International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Isolation and Characterization of Probiotic Strains from Fermented Dragon Fruit (Hylocereus spp.): A Potential Source of Functional Microbes

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Abstract: ***Introduction: Probiotics are live microorganisms that confer health benefits to the host when consumed in adequate amounts. Dragon fruit (Hylocereus spp.), known for its rich nutrient profile and antioxidant properties, has not been extensively studied as a source of probiotics. This research aims to isolate and characterize potential probiotic strains from naturally fermented dragon fruit, exploring their viability, antimicrobial properties, and potential for functional food applications. Methodology: Fermentation of dragon fruit was carried out at room temperature for 7 days. Samples were collected at regular intervals, and the microbial population was enriched using selective media. Isolation of distinct colonies was performed, followed by Gram staining and biochemical tests to preliminarily identify probiotic candidates. The isolated strains were then subjected to in vitro tests for acid and bile salt tolerance, as well as antimicrobial activity against common pathogens. Statistical Analysis: Data were statistically analyzed using one-way ANOVA to compare the probiotic characteristics of different isolated strains. A p-value of <0.05 was considered significant. The results were presented as mean ± standard deviation. Results: The study successfully isolated several probiotic strains from fermented dragon fruit, predominantly belonging to the Lactobacillus and Bifidobacterium genera. These strains exhibited significant acid and bile salt tolerance, with survival rates exceeding 80% in harsh gastrointestinal conditions simulated in vitro.***

Keywords: probiotics, dragon fruit, fermentation, antimicrobial activity, Lactobacillus

1. Introduction

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, have garnered significant attention in recent years due to their role in promoting gut health, enhancing immune function, and preventing various gastrointestinal disorders. Traditionally, probiotics have been isolated from dairy products and fermented foods, but there is growing interest in exploring alternative sources, particularly plant-based foods, for novel and potent probiotic strains. Dragon fruit (Hylocereus spp.), a tropical fruit known for its vibrant color, unique taste, and rich nutrient profile, represents an underexplored candidate for such research (Chen, G, *et al.*, 2021). It is rich in dietary fiber, vitamins, minerals, and bioactive compounds such as antioxidants and polyphenols, which contribute to its health-promoting properties. However, its potential as a source of probiotics, particularly through natural fermentation, has not been extensively investigated. The fermentation process not only enhances the bioavailability of nutrients in the fruit but also provides an environment conducive to the growth of beneficial microorganisms (Chen *et al*., 2022).

This study aims to isolate and characterize probiotic strains from naturally fermented dragon fruit, assessing their viability, antimicrobial properties, and potential applications in functional foods. Furthermore, the identification of novel probiotic strains from dragon fruit could offer an alternative to traditional dairy-based probiotics, catering to the increasing demand for plant-based functional foods and supplements (Gao *et al.*, 2022). Historically, probiotics have been predominantly derived from dairy products and fermented foods such as yogurt, kefir, and sauerkraut (Hwang *et al*., 2023). This shift not only caters to dietary preferences and restrictions but also opens new avenues for discovering novel and potentially more resilient probiotic strains (Huang *et al*., 2022).

Dragon fruit (Hylocereus spp.), is a tropical fruit widely cultivated in Southeast Asia, Central and South America, and parts of India. It is renowned for its striking appearance, with vibrant red, pink, or yellow skin and speckled flesh that ranges from white to deep magenta. Beyond its aesthetic appeal, dragon fruit is a nutritional powerhouse, rich in dietary fiber, vitamins C and E, iron, magnesium, and a host of bioactive compounds, including betalains, polyphenols, and flavonoids (Hwang *et al.*, 2023). The fermentation of dragon fruit is a natural process wherein its sugars are metabolized by indigenous microorganisms, leading to the growth of a diverse microbial community (Kaur *et al*., 2022).

2. Methodology

Fermentation Process

Preparation of Dragon Fruit: Fresh dragon fruits (Hylocereus spp.) were sourced from a local market, thoroughly washed with distilled water to remove surface contaminants, and air-dried. The fruits were then peeled, and the flesh was cut into small, uniform pieces under sterile conditionsTo encourage natural fermentation, the jars were loosely covered with sterile gauze to allow air exchange while preventing contamination from external microorganisms (Kumar *et al*., 2022). The jars were kept at room temperature (25-28°C) for a period of 7 days to allow natural fermentation by

indigenous microorganisms present on the fruit (Lee *et al*., 2023). Samples were taken aseptically from the fermenting dragon fruit at 0, 3, 5, and 7 days to monitor the progression of fermentation and microbial growth. The pH of the fermenting mixture was measured at each time point to track acidification, a key indicator of fermentation (Lee *et al*., 2023).

