisole are used, concerns about their safety have led to a preference for natural alternatives (Yang Wang et al., 2017).

Oxygen is essential for energy production but also generates ROS, creating toxic stress. When ROS production exceeds the cell's antioxidant capacity, oxidative stress results (Jorge Limón-Pacheco and María E. Gonsebatt, 2009). ROS and RNS play dual roles—they contribute to cancer progression but can also induce cellular senescence and apoptosis, thus acting as anti-cancer agents (M. Valko et al., 2006).

Radiation therapy, though effective in treating cancer, can damage surrounding healthy tissues and disrupt gut microbiota, leading to long-term gastrointestinal complications (Basileios G. Spyropoulos et al., 2011). Probiotics—beneficial microorganisms such as Lactobacillus and Bifidobacterium—along with prebiotics, form synbiotics that help restore microbial balance, enhance gut integrity, inhibit pathogens, and support immune function (V. Gupta and R. Garg, 2009; Jai K. Kaushik et al., 2009 & Koushik, A. K., & Alva, R. C. (2021).). These strains must meet key safety and functional criteria, including resistance to acid and bile, antibiotic susceptibility, and antimicrobial activity (Kridsada Unban et al., 2021). Probiotics can combat oxidative stress by producing antioxidant compounds like vitamins C and E, superoxide dismutase (SOD), and lactic acid, which help neutralize reactive oxygen species (ROS) (Yadav et al., 2007; De Vries et al., 2006). This antioxidant potential is significant in preventing diseases such as diabetes, atherosclerosis, and inflammatory conditions (Arkadiusz Hoffmann et al., 2019). Moreover, probiotics may suppress harmful oral bacteria like Streptococcus mutans, reducing biofilm formation and dental caries (Reham Wasfi et al., 2018). Although studies suggest that strains like Lactobacillus rhamnosus GG can modulate immunity, reduce inflammation, and enhance chemotherapy outcomes, no probiotic has yet been approved by regulatory agencies such as the FDA for cancer treatment. Thus, while promising, probiotics should be considered complementary to a healthy lifestyle and used under medical guidance, especially during cancer therapy (Chen Y.P. et al., 2019).

2. Materials and Methods

Sample Collection and isolation of bacteria

The "yakult" was purchased from the dmart supermarket and stored in the optimal 4℃ temperature of "temperature". The purchased probiotic milk was opened in the aseptic condition in the laminar air flow chamber. The bacteria were isolated using serial dilution method. Briefly, 1 ml of the probiotic milk was diluted in 9 ml of sterile distilled water which is labelled as 10-1 dilution. Then from this tube, 1 ml was transferred to the next tube with 9 ml of sterile distilled water, i.e., 10-2 dilution. The step is repeated till 10-6 dilutions. From this 1 ml of the sample was plated in the sterile nutrient agar plates using spread plate technique and incubated at 28 ℃ for 24 h. The bacterial isolates were purified using quadrant streaking method and stored in glycerol stock at -20 ℃ until further use.

Antioxidant property

DPPH radical scavenging assay

- 1 ml of Intact cells/ Cell free supernatant + 0.2 mM of freshly prepared DPPH solution, mixed thoroughly and was incubated for 30 min in dark condition. Deionised water was used as the blank. The decrease in absorbance at OD517 was

$$\text{DPPH scavenging \%} = 1 - \frac{\text{sample OD}}{\text{blank OD}} \times 100$$

used to monitor the scavenging of DPPH by the

$$\text{Hydroxyl radical scavenging \%} = \frac{(\text{sample} - \text{blank})}{(\text{control} - \text{blank})} \times 100$$

isolates.

- The DPPH free radical scavenging assay was done in triplicates. The mean value and ± Standard deviation were calculated from three parallel experiments.

Free radical scavenging ability of selected strain

Hydroxyl radical scavenging assay

- 1 ml intact cells and CFS in different tubes were added with 1 ml of 2.5 mM 1, 10-Phenanthroline, 1 ml of PBS (pH 7.4), and 1 ml of 2.5 mM FeSO4 and mixed well.
- Addition of 1 ml of 20 mM H2O2 starts the reaction and then the mixture is incubated at 37 ˚C for 90 min. The ability of the strain to scavenge hydroxyl radical was then determined

by measuring the increase in absorbance of the sample at OD536. The scavenging ability was calculated as follow:

- The hydroxyl radical scavenging activity of I7B strain were done in triplicates. The mean value and ± Standard deviation were calculated from three parallel experiments.

Hydrogen peroxide

A solution of hydrogen peroxide (40Mm) was prepared in phosphate buffer 0.1 ml of various concentration of broth culture 1ml in 1.6ml phosphate buffer (ph 7.4) was added to 0.6ml of 40 nm hydrogen peroxide solution.

