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# Isolation and characterization of beneficial bacteria from probiotic drinks and assessment of their antibacterial activity against natural and artificial sugars

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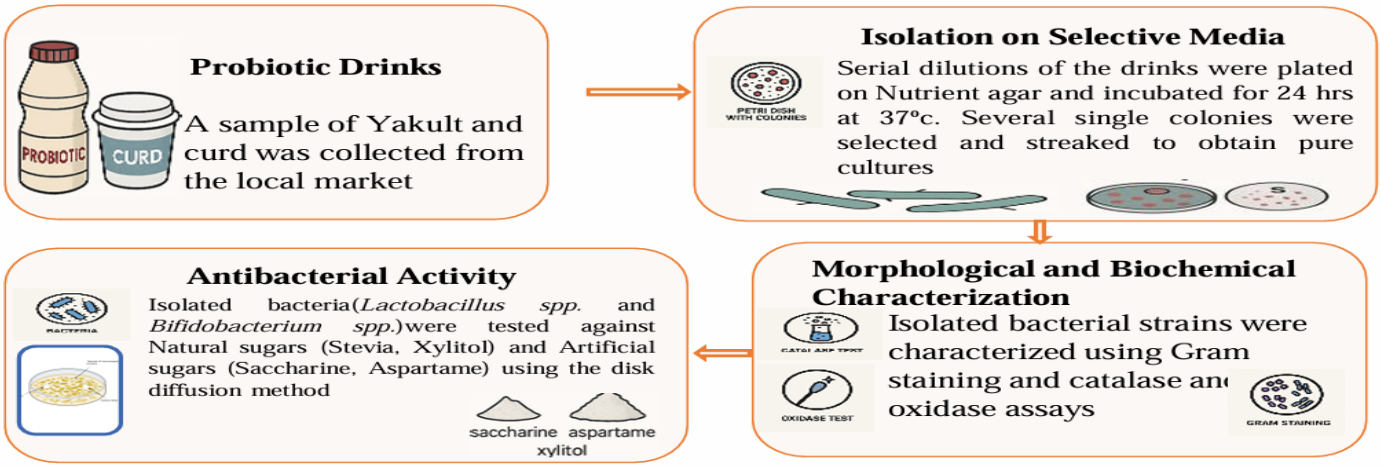
## ABSTRACT

Probiotic drinks are well-known fermented beverages that include live strains of yeast and helpful bacteria. These beverages are well-known for their possible health advantages, which include strengthening the immune system and enhancing digestion. The probiotic potential of the bacterial strains found in probiotic drinks makes them particularly interesting. Isolating bacteria from probiotic beverages, characterizing them biochemically, and assessing their antibacterial effectiveness against both natural and artificial sugars are the objectives of this study. Probiotic yogurt and Yakult samples will be collected from the local market. Nutrient agar plates were plated with serial dilutions of each beverage, which were then incubated for 24 hours at 37°C. Gram staining, catalase test, and oxidase test were performed to observe the isolated colonies for their morphology and characterize the isolated bacterial strains. Antibacterial Activity testing on the isolated bacteria was performed by the double disc diffusion method to determine their resistance patterns. Gram staining, catalase, and oxidase assays were among the biochemical tests used to describe the isolated bacterial strains. The disc diffusion method was utilized to ascertain the bactericidal activity of the isolated bacteria against both natural sugars (stevia and xylitol) and artificial sugars (saccharine and aspartame). Bacterial strains were isolated from the probiotic drinks, including Lactobacillus spp., Bifidobacterium spp. The biochemical tests confirmed the identity of the bacterial strains. The antibacterial activity of the isolated strains varied against different sugars. Lactobacillus spp. showed the highest antibacterial activity against stevia and xylitol, while Bifid bacterium spp. showed maximum antibacterial activity against saccharine and aspartame.




**Keywords:** Antibiotics, Drug discovery, Artificial intelligence, Innovation, Clinical trials.

## INTRODUCTION

Probiotics are live microorganisms that are thought to have health advantages when ingested in sufficient doses. Probiotic drinks, kefir, yogurt, and other fermented foods and beverages frequently contain them. In particular, probiotic drinks have gained popularity as a simple method of incorporating beneficial bacteria into one's diet. However, since some of the bacterial strains linked to these drinks might vary greatly, they might only be helpful to the extent that they survive in the gastrointestinal environment and have antimicrobial effects against hazardous pathogens [1].