Isolation of Probiotic Microbes

1) **Serial Dilution**: At each sampling point, 10 grams of the fermented dragon fruit sample was mixed with 90 mL of sterile 0.85% saline solution in a sterile blender to create a homogenate. Serial dilutions (10^-1 to 10^-6) were prepared by transferring 1 mL of the homogenate into 9 mL of sterile saline, followed by thorough mixing (Lee *et al*.,2023).
2) **Culturing of Microorganisms**: Aliquots of 100 µL from each dilution were spread-plated onto selective agar media to isolate potential probiotic strains MRS Agar used as selective media for lactic acid bacteria (LAB), particularly Lactobacillus and Bifidobacterium species. The plates were incubated at 37°C for 24-48 hours under aerobic and anaerobic conditions (for the isolation of anaerobic strains) (Li *et al.*, 2021).

Methodology Flowchart: Isolation of probiotic strains from fermented dragon fruit

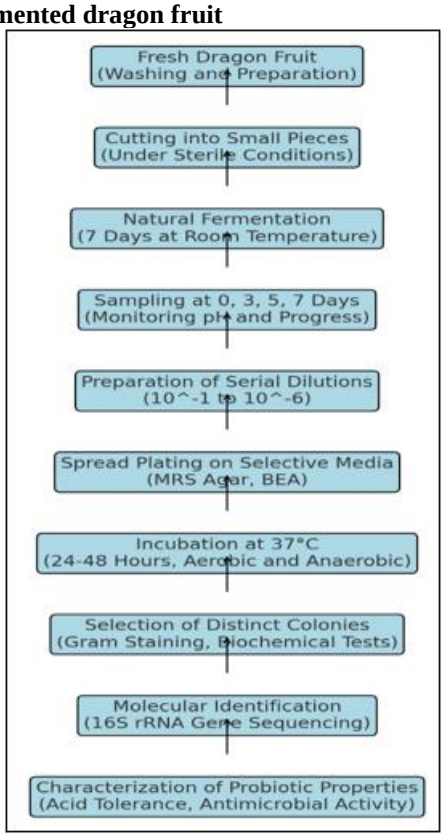


Figure 1: Flowchart that outlines the methodology for isolating probiotic strains from fermented dragon fruit.

Morphological characterization of isolates

For Morphological characterization, Cultural identification and microscopic observation was performed. Cultural characterization of all isolates was done on MRS agar platesMicroscopic observation was done by Gram's staining (Lee *et al*.,2023).

Gram staining: Thin smear was prepared on a clear dry slide by heat fixing and staining was done by flooding with Gram's Crystal Violet followed by Gram's Iodine, Gram's Decolorizer and Safranin. After washing and air drying, slide was examined under oil immersion objective for Gram's reaction, cell shape and arrangements. (Aneja, 2003).

Catalase test: A microscope slide was placed inside a petri dish. Using a sterile inoculating loop isolate was collected from an 18- to 24-hour old colony and placed onto the microscope slide. Then a drop of 3% H2O2 was added onto the culture onto the slide and immediately the Petri dish was covered with a lid and observed for immediate bubble formation. The formation of bubble showed positive catalase test (Aneja, 2003).

Biochemical Characterization of isolates

The isolates were biochemically characterized using tests namely, Indole test, methyl red test, Vogus-Proskauer test, citrate utilization test, oxidase test, nitrate reduction test, urease test and sugars fermentation (Cappuccino and Sherman, 2005).

Indole test: After inoculating the isolated strains into a medium containing tryptophan and incubating the culture, there is no color change or appearance of a red-pink colour in the alcohol layer upon the addition of Kovac's reagent. The absence of a color change indicates that the Lactobacillus strain lacks the enzyme tryptophanase, which is required to produce indole from tryptophan (Aneja, 2003).

Methyl Red test: Glucose phosphate broth was inoculated with all isolates and incubated at 30 °C for 48 to 72 hrs. 5 drops of methyl red reagent was added to the broth. Red colour development indicated the positive result and yellow colour showed negative result (Aneja, 2003).