The absorbance value of the reaction mixture was recorded at 230nm

The percentage of hydrogen peroxide scavenging of broth culture was calculate using the formula:

$$\% = \frac{(\text{absorbance of the sample} - \text{absorbance of the control})}{\text{absorbance of the control}}$$

In vitro analysis of probiotic of selected strain

The acid tolerance test

To determine acid tolerance ability of potent 17B strain in the host gastrointestinal tract, acid tolerance test was performed by Pradhan and Tamang. (2020). The potent 17B strain was inoculated in 10 ml of nutrient broth sterilized in autoclave at 121℃ for 15 min and incubated at 37℃ for 12 hours. Approximately 1x 108 CFU/ml of 12 hours old culture is inoculated at 37℃. The bacteria growth count was performed at 0,1,2, and 3 hours interval by inoculating 10µl of culture using spread plate technique in the sterile agar plate prepared by nutrient agar sterilized at 121℃ for 15 min and incubated at 37℃ for 24 hours.

$$\text{Cell viability (\%)} = \frac{\text{log CFU of viable cells survived}}{\text{Log CFU of initial cells}} \times 100$$

The acid tolerance test was done in triplicates. The mean value and = standard deviation were calculated from three paralle experiment.

Aggregation activity

Auto-aggregation and co-aggregation of potent 178 strain was assessed in accordance with the method of Breyer et al. (2021). Pathogenic strain (Staphylococcus aureus) and the selected 17B strain was inoculated separately in the nutrient broth sterilised at 121 °C for 15 min and incubated at 37 °C for 18 hours and then the cells were harvested by centrifugation at 10,000 rpm for 10 min. The cells were washed twice and resuspended in PBS of approximately 1 x 10" CFU/ml was used for the experiment. For determining auto-aggregation ability, 4 ml of the isolate suspension was individually added into sterile tubes and suspended thoroughly. The tubes were placed at room temperature with no agitation and a 150 µl aliquot of the upper suspension was taken after an incubation of 5 hours. The absorbance was determined at 600nm by using a microplate reader. Percentage Auto aggregation was calculated using the formula:

$$\text{Autoaggregation (\%)} = 1 - \frac{A0}{At} \times 100$$

Where Ao corresponds to the absorbance at 0 h, and A. corresponds to the absorbance of upper suspension after 5 hours.

For determining the coaggregation of isolated strains with the pathogens, equal amounts (2 ml) of isolates and pathogen cultures were mixed in sterile tubes and vortexed thoroughly. These tubes were placed at room temperature with no agitation and a 150 µl aliquots of upper suspension was taken at a time interval of 0 and 4 hours and the absorbance was determined at 600 nm absorbance by using a microplate reader. Coaggregation percentage was calculated as follows:

$$\text{Coaggregation percentage (\%)} = \frac{(\text{A pat} + \text{A probio}) - 2 \times \text{A mix}}{(\text{A pat} + \text{A probio})} \times 100$$

Where A pat is the absorbance of the pathogens and A probio are the absorbance of the isolate at 0 hour and A mix is the absorbance of the mixed suspensions at 4 hours.

The auto-aggregation and co-aggregation activity of 17B strain were done in triplicates. The mean value and Standard deviation were calculated from three parallel experiments.

3. Results and Discussions

Sample Collection and isolation of bacteria

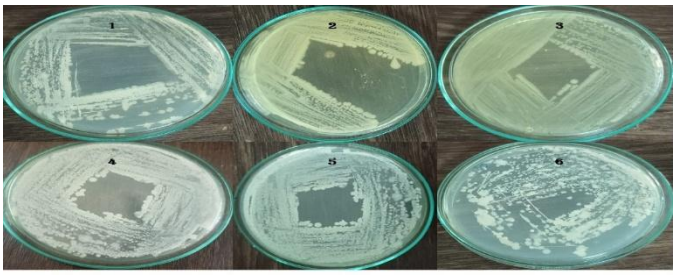

Fig -1: Isolation and preservation of bacterial strains from Yakult showing distinct colony morphology and glycerol stock preparation.

A total of six distinct bacterial isolates were successfully obtained from the commercial probiotic drink Yakult. These isolates were designated as Isolate 1, 2, 3, 4, 5, and 6, respectively. The isolation was carried out under sterile conditions, and each bacterial colony was subjected to purification using the quadrant streaking method. This approach ensured the acquisition of pure single colonies, which is essential for reliable downstream analyses.

Post-purification, all isolates were preserved in 15% glycerol stocks and stored at –4 °C for long-term maintenance. Glycerol acts as a cryoprotectant, preventing cell damage during freezing and thawing cycles, thereby maintaining the viability of the isolates for future experiments. This method is a widely accepted and effective technique for preserving microbial cultures over extended periods without significant loss of functional properties.