Drinks that have undergone fermentation and include live yeast and beneficial bacteria are called probiotics. These beverages have gained popularity because of their possible health advantages, which include immune system stimulation and digestive tract support. The probiotic potential of the bacterial strains found in probiotic drinks is one particularly noteworthy statistic. This study aims to separate bacteria from probiotic drinks, identify them biochemically, and evaluate how well they work as antibacterial agents against both natural and artificial sugars [2].

Probiotic drinks have recently attracted attention due to their associated health benefits, mainly attributable to the presence of beneficial bacteria, including the lactic acid bacteria (LAB) and Bifidobacterium species. These microorganisms help promote gut health and modulate the immune system, and they exhibit antimicrobial activity against various pathogens. The composition of sugar, such as natural (stevia or xylitol) and artificial (aspartame or saccharin), in addition to that in probiotic beverages, can lead to differences in bacterial growth, metabolism, and antibacterial activity. For the development of probiotic formulations and to enhance the functional properties, it is essential to know how the probiotic bacteria grow on other sugars and what the antibacterial effect looks like under those conditions [3].

**Importance of Probiotic Bacteria and Their Role in Health:**

The Role and Importance of Probiotic Bacteria in Health. Probiotic bacteria are small living things that, when administered appropriately, provide their host with healthy effects. These useful microorganisms are the ones that are the representatives of the genera Lactobacillus, Bifidobacterium, and Saccharomyces, and they play a crucial role in the maintenance of our microbiota, improving immunity, and preventing the appearance of pathogens. Probiotics are popular research targets because they can keep a healthy lipid profile and influence the therapeutic use of antibiotics, the ways of different gut diseases, metabolic pathways, and a variety of infections without antibiotics [4].

Gut Microbiota Balance and Digestive Health: Gut microbiota is vital for digestion and the absorption of food nutrients. The probiotic bacteria act as the builders of good digestion, who extract and use the nutrients, which in turn ensures good digestion and absorption of nutrients. In contrast to them are pathogenic bacteria, the ones that inhibit digestion and cause disease.

Probiotics are those that help digestion and Add back to the body in the form of nutrients. (Read more on) Also, through the activation of antibodies and other immune cells, the probiotic bacteria can improve the immune system. The study has also confirmed the fact that probiotic bacteria can lead to the reduction of diarrhea symptoms and prevent future infections [5]. The probiotics are those friendly to humans and are responsible for displacing the pathogenic bacteria like E. coli. Difficile, and Salmonella spp. This is a natural way bacteria that can be used to fight off bacteria. Besides, lactic acid bacteria (LAB) are the primary cells of organic acids, Bacteriocins, and hydrogen peroxide that create the unfavourable conditions for pathogens [6].

The modulation of the immune system: Probiotics help in the functioning of dendritic cells, macrophages, and natural killer (NK) cells, which aid the body in fighting infections. Some strains alter cytokine production related to autoimmune and allergy inflammation, reducing inflammation. Probiotics augment the production of secretory IgA (sIgA) to encourage mucosal immunity and protect the body against gastrointestinal infections. Probiotics are believed, based on clinical research, to be useful in reducing the duration and severity of some diseases, such as respiratory disease, allergies, and some inflammatory forms of dermatitis [7].

By promoting the synthesis of antibodies and other immune system cells, probiotics improve the effectiveness of the immune system.

Promoting mental functioning: Research on the relationship between these two fields of study is beginning to take shape. It has been demonstrated that the use of probiotic bacteria improves mood states and reduces symptoms of anxiety or depression.

Correcting bacterial imbalance: Probiotics can help treat a state of disturbed equilibrium of gut microbes, which can be caused by antibiotic therapy or digestive issues.

Probiotic bacteria are essential for preserving good health, especially with immune system regulation, diarrhea therapy, psychological welfare, gut flora alteration, and appetite-related digestion of appertaining tissues. By consuming probiotic meals or supplements, one can guarantee proper intake of these bacteria [8].

**Goals for the research**

The main goals of this research are to–

To isolate bacterial strains from probiotic drinks.

To characterize the bacteria isolated from these drinks

To assess the antibacterial activity of the isolated bacteria against both natural and artificial sugars.