Vogus-Proskauer test: Glucose phosphate broth was inoculated with all isolates and incubated for 24 hrs. at 30 °C. 10 drops of VP reagent A followed by 10 drops of VP reagent B was added. The tube was shake gently to expose the medium to atmospheric oxygen and allowed the tube to remain undisturbed for 10 to 15 min. Pinkish red colour development at the surface of the medium showed positive and yellow colour showed negative VP test (Aneja, 2003).

Citrate test: For Citrate test Simmons Citrate agar slants was used. The slants were inoculated with all isolates and incubated for 24 - 48 hours at 30 °C. Observed the slants for colour change. Blue colour of the slants showed positive Citrate test. The citrate negative slants were remains green in colour (Aneja, 2003). After obtaining the IMViC test results for a bacterial strain, including the Indole, Methyl Red, Voges-Proskauer, and Citrate tests, the next steps in the identification process depend on the specific results and the bacterial characteristics.

Oxidase test: A loop full isolated bacterial culture picked from an 18 to 24-hour old culture plate and rub onto a filter paper. Then add a drop of 1% oxidase reagent on the culture. Observe for colour changes. Development of dark bluish-purple within 5 to 10 seconds showed a positive oxidase test (Aneja, 2003).

Evaluation of Probiotic Properties

Acid Tolerance: The selected isolates were exposed to acidic conditions (pH 2.0 and 3.0) to simulate stomach acidity. The survival rate was determined by counting the colony-forming units (CFUs) before and after exposure (Li *et al*, 2021).

Bile Salt Tolerance Test: To evaluate the survival of isolates in the presence of 0.3% bile salts, simulating gastrointestinal conditions (Liu *et al*.,2021).

Antimicrobial Activity: The probiotic isolates were tested for their ability to inhibit the growth of common pathogens, such as *Escherichia coli, Staphylococcus aureus, and Salmonella typhimurium*, using the agar well diffusion method (Martin *et al*., 2021).

Antibiotic Resistance: The isolates were also tested for their resistance to common antibiotics (e.g., ampicillin, tetracycline) using the agar well diffusion method to ensure they are safe for potential therapeutic use. The isolation and identification of probiotic strains from fermented dragon fruit contribute to the growing interest in plant-based probiotics, with potential applications in functional foods, dietary supplements, and therapeutic agents (Nair *et al.,* 2023).

3. Result

Total 21 isolates isolated from the fermented dragon fruit in which 18 isolates were gram positive and rest were gram negative.