The successful isolation and storage of these bacterial strains provide a valuable foundation for further characterization. These isolates can now be investigated for their probiotic properties, including acid and bile tolerance, antimicrobial activity, and antioxidant potential. Such studies are essential to identify promising strains for therapeutic or functional food applications. Particularly, understanding strain-specific differences will help in selecting the most a ve candidates for in vivo and clinical studies.

Antioxidant property

DPPH radical scavenging assay

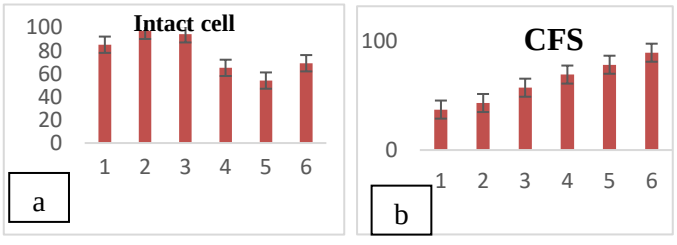

Fig-2: a) DPPH radical scavenging activity of intact cells of six bacterial isolates showing highest activity in Isolate 2 & 6. b) DPPH radical scavenging activity of CFS cells of six bacterial isolates showing highest activity in Isolate 2 & 6.

The antioxidant potential of all six bacterial isolates was assessed using the DPPH radical scavenging assay in both whole cells and cell-free supernatants (Fig-2 a,b). The results revealed that all isolates exhibited higher DPPH activity in the cell-free supernatant compared to their intact cells, indicating that the antioxidant compounds are likely secreted into the extracellular environment.

Among the intact cells, Isolate 2 demonstrated the highest DPPH scavenging activity, followed by Isolates 3, 1, 6, 4, and 5, respectively. This suggests that certain strains possess cell-bound antioxidant components, although to a lesser extent than their extracellular counterparts. In contrast, the cell-free supernatants showed a different trend, with Isolate 6 exhibiting the highest antioxidant activity, and Isolate 1 displaying the lowest.

These findings suggest that the antioxidant activity is both strain-dependent and significantly influenced by the release of metabolites into the surrounding medium. The superior activity in cell-free supernatants highlights the potential of these isolates, especially Isolate 6, in producing extracellular antioxidant compounds, which could be further explored for therapeutic or functional food applications.

Hydroxyl radical scavenging assay

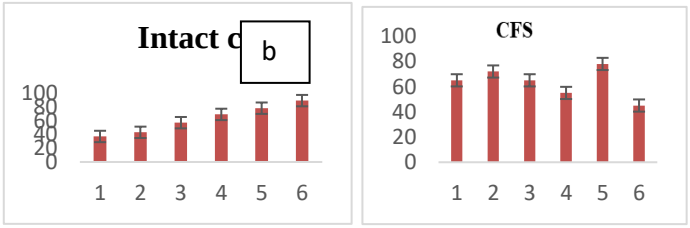

Fig – 3: a) Hydroxyl radical scavenging activity of intact cells from six isolates. b) Hydroxyl radical scavenging activity of cell-free supernatants (CFS) from six isolates.

The hydroxyl radical scavenging activity of both intact cells and cell-free supernatants of six bacterial isolates was assessed. The results revealed that all isolates exhibited higher hydroxyl radical scavenging activity in their intact cell form compared to their respective cell-free supernatants (Fig-3 a,b). . Among the intact cells, Isolate 6 demonstrated the highest hydroxyl radical

scavenging ability, followed by Isolates 5, 2, 3, 4, and the least activity observed in Isolate 1. In the case of cell-free supernatants, Isolate 5 showed the greatest activity, followed by Isolates 2, 3, 6, 4, and 1, in descending order.

These findings suggest that the structural integrity of bacterial cells may play a significant role in their hydroxyl radical scavenging ability, possibly due to cell-bound antioxidant compounds. The reduced activity in the cell-free supernatant indicates that extracellular antioxidant components are comparatively less effective or present in lower concentrations. Overall, the antioxidant potential appears to be strain-specific, highlighting the importance of selecting appropriate probiotic strains with potent intracellular antioxidant activity for therapeutic and nutraceutical applications.

Hydrogen peroxide

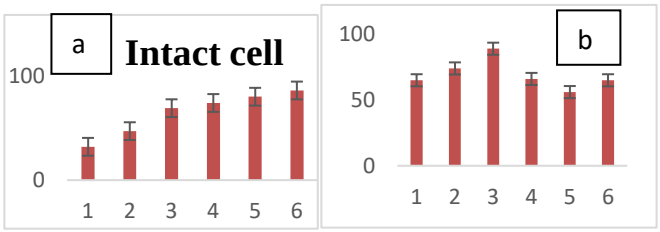

Fig – 4: a) Hydrogen peroxide scavenging activity of intact probiotic cells across six isolates. b) Hydrogen peroxide scavenging activity of cell-free supernatant (CFS) across six isolates.