## MATERIALS AND METHODS

### Sample Collection and Preparation

Samples of Yakult and probiotic yogurt were gathered from the neighbourhood grocery. Give the label to every probiotic drink. Using a sterile pipette, transfer 1 mL of each probiotic beverage into separate sterile test tubes. After that, the samples were kept at 4°C until they could be examined further. Next, to guarantee that the microbes are distributed evenly, give the probiotic drink bottle a good shake. To do serial dilutions ($10^1$ to $10^6$), we aseptically transfer 1 mL of the probiotic drink into 9 mL of sterile saline water or distilled water. Before plating, we vortex the samples for 30 seconds to homogenize the bacterial content.

### Isolation of Bacteria from Various Probiotics-based Drinks

### Selection of Culture Media

Media to isolate particular bacterial groups are

**Table 1:** Overview of Bacterial Culture Media and Incubation Requirements

| Media | Purpose | Incubation Conditions |
|---|---|---|
| De Man, Rogosa, and Sharpe (MRS) Agar | Selective for lactic acid bacteria (LAB) | Anaerobic, 37°C, 24-48 hours |
| Nutrient Agar (NA) | General purpose of non-LAB bacteria | Anaerobic, 37°C, 24 hours |

### Inoculation onto Agar Plates

Use the spread plate technique to spread100µl of each dilution for each beverage onto the corresponding agar media. Using an aseptic glass rod, pass the samples over the agar surface in a gentle mixing motion. Anaerobic (e.g., LAB) incubate plates in an anaerobic jar with gas packs to suck oxygen. Incubate plates in anaerobic conditions, 37°C, for 24-48 h [9].

### Colony Observation and Selection

After incubation, observed bacterial colonies based on:

Morphology – shape, size, color, edge, texture.

Opacity- transparent, translucent, or opaque.

Colony elevation- raised, convex, flat.

We select distinct bacterial colonies for further study [10].

### Purification of Bacterial Isolates

Stab a sterile inoculating loop through the slants to isolate an individual colony. For pure cultures, perform streak plates on MRS and NA agar plates. They incubate for 24 h at 37°C [11].

### Morphological and Biochemical Characterization of Isolated Bacteria

### Colony Morphology (Macroscopic Observation)

Observation of bacterial colonies in MRS, Nutrient Agar (NA), grown for 24-48 hrs at 37°C.

### Cell Morphology (Microscopic Observation)

Gram staining was followed by morphological analysis of the isolated colonies. Gram staining is a differential staining technique that uses the differences in the makeup of the cell walls to classify bacteria as either gram-positive (purple) or gram-negative (pink). In microbiology, it is frequently used to identify bacteria. Gram-positive bacteria have a thick peptidoglycan cell wall that holds the crystal violet dye, giving them a purple appearance under a microscope. Gram-negative bacteria have an outer membrane and a thin coating of peptidoglycan. They tolerate the counterstain (Safranin) because they lose crystal violet, and they turn pink following decolourization [12].

**Table 2:** Morphology-Based Classification of Bacterial Colonies

| Colony Characteristic | Description |
|---|---|
| Shape | Circular, irregular, filamentous |
| Size | Small, medium, large |
| Elevation | Flat, raised, convex, umbonate |
| Margin (edge) | Entire, undulate, lobate, curled |
| Surface Texture | Smooth, rough, wrinkled, mucoid |
| Opacity | Transparent, translucent, opaque |
| Pigmentation | White, cream, yellow, pink, etc. |

### Biochemical Characterization of Isolated Bacteria

Catalase and oxidase tests were performed to characterize the isolated bacterial strains.

### Catalase Test

Oxygen and water (H2O) produced from the catalase reaction indicate the breakdown of hydrogen peroxide (H2O2) into water and oxygen gas. Catalase-positive strains can be recognized by the gas bubble generated during the addition of hydrogen peroxide to the broth.

### Materials Required

3 percent v/v hydrogen peroxide (H2O2), sterile glass slide or petri dish, inoculation rod, bacterial culture grown in nutrient agar medium or slant.

### Procedure

Place a drop of 3% hydrogen peroxide on a clean glass slide. Using a sterile inoculating loop, we pick a small amount of the bacterial colony and mix it with the hydrogen peroxide drop. Then we observe for immediate bubble formation (oxygen release) [13].