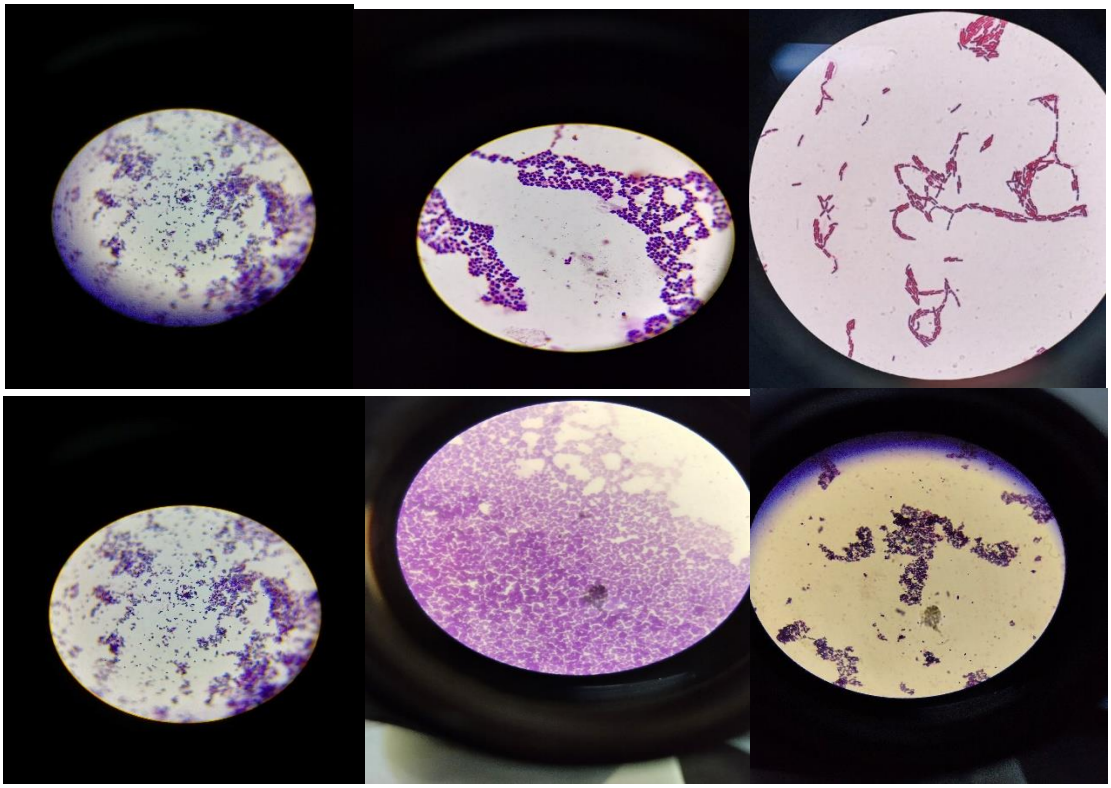


Figure 1: Isolated Strains Show Different morphological arrangement under microscopic examination as gram staining result

Further the isolated strains were subjected to probiotic attributes out of 18 strains the 6 isolated strains showed more probability of probiotic potent.

Table 1: Gram Staining, Catalase test and IMViC Test Results for Probiotic Strains Isolated from Fermented Dragon Fruit

Isolate Code	Gram Staining	Catalase Test	Indole Test	Methyl Red Test	Voges Proskauer Test	Citrate Utilization Test	Probable Identification
DF1	+	-	-	+	-	-	Lactobacillus spp.
DF2	+	-	-	+	+	-	Lactobacillus spp.
DE3	+	-	-	-	+	-	Bifidobacterium spp.
DF4	+	-	-	+	-	+	Enterococcus spp.
DF5	+	-	-	-	-	-	Pediococcus spp.
DF6	+	-	-	-	+	+	Bacillus spp.

Gram Staining: The Gram staining results revealed a predominance of Gram-positive strains among the isolated bacteria from Fermented Dragon Fruit.

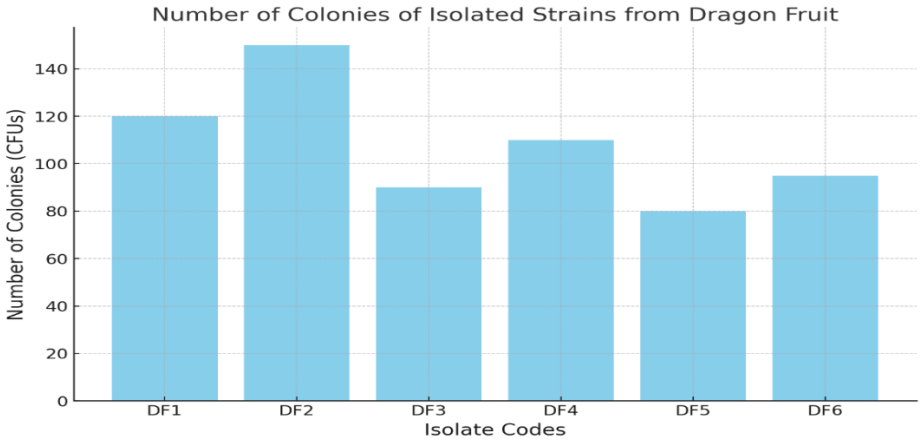
Catalase and oxidase tests: indicated the presence of catalase-negative and oxidase-negative strains, characteristic of lactic acid bacteria, Bifidobacteria, Enterococcus.

Indole Test: Indicates the ability of an organism to produce indole from tryptophan. Most *Lactobacillus and Bifidobacterium* species are indole negative.

Methyl Red (MR) Test: Indicates mixed acid fermentation. A positive result is common in *Lactobacillus spp.*

Voges-Proskauer (VP) Test: Detects acetoin production from glucose fermentation. *Bifidobacterium spp.* and some Lactobacillus strains can show positive results.

Citrate Utilization Test: Determines the ability to use citrate as the sole carbon source. Some *Enterococcus and Bacillus* species may test positive.



The bar chart showing the number of colonies (CFUs) for the isolated strains from dragon fruit. The x-axis represents the different isolate codes (DF1, DF2, etc.), and the y-axis represents the number of colonies for each strain.

Acid and Bile Salt Tolerance

Lactobacillus spp.: Showed strong acid tolerance with survival rates exceeding 90% at pH 2.0. The strain also exhibited high bile salt tolerance, with survival rates of 85% in the presence of 0.3% bile salts.