The hydrogen peroxide scavenging activity was evaluated for both whole cell and cell-free supernatant (CFS) of six different isolates. The results demonstrated that all six isolates exhibited higher hydrogen peroxide scavenging activity in the CFS compared to their corresponding intact cells, indicating that the antioxidant compounds responsible for this activity are more abundant or more active in the extracellular environment (Fig-4 a,b)..

Among the intact cells, isolate 6 showed the highest hydrogen peroxide scavenging activity, followed by isolates 2, 3, 5, 4, and 1 in descending order. Conversely, in the CFS, the highest activity was observed in isolate 3, while isolate 5 exhibited the lowest scavenging ability. This trend further supports the notion that secreted metabolites or enzymes in the supernatant significantly contribute to the overall antioxidant potential.

The enhanced activity in the CFS may be attributed to the secretion of antioxidant compounds such as catalase, peroxidase, or other reactive oxygen species (ROS)-neutralizing enzymes by probiotic strains. These findings align with earlier studies suggesting that certain probiotic strains can release bioactive compounds capable of reducing oxidative stress. The variation among isolates also suggests that strain-specific properties play a critical role in antioxidant effectiveness, underlining the importance of selecting potent strains for potential therapeutic or functional food applications.

In vitro analysis of probiotic of selected strain

The acid tolerance test

Isolates	1st Hour	2nd Hour
1	**67**	**72**
2	**59**	**65**
3	**67**	**75**
4	**68**	**80**
5	**36**	**58**
6	**58**	**65**

Table 1: Acid tolerance activity of probiotic isolates at 1 and 2 hours of incubation.

The acid tolerance assay demonstrated that all selected probiotic isolates exhibited the ability to survive under acidic conditions, indicative of their potential to withstand the harsh gastric environment. Among the six isolates tested, the 4th strain showed the highest survival rate at the 1-hour mark, suggesting early adaptation to low pH. Notably, an increase in activity was observed in the 2nd hour across most strains, indicating enhanced acid resistance over time. The 5th strain consistently showed the lowest tolerance at both time intervals, suggesting it may be less suitable for surviving gastrointestinal passage. Overall, these findings support the potential of certain isolates, particularly strain 4, for probiotic application due to their strong acid resistance.

Co aggregation

Isolates	1st Hour	2nd Hour	3rd Hour	4th Hour
1	**84**	**80**	**75**	**67**
2	68	74	58	49
3	57	54	52	43
4	80	89	90	69

Table 2: Co-aggregation activity of probiotic isolates measured over 1 to 4 hours of incubation.

The co-aggregation ability of probiotic strains is crucial for inhibiting pathogenic microorganisms through competitive exclusion and biofilm formation. The results of the co-aggregation assay revealed time-dependent variability among the isolates. At the 1st hour, the highest co-aggregation activity was observed in strain 1, suggesting a strong initial interaction with other microbial cells. However, from the 2nd to 4th hour, strain 4 consistently exhibited the highest co-aggregation potential, indicating its robust and sustained ability to associate with other microorganisms over time. In contrast, strain 3 showed the lowest co-aggregation across all time points, implying limited interaction capacity. These findings suggest that strain 4 holds significant promise as a probiotic candidate due to its strong and prolonged co-aggregation ability, which may contribute to its efficacy in preventing colonization by harmful pathogens.

3. CONCLUSIONS

In this study, six bacterial isolates were obtained from the probiotic drink “Yakult” and assessed for their antioxidant and probiotic properties. All isolates exhibited varying levels of antioxidant activity through DPPH, hydroxyl radical, and hydrogen peroxide scavenging assays. Notably, antioxidant activity was generally higher in the cell-free supernatants than in the intact cells, indicating that these bacteria may release active compounds capable of neutralizing free radicals.

Among the six isolates, isolate 3 demonstrated consistently strong performance across both antioxidant and probiotic parameters. It showed good acid tolerance, effective co-aggregation ability, and strong antioxidant activity, suggesting that it may be a promising candidate for functional probiotic applications.

These findings highlight the potential of naturally occurring probiotics from commercial products in contributing to health-promoting benefits, especially through antioxidant mechanisms. However, further in vivo studies and molecular characterization are essential to confirm the safety, efficacy, and mechanisms of action of isolate 3 under physiological conditions. Overall, this study provides a foundational step toward developing probiotic strains with enhanced functional properties for possible use in nutraceuticals and therapeutic formulations.

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