### Oxidase Test

The oxidase test is performed for the identification of cytochrome c oxidase, which is the enzyme of the electron transport chain of the cell. It is now known that these bacteria will have a certain enzyme if they turn a reagent of tetraethyl-p-phenylenediamine into a dark purple or blue solution in less than 30 seconds.

### Materials Required

Sterile filter paper or oxidase strips, sterile inoculating loop, and Bacterial culture on Na agar (oxidase reagent [1%tetramethyl p-phenylene diamine dihydrochloride].

### Procedure

A drop of oxidase reagent is placed on a filter paper or an oxidase strip. Grab a fresh bacterial colony with a sterile loop, stab it into the reagent drop, and wait to see the color change for thirty seconds [14].

### Antibacterial Activity Testing of Isolated Bacteria against Natural and Artificial Sugars

### Test Sugar Samples

**Natural sugars –**Stevia, Xylitol.

**Artificial Sugars –** Aspartame, Saccharine.

### Preparation of Sugar Solutions

Prepared 10% (w/v) stock solutions by dissolving 10 g of sugar/sweetener in 100 mL of distilled water. For lower concentrations (e.g., 5%, 1%), we dilute accordingly. Then, transfer the sugar into a sterile conical flask or bottle. After that, add pre-measured sterile distilled water. Stir using a magnetic stirrer or vortex until fully dissolved. Use a pH meter to measure the pH of each sugar solution. Adjust the pH to neutral (7.0) or physiological pH (~6.8-7.2) using sterile NaOH (0.1M) or HCl (0.1M). Store the sterile sugar solutions in airtight, sterile bottles at 4°C for up to one week.

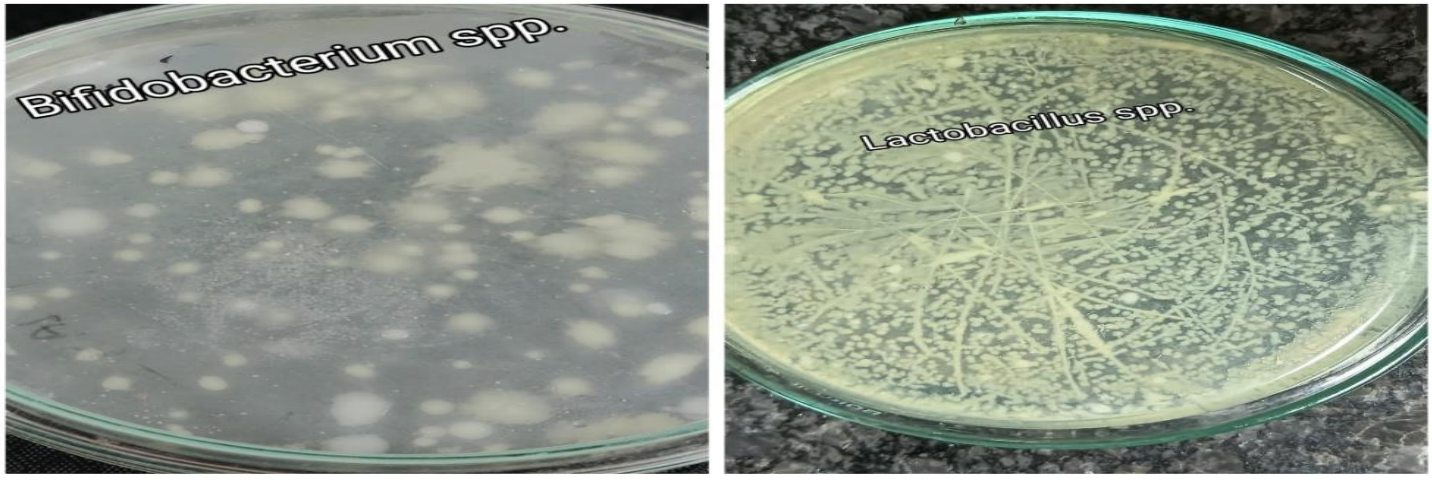
**Application in Antibacterial Activity Assays**

After preparing the sugar solutions, they can be tested against the isolated probiotic bacteria bythe double disc diffusion method to determine their resistance patterns. Pour nutrient agar or MRS agar into sterile Petri dishes. Swab probiotic bacterial culture onto the agar surface. Then prepare sterile filter paper disks 6 mm, soak them with different sugar solutions, and place them in the agar. Then add 100 µL of sugar solution into each well. Incubate at 37°C for 24-48 hours. Then measure the zone of inhibition of the bacteria [15].