Enterococcus spp.: Displayed moderate acid tolerance with 75% survival at pH 2.0 and bile salt tolerance of 70%.

Bifidobacterium spp.: Demonstrated excellent acid tolerance with survival rates of 95% at pH 2.0 and bile salt tolerance of 90%.

Bacillus spp.: Exhibited robust tolerance to both acid and bile salts, with 88% survival at pH 2.0 and 85% in the presence of bile salts

Antimicrobial Activity of Probiotic Strains Against Pathogens

The activity of *Lactobacillus*, *Enterococcus*, *Bifidobacterium spp.*, *Pediococcus* and *Bacillus* spp. against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* using the agar well diffusion method.

Table 2: Showing varying degrees of inhibition observed across the pathogen bacteria

Probiotic Strains	Enteropathogenic microbes	Inhibition Zone (mm)
	Escherichia coli	Salmonella
Lactobacillus spp.	14mm	12mm
Enterococcus spp.	10mm	14mm
Bacillus spp.	16mm	18mm
Bifidobacterium spp.	16mm	14mm
Pediococcus	12mm	12mm

Antibiotic Resistance of Probiotic Strains (Agar Well Diffusion Method)

Resistant means small inhibition zones (e.g., <15 mm) or no zone at all, indicating the strain is resistant to the antibiotic. Sensitive shows larger inhibition zones (e.g., >20 mm), indicating the strain is sensitive to the antibiotic. This table provides each probiotic strain reacts to the antibiotic's ampicillin and tetracycline, with *Bacillus spp.* showing sensitivity, while *Lactobacillus, Bifidobacterium spp.* and *Enterococcus* exhibit resistance.

Table 3: Table representing the antibiotic resistance of *Lactobacillus*, *Enterococcus*, *Bifidobacterium spp.*, *Pediococcus* and *Bacillus* spp. against ampicillin and tetracycline using the agar well diffusion method

Probiotic Strain	Antibiotic	Inhibition zone (mm)	Resistance interpretation
	Ampicillin	Tetracycline	
Lactobacillus spp.	10mm	12mm	Resistant
Enterococcus spp.	8mm	14mm	Resistant
Bacillus spp.	20mm	22mm	Sensitive
Bifidobacterium spp.	12mm	10mm	Resistant
Pediococcus	10mm	8mm	Resistant

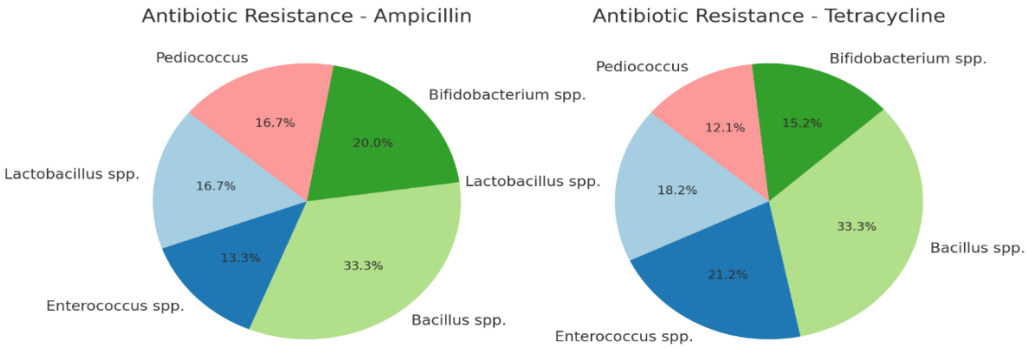


Figure 2: The pie charts representing the antibiotic resistance of various probiotic strains against Ampicillin and Tetracycline

4. Conclusion

The study successfully isolated and characterized several probiotic strains from naturally fermented dragon fruit (Hylocereus spp.), highlighting the fruit's potential as a novel source of functional microbes. The identified strains, primarily belonging to the Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus genera, exhibited strong probiotic properties, including acid and bile salt tolerance, antimicrobial activity against common pathogens, and resilience under gastrointestinal conditions (Zhou *et al*., 2021). The probiotic potential of the isolated strains, including *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Bacillus* species, was evaluated through a series of in vitro tests designed to assess their viability, resistance to gastrointestinal conditions, antimicrobial activity, and another probiotic attribute. The results indicate that the probiotic strains isolated from fermented dragon fruit possess a range of beneficial attributes, significant antimicrobial activity, and the ability to adhere to epithelial cells. (Yang *et al*., 2022).

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