## RESULTS AND DISCUSSION

We successfully isolated two main bacterial species, those are- A***)*** *Lactobacillus spp*. B) *Bifidobacterium spp.*

**Figure 1: (**A) *Bifidobacterium spp. and* (B*) Lactobacillus spp.*




**(A)** ***Bifidobacterium spp***. Irregular, convex, white colonies on Nutrient agar.

**(B)** ***Lactobacillus spp.*** Small, creamy, circular, raised colonies on MRS agar.

**Morphological and Biochemical Characterization of Isolated Bacteria**

**Morphological Characterization of Isolated Bacteria**

Below is a detailed description of the morphological characteristics of *Lactobacillus spp*. and *Bifidobacterium spp.* When grown on selective media such as MRS agar.

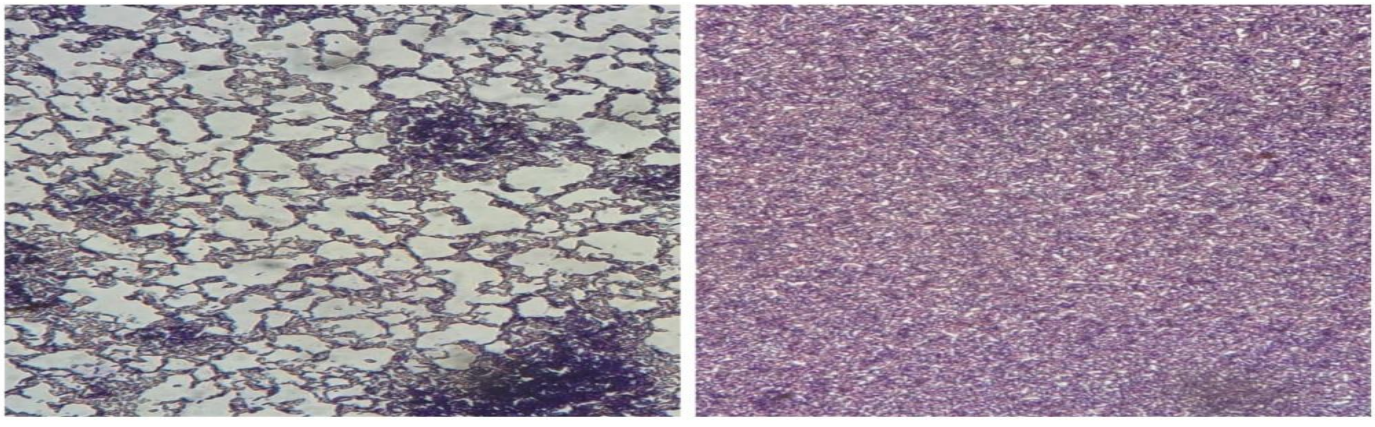
**Table 3:** Comparative Colony Morphology of *Lactobacillus spp.* and *Bifidobacterium spp*.

| Characteristic | *Lactobacillus spp.* | *Bifidobacterium spp.* |
|---|---|---|
| Shape | Circular | Irregular |
| Size | Small | Medium |
| Elevation | Raised | Convex |
| Margin (edge) | Entire | Undulate |
| Surface Texture | Smooth, moist | Rough |
| Opacity | Opaque to translucent | Opaque |
| Pigmentation | Cream | White |

**Microscopic Observation (Gram Staining)**

Gram staining was performed to determine the morphology and Gram reaction of bacterial isolates from probiotic drinks. The results are summarized below:

**Figure 2:** Microscopic view of (A) Bifidobacterium spp. (B) Lactobacillus spp



(A) (B)

**Table 4:** Microscopic view of isolated bacteria

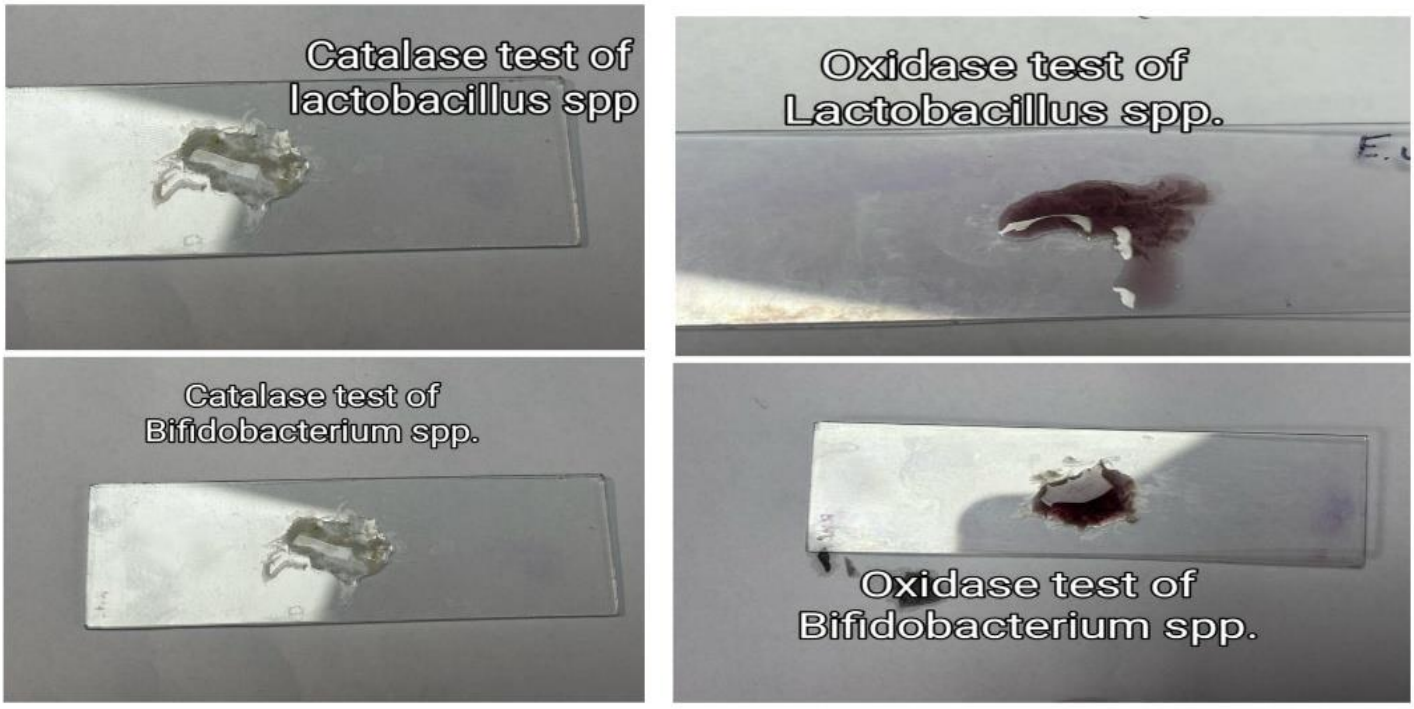
| Isolated Bacteria | Gram Reaction | Cell shape | Arrangement |
|---|---|---|---|
| Lactobacillus spp. | Positive (+) | Rod-shaped ( Bacilli) | Single, short chains |
| Bifidobacterium spp. | Positive (+) | Rod-shaped, Y-shaped, or Branched rods | cluster, V- or Y-shape |

Lactobacillus spp.— Gram-positive rods, usually single or in short chains, Bifidobacterium spp.-- Gram-positive branched rods appeared to be round, often arranged in a V - or Y-shaped pattern. Neither Gram-negative bacterium was observed (positive probiotic strains are there). No longer had Gram-negative bacteria washed out, ensuring probiotic strains were present. This is consistent with the bacteria in the probiotic drinks being predominantly Gram-positive, rod-shaped lactic acid bacteria (LAB) with a probiotic profile, which speaks to the claim.

**Biochemical Characterization of Isolated Bacteria**

Screening for the biochemical profile of the isolated probiotic bacteria was confirmed by biochemical tests. The main results of the Catalase and oxidase tests are mentioned below:

**Figure 3:** (A) Catalase test of *Lactobacillus spp*. and *Bifidobacterium spp.,* (B) Oxidase test of *Lactobacillus spp*. and *Bifidobacterium spp*.




(A) (B)

**Table 5:** Biochemical Characteristics of Isolated Bacteria

| Isolated Bacteria | Catalase Test | Oxidase Test |
|---|---|---|
| Lactobacillus spp. | Negative (-) | Negative (-) |
| Bifidobacterium spp. | Negative (-) | Negative (-) |

Catalase Test: Everything Isolate was- (-) to confirm they are lactic acid bacteria (LAB). However, this is a trait that LABs do not have since they are fermentative rather than aerobic.

Oxidase Test: (-) For all isolates, look for cytochrome c oxidase indicative of the absence of this trait in facultative, or facultative/obligate anaerobic bacteria, *Lactobacillus,* and *Bifidobacterium.*

Negative catalase and oxidase: these results also suggest the isolates of probiotic drinks follow obligatory or facultative anaerobic lactic acid bacteria (LAB), which aligns with their probiotic nature.

**Antibacterial Activity Testing of Isolated Bacteria against Natural and Artificial Sugars**

The antibacterial activity of isolated bacteria was performed by the disk diffusion method. The antibacterial activity of the isolated strains varied against different sugars. Lactobacillus spp. showed the highest antibacterial activity against stevia and xylitol, while Bifidobacterium spp. showed maximum antibacterial activity against saccharine and aspartame.

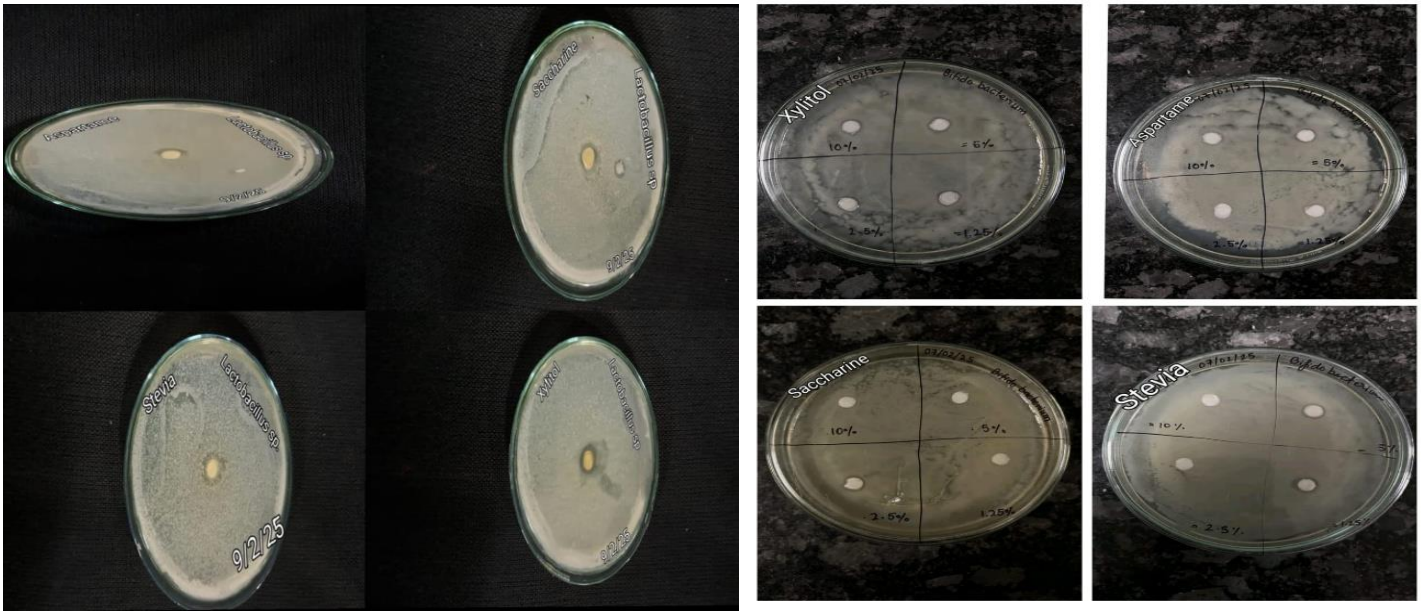
**Antibacterial Activity of Isolated Bacteria in the Presence of Natural & Artificial Sugars**

Influence of Natural and Artificial Sweeteners in the Inhibition of Isolated Bacteria Evaluated by Zone of Inhibition Using the Graph. Using this graph, we can demonstrate the probiotic potential of bacterial strains in enhancing the inhibitory effect of probiotic bacteria against natural sugars, stevia, and xylitol. The restriction of probiotic bacteria functions by artificial sugars such as aspartame, fructose, and saccharin. This indicates that ingesting artificial sugars could minimize the health benefits of gut-bacterial probiotics.

Table 6: Antibacterial Activity of Isolated Bacteria against Natural and Artificial Sugars

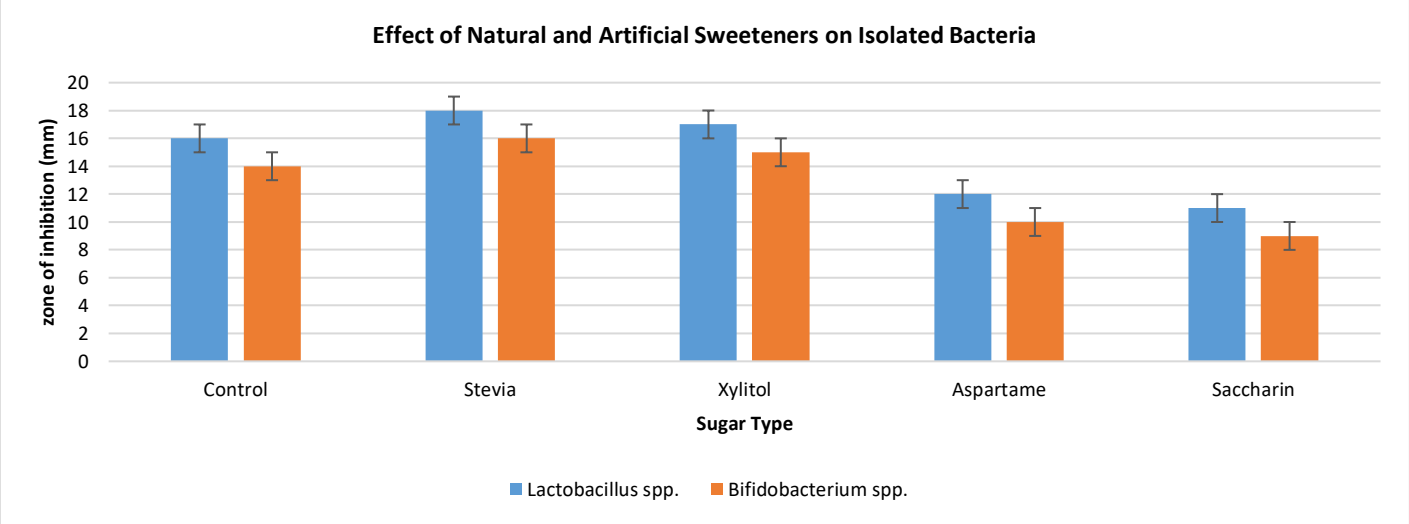
| Bacterial Strain | Stevia | Xylitol | Aspartame | Saccharine |
|---|---|---|---|---|
| *Lactobacillus spp.* | Strong | Strong | Moderate | Moderate |
| *Bifidobacterium spp.* | Moderated | Moderate | Strong | Strong |

**Figure 4:** Antibacterial Activity of (a) Lactobacillus sp., and (b) Bifidobacterium sp., Against Natural and Artificial Sugars




(A) (B)

**Figure 5:** Antibacterial activity of isolated bacteria in the presence of natural & artificial sugars




## CONCLUSION

The study focuses on the *lactobacillus* or *bifidobacterium* genera to identify probiotic bacterial strains from specific probiotic drinks. These strains can be recognized and connected to gut health based on their appearance, biochemistry, and molecular makeup. These bacteria's microbiome exhibits remarkable persistence and flexibility. The impact of artificial and natural sugar sources on probiotic strains was also investigated in this study. The growth and activity of probiotic bacteria were enhanced by natural sugars, whilst some strains were suppressed by artificial sweeteners. This raises questions regarding the safety of artificial sweeteners and their effects on the balance of the gut microbiota. Depending on the type of sugar available, probiotics' antibacterial capabilities against harmful bacteria differed. Certain natural sugars promoted the production of Bacteriocins and organic acids.

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